MATERIALS AND METHODS

CRP Transfection to PEC

cDNA of transmembrane-type CRP, MCP, and glycosylphosphatidylinositol-anchored CRP, DAF, and CD59 were prepared. Stable PEC lines expressing these CRPs were produced by transfection of the cDNA. The expression of the each transfected molecule was verified by flow cytometry.

PERV and *LacZ* Gene Transfection to PEC

The PEC lines with human CRP were next transfected with the *LacZ* gene with the packaging signal of the murine leukemia virus (MuLV) under the control of the long-terminal repeat of MuLV by a pseudotype infection. The PEC lines were further infected with PERV subtype B (PERV-B).[4] The established PEC lines, PEC(Z)/PERV-B with CRP, at approximately passage 10 from the original stable PEC lines with CRP, were used in the *LacZ* pseudotype assay. The passage number did not affect the growth of these PEC lines.

LacZ Pseudotype Assay

Culture supernates of the infected PEC cells were inoculated to HEK (human embryonic kidney) 293 cells with or without 10% human serum. The inoculated HEK293 cells were histochemically stained to count *LacZ*-positive blue foci. The rate of reduction of *LacZ*-positive cells by serum was then calculated.[5]

Statistical Analysis

Data are presented as the mean value ± standard error (SEM). Student *t*-test was used to ascertain the significance of differences, which were considered statistically significant when $P < .05$.

RESULTS

The Expression of CRPs on PEC

The expression of MCP, DAF, and CD59 on PEC (LacZ)/PERV-B were determined by FACS analysis. Stable transfectants effectively expressed each CRP molecule on their surface (data not shown).

The Difference in PERV Infectivity Among the PEC Lines with Human CRP and Control PEC by 10% Human Serum Treatment

A difference in PERV transmission to HEK 293 cells among the PEC (LacZ)/PB transfectants with each CRP was observed. The number of infected HEK 293 cells was more significantly reduced by inoculation with the supernates from MCP-transfectants (237.0 ± 37.9 vs 64.0 ± 6.0; $P = .003$) than those from DAF transfectants (70.0 ± 21.1 vs 41.5 ± 13.6; $P = .03$) or CD59 transfectants (52.5 ± 19.1 vs 31.5 ± 11.1; $P = .06$) transfectant (Fig 1a).

The Difference in Each PERV Resistance to Human Serum

The resistance of the PERV from each PEC with CRP to 10% human serum was assessed. PERV from the PEC with DAF or CD59 showed resistance to 10% human serum in comparison with those from control PEC (DAF: 59.6% ± 5.3%, CD59: 61.1% ± 3.9% vs control: 31.3% ± 3.6%; $P <$ .01). However, PEC with MCP did not produce this effect (28.8% ± 2.5%; Fig 1b).

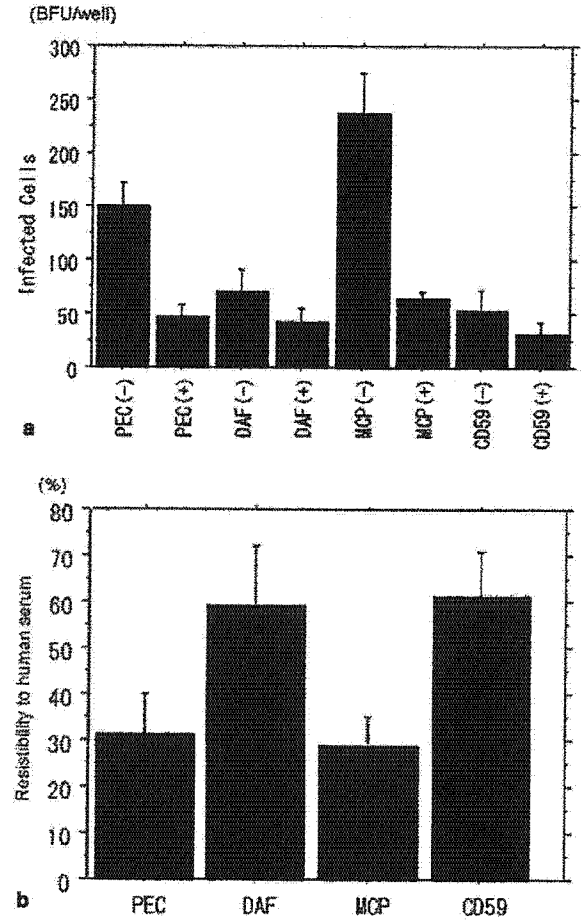

Fig 1. **(a)** The number of infected HEK 293 cells were more significantly reduced in the inoculation with the supernatant from the MCP-transfected PEC ($P = .003$) than those from the DAF- ($P = .03$) and CD59- ($P = .06$) transfected PECs. **(b)** PERV from DAF and CD59 transfectants showed resistance to 10% human serum in comparison with those from control PEC, while MCP transfectant did not cause such resistance. BFU: blue focus forming unit; +, 10% human serum added; −, 10% human serum not added.

DISCUSSION

To perform clinical xenotransplantation safely, it is important to prevent PERV transmission to human beings. In view of overcoming hyperacute rejection, it is indispensable to produce a pig with low antigenicity and resistance to the human immunological system, by knocking out the Galα1-3Gal (α-Gal) epitope and by gene transduction of human CRP. However, at the same time, these genetic modifications might make the PERV particle from pig cells more resistant to human serum, which enlarges the new risk of PERV transmission to humans.

Recently, it has been reported that the PERV released

from the α-Gal-negative PEC or the PEC with DAF show low sensitivity to human sera.[6] In this study, the PERV from the PEC with DAF or CD59 also acquire the resistance to human sera. These findings might indicate an increased risk of PERV transmission to human cells by CRP transfection. This public health problem must be solved for clinical xenotransplantation. On the other hand, PERV from the PEC with MCP did not show changes in the resistance to human sera. We will try to perform further analyses to verify this discrepancy among CRPs in modulating PERV resistance to human sera. The findings herein may be useful to make clinical xenotransplantation free from PERV infection. In conclusion, DAF and CD59 expression on PEC reduced PERV resistance to human sera, while MCP did not have such an influence.

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Prevention of PERV Infections in Pig to Human Xenotransplantation by the RNA Interference Silences Gene

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The possibility of preventing the transmission of porcine endogenous retrovirus (PERV) to human cells using short interfering RNAs (siRNA) was investigated. The siRNA for the p30 of PERV gag region was cloned into pSUPER, the polymerase-III H1-RNA gene promoter. A green fluorescence protein (GFP) was also cloned into pSUPER to establish pSXGH. Pig endothelial cells (PEC) were transduced with the *LacZ* gene by pseudotype infection, and infected with PERV subtype B, resulting in the formation of PEC(*LacZ*)/PB. The PEC(*LacZ*)/PB was next transfected with pSXGH-siRNA. The expression of siRNA was provisionally checked by determining the level of expression of GFP. Culture supernatants of infected cells were then inoculated into HEK293 cells. The siRNA clearly destroyed the PERV infectivity of PEC(*LacZ*)/PB in both transient cell lines and stable clones. Moreover, the decreased levels of mRNA and gag protein were evidenced in the stable clones by real-time PCR and Western blotting, respectively. The final goal of our study was to establish a transgenic pig expressing the siRNA for PERV. The results suggest that siRNA represents a novel approach for controlling PERV infections in clinical xenotransplantation.

Key words: PERV, pig endothelial cell, pseudotype infection, siRNA, xenotransplantation.

Abbreviations: PERV, porcine endogenous retroviruses; siRNA, short interference RNA ; PEC, pig endothelial cells ; GFP, green fluorescence protein; PB, PERV-B; BFU, blue focus forming unit.

The pig represents an ideal source of xenogeneic organs because of their plentiful supply and their numerous anatomical and physiological similarities to their human counterparts. However, the discoveries that porcine endogenous retroviruses (PERV) can infect human cells *in vitro* and SCID mouse tissue *in vivo* have stimulated discussions concerning the degree of infectious risk in such xenotransplantations (*1–4*). At the present time, although PERV-related infections have not been detected in humans or non-human primates after exposure to pig xenografts (*5*, *6*), the possibility that they could arise as a consequence of xenotransplantation cannot be excluded, especially when genetically modified pigs are used based on recent advances in nuclear transfer technology (*7*, *8*).

In order to prevent PERV transmission, several strategies have been developed, such as the selection of animals that do not release PERVs (*9*), treatment of the recipient with an antiviral vaccine (*10*), as well as others (*11*, *12*). However, in pigs, at least 50 proviral copies of PERV are present in the genome (*13*). Therefore, the production of pigs that are completely devoid of all PERV related elements is unrealistic (*14*). A neutralizing antibody against the env protein of PERV would be a useful approach, but might carry the risk of activating host pig cells that express the env protein on the membrane.

A short interference RNA (siRNA) represents a mechanism of post-transcriptional gene silencing (PTGS), and has been described in plants, invertebrates and, more recently, in mammalian cells (*15–17*). It has been applied to inhibit the pre and/or post integration of HIV-1, and can be used as a possible therapeutic strategy to inhibit HIV-1 replication in host cells (*18*).

In this study, the possibility of preventing the transmission of PERV from pig cells to human cells by a stable siRNA expression system was investigated on the assumption that it is possible to establish a transgenic pig expressing siRNA.

MATERIALS AND METHODS

Cell Cultures—A PEC line, MYP30, and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Sigma Chemical Co., MO) supplemented with 10% heat-inacti-

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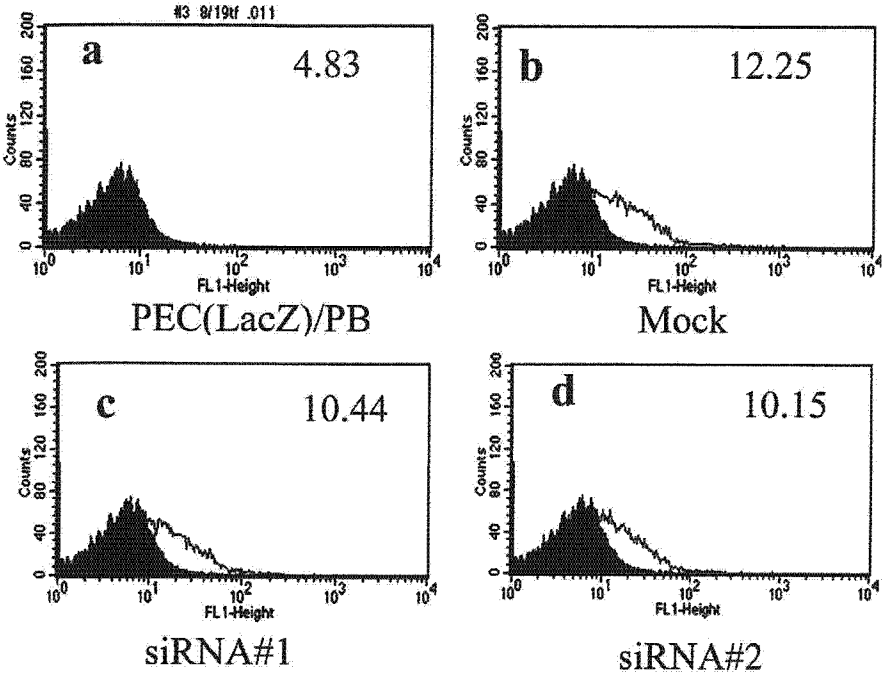

Fig. 1. FACS analysis of GFP expression in PEC(*LacZ*)/PB transfected with pSXGH-siRNA. An expression vector containing GFP and siRNA was transfected into PEC(*LacZ*)/PB cells. The level of expression of GFP on the cell surface was checked as an alternate to assessing the expression of siRNA by flow cytometry. Typical flow cytometric histograms for these transfectants after 1 week of selection with hygromycin are shown (open histogram). a, parental PEC(*LacZ*)/PB (closed histogram); b, PEC(*LacZ*)/PB with pSXGH (mock); c, PEC(*LacZ*)/PB with pSXGH-siRNA#1; d, PEC(*LacZ*)/PB with pSXGH-siRNA#2. The mean shift values for GFP expression in transfectants and naive PEC are indicated in each panel (a–d).

vated fetal bovine serum (FBS) with L-glutamine and kanamycin/amphotericin. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37°C (*19*).

Construction of the Modified Genes—To clone pSUPER, the polymerase-III H1-RNA gene promoter was amplified by PCR using the recommended primers: 5′-CCATggAATTCGAACGCTgACgTC-3′ (sense) and 5′-gCAAgCTTAgATCTgTggTCTCATACAgAACTTATAAgATTCCC-3′ (antisense). The PCR product was digested with *Eco*RI and *Hin*dIII enzymes and then cloned into the sites of pBluescript IISK(+) (*20*). A green fluorescence protein (GFP) subcloned into pCX, a β-actin promoter with a CMV enhancer (*21*), was digested with *Sal*I–*Hin*dIII enzymes, and cloned into the sites of pSUPER. A hygromycin resistance gene under a thymidine kinase (TK) promoter was also cloned into the *Eco*RV site of pSUPER, to establish pSXGH.

To insert the targeting sequence, DNA oligos in the p30 site of PERV were designed and cloned into the *Bgl*II–*Hin*dIII sites of pSXGH. siRNAs with the following sense and antisense sequences were used: 5′-gATCCCCggCAATAggACCCCACTCgACTTCAAgAgAgATCgAgTggggTCCTATTgCCTTTTTggAAA-3′ (sense), 5′-AgCTTTTCCAAAAAggCAATAgg ACCCCACTCgACTCTCTTgAAgTCgAgTggggTCCTATTgCCggg-3′ (antisense) (*20*). The siRNAs were synthesized by Nisshinbo (Chiba, Japan).

Preparation of PERV-Producing Cells—To determine PERV infectivity, we introduced a MFGnls*LacZ* plasmid that encodes the *LacZ* gene with the packaging signal of the murine leukemia virus (MuLV) under the control of the long terminal repeat of MuLV into PEC by a pseudotype infection, and prepared PEC(*LacZ*), as described previously (*22*). To establish a PEC(*LacZ*) that produces PERV-B, PEC(*LacZ*) was infected with PERV-B produced from HEK293 cells that had been persistently infected with PERV-B; hereafter this is referred to as PEC(*LacZ*)/PB. Eighteen to 32 days after infection, the viral titers of pseudotypes of PERV-B containing the *LacZ* gene, PERV-B(*LacZ*), that were released from PEC(*LacZ*)/PB were measured in naive HEK293 cells (*23*).

Flow Cytometry—The transduction of siRNA into the PEC(*LacZ*)/PB was provisionally checked by the expression of GFP. The expression of GFP was confirmed by FACS Calibur flow cytometry (BECTON DICKINSON) for each transient cell line and a stable clone. Parental PEC(*LacZ*)/PB cells were used as controls.

LacZ Assay—HEK 293 cells were seeded at 2×10^5 cells per well in 24-well plates one day prior to infection. Culture supernatants containing pseudotype viruses of PERV-B were incubated with 8 μg/ml of polybrene for 30 min after filtration through a Millipore filter (pore size 0.80 μm) and inoculated into the HEK 293 cells. Four hours after the inoculation, the medium was replaced with fresh D-MEM supplemented with 10% FBS, the culture was incubated for an additional 2 days, and the cells were then stained with 5-bromo-4-chrolo-3-indolyl-β-D-galactopyranoside. The number of *LacZ*-positive blue focus forming units (BFU) was counted under a microscope (*24*).

Quantitative Real-Time RT-PCR—Total RNA was collected from the stable clones and mock control clone, using the TRIZOL LS Reagent (Invitrogen, CA, USA). The total RNA was used in a reverse transcriptase reaction.

To evaluate the degradation of PERV gag mRNA, SYBR-Green real-time PCR was performed with Smart Cycler II System (Takara) and the SYBR premix Taq (Takara). The PERV sequence was amplified using two primer pairs specific to the target site in the gag p30 region. RT was carried at 42°C for 15 min, followed by 95°C for 2 min using random primers, followed by PCR for 45 cycles of 95°C for 5 s and 60°C for 20 s.

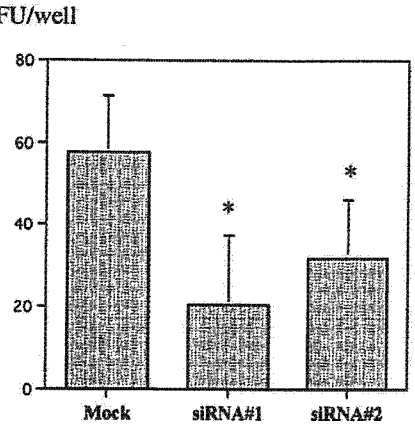

Fig. 2. **siRNA directed silencing on PERV infectivity.** PEC(*LacZ*)/PB was transfected with pSXGH-siRNA, and the culture supernatants collected from them were added to the medium of HEK293 cells. Target HEK293 cells were histochemically stained, and *lacZ*-positive BFU were counted, to determine viral titers. The infectivity of each culture supernatant from PEC(*LacZ*)/PB with pSXGH-siRNA was calculated, and the data are expressed as the mean ± SEM of eight independent experiments. An asterisk indicates a significant difference (* $p < 0.05$ *vs.* mock).

The amount of gag RNA in the transfectants was normalized to the level of GAPDH RNA. The amount of PERV mRNA degradation in the stable clones with siRNA was calculated with the average of the control clones with the pSXGH.

Western Blotting—The protein content of transfectant and naive cell lysates was quantified by the BCA method (Pierce), and approximately 30 μg aliquots of the obtained proteins were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The separated proteins were then electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked by treatment with 5% skim milk in Tris-buffered saline/0.05% tween 20 (TBST) for 1 h at 25°C, and then incubated in 1% bovine serum albumin (BSA)/0.5% skim milk/TBST with a rabbit anti-gag p30 peptide, LRGASRRPTNLAKVC, antibody for 1 h at 25°C. After washing, the blots were incubated with horseradish peroxidase conjugated secondary antibody, porcine anti-rabbit Ig (CPL), and the signal was developed using an ECL detection system (Amersham) (*25*).

Statistical Analysis—Data are presented as the mean ± SEM. Student's *t* test was used to ascertain the significance of differences within groups. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

GFP Expression in Transient Cell Lines of PEC(LacZ)/PB with pSXGH-siRNA—Hairpin siRNA, corresponding to the targeted site, was designed in the pSXGH vector based on the polymerase III H1-RNA promoter. A closed circular plasmid, pSXGH-siRNA, was established and introduced into the PEC(*LacZ*)/PB by means of lipofectamine. After the selection of PEC(*LacZ*)/PB with hygromycine for one week, the transient expression of the GFP gene in PEC(*LacZ*)/PB with pSXGH was estimated by FACS analysis as a provisional index of the extent of gene transduction. After drug selection, the FACS values for GFP expression were clearly shifted in both the siRNA#1 and siRNA#2 samples (Fig. 1).

Effect of siRNA Directed Silencing on PERV Infectivity—The viral titers of PERV-B released from PEC(*LacZ*)/PB were next measured in HEK293 cells. HEK293 cells were inoculated by incubation with the culture supernatants of transfected cells, and a *LacZ* assay was carried out. While the PERV from the control PEC(*LacZ*)/PB and mock cells were easily transmited to HEK293, the extent of PERV infection from PEC(*LacZ*)/PB with pSXGH-siRNA was limited. Virus titers for PERV-B released from PEC(*LacZ*)/PB with pSXGH-siRNA and control mock clones were measured using HEK293 cells (Fig. 2). The data clearly show a significant

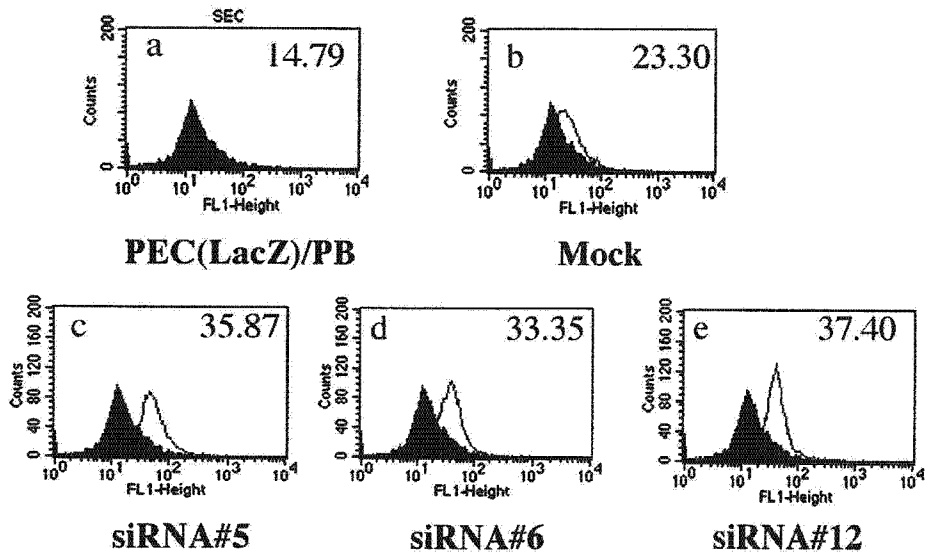

Fig. 3. **FACS analysis of GFP expression in stable clones of PEC(*LacZ*)/PB transfected with pSXGH-siRNA.** The expression levels of GFP on the cell surface of single PEC(*LacZ*)/PB clones were checked by flow cytometry. Typical flow cytometric histograms for each established clone are shown (open histogram). a, parental PEC (*LacZ*)/PB (closed histogram); b, PEC(*LacZ*)/PB clones with pSXGH (mock); c, PEC(*LacZ*)/PB clones with pSXGH-siRNA#5; d, PEC(*LacZ*)/PB clones with pSXGH-siRNA#6; e, PEC(*LacZ*)/PB clones with pSXGH-siRNA#12. The mean shift values of GFP expression in transfectants and parental PEC are indicated in each panel.

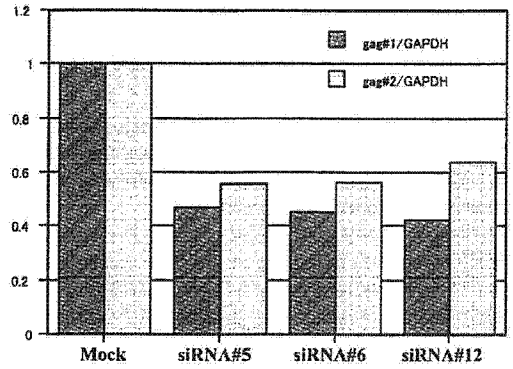

Fig. 4. **Real time PCR for the mRNA of a PERV in a PEC(*LacZ*)/PB transfectant.** To evaluate the degradation of PERV gag mRNA, SYBR-Green real-time PCR was performed with the Smart Cycler II System. The amount of gag mRNA for each of the PEC(*LacZ*)/PB transfectants was measured using two pairs of primers for the gag p30 region, gag#1 and gag#2, and normalised by comparison with the level of each GAPDH mRNA. The amount of PERV mRNA degradation in clones with siRNA was calculated with reference to the mock clone. Compared with the mock transfectants, PEC(*LacZ*)/PB with pSXGH-siRNA clones, #5, #6 and #12, showed a decreased mRNA expression for the gag region.

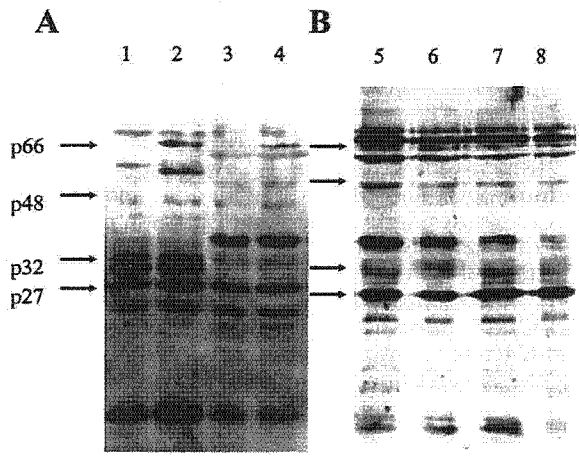

Fig. 5. **Western blot analysis of PERV p27gag expression in the stable transfectants with pSXGH-siRNA.** PEC, PEC(*LacZ*)/PB transfectants and HEK293 cells were solubilized in SDS. For each lane, 30 μg of tatal cell lysate was loaded, and stained with the anti-gag peptide, LRGASRRPTNLAKVC, antibody. Specific bands for the gag polyprotein (p66), intermediate (p48), and mature capside (p27) are indicated. 1, naive HEK293; 2, HEK293 with PERV-B; 3, naive PEC; 4, PEC(*LacZ*)/PB; 5, PEC(*LacZ*)/PB with pSXGH (mock); 6, PEC(*LacZ*)/PB with pSXGH-siRNA#5; 7, PEC(*LacZ*)/PB with pSXGH-siRNA#6; 8, PEC(*LacZ*)/PB with pSXGH-siRNA#12.

decrease in PERV infectivity (mock: 57.7 ± 13.6, siRNA#1: 20.6 ± 17.0, siRNA#2: 32.1 ± 14.0, $n = 8$).

FACS Profiles of GFP Expression in Stable Clones of PEC(LacZ)/PB with pSXGH-siRNA—After selection with hygromycine, stable PEC(*LacZ*)/PB transfectants with pSXGH-siRNA and pSXGH (mock control) were next established. The expression of GFP in the stable clones was then checked by FACS. The FACS values for GFP

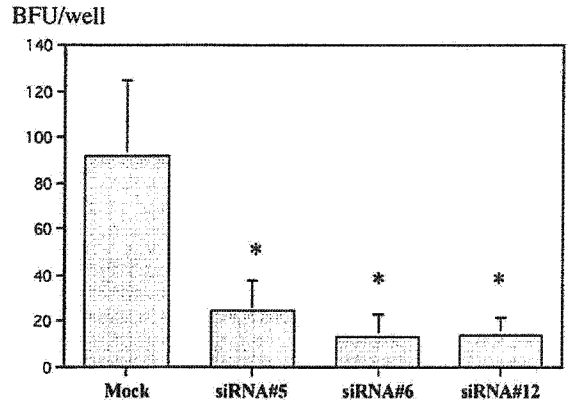

Fig. 6. **The effect of siRNA directed silencing on PERV infectivity Culture supernatants collected from PEC(*LacZ*)/PB transfectants with pSXGH-siRNA were added to the medium of HEK293 cells.** Target HEK293 cells were histochemically stained, and *lacZ*-positive BFU were counted to determine the viral titers. The infectivity of each culture supernatant from the PEC(*LacZ*)/PB clone with pSXGH-siRNA was calculated, and the data are expressed as the mean ± SEM of eight independent experiments. Compared with the mock transfectant, PEC(*LacZ*)/PB with pSXGH-siRNA (#5, #6 and #12) showed a decreased PERV infectivity. An asterisk indicates a significant difference (* $p < 0.05$ *vs.* mock).

expression were clearly shifted in both the representative standard siRNA clones and the mock controls (Fig. 3).

Real-Time PCR for mRNA of PERV in the PEC(LacZ)/PB Transfectant—Real-time PCR was performed to detect any alterations in the mRNA of PERV-B production targeted by the siRNA in each clone. mRNA levels of the gag region were measured individually by means of a SYBR green system and normalized to GAPDH. The introduction of pSXGH-siRNA into PEC(*LacZ*)/PB decreased the amount of mRNA in the representative standard clones in comparison with mock clones (siRNA#5: 0.472 and 0.561, siRNA#6: 0.456 and 0.568, siRNA#12: 0.426 and 0.642) (Fig. 4).

PERV gag Protein Expression Measured by Western Blotting—To confirm the results showing the inhibition of PERV protein expression in PEC, Western blotting for the gag protein was performed in stable PEC(lacZ)/PB clones. Naive HEK293, HEK293 infected with PERV-B, PEC(*LacZ*) and PEC(*LacZ*)/PB were first checked with a peptide antibody to ascertain the gag protein bands. Each stable clone was then checked. Clones with siRNA showed a visible reduction in gag protein expression compared with mock clones without siRNA (Fig. 5).

The Effect of siRNA Directed Silencing on PERV Infectivity—The viral titers of PERV-B released from each PEC(*LacZ*)/PB clone were next measured in HEK293 cells. HEK293 cells were inoculated by incubation with culture supernatants of each clone, and a *LacZ* assay was carried out. While the PERVs from the control PEC(*LacZ*)/PB and mock clones were easily transmited to HEK293, the extent of PERV infection from PEC(*LacZ*)/PB with pSXGH-siRNA was limited. We studied the inhibition of PERV-B infectivity of HEK293 cells by siRNA and these data are summarized in Fig. 6. The data clearly show a significant decrease in PERV

infectivity that is related to the downregulation of mRNA expression (Mock: 91.9 ± 32.8, siRNA#5: 24.3 ± 13.6, siRNA#6: 13.1 ± 9.7, siRNA#12: 14.1 ± .7, $n = 8$).

DISCUSSION

We first presented our findings on the suppressive effect on PERV infectivity by siRNA at the Xenotransplantation Association Meetings in 2003 (*26*). Subsequently, a similar study was reported by Karlas *et al.* (*27*). However, different from our present study, they investigated the effect of siRNA on PERV-infection of a human cell line, which from a clinical point of view is not related to the suppression of the primary PERV release from pig cells but, rather, is related to the secondary release from infected human cells. In addition, it is relatively easy to study the downregulation of PERV infectivity by siRNA in human cells, including the choice of the siRNA target site and the PCR primers for real-time PCR, because human cells lack naive PERVs. Moreover, the extensive human gene database is available to check the off-target effect on other genes. Therefore, the selected siRNA sites for PERV in human cells might not be a suitable site for PERV in pig cells. In the present study, we investigated the suppression of primary PERV infection by siRNA using pig endothelial cells. Since our ultimate goal is the establishment of a transgenic pig expressing the PERV siRNA, the siRNA effect must be investigated using pig cells.

In our previous study, we reported that the PEC line, MYP-30, expresses both PERV-A and -B transcripts, but not PERV-C, as evidenced by RT-PCR, and does not produce any PERV capable of infecting HEK293 cells or ST-IOWA by pseudotype infection (data not shown). Therefore, we evaluated the effects of siRNA on PERV infectivity, using PEC that had been exogenously infected with PERV-B, PEC(*LacZ*)/PB.

Concerning the target site of PERV by siRNA, we followed previously published procedures (*20*). A 21 bp oligomer initiated by gg, which has a minimal homology to other DNAs, especially pig DNA, was searched for in the GenBank data base. A site for p30 of the gag region was then selected. During the procedure, we mainly searched the gag-pol region to suppress whole PERV-A, -B, and -C by siRNA, because the env region has a less conserved sequence. We also chose another siRNA site in the gag region and attempted to construct pSXGH, but these efforts were unsuccessful. We then selected this gag region as a target site in the present study.

At the moment, several services on the internet can be used to provide information concerning a suitable RNAi site and a construct. Unfortunately, this information is currently unorganized. For example, in addition to the model presented here, an oligomer nineteen bp in length initiated by just g, or nineteen bp in length initiated by aa and terminated by tt has become popular. An oligomer 27 bp in length with a blunt end is also recommended. In addition, searching for off-target conditions in the non-redundant mRNA set of the pig was difficult it because of the incomplete database relative to the pig gene.

Additionally, in this study we used are an siRNA containing a cccc structure, which sometimes introduces complications in the whole structure. However, the target site in the present study was easily prepared and a strong effect of siRNA was indicated.

In the experiment using the transient cell line, the efficiency with respect to the suppression of PERV infectivity by the siRNA for p30 was not extremely high, because not all the PEC(*LacZ*)/PB cells had been transfected with siRNA; that is, 20–30% of the parental PEC(*LacZ*)/PB may have remained intact, even after drug selection (Fig. 1). However, compared with other reports of transient suppression related to genetic therapy by siRNA for HIV, the siRNA for p30 appears to be an effective site for targeting (*18, 28*).

We next extended the analysis of the siRNA effect, using single PEC clones. The clones and mock clones showed some diversity in terms of PERV infectivity. The average PERV reduction of the whole PEC clones with siRNA vs. whole mock clones might be close to those of the transient lines (data not shown). However, in some representative standard clones, extremely suppressive effects on infectivity by siRNA were found.

The SYBR green system was used for real-time PCR. This procedure requires quantification of a housekeeping gene as an endogenous standard, and the detection of a suitable site in each mRNA for PCR. Several studies of the real-time PCR for pig mRNAs have been reported. However, most of the PCR primers for pig GAPDH reported in these papers do not indicate a suitable site to make using the SYBR green system possible.

Concerning the correlation between the results of infectivity and the real-time PCR, while each clone indicated a 75–80% suppression of infectivity, the suppression rate of the mRNA of p30 was relatively mild, approximately 50%. In addition, other clones also showed the same tendency (data not shown). The results of the real-time PCR for the p30 mRNA might be affected by naive competent and non-competent PERV proviruses in pig cells. However, the siRNA on p30 had a strong effect on PERV infectivity.

In the case of Western blotting, the levels of gag protein (p66) and intermediate (p48) could be analysed by the peptide antibody. Unfortunately, nonspecific (p32) and mature capside (p27) were overlapped by non-specific bands in our system. However, the visible down regulation of the PERV gag protein could be verified, supporting the real-time PCR data.

Further examination of, for example, multiple site targeting by siRNA, will be required to attain the perfect or semi-perfect suppression of PERV infectivity. The results reported here suggest that siRNA could be useful in a new approach to addressing the issue of PERV infections in clinical xenotransplantation.

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Original article

Cell-binding properties of the envelope proteins of porcine endogenous retroviruses

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Abstract

To examine the binding properties of the envelope glycoproteins of porcine endogenous retrovirus subgroups A and B (PERV-A and PERV-B), we produced two forms of soluble envelope proteins, termed Env-ST and Env-SU, using a baculovirus expression system. Env-ST and Env-SU encompass one-third of the N-terminal and the entire surface unit (SU) of the envelope protein, respectively. Using these proteins, binding assays were performed in various mammalian cell lines. The binding properties of the Env-STs that contain the putative receptor binding domain (RBD) did not correlate with the susceptibility to the pseudotype viruses having PERV envelopes, whereas those of the Env-SUs correlated fairly well. These results suggested that the Env-SUs but not Env-STs interacted with their receptors in various cell lines. Interestingly, PERV-A Env-SU did not bind to a mink cell line (Mv1-Lu cells) that is highly susceptible to the PERV-A pseudotype virus. In addition, PERV-B Env-SU did not interfere with the PERV-B pseudotype virus on Mv1-Lu cells. These results suggest the existence of a cognate receptor-independent entry pathway as demonstrated in an immunodeficiency-inducing variant of feline leukemia virus FeLV.

Keywords: Porcine endogenous retroviruses; Receptors; Xenotransplantation

1. Introduction

Pigs are considered the most suitable donor of organs or tissues in xenotransplantation to humans [1]. However, endogenous retroviruses are present in the porcine genome widely [2,3] and complete particles of porcine endogenous retroviruses (PERVs) [4], once thought to be uninfectious to human cells [5], have recently been shown to infect and replicate in human cells in vitro [6]. Therefore, the potential risk of PERV infection [7] has became an issue in pig-to-human xenotransplantation.

PERVs consist of three subgroups (PERV-A, B and C) according to the sequences of their envelope proteins, with both PERV-A and PERV-B exhibiting tropism to human cells in vitro [8]. PERVs are classified into the family Retroviridae, genus *Gammaretrovirus* [5], and gammaretroviral envelope proteins are synthesized as polyproteins that are proteolytically processed into surface unit (SU) and transmembrane glycoproteins to acquire infectivity [9]. The receptor binding domain (RBD) is present at the N-terminal of the SU envelope glycoprotein. The transmembrane envelope glycoprotein is responsible for the membrane anchoring and fusion activity that is required for viral entry into host cells [10]. Le Tisser et al. (1997) defined two variable regions (VRs), VRA and VRB, followed by a proline-rich region

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FeLV, feline leukemia virus; GaLV, gibbon ape leukemia virus; HEK, human embryonic kidney; MAb, monoclonal antibody; MLV, murine leukemia virus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PERV, porcine endogenous retrovirus; PRR, proline-rich region; RBD, receptor binding domain; SDS, sodium dodecyl sulfate; Sf9, *Spodoptera frugiperda* 9; SU, surface unit; VR, variable region; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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(PRR) in the SU envelope of PERV based on analogy with the envelope of murine leukemia virus (MLV), a prototype of the gammaretroviruses. The RBD of MLV consists of VRA and VRB and plays a dominant role in the choice of receptor [11–13], while the PRR of MLV is considered to contribute to the envelope's conformation and fusogenicity [14,15].

The host ranges of PERVs have been well characterized by infection assays using pseudotype MLV harboring PERV envelopes and it has been revealed that various mammalian cell lines possess functional receptor molecules permitting pseudotype viral infections [16]. On the other hand, the basic properties of PERV envelope proteins are still unclear. In this study, to examine the binding properties of envelope glycoproteins for PERV receptors, we expressed two forms of soluble envelope protein, termed Env-ST and Env-SU, using a baculovirus expression system. Env-ST covered one-third of the N-terminal SU envelope protein containing VRA and VRB. Env-SU covered all of the SU of envelope protein. Using these proteins, binding and interference assays were performed in various mammalian cell lines.

2. Materials and methods

2.1. Cells

Mammalian cells, human embryonic kidney (HEK) 293, HeLa (human cervix adenocarcinoma), HT1080 (human fibrosarcoma), NIH3T3 (mouse embryonic fibroblast), *Mus dunni* (mouse fibroblast) and Mv1-Lu (mink lung) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MI, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml). TELCeB/pFBPERV-A and TELCeB/pFBPERV-B cells were used as a source of PERV pseudotype viruses and maintained in DMEM. Both MOLT-4 (human T lymphoblast) and 3201 (feline thymic lymphoma) cells were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics. An insect cell line *Spodoptera frugiperda* (*Sf*)9 was cultured in TC100 insect medium (Sigma) supplemented with 10% FCS. Another insect cell line, High-Five™, derived from *Trichoplusia ni*, was cultured in EX-CELL™ 405 medium (JRH BIOSCIENCES, Lenexa, KS, USA) in the absence of FCS.

2.2. Construction of mammalian expression plasmids and production of recombinant baculoviruses

According to the alignment of the amino acid sequences of the PERV envelope proteins (Fig. 1a), two soluble envelope proteins were designed. The DNA fragments encoding the N-terminal (Env-ST) and entire region (Env-SU) of SU of envelopes (Fig. 1b) were amplified by polymerase chain reaction (PCR) from the envelope expression plasmids pFBPERV-A and pFBPERV-B [16] as templates. PCR was performed using platinum® *pfx* polymerase (Invitrogen, Bedfold, MA, USA) and the primers listed in Table 1. Amplified fragments were digested with *Xba*I and *Sac*II, and then cloned into *Xba*I/*Sac*II sites of pcDNA3.1/myc-His B (Invitrogen) to add myc and His tags at the C-terminal of the SU proteins. DNA fragments encoding Env-ST and Env-SU tagged with myc-His epitopes, were excised with *Eco*RI/*Pme*I digestion and cloned into *Eco*RI/*Pme*I sites of the vector pCAG to produce mammalian expression vectors, pCAG/Env-STs {pCAG/PERVA(ST) and pCAG/PERVB(ST)} and pCAG/Env-SUs {pCAG/PERVA(SU) and pCAG/PERVB(SU)}. These fragments were also cloned into *Eco*RI/*Bgl*II sites of the vector pSG5 (Stratagene, La Jolla, CA, USA), and then *env* sequences followed by the SV40 polyA tail signal were cut out by *Xba*I digestion and cloned into the *Xba*I site of the baculovirus transfer vector pVL1392 {Becton Dickinson (BD), San Jose, CA, USA} to produce transfer vectors termed pVL/PERVA(SU), pVL/PERVB(SU), pVL/PERVA(ST) and pVL/PERVB(ST). To obtain recombinant baculoviruses, AcPERVA(SU), AcPERVB(SU), AcPERVA(ST) and AcPERVB(ST), transfer vectors were transfected to *Sf*9 cells with linear BaculoGold™ (BD). The recombinant baculoviruses were purified by plaque purification and inoculated onto *Sf*9 cells to obtain stocks of virus with higher titers. The DNA fragment encoding Env-SU of the amphotropic MLV (MLV-A) envelope was amplified by PCR from the envelope expression plasmid pFBASALF [17]. The amplified fragment was cloned into pcDNA3.1/myc-His B and excised by *Bam*HI/*Pme*I digestion. This fragment was cloned into *Bam*HI/*Bgl*II sites of the vector pSG5 and excised by *Xba*I digestion to produce pVL/MLVA(SU) as described above. The recombinant baculovirus Ac/MLVA(SU) was obtained by co-transfection of pVL/MLVA(SU) and linear BaculoGold™.

2.3. Expression and immunoblot analyses of the recombinant proteins

Mammalian expression plasmids, pCAG/Env-SUs, were transfected to HEK293T cells using FuGENE6 transfection reagent (Roche, Basel, Switzerland). Forty-eight hours after transfection, the culture supernatants and cells were harvested. Culture supernatants were centrifuged at 3500 rpm for 5 min to clarify the cell debris. Cells were washed twice with ice-cold phosphate-buffered saline {PBS(−)}, lysed with a lysis buffer (10% Triton X-100, 10% glycerol, and 0.5% CHAPS), and then soluble fractions were collected.

Stocks of recombinant baculoviruses were inoculated onto HighFive™ cells at a multiplicity of infection of 10. One hour after infection, cells were washed and cultured in non-serum EXCELL™ 405 medium. Three days after infection, culture supernatants and cells were harvested as described above.

Each sample was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were detected by immunoblotting using an anti-hexa-His monoclonal antibody (MAb) (TECHNE, Minneapolis, MN, USA) as described elsewhere.

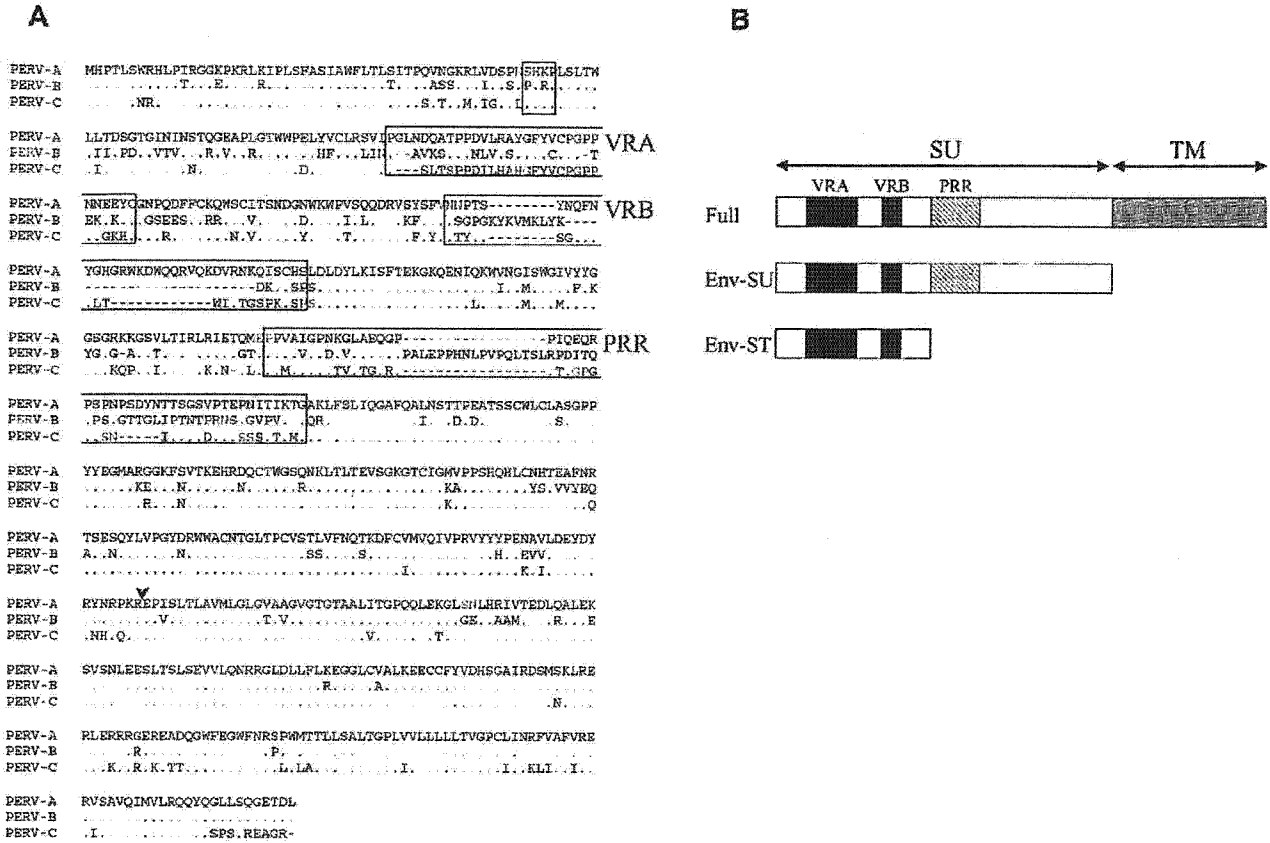

Fig. 1. (a) Comparison of the amino acid sequences of envelope proteins of PERV-A, PERV-B and PERV-C. Identical amino acids are shown with a dot and gaps are indicated with a dash. Two VRs (VRA and VRB), PRR and putative PHQV motif are enclosed with boxes. The cleavage site between SU and transmembrane envelope glycoproteins is indicated with an arrowhead. (b) Schematic representation of a full-length PERV envelope protein (Full) and truncated soluble envelope proteins (Env-ST and Env-SU) used in this study.

Table 1
Oligonucleotide primers used for PCR

Forward	PERV Fwd	5′-GCTCTAGACCACCATGCATCCCACGTTAAGCTG-3′
	MLV-A Fwd	5′-GCTCTAGACCACCATGGCGCGTTCAACGCTCTC-3′
Reverse	PERV-A ST	5′-TCCCCGCGGTTCCATCTGAGTTTCTATTCTGAGGCG-3′
	PERV-A SU	5′-TCCCCGCGGTCTTTTTGGCCGATTATATCTATAG-3′
	PERV-B ST	5′-TCCCCGCGGGGGTCCCACTGCCACAGGGG-3′
	PERV-B SU	5′-TCCCCGCGGATAGTCATATTCATCAAGGACC-3′
	MLV-A SU	5′-TCCCCGCGGTCTTTTATATTTGGTACGCTGTTCAAGC-3′

The PERV Fwd is a universal primer for all target sequences of PERVs. Underlines represent restriction enzyme recognition sites.

2.4. Deglycosylation analyses

Harvested protein samples were deglycosylated using endoglycosidases, EndoH and PNGaseF (New England Biolabs, Beverly, MA, USA), according to the manufacturer's instructions. In brief, samples were denatured by boiling for 10 min at 100 °C in the presence of 0.5% SDS and 1% β-mercaptoethanol. Then, reaction buffer and the endoglycosidase were added to the samples and incubated for 1 h at 37 °C. PNGaseF treatment was carried out in the presence of 1% Nonidet P-40. Following the deglycosylation, each sample was subjected to 12% SDS-PAGE and separated proteins were detected with the anti-His MAb.

2.5. Titration of PERV pseudotype viruses

The titration of PERV pseudotype viruses was performed as described previously [18] with a slight modification. In brief, target cells were seeded at a concentration of 2.5×10^4 cells in 0.25 ml per well of 48-well plates the day before infection. Culture supernatants from producer cell lines were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) and serially diluted. These diluted samples were inoculated onto target cells in the presence of 8 μg/ml polybrene. Four hours after inoculation, the cells were washed once and incubated with fresh medium for a further 2 days. Cells were

stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and *lacZ*-positive foci were counted.

2.6. Binding assays of soluble envelope proteins

Target cells were detached from culture flasks by treatment with PBS(–) containing 0.05% EDTA. Approximately 10^6 cells were suspended in 500 μl of culture medium, then mixed with an equal volume of culture supernatant from HighFive™ cells infected with recombinant or wild-type baculoviruses. The mixtures were incubated at 4 °C for 1 h with gentle rotation. After three washes with PBS(–) containing 1% FCS, the cells were incubated with 100 μl of 1:200 diluted anti-tetra-His MAb (Qiagen, Hilden, Germany) for 1 h on ice and washed. Then, cells were incubated with 100 μl of 1:200 diluted anti-mouse IgG (H + L) conjugated with phycoerythrin (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h on ice. After washing, fluorescence intensity was analyzed with a cytofluorometer FACSCalibur (BD). Then, mean fluorescent intensity was read from histograms and relative binding intensity was calculated based on control mean fluorescent intensity.

2.7. Purification of Env-SU proteins and interference assays

Env-SU proteins of PERV-B and MLV-A were purified from culture supernatants of HighFive™ cells by using Ni-NTA agarose beads (Qiagen). In brief, 100 ml of supernatant was added to 500 μl of 50% Ni-NTA slurry and incubated for 14 h at 4 °C with gentle rotation. Then, the beads were collected using an empty column and washed twice with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). After the wash, Env-SU proteins were eluted with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) and flow-through fractions were collected. The collected proteins were dialyzed to remove the imidazole and analyzed by SDS-PAGE to check their purity.

The interference assays of pseudotype viruses were performed as described previously [19] with a slight modification. In brief, Mv1-Lu cells were seeded at a concentration of 2.5×10^4 cells in 0.25 ml per well of 48-well plates the day before infection. The purified proteins were serially diluted and added to the target cells prior to infection. After 30 min of pre-incubation, DMEM containing 100 focus-forming units (ffu) of PERV-B or MLV-A pseudotype virus was inoculated onto the target cells. Four hours after inoculation, the cells were washed once and incubated with fresh DMEM for a further 2 days. Cells were stained with X-gal and *lacZ*-positive foci were counted.

3. Results

3.1. Alignment of the amino acid sequences of PERV envelope proteins

The alignment of the amino acid sequences of envelope proteins of PERV-A, PERV-B and PERV-C are shown in Fig. 1a. There are two VRs, VRA and VRB, in the N terminus of the SU region followed by PRR. PRR has extensive variations and the sequence identity of this region is even lower than that of VRA.

3.2. Expression of recombinant envelope proteins

Culture supernatants and cell lysates of HEK293T cells transfected with expression plasmids and HighFive™ cells infected with recombinant baculoviruses were harvested. Each sample was separated by SDS-PAGE under reducing conditions, and then the separated proteins were detected by immunoblotting (Fig. 2). The Env-ST proteins of both PERV-A and PERV-B were detected in cell lysates and efficiently released into culture supernatants of HEK293T cells (Fig. 2a, upper panel). The approximate molecular weights were 27 kDa, coinciding with a previous report on MLV [20]. However, though Env-SU proteins from HEK293T were detected in the cell lysates, they were not released into culture supernatants efficiently (Fig. 2a, lower panel). On the other hand, in the baculovirus expression system, both Env-SU and Env-ST proteins were detected in the culture supernatants and cell lysates (Fig. 2b).

To examine the glycosylation-properties, the proteins from culture supernatants of HighFive™ cells infected with recombinant baculoviruses were deglycosylated by EndoH and PNGaseF. Immunoblot analyses after deglycosylation revealed that Env-ST and Env-SU were attached with carbohydrates after translation (Fig. 3). Env-ST proteins were attached with EndoH susceptible high-mannose-typed carbohydrates. On the other hand, almost all carbohydrates attached to Env-SU proteins were resistant to EndoH and partially digested by PNGaseF.

Deglycosylation analyses were also carried out using soluble envelope proteins expressed in HEK293T cells. Cell lysates of HEK293T cells transfected with expression plasmids were treated with endoglycosidases. Immunoblot analyses after deglycosylation showed the same glycosylation pattern with the baculovirus expression system (data not shown).

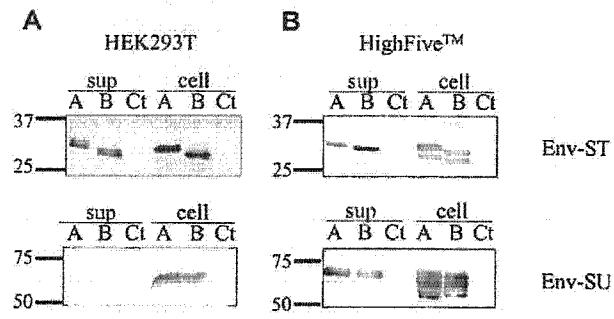

Fig. 2. Immunoblot analyses of Env-ST (upper panel) and Env-SU (lower panel) proteins expressed by mammalian (a) and baculovirus expression systems (b). (a) HEK293T cells was transfected with mammalian expression plasmids (pCAG) of envelopes of PERV-A (lane A) and PERV-B (lane B). pCAG empty plasmid was used as a control (lane Ct). (b) HighFive™ cells was infected with recombinant baculoviruses expressing envelopes of PERV-A (lane A) and PERV-B (lane B). The baculovirus AcNPV was used as a control (lane Ct).

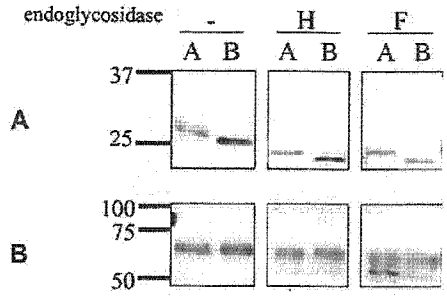

Fig. 3. Immunoblot analyses of Env-ST (a) and Env-SU (b) proteins in the culture supernatants from HighFive™ cells after the treatment with endoglycosidases, EndoH (H) and PNGaseF (F). Soluble envelope proteins of PERV-A (lane A) and PERV-B (lane B) were digested with the endoglycosidases indicated at the top of each panel.

3.3. Susceptibilities of various mammalian cell lines to PERV pseudotype viruses

PERV pseudotype viruses were titrated in various mammalian cell lines (Table 2). Pseudotype viruses possessing Moloney MLV cores were used to avoid post-entry restriction [21]. Consistent with a previous report [16], Mv1-Lu and HEK293 cells were susceptible to both PERV-A and PERV-B pseudotype viruses. Murine cell lines, NIH3T3 and *M. dunni*, showed susceptibility to PERV-B but not to PERV-A pseudotype viruses. HEK293 cells persistently infected with PERV-A or PERV-B were resistant to superinfections of each pseudotype virus. Interestingly, the HEK293 cells infected with PERVs were significantly more susceptible to the pseudotype viruses of heterologous subtypes than unifected HEK293 cells. Though the reason for the phenomenon is unknown at present, PERV may assist the infection of heterologous subtype viruses with each other.

3.4. Binding properties of soluble envelope proteins in mammalian cells

The binding assays were conducted on a PERV-susceptible cell line (HEK293) and unsusceptible cell line (MOLT-4) by using Env-ST and Env-SU proteins expressed by the recombinant baculoviruses (Fig. 4). PERV-B Env-ST efficiently bound to both HEK293 and MOLT-4 cells, whereas PERV-A Env-ST did not bind to either. In contrast, PERV-B Env-SU strongly bound to HEK293 but not to MOLT-4 cells, and PERV-A Env-SU bound to HEK293, but not to MOLT-4 cells. To know the binding specificity, we carried out binding assays on HEK293 cells persistently infected with PERV-A or PERV-B. Irrespective of the persistent infection of PERVs, PERV-A Env-ST did not bind to HEK293 cells and PERV-B Env-ST bound to HEK293 cells. On the other hand, Env-SU proteins of PERV-A and PERV-B did not bind to HEK293 cells infected with PERV-A (293/PERV-A) and PERV-B (293/PERV-B), respectively (Fig. 4).

Table 2
The titers of pseudotype virus in various target cells

Target cells	Source	Titer (ffu/ml) of *lacZ* pseudotype[a]	
		PERV-A	PERV-B
Mv 1-Lu	Mink	55,000	26,500
NIH3T3	Mouse	<10	150
M. dunni		<10	1300
3201	Feline	<10	<10
HEK293	Human	1000	200
293/PERV-A[b]		<10	3050
293/PERV-B[b]		6050	<10
HeLa		400	30
MOLT-4		<10	<10
HT1 080		<10	<10

[a] Averages of titers expressed as ffu of *lacZ* pseudotype viruses in two independent experiments are shown.
[b] HEK293 cells persistently infected with PERV-A or PERV-B.

The binding assays were extended to various mammalian cell lines and the relative binding intensity was calculated (Table 3). There was no binding of PERV-A Env-ST in the cell lines as observed in HEK293 and MOLT-4 cells. PERV-B Env-ST showed non-specific binding properties to non-susceptible cell lines such as 293/PERV-B and MOLT-4 cells. PERV-A Env-SU showed specific binding to all PERV-A-susceptible cell lines except for Mv1-Lu cells. Although PERV-A pseudotype virus infects Mv1-Lu cells very efficiently (Table 2), there was no binding between PERV-A Env-SU and Mv1-Lu cells. PERV-B Env-SU bound to various cell lines susceptible to the PERV-B pseudotype virus.

3.5. Interference of pseudotype viruses with soluble envelope proteins

The inhibition assays on HEK293 and Mv1-Lu cells were performed using purified Env-SUs of PERV-B and MLV-A. In HEK293 cells, MLV-A Env-SU specifically inhibited the infection of MLV-A pseudotype virus efficiently and PERV-B Env-SU also inhibited the infection of PERV-B pseudotype virus (about 70% reduction by addition of 10 μg/ml of purified PERV-B Env-SU) (data not shown). In Mv1-Lu cells, the infection of MLV-A pseudotype virus was efficiently inhibited by MLV-A Env-SU (Fig. 5a). This inhibition was specific because the envelope did not interfere with PERV-B pseudotype virus infection (Fig. 5b). However, Env-SUs of PERV-B and MLV-A did not interfere with PERV-B pseudotype virus on Mv1-Lu. These inhibition assays were repeated with crude Env-SU proteins from culture supernatants of HighFive™ cells infected with Ac/PERVB(SU) and Ac/MLVA(SU) and similar results were obtained (data not shown).

4. Discussion

The glycosylation of envelope protein is essential for the proper folding of receptor binding sites in human immunodeficiency virus [22,23]. In addition, in Friend MLV, secretion of Env-SU was prevented when N-linked oligosaccharide was eliminated [24]. Previously we found that PERV infection requires N-linked carbohydrates in the envelope pro-

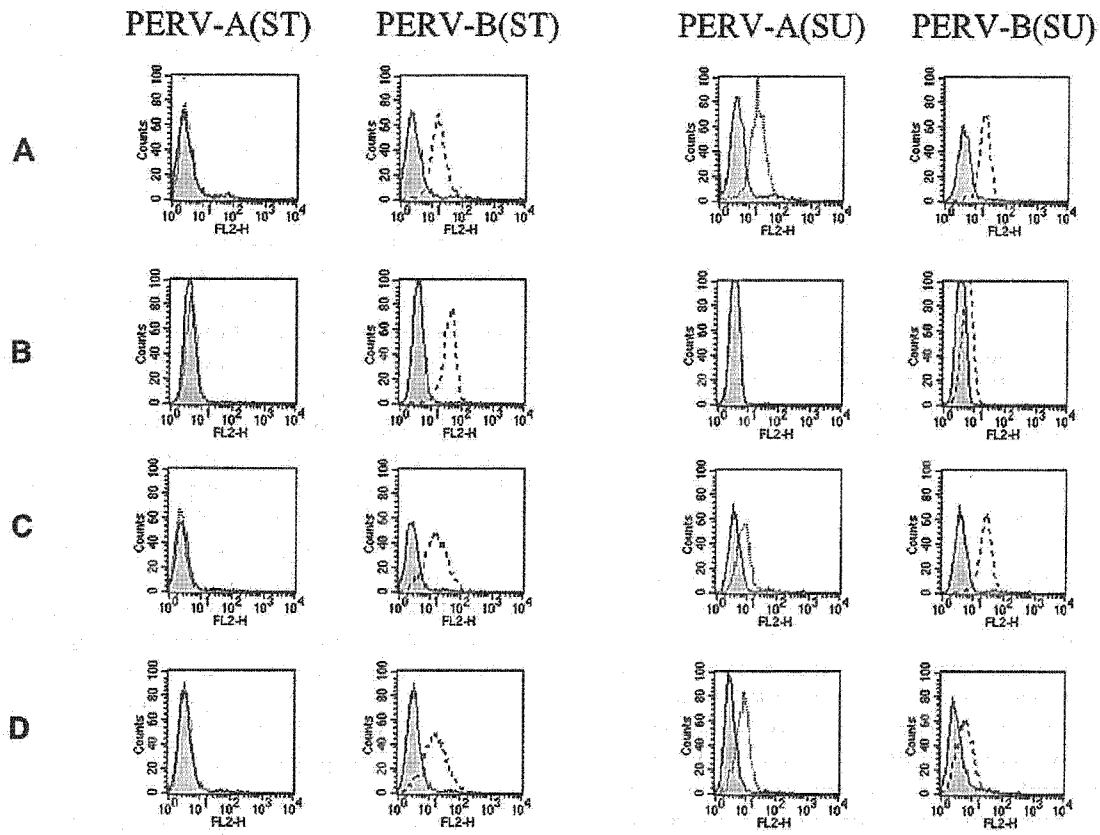

Fig. 4. Binding properties of PERV envelope proteins to HEK293 (a) and MOLT-4 (b). HEK293 cells persistently infected with PERV-A (c) and PERV-B (d) were also used as target cells. The *X*-axis represents the fluorescence intensity of control (gray filled), PERV-A Env (dotted line) and PERV-B Env (broken line). The soluble envelope proteins used are indicated at the top of each panel.

teins [25]. Although there are differences in the glycosylation pathways between insect and mammalian cells [26], many groups have used recombinant glycoproteins expressed by baculovirus systems. By using the recombinant proteins, binding properties of envelope proteins of amphotropic MLV, dengue virus and feline leukemia virus (FeLV) have been investigated [19,27,28]. As the first step in this study, deglycosylation experiments were performed using two endoglycosidases (EndoH and PNGaseF) to reveal the glycosylation properties of the recombinant proteins with a baculovirus expression system. EndoH digests high-mannose-typed carbohydrates and PNGaseF digests both high-mannose-typed and complex-typed carbohydrates [29]. Immunoblot analyses after endoglycosidase treatments revealed that both Env-ST and Env-SU had been modified with carbohydrates. The carbohydrates of Env-ST proteins were completely removed by EndoH treatment, indicating that high-mannose-typed carbohydrates were attached to the proteins. On the other hand, the great majority of carbohydrates attached to Env-SU was resistant to EndoH and only partially removed by PNGaseF treatment, indicating that these proteins were modified with O-linked carbohydrates. The reason for this different modification is unclear at present. However, it is possible that the conformations of the truncated Env-ST proteins are different from those of Env-SU proteins, influencing the carbohydrate-modification in the cells. Previously, it had been reported that retroviral envelope glycoproteins were modified with both N-linked and O-linked carbohydrates [30,31]. Since we needed large amounts of soluble envelope proteins modified with carbohydrates for the assays, we used recombinant envelope proteins expressed by the baculovirus expression system.

Table 3
Relative binding intensities of PERV envelopes in various mamalian cells

	PERV-A[a]		PERV-B[a]	
Target cells	Env-ST	Env-SU	Env-ST	Env-SU
Mv1-Lu	–	–	++	++
NIH3T3	–	–	+	+
Mus dunni	–	–	++	+
3201	–	–	–	–
HEK293	–	+	+	+
293/PERV-A	–	–	+	+
293/PERV-B	–	+	+	–
HeLa	–	+	+	+
MOLT-4	–	–	++	–
HTI080	–	–	–	–

[a] Represents relative binding intensity under 2.5; +, 2.5–10.0; ++, > 10.0. At least two independent experiments were carried out.

First we used Env-ST proteins for the binding assays because Env-ST contains the putative RBD and Env-ST proteins have been used for binding assays in MLV studies [27]

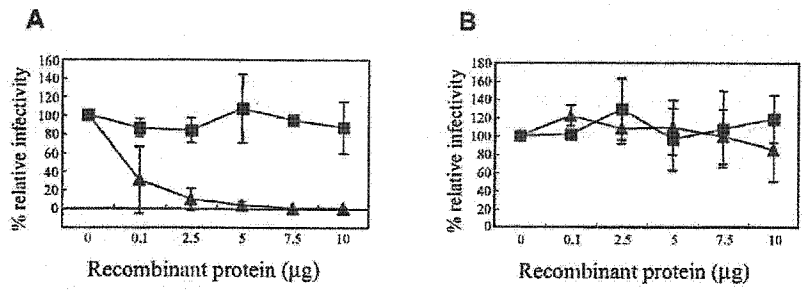

Fig. 5. Interference assays using purified Env-SU of MLV-A (a) and PERV-B (b). One hundred focus forming units of PERV-B (■) and MLV-A (▲) pseudotype viruses were inoculated onto the Mv1-Lu cells in the presence of serially diluted purified Env-SU proteins. Two days after infection, cells were stained with X-gal and *lacZ*-positive foci were counted.

and receptor cloning for PERV-A [32]. HEK293 and MOLT-4 cells were selected as representative target cells that showed high and no susceptibility to PERV pseudotype viruses, respectively. Contrary to our expectation, PERV-A Env-ST did not bind either cell line whereas PERV-B Env-ST bound both. These results suggest that the conformation of RBD might not be retained properly in the Env-ST form. Then, we also used Env-SU proteins in the binding assays. Env-SUs from both PERV-A and PERV-B bound to HEK293 cells but not to MOLT-4 cells, suggesting that there was a specific interaction between PERV Env-SUs and receptor molecules. The binding specificity was confirmed in assays using HEK293 cells persistently infected with PERVs. Previously, two molecules were cloned as cellular receptors for PERV-A from HeLa cells, and designated as HuPAR-1 and HuPAR-2 [32]. PERV-A preferred HuPAR-2 to HuPAR-1, and PERV-A Env-ST bound specifically to HuPAR-2 but not HuPAR-1 [32]. It is possible that PERV-A Env-ST binds specifically to HuPAR-2 but this binding does not reflect the natural condition. In the present study, HEK293 cells may predominantly express only HuPAR-1 since PERV-A Env-ST bound to HEK293 cells transfected with the expression plasmid for HuPAR-2 (data not shown). PERV-A Env-SU might have bound to HuPAR-1 expressed on HEK 293 cells.

Next, we applied the binding assays to various mammalian cell lines. The binding properties of PERV-A Env-SU correlated fairly well with the susceptibility to PERV-A pseudotype virus and those of PERV-B Env-SU completely coincided with the susceptibility to PERV-B pseudotype virus (Table 3). However, there was one exception; PERV-A Env-SU did not bind to Mv1-Lu cells, which are highly susceptible to the pseudotype virus. From the results obtained in HEK293 and 293/PERV-A, PERV-A Env-SU surely interacts with the PERV-A receptor(s). There are two possible explanations for this phenomenon. First, the affinity of PERV-A Env-SU prepared in this study for the PERV-A receptor(s) of minks was very low. Another possibility is that PERV-A infected the cells via an alternative pathway (i.e. cognate receptor-independent pathway) by virtue of the envelope protein of an endogenous retrovirus as reported in an immunodeficiency-inducing variant of FeLV [33,34].

Finally, we performed interference assays with the pseudotype viruses in HEK293 and Mv1-Lu cells by using purified recombinant envelope proteins of PERV-B and MLV-A. In HEK293 cells, both recombinant proteins inhibited the infection of the homologous pseudotype viruses. However, PERV-B Env-SU failed to block the infection by PERV-B pseudotype virus on Mv1-Lu even at high concentrations. Although the reason for this phenomenon is unclear, PERV-B pseudotype virus might have infected the cells via an alternative pathway instead of through its cognate receptor(s) on Mv1-Lu. Quite recently Lavillette et al. [35] reported that both PERV-A and PERV-B can infect non-susceptible cells when the envelope protein of gibbon ape leukemia virus (GaLV) is added in trans. It is possible that Mv1-Lu cells express an endogenous retrovirus similar to GaLV. Actually, Mv1-Lu cells have been reported to express an endogenous retrovirus, although the characteristics of the virus are unknown [36]. GaLV utilizes Pit-1 as a receptor sharing with subgroup B FeLV (FeLV-B) [37] and we also reported that Mv1-Lu cells were partially resistant to FeLV-B pseudotype virus [38]. The Mv1-Lu cells seem not to express envelope protein of endogenous FeLV-related virus that interferes with FeLV-B because endogenous FeLV-dependent FeLV-T does not infect Mv1-Lu cells (data not shown). These data may suggest that Mv1-Lu cells express GaLV-like endogenous retrovirus [39] but not FeLV-like one. Molecular cloning of the endogenous retroviruses expressed in the Mv1-Lu cells is needed to elucidate the mechanism of PERV infection to the cells.

In this study we revealed that PERV Env-STs do not bind to PERV receptors specifically and that the entire region of SU Env is required for a specific binding. These properties are similar to those observed in xenotropic and polytropic MLVs but not those in ecotropic and amphotropic MLVs [13]. As our results suggested that both PERV-A and PERV-B infected Mv1-Lu cells via a cognate receptor-independent pathway, PERVs may infect non-susceptible cells efficiently by virtue of endogenous retrovirus expressed in humans. Further studies are required to verify this possibility and to evaluate the risks of PERV infections in the xenotransplantation of pig organs to humans.

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特集 「異種移植」

ブタ内在性レトロウイルスと異種移植

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はじめに

ヒトおよび類人猿と旧世界ザルでは，進化の過程で約3,000万年前に糖鎖を合成する酵素の一つであるα-1,3ガラクトース転移酵素（α-1,3GT）の遺伝子が不活化された[1]。そのため，ヒトはα-ガラクトース（α-Gal）抗原を持たず，外来抗原として認識し，自然界に存在するα-Gal抗原の曝露により抗α-Gal抗体が大量に誘導されている。この抗体は，他の動物由来のエンベロープウイルスの感染防御に役に立っている[2]。他の動物の細胞で増殖したエンベロープウイルスは，エンベロープにα-Gal抗原を持ち，ヒトがこのウイルスに曝露されたとしても，抗α-Gal抗体と補体により速やかにウイルスが中和され，感染は成立しにくいのである。

現在，臓器移植のための臓器不足の解消や，新しい細胞移植治療法の開発に向けて，ブタの臓器や細胞を異種間移植する方法が活発に研究されている[3]。ブタの細胞はα-Gal抗原を大量に発現しているため，ヒトの抗α-Gal抗体と補体を介する超急性拒絶反応が起こるが，これを抑えるために補体制御遺伝子や糖転移酵素遺伝子を導入したブタ，さらにはα-1,3GTそのものをノックアウトしたブタなどが開発されている[4-7]。しかし，そのような手法は移植を成立させる一方で，抗α-Gal抗体を介したウイルスに対する自然抵抗性を減弱させてしまう。そのため，これらの遺伝子改変ブタからのウイルスが患者に感染し，さらにはヒト社会に広まる危険性がある。

ヒトに感染する可能性があるブタのウイルスのうち最大の問題は，ブタ内在性レトロウイルス（porcine endogenous retrovirus：PERV）である[8]。内在性レトロウイルスは生まれつきすべての体細胞のゲノムに存在するため，ブタから取り除くことは現時点では不可能である。1996年にヒトの細胞に感染するPERVが2種類（PERV-A，PERV-B）発見された（AとBはそれぞれ異なる受容体を認識する）[9]。PERVはヒトの細胞で増殖はするが，一般にその増殖力は弱い。しかし，わずかな変異によりヒトに馴化したウイルスが生じたり，ヒトの内在性レトロウイルスと組み換わることで，より増殖性の高いウイルスが出現する可能性もある[10]。PERVはブタでは病原性を持たないが，突然変異や組み換えにより，病原性を発現する可能性がある。そして，その感染は移植患者だけではなく，社会全体にも広がることも危惧される。従って，ブタを用いた異種間臓器移植の実用化には，PERVの制御が必要不可欠である。そのためには，PERVの産生抑制法の開発，感染メカニズムの解析および高感度検出系の確立が求められる（**図1**）。

PERVの考えられる制御法

PERVは，ブタのゲノムからmRNAとして転写され，mRNAからウイルス構成蛋白が合成され，最

終的に感染性のあるウイルス粒子として放出される。PERVのウイルスゲノムは，ブタのゲノムのなかに50カ所以上存在するが，完全な感染ウイルス粒子として翻訳されるmRNA転写部位は数カ所である。しかしながら，PERVも含めたレトロウイルスは不完全なmRNAゲノム同士で組み換えを起こし，不完全な部分をお互いに補い合うことがある。従って，不完全なmRNAゲノム転写物と言えども，極力抑えることが重要である。

mRNAの転写物を抑える方法には，①RNA interference（RNA干渉）を利用する方法，②リボザイムによるmRNAの破壊がある。これは，すでに確立されている手法であるが，実際には標的部位の選択が重要である。RNA干渉を利用して，PERVのmRNAの発現を抑制したとの結果を，ドイツのDenner博士らのグループがすでに発表している[12]。国内でも同様の手法で，大阪大学と帯広畜産大学の研究グループがRNA干渉を用いた方法によるPERV mRNAの発現抑制を報告している[13]。リボザイムによるmRNAの破壊については，PERVへの応用例は国内外ともにまだ報告されていない。東京大学工学部の多比良和誠教授らが開発したマキシザイム（maxizyme）は，標的となるmRNAへの特異性が高い上に分解効率も高いので，PERVへの応用が期待できる[14,15]。

mRNAが産生されると，次いでウイルス蛋白が産生される。ウイルスのコア蛋白（Gag蛋白）のなかにPERVのゲノムRNAが組み込まれ，細胞外に放出される。レトロウイルスのGag蛋白のlate assembly（L）domainは，細胞からウイルスが放出される際に重要である。L domainに結合する細胞側の蛋白であるlate domain interacting（LDI）蛋白は，ウイルスの放出を助ける。LDI蛋白のドミナントネガティブ変異体は，ウイルスの放出を阻害する。この現象を利用し，PERVのL domainに結合する細胞側の蛋白のドミナントネガティブ変異体を作製することにより，ウイルスのコア蛋白が産生されても，ウイルスが放出されないようにすることができる。

ウイルスの内部には，RNAのウイルスゲノムがパッケージングされる。通常のウイルスゲノムRNAはウイルス粒子のなかでコア蛋白によって守られている。しかし，このコア蛋白にRNAを分解する酵素を融合させることにより，取り込まれたRNAを積極的に分解させることが可能である。実際に同じガンマレトロウイルスで，この手法によりウイルスの産生を抑制に成功した例が報告されており，PERVへの応用が期待される[16-21]。

ヒトは，NIHマウスに感染するマウス白血病ウイルス（N-tropic MLV）に対して抵抗性を有している[22]。この抵抗性は*Ref-1*因子と名付けられている。*Ref-1*因子の本態は長らく不明であったが，最近それがTRIM5αという分子であることが明らかとなった[23,24]。TRIM5αはN-tropic MLVのコア蛋白と相互作用して，細胞内に侵入したウイルスのコアを分解経路に導くと考えられている（図2）。TRIM5αが認識するウイルス

図1　豚内在性レトロウイルス（PERV）の制御法の開発

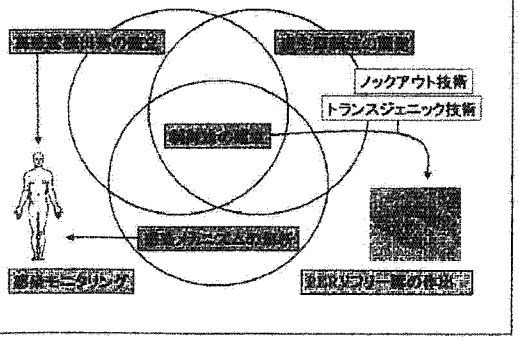
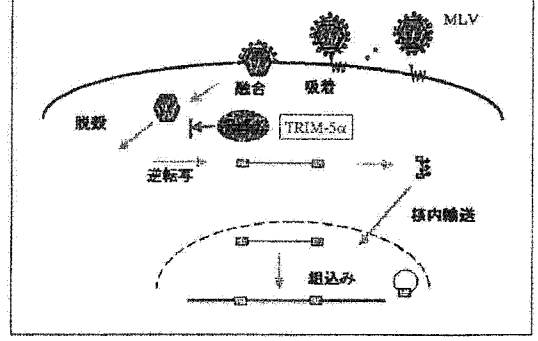


図2　*Ref-1*（TRIM-5α）の作用機序



コアの高次構造はまだ分かっていないが，PERVのコア蛋白に変異を導入することで，TRIM5αに認識されるコア蛋白を作出できる可能性がある。TRIM5αに認識されるPERV改変コア蛋白を遺伝子改変ブタに発現させれば，そのブタから産生されるPERV粒子は改変コア蛋白を取り込み，たとえヒトの細胞内に侵入したとしても，TRIM5αに認識され分解されると考えられる。

これ以外にもPERVを制御する方法がいくつか考えられる。どの方法がPERVの制御において最も有効であるかは，今後の研究で明らかにされるであろう。トランスジェニック技術では，有効な方法を組み合わせることも可能である。従って将来的には，移植した臓器から産生されるヒトに感染するウイルス量を理論上ゼロにすることも可能であると思われる。しかし，ヒトに感染するPERVの産生を理論上「ゼロ」に抑えたとしても，安全性の検証には実験動物モデルが必要である。現在までに，PERV-Aの受容体のクローニングが成功しており[25]，トランスジェニック技術によりマウスで感染実験モデルの作出が試みられている。今後PERV-Bの受容体のクローニングが成功すれば，ヒトに感染するすべてのPERV（PERV-AおよびPERV-B）の実験動物モデルも作出可能になると思われる。

最近，PERVの感染がギボンザル白血病ウイルス（GaLV）のエンベロープ蛋白により増強される現象が発見された[26]。さらに，PERVの受容体が細胞表面上に存在しなくても，GaLVのEnvを利用して細胞内に侵入可能であることも明らかとなった。同様に，ミンクの肺由来細胞であるMv1-Lu細胞にPERV-Aが感染するときも，PERV受容体非依存性の感染が示唆されている[27]。この現象を利用して，新たなPERV感染動物モデルの作出につながる可能性があり，今後の研究が期待される。

おわりに

今後数年以内に感染性のあるPERVが計算上産生されないブタが産出される可能性が高い。PERVの産生が完全に制御されれば，ブタを用いた異種移植への道は開かれるものと考えられる。PERVの産生が検出されないブタの作出に成功したとしても，PERVの産生を完全に否定することはできない。また，PERV以外のヒトに感染性のある内在性レトロウイルスは，今のところ見つかってはいないが，存在しないことを証明することは事実上不可能である。この点については，PERVを理論上産生しないブタの臓器や組織，あるいは細胞を実際に感受性のある動物に接種して，安全性を検定する以外に現時点では方法はないと考えられる。

現時点で考えられるすべての可能性に対して万全の備えをし，万が一，PERVやその他の未知のウイルスがヒトに感染したときにも対応できるように，追跡システムの確立も重要であると思われる。

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