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Identification of Exogenous Forms of Human-Tropic Porcine Endogenous Retrovirus in Miniature Swine

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The replication of porcine endogenous retrovirus subgroup A (PERV-A) and PERV-B in certain human cell lines indicates that PERV may pose an infectious risk in clinical xenotransplantation. We have previously reported that human-tropic PERVs isolated from infected human cells following cocultivation with miniature swine peripheral blood mononuclear cells (PBMC) are recombinants of PERV-A with PERV-C. Here, we report that these recombinants are exogenous viruses in miniature swine; i.e., they are not present in the germ line DNA. These viruses were invariably present in miniature swine that transmitted PERV to human cells and were also identified in some miniature swine that lacked this ability. These data, together with the demonstration of the absence of both replication-competent PERV-A and recombinant PERV-A/C loci in the genome of miniature swine (L. Scobie, S. Taylor, J. C. Wood, K. M. Suling, G. Quinn, C. Patience, H.-J. Schuurman, and D. E. Onions, J. Virol. 78:2502–2509, 2004), indicate that exogenous PERV is the principal source of human-tropic virus in these animals. Interestingly, strong expression of PERV-C in PBMC correlated with an ability of the PBMC to transmit PERV-A/C recombinants in vitro, indicating that PERV-C may be an important factor affecting the production of human-tropic PERV. In light of these observations, the safety of clinical xenotransplantation from miniature swine will be most enhanced by the utilization of source animals that do not transmit PERV to either human or porcine cells. Such animals were identified within the miniature swine herd and may further enhance the safety of clinical xenotransplantation.

Xenotransplantation from swine has been proposed to alleviate the shortage of human donor organs for allotransplantation (28). Transmission of pig-derived infections to xenograft recipients and to the community at large has been raised as a potential risk of xenotransplantation. Many potential infectious agents can be prospectively excluded from herds of donor animals (specific-pathogen-free pigs) to improve the safety of clinical xenotransplantation beyond that normally achieved in clinical allotransplantation (11). Although significant microbiological advantages can be gained with the use of specific-pathogen-free pigs, porcine endogenous retrovirus (PERV) represents a unique concern associated with the transplantation of pig cells, tissues, or organs (2). While there is no evidence of PERV transmission to humans exposed to living porcine tissues (6, 8, 12, 13, 22, 23, 25), concerns about this safety aspect persist (36).

Infectious PERVs are limited to three subgroups of PERV that have been identified in the genomic DNA of pigs (9, 16, 24). Two of these, PERV-A and -B, can infect human and pig cells in vitro (31). The third subgroup, PERV-C, is ecotropic and infects porcine cells only (31). PERV-A and PERV-B isolates obtained from porcine cell lines possess significantly greater replication competence than those isolated from primary pig cells (17, 20). Because PERV-B has never been isolated in human cell transmission assays with primary pig cells as the source of PERV, most microbiological risk has been ascribed to PERV-A.

Previously, we have conducted in vitro PERV transmission studies using a herd of miniature swine that are inbred at the swine leukocyte antigen locus, the porcine equivalent of the major histocompatibility complex (21). Within this herd we identified animals from which peripheral blood mononuclear cells (PBMC) either do or do not transmit PERV to human cells in vitro. Such animals were termed either transmitters or nontransmitters as determined by their transmission phenotype for human cells. All of the pig-derived PBMC transmitted PERV-C to porcine cells. In all instances, human-tropic PERVs isolated from the PBMC of transmitting animals were recombinants between PERV-A and PERV-C sequences. Although the site of recombination varied, viral sequences were derived from the recombination of PERV-A elements with the post-VRA (envelope) region of PERV-C (21, 37). Accordingly, although PERV-C is not capable of infecting human cells, it appears to be an essential component of human-tropic PERV from these swine. Therefore, PERV-C may be an im-

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portant factor in the assessment of infectious risk associated with xenotransplantation.

In this study, we extend previous investigations to report the incidence of PERV transmission in litters of pigs derived from the mating of animals with known PERV transmission phenotypes in vitro. We demonstrate that human-tropic PERVs are not a product of in vitro recombination but rather that PERV-A/C recombinants exist in vivo as exogenous viruses. These studies also identified a group of miniature swine that do not carry PERV that infects either human or pig cells; these animals are referred to has having a PERV-null transmission phenotype.

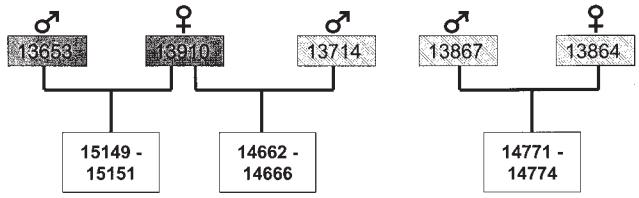

FIG. 1. Schematic showing the matings that were initiated to investigate the incidence of PERV transmission in vitro. Human-tropic transmitting animals are indicated with dark fill; nontransmitting animals are indicated with light fill. Note all parental animals produced PERV that infected pig cells in vitro. The transmission phenotypes of the offspring animals are not indicated but are presented in Tables 1 to 3.

MATERIALS AND METHODS

Miniature swine. Details of the derivation of the herd of inbred MGH major histocompatibility complex-defined miniature swine from two founder animals have been described previously (27). The ongoing inbreeding program focuses on increasing the coefficient of inbreeding and maintaining distinct swine leukocyte antigen haplotypes within the herd. Care of animals was in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Isolation of PBMC. PBMC were isolated from approximately 25 ml of heparinized whole blood by centrifugation over lymphocyte separation medium (ICN Biomedicals, Aurora, Ohio). Prior to coculture with target cells, the PBMC were stimulated for 5 days in 20 to 30 ml of Aim-V medium supplemented with 20% fetal bovine serum, 2.5 μg of phytohemagglutinin-P (PHA-P) per ml, 1 ng of phorbol 12-myristate-13-acetate per ml, 20 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. When appropriate, PHA-P and phorbol 12-myristate-13-acetate were replaced by either tissue plasminogen activator (4 μM), bromodeoxyuridine (1.5 μM), iododeoxyuridine (1.5 μM), or 5-aza-cytidine (6 μM) for the duration of the stimulation.

Cell lines. The human 293 (kidney epithelium) and porcine ST-IOWA cell lines were obtained from the American Type Culture Collection and maintained in culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and glutamine, penicillin, and streptomycin at the concentrations given above).

In vitro transmission assays. PBMC transmission assays followed published methodologies (21, 37). Briefly, approximately 10^8 mitogen-stimulated PBMC and the associated stimulation medium were cocultured with subconfluent target cells in a 75-cm^2 flask. The PBMC were kept in contact with the target cells for 4 to 5 days, after which the culture medium and PBMC were removed and the target cell cocultures were maintained by subculturing as necessary. PERV infection of target cells was determined by the presence of reverse transcriptase (RT) activity in the culture supernatants and was assayed on a weekly basis by using an indirect enzyme-linked immunosorbent assay-based system optimized for the detection of PERV RT according to the manufacturer's protocol (HS-kit Mn^{2+} RT kit; Cavidi Tech AB, Uppsala, Sweden). Transmission assays were maintained for a minimum of 60 days before being considered negative.

RT-PCR and sequence analysis. RNA was extracted from cell lines and PBMC by using Trizol (Invitrogen Life Technologies, Baltimore, Md.) according to the manufacturer's instructions. Single step RT-PCRs were performed on 0.3 to 0.7 μg of total RNA by using the OneStep RT-PCR kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. The PCR cycling conditions for PERV-A/C recombinant sequences consisted of 30 min at 50°C followed by 40 cycles of 96°C for 2 s, 55°C for 30 s, and 72°C for 2 min. Two sets of primer pairs were used to detect PERV-A/C sequences: primer pair 1, sense 5′-CCTACCA GTTATAATCAATTTAATTATGGC-3′ (PERVA-VRBF) and antisense 5′-C TCAAACCACCCTTGAGTAGTTTCC-3′ (PERVC-TMR); primer pair 2, sense 5′-ATGTCTGCCTTCGATCAGTAATCCC-3′ (PERVA-VRAF) and antisense PERVC-TMR. The combination of PERVA-VRAF and PERVC-TMR detects a wider range of recombinant PERV than the primer pair PERVA-VRBF and PERVC-TMR because of the different locations of the sense primers (VRA and VRB, respectively). The cycle conditions for porcine glyceraldehyde 3-phosphate dehydrogenase PCR were the same except that 20 amplification cycles were performed with the primers pGAPDH sense (5′-CGTCAAGCTCA TTTCCTGGTACG-3′) and pGAPDH antisense (5′-GGGGTCTGGGATGGA AACTGGAAG-3′). All PCRs were performed with a 2400 thermocycler (Perkin-Elmer BioSciences, Atlanta, Ga.). DNA sequencing was performed with a CEQ 2000 XL instrument (Beckman Instruments, Palatine, Fla.) and associated Dye Terminator cycle sequencing with quick-start kit (Beckman Instruments) according to the manufacturer's instructions.

Detection of PERV RNA expression by tyramide fluorescence in situ hybridization. PBMCs (10^6 cells) were isolated from miniature swine blood, washed once with phosphate-buffered saline, spread on Superfrost Plus glass slides (Fisher Scientific, Atlanta, Ga.), air dried, and stored at −70°C until use. Probes for PERV-C were prepared from cloned PERV-C plasmid by using primers PERV-CF (5′-CTGACCTGGATTAGAACTGGAAG-3′) and PERV-CR (5′-T ATGTTAGAGGATGGTCCTGGTC-3′) to amplify an envelope fragment that was then nick translated in the presence of biotin (kit from Perkin-Elmer, Boston, Mass.). Probes were precipitated in the presence of 0.4 μg of cot-1 DNA, 0.20 μg of yeast tRNA, and 0.6 μg of salmon sperm DNA per μl. The probe cocktail was prepared in Hybridsol VII (Ventana Medical Systems, Tucson, Ariz.) (35).

Detection of nascent viral RNA transcripts was performed on cells without prior denaturation of nucleic acids (15). Cells were permeabilized with a cytoskeleton solution (10) for 3 min at 4°C, fixed with 4% paraformaldehyde–1 mM $MgCl_2$ (pH 7.2) for 10 min, dehydrated in ethanol, and hybridized with denatured probes. After overnight incubation at 37°C, slides were washed three times for 10 min in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), followed by one 30-min wash in 1× SSC, all at 37°C. The slides were then transferred to room temperature washes of 1× SSC–3% H_2O_2 for 15 min and 1× SSC for 15 min and then equilibrated in 4× SSC. Slides were then incubated with a 1:100 dilution of streptavidin-horseradish peroxidase conjugate in 4× SSC–1% casein at 37°C for 1 h and washed. Signals were revealed with a tyramide-fluorophor (1:100) in the amplification diluent (NEN, Boston, Mass.). Cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (0.05 mg/ml; Sigma) and mounted in Antifade mounting fluid (Molecular Probes). Slides were viewed with an Olympus AX70 microscope equipped with a Speicher filter set (Chroma, Brattleboro, Vt.). Photographs were taken with a Zeiss Axiocam and Zeiss software. Quantification was conducted on at least 200 cells per slide.

RESULTS

In vitro PERV transmission characteristics of families of miniature swine. The in vitro PERV transmission characteristics of miniature swine families were investigated over extended time periods by using coculture studies with human (293) and porcine (ST-IOWA) target cells. The PERV transmission phenotypes of animals were categorized as follows: (i) transmitting, i.e., transmits PERV to human and porcine cells; (ii) nontransmitting, i.e., does not transmit PERV to human cells but does transmit PERV to porcine cells; and (iii) PERV-null, i.e., does not transmit PERV to porcine or human cells. The families that were investigated were derived from three matings of miniature swine with known transmission phenotypes: (i) transmitter × transmitter, (ii) transmitter × nontransmitter, and (iii) nontransmitter × nontransmitter (Fig. 1).

Following the mating of two nontransmitting animals (ani-

TABLE 1. Inheritance of PERV transmission phenotypes within the nontransmitter × nontransmitter miniature swine family

Pig no.[a]	Target cell	Day of positive transmission result[b] for animal at the following age (mo)								
		5	13	16	20	22	28	36	39	43
13867	Pig					17	13	14	18	14
	Human					—	—	—	—	—
13864	Pig					17	13			
	Human					—	—			
14771	Pig	13	14	18	14					
	Human	—	—	—	—					
14772	Pig	13	14	18	14					
	Human	—	—	—	—					
14773	Pig	13	14	18	14					
	Human	—	—	—	—					
14774	Pig	13	14	18	14					
	Human	—	—	—	—					

[a] Boldface numbers indicates parental animals.
[b] Transmission phenotypes for pig and human cells are reported. Blank fields indicate that the animal was not tested at that age. —, no transmission was detected. Positive transmission results are reported as the day after coculture that positive RT results were first obtained.

mals 13867 and 13864), four offspring were born (animals 14771 to 14774). The transmission characteristics of these animals were analyzed over a 15-month period, between 5 and 20 months of age. All four offspring, as well as the parental animals, possessed a nontransmitting phenotype for the duration of the monitoring period (Table 1). Following the mating of two transmitting animals (animals 13653 and 13910), three offspring were born (animals 15149 to 15151) (Table 2). These animals were analyzed over a 6-month period, between 3 and 9 months of age. Two of the animals (15149 and 15150) were transmitting animals, and the third (15151) proved to be a nontransmitting animal. The mating of a nontransmitting (13714) with a transmitting (13910) miniature swine resulted in

TABLE 2. Inheritance of PERV transmission phenotypes within the transmitter × transmitter miniature swine family

Pig no.[a]	Target cell	Day of positive transmission result[b] for animal at the following age (mo)																	
		3	5	9	19	21	26	28	30	32	34	36	37	39	40	41	43	47
13653	Pig															21	11	13
	Human															21	17	27
13910	Pig				20	11			14	14	21	13			14			
	Human				20	11			14	14	30	26			42			
15149	Pig	21	11	13														
	Human	—	30	48														
15150	Pig	21	11	13														
	Human	21	11	41														
15151	Pig	21	17	13														
	Human	—	—	—														

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.

five offspring (animals 14662 to 14666), which were analyzed over a 21-month period, between 3 and 24 months of age (Table 3). One animal (14665) was a transmitter, and the remaining animals (14662 to 14664 and 14666) did not infect either human or pig cells. These animals represented a novel transmission phenotype not previously identified in miniature swine, i.e., the PERV-null phenotype. While extended analysis of the PERV transmission phenotype of animals 14663 and 14664 was not possible due to death of the animals, animal 14666 retained its PERV-null phenotype over at least an 18-month period.

It is notable that the transmission phenotypes of the miniature swine typically remained constant over extended time periods. Interestingly, however, both exceptions to this observation involve the conversion of young nontransmitting animals to the transmitter phenotype (animal 15149 [conversion between 3 and 5 months] [Table 2] and animal 14665 [conversion between 3 and 14 months] [Table 3]).

Identification of exogenous human-tropic recombinant PERV. Human-tropic PERVs isolated from miniature swine by using in vitro transmission assays are PERV-A/C recombinants with recombination within the *env* region (21, 37). It was thought that these viruses were formed during the in vitro coculture because (i) these recombinant viruses were not detected in the genomes of miniature swine PBMC by DNA PCR and (ii) PERV-C, a virus that has no tropism for human cells, was transiently detected in the human 293 target cells and was therefore available for recombination events. We evaluated this assumption by developing an RT-PCR assay specific for the detection of PERV-A/C recombinant viruses, using primers that spanned the region in which the PERV-A/C recombinations occurred. Using this assay, PERV-A/C recombinants were detected in unstimulated PBMC of transmitting miniature swine by RT-PCR (Fig. 2A), as well as in the associated 293 cell transmission assay according to DNA PCR and RT-PCR (Fig. 2A). In contrast, PERV-A/C recombinants were not detected in genomic DNAs of PBMC from the same transmitting animals when tested by DNA PCR. The identification of PERV-A/C recombinants in vivo raised the possibility that these viruses were endogenous. However, plasmid-spiking experiments (Fig. 2B) indicated that the sensitivity of the PERV-A/C DNA PCR is less than 0.5 copies per cell equivalent, i.e., is of sufficient sensitivity to detect a single endogenous provirus. Therefore, these results indicate that the PERV-A/C recombinants are not endogenous.

Additional RT-PCR analysis was performed to determine the distribution of the PERV-A/C recombinants in miniature swine (Table 4). PERV-A/C recombinants were identified in the RNAs of PBMC from 20 out of 24 transmitting miniature swine. In each of the four negative cases, the associated PERV-infected 293 cell transmission assay tested positive for PERV-A/C recombinants by DNA PCR, indicating that the negative direct analysis of the PBMC from these animals was most likely due to the copy numbers of the PERV-A/C recombinants in vivo being below that detectable by the assay. PBMC from nontransmitting animals did not contain detectable levels of the PERV-A/C recombinants (data not shown). However, to enhance the sensitivity of the PERV-A/C RT-PCR assays, a second primer pair was developed (pair 2 [see Materials and Methods]). Using this primer pair, we were able to detect

TABLE 3. Inheritance of PERV transmission phenotypes within the transmitter × nontransmitter miniature swine family

Pig no.[a]	Target cell	Day of positive transmission result[b] for animal at the following age (mo)																	
		3	11	12	13	14	16	18	20	21	27	32	34	36	38	40	42	43	46
13714	Pig		18	18	19		22	14	13		11			14	14	21	17	20	14
	Human		—	—	—		—	—	—		—			—	—	—	—	—	—
13910	Pig							20		11	14	14	21	13	20	14			
	Human							20		11	14	14	30	26	20	22			
14662	Pig	—		—															
	Human	—																	
14663	Pig	—																	
	Human	—																	
14664	Pig	—																	
	Human	—																	
14665	Pig	32				14	21	11	20	14									
	Human	—				21	21	11	20	42									
14666	Pig	—		—		—	—	—	—	—									
	Human	—		—		—	—	—	—	—									

[a] See Table 1, footnote *a*.
[b] See Table 1, footnote *b*.

PERV-A/C RNA in approximately 50% of nontransmitting animals (Table 4). These results indicate that PERV-A/C recombinants can be detected in all transmitting miniature swine as well as in some nontransmitting animals.

PERV expression in miniature swine PBMC. RT-PCR analysis has shown that basal levels of PERV RNA expression are low in primary cells from swine but can be increased by mitogenic and immunological stimuli in vitro and in vivo (33, 37).

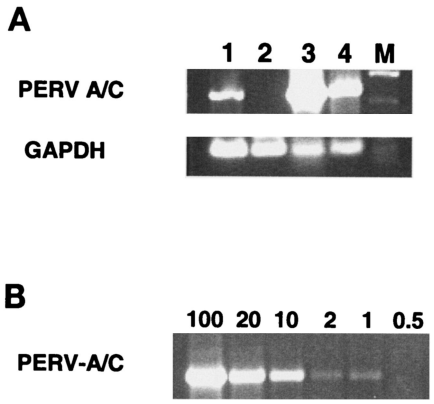

FIG. 2. PERV-A/C recombinants can be identified in the PBMC of miniature swine and are transmitted to 293 cells. (A) PCR and RT-PCR assays specific for the detection of PERV-A/C recombinant sequences were performed on miniature swine PBMC and PERV-infected 293 cells with primer pair 1 (see Materials and Methods). Representative samples from a transmitting animal and the associated in vitro 293 cell transmission assay are presented. Lanes: 1, PBMC RT-PCR; 2, PBMC PCR; 3, 293 cell RT-PCR; 4, 293 cell PCR; M, marker. (B) Determination of the sensitivity of the PERV-A/C PCR, indicating that PERV-A/C recombinant viruses are not endogenous. PERV-A/C plasmids were spiked into approximately 5,000 cell equivalents (100 ng) of porcine DNA at the copy number per cell equivalent indicated.

While informative, these analyses do not produce data pertaining to the expression of PERV in individual cells. Because the infectious phenotype of an animal may be dictated by a minor population of cells that contain transcriptionally active, perhaps exogenous, PERV, we addressed this possibility by using a fluorescence in situ hybridization-based assay (34) and probing the unstimulated PBMC of three transmitting animals and three nontransmitting animals for PERV expression in individual cells. Strong expression of PERV-C was detected in approximately 1% of the PBMC of the three transmitting animals (Fig. 3). In contrast, expression of PERV-C was not detected in three nontransmitting animals tested (Fig. 3). Two of the three nontransmitting animals (14771 and 14774) were positive for PERV-A/C sequences according to RT-PCR (data not shown). These results indicate that a correlation between the ability to isolate PERV-A/C recombinants in vitro with strong expression of PERV-C may also exist.

Identification of PERV-null animals within the miniature swine herd. Having identified an animal (14666) that repeatedly displayed a PERV-null phenotype, additional transmission assays were performed on other animals that had been identified previously as having the PERV-null transmission phenotype (approximately 4% of the animals tested). As shown in Table 5, a number of these animals retained a PERV-null phenotype over, in some instances, up to a 3-year period.

TABLE 4. Distribution of PERV-A/C recombinants among miniature swine[a]

In vitro transmission phenotype	No. tested	No. PERV-A/C positive	No. PERV-A/C negative
Transmitter	24	20	4
Nontransmitter	14	7	7

[a] PBMC were isolated from miniature swine with known 293-cell transmission phenotypes and tested by RT-PCR for the presence of PERV-A/C recombinants.

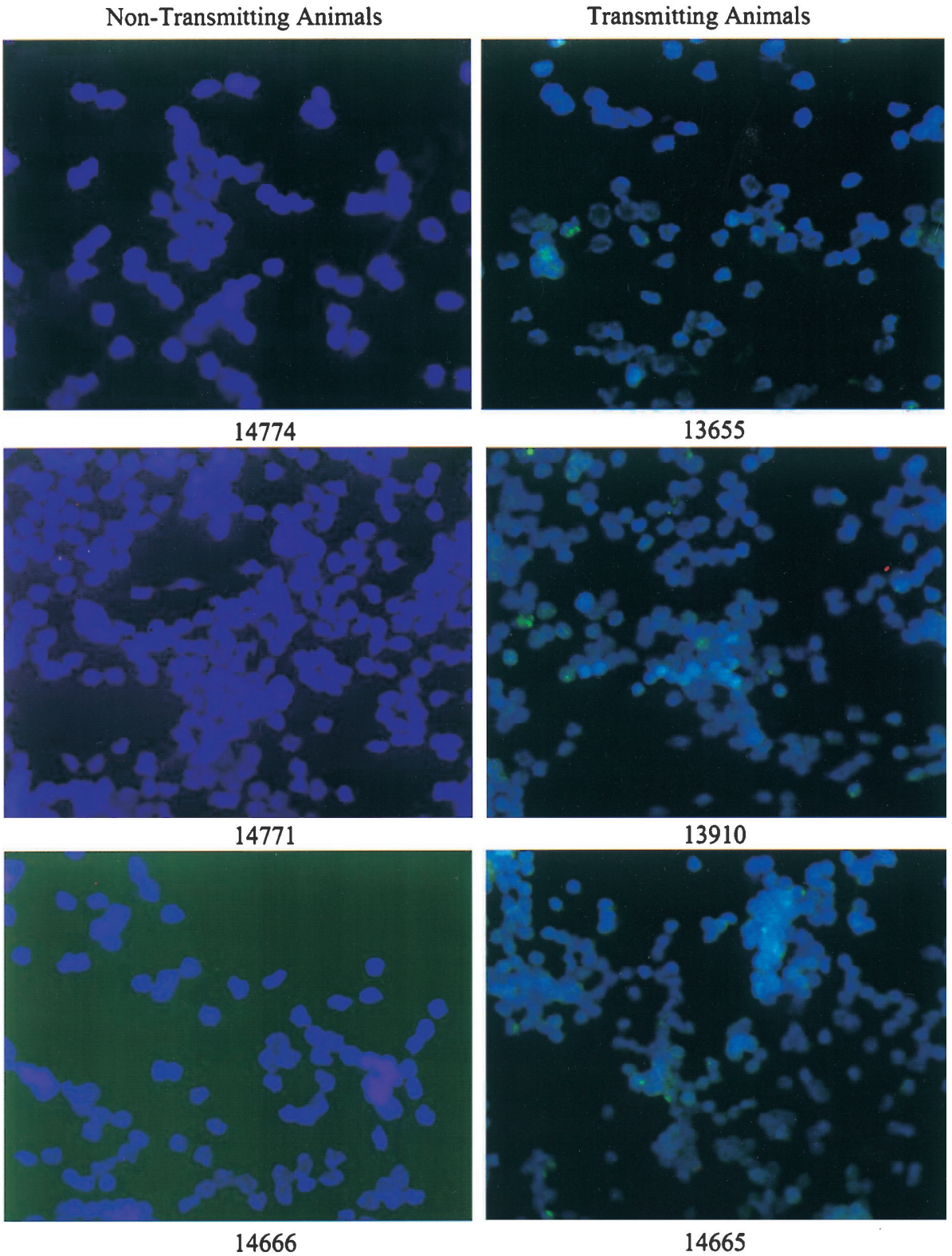

FIG. 3. Fluorescence in situ hybridization for PERV-C RNA expression. Strong expression of PERV-C can be detected in a minority of PBMC from transmitting, but not nontransmitting, miniature swine. Miniature swine PBMC were analyzed by in situ hybridization for PERV RNA expression with a probe for PERV-C envelope sequences.

TABLE 5. Identification of PERV-null miniature swine

Pig no.	Target cell	Transmission result[a] for animal at the following age (mo)																
		4	10	11	12	15	17	19	20	23	33	41	45	51	53	59	81	87
12190	Pig													—	—	—	—	—
	Human													—	—	—	—	—
13752	Pig		—	—	—	—						—	—					
	Human		—	—	—	—						—	—					
14335	Pig		—	—							—							
	Human		—	—							—							
14666	Pig	—			—	—		—	—	—								
	Human	—			—	—		—	—	—								

[a] See Table 1, footnote *b*.

Additional transmission assays were performed with a variety of agents that can stimulate ERV expression (bromodeoxyuridine, iododeoxyuridine, and 5-aza-cytidine), as an alternative to PHA. These agents were unable to induce PERV production in cells from PERV-null animals (data not shown). Detailed analyses of transmission assays associated with PERV-null animals indicated that the inability to infect porcine cells was not due to failed mitogen stimulation, as, based on cell counts, the PBMC proliferated at a rate comparable to that for transmitting animals analyzed within the same transmission assay (data not shown). However, comparison of the relative amounts of PERV released from the PBMC indicated that while transmitting and nontransmitting animals were comparable in their production of PERV, in contrast, virus production from PERV-null animals was nearly undetectable and was indistinguishable from the background RT activity found associated with uninfected pig cells (ST-IOWA) (Fig. 4). Taken together, the results suggest that the PERV-null phenotype is due to low or absent virus production from the PERV-null miniature swine cells.

DISCUSSION

Despite encouraging indications of safety from clinical trials of porcine xenotransplantation, porcine endogenous retrovirus that is infectious for human cells remains a major theoretical complication for clinical xenotransplantation. Molecular mapping studies have demonstrated the existence of three subgroups of PERV proviruses in the genomic DNA of pigs and pig cells (5, 14, 18, 20, 26). In vitro transmission assays have demonstrated an ability of primary cells from some pigs to transmit PERV to certain human target cell lines (19, 21, 37). For inbred miniature swine it has been shown that these viruses are recombinants between PERV-A and PERV-C. We hypothesized that these viruses were a manifestation of in vitro assay systems. In the present study, we provide evidence that these PERV-A/C recombinants are exogenous viruses in the PBMC of transmitting and some nontransmitting miniature swine. These observations suggest that the assessment of the infectious risks of clinical xenotransplantation should include the impact of exogenous retroviruses rather than focus solely on endogenous loci.

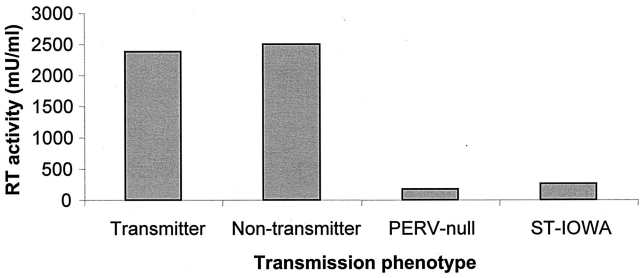

FIG. 4. PERV-null animals produce significantly less virus that transmitting and nontransmitting miniature swine. PBMC were stimulated with PHA for 72 h, at which point the supernatant was tested for RT activity and the PBMC were used in transmission assays.

The conclusion that PERV-A/C recombinants exist as exogenous viruses is based on a number of independent observations. First, recombinant PERV-A/C sequences were detectable in miniature swine PBMC RNA. Second, genomic DNA PCR (this paper) and mapping studies (28a) of transmitting and nontransmitting animals did not detect the presence of endogenous PERV-A/C recombinant loci. While these results are consistent with PERV-A/C sequences being exogenous, they do not unequivocally prove that these sequences are components of replication-competent PERV. However, because (i) all transmitting miniature swine possessed these recombinant sequences and (ii) cloned PERV-A/C recombinant envelope sequences all possess full-length open reading frames (21), we conclude that they are likely to reflect the presence of replication-competent PERV.

As discussed above, in vitro transmission analysis has been a useful technique for the assessment of the transmission phenotypes of pigs (21). However, two observations reported in this study suggest that although transmission assays are sensitive methods for the detection of replication-competent virus, they may not have the sensitivity needed to detect the presence of low levels of PERV-A/C recombinants. For example, the presence of PERV-A/C recombinants in the PBMC of 7 of 14 nontransmitting animals suggests that these sequences may be more widely distributed than transmission assays have indicated. The possible conversion with age of nontransmitting miniature swine into transmitting animals also supports this conclusion. Therefore, while it is possible that PERV-A/C recombinants are ubiquitous within the herd, the observation

that certain miniature swine retain a negative transmission phenotype suggests that some animals are free of PERV-A/C recombinants. This hypothesis merits further exploration such as the use of techniques with greater sensitivities, e.g., sequence-capture real-time PCR (29). We conclude that molecular analyses can be used to determine whether miniature swine have the potential to transmit human-tropic replication-competent PERV in vitro at a sensitivity that exceeds that achieved by in vitro transmission assays.

From the perspective of the safety of clinical xenotransplantation, it is important to determine the mechanism by which human-tropic PERV-A/C recombinant viruses are generated. Several possibilities exist, based on analogy to other retroviruses. PERV-A/C recombinants may exist as exogenous viruses that are transmitted congenitally and/or horizontally between animals. Alternatively, PERV-A/C recombinants might be generated de novo. Mechanisms for production of PERV-A/C in vivo might include recombination between two endogenous PERV loci that are independently replication defective (3, 4), recombination of a replication-competent endogenous virus with a defective endogenous locus, or the recombination of defective endogenous loci with replication-competent exogenous PERV. Support for the latter mechanism can be taken from analogy to the formation of mink cell focus-forming viruses in mice (7) as well as from the exogenous B subgroup of feline leukemia virus (FeLV-B) (1, 30, 32). Xenotropic FeLV-B is generated via the recombination of exogenous ecotropic virus (FeLV-A) with defective endogenous FeLV-related sequences, and as a result, FeLV-B is only found associated with cats infected with FeLV-A. By analogy, infectious recombinant human-tropic PERV-A/C loci have also not been identified in the genome of miniature swine, and human-tropic PERV has never been identified in miniature swine that lack ecotropic PERV-C. Moreover, because we (28a) did not identify PERV-A loci in primary miniature swine cells that possess replication competence, the most likely mechanism for the generation of PERV-A/C recombinants would be the recombination of a defective endogenous PERV-A locus with replication-competent PERV-C (either exogenous or endogenous). This conclusion is also supported by the studies of Niebert et al. (20), who reported that the PERV-A proviruses present in the genomic DNA of Large White pigs possess only minimal replication competence. Accordingly, if this recombinatorial mechanism proves to be correct, the identification of animals that do not produce infectious PERV-C should greatly reduce the rate of formation of human-tropic recombinant PERV in vivo. The PERV-null animals identified within the miniature swine herd might represent such animals.

Miniature swine carry a higher copy number of germ line PERV-C elements than some other breeds of pig, e.g., Large White, without any apparent health consequences. Prior to the identification of PERV-null animals, complex breeding programs or knockout approaches would have been needed to remove replication-competent PERV-C loci. Because many of these loci are likely to be homozygous as a result of the highly inbred nature of the miniature swine herd, the identification of animals that do not carry replication-competent PERV-C is suggestive of either a low number of replication competent PERV-C loci in the germ line or the existence of variable amounts of exogenously acquired PERV-C. In this regard, while our results demonstrate that the PERV-null phenotype can remain stable over extended periods (up to at least 3 years), it will prove interesting to determine whether it is inherited in a Mendelian manner or whether it is subject to exogenous influences.

In summary, the safety concerns for clinical xenotransplantation associated with PERV have been based on the assumption that replication-competent human-tropic PERV loci are present in the germ line DNA of pigs. The present study indicates that human-tropic PERV recombinants exist as exogenous agents in miniature swine. Thus, recombination is a critical factor in the generation of human-tropic PERV. Further studies of the molecular mechanisms governing this phenomenon will enhance the safety of clinical xenotransplantation from miniature swine.

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