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Molecules and Cells

Analysis of the Molecular and Regulatory Properties of Active Porcine Endogenous Retrovirus Gamma-1 Long Terminal Repeats in Kidney Tissues of the NIH-Miniature Pig

Sang-Je Park[1,2], Jae-Won Huh[2], Dae-Soo Kim[2], Hong-Seok Ha[1], Yi-Deun Jung[1], Kung Ahn[1], Keon Bong Oh[3], Eung-Woo Park[3], Kyu-Tae Chang[2], and Heui-Soo Kim[1,*]

The pig genome contains the gamma1 family of porcine endogenous retroviruses (PERVs), which are major obstacle to the development of successful xenotransplantation from pig to human. Long terminal repeats (LTRs) found in PERVs are known to be essential elements for the control of the transcriptional activity of single virus by different transcription factors (TFs). To identify transcribed PERV LTR elements, RT-PCR and DNA sequencing analyses were performed. Twenty-nine actively transcribed LTR elements were identified in the kidney tissues of the NIH-Miniature pig. These elements were divided into two major groups (I and II), and four minor groups (I-1, I-2, I-3, and II-1), by the presence of insertion and deletion (INDEL) sequences. Group I elements showed strong transcriptional activity compared to group II elements. Four different LTR elements (PL1, PL2, PL3, and PL4) as representative of the groups were analyzed by using a transient transfection assay. The regulation of their promoter activity was investigated by treatment with M.SssI (CpG DNA methyltransferase) and garcinol (histone acetyltransferase inhibitor). The transcriptional activity of PERV LTR elements was significantly reduced by treatment with M.SssI. These data indicate that transcribed PERV LTR elements harbor sufficient promoter activity to regulate the transcription of a single virus, and the transcriptional activity of PERV LTRs may be controlled by DNA methylation events.

INTRODUCTION

Among the human organs used in transplantation, the kidney is the most well known and commonly used. The number of kidney donors has increased dramatically from 8717 in 1996, to 13,266 in 2005 (Andreoni et al., 2007). Increasing numbers of living donor kidney transplants, an improvement in graft survival rates, and a decline in acute rejection rates are cause for optimism for the many patients waiting for kidney transplantations. However, there are about 75,000 patients on the kidney transplant waiting list in the United States (Sprangers et al., 2008). Thus, xenotransplantation of organs from pig to human holds great promise as a potential means to solve the kidney shortage problem.

Unfortunately, many studies have indicated that porcine endogenous retroviruses (PERVs) in the pig genome could be a potential infectious risk factor in xenotransplantation. PERVs could be composed of a functional gene and untranslated region (UTR). Specifically, some PERVs consist of the functional genes *gag*, *pol*, and *env*, along with LTRs located at the end of both sides of the element (Blusch et al., 2002). PERVs have been reported to release viral particles from porcine kidney cell lines, and they showed infectious activity in human 293 cells (Czauderna et al., 2000; Le Tissier et al., 1997; Martin et al., 1998; Patience et al., 1997). The PERV particle also contains galactosyl-α-1,3-galactose residues, which could be a potentially harmful element causing immunological rejection (Phelps et al., 2003; Platt et al., 2000).

PERVs have been classified into betaretroviruses and gammaretroviruses. The gammaretroviruses are composed of 10 subgroups (Klymiuk et al., 2002; Patience et al., 2001). Among the 10 subgroups, only the gamma-1 family is replication competent as an active provirus. Fifty copies of these proviruses are present in the pig genome (Patience et al., 2001). Therefore, if pig organs are to be used in xenotransplantation, replication competent proviruses need to be removed from the pig genome using advanced biotechnology methods (Takeuchi et al., 1998).

Gamma1 retroviruses are divided into PERV-A, -B and -C subfamilies based on differences in *env* gene sequences, while the other internal genes, including *gag* and *pol*, are highly conserved (Magre et al., 2003). PERV-A and -B are competent to infect both human and porcine cell lines. While PERV-C cannot replicate in human cells, it is competent to infect porcine cell

[1]Department of Biological Sciences, College of Natural Sciences, Pusan National University, Busan 609-735, Korea, [2]National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang 363-883, Korea, [3]Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Suwon 441-706, Korea
*Correspondence: khs307@pusan.ac.kr

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lines (Huh et al., 2009; Takeuchi et al., 1998).

Once PERVs were found to be infectious in human cell lines, many strategies were developed to control PERV activity. These included the elimination of proviral loci containing replication competent PERVs from the pig genome (Clark et al., 2007), the selection of low-virus-producer animals (Karlas et al., 2004; Oldmixon et al., 2002), and the development of an antiviral vaccine and antiviral therapy (Griffiths et al., 2000). The inhibition of PERV expression by RNA interference has also been attempted (Dieckhoff et al., 2008; Karlas et al., 2004).

The U3, R, and U5 LTR regions were shown to be important for PERV transcriptional activity (Maksakova et al., 2005). The U3 region is the promoter region for the transcriptional regulation of PERV, and it contains several types of transcription factor (TF) binding sites (Huh et al., 2009; Krach et al., 2001; Wilson et al., 2003). Thus, PERV expression may be regulated by the transcriptional activity of LTRs (Maksakova et al., 2005). Epigenetic modifications are one of the most effective methods for the regulation of transcription activity (Ooi et al., 2008). Therefore, we examined the regulation of promoter activity driving transcription of the PERV LTR using M.SssI (CpG DNA methyltransferase) and garcinol (histone acetyltransferase inhibitor).

MATERIALS AND METHODS

Total RNA extraction and RT-PCR

Total RNA was extracted from kidney tissues of the NIH-miniature pig using the Trizol reagent (Invitrogen). The turbo DNA-free™ kit (Ambion) was used to eradicate DNA contamination in total RNA preps. To confirm the absence of DNA contamination from total RNA, PCR amplification of DNase-treated total RNA samples was performed without reverse-transcription (No-RT experiment). The lack of PCR products indicated complete elimination of DNA after DNase treatment. cDNA was synthesized from total RNA by reverse transcription with Moloney-murine leukemia virus reverse transcriptase and an RNase inhibitor (Promega). The transcripts of the PERV-LTR elements were analyzed by RT-PCR (Fig. 1). RT-PCR products were amplified using the primer pair: PERV-S (5′- GAT GAA AAT GCA ACC TAA CCC -3′) and PERV-AS (5′- CCC CAA ATC ACT CAC GAG AA -3′) (Huh et al., 2009). As a control, the GAPDH gene was amplified using GAPDH-S (5′- GAA ATC CCA TCA CCA TCT TCC AGG -3′) and GAPDH-AS (5′- GAG CCC CAG CCT TCT CCA TG -3′) primers targeting human GAPDH (GenBank accession no.NM_002046). RT-PCR reactions were carried out for 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 40 s.

Molecular cloning and DNA sequencing analysis

RT-PCR products were separated on a 1.5% agarose gel, purified using the QIAquick gel extraction kit (QIAGEN), and cloned into the pGL-4.11 vector (Promega). DNA was isolated using the High Pure plasmid isolation kit (Roche). Sequencing services were performed by Macrogen (KOREA) using the pGL-4.11 primer pair: pGL-4S (5′- CTA GCA AAA TAG GCT GTC CCC AG -3′) and pGL-4AS (5′- CGT CTT CGA GTG GGT AGA ATG G -3′). Multiple alignment and comparative analysis of sequencing data was performed with (http://prodes. toulouse.inra.fr/multalin/multalin.html) and manually inspected using the BioEdit program. All nucleotide sequences reported in this paper were deposited in the DDBJ nucleotide sequence database (Supplementary Table S1).

Cell culture and transient transfection assay

Porcine kidney (PK15), Human embryonic kidney (HEK293), and African green monkey kidney (Cos7) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS), and were grown at 37°C and 5% CO_2 in a cell culture incubator. Cells were seeded at a density of 2×10^4 cells per well in 24 well plates. When cells reached 60-70% confluence, they were transfected with 100 ng of pGL4.11 containing each sequence construct (pGL4.11-PL1, 2, 3, and 4). The 20 ng of pRL-TK vector was used to normalize transfection efficiency using polyethyleneimine (PEI; Sigma-Aldrich) according to the manufacturer's instruction. At 24 h post-transfection, the activities of firefly and *Renilla* luciferase were measured using the Dual Luciferase Reporter Assay System (Promega) and a luminometer. All values were normalized against pRL-TK vector activity. Each experiment was performed in triplicate.

Plasmid methylation analysis

To methylate the three plasmids (PL1, 2, and 3), each plasmid was treated with M.SssI methyltransferase (New England Biolabs) according to the manufacturer's instructions. Briefly, the three plasmids were incubated with M.SssI (1 U/μg DNA), 10X NEBuffer 2, and 1600 μM S-adenosylmethionine at 37°C for 2 h, then M.SssI was heat inactivated at 65°C for 20 min. M.SssI treated plasmids were purified using the Microcon Ultracel YM-100 (Millipore) system. The efficiency of M.SssI was confirmed by HhaI digestion and gel electrophoresis. Finally, methylated and non-methylated plasmids were transfected and analyzed as described above for a comparative analysis.

MTT assay

PK15, 293T, and Cos7 cells were cultured as described above. When cells reached 50% confluence, they were treated with garcinol (BIOMOL) at final concentrations of 0 μM, 2 μM, 4 μM, 6 μM, 8 μM and 10 μM for 12 h. Next, PBS [250 μl of 2.5 mg/ml MTT (Amresco) in pH 7.4] was added to each well. The plate was incubated for 3 h at 37°C and then dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured using an ELISA reader at 540 nm.

Garcinol treatment as a histone acetyltransferase inhibitor

To analyze the inhibition effect of histone acetylation, we treated cells with garcinol. PK15, HEK293, and Cos7 cells were transfected with one of three plasmids (PL1, 2, and 3) as described above. Approximately 24 h post-transfection, cells were treated with garcinol, at 4 μM (PK15 and HEK293) and 6 μM (Cos7) concentrations. After 12 h, firefly and *Renilla* luciferase activities were measured as described above.

Data analysis

Potential TF binding sites in transcribed PERV LTR sequences were predicted using MATCH in Transfac 8.0 (http://www.generegulation.com). CpG islands in PERV LTR elements were identified using the CpG Plot/CpGreport program (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html).

RESULTS

Identification and molecular characterization of transcribed PERV LTR elements in kidney tissues of the NIH-miniature pig

Using PERV LTR specific RT-PCR, actively transcribed PERV LTR elements were amplified from mRNAs of the NIH-miniature pig kidney tissue (Fig. 1). The different-sized RT-PCR products indicate the presence of multiple actively transcribed LTR elements in the kidney tissues of the NIH-miniature pig. To

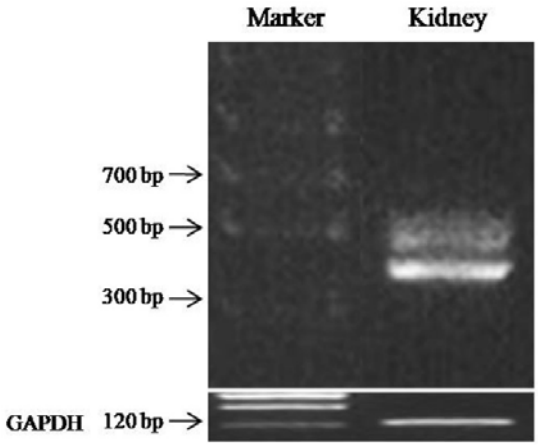

Fig. 1. RT-PCR amplification of PERV LTR elements in kidney tissue of the NIH-miniature pig. Different-sized PCR products were detected from 300-700 bp. GAPDH (120 bp) indicates the positive control.

analyze these transcript sequences, the RT-PCR products were randomly inserted into the pGL-4.11 vector.

To identify and analyze these different-sized PERV LTR elements, multiple alignment analysis was performed (http://prodes.toulouse.inra.fr/multalin/multalin.html). We corrected sequences manually using the BioEdit program. Among 40 randomly selected sequenced clones, 29 different LTR elements were identified. A phylogenetic analysis using the neighbor-joining method indicated that the 29 LTR elements could be divided into two major groups (I and II) and subdivided into four minor groups (I-1, I-2, I-3 and II-1). This data is presented in Figure S1. Based on these results, we selected four different clones (PL1, PL2, PL3, and PL4) for further structural and functional analyses (Fig. 2).

Comparative INDEL analysis of the PERV LTR elements

Within these four clones (PL1, PL2, PL3, and PL4), the DNA sequences of the R and U5 regions were highly conserved. However, compared to the other three elements, the PL4 element had a lower sequence similarity of only 45-56% in the U3 region. INDEL analysis of the PL1, PL2, PL3, and PL4 elements demonstrated that in the U3 region the PL1 element contains inserted sequence of "CTC CAG CTT CCT AAA AAG CCC TAG", with an additional sequence, "GCC AGT AA", also present in the PL2 element. A deleted sequence of 96 bp was also identified in the PL3 element. Each PL element has its own specific repeated sequences in the tandem repeated region (Fig. 2 and Supplementary Fig. S2). The PL1 element has S3/S5 and S1-1 sequences (the hyphenated number indicates the subtype of original sequences), the PL2 element has S1/S5-1, S1/S5-2, and S1-2 sequences, the PL3 element has S2/S6, S1-4/S5-3, and S1-1 sequences, and the PL4 element has S1-5/S5-4, S8, and S1-3 sequences. The PL1 element has less repeat sequences than the other three elements. The PL3 and PL4 elements have specific S6 sequences (truncated sequences of S5) and S8 sequences, respectively.

This demonstrates that independent clones of the U3 region of the PERV LTR contain different tandem repeated sequences (S1, S1-1, S1-2, S1-3, S1-4, S1-5, S2, S3, S5, S5-1, S5-2, S5-3, S5-4, S6, and S8), and sequence specific INDELs.

Prediction of TF binding sites and transient transfection analysis of four PERV LTRs

PERV LTR sequences are important regions for the transcriptional control of functional PERV genes. The U3 region of PERV LTR elements is known to contain many binding sites for TFs. Therefore, the presence and absence of specific TF binding sites or CpG islands could have an effect on the transcriptional activity of the PERV LTR elements. To determine the differences in potential TF binding sites and CpG islands between the four LTR sequences, the TRANSFAC v8.0 and CpG Plot/CpG report programs were used. The results of this analysis indicated that PL1, PL2, and PL3 elements had similar TF binding sites, including sites for GATA-4, Hand1:E47, and TGIF. CpG islands were also identified. However, the PL4 element showed completely different TF binding sites (EIK-1, SF-1, PR, HSF, Barbie Box, AREB6, and CACCC-binding factor), and did not harbor a CpG island (Fig. 3).

To investigate the promoter activity of the four PERV LTRs, we performed a transient transfection assay using pGL4.11-PL1, pGL4.11-PL2, pGL4.11-PL3, and pGL4.11-PL4 plasmids in PK15, HEK293, and Cos7 cell lines (Fig. 4). The promoter

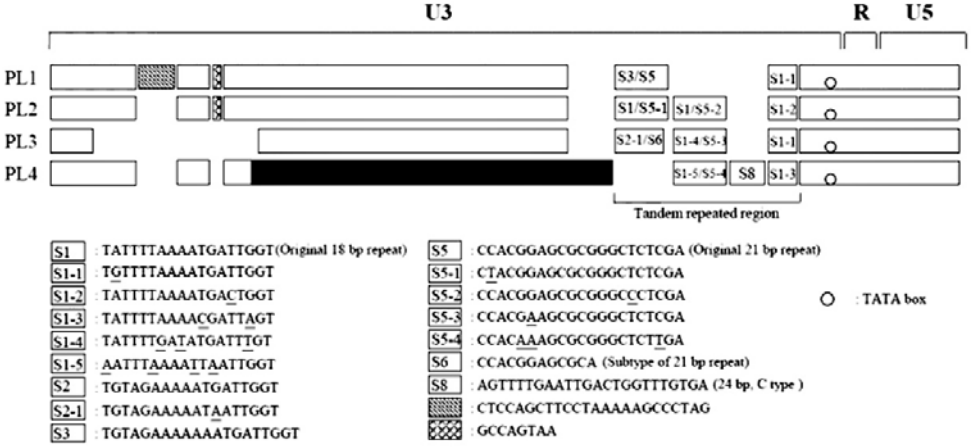

Fig. 2. Schematic representation of four PERV LTR elements and their consensus sequences. White boxes indicate conserved sequences within PL1-4 elements, labeled boxes indicate specific sequences, and the closed box indicates the highly divergent sequences. Different box numbers indicate different tandem repeat sequences. Open circles indicated the TATA box.

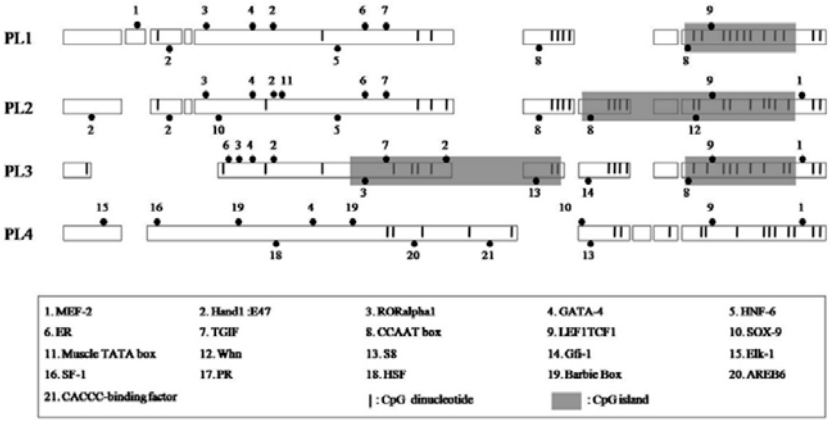

Fig. 3. Analysis of CpG islands and TF binding sites in the PERV LTR elements. Closed circles represent TF binding sites. Vertical bars and gray boxes indicate CpG dinucleotides and CpG islands, respectively.

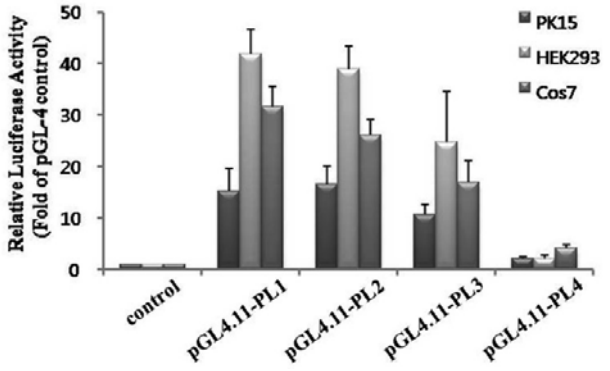

Fig. 4. Transient trasnfection assay of four PERV LTR elements. pGL4.11-PL1, pGL4.11-PL2, pGL4.11-PL3, and pGL4.11- PL4, derived from the kidney tissues of the NIH-miniature pig, were analyzed in PK15, HEK293, and Cos7 cells. Relative luciferase activity is indicated in the schematic diagram. Results are expressed as ratios of the luciferase activity to that of the promoterless pGL4.11 reporter plasmid. The standard error of the mean are presented as error bars.

activity of the pGL4.11-empty plasmid was used as a control. Overall, the pGL4.11-PL1, pGL4.11-PL2, and pGL4.11-PL3 plasmids showed the highest promoter activity in all tested cell lines. This is in contrast to the promoter activity of pGL4.11-PL4, which seemed to be very low. Among the four tested plasmids, pGL4.11-PL1 had the highest activity, followed by pGL4.11-PL2, pGL4.11-PL3, and pGL4.11-PL4.

CpG methylation of the PERV LTR may significantly suppress promoter activity

To investigate the effect of CpG methylation on the promoter activity of the transcribed PERV LTR elements, we estimated promoter activity of the three LTR elements (pGL4.11-PL1, pGL4.11-PL2, and pGL4.11-PL3) in PK15, HEK293, and Cos7 cells after treatment with M.SssI. The enzymatic activity of M.SssI treatment was validated by test cleavage with HhaI, an enzyme known to digest the specific sequence of non-methylated GCGC (Supplementary Fig. S3). Our sequence analysis indicated that the three LTR sequences contained numerous GCGC HhaI enzyme cleavage sites. Thus, if the M.SssI enzyme properly methylated the pGL4.11-PL1, pGL4.11-PL2, and pGL4.11-PL3 plasmids, HhaI should not digest the GCGC sequences in the LTRs. We found that all M.SssI treated plasmids

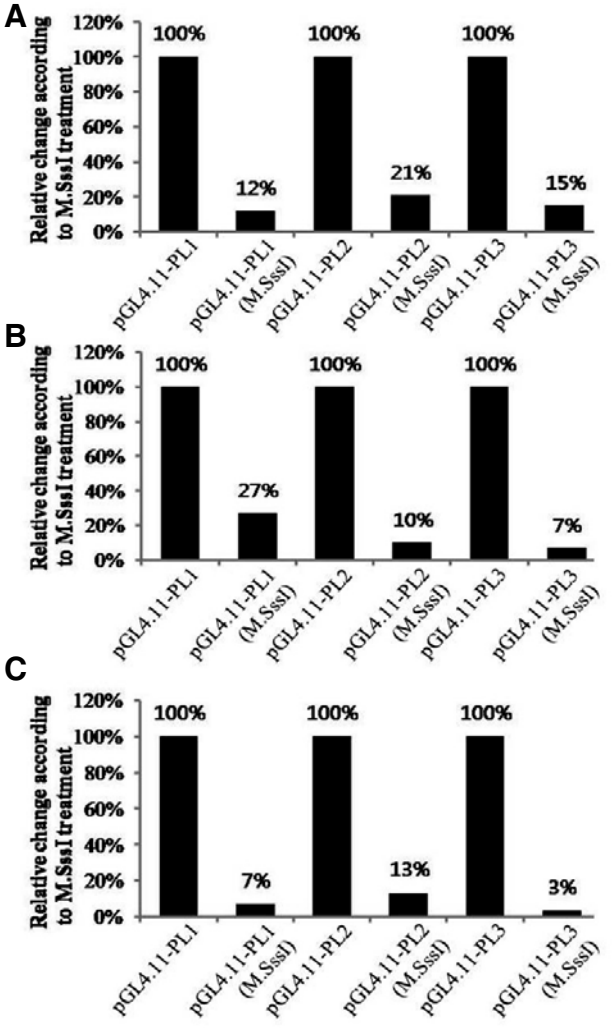

Fig. 5. Comparative transient transfection assay of methylated and unmethylated PERV LTR elements in PK15 (A), HEK293 (B), and Cos7 (C) cells. Methylated plasmids were treated with the M.SssI enzyme. Relative activities of luciferase between methylated and non-methylated plasmids in the transient transfection assay are indicated. Results are expressed as ratios of the luciferase activity of an unmethylated control plasmid to that of a methylated plasmid.

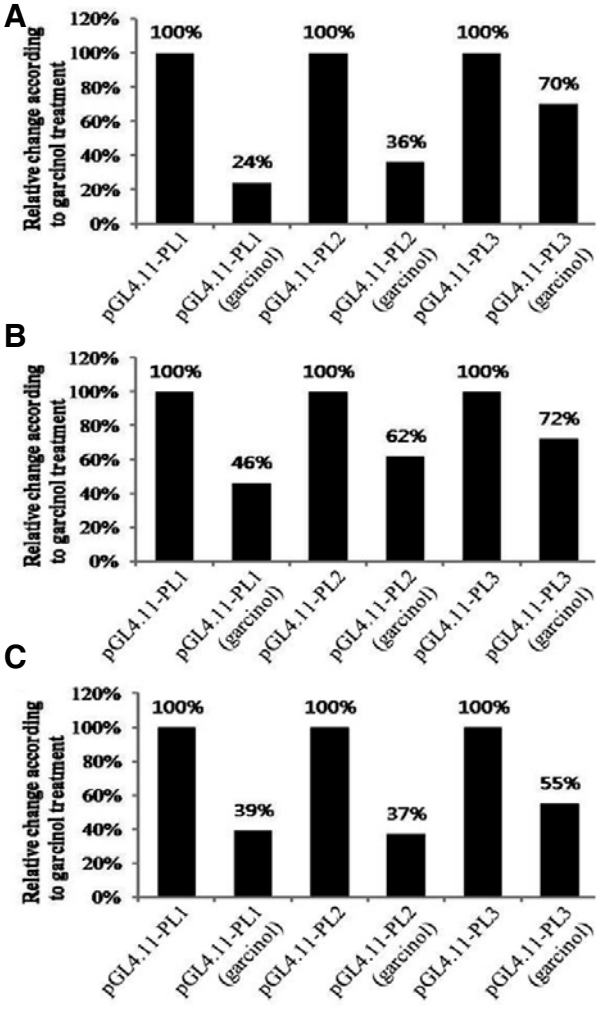

Fig. 6. Comparative transient transfection assay after treatment garcinol in PK15 (A), HEK293 (B), and Cos7 (C) cells. Relative activity between treated and non-treated cells in the transient transfection assay is indicated. Results are expressed as ratios of the luciferase activity of untreated control cells to that of treated cells.

were resistant to HhaI enzyme activity, whereas the non-methylated plasmids were readily digested into several fragments by HhaI treatment (Supplementary Fig. S3). The promoter activity of the unmethylated and methylated plasmids was then examined in PK15, HEK293 and Cos7 cells. Interestingly, we found that the promoter activity of methylated plasmids was significantly suppressed compared to that of unmethylated plasmids (Fig. 5).

Inhibition of histone acetylation may reduce the promoter activity of PERV LTR elements

Histone deacetylation is an epigenetic modification that has been shown to lead to suppressed transcriptional activity of a gene (Pazin et al., 1997). "Garcinol" is a histone acetyltransferase that has been shown to induce histone deacetylation. We hypothesized that treatment with garcinol would affect the transcriptional activity of PERV LTR elements.

To assess the effect of garcinol on promoter activity, we treated PK15, HEK293, and Cos7 cells with garcinol. To determine the effect of garcinol treatment on cell viability, a MTT assay was performed (Supplementary Fig. S4). PK15, HEK293, and Cos7 cells were treated with garcinol at concentrations of 4 μM, 4 μM, and 6 μM, respectively, after transient transfections of pGL4.11-PL1, pGL4.11-PL2, and pGL4.11-PL3 plasmids. The promoter activity in treated and untreated cells was then estimated (Fig. 6). In these experiments, the promoter activity of garcinol treated cells was decreased by approximately 52%, 41%, and 55% for the PK15, HEK293, Cos7 cells, respectively. While the suppression of promoter activity was less dramatic with garcinol treatment compared to M.SssI treatment, we found that garcinol treatment also reduced the transcriptional activity of the PERV LTRs elements.

DISCUSSION

The miniature pig is a specialized organism that has been used to solve the problem of organ shortage by allowing xenotransplantation from human to pig. Among pig organs, the kidney is most commonly used for xenotransplantation. In this study, we analyzed the molecular and transcriptional properties of transcribed LTR elements in kidney tissue of the NIH-miniature pig. These LTR elements were compared with other LTR sequences derived from hybrid Korean domestic pig, Yorkshire, Korean domestic pig, and wild pig breeds (Huh et al., 2009). Previously reported LTR sequences were also analyzed (Scheef et al., 2001). In this study, many different PERV LTR sequences in the NIH-miniature pig were identified including U3, R and U5 regions. Fundamentally, it is impossible for the transcribed PERV LTR to contain the U3 region. However, many LTR sequences including the U3 region were transcribed in various pig tissues by RT-PCR amplification (Huh et al., 2009). Although this phenomenon cannot explain the exact mechanism, the U3 containing LTR element might be transcribed by other promoters, not by their own promoter activity. The elimination of genomic DNA contamination in RNA samples was confirmed by control reaction that lacked RT. We thus used full-length LTR sequences for our investigation. As shown in Fig. 2, we identified 96 bp deleted in PL3 elements. The PL1, PL2, and PL4 elements had sequence similarities of about 99.6%, 98.4%, and 100%, respectively, with previously reported sequence accession no. AB280706, AB280709, and AB280703. The PL2 element showed the highest sequence similarity to LTR sequence of PERV-B (43), which has been shown to infect human 293 cells (Scheef et al., 2001). The LTR sequence of the PL4 element belongs to the PK15-PERV-A (58) and Bac-PERV-A (151B10) families. Therefore, in addition to molecular characterization, an investigation into the epigenetic control of the PL1, PL2, and PL3 elements could provide valuable information for xenotransplantation of pig to human.

To investigate the promoter activity of these elements, TF binding sites were analyzed using the TRANSFAC v8.0 program, and transient transfection assays were performed. Interestingly, the composition of TF binding sites was similar between the PL1, PL2 and PL3 elements. This was in contrast to the PL4 element, which had completely different TF binding sites (Fig. 3). Of the many TF binding sites identified in the PL4 element, the Hand1:E47, RoRalpha1, ER, TGIF, and CCAAT box were also found in the PL1, PL2, and PL3 elements. However, several other TF binding sites, including Elk-1, SF-1, PR, HSF, Barbie Box, AREB6, and CACCC-binding factor, were identified in only the PL4 element (Fig. 3). Among these identified TF binding sites, Hand1:E47 and RoRalpha1, are known to be positive elements for the regulation of several genes (Coste et al., 2002; Morin et al., 2005). However, Elk-1 and AREB6, which were identified only on the PL4 element, are negative

regulators of transcription (Ikeda et al., 1995; Sharrocks et al., 2001). The PL4 element also did not harbor the CCAAT box, which is a positive regulator of gene expression and functions by enhancing the transcription of RNA polymerase II (Fang et al., 2004; Raymondjean et al., 1988). Our prediction that different TF binding sites in the PL4 element would lead to altered levels of transcription was validated by the transient transfection assay results obtained using pGL4.11-PL1, pGL4.11-PL2, pGL4.11-PL3, and pGL4.11-PL4 in three different cell lines (Fig. 4).

Since the emergence of infectious PERVs, various methods have been developed to prevent and limit PERV activity, including antiretroviral drugs, complementary control, and RNA interference (Dieckhoff et al., 2007; Karlas et al., 2004; Takefman et al., 2002). In addition, previous studies have been conducted to identify and select low-virus-producing pigs (Lee et al., 2002; Mang et al., 2001; Niebert et al., 2002). However, the suppression of transcribed PERVs using epigenetic approaches has not yet been tested. Interestingly, in the case of other retroviruses, including HIV-1 provirus and human endogenous retrovirus (HERVs), the transcription level of viral RNA can be effectively suppressed by epigenetic modification (Ishida et al., 2006; Lavie et al., 2005). In particular the transcription level of the HERV LTR element was significantly suppressed by DNA methylation using the M.SssI (Lavie et al., 2005). It was also found that 1631 genes were transcriptionally down regulated after treatment of HeLa cells with garcinol (Balasubramanyam et al., 2004). Based on these results, we investigated the ability of a DNA methyltransferase (M.SssI) and a histone acetyltransferase inhibitor (garcinol) to regulate the transcription of PERV LTRs. Interestingly, only DNA methyltransferase (M.SssI) resulted in significant suppression of PERV LTR transcription (Figs. 5 and 6). This result could be explained by the presence of CpG islands and the distribution of CpG dinucleotides in the LTR sequences (Fig. 3). PL1, PL2, and PL3 elements have a common CpG island in their R and U3 regions, and most CpG dinucleotides are located in the R and U5 regions (Fig. 3). The CpG related region overlapped with many TF binding sites (Hand1:E47, RORalpha1, TGIF, CCAAT box, LEF1TCF1, Whn, and S8).

In conclusion, we have identified novel transcribed PERV LTR elements in the kidney tissue of the NIH-miniature pig, and we confirmed their transcriptional activity. We then validated the epigenetic regulation of transcribed PERV LTR elements by CpG methylation and histone acetylation inhibition. These results may contribute to the development of useful methods to control PERVs and allow for safe xenotransplantation.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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