

Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

SCIENCE @ DIRECT®

Virology 325 (2004) 18–23

VIROLOGY

[www.elsevier.com/locate/yviro](http://www.elsevier.com/locate/yviro)

# Inhibition of porcine endogenous retroviruses by RNA interference: increasing the safety of xenotransplantation

Alexander Karlas, Reinhard Kurth, and Joachim Denner\*

*Robert Koch-Institute, D-13353 Berlin, Germany*

Received 7 January 2004; returned to author for revision 17 March 2004; accepted 1 April 2004

Available online 2 June 2004

## Abstract

Transplantation of porcine xenografts into human recipients is a realistic option to overcome the growing worldwide shortage of suitable allogeneic organs. However, there remains the risk of infection by porcine endogenous retroviruses (PERVs) that cannot be eliminated like that by other microorganisms by breeding pigs under specified pathogen-free conditions. To reduce the release of PERVs by porcine transplants, a new approach, RNA interference (RNAi), was applied. Here, we show significant reduction of PERV expression by synthetic short interfering RNAs (siRNAs) corresponding to different parts of the viral genes *gag*, *pol*, and *env*. The most inhibitory sequences were selected and expressed as short hairpin RNAs (shRNAs) by a polymerase III vector system leading to persistent suppression of PERV replication. Cells or organs from transgenic pigs producing such shRNAs should increase the safety of xenotransplantation.

© 2004 Elsevier Inc. All rights reserved.

**Keywords:** Porcine endogenous retrovirus; Xenotransplantation; RNA interference; Short interfering RNA

## Introduction

Xenotransplants from pigs may offer a potential solution to the lack of allotransplants (Cooper, 2003). However, this requires prevention of the immunological rejection and a better physiological compatibility of the xenotransplant as well as prevention of transmission of porcine microorganisms. The porcine endogenous retroviruses (PERVs) are particularly problematic because they are integrated into the genome of all pigs (Patience et al., 2001) and can infect human cells in vitro (Patience et al., 1997; Specke et al., 2001). Knock-out animals may prove difficult to produce due to the presence of several replication-competent and numerous defective proviruses capable of complementing each other. Although PERVs infect human cells in vitro, no virus transmission was observed in initial clinical xenotransplantations carried out to treat diabetes or acute liver failure (Irgang et al., 2003; Paradis et al., 1999). In these trials, the porcine cells were encapsulated, the contact time was very short, or the

immunosuppression of the transplant recipient was very weak. In contrast, the conditions envisaged for future xenotransplantation include long-term transplantation of porcine cells or whole organs, direct contact between porcine and human cells, and pronounced immunosuppression. In the meantime, several experimental pig to nonhuman primate xenotransplantations and infection experiments using high doses of virus in small animals and nonhuman primates under immunosuppression have been performed. In none of these was transmission of PERV observed (Denner et al., 2001; Loss et al., 2001; Specke et al., 2000). Nevertheless, the risk of transmission of porcine viruses to transplant recipients remains and with it the risk of retrovirus-induced tumours or immunodeficiencies (Denner, 1998).

To minimize the possibility of PERV transmission, different strategies may be developed such as the selection of low-virus-producer animals (Oldmixon et al., 2002) or the development of an antiviral vaccine (Fiebig et al., 2003). In addition, new approaches such as the use of RNA interference (RNAi), based on the sequence-specific degradation of target mRNA induced by short double-stranded RNA, should be followed. The mechanism of RNAi is highly conserved among plants, flies, worms, and mammals (Caplen et al.,

\* Corresponding author. Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany. Fax: +49-30-4547-2801.

E-mail address: [DennerJ@rki.de](mailto:DennerJ@rki.de) (J. Denner).

2001). After processing by the ribonuclease III (RNase III)-like enzyme Dicer, double-stranded RNA is cleaved into small double-stranded fragments (21–25 nt) (Hammond et al., 2000). In the cytoplasm, these short interfering RNAs (siRNA) interact with the RNA-induced silencing complex (RISC), which recognises the target mRNA, homologous to the sequence of the siRNA. Nucleases, as part of the complex, degrade the target RNA and prevent protein translation (Billy et al., 2001; Yang et al., 2000). Transfecting mammalian cells with longer dsRNA (>30 nt) was found to be problematic because the induced interferon response led to unspecific degradation of mRNA and finally to apoptosis. The Dicer processing step can be bypassed by transfecting cells with synthetic siRNAs (Elbashir et al., 2001). As these siRNAs within the cells are diluted out during cell division, the time of gene silencing activity is limited to approximately four to eight cell doublings (McManus et al., 2002; Stein et al., 2003). Expressing short hairpin RNA (shRNA), which contains a short loop sequence joining the forward and reverse strand, can overcome this limitation. Defined shRNA without any cap or polyA structure can be expressed by polymerase-III-dependent promoters such as the murine or human U6 snRNA or the human RNase P (H1) RNA promoters (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002). In addition, cells with stable chromosomal integration and permanent inhibition of target genes can be obtained if adequate selection markers are used.

Here, we show inhibition of the expression of PERVs by siRNAs at the level of RNA, protein, and virus release. This

is to our knowledge the first report showing suppression of a retrovirus other than HIV-1 and the first report showing suppression of an endogenous retrovirus. The approach could lead to the generation of transgenic pigs providing safe xenotransplants and is therefore of considerable medical importance.

## Results

### *Inhibition of PERV expression by synthetic siRNAs and selection of potent siRNA target sequences*

Synthetic siRNAs targeting the genes *gag*, *pol*, and *env* of PERV were designed according to published guidelines (Fig. 1A) (Elbashir et al., 2002). The siRNAs *gag1*, *gag2*, *pol2*, *pol4*, and *pol5* should be able to inhibit PERV-A, PERV-B, and PERV-C due to conserved sequences. The siRNAs *pol1*, *pol3*, *env1*, and *env2* are specific for PERV-B. The siRNAs were transfected into PERV-B-infected 293 cells and the inhibition of gene expression was measured by real-time reverse transcription (RT) PCR with different primer pairs flanking the siRNA target region (Fig. 1A). Some of the selected siRNAs failed to induce any silencing activity (e.g., *gag1*), whereas others suppressed viral gene expression to as little as 18% (Fig. 1B). Based on these data, the most effective inhibitory construct in terms of silencing PERV expression (siRNA Pol2) was selected.

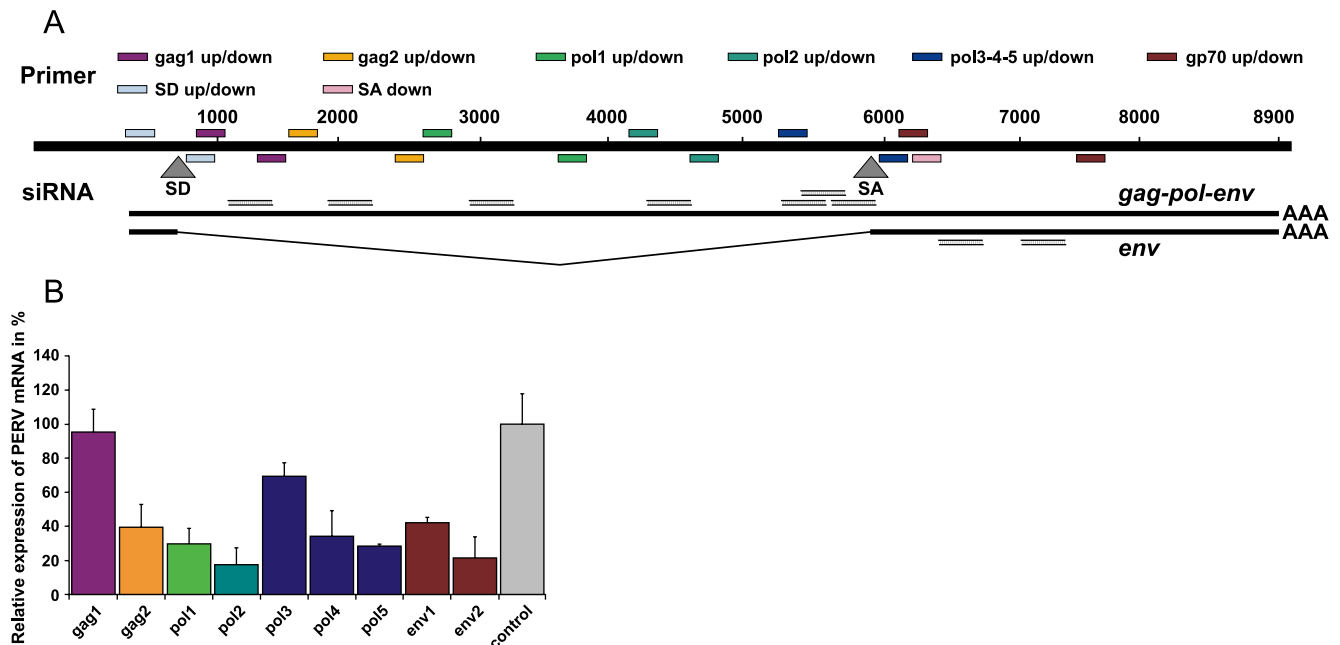


Fig. 1. Localisation and efficacy of siRNAs targeting PERV. (A) Schematic presentation of the PERV genome with the localisation of siRNA target sequences and oligonucleotide primers used for detection of viral RNA in PCR assays. SD and SA indicate splice donor and acceptor sites. (B) Expression of viral RNA as measured by quantitative real-time RT-PCR after transfection of synthetic siRNAs into PERV-B-infected human 293 kidney cells, expressed as percentage of mRNA synthesis in PERV-B-infected 293 cells transfected with an irrelevant control siRNA.

### Inhibition of PERV expression after stable transfection of a shRNA-expressing plasmid

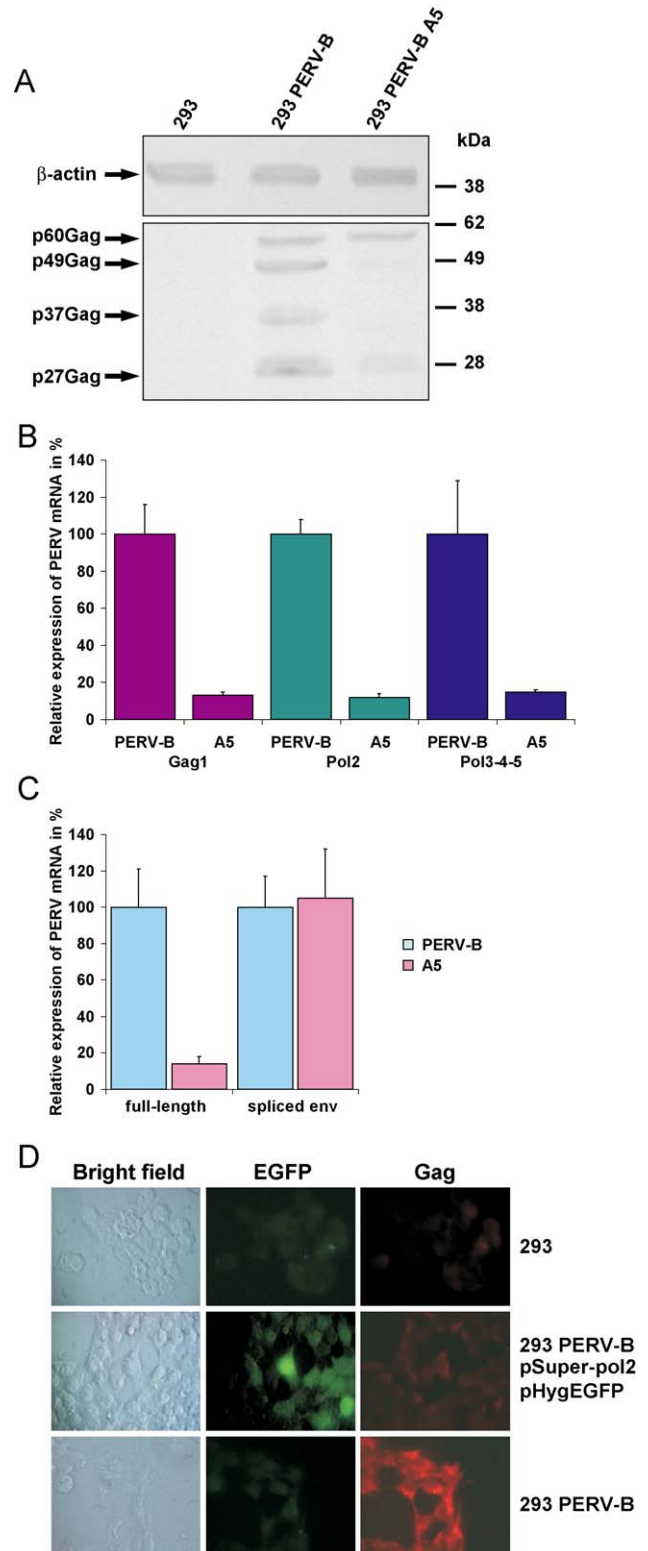
To achieve permanent expression of this *pol*-specific siRNA, oligonucleotides corresponding to the *pol2* region were ligated into the vector pSuper (Suppression of endogenous RNA) containing the polymerase III H1-RNA gene promoter (Brummelkamp et al., 2002). The plasmid was co-transfected into PERV-B-infected 293 cells together with the plasmid pHygEGFP expressing the enhanced green fluorescent protein (EGFP) and providing hygromycin resistance.

Some of the isolated cell clones showed a strongly reduced expression of p27Gag protein, as shown by Western blot analysis. A strong reduction up to 90% was found in the cell clone A5. Although the target region is located within the *pol* gene, the degradation of the complete full-length mRNA coding for both Gag and Pol resulted in a concomitant decrease of Gag protein synthesis. Because the viral protease (also encoded by the *pol* gene) was silenced at the same time, the Gag protein was not processed, leading to a relative accumulation of the Gag precursor protein (60 kDa), whereas the other Gag proteins partially or completely disappeared (Fig. 2A). In addition, this effect may be explained by the fact that core assembly needs a distinct threshold of gag protein to occur. Because the level of Gag was reduced by RNAi, core assembly, essential for budding and for the proteolytic cleavage of Gag, cannot take place. Virtually the same degree of inhibition could be demonstrated at the RNA level (Fig. 2B). This was shown by a quantitative real-time PCR using the primer pairs (*pol2* up/down) flanking the shRNA binding site. However, when other oligonucleotide primers were employed located up- and downstream of the shRNA binding site, the level of viral RNA was not significantly higher, indicating a rapid decay of the full-length RNA molecule by the RISC complex.

Fig. 2. Inhibition of PERV p27Gag expression after stable transfection of pSuper-pol2 shRNA. (A) Western blot assay measuring viral Gag protein in untreated 293 cells, in PERV-B-infected 293 cells and in PERV-B-infected 293 cells stably transfected with pSuper-pol2 (cell clone A5). Gag protein expression was significantly inhibited by the siRNA and due to the inhibition of the viral protease, a relative accumulation of the precursor Gag protein was observed. (B) Viral RNA was processed as measured by real time RT-PCR with different primer pairs (*gag1* up/down in violet, *pol2* up/down in green, *pol3-4-5* up/down in blue). PERV expression was calculated as the percentage of mRNA expression in PERV-B-infected 293 cells. (C) The expression rate of spliced and unspliced viral RNA was quantified in PERV-B-infected 293 cells and clone A5 cells expressing pSuper-pol2 shRNAs using primers flanking the splice donor and splice acceptor site. The results demonstrate the effective inhibition of full-length but not spliced *env* RNA. (D) Immunofluorescence assays also demonstrate significant inhibition of p27Gag expression in PERV-B-infected 293 cells transfected with pSuper-pol2 in comparison to untreated PERV-B-infected 293 cells. Cells were treated with an antiserum against p27Gag and a TRITC-labelled secondary antibody. The expression of EGFP indicates stable co-transfection of pSuper-pol2 and pHygEGFP.

### Inhibition of expression of full-length, but not spliced *env* RNA

Using a PCR method which allows to discriminate between full-length and spliced *env* RNA, it was shown that the synthesis of the spliced *env* mRNA was not



influenced by the expression of pol2 shRNA in clone A5 cells but the full-length mRNA was (Fig. 2C). The viral *env* message is spliced in the nucleus and the viral precursor RNA cannot be processed by the RISC complex in the cytoplasm (Zeng and Cullen, 2002). This result also shows that the spliced *env* RNA is expressed at similar levels in PERV-B-infected 293 cells and A5 cells, indicating that the inhibition of expression of the full-length mRNA in A5 cells up to 14% is not due to a reduced promoter activity but due to the expression of pol2 shRNA.

After culturing these A5 cells for several months, the gene silencing activity was still present and the plasmid pSuper was always detected by PCR in these cells (data not shown). The morphology and proliferation rate of these cells were not changed compared to the untransfected cells.

#### *Inhibition of PERV protein expression*

To confirm results showing inhibition of PERV protein expression (Fig. 2A), an immunofluorescence assay was performed, measuring the expression of p27Gag in PERV-B-infected cells, in cells from clone A5 and in uninfected 293 cells (Fig. 2D). Because the A5 cells were co-transfected with the plasmid pHygEGFP, these cells stably express EGFP together with the shRNA pol2. Clone A5 cells showed a strong reduction of p27Gag expression compared to the untreated PERV-B-infected cells and at the same time EGFP is expressed (Fig. 2D).

#### *Inhibition of virus release*

Finally, to study the release of infectious PERV by pSuper-pol2-transfected PERV-B-infected cells, the titres of infectious particles in the supernatant of PERV-B-infected cells and of clone A5 cells were determined. When equal volumes of the supernatants produced by an equal number of cells in the same time span were titrated on uninfected 293 cells, the TCID<sub>50</sub>/ml in the supernatant of cells expressing the shRNAs against PERV *pol* was more than 1 log lower than that of untreated PERV-B-infected 293 cells (TCID<sub>50</sub>/ml<sub>PERV-B</sub> = 190; TCID<sub>50</sub>/ml<sub>A5</sub> = 17).

## Discussion

Using synthetic siRNAs corresponding to different parts of the viral genes *gag*, *pol*, and *env*, several suitable target regions for RNAi within the PERV genome were identified, among them the pol2 sequence that proved to be the most effective in reducing virus expression. This is the first report showing suppression of a retrovirus other than HIV-1 and the first report showing suppression of an endogenous retrovirus. Because these experiments were performed in PERV-infected 293 cells, which produce viral particles at a relatively high level, the suppression of PERV by RNAi may well be more effective in pig cell lines or primary

porcine cells producing less virus as shown previously (Denner et al., 2001).

The RNAi strategy used here could lead to the generation of transgenic pigs providing safe xenotransplants and is therefore of considerable medical importance. Transgenic mice have recently been described (Carmell et al., 2003) in which expression of shRNA lead to the silencing of the Neill gene, a DNA *N*-glycosylase, in all organs tested demonstrating the feasibility of this concept. To generate transgenic pigs expressing shRNA-pol2, a lentiviral vector may be useful. As has been shown previously, pig strains and single individuals from the same strain differ in their ability to release PERV from mitogen-stimulated normal blood cells (Tacke et al., 2003). Conventional breeding and selection of such low-virus-producer pigs together with the creation of transgenic animals may result in a highly reduced PERV expression in cells and organs suitable for safe xenotransplantations. The siRNA corresponding to a highly conserved sequence of the *pol* gene of all PERVs will also suppress expression of defective proviruses, thus preventing complementation. To reduce the virus release to zero, additional shRNA targeting PERV at different conserved regions will be useful. Because the RNAi mechanism seems to be sequence specific, gene silencing of vital cellular proteins should not occur in transgenic pigs, although this has still to be demonstrated experimentally as some RNAi off-target effects have been recently reported (Jackson et al., 2003). In addition to the work on other strategies to prevent PERV transmission (Fiebig et al., 2003; Oldmixon et al., 2002; Tacke et al., 2003), extensive virological characterisation of PERVs adapted to human cells (Denner et al., 2003), investigation of the in vitro and in vivo host range (Denner et al., 2001; Loss et al., 2001; Patience et al., 1997; Specke et al., 2000, 2001), as well as sensitive and specific detection methods were developed to facilitate monitoring of PERV transmission in future clinical xenotransplantations (Tacke et al., 2001). Together, these investigations allow a reliable evaluation of the potential risk of PERVs in xenotransplantation.

## Materials and methods

### *Synthetic siRNAs*

The siRNAs gag1, pol2, pol4, and pol5 were purchased from Dharmacon (Lafayette, CO); gag2, pol1, pol3, pol4, env1, env2, and the control siRNA (fluorescence labelled, without significant homology to mammalian genes) from Qiagen (Hilden, Germany).

### *Plasmids*

The plasmid pSuper (*Suppression of endogenous RNA*) was purchased from Oligoengine (Seattle, WA).

Oligonucleotides were ordered from Sigma-Genosys (Steinheim, Germany): (sense: 5'-GATCCCCGGACGCTGACAAATTGACTTTTCAAGAGAAGTCAATTTGT-CAGCGTCCTTT-TTGAAA-3' and antisense 5'-AGCTTTTCCAAAAAGGACGCTGACAAATTGACTTCTTTGAAAGTCAATTTGT-CAGCGTCCGGG-3'). Oligonucleotides were annealed and ligated to the plasmid pSuper, previously digested with *Bgl*III and *Hind*III, resulting in pSuper-pol2.

#### Cells and transfection

Uninfected and PERV-infected 293 human kidney cells were cultured in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

pSuper-pol2 (12 µg) and pHygEGFP (1 µg, BD Biosciences, Franklin Lakes, NJ) plasmid DNA were transfected with TransFast transfection reagent (Promega, Madison, WI) into  $5 \times 10^6$  PERV-infected 293 cells according to the manufacturer's protocol. PERV-B was kindly provided by David Onion, Q-One Biotech Ltd, University of Glasgow, Glasgow, UK. Forty-eight hours post transfection, hygromycin (200 µg/ml) was added, and after 10 days, individual clones were isolated using cloning discs (Sigma, Munich, Germany).

For transfection of synthetic siRNAs, 7500 cells/well were seeded in a 96-well plate. The next day, medium was replaced by 100-µl fresh DMEM containing 10% FCS. siRNAs were diluted in 25 µl Opti-MEM (Invitrogen) and 1 µl of the transfection reagent GeneEraser (Stratagene, La Jolla, CA) was added. After 15 min incubation at room temperature, the mixture was added to the cells resulting in a final siRNA concentration of 20 nM. All assays were performed in triplicate.

#### Western blot analysis

Cells were lysed with RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 2 µg/ml leupeptin, 100 µM sodium orthovanadate, 10 mM *p*-nitrophenylphosphate) and proteins were subjected to preparative SDS-PAGE (10 µg cell lysate per lane) and transferred to PVDF membranes by electroblotting. The level of PERV Gag expression was analysed by Western blot techniques using a goat anti-p27Gag antiserum (Irgang et al., 2003). Loading of equal amounts of protein was verified using antibodies against β-actin (Sigma).

#### Immunofluorescence

Stably transfected cells ( $2 \times 10^5$ /well) were seeded in a six-well plate onto glass coverslips. The next day, cells were washed with PBS, fixed with 3% formaldehyde, and incubated for 30 min with Triton X-100. After treatment

with the primary (anti-p27Gag, 1:100) (Irgang et al., 2003) and secondary antibody (anti-goat IgG TRITC conjugate, 1:2000, Sigma) at room temperature for 1 h, cells were covered with the ProLong Antifade Kit (Molecular Probes, Eugene, OR) and mounted on slides upside down.

#### Quantitative real-time RT-PCR

Cellular RNA was extracted from PERV-infected cells using the RNeasy Kit (Qiagen) according to the manufacturer's protocol or (for the 96-well format) by lysing cells with 100 µl/well "cells-to-cDNA II cell lysis buffer" (Ambion, Austin, TX). DNA was removed by adding DNaseI (Hoffmann-La Roche, Basel, Switzerland) (0.04 U/µl final, 37 °C, 30 min), which was inactivated by incubating at 75 °C for 10 min. cDNA from 5-µl lysate was synthesised with the RT-Kit (Applied Biosystems, Foster City, CA) using random decamers (Ambion). To evaluate the degradation of PERV *gag-pol* mRNA, a SYBR-Green real-time PCR was performed with the real-time PCR cycler MX-4000 (Stratagene) and the Brilliant SYBR Green QPCR Master-Mix (Stratagene). The amount of *gag-pol* and *env* was calculated relatively to the level of *GAPDH* RNA using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). The amount of PERV mRNA degradation after siRNA treatment was normalised to cells transfected with the control siRNA.

#### In vitro infection assay

Equal amounts of PERV-B-infected 293 cells and PERV-B-infected 293 cells stably transfected with pSuper-pol2 were cultured. Three days later, the supernatant from these cells was collected, sterile filtrated, and incubated with 5000 uninfected 293 cells in 96-well plates for 4 days. Supernatants were removed and cells were lysed by three freeze–thaw cycles and 3-h incubation with proteinase K in  $1 \times$  PCR buffer at 56 °C. To inactivate proteinase K, the plate was incubated at 95 °C for 20 min. PCR was performed using cell lysate, *pol*-specific primers (Pol-PK1/6) and the AmpliTaq Gold Polymerase (Applied Biosystems). TCID<sub>50</sub> was calculated using the Spearman–Karber method. Titration was performed in four replicates, 50 µl virus-containing supernatant was diluted 1:2.

#### Acknowledgments

We thank S. Norley for comments on the manuscript; D. Onion for the PERV-B strain; S. Neitzel and P. Zander for technical assistance; and J. Wittmann for useful discussions and support. This work was partially funded by the German Ministry of Health and Social Security.

## References

- Billy, E., Brondani, V., Zhang, H., Muller, U., Filipowicz, W., 2001. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. U.S.A.* 98 (25), 14428–14433.
- Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296 (5567), 550–553.
- Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. U.S.A.* 98 (17), 9742–9747.
- Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J., Rosenquist, T.A., 2003. Germline transmission of RNAi in mice. *Nat. Struct. Biol.* 10 (2), 91–92.
- Cooper, D.K., 2003. Clinical xenotransplantation—How close are we? *Lancet* 362 (9383), 557–559.
- Denner, J., 1998. Immunosuppression by retroviruses: implications for xenotransplantation. *Ann. N. Y. Acad. Sci.* 862, 75–86.
- Denner, J., Specke, V., Schwendemann, J., Tacke, S.J., 2001. Porcine endogenous retroviruses (PERVs): adaptation to human cells and attempts to infect small animals and non-human primates. *Ann. Transplant.* 6 (3), 25–33.
- Denner, J., Specke, V., Thiesen, U., Karlas, A., Kurth, R., 2003. Genetic alterations of the long terminal repeat of an ecotropic porcine endogenous retrovirus during passage in human cells. *Virology* 314 (1), 125–133.
- Elbashir, S.M., Lendeckel, W., Tuschl, T., 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15 (2), 188–200.
- Elbashir, S.M., Harborth, J., Weber, K., Tuschl, T., 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26 (2), 199–213.
- Fiebig, U., Stephan, O., Kurth, R., Denner, J., 2003. Neutralizing antibodies against conserved domains of p15E of porcine endogenous retroviruses: basis for a vaccine for xenotransplantation? *Virology* 307 (2), 406–413.
- Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 404 (6775), 293–296.
- Irgang, M., Sauer, I.M., Karlas, A., Zeilinger, K., Gerlach, J.C., Kurth, R., Neuhaus, P., Denner, J., 2003. Porcine endogenous retroviruses: no infection in patients treated with a bioreactor based on porcine liver cells. *J. Clin. Virol.* 28 (2), 141–154.
- Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., Linsley, P.S., 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21 (6), 635–663.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25 (4), 402–408.
- Loss, M., Arends, H., Winkler, M., Przemeczek, M., Steinhoff, G., Rensing, S., Kaup, F., Hedrich, H., Winkler, M., Martin, U., 2001. Analysis of potential porcine endogenous retrovirus (PERV) transmission in a whole-organ xenotransplantation model without interfering microchimerism. *Transplant Int.* 14 (1), 31–37.
- McManus, M.T., Haines, B.B., Dillon, C.P., Whitehurst, C.E., van Parijs, L., Chen, J., Sharp, P.A., 2002. Small interfering RNA-mediated gene silencing in T lymphocytes. *J. Immunol.* 169 (10), 5754–5760.
- Oldmixon, B.A., Wood, J.C., Ericsson, T.A., Wilson, C.A., White-Scharf, M.E., Andersson, G., Greenstein, J.L., Schuurman, H.J., Patience, C., 2002. Porcine endogenous retrovirus transmission characteristics of an inbred herd of miniature swine. *J. Virol.* 76 (6), 3045–3048.
- Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., Conklin, D.S., 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16 (8), 948–958.
- Paradis, K., Langford, G., Long, Z., Heneine, W., Sandstrom, P., Switzer, W., Chapman, L., Lockey, C., Onions, D., Otto, E., 1999. Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. *Science* 285 (5431), 1236–1241.
- Patience, C., Takeuchi, Y., Weiss, R., 1997. Infection of human cells by an endogenous retrovirus of pigs. *Nat. Med.* 3 (3), 282–286.
- Patience, C., Switzer, W., Takeuchi, Y., Griffiths, D., Goward, M., Heneine, W., Stoye, J., Weiss, R., 2001. Multiple groups of novel retroviral genomes in pigs and related species. *J. Virol.* 75 (6), 2771–2775.
- Paul, C.P., Good, P.D., Winer, I., Engelke, D.R., 2002. Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* 20 (5), 505–508.
- Specke, V., Tacke, S., Boller, K., Schwendemann, J., Denner, J., 2000. Porcine endogenous retroviruses: in vitro host range and attempts to establish small animal models. *J. Gen. Virol.* 82 (Pt. 4), 837–844.
- Specke, V., Rubant, S., Denner, J., 2001. Productive infection of human primary cells and cell lines with porcine endogenous retroviruses. *Virology* 285 (2), 177–180.
- Stein, P., Svoboda, P., Anger, M., Schultz, R.M., 2003. RNAi: mammalian oocytes do it without RNA-dependent RNA polymerase. *Rna* 9 (2), 187–192.
- Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., Forrester, W.C., 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 99 (8), 5515–5520.
- Tacke, S., Bodusch, K., Berg, A., Denner, J., 2001. Sensitive and specific immunological detection methods for porcine endogenous retroviruses applicable to experimental and clinical xenotransplantation. *Xenotransplantation* 8 (2), 125–135.
- Tacke, S., Specke, V., Denner, J., 2003. Differences in release and determination of subtype of porcine endogenous retroviruses (PERV) produced by stimulated normal pig blood cells. *Intervirology* 46, 17–24.
- Yang, D., Lu, H., Erickson, J.W., 2000. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.* 10 (19), 1191–1200.
- Zeng, Y., Cullen, B.R., 2002. RNA interference in human cells is restricted to the cytoplasm. *Rna* 8 (7), 855–860.