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Analysis of potential porcine endogenous retrovirus (PERV) transmission in a whole-organ xenotransplantation model without interfering microchimerism

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Abstract The question whether porcine xenografts can lead to porcine endogenous retrovirus (PERV) infection of recipients is critical for the evaluation of the safety of pig-to-man xenotransplantation. Unfortunately, polymerase chain reaction (PCR)-based analysis of potential PERV infections in nonhuman-primate whole-organ xenotransplantation models is hampered by false positive results due to chimeric porcine cells. To avoid the inherent analytical problem of xenomicrochimerism, we developed a non-life-supporting pig-to-primate kidney xenotransplantation model: porcine kidneys were transplanted, whereas the functioning recipient kidneys remained in situ. Subsequent to rejection (after 2 hours to 15 days), xenografts were removed, and recipients remained alive for up to 287 days. Immunosuppressive therapy based on cyclophosphamide, cyclosporine, and steroids was maintained for 28 days after transplantation. Using appropriate PCR assays, xenochimerism was found in tissue samples and partly even in peripheral blood leukocytes (PBLs) while the porcine kidneys were in situ. After graft removal, xenochimerism was no longer detectable, thus allowing analysis for possible PERV transmission.

Keywords Xenotransplantation · Porcine endogenous retrovirus · Chimerism · Pig-to-primate · Kidney transplantation

Abbreviations *PBL* Peripheral blood leukocyte · *PCR* polymerase chain reaction · *PERV* porcine endogenous retrovirus · *RT* reverse transcriptase

Introduction

Recent progress in the development of transgenic donor organs [7] has led to a renewed interest in xenotransplantation as a possible solution for the current donor organ shortage. Among the different species under consideration as suitable organ donors, pigs are the most likely donor animals [5, 6]. Currently, the potential for infection of xeno-organ recipients with xenozoonoses and, especially, the subsequent risk of transmission of any infective agent from the xenotransplant recipient to the general population are major concerns [1, 2, 4, 29]. In the xenotransplant setting, postoperative immunosuppression and immunomodulation [8] may lead to an enhanced susceptibility of recipients to any potential infective agent spread by the xeno-organ. Porcine en-

dogenous retroviruses (PERVs) are currently the main agents under investigation [21, 31]. This is due to the fact that PERV is carried in the porcine germline and cannot be eliminated from donor organs by classic means such as specific-pathogen-free animal housing. PERVs are detectable in all cell types and all pig strains analyzed so far [3, 14, 21, 34].

In vitro infection of various human cell lines with PERV during cocultivation with porcine cell lines or primary cell cultures has been demonstrated by several groups [16, 21, 28, 34]. In vivo, the available data on possible PERV infection in patients treated with living pig cells or tissues suggest that infection does not take place [11, 20, 22]. However, the viral load in the patients analyzed in these studies might have been low compared to solid-organ transplantation. In addition, only a small subset of patients had been treated with immunosuppressive therapy during xenotissue exposure. Therefore, although the available clinical data so far do not provide any evidence for cross-species transmission of PERV to humans, a definite conclusion concerning the risk of transmission of endogenous retroviruses to xeno-organ recipients under intense immunosuppression cannot be drawn from these data [32]. Consequently, additional data on this issue using solid-organ transplantation models are needed.

Unfortunately, classic animal models in solid-organ xenotransplantation (creating life-supporting situations) are associated with the inherent problem of xenomicrochimerism, leading to false positive PERV detection by polymerase chain reaction (PCR). It is well known from allotransplantation that the existence of microchimerism is dependent on the presence of the graft [13]. After graft removal (e.g., re-transplantation), chimeric cells (e.g., derived from the original graft) usually are no longer detectable in the recipient [12, 13]. We therefore developed a solid-organ discordant non-life-supporting kidney xenotransplantation model with special emphasis on long-term survival of the organ recipient after graft removal. In this setting, the recipients, which were under immunosuppression for 28 days after transplantation, were exposed to the porcine grafts for up to 2 weeks. After graft nephrectomy, the recipients were followed up for 28–272 days.

Materials and methods

Selection of donors

Twelve nontransgenic large white Landrace pigs were used. Pigs were 3–12 weeks old and weighed 4.1–25.0 kg. Pigs were obtained from Schweinezuchtverband Weser-Ems, Oldenburg, Germany and were transferred to the central animal operation facility at Hannover Medical School on the day of operation.

Selection of recipients

Twelve cynomolgus monkeys (*Macaca fascicularis*) weighing 3.8–9.0 kg and between 1.5 and 3.5 years of age were used. Animals were purchased from the German Primate Center at Göttingen. Preoperative sera from all monkeys were screened for antiporcine antibody titer using flow-cytometric assay analysis (see below).

Surgical technique

Donor pigs were anesthetized with ketamine and intubated. Anesthesia was maintained with isoflurane and N_2O/O_2. Through a midline laparotomy and after anticoagulation with 300 IU/kg b.w. heparine, the abdominal aorta was cannulated, and the kidneys were perfused in situ with cold preservation solution (HTK, Köhler-Chemie GmbH, Alsbach-Höhnlein, Germany). After perfusion, the kidneys were dissected, removed, and stored in ice-cold perfusion solution until they were used. The cold ischemia time was between 75 and 310 min. Recipient cynomolgus monkeys were anesthetized using ketamine and propofol and intubated. Anesthesia was maintained with isoflurane and N_2O/O_2 and supplemented with boli of buphrenorphine.Through a midline abdominal incision, the inferior aorta and inferior vena cava were exposed. Transplantation was carried out by end-to-side anastomosis of the donor vessels to the aorta and inferior vena cava. The ureter was implanted into the bladder using a submucosal tunnel. In group A ($n = 7$) both native kidneys were left in place, thus creating a non-life-supporting model. In group B ($n = 5$), a life-supporting model was generated by ligature of the recipient ureters.

Immunosuppressive therapy

Immunosuppression was commenced on the day before the operation and continued until day 28 of the experiment. Immunosuppression consisted of cyclosporine, cyclophosphamide, and prednisolone (reducing dose). Cyclosporine was used to maintain the blood level from 400–600 ng/l. Cyclophosphamide was administered at a dose sufficient to suppress the leukocyte count down to $4–2 \times 10^9$/l [9, 30].

Postoperative monitoring

Blood samples were taken every day for assessment of full blood count, urea, creatinine, and electrolytes. Cyclosporine blood levels were determined using a monoclonal-antibody-based method (EMIT 2000, Behringwerke, Liederbach, Germany). Serum was prepared by centrifugation at 3400 *g* for 10 min at 20 °C. For detection of antiporcine xenoreactive antibodies, a flow-cytometric assay was utilized: frozen aliquots of porcine peripheral blood leukocytes (PBLs) obtained from an individual large white pig were thawed, and 0.5×10^5 cells were stained by 20 µl of the respective cynomolgus serum in different solutions. After 20 min of incubation at 4 °C, cells were washed twice with phosphate-buffered saline containing 1 % bovine serum albumin and 0.1 % sodiumazide. Bound cynomolgus antibody was detected with goat antihuman fluorescein isothiocyanate secondary antibodies, detecting IgG and IgM (both by Dianova, Hamburg, Germany), respectively. These antibodies are known to cross-react with cynomolgus immunoglobulins. The antibodies had been preabsorbed using porcine serum. After incubation for 20 min at 4 °C, cells were washed again

twice and then analyzed on a FACScan (Becton Dickinson, Mountain View, Calif.) cytometer. Using list mode data, 5×10^3 cells were analyzed.

PCR detection of PERV

PBLs of the transplant monkeys were prepared on days 0, 4, 7, 14, 21, and 28; in one animal additional PBL samples were collected on days 35, 49, 196, and 250. Furthermore, tissue samples were taken upon autopsy of the animals.

The cynomolgus PBLs ($1 \times 10^{6)}$ were lysed in 100 µl of 200 µg/ml proteinase K in PCR buffer for 3 h at 56 °C, followed by 10 min of inactivation at 95 °C. Of these crude extracts, 3.5 µl served as template for PCR with PERV pol-specific primers [21, 27]. Porcine β-globin [10] and mitochondrial gene-specific primers [11] were used as controls for chimerism. Baboon cytochrome oxidase-specific primers were used for internal positive controls [10]. These primers were shown to cross-react with cynomolgus cytochrome oxidase sequences.

PCR sensitivity was determined using PK15, a porcine kidney cell line which has been shown to express PERV [21]: different quantities of lysed PK15 cells were mixed with lysed cynomolgus cells. The sensitivity of the assays is shown in Table 1. PCR analysis for detection of PERV sequences was performed on the lysates of PBL and various tissue homogenates using primers specific for PERV pol, PERV env A, and PERV env B. Using the PERV pol- or env A-specific PCR, one PK15 cell could be detected in 10^6 cynomolgus PBLs. In contrast, the env B-specific PCR was less sensitive: one PK15 cell could be detected within 10^4 cynomolgus PBLs. The sensitivity of the pig β-globin-specific PCR was one PK15 cell in 10^4 cynomolgus cells; the sensitivity of the porcine mitochondrial gene-specific PCR was substantially higher, with one porcine cell detected in 10^7 cynomolgus cells (Table 1).

PERV pol-specific reverse transcriptase (RT)-PCR of serum samples

RNA was extracted by treating 1 ml serum with four volumes of a lysis solution containing 5.75 M guanidinium thiocyanate, 50 mM TRIS (pH 7.5), 100 mM β-mercaptoethanol, and 1 µg of poly (rA) per ml. The resulting lysates were incubated at 65 °C for 10 min. The RNA was precipitated with 1 ml of isopropanol at room temperature at 14,000 *g* for 30 min, washed with 70% ethanol, and resuspended in 100 µl diethylpyrocarbonate-treated H_2O. Samples were stored on ice until amplification [18]. Of each sample, 5 µl was used for cDNA generation with AMV-RT (Boehringer Mannheim, Germany), as described by the manufacturer. The cDNA synthesis was randomly primed. The following PERV pol gene-specific PCRs were performed as described above, using 0.5 µl cDNA as template. To exclude false positive results due to contamination with genomic DNA, RT reactions were performed without RT. RT-PCRs with primers specific to pig mitochondrial sequences [11] ruled out interfering porcine cellular RNA.

Table 1 Sensitivity of the various PCR assays used. Data are given as the number of PK15 cells still detectable if diluted in cynomolgus cells (*mtDNA* mitochondrial DNA)

Primer	Sensitivity
PERV pol	$1/10^6$
PERV env A	$1/10^6$
PERV env B	$1/10^4$
Pig β-globin	$1/10^4$
Pig mtDNA	$1/10^7$

Table 2 Survival and exposure time of graft recipients in group A *(upper part)* and group B *(lower part)*

	Animal	Survival	Reason for death	Graft in situ
Group A	1/162	28 days	Killed (planned)	2 h
	2/689	25 days	Killed (planned)	9 days
	3/660	29 days	Killed (planned)	2 h
	4/232	4 days	Killed (pneumonia)	2 h
	5/023	287 days	Killed (planned)	15 days
	6/240	28 days	Killed (planned)	6 days
	7/411	4 days	Killed (arterial thrombosis of the leg)	1 h
Group B	1/143	3 days	Uremia	3 days
	2/323	2 days	Killed (uremia)	2 days
	3/1363	1 day	Pneumonia	1 day
	4/135	4 days	Killed (pneumonia)	4 days
	5/343	11 days	Killed (uremia)	11 days

Results

In animals 1–7 (group A, non-life-supporting model), kidney grafts were left in situ for 1 h to 15 days (Table 2). All long-term survivors (animals 1, 2, 3, 5, and 6) remained apparently healthy, and the autopsies showed no signs of intra-abdominal lymphoproliferative disease. To obtain more long-term data, animal 5 was kept alive for 287 days after transplantation and was then killed.

In group B (with a life-supporting xenotransplant, Table 2), the recipients survived between 1 and 11 days. Animals 1, 2, and 5 died or were killed due to uremia, animals 3 and 4 due to pneumonia.

All recipients showed an initial sustained decrease of antiporcine antibody titer lasting for several days after transplantation. In the later course, a profound increase in antiporcine antibody titer for IgM and IgG was observed in five animals of group A despite immunosuppressive therapy.

Analysis of tissue samples by porcine mitochondrial gene-specific PCR demonstrated xenomicrochimerism in all animals of group B, thus excluding analysis for PERV release or transmission by standard PERV-PCR (Fig. 1). In contrast, when pig β-globin-specific primers were used, no xenomicrochimerism was detected, probably due to the lower sensitivity of these primers (data not shown). When PBLs of these animals were analyzed, PERV pol-specific primers as well as pig mito-

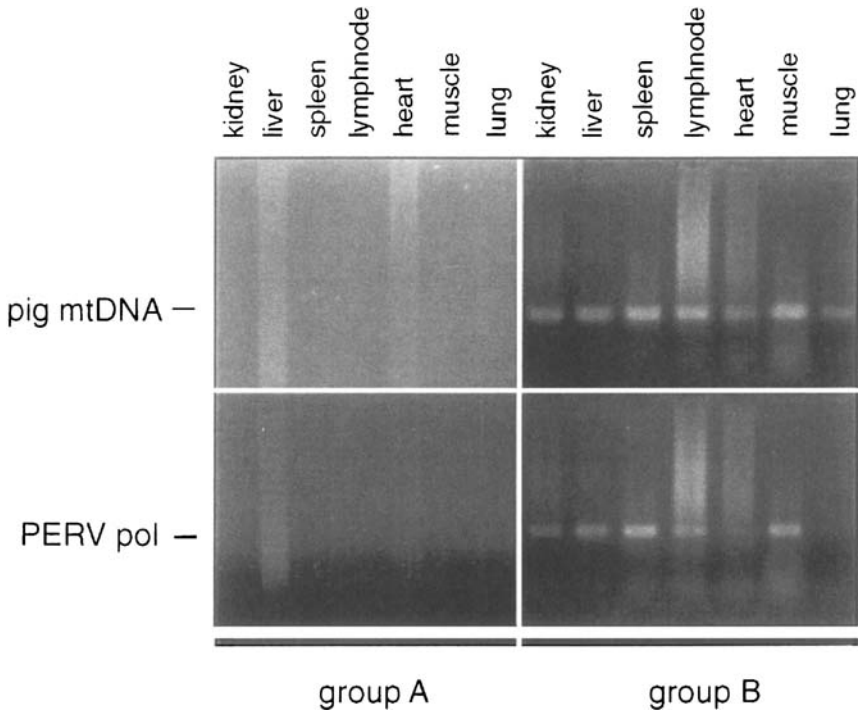

Fig. 1 Demonstration of xenomicrochimerism in animals of group B (*right panel*), but not in animals of group A (*left panel*), by PCR. PCR was performed using pig mitochondrial gene-specific primers (*pig mtDNA, upper panel*) and PERV pol-specific primers (*lower panel*). Results of tissue samples obtained from two representative animals killed on day 11 (group B; animal 343, *right panel*) and day 28 (group A; animal 240, *left panel*) are shown. All baboon cytochrome oxidase-specific control PCRs showed positive results (data not shown)

chondrial gene-specific primers inconsistently showed xenomicrochimerism for the time the xenograft was in situ (data not shown).

Porcine mitochondrial gene-specific PCRs as well as PERV pol-specific PCRs of various tissue samples from one representative animal of group A are shown in Fig. 1. These samples were obtained upon autopsy 22 days after graft removal. All samples were negative in the pig mitochondrial gene-specific PCR as well as for the PERV pol sequences. In addition, using these primers the tissue samples of all other animals in this group (4–235 days after graft removal) were negative. Likewise, neither the env A- nor env B-specific PCRs could detect PERV DNA in these animals (data not shown). All internal positive control reactions using primers specific for cytochrome oxidase showed positive results (data not shown).

In order to screen for retroviral particles in porcine and cynomolgus serum samples, we performed RT-PCR reactions using primers specific for PERV pol. Serum samples of several pigs were tested and showed PERV pol RNA expression (Fig. 2). Internal controls without RT were negative and thereby excluded false positive results due to contamination by pig genomic DNA. In order to test whether generation of PERV cDNA could be based on contamination of cellular mRNA in serum and not on RNA of retroviral particles, we performed control RT-PCRs with pig mitochondrial gene-specific primers: no porcine mitochondrial RNA could be detected in the pig serum samples (data not shown). Pig mitochondrial gene-specific RT-PCR using total RNA of porcine endothelial cells served as positive control (data not shown). In contrast to the pig serum samples, none of the cynomolgus serum samples showed evidence of PERV particles (Fig. 2). As internal positive control, all cynomolgus serum samples were spiked with a definite amount of PK15 cell culture supernatant. All of these spiked samples resulted in positive results.

Discussion

PERV has been shown to infect several human cell lines in vitro [3, 22, 34]. However, retroviral in vitro infection does not necessarily implicate similar results in vivo since host defense mechanisms in the latter render retroviral infection more unlikely than in the former. Unfortunately, recent strategies to overcome hyperacute rejection in xenotransplantation do impair a major natural barrier against foreign pathogens. In humans and all old-world monkeys there is a special defense mechanism against different parasites, bacteria, and enveloped viruses of other mammalian species: due to the absence of a functional αa1,3-galactosyltransferase gene and, presumably, because of the continuous contact to galactose αa1,3-galactose (Galαa1,3-Gal)-bearing intestinal bacteria, the sera of all old-world monkeys and human beings contain relatively high titers of anti-Galαa1,3-Gal antibodies [19]. These antibodies constitute the ma-

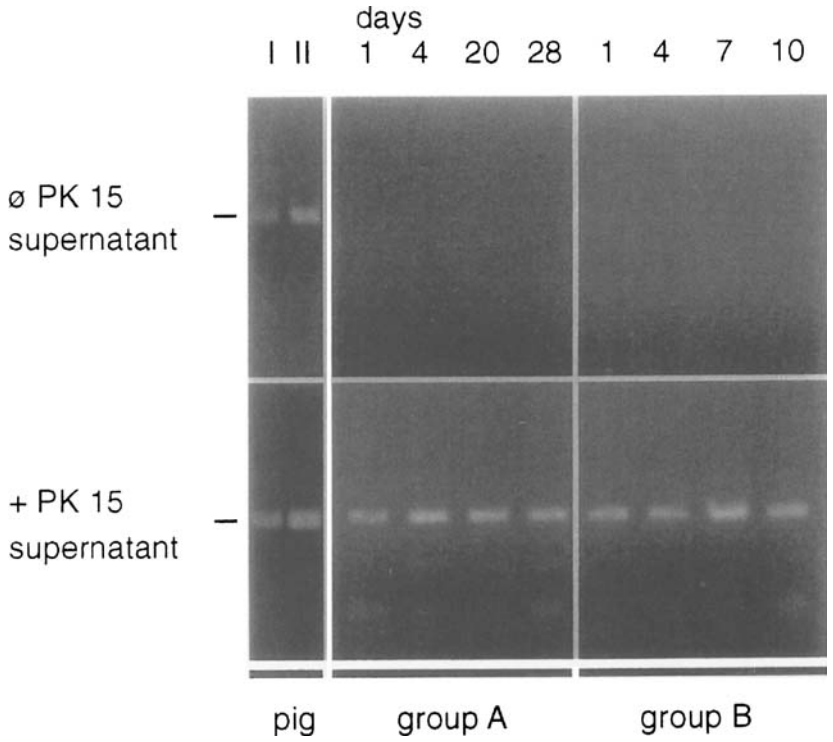

Fig. 2 Analysis of porcine and cynomolgus serum samples by PERV pol-specific RT-PCR. *Upper panel* Without exogenous PK15 culture supernatant. *Lower panel* Samples spiked with PK15 culture supernatant. False-positive results due to contamination with pig genomic DNA or cellular RNA were excluded by internal controls without RT and by pig mitochondrial gene-specific RT-PCR, respectively (data not shown)

jority of natural antiporcine immunoglobulins. Anti-Galαa1,3-Gal antibodies contribute to an important humoral host-defense mechanism against bacteria and also against viruses from other (non-old-world monkey) mammalian species: murine retroviruses and also HIV, if grown in Galαa1,3-Gal-expressing cells, have been shown to bear Galαa1,3-Gal epitopes on their envelope membrane [23, 24, 25, 26, 33]. These retroviruses are effectively eliminated from human serum by binding of anti-Gal antibodies and subsequent complement-mediated viriolysis. This mechanism probably also prevents infection of human beings by porcine endogenous retroviruses, at least in the normal in vivo setting .

Recent investigation of patients after limited contact with porcine cells or tissues did not provide any evidence of PERV infection [11, 20, 22]. However, although those patient samples are the most suitable currently available to assess PERV transmission, these retrospective studies had several shortcomings: no whole-organ transplantations were performed, and most of these patients did not undergo pharmacological immunosuppression. Also, the antiviral mechanism mentioned above was not released in the majority of patients (e.g., no cells or tissues transgenic for human immunoregulators were used) [11, 20, 22].

All of these subjects could be addressed in suitable nonhuman primate models. However, first preliminary results of in vitro infection experiments using a very limited number of cell lines of 5 different primate species suggested nonhuman primate cells not to be permissive to PERV infection. Contrarily, recent data on a much broader experimental basis supplied evidence that apes, baboons, rhesus, and maybe cynomolgus monkeys are susceptible to PERV infection [15, 17]. In the light of these new findings, appropriate in vivo models simulating whole-organ xenotransplantation in nonhuman primates should be applied for analysis of potential PERV in vivo infection.

Therefore, we developed a solid-organ discordant kidney xenotransplantation model with special emphasis on long-term survival of the organ recipient after graft removal. In our transplantation model, PERV transmission may be supported by application of a profound pharmacological immunosuppression. Furthermore, the immediate lysis of PERV particles in the recipients' sera is affected by adsorption of natural anti-Gal antibodies to the porcine kidneys, which has been described by other investigators as well [35]. In all monkeys, transplantation of the xenografts resulted in a profound decrease of antiporcine antibody titer for several days. One could speculate that, due to the initial decrease in anti-porcine antibody titer, the viriolysis of PERV particles by activation of the classic complement pathway would be reduced substantially.

Unfortunately, classic animal models in solid-organ xenotransplantation (creating a life-supporting situation) are associated with the inherent problem of xenomicrochimerism leading to false positive PERV detec-

tion by PCR. We were able to confirm the presence of chimeric porcine cells (or at least porcine DNA fragments) in our life-supporting model: PCRs specific to pig mitochondrial DNA showed positive results in all tissue samples of the animals in group B. In correlation with the results obtained with pig mitochondrial gene-specific primers, most of the tissues also showed positive results with the PERV pol-specific primers, probably on account of chimeric cells. In contrast, all tissue samples showed negative results using β-globin-specific primers, presumably due to the lower sensitivity of the β-globin-specific PCR versus pig mitochondrial DNA and PERV pol-specific PCR (Table 1). In the case of PBL samples, the frequency of chimeric cells was hardly enough to be detected, even when using the pig mitochondrial gene-specific primers: only few of the PBL samples taken before graft removal were weakly positive. It is likely that the very infrequent chimeric cells in the circulation get enriched in the microvascularized tissues due to trapping by cellular adhesion.

To solve the problem of microchimerism, we have established a non-life-supporting "piggy-bag" xeno-kidney transplantation model in the cynomolgus monkey. Using this setting, all recipient animals had at least a 2-h exposure to the porcine kidney; the longest exposure was 15 days. None of the tissue and cell samples analyzed more than 14 days after xenograft removal showed any evidence of chimeric porcine cells possibly interfering with PERV analysis.

All animals studied, including one recipient investigated as long as 250 days after transplantation, remained negative for PERV sequences in their peripheral blood lymphocytes and the analyzed tissue homogenates.

In order to get additional evidence for the absence of a productive PERV infection, we tried to identify PERV RNA from retroviral particles in the recipients' sera. Our RT-PCR assay was sensitive enough to detect PERV RNA in sera of a variety of pigs. In order to analyze whether amplification of PERV cDNA could be due to persisting porcine cellular RNA in the sera and not due to retroviral particles, we performed pig mitochondrial gene-specific RT-PCRs of the porcine sera, which did not result in amplification of pig-specific cDNA. However, further investigations are necessary to clarify whether the detected PERV pol RNA indeed derived from PERV particles or from destructed porcine cells.

In contrast to the tested pig sera, it was not possible to detect PERV RNA in the sera of the transplant monkeys. For internal positive control, we spiked the monkey sera with a fixed amount of PK15 culture supernatant. PERV RNA was found in all these spiked samples.

In conclusion, no evidence of PERV infection could be found either in the tested cynomolgus leukocytes and tissues or in the tested sera. Although Long et al. have recently reported PERV transmission to cynomolgus cells, it is currently controversial whether this species is susceptible to PERV infection [15]. Therefore, the absence of PERV infection in the animals with transplants might be due to a missing permissivity of cynomolgus cells to PERV infection. However, independent of the question of whether cynomolgus monkeys are susceptible to PERV infection, our non-life-supporting kidney transplantation model should be useful for further PERV in vivo infection studies with other more suitable primate species, e.g., baboons.

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