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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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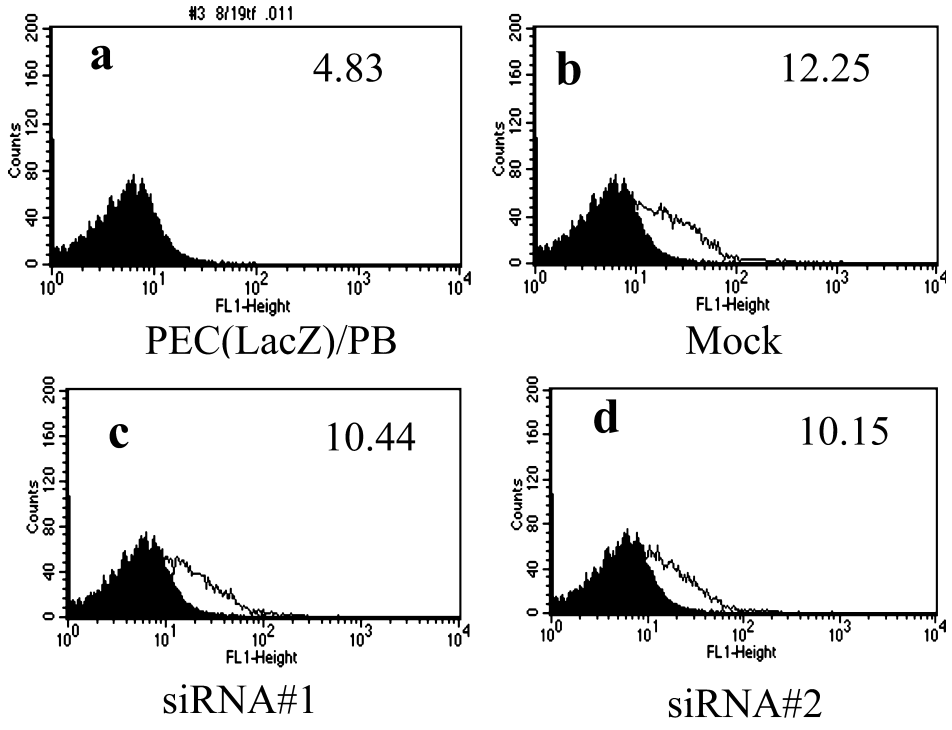

Fig. 1. **FACS analysis of GFP expression in PEC(*LacZ*)/PB transfected with pSXGH-siRNA.** An expression vector containing GFP and siRNA was transfected into PEC(*LacZ*)/PB cells. The level of expression of GFP on the cell surface was checked as an alternate to assessing the expression of siRNA by flow 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.

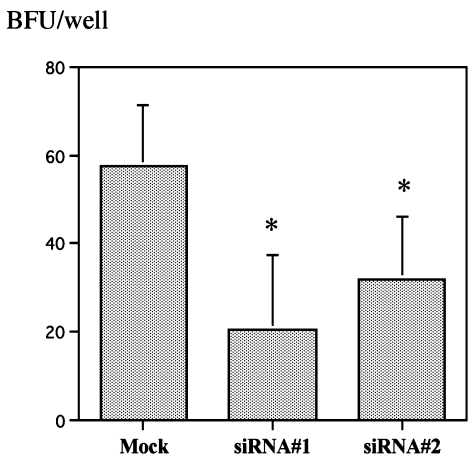

Fig. 2. **siRNA directed silencing on PERV infectivity.** PEC(*LacZ*)/PB was transfected with pSXGH-siRNA, and the culture supernatants collected from them were added to the medium of HEK293 cells. Target HEK293 cells were histochemically stained, and *lacZ*-positive BFU were counted, to determine viral 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

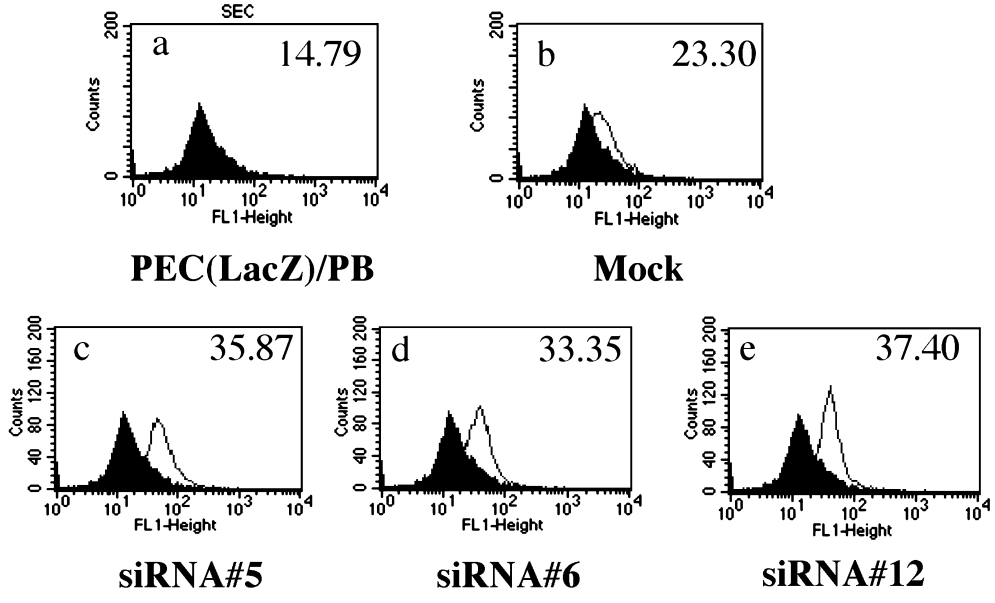

Fig. 3. **FACS analysis of GFP expression in stable clones of PEC(*LacZ*)/PB transfected with pSXGH-siRNA.** The expression levels of GFP on the cell surface of single PEC(*LacZ*)/PB clones were checked by flow cytometry. Typical flow cytometric histograms for each established clone are shown (open histogram). 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.

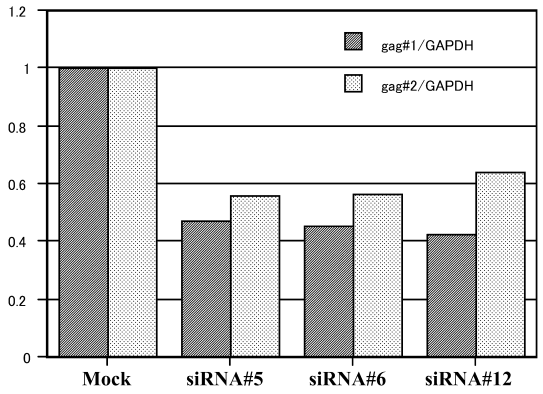

Fig. 4. **Real time PCR for the mRNA of a PERV in a PEC(*LacZ*)/PB transfectant.** To evaluate the degradation of PERV gag mRNA, SYBR-Green real-time PCR was performed with the Smart Cycler II System. The amount of gag mRNA for each of the PEC(*LacZ*)/PB transfectants was measured using two pairs of 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.

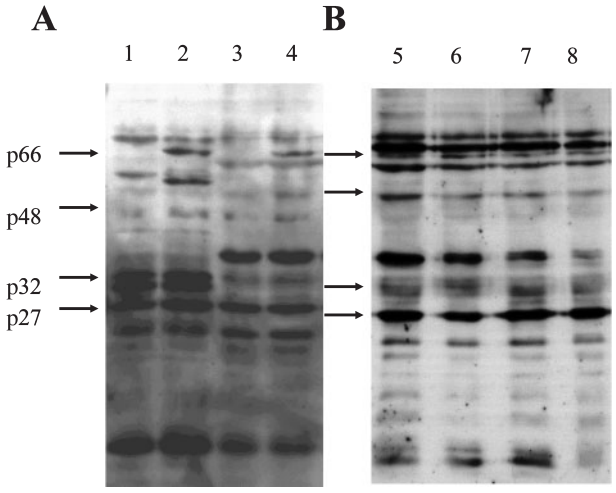

Fig. 5. **Western blot analysis of PERV p27gag expression in the stable transfectants with pSXGH-siRNA.** PEC, PEC(*LacZ*)/PB transfectants and HEK293 cells were solubilized in SDS. For each lane, 30 μg of tatal cell lysate was loaded, and stained with the anti-gag peptide, LRGASRRPTNLAKVC, antibody. 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

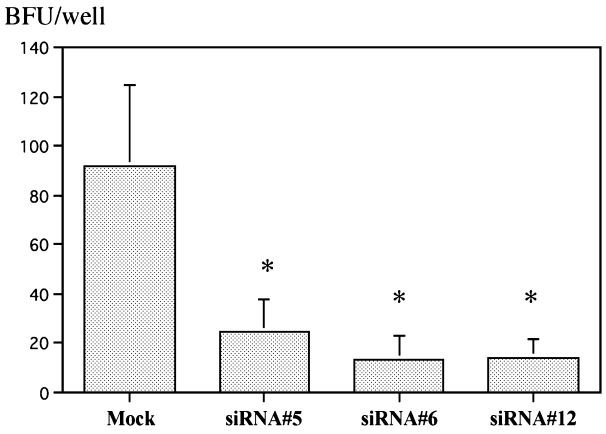

Fig. 6. **The effect of siRNA directed silencing on PERV infectivity Culture supernatants collected from PEC(*LacZ*)/PB transfectants with pSXGH-siRNA were added to the medium of HEK293 cells.** Target HEK293 cells were histochemically stained, and *lacZ*-positive BFU were counted to determine the viral 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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