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ORIGINAL ARTICLE

A newly cloned pig dolichyl-phosphate mannosyltransferase for preventing the transmission of porcine endogenous retrovirus to human cells

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Keywords
dolichyl-phosphate mannosyltransferase, high mannose type, porcine endogenous retrovirus, xenotransplantation

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Summary

Porcine endogenous retrovirus (PERV) is a major problem associated with successful clinical xenotransplantation. In our previous study, reducing the high mannose type of *N*-glycan content proved to be very effective in downregulating PERV infectivity. In this study, dolichyl-phosphate mannosyltransferase (D-P-M), an enzyme related to the early stages of *N*-linked sugar synthesis was studied. The pig cDNA of the encoding D-P-M was newly isolated. The RNA interference (siRNA) for the D-P-M was applied and transfected to PEC(Z)/PB cells, a pig endothelial cell line with the *Lac* Z gene and PERV-B, to reduce the levels of high mannose type *N*-glycans. Compared with the mock line, the temporary PEC(Z)/PB lines showed a decreased mRNA expression for pig D-P-M, and each line then showed a clear destruction of PERV infectivity to human cells in the *Lac* Z pseudotype assay. The PEC(Z)/PB was next transfected with pSXGH-siRNA, H1-RNA gene promoter. The established PEC(Z)/PB clones with pSXGH-siRNA clearly led to the downregulation of PERV infectivity, as evidenced by the decreased levels of the mRNA for pig D-P-M. Reducing D-P-M enzyme activity represents a potentially useful approach to address the problem of PERV infections in clinical xenotransplantations.

Introduction

Xenotransplantation offers a potential solution to the worldwide shortage of available organs for transplantation and the pig represents an ideal source of such organs [1]. However, the discoveries that porcine endogenous retroviruses (PERV) are able to infect human cells *in vitro* and mice that are transgenic for human PERV-A receptor 2 *in vivo* have stimulated considerable discussion regarding the infectious risk associated with such xenotransplantations [2–4]. At least several proviral copies of PERV-A, B, and C are present in the genome of the pig [5]. In addition, possible recombinations between PERV-A and PERV-C cannot be excluded [6]. Therefore, the production of pigs that are completely devoid of all PERV-related elements is unlikely ever to be realized.

The infectivity of PERV released from pig cells to human cells has been investigated by many researchers [7,8]. Currently, although PERV-related infections have never been detected in human and nonhuman primates that have been exposed to pig xenografts [9,10], the possibility that they could arise as a consequence of xenotransplantation still remains.

In our previous work, we demonstrated the effects of the remodeling of pig cell-surface glycoproteins, especially *N*-glycans, with reference to not only the susceptibility of PERV to human serum but the infectivity to human cells as well, using pig endothelial cell (PEC) transfectants that

produce different glycosyltransferases [11]. As the next step, we also showed that the high mannose type of *N*-glycan, contained by the envelope glycoprotein, is closely related to PERV infectivity with respect to human cells. Inhibition of the terminal α-glucose residues from the precursor *N*-glycan by treatment with castanospermine, 1-deoxynojirimycin attenuated PERV infectivity, whereas both mannose inhibitors, 1-deoxymannojirimycin and swainsonine, upregulated infectivity. In addition, treatment with α-mannosidase and incubation with concanavalin A completely abrogated the transmission of PERV to human cells [12]. We also addressed the effects of the remodeling of pig cell-surface glycoproteins, especially the high mannose type of *N*-glycan, on the susceptibility of PERV by the processing enzymes [13].

In this study, as an alternate strategy, dolichyl-phosphate mannosyltransferase (D-P-M) [14], an enzyme that is associated with the early stages of *N*-linked sugar synthesis, was examined in a strategy directed at preventing PERV infectivity.

Materials and methods

Cell cultures

Pig endothelial cells and human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) with L-glutamine and kanamycin/amphotericin. The cultures were maintained in a 5% CO_2/95% air atmosphere at 37 °C [15].

Cloning of pig dolichyl-phosphate mannosyltransferase polypeptide

The DNA sequence of pig D-P-M was queried in dbEST (NCBI) using the TBLASTIN program to search for cDNA fragments of D-P-M. Primers were identified, based on the expressed sequence tag (EST) cloning data. Total RNA was then isolated from a fresh pig liver using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). An oligoTex-dT30 mRNA purification kit was used for the mRNA preparation, following the manufacturer's recommended protocol (Takara, Tokyo, Japan). A portion of purified liver mRNA was then reverse-transcribed using RevertraAce reverse-transcriptase (TOYOBO, Osaka, Japan) and a random oligo primer. A PCR experiment was performed and candidate cDNA clones were isolated using pyrobest DNA polymerase (TOYOBO) with the following primers for pig D-P-M: (sense primer) #1: 5′-AACTATgAgATTATAATCATA-3′ and #2: 5′-CAAAATTTATTCCTgAATTCA-3′, (antisense primer) #3: 5′-TTTCTTTTATgTRgTAgCAAA-3′.

The amplified DNA fragments were subcloned into the *Eco*RV site of the pBluescript II SK(−) cloning vector via a TA-cloning method. The nucleotide sequences were determined by the dideoxy chain termination method using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) [16].

Preparation of PERV-produce 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 pseudotype infection, and prepared PEC(Z) (a gift from Prof. Yasuhiro Takeuchi, The Institute of Cancer Research, London), as described in a previous report. To establish PEC(Z) that produces PERV-B, PEC(Z) were infected with PERV-B, produced from HEK293 cells that had been persistently infected with PERV-B, and are referred to as PEC(Z)/PB. At 18–32 days after infection, the viral titers of the pseudotypes of PERV-B containing the *LacZ* gene, PERV-B(Z), released from PEC(Z)/PB were measured on naive HEK293 cells [12].

For the transfection of synthetic siRNA

PEC(Z)/PB cells were seeded in a 10 cm dish with D-MEM containing 10% FBS. The medium was replaced by FBS free D-MEM the next day. The nominated siRNA#1: GGCUGCUGGUGAAGAGCUUUUTT & AAAAGCUCU UCACCAGCAGCCTT, siRNA#2: AGCACGCCACAG GAAACUACAUTT & AUGUAGUUUCCUGUGGCGUGC UTT and a siRNA with several mutation (mock control): AAGCCCAUGGAAGGUAAUUUUTT & AAAAUUAC-CUUCCAUGGGCUUTT were diluted with Opti-MEM and Lipofectamine 2000 (Invitrogen), and incubated for 20 min at room temperature, and added to the cells, resulting in a final siRNA concentration of 20 nM. The medium was replaced with D-MEM containing 10% FBS after 4 h.

Quantitative real-time PCR

Total RNA was collected from the PEC transfectants and the culture medium, using the TRIZOL LS Reagent (Invitrogen). The total RNA was used in a reverse transcriptase reaction.

To evaluate the expression of the transgene mRNA, SYBR-Green real-time PCR was performed with a Smart Cycler II System and SYBR premix Taq (Takara). Each glycosyltransferase sequence was amplified using the specific primer pairs: 5′-CACCTACAATGAGCGCGAGA-3′

(sense), 5′-ACCACCTGTTCAGCAATGTCC-3′ (antisense). Reverse transcription (RT) was carried at 42 °C for 15 min, followed by 95 °C for 2 min, using random primers, followed by PCR at 45 cycles at 95 °C for 5 s and 60 °C for 20 s. The amount of mRNA in the transfectants was normalized with the level of GAPDH mRNA [17].

Lac Z pseudotype assay

HEK 293 cells were seeded at 2×10^5 cells per well in 24-well plates 1 day prior to infection. Culture supernatants containing pseudotype PERV-B viruses 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. The medium was replaced with fresh D-MEM supplemented with 10% FBS. Four hours after the inoculation, the culture was incubated for additional 2 days after which they were then stained with 5-bromo-4-chrolo-3-indolyl-β-D-galactopyranoside. The number of *Lac*Z-positive blue focus forming units (BFU) was counted under a microscope [11].

Construction of the modified genes

To clone pSUPER, the polymerase-III H1-RNA gene promoter was amplified by PCR, using the recommended primers. The PCR product was digested with *Eco*RI and *Hind*III enzymes and then cloned into the sites of pBluescript IISK(+). A green fluorescence protein (GFP) subcloned into pCX, a β-actin promoter with a CMV enhancer [18], was digested with *Sal*I-*Hind*III enzymes, and cloned into the sites of the pSUPER. A hygromycin resistance gene under a thymidine kinase (tk) promoter was also cloned into the *Eco*RV site of the pSUPER, to establish pSXGH [17].

To insert the targeting sequence, DNA oligos in the D-P-M site were designed and cloned into the *Bgl*II-*Hind*III sites of pSXGH. siRNA with the following sense and antisense sequences were used: 5′-gATCCCCAgCACgCCACAggAAACTACATTTCAAgAgAATgTAgTTTCCTgTggCgTgCTTTTTTggAAA-3′ (sense), 5′-AgCTTTTCCAAAAAAgCACgCCACAggAAACTACATTCTCTTgAAATgTAgTTTCCTgTggCgTgCTggg-3′ (antisense) [17]. The siRNA was synthesized by Nisshinbo (Chiba, Japan).

Flow cytometry

The transduction of siRNA into the PEC(Z)/PB was provisionally verified by the expression of GFP. The expression of GFP was confirmed by FACS Calibur flow cytometry (Nippon Becton Dickinson, Tokyo, Japan) for each stable clone. Parental PEC(Z)/PB cells were used as controls.

Statistical analysis

Data are presented as the mean ± SD. The Student *t*-test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $P < 0.05$.

Results

Isolation of pig D-P-M cDNA

To start this study, the sequence data for pig D-P-M was investigated. An EST sequence with similarity to human D-P-M was then identified from the DNA database by a BLAST search. Oligo DNA primers were then designed, and the pig D-P-M sequence was amplified from pig liver cDNA. The cDNA fragments containing the start and stop codons were subcloned into the plasmid vector and the sequence was analyzed by testing multiple independent clones (Fig. 1a).

From the nucleotide sequences, the predicted open reading frame for pig D-P-M consisted of 259 AA. Alignment of the AA sequences from the pig was next compared with those for the human, mouse, rat, and cattle (Fig. 1b). The overall identity of the deduced AA sequence of pig D-P-M with the human, mouse, rat, and cattle was found to be 94.2%, 91.5%, 91.2% and 93.9%, respectively.

Real-time PCR for mRNA of D-P-M in PEC(Z)/PB transient transfectants

Real-time PCR was performed to detect any alterations in the mRNA of the D-P-M targeted by siRNA#1 or siRNA#2 in the transient transfectants. mRNA levels of D-P-M were measured individually by means of a SYBR green system and normalized to GAPDH. The introduction of siRNA#1 or siRNA#2 into PEC(Z)/PB decreased the amount of the mRNA produced by the transient transfectants by one-fifth compared to parental and mock lines (Fig. 2b).

Transgene expression in the transient siRNA transfection into cell lines

The viral titers of PERV-B released from PEC(Z)/PB with siRNA#1 or #2 were next measured in HEK293 cells. HEK293 cells were inoculated by incubation with culture supernatants of the transfected cells, and a *Lac* Z pseudotype assay was carried out. While the PERV from the mock cells were easily transmitted to HEK293 (86.3 ± 22.1% vs. parental), the extent of PERV infection from PEC(Z)/PB with the transgenes was limited (siRNA#1: 45.6 ± 24.1% and siRNA#2: 12.5 ± 15.1% vs. parental) (Fig. 3).

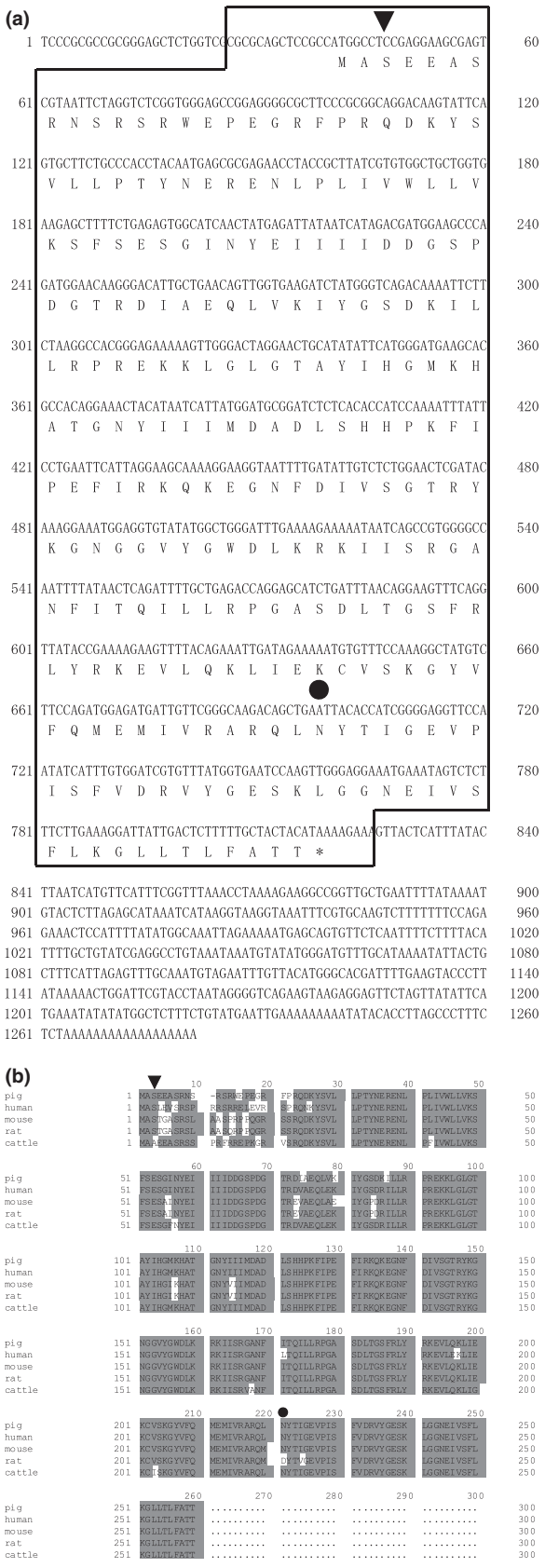

Figure 1 Sequences of pig D-P-M. (a) The cDNA of pig D-P-M is shown. The pig D-P-M consists of 1263 bp. The open reading frame for pig D-P-M consists of 777 bp (259aa). (b) Comparison of amino acid sequences among pig D-P-M, human D-P-M [NP_003850.1], mouse D-P-M [NP_034202], rat D-P-M [NP_001100014.1] and cattle D-P-M [NP_001069481.1]. The predicted *N*-glycosylation sites identified by the NetNGlyc program (●) and *O*-glycosylation sites found by the NetOGlyc program (▼) are indicated.

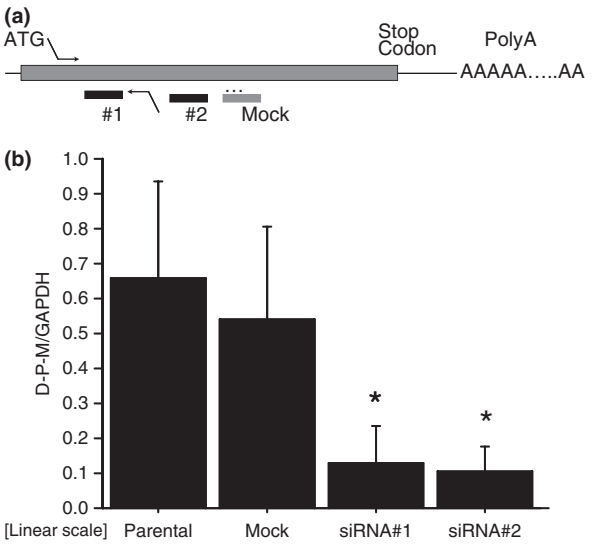

Figure 2 Real time PCR for mRNA of the D-P-M in PEC(Z)/PB transfectant. (a) Schematic presentation of the D-P-M mRNA with the localization of siRNA target sequences (#1, #2 and mock with mutations) and oligonucleotide primers (arrows) used for the detection of D-P-M mRNA in PCR assays. (b) To evaluate the degradation of D-P-M mRNA, SYBR-Green real-time PCR was performed with the Smart Cycler II System. The amount of D-P-M mRNA for each of the PEC(Z)/PB transfectants with siRNA was measured, using the pair of primers for the D-P-M, and normalized in comparison with the level of each GAPDH mRNA. The extent of D-P-M mRNA degradation in a transfectant with siRNA was calculated with reference to parental PEC(Z)/PB. Compared with parental and mock cells, all PEC(Z)/PB with siRNA, especially #2, showed a decreased mRNA expression for the D-P-M by one-fifth. The data are expressed as the mean ± SD of three to four independent experiments. An asterisk indicates a significant difference (*$P < 0.05$ vs. parental).

GFP expression in transient cell lines of PEC(Z)/PB with pSXGH-siRNA

Considering the results of the *Lac* Z assays for the transient cell lines, hairpin siRNA, corresponding to targeted site #2, was designed in the pSXGH vector, based on the polymerase III H1-RNA promoter (Fig. 4A). A closed circular plasmid, pSXGH-siRNA#2, was established and introduced into the PEC(Z)/PB by means of lipofectamine. After the selection of PEC(Z)/PB with hygromycine

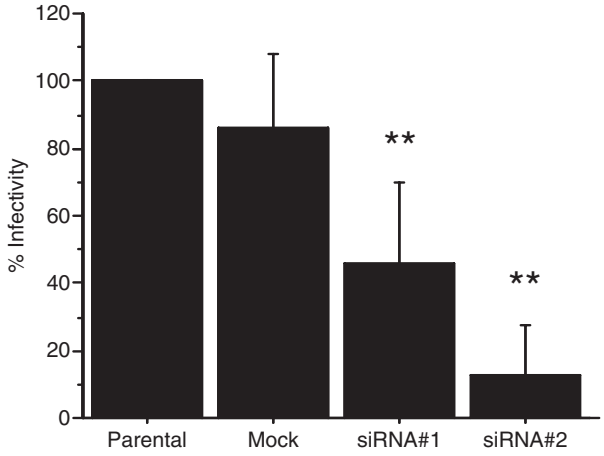

Figure 3 siRNA directed silencing of PERV infectivity. PEC(Z)/PB was transfected with the siRNA for pig D-P-M, and the culture supernatants collected from them were added to the medium of the HEK293 cells. Target HEK293 cells were histochemically stained, and *Lac* Z-positive BFU were counted, to determine viral titers. The infectivity of each culture supernatant from PEC(Z)/PB with siRNA was calculated, and the data are expressed as the mean ± SD of five independent experiments. An asterisk indicates a significant difference (**$P < 0.05$ vs. parental and mock).

for approximately 2 weeks, the transient expression of the GFP gene in PEC(Z)/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 the cases of clones #5, #6, #9 and bulk line (Fig. 4B).

Real-time PCR for mRNA of D-P-M in the PEC(Z)/PB clone

Real-time PCR was performed to detect any alterations in the mRNA of D-P-M targeted by the siRNA in each clone. The mRNA levels of D-P-M were measured individually by means of a SYBR green system and normalized to GAPDH. The introduction of pSXGH-siRNA#2 into PEC(Z)/PB led to a decrease in the amount of the mRNA of D-P-M in the bulk line and representative standard clones, compared with parental and mock lines (Fig. 4C).

siRNA directed silencing on PERV infectivity

The viral titers of PERV-B released from each PEC(Z)/PB clone were next measured on HEK293 cells. HEK293 cells were inoculated by incubation with culture supernatants of each clone, and a *LacZ* assay was carried out. While the PERV from the parental PEC(Z)/PB was easily transmitted to HEK293, the extent of PERV infection from PEC(Z)/PB with pSXGH-siRNA was limited. These data clearly indicate that a significant decrease in PERV infectivity was achieved, which is related to the downregulation of mRNA expression (Fig. 5).

Discussion

In our previous study, an analysis of *O*-glycans was not performed, because it is less relevant to general retrovirus infectivity. On the other hand, most of the individual consensus *N*-linked glycosylation sites are required for viral infectivity. For example, the carbohydrate moiety of the human immunodeficiency virus (HIV)-gp120 that binds to CD4 molecules consists of 24 *N*-linked oligosaccharides, 11 of which are believed to be of the high-mannose or hybrid type. The five consensus *N*-linked glycosylation sites that are likely to play important roles in infectivity are located in the amino-terminal half of gp120 [19–22]. It is also noteworthy that PERV-A and -B contain only seven and five *N*-linked sites, respectively, on the surface of the envelope protein [5]. The number of sites for an *N*-glycan to become attached to the surface envelope does not directly imply that they are relevant to virus infectivity. In addition, the types of *N*-glycans that are attached at each *N*-linked site, such as the high mannose or complex-type, also remain unknown. However, the *N*-glycan is considered necessary for forming the structure of the ligand glycoprotein, gp70, which corresponds to the PERV receptor [5,22].

To diminish PERV infectivity, we focused on altering the compatibility between the PERV ligand and human PERV receptors by modifying glycosylation reactions. An *N*-linked sugar was assumed to be the main target for effectively altering PERV infectivity. Therefore, the elimination of an *N*-linked sugar by the knocking out of a glycosyltransferase, such as the 3-D-mannoside-β-1,2-*N*-acetylglucosaminyltransferase I (GnT-I), was first considered. However, it was reported that the knockout (KO) of this gene became lethal. We then attempted to accelerate the processing of an *N*-linked sugar by overexpressing the processing enzymes. Therefore, the transfection of genes for both α 1,2 mannosidase IB (Man-IB), which converts Man9GlcNAc to Man5GlcNAc, and GnT-I, which catalyzes the first step in the conversion of an oligomannose to complex or hybrid *N*-glycans of glycoproteins, to pig cells proved to be quite effective in reducing PERV infectivity in human cells. In addition, we also addressed the effects of remodeling pig cell-surface glycoproteins, especially the high mannose type of *N*-glycan, on the susceptibility of PERV by α 1,2 mannosidase II (Man-II), which controls the conversion of high mannose to complex *N*-glycans. Man II occupies a central position in the *N*-linked oligosaccharide processing pathway, functioning as the committed step in the synthesis

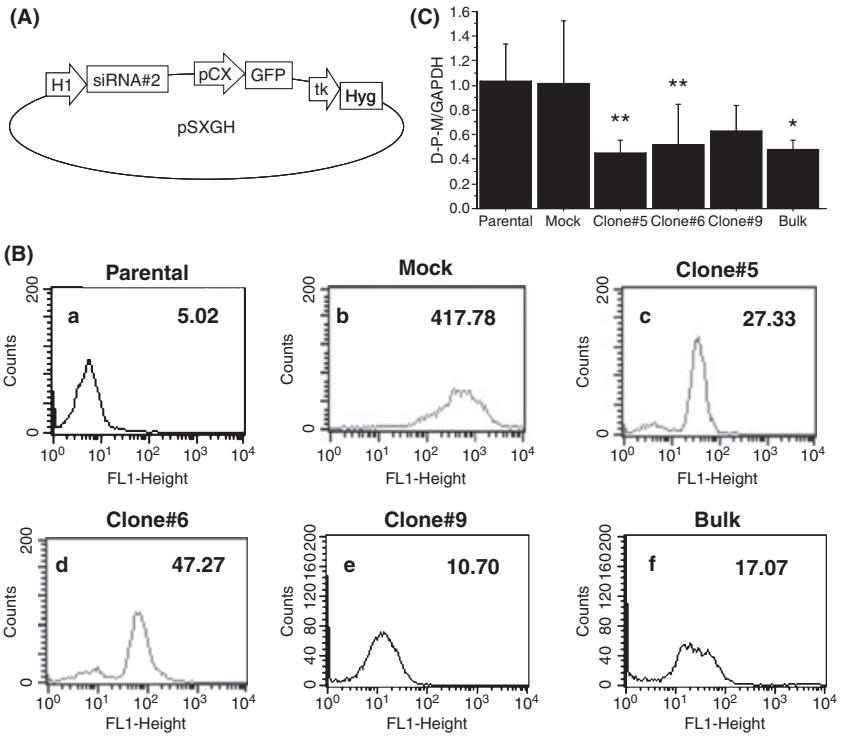

Figure 4 FACS analysis of GFP expression in stable clones of PEC(Z)/PB transfected with pSXGH-siRNA. (A) Schematic diagram of pSXGH-siRNA. (B) The expression levels of GFP on the PEC(Z)/PB clones were determined by flow cytometry. Typical flow cytometric histograms for each of the established clones are shown. a, parental PEC(Z)/PB; b, mock: PEC(Z)/PB clone with pSXGH; c, clone #5: PEC(Z)/PB clone with pSXGH-siRNA; d, clone #6: PEC(Z)/PB clone with pSXGH-siRNA; e, clone #9: PEC(Z)/PB clone with pSXGH-siRNA; f, bulk: PEC(Z)/PB bulk line with pSXGH-siRNA. The mean shift values of GFP expression in clones and parental PEC(Z)/PB are indicated in each panel. (C) Real-time PCR for the mRNA of PERV in the PEC(Z)/PB transfectant. To estimate the degradation of D-P-M mRNA, SYBR-Green real-time PCR was performed with the Smart Cycler II System. The amount of pig D-P-M mRNA for each of the PEC(Z)/PB clone was measured, using two pair of primers for the pig D-P-M, and normalized in comparison with the level of each GAPDH mRNA. The extent of pig D-P-M mRNA degradation in clones with siRNA was calculated with reference to parental lines. Compared with parental cells, PEC(Z)/PB with pSXGH-siRNA, clones #5, #6, #9 and bulk line, showed a decreased mRNA expression for the D-P-M, as the data are expressed as the mean ± SD of four independent experiments. An asterisk indicates a significant difference (*$P < 0.05$ vs. parental, **$P < 0.05$ vs. parental and mock).

of complex type structures. The strategy was very effective, but not perfect in preventing PERV infectivity [13].

As an alternate strategy for suppressing PERV involved *N*-glycan production, we identified the Carbohydrate-Deficient Glycoprotein (CDG) Syndrome, especially type IV [23]. CDG type IV is characterized by the neonatal onset of a convulsive disease with almost no psychomotor development, including microcephaly. As a feature of the disease, it was reported that the $Man_5GlcNAc_2$-PP-Dol intermediate can be glucosylated and that the resulting $Glc_3Man_5GlcNAc_2$ oligosaccharides can be transferred by an oligosaccharyltransferase complex. The presence of $Man_5GlcNAc_2$ oligosaccharides in newly synthesized glycoproteins demonstrated the utilization of this alternative *N*-glycosylation pathway in CDG type IV. However, neither the residual mannosyltransferase activity nor the alternative *N*-glycosylation pathway which utilizes truncated oligosaccharides can ensure the glycosylation of the asparagine residues in CDG type IV that are normally utilized. On the other hand, other reports revealed that the patient's fibroblasts are able to synthesize mature $Glc_3Man_9GlcNAc_2$, but to a decreased extent. This can only be due to α1,3mannosyltransferase (ALG3) proteins coded by the small amount of normal transcripts found in the patient or by a partial bypass of the ALG3 [24–30].

After all, in spite of the features of the disease, the patient lived and the content of high-mannose type of *N*-linked sugars was diminished. However, production of the D-P-M-KO pigs might not constitute an ideal solution, because of the severe symptoms of this disease. Therefore, a tissue or organ specific KO or knockdown (KD) of D-P-M gene may be a more reasonable route to producing immuno-modified pigs with low PERV infectivity.

Finally, the hyperacute rejection can be overcome by the generation of a pig strain that is resistant to the human immune system by knocking out the α1,3

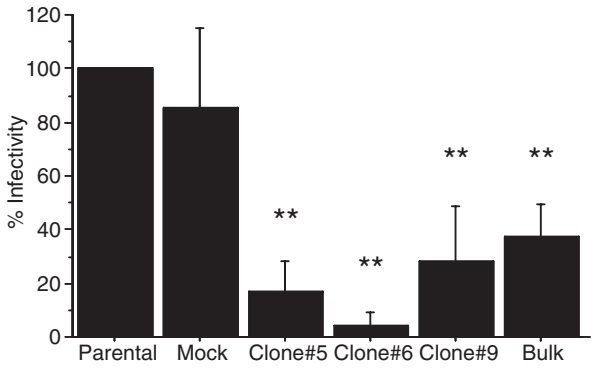

Figure 5 siRNA directed silencing on PERV infectivity. Culture supernatants, collected from PEC(Z)/PB clones with pSXGH-siRNA, were added to the medium of the HEK293 cells. Target HEK293 cells were histochemically stained, and *Lac*Z-positive BFU were counted to determine the viral titers. The infectivity of each culture supernatant from the PEC(Z)/PB clones was calculated, and the data are expressed as the mean ± SD of five independent experiments. Compared with parental and mock cells, PEC(Z)/PB with pSXGH-siRNA, clones #5, #6, #9 and bulk line, showed a decreased PERV infectivity. An asterisk indicates a significant difference (**$P < 0.05$ vs. parental and mock).

galactosyltransfearse (α1,3GT) and transduction of the human complement regulatory gene, such as CD55 [31–34]. However, these same genetic modifications might make PERV particles from pig cells more resistant to human serum, thus creating a new risk of PERV transmission to humans [35]. On the other hand, safe clinical xenotransplantation requires prevention of PERV transmission to human patients. Therefore, the reduction in high mannose type *N*-glycans on pig cell surface glycoproteins by KD or KO of D-P-M represents a novel and potentially useful strategy for reducing PERV transmission to humans. In the next step for this study, an analysis of changes in the glycosylation of D-P-M-KD cells will be performed using a lectin blot panel or HPLC. Our plans also involve the production of D-P-M-KD pigs, using a intracytoplasmic sperm injection-mediated gene transfer or somatic cell nuclear transfer (SCNT) technique [36].

Authorship

AY: performed the research and analyzed the data. SN: contributed to the data (siRNA study). AK: contributed to the data (RT-PCR) and supervised the experiment. TA: contributed to the data (cDNA cloning). MO and MF: supervised the experiment. SM: designed the study and supervised the experiment.

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