Original Article

Porcine Endogenous Retrovirus (PERV) Infection of HEK-293 Cell Line Alters Expression of Human Endogenous Retrovirus (HERV-W) Sequences

(transplantation / heterologous / endogenous retroviruses / recombination / genetic)

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Abstract. The risk of infections of human recipients after xenotransplantations is now mainly represented by porcine endogenous retroviruses (PERVs) as these particles are part of the porcine genome. As in all vertebrates, human genome harbours its own numerous genetic sequences of retroviral origin; it is estimated that they comprise about 8 % of the human genome. Because some of them play an important role in human physiology, it is valuable to estimate whether the presence of PERVs in human cells influences homeostasis of the human endogenous retrovirus (HERV) expression pattern. The aim of the study was to evaluate whether the expression profile of HERV-W genes changes after infection of cells by porcine endogenous retroviruses. In the experimental settings, human embryonic kidney cell line (HEK-293) was infected by PERV particles and cultivated up to 22th passage after infection. HERV-W *gag*, *pol* and *env*, as well as *env* from locus 7q21.2 gene expression was monitored by means of real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot techniques. We found that the expression level of HERV-W genes differs in PERV-infected HEK-293 cell cultures in comparison with that from non-infected cultures. Relative HERV-W gene expression also differed significantly between particular passages ($P < 0.05$). Moreover, we have noticed a high correlation between the HERV-W Env(7q21.2) mRNA and protein level (Spearman rank $r = 0.65$; $P < 0.05$) during the course of the experiment. As previously hypothesized, human genomic sequences of retroviral origin may be changed by the presence of porcine endogenous retroviruses.

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Abbreviations: BSA – bovine serum albumin, Env – [viral] envelope, Gag – group-specific antigen, *GAPDH* – glyceraldehyde 3-phosphate dehydrogenase gene, HEK-293 – human embryonic kidney cell line, HIV-1 – human immunodeficiency virus, HERV – human endogenous retrovirus, IOD – integrated optical density, LTRs – long terminal repeats, MS – multiple sclerosis, MSRV – MS-associated retrovirus, mt DNA – mitochondrial DNA, ORF – open reading frame, PCR – polymerase chain reaction, PERV – porcine endogenous retrovirus, pGAPDH – porcine glyceraldehyde 3-phosphate dehydrogenase, Pol – [viral] polymerase, qPCR – quantitative polymerase chain reaction, RT-qPCR – reverse transcription-qPCR, VLPs – virus-like particles.

Introduction

Xenotransplantation is currently a subject of intensive research all over the world due to a permanent shortage of allogeneic organ donors. Domestic pig (*Sus scrofa*) is considered to be the most appropriate organ donor species in xenotransplantation, having many advantageous features such as similar organ size and physiology to humans, relative short life span, and well-known and high breeding potency with possible further manipulations. Unfortunately, the most serious problem in xenotransplantation is the existence of numerous pathogens capable of infecting human recipients, especially when they undergo immunosuppression (Garkavenko et al., 2004). Although careful screening of pig herds allow elimination of the majority of pathogens, the ubiquitous presence and replication activity of porcine endogenous retroviruses (PERVs) may pose a severe threat to human health. PERV genomes are integrated within the host (porcine) DNA (LeTissier and Stoye, 1997), and therefore there is no possibility to eliminate them by means of conventional breeding methods, such as animal selection. It has been shown that swine cells release active, replication-competent vi-

ral particles that are able to infect human cells *in vitro* (Kim et al., 2009). Since no paper confirming human infection with PERV *in vivo* has been published so far (Di Nicuolo et al., 2010, also reviewed in Denner, 2011), in fact, nothing is known about a possible influence of PERV infection on human health.

One of the hypothesized pathogenic effects of PERVs is their capability of recombination or pseudotyping with other genetic elements of retroviral origin already existing in the recipient's genome, i.e. human endogenous retroviruses (HERVs). Such elements are widely distributed throughout the human genome, encompassing about 5–8 % of total chromosomal DNA (Griffiths, 2001). HERV sequences belong to 27 distinct recognized retroviral lineages classified according to the type of tRNA bound by the primer binding site during replication. Phylogenetic analysis of HERV families in regard to the known retroviral genera has shown that class I HERVs cluster phylogenetically together with γ- and ε-retroviruses. Although most proviral HERV elements (open reading frames, ORFs) are truncated due to accumulated stop codons, frame-shift mutations and deletions, some of them retain transcriptional activity and even play an important role in human physiology as well as in pathogenesis (Perron et al., 1997; An et al., 2001). For example, a particular locus of the human endogenous retrovirus W family, 7q21.2, encodes functionally active envelope (Env) polyprotein, termed syncytin-1, which is necessary for syncytiotrophoblast formation during placental morphogenesis (Mi et al., 2000; Noorali et al., 2009). In fact, tissue-specific high levels of syncytin-1 expression in placenta are rather an exception to the rule, being a result of strong positive selection pressure, while most of HERV-encoding genes, conversely, undergo a strong negative selection resulting in rapid accumulation of mutations in the course of evolution. In the case of syncytin-1, several lines of evidence suggest its role in an autoimmune mechanism of multiple sclerosis (MS) pathogenesis, where syncytin-1 expression in astrocytes mediates neuroimmune activation, synthesis of reactive oxygen species and death of oligodendrocytes (Antony et al., 2004).

Based primarily on HIV-1-infected patients, it has been observed that the host organism develops different mechanisms that prevent transposable elements from being retrotransposed or expressed. For example, it has been demonstrated that the most potent antiviral protein, cytidine deaminase family member APOBEC3, is able to restrict endogenous retroviral elements as well (Esnault et al., 2008; Dorrschuck et al., 2011). APOBEC3G is a single-stranded DNA cytosine deaminase best understood as a potent inhibitor of HIV-1 replication. During HIV-1 infection the viral regulatory Vif protein performed activity to disrupt the APOBEC3G protein (Stopak et al., 2003). This state may, in turn, lead to enhanced activity of some endogenous retroviruses. Indeed, it has been observed that HERV-K expression, measured at the RNA transcript level, was enhanced in the sera of HIV-1-infected individuals (Contreras-Galindo et al., 2006). In HEK-293 cells co-transfected with a porcine endogenous retrovirus molecular clone together with an APOBEC3-expressing vector, strong repression of PERV replication, ranging between 60–90 %, was reported (Lee et al., 2011). However, if barriers preventing endogenous retroelements from expression (such as APOBEC3) fail, it may lead to the development of particular disorders, mainly on the (auto-)immunological basis. Such events have been broadly documented and described in research papers. For example, increased expression of the HERV-W Env polyprotein (measured at both the mRNA and protein level) is correlated with the development of some neuroinflammatory disorders, e.g. MS or schizophrenia (Alliel et al., 1998).

In case of xenotransplantations it is apparent that porcine PERVs will be introduced together with the graft directly into the immunocompromised recipient host. Since three classes of PERV (PERV-A, -B and -C) belong to the γ-retroviral family, the possibility of a recombination between PERV and HERV (especially class I) cannot be excluded if both elements are situated in close proximity, potentially giving rise to new viruses with unknown pathogenic potential. In theory, recombination between PERVs and HERVs could occur during a productive infection as a result of co-packaging HERV and PERV transcripts into a single retroviral particle. Phylogenetic evaluations have shown that the most probable recombination events might take place between PERVs and HERV-R or PERVs and HERV-E genes as these families are closely related (Klymiuk et al., 2006). However, to date, no recombination events have been observed in studies performed *in vitro* (Suling et al., 2003), although published data is limited.

Because the HERV-W family is of particular importance due to its physiological role in human development, we investigated whether PERV expression in *de novo* infected HEK-293 cell line *in vitro* would influence the overall level of HERV-W mRNA sequences from different loci. Thereafter, HERV-W *env* expression from locus 7q21.2 was examined both at the mRNA level and as its protein product syncytin-1 abundance.

Material and Methods

Cell cultures and infection

The following cell lines were used: HEK-293, human embryonic kidney cell line (Health Protection Agency Culture Collection (HPACC) cat. No. 85120602); PK(15) porcine embryonic kidney cell line (a gift from Dr. Andrzej Lipowski, National Veterinary Research Institute, Puławy, Poland); 293-PERV-PK-CIRCE, human embryo kidney cells infected with a porcine endogenous retrovirus (HPACC No. 97051411). Cell lines were propagated in Dulbecco's modified MEM medium (PAA, Pasching, Austria) supplemented with 10 % foe-

tal bovine serum (PAA) upon 80% confluence, and then split 1 : 6. The PERV infection procedure of HEK-293 cells was performed as previously described (Kuddus et al., 2003 and Dr. Kuddus's personal communication). Briefly, supernatant from PK(15) cell line was harvested after 48 h of cultivation, passed through a 0.45µm-pore syringe filter (Sartorius AG, Goettingen, Germany), and polybrene (hexadimethrine bromide, Sigma-Aldrich, St Louis, MO) was added to the final concentration of 800 µg/ml. This solution served as virus stock. HEK-293 cells were seeded into a 75 ml bottle one day before infection in DMEM medium as usual. On the day of the experiment culture medium was removed, cells were washed with PBS and 25 ml of virus stock was added. Cells were then cultivated in the presence of virus-containing medium for 4 h under conventional conditions. Thereafter the medium was removed, cells were washed with fresh DMEM, and the culture growth was continued as described above. At every culture passage a cell stock was collected in triplicate for Western blot analysis and for quantitative polymerase chain reaction as described below. Samples were then stored at -80 °C for further experiments. The cultivation of PERV-infected HEK-293 PERV was continued for 22 passages. Control cells were maintained in the same way as and parallel with the PERV-infected cell line. Both cell lines were polybrene-treated and split at the same cell confluence.

DNA extraction and PCR

DNA was extracted from HEK-293 cells after each cell passage. The DNA extraction technique was based on proteinase K lysis and phenol/chloroform extraction; it was performed according to the producer's protocol (Purification of DNA from Cultured Eukaryotic Cells with Proteinase K, Fermentas, Thermo Scientific, Vilnius, Lithuania). Quantitative polymerase chain reaction (qPCR) was carried out in order to verify the presence of PERV-specific sequences in HEK-293 DNA. Subsequently, the same samples were analysed for the absence of swine-specific genes and thus for the exclusion of contamination by porcine cells. Primer sequences specific for group-specific antigen gene fragment of PERV (*gag*) were used for PCR as described by Paradis et al. (1999). Primer sequences were as follows: PERV_F: 5'-TGA TCT AGT GAG AGA GGC AGA G-3' and PERV_R: 5'-CGC ACA CTG GTC CTT GTC G-3'. Primers for swine-specific mitochondrial DNA (mt DNA) were PMITF1: 5'-CAC CCG TTC ATC ATC ATC GGC CA-3'; PMITR2 5'-GGT GGT GAT ATG CAT GTT GAC TG-3' (Kuddus et al., 2003). Appropriate PCR product lengths for PERV *gag* and mt DNA were 262 bp, and 547 bp, respectively. The PCR cycling conditions for PERV and mt DNA sequences consisted of 5 min at 95 °C followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 40 s (Ruprecht et al., 2006). All PCR reactions were carried out using a Mastercycler ep PCR apparatus (Eppendorf, Hamburg, Germany).

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from cell cultures using TRI Reagent (MRC, Cincinnati, OH) according to the one-step extraction method described by Chomczynski and Sacchi (1987). One ml of TRI Reagent was added to lyse cells from the 75-cm^3 bottle. RNA precipitates were dissolved in 100 µl of nuclease-free water. Each RNA sample was examined spectrophotometrically at 260 and 280 nm wavelengths. Directly before reverse transcription 1 U of *E. coli* DNAse I, RNAse-free (New England Biolabs INC, Beverly, MA) was added per 1 µg of total RNA in order to remove the residual DNA. This step was crucial for interpretation of results because HERV sequences of both cDNA and genomic DNA origin are identical in length, and there is no possibility to distinguish between them by intron-exon spanning primers. Despite the use of intron-exon spanning primers, DNAse I treatment of RNA samples before reverse transcription (RT) is the second possibility of choice recommended by the manufacturer of the TRI reagent used in RNA extraction (Molecular Research Center, Inc., Cincinnati, OH). Next, 1 µg of total DNA-free RNA was reverse transcribed by GoScript Reverse Transcription System (Promega Corporation, Madison, WI). The thermal profile was: 22 °C for 5 min., 42 °C for 15 min. and 95 °C for 5 min. Finally, the RT reaction mixture was diluted 1 : 4 with DNAse-/RNAse-free water.

Quantitative analysis of HERV-W *gag*, *pol env* and *env* mRNA from the 7q21.2 locus in both PERV-infected and non-infected HEK-293 cells was carried out by real-time qPCR assay. An amount of 5 µl of RT reaction mixture (i.e. an equivalent of 50 ng of total RNA) was used as template in each reaction. Real-time PCR was performed using GoTaq qPCR Master Mix (Promega). The reaction mixture (total volume of 25 µl) contained 12.5 µl 2× Master Mix, and 200 nM of each forward and reverse primer. Primer sequences for HERV-W *env* 7q21.2 mRNA were described by Menendez et al. (2004), primers for HERV-W *gag* (designed to amplify 44 targets) were described by Nellåker et al. (2006), primers for HERV-W *env* (designed to amplify 11 targets) were previously described by Yao et al. (2008). Primer pairs for the amplification of the HERV-W *pol* gene fragment from various loci were adopted from Yi et al. (2004) and modified according to qPCR reaction requirements. Primers that amplify the 235-length syncytin-1 transcript fragment (*env*(*7q21.2)*) are syncytin-1-specific, which was proved by means of dideoxy sequencing. Briefly, the appropriate qPCR products were resolved in agarose gel, extracted, purified by means of a silica-based column (Invisorb Fragment CleanUp, InviTec, Berlin, Germany) and sequenced (Genomed SA, Warsaw, Poland). BLAST analysis of three obtained sequences showed high specificity only to syncytin-1-derived clones found in the GenBank database. Appropriate multiple alignment of three 235 bp-long qPCR frag-

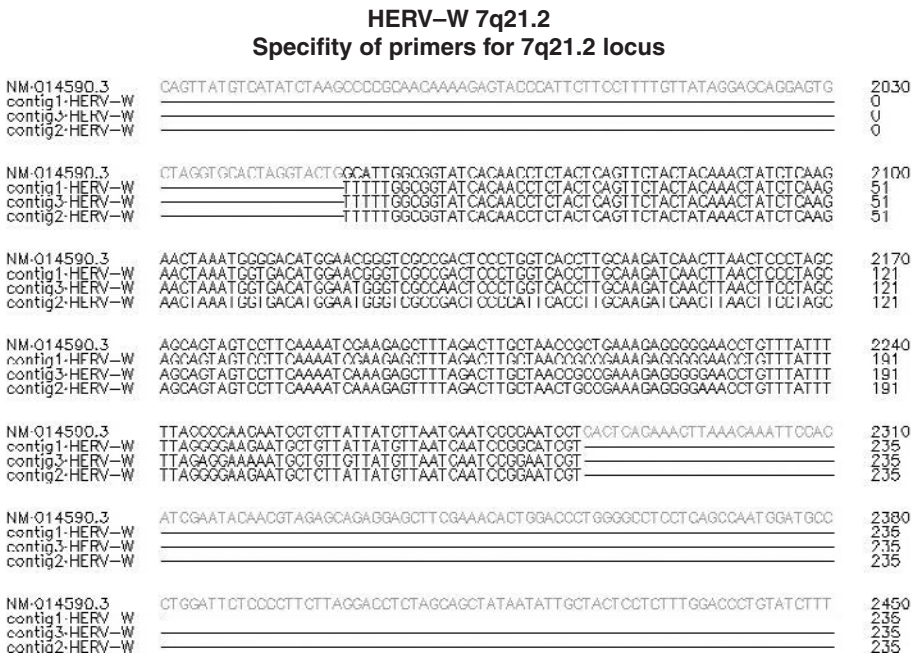

Fig.1. Sequencing of 235 bp qPCR fragment for HERV-W *env(7q21.2)* Multiple alignment of three purified samples shows their high degree of identity with the syncytin-1 clone obtained from the GenBank database (accession number: NM_014590). The alignment was constructed using Emma software from the EMBOSS package (Rice et al., 2000).

ments together with syncytin-1 mRNA (GenBank accession nr. NM_014590) was done and it is shown in Fig. 1. All sequences of qPCR primers used in the experiment are summarized in Table 1.

Additionally to HERV-W-specific primers, sequences specific for human glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) were used. We chose a relative quantification experiment type based on SYBR-Green I chemistry for parallel amplification of HERV-W genes and *GAPDH* as endogenous normalizer. The level of GAPDH mRNA expression is thought to be the most frequently used as the reference, "housekeeping" gene together or parallel with the β-actin gene. In addition, primers for polymerase that span the intron-exon boundary in the *GAPDH* gene give the guarantee that no amplification of genomic DNA occurs. Therefore, we used primers well described previously and collected in the primer database (RTPrimerDB; Primers' ID: 2045). qPCR was performed in a Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics Polska, Warsaw, Poland). The real-time PCR thermal profile was: 95 °C for 3 min (Ruprecht et al., 2006), cycles of 94 °C for 30 s, 58 °C for 1 min, followed by melting curve analysis. The specificity of each reaction was confirmed by melting-curve analysis and by gel electrophoresis in 1.5% agarose gel (Agagel mini, Biometra, Göttingen, Germany) and, in case of shorter products, by polyacrylamide gel electrophoresis (SE260, Hoefer, San Francisco, CA).

Protein expression analysis

The semi-quantitative measurement of HERV-W Env protein (syncytin-1) abundance was based on Western blot analysis utilizing syncytin-1-specific polyclonal antibodies (Thermo Scientifics, Rockford, IL) and actin-specific antibodies (Abcam Corp., Cambridge, MA). The protein extraction procedure for Western blot was as follows: on every passage, 250 µl of ice-cold RIPA buff-

Table 1. HERV-W-specific primers used in qPCR assays

Target gene	Forward primer	Reverse primer	Product length	Reference
gag	TCAGGTCAACAATAGGATGACAACA	CAATGAGGGTCTACACTGGGAACT	81 bp	Nellåker et al. (2006)
pol	CCTGTGGCTACAAGGTTTCC	CCCTGGTGCCTTTGGATAAT	90 bp	Yi et al. (2004) (modified)
env	CCAATGCATCAGGTGGGTAAC	GAGGTACCACAGACAAAAAATATTCCT	78 bp	Yao et al. (2008)
syncytin-1 (*env* 7q21.2)	TTGGCGGTATCACAACCTCT	GTGACGATTCCGGATTGA	235 bp	Menendez et al. (2004)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226 bp	RTPrimerDB; ID: 2045

er supplemented with 1/100 volume of Protease Inhibition Cocktail (both from Sigma) was added to the cells directly into the bottle and mixed continuously on ice for 5 min on a shaking plate. After scraping cells into 1.5 ml tubes and centrifugation (13 000 *g*, 15 min at 4 °C), supernatant was collected for further analysis. Total protein concentration was measured in each sample by modified micro Bradford's (1976) technique and total protein concentration was calculated according to the standard curve based on bovine serum albumin (BSA) solutions of known protein concentrations (Fermentas, Lithuania). Proteins were separated by 10% SDS-PAGE in 10% polyacrylamide gel (Mighty Small electrophoresis unit, Hoefer INC.). An amount of 20 μg of total protein was loaded into each lane. All samples were analysed in duplicates. Next, proteins were electroblotted (TE22 blotting unit, Hoefer INC.) onto PVDF membrane (Pall Corporation, Port Washington, NY). Blotted proteins were quickly visualized by Ponceau red staining in order to confirm the transfer efficiency.

After blotting, membranes were immediately blocked by incubation in 2% BSA solution in Tris-buffered saline (1 x TBS, BioRad, Hercules, CA) for 45 min with constant rocking. HERV-W Env and actin protein bands were detected by incubation with primary antibody solutions: TTBS (Tris-buffered saline supplemented with 0.05% of Tween 20) solution containing 500-fold diluted polyclonal rabbit anti-HERV-W Env (syncytin-1) or 2000-fold diluted rabbit polyclonal anti-actin antibodies. Incubations were performed overnight at 4 °C with gentle rocking. After incubation with primary antibodies, blots were washed twice (5 min each) in TTBS and incubated in a solution containing HRP-conjugated secondary antibody (goat anti-rabbit-HRP, SantaCruz Biotechnology, Santa Cruz, CA). Finally, ECL substrate was added (Pierce ECL Western Blotting Substrate, Thermo Scientifics) and light signals were detected on Kodak BioMax film (Eastman Kodak, Rochester, NY). Pictures were scanned and semi-quantitative analysis was performed using ImageJ software (Abramoff et al., 2004).

Results

Verification of PERV infection and PERV load in cultured cells

The PERV *gag* gene-specific PCR fragment was detected in agarose gel for the first time after the 5th cell passage and after every following passage. None of the analysed samples contained the PCR product specific for porcine mitochondrial DNA, confirming that no cell contamination occurred. No statistically significant differences in PERV *gag* DNA load were observed in any of the HEK-293, PERV-infected cell line passages ($P = 0.198$) (Fig. 2).

Relative expression of HERV-W genes

We chose the $2^{-\Delta\Delta Ct}$ formula for the explanation of changes in gene expression at particular passages (Livak and Schmittgen, 2001). Relative expression values were calculated using a calibrator, i.e. HERV-W *pol, gag, env* and *env(7q21.2)* expression level in uninfected HEK-293 cells, which was considered 1. Data are presented as the median and interquartile range. Differences in the expression of HERV-W genes were analysed by ANOVA Kruskal-Wallis test, and the changes in expression levels between control cells and cells in each passage were calculated by Mann-Whitney U test.

Our results showed that the expression of HERV-W *gag*, *pol* and *env* genes varied in the cell cultures during

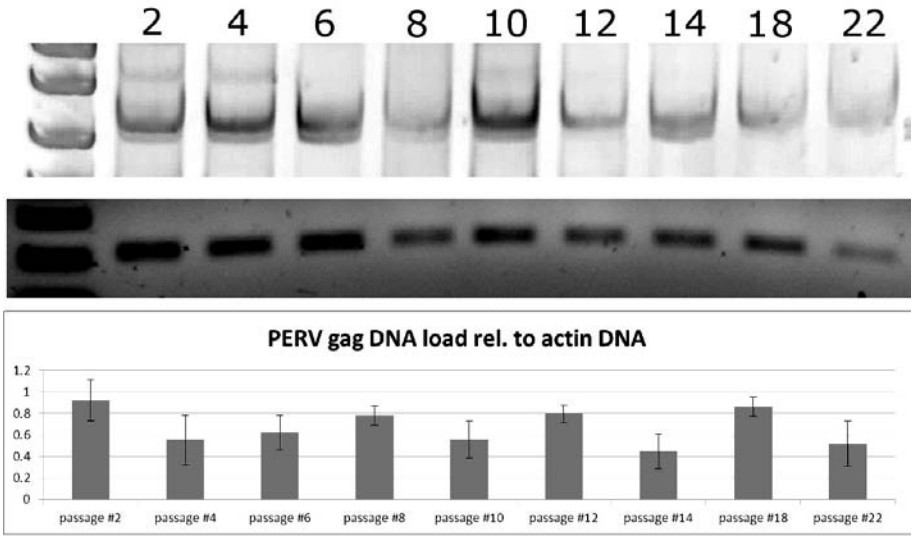

Fig. 2. Semi-quantitative assay of PERV DNA load in HEK-293 cell passages after infection by porcine endogenous retroviruses. The cycle number of PERV *gag*-specific PCR was limited to 25, allowing analysis of amplimers' abundance during the logarithmic phase of PCR. From the top: PCR products for the PERV *gag* gene; PCR products for β-actin as DNA loading control; relative abundance of PERV *gag* DNA relative to β-actin DNA at particular passages. The number of each cell passage is depicted above the figure.

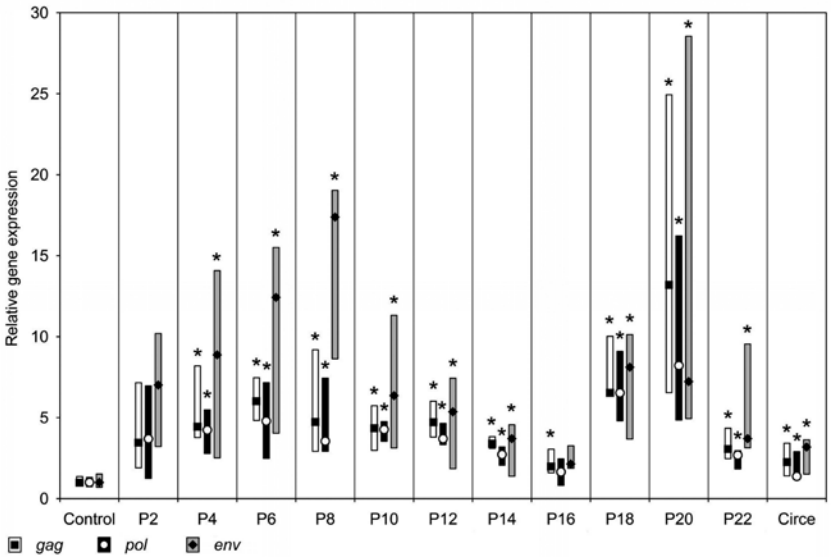

Fig. 3. Relative expression of HERV-W genes in HEK-293 cells after infection by PERV
Control: Uninfected HEK-293 cells; P2-P22: consecutive cell passages after PERV infection; Circe – stably infected HEK-293/PERV cell line derived from cell culture collection. Median values for each gene are indicated by tags and interquartile ranges by boxes. Results show the comparison with the control: *P < 0.05.

cultivation in the presence of PERVs in comparison to non-infected control cells (Fig. 3).

In detail, comparing to the control: the *gag* gene expression significantly differed in all subsequent passages, that of the *pol* gene differed significantly in all but 4th and 16th passages, and the *env* gene expression differed significantly in all but 2nd and 16th passages. We also found that the HERV-W expression differed at particular passages (Kruskal-Wallis test, P < 0.05, detailed results are not shown but are summarized in a table and available on request).

In subsequent analysis of the *env(7q21.2)* locus we found that the relative expression of this element differed significantly between particular passages (P < 0.05) (Fig. 4). The highest HERV-W *env(7q21.2)* gene expression was observed in the cells of 6th and 16th pas-

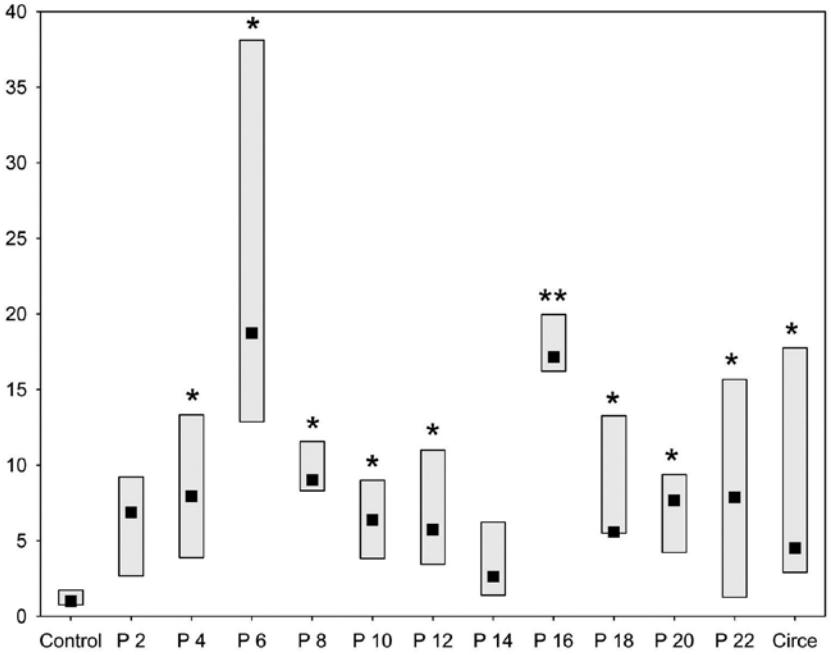

Fig. 4. Relative expression of the HERV-W *env(7q21.2)* gene in HEK-293 cells after infection by PERV. Control: Uninfected HEK-293 cells; P2-P22: consecutive cell passages after PERV infection; Circe – stably infected HEK-293/PERV cell line derived from the cell culture collection. Median values are indicates by black squares and interquartile ranges by gray boxes. Results show the comparison versus control: *P < 0.05, **P < 0.01.

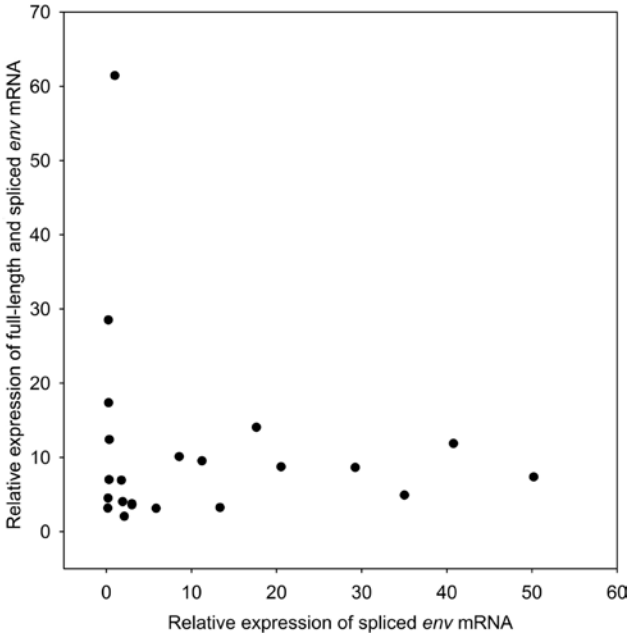

Fig. 5. Spearman rank correlation test of total (i.e. spliced and non-spliced *env* mRNA) and spliced *env* mRNA alone. No correlation has been found, thus permitting the conclusion that the observed increase in total *env* mRNA expression is due to the changes in the number of molecules of *env* mRNA expressed from a locus that is distinct in respect to *7q21.2.*

sages (median: 18.74 and 17.15-fold increase vs. control cells, respectively, $P < 0.01$). In all but two passages (2nd and 14th) the HERV-W *env*-syncytin-1 expression was significantly higher than in control cell cultures ($P < 0.05$). The Spearman rank correlation coefficient (r) test was used to assess the relationships between the mRNA and protein levels of HERV-W Env(7q21.2); $P < 0.05$ was considered significant. All calculations were performed using STATISTICA v. 9.0 (StatSoft Polska, Krakow, Poland)

A special concern of HERV-W expression is the quantitative evaluation of spliced *env* mRNA molecules. To measure the level of spliced *env* mRNA exclusively, we adopted a specific primer set and real-time PCR assay conditions described previously. ERVWE1 splicing was extensively examined by Trejbalová et al. (2011) and also by Gimenez et al. (2010). According to the methodology described by Gimenez et al. we observed predicted, 419 bp-long PCR amplimers specific for spliced *env* mRNA in all samples after the electrophoresis of real-time RT-qPCR products. However, statistical analysis of the quantitative results (Spearman rank correlation test) showed that there was no significant correlation in the expression level between total HERV-W *env* mRNA and spliced HERV-W *env* mRNA only ($r = 0.064$, $P = 0.773$), see Fig 5.

The specificity of HERV-W *gag, pol* and *env*, as well as of *GAPDH* real-time RT-PCR was confirmed by

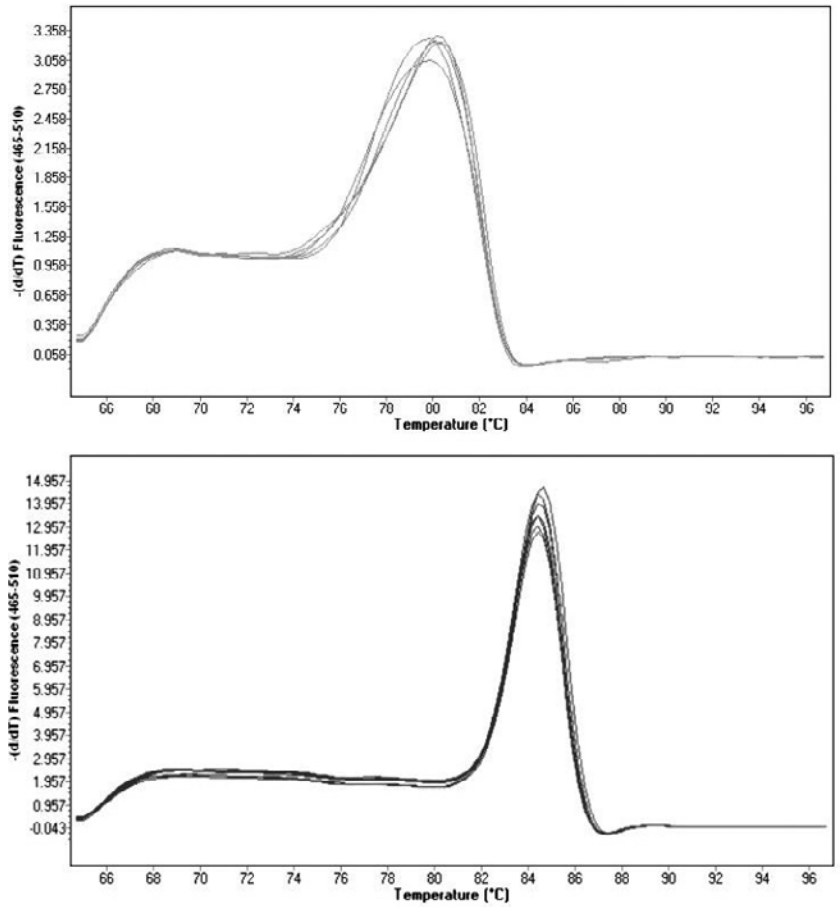

Fig. 6. Melting curve analysis of HERV-W *gag* qPCR products (above) and of the human *GAPDH* gene product. Differences in the shape of melting curves are discussed in the text (see Discussion).

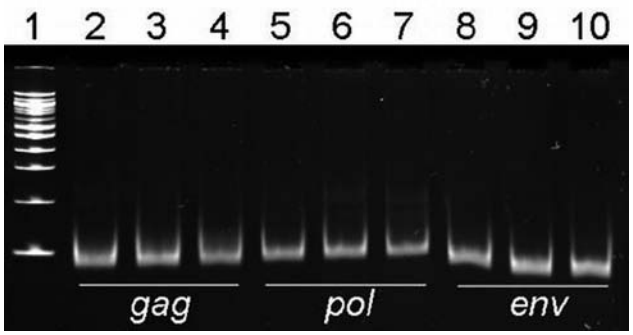

Fig. 7. Acrylamide gel electrophoresis of real-time RT-PCR products after quantitative analysis

melting curve analysis (Fig. 6) and by polyacrylamide gel electrophoresis (Fig. 7).

There are specific bands of 81, 90 and 78 bp for HERV-W *gag*, *pol* and *env* genes, respectively. No unspecific reaction products were formed. 1: PCR 100 bp Low Ladder (Sigma); 2, 5, 8: uninfected HEK-293 cells; 3, 6, 9: HEK-293 cells after 10th passage; 4, 7, 10: HEK-293 cells after 20th passage.

Western blot of syncytin-1 protein

Specific bands for syncytin-1, a HERV-W *env(7q21.2)*--encoded glycoprotein (molecular weight ~55 kDa), and for β-actin (MW = 42 kDa) were detected in all analysed samples derived from the studied cell cultures, as well as from human placenta (positive control for syncytin-1 expression). Semi-quantitative assay of syncytin-1 protein was carried out using the standard immunodetection procedure and signal intensities were expressed as the integrated optical density (IOD) value of specific bands. Results obtained for syncytin-1 were normalized (divided by) to that for β-actin in the same sample in order to normalize possible variations in protein concentrations between samples. We observed differences in abundance of syncytin-1 protein in HEK-293 cells during cultivation after PERV infection. The highest syncytin-1 level was noted in the 4th–6th passage (Fig. 8). Correlation analysis for syncytin-1 mRNA and protein abundance in the cells of particular passages showed high positive correlation (Spearman rank correlation test $r = 0.65$; $P < 0.05$).

Discussion

Despite the fact that the majority of HERV genomes harbour numerous mutations and are transcriptionally defective, some HERV elements retain their genetic activity. Moreover, an unusual HERV expression pattern has been linked to numerous disorders such as MS, schizophrenia, lupus erythrematosus and several types of cancer. The potential involvement of HERVs in various pathological states may be directly linked with the proteins that they express (Antony et al., 2007), superantigen activity (Sicat et al., 2005; Perron et al., 2008), molecular mimicry (Joliver-Reynaud et al., 1999) or any HERV influence on the host immune response such as induction of release of various cytokines (Rolland et al., 2005). On the other hand, different studies have indicated that HERV transcription can be modulated and activated by a number of factors, such as UV light irradiation (Hohenadl et al., 1999), cell infection (Nellåker et al., 2006), cytokines (Nelson et al., 2004), and also by genotoxic and cytotoxic drugs (Taruscio and Mantovani, 1998). In respect of inter-viral relationships, An et al. (2001) have demonstrated that HERV-W envelope glycoprotein retains its primary retroviral features, which renders pseudotyping between HERV-W and human immunodeficiency virus type 1 possible. These pseudotyped virions confer the tropism for CD4-negative cells. Other researchers have confirmed that retroviral activation may result from different viral infections, which has

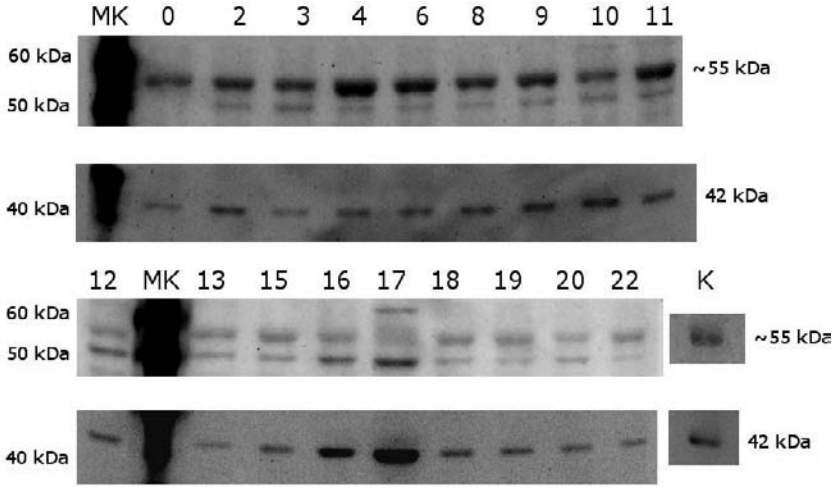

Fig. 8. Western blot analysis of syncytin-1, a protein product of the HERV-W *env(7q21.2)* locus in subsequent passages after PERV infections. Specific bands for syncytin-1 and β-actin (~55 kDa and 42 kDa, respectively) are depicted on the right. Numbers from 2 to 22 indicate cell passages after infection by PERVs; 0 – uninfected HEK-293 cells; K – control sample: cell lysate from human placenta. MK – SuperSignal Molecular Weight Protein ladder (Thermo Scientific), the length of appropriate marker's band is shown on the left.

been demonstrated for *Herpes* and both exogenous (Moriuchi and Moriuchi, 2002; Ruprecht et al., 2006) and endogenous retroviruses (Laderoute et al., 2007; Hsiao et al., 2009). Thus, it seems possible that the formation of HERV-W/PERV pseudotypes may lead to unknown infective potential in case of human cell infection by PERVs. On the other hand, Laderoute et al. (2007) documented in their work that a provirus exclusive to humans, HERV-K102, may be induced and may replicate in association with HIV infection, but this mechanism is potentially thought as a novel host protective defence from retroviral replication.

Recently, Contreras-Galindo et al. (2013) have demonstrated the activation of proviral HERV sequences belonging to a certain HERV-K (HML-2) family in HIV-infected patients and in persons with certain types of cancers, such as lymphoma and breast cancer. The activation of proviral HERVs resulted in production of viral RNA and proteins as well as virus-like particles (VLPs) observed in the blood of these individuals. Our results showed that HERV-W gene expression changed after infection of HEK-293 cells by porcine endogenous retroviruses. Both RNA transcripts, and in the case of *env(7q21.2)*, also protein abundance was higher in PERV-infected cells than in the control cultures.

As described by Pavlicek et al. (2002) and Paces et al. (2002), the human genome contains 654 HERV-W elements, but the majority of them are comprised of only long terminal repeats (LTRs) with no internal sequences. The other HERV-W elements were divided into two major categories: a total of 77 retroelements of proviral structure containing intact LTR sequences and complete or at least partial internal sequences (such as *gag*, *pol* and *env* genes). The remaining elements have been suggested to be non-transcribed due to the absence of regulatory promoter regions. Other authors estimate HERV-W as a multi-copy family with about 70 *gag*, 100 *pro* and 30 *env* genes dispersed throughout the genome (Voisset et al., 2000).

Although the HERV-W retroviral family is represented in numerous loci in the human haploid genome, not all of them retain their coding capacity for intact Env glycoprotein (Christensen, 2010). An ERVWE-1 locus, a HERV-W insertion that codes for syncytin-1, a highly fusogenic membrane glycoprotein whose expression is found primarily in the placenta but may also appear in other cell types, is located on human chromosome 7 (7q21.2) and is of particular importance and warrants more attention (An et al., 2001). ERVWE-1 as well as MSRV, an MS-associated retrovirus, were both likely to be implicated in the neuroinflammation of MS. As described by Antony et al. (2007), ERVWE-1 and MSRV share 81 % identity of their *env* ORFs and more than 90 % identity in their *pol* sequences. According to Mameli and al. (2009), MSRV *env* has only a 12-nucleotide insertion with respect to syncytin-1.

In our assay settings, we first analysed multiple loci of known HERV-W genes, as described previously (Menendez et al., 2004; Yi et al., 2004; Yao et al., 2008) and showed that the overall expression of HERV-W undergoes unusual up-regulation. Subsequently, we focused our attention on the *env(7q21.2)* product as it is the most important one in human physiology and pathology. Although polyclonal HERV-W Env antibodies were synthesized using syncytin-1, they presumably showed reactivity against syncytin-1 as well as against other HERV-W Env proteins. Thus, the strong correlation observed between syncytin-1 protein abundance and mRNA level elevation suggest that the expression of the *7q21.2* locus is altered in the course of PERV infection, but the mRNA expression from other loci (such as MSRV) may be changed as well. Specific analysis of transcripts from *7q21.2* (syncytin-1 coding locus) performed by Nellåker et al. (2006) showed that this particular proviral element was transactivated by the influenza A/WSN/33 virus in all cell lines tested in their work, i.e. 293F cell line as well. Bhat et al. (2011) presented expression diversity of HERV-W. They have noted that HERV-W Env sequences were encoded by multiple chromosomal coding loci in primary human neurons, but less chromosomal diversity was observed in astrocytes and microglia. If so, the influence or PERV infection may alter more different loci, and not only *7q21.2*.

Since no PERV infection of human cells has been documented *in vivo*, little is known about the infection course of these viruses in human recipients, and nothing is known about how PERVs change the metabolism of infected cells. Kuddus et al. (2003) have demonstrated that up to the 72nd passage after infection of HEK-293 with PERV cell morphology, the cell doubling time, DNA content, and expression pattern of HERV-K transcripts were not altered. Similarly, no differences in morphology, growth, apoptosis and no influence on HERV genes were observed by Yu et al. (2005). In the last assay, cultivation of PERV-infected HEK-293 cells lasted over six months. In our experiment the highest levels of HERV-W-derived mRNAs were observed in the 6th and 16th passages after PERV infection. This, in part, correlates with the protein level which was highest at 4th to 6th passages. Interestingly, HERV-W *env(7q21.2)* expression in stably infected 293-PERV-PK-CIRCE cells was slightly higher but comparable with the expression in non-infected HEK-293 cell cultures ($P > 0.5$).

HERVs are known to retain the retroviral type of RNA splicing where only the spliced *env* mRNA serves for translation of the envelope glycoprotein. Both splicing mRNA forms have been observed in human placenta as well as in testicular cancer cells (Mi, 2000; Gimenez, 2010). If so, the observed increase of the *env* mRNA expression level that is depicted in Fig. 5 seems to be due to the changes of full-length mRNA copy number but not to the changes of the spliced mRNA form expressed from the ERVWE1 locus. On the other hand, at the level of syncytin-1 glycoprotein, we have demonstrated changes during consecutive passages of HEK-293 cells in the presence of PERVs. Given the multiple

insertion sites of HERV genes as complete and incomplete open reading frames, together with their differing capacity to be expressed, we suggest that the observed Env glycoprotein level may reflect altered expression activity from other HERV-W members located distantly to that from the ERVWE1 locus. This phenomenon is not to be ruled out in the immortalized HEK-293 cell line and shows that PERV infection possibly influences HERV-W expression at undefined localization of at least partially active *env* gene. The spliced mRNA quantification methodology used in our study was ERVWE1-specific, which was documented by the sequencing of RT-qPCR products (see Fig. 1). We therefore cannot tightly connect our results obtained at the mRNA and protein levels, and further investigation by other groups will be meaningful in order to exclude any unspecific antibody binding or other technical flaws.

In quantitative PCR assays in which intercalating dyes are used such as SYBR-Green, melting curve analysis is strongly recommended, as it validates the specificity of the qPCR product. The other way is to run electrophoresis of the reaction mixture after qPCR assay has been performed. In our case, both techniques were done, because the melting curve alone may suggest that a non-specific reaction product was present. The unusual melting curve shape observed in qPCR products from infected HEK-293 cells might reflect the fact that qPCR products derive from heterogeneous, differently mutated HERV-W *env* loci, i.e. they comprise a non-homogenous population of molecules. Conversely, a strong, sharp peak observed in the melting curve obtained for the *GAPDH* gene indicated that PCR products originate from one specific locus. Nevertheless, in both cases gel analysis as well as dideoxy-sequencing of qPCR products confirmed that the reactions were specific and no undesired products were amplified (Fig. 1 and Fig. 7).

Numerous papers reported that the viral titre of PERV particles increases in long-term culture (measured as qPCR-estimated provirus number) and its structure undergoes changes (Karlas et al., 2010). Some authors, however, performed quantitative PCR assay only to confirm the presence of PERV in infected cultures (Kuddus et al., 2003; Yu et al., 2005).

Although we carefully followed previously published assays, in our infection procedure of HEK-293 cell line with porcine endogenous retroviruses we decided to perform semi-quantitative confirmation of the PERV DNA load at particular passages after infection. To resolve this issue, PCR and polyacrylamide gel electrophoresis stained with SYBR-Green nucleic acid stain was chosen. Bearing in mind that the end-point PCR cannot be validated as a quantitative method because of the plateau phase at its end, we limited the number of reaction cycles to 25 in order to stop the amplification being in its logarithmic phase. In this case, the intensity of the bands on the gel, measured as IOD, can be treated as a semi-quantitative value and may be compared to each other. We amplified the DNA fragment of the PERV *gag* gene and the β-actin gene fragment as loading control of the DNA amount used in the reaction. No statistically significant differences in the PERV Gag load were observed in any of the PERV-infected HEK-293 cell line passages ($p = 0.198$)(Fig. 2).

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