Role of DNA Methylation in Expression and Transmission of Porcine Endogenous Retroviruses

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Porcine endogenous retroviruses (PERV) represent a major safety concern in pig-to-human xenotransplantation. To date, no PERV infection of a xenograft recipient has been recorded; however, PERVs are transmissible to human cells *in vitro*. Some recombinants of the A and C PERV subgroups are particularly efficient in infection and replication in human cells. Transcription of PERVs has been described in most pig cells, but their sequence and insertion polymorphism in the pig genome impede identification of transcriptionally active or silenced proviral copies. Furthermore, little is known about the epigenetic regulation of PERV transcription. Here, we report on the transcriptional suppression of PERV by DNA methylation *in vitro* and describe heavy methylation in the majority of PERV 5' long terminal repeats (LTR) in porcine tissues. In contrast, we have detected sparsely methylated or nonmethylated proviruses in the porcine PK15 cells, which express human cell-tropic PERVs. We also demonstrate the resistance of PERV DNA methylation to inhibitors of methylation and deacetylation. Finally, we show that the high permissiveness of various human cell lines to PERV infection coincides with the inability to efficiently silence the PERV proviruses by 5' LTR methylation. In conclusion, we suggest that DNA methylation is involved in PERV regulation, and that only a minor fraction of proviruses are responsible for the PERV RNA expression and porcine cell infectivity.

Pig-to-human xenotransplantation has been preclinically tested with the aim of alleviating the shortage of human donor organs (1, 2). Together with physiological incompatibilities and the immunological rejection of xenograft, there is also a risk of zoonotic transmission of porcine pathogens (3–5). In particular, the porcine endogenous retrovirus (PERV) cannot be eliminated due to its presence in the pig genome (6, 7). The abundance of γ 1-PERVs (6) prevents selection of PERV-free animals, and human-tropic subgroups PERV-A and PERV-B are known to infect human cells *in vitro* (8–10). The subgroup PERV-C is ecotropic but often recombines with PERV-A, and certain PERV-A/C copies are highly efficient in infection and replication in human cells (11–15).

Analysis of different porcine breeds and cell lines has revealed the presence of 30 to 60 γ 1 PERV proviral copies dispersed across the genome (8, 10, 15, 16). The total numbers of PERVs and the presence of particular proviral sequences vary among breeds and even individual animals within one breed (17). The recent draft of the pig whole-genome sequence (18) identified 20 almost intact PERV copies, none of which was found to be conserved in more than 120 pigs tested. This indicates an extraordinary rate of insertion polymorphisms and suggests that rare replication-competent proviruses exist in individual pigs or could easily arise by complementation and recombination of defective members. A full-length 8-kb mRNA has been documented in all PERV-producing cells (19), but the source of these transcripts remains unidentified.

Considering the diversity of sequence and insertion polymorphisms of PERV copies, the transcription of PERV mRNA may reflect the transmission capacity of pig cells. It is known that PERV transcription differs in individual pigs, tissues, or cell lines (20, 21). The expression of particular PERV copies has been shown to depend on the sequence of the long terminal repeat (LTR) (22–24) and the availability of various transcription factors (25–27). We,

however, assume that epigenetic control at the respective integration site is crucial for either provirus expression or silencing. DNA methylation at CpG dinucleotides is a hallmark of silenced retroviruses, both exogenous (28–30) and endogenous (31–34). Modifications of histone molecules associated with retroviral LTR are additional controllers of retroviral transcription or silencing (35–37). Despite the consensus that the DNA methylation of endogenous retroviruses is a general defense mechanism against genomic parasites (38), little is known about PERV DNA methylation (37, 39) in donor animals and xenografts.

Here, we investigate the role of DNA methylation in the regulation of PERV expression in detail. We checked the CpG methylation status of PERV 5' LTRs in porcine organs and in cell lines and correlated this with provirus expression at the level of transcription or virus production. The basic challenge in this PERV regulation study is the high variability of PERV sequences and integrations among individual pigs. To compensate for this and report the DNA methylation in at least the majority of provirus loci, we combined the quantitative and qualitative methylation analyses. Our results indicate that PERV LTR-driven transcription is sensi-

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tive to methylation, and most PERV proviruses in pig tissues are stably silenced by DNA methylation. A small fraction of PERV loci remain sparsely methylated or nonmethylated, and the extent of PERV expression partially correlates with the number of these hypomethylated PERV copies. We hypothesize that the epigenomic analysis of PERV loci is useful in identification of proviruses critical for PERV transmission and in screening for safer donors of xenografts.

MATERIALS AND METHODS

Cell cultures. PK15 and ST-IOWA cells were grown in F-12 and Dulbecco's modified Eagle medium (MEM-D; Gibco) mixed 1:1 and supplemented with 1% NaHCO₃, 10% fetal calf serum, a penicillin-streptomycin mix (100 µg/ml each), and 2.5 µg/ml amphotericin B. Human embryonic kidney 293T cells and TE671 rhabdomyosarcoma cells were maintained in MEM-D supplemented with 10% fetal bovine serum (BioSera). The treatment of ST-IOWA cells with 5-azacytidine (azaC) and trichostatin A (TSA) was carried out at 70% confluence. Respective concentrations of azaC and TSA (Sigma) were added, and cells were harvested for DNA analysis after 48 h.

Animals and organ samples. Samples of pig skin, pancreas, lung, heart, brain, kidney, ovary, liver, and muscle were taken from three animals, the testes were taken from another four animals, and blood together with skin samples were taken from 20 6-month-old pigs. The animals were from a Large White/Landrace/Goland commercial herd slaughtered in the Czech Republic. Small organ samples were taken immediately after slaughtering and frozen in liquid nitrogen. Samples of terminal pig placenta were obtained from two new-born experimental Göttingen-Minnesota miniature pigs bred at the Institute of Animal Physiology and Genetics, Liběchov, Czech Republic, and frozen on dry ice. Whole blood samples were collected into BD Vacutainer CPT tubes (BD), and mononuclear cells were separated according to the manufacturer's instructions for DNA preparation. The obtaining of animal samples was organized and performed in accordance with the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

Reporter plasmids and *in vitro* DNA methylation. Reporter vectors spLTR-A-luc and pLTR-B-luc were prepared by replacement of the SmaI-HindIII promoter region of the luciferase reporter vector pGL3-promoter (Promega, Madison, WI) with PERV-A 5'LTR or PERV-B 5'LTR, respectively. PERV-A provirus (EU789636.1) (40) was amplified using primers for3 (5'-AGTGTCTGAGAATTGCTTGGACC-3') and rev1 (5'-CAAATGCCTTCTGGTGTCTCA-3') from porcine chromosomal DNA and cloned into the pGEM-T Easy vector. PCR was performed with long-distance polymerase (Roche) according to the manufacturer's recommendations in 35 cycles of 95°C for 15 s, 61°C for 30 s, and 68°C for 8 s. The 5'LTR was excised by PvuI and HindIII, the PvuI site was blunted by T4 polymerase, and the resulting fragment was used to replace the SmaI-HindIII fragment of pGL3-promoter. The 5'LTR of subgroup B was excised by KpnI and HindIII from the A/B PERV recombinant PERV60 (AY099323) cloned in the pCR PERV60 plasmid (40). The KpnI site was blunted by T4 polymerase, and the resulting fragment was used for promoter replacement of pGL3-promoter. Reporter plasmid DNAs prepared at transfection quality using the Qiagen plasmid midi columns (Qiagen, Hilden, Germany) were *in vitro* methylated by SssI methylase (CpG methyltransferase; NEB). Incubation of 10 µg DNA was done in the recommended buffer containing 160 µM S-adenosyl-L-methionine for 2 h at 37°C. The efficiency of *in vitro* methylation was controlled by digestion of the methylated plasmid DNA by the methyl-sensitive restriction enzyme HpaII.

DNA transfection and reporter expression assays. 293T cells were grown on 35-mm petri dishes to 50 or 70% confluence, and 1 µg of methylated and mock-methylated reporter plasmid DNA was applied using the FuGene-6 lipofection reagent (Roche) at a DNA/reagent mixture ratio of 1:3 in OPTI-MEM (Invitrogen). An equal amount of plasmid pCMV-β-gal (Stratagene) bearing the cytomegalovirus promoter-driven

β-galactosidase (β-gal) was cotransfected with either pLTR-A-luc or pLTR-B-luc. OPTI-MEM was replaced with standard cultivation medium 24 h posttransfection. Cell lysates were prepared 48 h after transfection using lysis buffer containing 150 mM NaCl, 20 mM Tris, pH 7.5, 1% Triton X-100, and 2 mM EDTA. Luciferase activity was measured in the mix of 10 µl of lysate and 30 µl of luciferin solution (Promega) using a Promega DLReady luminometer with the Berthold detection system. To normalize the transfection efficiency, β-gal activity was measured in the mixture of 30 µl of cell lysate, 200 µl of 100 mM phosphate buffer, 3 µl of 100 mM magnesium buffer, 1 µl of β-mercaptoethanol, and 66 µl of 0.4% O-nitrophenyl β-D-galactopyranoside (Sigma). After 10 to 20 min of incubation at 37°C, the reaction was stopped by 500 µl of 1 M Na₂CO₃, and the β-gal activity was measured spectrophotometrically at a wavelength of 420 nm (Beckman DU 640).

MS-qPCR. DNA samples for analysis of CpG methylation were isolated by phenol-chloroform extraction. Sodium bisulfite treatment of genomic DNA was performed with either an EZ DNA methylation kit (ZymoResearch) or EpiTect bisulfite kit (Qiagen) according to the manufacturer's instructions. Fifty ng of converted DNA then was used for methyl-specific quantitative PCR (MS-qPCR) based on the Mesa Green qPCR MasterMix plus for SYBR assay kit (Eurogentec) and Chromo4 system (Bio-Rad) for real-time PCR detection. We separately quantified the total, hypomethylated, and hypermethylated PERVs together with the porcine ELF2 as a unique reference sequence. Primers for amplification of 5'LTRs and leader sequences were designed to be complementary to 16 different PERV LTRs, including 3 representatives from subgroup A, 10 from subgroup B, and 3 from subgroup C (accession numbers AJ279056.1, AY570980.1, AF435967.1, AJ133817.1, AF435966.1, AJ293656.1, AY099323.1, AJ279057.1, AJ293657.1, AJ133816.1, AJ133818.1, AY099324.1, HQ540593.1, AF038600.1, and AF038599.1). Primers for selective amplification of either hypomethylated or hypermethylated PERV sequences were designed with converted or nonconverted cytosines within CpG dinucleotides, respectively. Stringent discrimination between CpG and TpG dinucleotides was achieved by localization of the crucial complementary thymine or guanine to the very 3' end in the forward primer and adenine or cytosine in the reverse primer. Furthermore, all bases distinguishing the nonmethylated or methylated CpGs were on the locked nucleic acid backbone (LNA) to improve the specificity of primers (41). For quantification of hypomethylated 5'LTRs, the following primers were employed (LNA bases are in lower-case): quantBIS_n/F (5'-TATTtRGGGTtGTAGTTTTTATTTTTGt-3' and quantBIS_n/R 5'-AAAAACAACtACAAACCaAAAACaCa-3'); for total 5'LTRs, seqBIS/F (5'-GGTTTTGTGTGAATTTATAAAAGTTG TTT-3') and seqBIS/R (5'-AACACCTATAATCCTCCTCAACAC-3'); and for ELF2, qbisELF/F (5'-GAAGGGAGTTGGGATAAGGTGGAGTAA ATTT-3') and qbisELF/R (5'-CCCCAAATACCTCAATTCCCCTATA CCATA-3'). Reaction mixtures were then heated at 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 60°C for 3 min, and 72°C for 20 s. For quantification of hypermethylated 5'LTR, we used primers quantBIS_m/F (5'-TAT TcRGGGTcGTAGTTTTTTATTTTTGc-3') and quantBIS_m/R (5'-AAAA ACAACTACAAACGcAAAAACGc-3'), and the reaction mixture was heated at 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 63°C for 3 min, and 72°C for 20 s. The volume of the reaction mixture was 25 µl, with a 300 nM final concentration of each primer. PCRs were supplemented with 0.9 M betaine and 0.9% dimethyl sulfoxide (DMSO). Measurements were carried out in triplicates. The negative controls included water or converted DNA negative for the analyzed sequence as a template. The specificity of the amplified PCR products was confirmed by melting curve analysis and by sequencing the PCR products. The number of PERV copies per cell was obtained by normalization to the reference sequence using the following formula: $2^{-\Delta CT} = 2^{-(CT_{\text{sample}} - CT_{\text{reference}})}$ (42). The specificity for the hypomethylated LTRs was confirmed by sequencing the representative clones. 5'LTRs amplified from PK15 contained only 2.7% methylated CpG, which is 14 times less than the level with nonselective seqBIS primers (data not shown). The MS-qPCR technique with LNA primers is a modification of a previously published procedure (43).

Bisulfite sequencing. DNA samples were prepared and treated with sodium bisulfite as described for MS-qPCR. Fifty ng of converted DNA was amplified by OneTaq polymerase (New England BioLabs) according to the manufacturer's recommendations. The volume of the reaction mixture was 25 μ l, with a 400 nM final concentration of each primer. We used primers seqBIS/F (5'-GGTTTTGTTGTGAATTTTATAAAAGTTGTTT-3') and seqBIS/R (5'-AACAACTATAATCCTCCTAACAC-3') for amplification of total PERV 5'LTR. The reaction mixture was heated at 95°C for 5 min, subjected to 10 cycles of 95°C for 20 s and 57°C for 1 min with a decrease of 0.5°C per cycle, and 68°C for 30 s, and then 30 cycles of 95°C for 20 s, 52°C for 1 min, and 68°C for 30 s. For amplification of PERV 3a and PERV14/220 5'LTR, we performed seminested PCR with rTaq polymerase (TaKaRa). The supplied buffer was supplemented with 0.9 M betaine, 0.9% DMSO, and 3.4 mM MgCl₂. We used primers bis3a/F1 (5'-GTTGTTAGTAAATAGGTAGAAGGTT-3'), bis3a/F2 (5'-TTTGGATTTTGTAAATTTGATTGGT-3'), bis3a/R (5'-AAAAATCCCTTTACCTCCAATC-3'), bis14/220/F1 (5'-TAGGTAAGAAAGATTAGGTTTTTTGTTG-3'), bis14/220/F2 (5'-GGGAGTTTTTAATTGTTTGTAGT-3'), and bis14/220/R (5'-ACTAAAAACAAACACTCAAAACAA-3') under the following conditions: 10 cycles of heating at 95°C for 20 s and 57°C for 1 min (with a decrease of 0.5°C per cycle) followed by 72°C for 30 s, and then 30 cycles of 95°C for 20 s, 52°C for 1 min, and 72°C for 30 s. One μ l of the product was repeatedly amplified with the second forward primer and the same reverse primer.

In order to amplify the 5'LTR of pLG vector (44) based on murine leukemia virus (MLV), we performed seminested PCR with OneTaq DNA polymerase (New England BioLabs). We used forward bisMLV/F1 (5'-GTTAAATAGGATATTTGTGGTAAGT-3') and reverse bisMLV/R (5'-ATAATCCCTAAACAAAAATCTCCAAA-3') primers under the following conditions: 95°C for 5 min and then 10 cycles of 95°C for 50 s, 58°C for 2 min, and 68°C for 1.5 min, followed by 25 cycles of 95°C for 45 s, 54°C for 2 min, and 68°C for 1.5 min, with an increase of 2 s per cycle. One μ l of the product was repeatedly amplified with a second forward primer, bisMLV/F2 (5'-GGTGTTTTAAAGGATTTGAAATGATTT-3'), and the same reverse primer under the following conditions: 95°C for 5 min, 12 cycles of 95°C for 50 s, 58°C for 2 min, and 68°C for 1.5 min, and then 8 cycles of 95°C for 45 s, 54°C for 2 min, and 68°C for 1.5 min with an increase of 2 s per cycle.

The seminested PCR products were subsequently cloned using the pGEM-T Easy vector cloning system (Promega). Individual PCR clones were sequenced (GATC-Biotech) with the universal pUC/M13 reverse primer. Only PCR clones with at least 95% conversion of C nucleotides outside CpGs were taken into account. Bisulfite-converted sequences were compared to the nonconverted genomic consensus PERV sequence. The consensus sequence was compiled from the 16 aforementioned PERV sequences of all three subgroups. All CpG dinucleotides within the analyzed region from all PERV sequences were included. Results were analyzed using tools at <http://quma.cdb.riken.jp/>.

RNA extraction and qRT-PCR. Total RNA was extracted from cultured PK15, ST-IOWA, 293T, and TE671 cells or from pig organ samples using RNeasy (MRC) according to the manufacturer's protocol. The tissue samples were first homogenized with Tissue Ruptor (Qiagen). RNA samples were treated with DNase I (Roche) for 15 min in Moloney-MLV (M-MLV) reverse transcriptase buffer (Promega). One μ g of RNA was reverse transcribed into cDNA using random hexanucleotides and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol. Quantitative reverse transcription-PCR (qRT-PCR) of PERV transcripts was performed using Mesa Green qPCR MasterMix plus (Eurogentec) with primers pervRNA/F (5'-AGTCCTCTACCCCTGCGTG-3') and pervRNA/R (5'-CTCCAAGTCGGTTCTCGGGTGT-3'), complementary to 5'LTR and leader sequences invariant in the 16 aforementioned PERV sequences from all three subgroups. The volume of the reaction mixture was 20 μ l with a 300 nM final concentration of each primer. The cycling conditions were 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. We used water as a negative

control. The level of PERV transcript was normalized to the transcription of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene amplified with primers *GAPDH*/F (5'-CGTCAAGCTCATTTCC TGGTACG-3') and *GAPDH*/R (5'-GGGGTCTGGGATGGAACTGGA AG-3'). qRT-PCR of the *GAPDH* gene was performed similarly to qRT-PCR of PERV. Plasmid with PERV-14/220 was used as an external standard for PERV transcripts; thus, strictly speaking, the RT-PCR assay is semiquantitative. The external standards for *GAPDH* were constructed by PCR using ST-IOWA cDNA and transcript-specific primer sets. Resulting PCR fragments were cloned into pGEM-T Easy (Promega) and verified by sequencing. Calibration curves were prepared by PCR of diluted plasmid samples ranging from 10³ to 10⁸ copies per reaction. The calibration curves were linear within this range.

For quantification of PERV proviruses in PERV 14/220-infected 293T and TE671 cells, we used the pervRNA/F and pervRNA/R primers under the same cycling conditions and with the same calibration curves. The provirus number was normalized to a single-copy gene, the porphobilinogen deaminase (*PBGD*) gene. The *PBGD* gene was quantified in the same cycling conditions using a master mix and the primers *PBGD*/F (5'-AAG GGATTCACCTCAGGCTCTTTC-3') and *PBGD*/R (5'-GGCATGTTCAA GCTCCTTGG-3') and a probe (5'-CCGGCAGATTGGAGAGAAAAAGC CTGT-3'). The reaction mixture was prepared in 20 μ l using the qPCR MasterMix plus (Eurogentec), 300 nM final concentration of each primer, and 100 nM final concentration of the probe. Calibration curves were prepared by PCR of serially diluted human chromosomal DNA, ranging from 2 μ g to 0.64 ng DNA per reaction. The calibration curves were linear within this range.

Virus production and infection. Proviral clones of replication-competent PERV-A 14/220 and PERV3a were described previously (11). PERV3a is a chimeric construct between A14/220 and PERV60, with LTRs originating from PERV60. Preparation of infectious PERV-A 14/220 was described before (45). PERV3a virus was prepared in 293T cells transfected with PERV3a plasmid (11) using FuGene-6. The supernatant of PERV 14/220-infected 293T cells was collected and filtered 48 h later and used for infection of 293T and TE671 cells. We plated 2.5 $\times$ 10⁴ cells per well of the 6-well plate, and 500 μ l of virus-containing supernatant with 8 μ g/ml Polybrene was added the next day.

The replication-defective MLV-based vector pLG and its propagation from a molecular clone were described previously (44). Infection of 293T and TE671 cells was done in the same way as that for the PERV 14/220.

RESULTS

PERV LTR-driven transcription is suppressed by CpG methylation. Epigenetic status, particularly the CpG methylation level of LTR, is a good correlate of provirus expression. In order to test the sensitivity of PERVs to CpG methylation, we prepared reporter constructs joining the firefly luciferase gene *luc* with either the 5'LTR from PERV-A (EU789636) or the 5'LTR from PERV-B (AY099323) (11, 40). Both 5'LTRs contain similar numbers of CpG dinucleotides. The reporter plasmids were *in vitro* methylated by the bacterial CpG-specific DNA methyltransferase and transiently transfected into human kidney 293T cells. The luciferase activity induced by the methylated plasmids reached only ca. 20% of that induced by control mock-methylated plasmids (Fig. 1). The PERV-B LTR-driven luciferase expression was higher, which correlates with the longer homologous repeat box (22) in our PERV-B LTR. This result suggests that PERV-A and PERV-B LTR-driven transcription is sensitive to CpG methylation.

Global CpG methylation of PERV 5'LTR in porcine tissues. The presence of multiple-insertion polymorphic and sequence-divergent PERV copies in each individual pig genome presents a problem for the analysis of PERV methylation status *in vivo* and its comparison between different pigs. From the point of view of

FIG 1 CpG methylation suppresses the transcriptional activity of PERV-A and PERV-B 5'LTR in 293T cells. The luciferase activity driven by mock-methylated (black columns) and CpG-methylated (white columns) constructs was measured 2 days after transfection and normalized to β -gal activity. Error bars correspond to standard errors (SE) calculated from three parallel experiments. **, $P < 0.01$.

proviral transcription and potential virus transmission, the PERV copies with sparsely methylated or nonmethylated 5'LTR are conceivably the most relevant, and we elaborated the MS-qPCR technique for quantification of these hypomethylated copies. This MS-qPCR is based on bisulfite conversion of nonmethylated cytosine and designing primers complementary to the converted CpG dinucleotides. In order to survey the broadest variance of PERV sequences, we designed the primers for the U3/R and the leader region invariably found in 3 PERV-As, 10 PERV-Bs, and 3 PERV-Cs (Fig. 2A). The reverse primer was localized into the leader region to discriminate between 5' and 3'LTRs. The analyzed part of the promoter properly reflects the whole 5'LTR, as indicated by the analysis of the whole promoter region from several particular PERV loci (46).

We compared the *in vivo* prevalence of hypomethylated PERV 5'LTRs using the MS-qPCR in 12 different organs of three individual pigs and in the cell lines PK15 and ST-IOWA, porcine cells with defined high and low transmission potential, respectively (8). In these cell lines, the hypermethylated 5'LTRs and the total numbers of 5'LTRs were also quantified. Selected samples and cell lines were analyzed by bisulfite sequencing using the seqBIS primers. Our quantitative approach revealed no major differences in PERV 5'LTR methylation in porcine tissues, while the prevalence of hypomethylated copies in PK15 was about five times higher and significantly ($P < 0.05$) differed from the levels in all other samples (Fig. 2B). More than half of the LTRs in one placenta sample were hypomethylated, in contrast to results with the PK15 cell line. That level three times higher than the average tissue methylation and nearly twice as much as the second highest value; however, the average methylation of 5'LTRs in placenta did not sig-

nificantly differ from those of other tissues. Detailed quantitative comparison of transmitter PK15 and nontransmitter ST-IOWA cell lines showed that in ST-IOWA, the prevalence of hypomethylated PERV 5'LTRs is ca. 10 times lower than that in PK15, whereas the hypermethylated PERV 5'LTRs are slightly but not significantly more abundant in ST-IOWA cells and the total PERV load is lower in ST-IOWA cells (Fig. 2C). These results suggest that only a minor fraction of PERV copies in PK15 and ST-IOWA cells are hypomethylated, and both cell lines differ mainly in this fraction of PERVs. The number of hypomethylated PERV copies corresponds to their PERV transmission capacity.

Standard bisulfite sequencing of selected organ samples and cell lines confirmed the high PERV 5'LTR methylation level in all tissues (Fig. 2D). Out of 72 sequences analyzed, all but two had more than half of their CpG methylated. One PERV promoter sequence of placenta origin was completely nonmethylated (Fig. 2D, placenta 2). No specific patterns of CpG methylation were observed in any group of PERV 5'LTR sequences. In PK15 and ST-IOWA cells, the distribution of CpG methylation was more complex. We have confirmed that PK15 cells contain more hypomethylated PERV 5'LTR than ST-IOWA, but the difference in the total percentage of methylated CpGs was rather small. Presumably, the highly selective quantBIS_n primers amplify only rare sequences, with all six complementary CpG dinucleotides nonmethylated. In our data set, we found these specifically hypomethylated sequences in PK15 cells and, rarely, in tissues but not in ST-IOWA cells (Fig. 2D).

PERV RNA expression in porcine tissues and cell lines. We analyzed the total PERV RNA expression by qRT-PCR in the same set of tissues and two cell lines and correlated it with DNA methylation. The pervRNA primers were designed for the transcribed part of the PERV γ 1 LTR and leader sequence conserved in all three subgroups (Fig. 2A). The highest total PERV RNA expression, 3-fold higher than that observed for the *GAPDH* housekeeping gene, was detected in the PK15 cell line, whereas in ST-IOWA cells, PERV expression was only 3% of that observed for *GAPDH*. In porcine organs, the RNA expression varied from less than 1% of *GAPDH* expression in the muscle, heart, and testes to over 10% in the liver, placenta, and blood and up to nearly 100% *GAPDH* expression in the lung (Fig. 3). The highest PERV expression in PK15 cells correlates with the lowest level of PERV 5'LTR methylation. In porcine tissues, the 5'LTR methylation was roughly equal in all samples (Fig. 2B), whereas the PERV expression varied between 1 and 100% of that of *GAPDH*. These results suggest that strong hypomethylation is a good predictor of PERV expression. On the other hand, slight differences in the level of PERV 5'LTR methylation in tissues do not correlate with the expression of PERV RNA.

The range of interindividual variability of PERV RNA expression and 5'LTR methylation. More detailed analysis of the interindividual differences in global PERV DNA methylation and PERV RNA expression could substantiate selection of the pig for transplantation. Therefore, we analyzed the PERV RNA level and 5'LTR methylation in samples of porcine skin and blood from 20 Large White pigs by qRT-PCR and MS-qPCR techniques (Fig. 4A). In general, the PERV RNA expression was always higher in blood than in the skin. The PERV RNA level varied from 12 to 113% (33% on average) of *GAPDH* in blood and from 2 to 16% (8% on average) of *GAPDH* in the skin. Only one blood sample obtained from pig LW1 significantly exceeded the PERV RNA

FIG 2 DNA methylation of PERV 5'LTR in porcine tissues and cell lines. (A) The scheme represents the complete PERV provirus and its 5'LTR region (enlarged). Arrows show the location of PCR primers for DNA methylation and RNA expression analysis (see Materials and Methods). White lollipops symbolize CpG dinucleotides distinguished by primers for MS-qPCR. (B) Ratios of hypomethylated PERV 5'LTRs in 12 pig organs are shown as a percentage of hypomethylated PERV 5'LTRs per genome in the PK15 cell line. Organs were collected from two to four pigs; MS-qPCR of each sample was performed in triplicate. The results represent the means $\pm$ SE. (C) ST-IOWA/PK15 ratio of all (gray), hypermethylated (white), and hypomethylated (black) PERV copies estimated by MS-qPCR and performed in triplicates. Means $\pm$ SE are shown. (D) DNA methylation status of the 5'LTR region was confirmed by bisulfite sequencing in four selected porcine tissues and two cell lines. The percentages of methylated CpGs are shown in parentheses. Methylated CpG dinucleotides are depicted by solid circles; nonmethylated CpG sites are indicated by open circles. The gray rectangles along the bottom scheme of the 5'LTR depict the quantBIS primer annealing sites. The arrows mark sequences containing quantBIS_n/F and quantBIS_n/R primer annealing sites.

FIG 3 Level of PERV RNA in 11 tissues and two cell lines estimated by qPCR is shown as the percentage of *GAPDH* RNA expression. The results are shown as means $\pm$ SE calculated from three pigs or three parallels of cell cultures and three triplicates of qRT-PCR. Vertical arrows mark samples with very low PERV RNA expression.

levels in the skin and blood samples of all other pigs. In 15 out of 20 blood samples, we succeeded in performing methylation analysis of the PERV 5'LTR via MS-qPCR. The prevalence of hypomethylated PERVs in Large White pig blood varied from 3 to 15% of the hypomethylated PERVs in PK15 (Fig. 4B). These results suggest minor differences in PERV 5'LTR methylation within the Large White pigs. The increased PERV RNA expression in one pig, LW1, was not accompanied by an increased number of hypomethylated 5'LTRs.

Stability of CpG methylation of the PERV 5'LTR. Cytosine demethylation might be a consequence of the oxidative stress induced by ischemia-reperfusion injury in clinical transplantation (47). Therefore, we analyzed the stability of CpG methylation within the PERV 5'LTR in ST-IOWA cells treated with inhibitors of DNA methyltransferases and histone deacetylases. We applied azaC alone or together with TSA (Fig. 5A), and the shift of global PERV 5'LTR methylation was measured by MS-qPCR. We de-

tected only a minor increase in the prevalence of hypomethylated PERV 5'LTRs in the treated cells irrespective of the combination or concentration of the inhibitors (Fig. 5B). The quantitative approach was confirmed by standard bisulfite sequencing of sample 4 (Fig. 5C). We observed 47% of methylated CpG in the 5'LTR sequences obtained from untreated ST-IOWA cells and 51% in the cells treated with the highest concentrations of inhibitors. Furthermore, we tested whether this treatment influences expression of PERV RNA. RNA from azaC- and TSA-treated ST-IOWA cells was isolated and quantified by qRT-PCR. The obtained values were related to the expression of *GAPDH*. We observed a slight decrease of PERV expression (Fig. 5D). This decrease could be associated with a side effect of inhibitors. Similar results have already been described (37).

Lack of provirus silencing in PERVs transmitted to human cells. Retroviral infection of a heterologous host often results in DNA methylation-mediated transcriptional silencing of the inte-

FIG 4 Interindividual variability of PERV RNA expression and the prevalence of hypomethylated PERVs in pigs. (A) The level of PERV RNA in skin (white) and blood (black) collected from 20 pigs, as estimated by qPCR, is shown as the percent *GAPDH* RNA expression. The dotted line depicts the average PERV RNA level in the skin, and the broken line depicts that in the blood. (B) The rate of hypomethylated PERV 5'LTRs per genome in the blood of 20 pigs, shown as the percentage of hypomethylated PERV 5'LTRs per genome in the PK15 cell line. All MS-qPCRs were performed in triplicate.

FIG 5 Effect of DNA methyltransferase and histone deacetylase inhibitors azaC and TSA on the CpG methylation and expression of PERVs in ST-IOWA cells. (A) Depicted concentrations of azaC alone or in combination with TSA were used in ST-IOWA cells. (B) The ratio of hypomethylated PERV 5′LTRs per genome in ST-IOWA cells treated with the respective concentrations of azaC and TSA, shown as the percentages of the hypomethylated PERV 5′LTRs per genome in the PK15 cell line. (C) DNA methylation status of PERV 5′LTR region in ST-IOWA sample treated with 100 μM azaC and 0.15 μM TSA, analyzed by bisulfite sequencing. Methylated CpG dinucleotides, solid circles; nonmethylated CpG dinucleotides, open circles. (D) The levels of PERV RNA expression in ST-IOWA cells treated with azaC and TSA, as estimated by qRT-PCR, are shown as the percentages of *GAPDH* expression. All qRT-PCRs were performed in triplicate.

grated virus. We infected the permissive human cell lines 293T and TE671 with PERV-14/220 or PERV-3a (11) and an MLV-based vector as a control. Infected cells were collected after different intervals from 2 days up to 2 months. We observed a very slow, slight increase of 5′LTR methylation after infection by PERV-3a (Fig. 6A) and PERV-14/220 in 293T cells (Fig. 6B). No methylated PERV LTR sequences were detected in long-term infected 293T cells used as a source of PERV-14/220 virus (Fig. 6B). Also, we did not observe any progression of PERV-14/220 5′LTR methylation in TE671 cells 3, 13, 27, and 63 days postinfection, with 0, 3.8, 0.8, and 0% methylated CpGs, respectively (Fig. 7). The number of proviruses significantly increased in TE671 cells in the course of the first month after infection. In contrast, the proviral load in 293T cells was high already 4 days postinfection and did not increase afterwards (Fig. 7A). This is in agreement with the measured total PERV RNA levels (Fig. 7B). Both cell lines produced infectious PERV particles, with the 293T cell line producing higher viral titers (Fig. 7C). These results suggest that provirus silencing and DNA methylation of PERVs transmitted to human cells is inefficient. Similarly, the MLV-based vector remained transcriptionally active, with almost no methylated 5′LTRs in both cell lines. After 2 months of culture, the 5′LTR methylation did not exceed 2% of CpGs (Fig. 7E).

DISCUSSION

Pig-to-human PERV transmission represents a potential risk in xenotransplantation, but the multicopy and polymorphic reservoir of PERV sequences cannot be easily eliminated from donor animals, and strategies diminishing the virus expression were shown to be inefficient (48–50). The present study brings detailed analysis of the epigenetic parameters of PERV regulation and points to the possible search for transcriptionally active PERV copies. We demonstrated the PERV LTR sensitivity to CpG methylation *in vitro* and a high level of PERV 5′LTR methylation in all porcine tissues. A small fraction of hypomethylated PERV sequences were detected in several pig organs and in PK15 cells, where it correlated with a high level of PERV expression. Altogether, we conclude that DNA methylation can control transcription of PERVs in the pig genome, and the increased prevalence of

FIG 6 CpG methylation of PERV 5′LTR after infection of human 293T cells. The upper schemes represent 5′LTRs and leader sequences of the examined PERVs with primers for the bisulfite sequencing (arrows). The CpG methylation status of the indicated regions of PERV3a (A) and PERV14/220 (B) was assessed at three or four time points, respectively, postinfection. Methylated CpG dinucleotides, solid circles; nonmethylated CpG dinucleotides, open circles. Days postinfection and percentages of methylated CpGs (in parentheses) are shown on the right side. Gray rectangles indicate the region of U3 repeats. Different sequences of PERV3a contain different numbers of U3 repetitions.

FIG 7 Progress of PERV 14/220 and control MLV infection in TE671 (light gray column) and 293T cells (dark gray column). (A) Numbers of PERV proviruses per genome were estimated by qPCR after the depicted number of days postinfection (dpi). (B) The level of PERV RNA in infected TE671 and 293T cells after the depicted dpi, as estimated by qPCR, is shown as a multiple of human RPII RNA expression. (C) The level of PERV RNA in 293T cells infected by virus collected from TE671 (white column) and 293T cells (black column) 30 dpi after PERV 14/220 infection, as estimated by qPCR, is shown as a multiple of human RPII RNA expression. RNA expression was measured 3 dpi. (D) The level of MLV RNA in infected TE671 and 293T cells after the depicted dpi, as estimated by qPCR, is shown as a multiple of human RPII RNA expression. (E) The CpG methylation in infected TE671 and 293T cells after the depicted dpi, as estimated by bisulfite sequencing, is shown as the percentage of methylated CpG within the sequenced region in PERV 14/220 and MLV. The sequenced region is depicted above each graph, together with the positions of primers used for analysis of TE671 (light gray arrow) and 293T cells (dark gray arrow). Vertical arrows mark samples with 0% methylated CpGs.

sparsely methylated PERV sequences indicates increased virus expression.

The regulation of PERV transcription is poorly understood. Wilson et al. (26) identified various DNA-binding factor sites via database search, but the only transcription factor experimentally proved to bind the PERV LTR sequences is the ubiquitously expressed NF-Y (25). Epigenetic aspects of PERV transcriptional control have been briefly reported by Park et al. (39) who, in agreement with our study, showed the sensitivity of the PERV 5'LTR to methylation *in vitro* and the promoter activity reduced by inhibition of histone acetylation. However, the correlation between LTR methylation and provirus silencing need not be absolute, and complex epigenomic features at the site of provirus integration play an important role. For example, proviruses integrated in the intergenic regions have been found to be transcriptionally silent in a DNA methylation-independent way (51). Further, Wolf et al. (37) showed the presence of repressive histone epigenetic marks and very low levels of activation marks on PERVγ1 provirus in two porcine cell lines, pEGC and PK15. In accordance with our study, the level of repressive epigenetic marks was lower in the PK15 cell line, while the level of activation marks was higher.

The main finding of this study is that only a very small fraction of PERV 5'LTRs in porcine tissues are nonmethylated or sparsely methylated. The observed PERV mRNA transcription can be attributed to these hypomethylated PERV loci, which is exemplified by the highest PERV transcription, virus production, and transmission in PK15 cells with the largest fraction of hypomethylated PERV copies (Fig. 2). Another example is the specific PERV locus

6SH, which has been shown to be sparsely methylated in animals that transmit to porcine and human cells (46). The alleviated suppression of hypomethylated PERV loci permits positive promoter regulation by transcription factors whose tissue specificity could be responsible for the different PERV RNA levels (25, 26). The increased fraction of hypomethylated PERVs in the PK15 cell line might be a consequence of superinfection and integration of new, probably replication-competent copies. The relatively poor superinfection resistance has already been described in PERVs (52, 53). Similarly, some nonmethylated PERV 5'LTRs detected in the tissues could be a result of somatic integration. ST-IOWA cells, however, do not conform to this hypothesis, because the bisulfite sequencing revealed hypomethylated PERV sequences but PERV expression was negligible (8, 20). This inconsistency could be explained by the different pattern of CpG methylation. Whereas completely or nearly nonmethylated 5'LTRs were found in PK15 cells, in ST-IOWA we could only observe LTRs with dispersed methylation that may not be sufficient for detection by MS-qPCR or maintenance of transcriptional activity. Previous work also suggests that ST-IOWA cells lack specific transcription factors necessary for efficient PERV 5'LTR activity (26).

The tissue-specific PERV transcription has already been studied with respect to the xenotransplantation use of pig organs. Our observation of increased PERV transcription in the lungs is in agreement with previous studies (21, 54). Dieckhoff et al. (20) detected the highest PERV RNA level in the spleen, which was not included in our organ panel. Moderately increased transcription of PERVs was also detected in peripheral blood mononuclear cells (PBMCs), ovary, placenta, and liver, but the increase in levels

compared to those in other organs was not accompanied by any significant decrease of 5'LTR methylation. Endogenous retroviruses often are found hypomethylated in the placenta (33, 55–58). We detected hypomethylated PERV sequences in one placenta sample; the other two placenta samples did not exceed the average considerably. These differences may originate from nonequal proportions of trophoblastic elements in various samples, because the layers of diffuse epitheliochorial placentas in the pig are hardly separable.

The interindividual comparison of PERV expression and 5'LTR methylation in the skin and PBMC of Large White pigs showed only moderate differences and no significant correlation between the expression and the number of hypomethylated sequences. In one case, we observed an exceptionally high level of PERV transcription in PBMCs but not in the skin, suggesting that rare animals with tissue-specific PERV expression exist and should be excluded from xenotransplantation breeding programs. The range of interindividual variability of PERV expression in PBMC is comparable to that in a previous study (20). This variability could result from the presence of different proviruses with variable regulatory sequences in individual pigs (24, 26).

The stability of PERV LTR methylation is of great importance, because fluctuations in PERV expression were observed in animals under different conditions and different levels of stress (59, 60). Furthermore, an increased PERV expression level was observed in diseased pigs (61). Because the xenograft handling and its survival in the artificial environment induce some kind of stress, we checked the resistance of PERV DNA methylation in cells cultured with inhibitors of DNA methyltransferases and histone deacetylases. The 5'LTR methylation remained at the same level. The low PERV RNA expression even decreased under these conditions, as shown previously (37, 62). Hypothetically, repressive factors might be activated by the methyltransferase and deacetylase inhibitors.

Although PERVs were shown to be able to infect human cells *in vitro* (8), to date there is no evidence of transmission to recipients of xenografts (63–68), apparently due to an efficient protective mechanism of the recipient. Epigenetic silencing is a common mechanism of host protection against retroviral infection; however, here we show that human cells are very inefficient in PERV silencing by DNA methylation. For example, the LTRs of Rous sarcoma virus or lentiviral vectors become silenced by methylation within weeks (69, 70), in contrast to PERV LTRs, which are not substantially methylated even after 2 months (Fig. 6). We observed similar resistance to DNA methylation in our control experiment with MLV. Silencing of retroviruses substantially depends on the site of integration (51), and our observation could be explained by similar integration preferences of PERVs and MLV into the promoter regions of active genes (71).

Our results suggest that most PERV proviruses are silenced by DNA methylation in porcine tissues and only a minority of PERVs can be transcribed. The activity of these nonmethylated PERV 5'LTRs depends on other regulatory factors. Interestingly, the methylation status largely differs between the porcine tissues and the analyzed cell lines. Higher and more continuous DNA methylation of PERV 5'LTR in the tissues underline the importance of PERV silencing in the organism, while there may be lower selection pressure in the cell lines. The observed methylation pattern could partially explain much higher infectivity of the PK15 cell line than that of any of the porcine tissues (72). In the future, it

would be interesting to identify the active PERV loci; however, identification of a particular nonmethylated locus is complicated due to the repetitive character of PERVs and the degenerated PERV sequences obtained by bisulfite sequencing. Therefore, other approaches to identification of active proviruses should be considered, such as their isolation by chromatin immunoprecipitation. Nevertheless, our results suggest that PERV 5'LTR hypomethylation can serve as a marker of active provirus.

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