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Knockdown of porcine endogenous retrovirus (PERV) expression by PERV-specific shRNA in transgenic pigs

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Background: Xenotransplantation using porcine cells, tissues or organs may be associated with the transmission of porcine endogenous retroviruses (PERVs). More than 50 viral copies have been identified in the pig genome and three different subtypes of PERV were released from pig cells, two of them were able to infect human cells in vitro. RNA interference is a promising option to inhibit PERV transmission.

Methods: We recently selected an efficient si (small interfering) RNA corresponding to a highly conserved region in the PERV DNA, which is able to inhibit expression of all PERV subtypes in PERV-infected human cells as well as in primary pig cells. Pig fibroblasts were transfected using a lentiviral vector expressing a corresponding sh (short hairpin) RNA and transgenic pigs were produced by somatic nuclear transfer cloning. Integration of the vector was proven by PCR, expression of shRNA and PERV was studied by in-solution hybridization analysis and real-time RT PCR, respectively.

Results: All seven born piglets had integrated the transgene. Expression of the shRNA was found in all tissues investigated and PERV expression was significantly inhibited when compared with wild-type control animals.

Conclusion: This strategy may lead to animals compatible with PERV safe xenotransplantation.

Introduction

Xenotransplantation using porcine cells, tissues or organs may offer a potential solution for the shortage of allogeneic human organs [1]. Pigs are the most favored donor species for several reasons: (i) similar physiology and size of organs, (ii) unlimited availability, (iii), short generation interval, high number of progeny (up to 15 to 18 piglets); (iv) relatively low costs, (v) maintenance under high hygienic conditions is possible, i.e., specified pathogen-free (spf) breeding and gnotobiotic delivery is possible, (vi) tools for the genetic modification to reduce the immunogenicity are available and (vii) somatic cloning is possible and thus production of transgenic animals can be significantly enhanced [2]. However, prior to the clinical use of porcine xenotransplants, three main hurdles have to be overcome: The immunological rejections, the physiological incompatibility and the risk of transmission of porcine pathogens. The premier immunological hurdle, the hyperacute rejection, which is due to the stimulation of the human complement system through the gal alpha 1,3 gal epitopes on the surface of porcine cells can be overcome in a clinically acceptable manner by either the production of Gal knock-out animals [3,4] or animals over-expressing human complement regulatory factors [5].

Specified pathogen-free breeding of pigs can prevent transmission of most porcine microbes. However, porcine endogenous retroviruses (PERVs) are integrated in the porcine genome [6,7], and can be released as infectious particles from normal pig cells [8–10] and can infect human cells in vitro [11– 15]. The three replication-competent subtypes of PERVs mainly differ in their receptor-binding site of the envelope protein [6,16]. The subtypes PERVA and PERV-B are polytropic and able to infect human cells in vitro, whereas the ecotropic PERVC infects only porcine cells.
Retroviruses are tumorigenic and may cause immunodeficiencies in infected hosts. The risk of PERV infection has to be carefully assessed specifically in view of the fact that the human immunodeficiency viruses HIV-1 and HIV-2, causing AIDS in humans, are the result of multiple trans-species transmissions of retroviruses from non-human primates [17,18]. However, in several clinical applications of porcine islet cells or ex vivo perfusions of pig liver cells such as bridging during acute liver failure, no virus transmission could be detected [19–22]. Likewise, in a cohort of 160 patients treated with various porcine tissues, no PERV transmission was observed [23,24]. Moreover, PERV transmission was not found after pig to non-human primate transplantation and PERV infection experiments [25–27]. However, the risk of PERV transmission associated with tumors or immunodeficiencies is still prevalent [28]. Removal of PERV sequences by knock-out technology would be the safest strategy, but may prove difficult due to the presence of numerous replication competent and defective proviruses capable of recombination and complementing each other.

Different strategies for the prevention of PERV transmission have been developed including the selection of low-producer animals based on sensitive PERV assays [29–31], the generation of an antiviral vaccine [32], and the inhibition of PERV expression by RNA interference (RNAi) [33–35]. In these first experiments efficient short interfering RNAs (siRNAs) were selected and inhibition of virus expression was studied in PERV infected human cells [33] and primary pig cells [36].

The mechanism of RNAi is highly conserved among different biological systems, including plants, flies, worms, and mammals [37]. After processing by the ribonuclease III-like enzyme Dicer, double stranded RNA is cleaved into small double stranded fragments (21 to 25 nt) [38]. In the cytoplasm, these siRNAs interact with the RNA induced silencing complex, which recognizes the target mRNA, that is homologous to the sequence of the siRNA. Nucleases, which are part of the complex, degrade the target RNA and prevent protein translation [39,40]. Transfection of mammalian cells with longer dsRNA (>30 nt) induced an interferon response associated with an unspecific degradation of mRNA and finally apoptosis. The Dicer processing step can be bypassed by transfecting cells with synthetic siRNAs [41]. The amount of siRNAs within the
cells is diluted out during cell division, thus limiting the time of gene silencing activity to approximately four to eight cell doublings [42,43]. Expression of sh (short hairpin) RNAs, that contain a short loop sequence linking the forward and reverse strand, can overcome this limitation. Defined shRNA without any cap- or polyAstructure can be expressed by polymerase-IIIdependent promoters such as the murine or human U6 snRNA- or the human RNase P (H1) RNA-promoters [44–47]. In addition, cells
with stable chromosomal integration and permanent inhibition of target genes can be obtained if adequate selection markers are used. In addition, retroviral vectors may be used to integrate the
siRNA producing vector [36]. The efficacy of RNAi has been investigated in vivo, until now mainly in mice, rats, and cattles [48–52].

Here, we show for the first time lentiviral vectormediated RNAi in pigs associated with a significant inhibition of PERV expression.

Materials and methods

Cell culture and lentiviral transfection

Primary pig fibroblasts were transduced with the lentiviral vector pLVTHM-pol2 (Fig. 1). The vector expressed a shRNA corresponding to the viral pol2 sequence able to inhibit PERV expression efficiently [36]. Transduced cells were grown as described [53], selected by FACS using the expression of the reporter gene GFP and used in nuclear transfer.

Generation of transgenic pig embryos by somatic nuclear transfer

Transgenic pigs were generated by somatic nuclear transfer cloning using in vitro matured abattoir oocytes [54]. Briefly, oocytes were enucleated by removing the first polar body along with adjacent cytoplasm containing the metaphase plate. The donor cells were arrested at G0/G1 of the cell cycle by contact inhibition and serum starvation [DMEM + 0.5% fetal calf serum (FCS)] for 48 h. Prior to nuclear transfer, cells were treated for 10 min with trypsin/EDTA and centrifuged (200 g/3 min) twice in a 10 ml tube containing 5 ml phosphate-buffered saline and 100 ll FCS. The supernatant was removed and the cells were resuspended in Ca2+-free TL-HEPES medium. A small fibroblast from the positive cell clone was placed in the perivitelline space in close contact with the oocyte membrane to form a couplet. After cell transfer, fusion was induced in Ca2+-free SOR2 medium [0.25 m Sorbitol, 0.5 mm Mg-acetate, 0.1% bovine serum albumin (BSA)]. A single electrical pulse of 15 V for 100 ls (Eppendorf Multiporator_, Eppendorf, Germany) between two electrodes was employed to fuse the membranes of the oocyte and the donor cell. The reconstructed embryos were activated in an electrical field of 1.0 kV/cm for 45 ls in SOR2 activation medium (0.25 m Sorbitol, 0.1 mm Ca-acetate, 0.5 mm Mg-acetate, 0.1% BSA) followed by incubation with 2 mm 6-dimethylaminopurine (DMAP; Sigma, Steinheim, Germany) in NCSU23 medium for 3 h prior to transfer to recipients.

Synchronization of recipients and embryo transfer

Peripuberal German Landrace gilts served as recipients. The gilts were synchronized by feeding 20 mg/pig Altrenogest (Regumate_; Janssen-Cilag, Neuss, Germany) for 13 days followed by an injection of 1000 IU Pregnant Mare Serum Gonadotropine (PMSG; Intergonan_, Intervet, Unterschleissheim, Germany) on the last day of Altrenogest feeding. Ovulations were induced by intramuscular injections of 500 IU human Chorion Gonadotropin (hCG; Ovogest_, Intervet) 80 h after PMSG administration. Nineteen hours later, 115 reconstructed embryos were surgically transferred into the oviducts of one recipient via mid ventral laparatomy under general anesthesia induced and maintained with thiopental sodium (Trapanal_, Altana, Konstanz, Germany). Maintenance of pregnancies was supported by administration of 1000 IU PMSG and 500 IU hCG on days 12 and 15 after surgery. Pregnancy was confirmed by ultrasound on days 25 and 35.

DNA isolation

DNA was isolated from ear biopsies using a salt chloroform extraction method. Sixty micrograms tissue were incubated in 500 ll HOM buffer (160 mm saccharose, 80 mm EDTA, 100 mm Tris–HCl pH 8.0) (all reagents in this chapter from Roth, Karlsruhe, Germany) and 20 ll of a proteinase K solution (20 mg/ml; Invitrogen, Karlsruhe, Germany) at 60 _C over night. After addition of 200 ll of a 4.5 m NaCl solution and 700 ll chloroform/isoamyl alcohol, DNA was extracted by centrifugation (10 min, 10 000 g, 4 _C). The aqueous phase containing genomic DNA was mixed with 700 ll isopropanol and centrifuged as described above. After washing with 70% (v/v) ethanol the precipitated DNA was resuspended in 70 ll H2O and stored at)20 _C until assayed.

Detection of transgene

Integration of the transgene was investigated by two independent PCR assays, using primers specific for (i) GFP (GFP for: 5¢-GATCACGAGACTAGCCTCGAGGT, GFP rev: 5¢-CCAGGATGTTGCCGTCCTC) and (ii) the shRNA expression cassette (pol2 for: 5¢-AACGCTGACGTCATCAAC, pol2 rev: 5¢-GGACGCTGACAAATTGAC) under the following temperature conditions: 95 _C, 10 min; 35 cycles (95 _C, 30 s; 45 _C and 54 _C respectively, 30 s; 72 _C, 1 min); 72 _C, 5 min.

Isolation of siRNA and total RNA

The siRNA and siRNA-depleted total RNA isolation from frozen tissues was carried out using the mirVana miRNA isolation Kit (Ambion, Huntingdon, UK) allowing the enrichment of RNAs smaller than 200 nucleotides according to the manufacturer's instructions and stored at)80 _C until assayed.

RNA probe construction

For the in liquid hybridization a short 32P (GE Healthcare, Freiburg, Germany)-labeled RNA probe (29 nt) was generated by in vitro transcription using the mirVana miRNA Probe Construction Kit (Ambion). A DNA oligonucleotide with the reverse complement sequence of the targetRNAwas used as template for the in vitro transcription (pol2- siRNA) and an additional T7 promotor sequence (5¢-CCTGTCTC-3¢) on it's 3¢end was used (5¢-AGTCAATTTGTCAGCGTCCcctgtctc- 3¢). The transcription reaction and the gel purification of the labeled probe were performed according to the manufacturer's instructions. Visualization was carried out by phosphor-imaging (Imagine Plate Type Bas-III and FLA-2000; Fujifilm, Düseldorf, Germany) with an exposition time of 30 to 60 s.

Detection of siRNA

Pol2-siRNA was detected with the mirVana miRNA Detection Kit (Ambion) according to the manufacture's instructions, detection was carried out by phosphor-imaging with an exposure time of 3 to 7 min.

One-step RT real-time PCR

Quantitative real-time RT PCR was performed using the SuperScript One-Step RT-qPCR System with Platinum_ Taq DNA polymerase (Invitrogen) and the MX4000 thermocycler (Stratagene, La Jolla, CA, USA). A FAM-labeled probe (5¢-FAM-AGAAGGGACCTTGGCAGACTTTCTBHQ1; Sigma) as well as primers specific for PERV gag, amplifying the viral full length RNA of all three subtypes (for: TCCAGGGCTCATAATTTGTC, rev: TGATGGCCATCCAACATCGA) were applied. PERV expression was normalized to the amount of total RNA as well as to the expression of the house-keeping genes glycerinaldehyd-3-phosphate-dehydrogenase (GAPDH), cyclophilin and hypoxanthine-guanine phosphoribosyltransferase (HPRT). A HEX-labeled probe and primers specific for porcine GAPDH (5¢-HEXCCACCAACCCCAGCAAGAGCACGC-BHQ1, for: 5¢-ACATGGCCTCCAAGGAGTAAGA, rev: GATCGAGTTGGGGCTGTGACT) (Operon, Cologne, Germany), a Cy5-labeled probe and primers specific for porcine cyclophilin (5¢-Cy5-TGCCAGGGTGGTGACTTCACACGCC-BHQ2, for: 5¢-TGCTTTCACAGAATAATTCCAGGATTTA, rev: 5¢-GACTTGCCACCAGTGCCATTA, Operon), as well as a FAM-labeled probe and primers specific for porcine HPRT (FAM-ATCGCCCGTTGACTGGTCATTACAGTAGCT- BHQ1, for: 5¢-GTGATAGATCCATTCCTATGACTGTAGA, rev: 5¢-TGAGAGA TCATCTCCACCAATTACTT were used [55]. For each reaction, 50 ng total RNA and the following temperature conditions were used: 50 _C, 15 min; 95 _C, 2 min; 45 cycles (95 _C, 15 s; 54 _C, 30 s). PERV expression in each organ was normalized to porcine GAPDH, porcine cyclophilin and total RNA, respectively, and compared with the expression in the corresponding organ of the control animal. Samples from control and experimental animals were always run together. Data were analyzed using the DDCTmethod [56].

Western blot analyzes

Western blot analyzes using sera specific for p15E and p27Gag of PERV were performed as described [36,57].

Results

shRNA-mediated inhibition of PERV expression in primary fibroblasts

Porcine fetal fibroblasts (primary cell line: P1 F10) were transduced using the lentiviral vector pLVTHM-pol2 (Fig. 1) expressing the PERV-specific shRNA pol2. In order to analyze the shRNAmediated inhibition of PERV-mRNA expression, total RNA was isolated from transduced and nontransduced fibroblasts and one-step RT real-time PCR was performed. PERV full-length mRNA expression was inhibited by 94.8% in fetal fibroblasts P1 F10 transduced with pLVTHM-pol2 as described previously [36]. The inhibition of PERV expression in transduced cells was stable and remained at 95% over months.

Somatic cell nuclear transfer and the production of shRNA transgenic pigs and control animals

The pLVTHM-pol2-transduced fibroblasts were cultured in vitro prior to being used for the production of transgenic pigs via somatic cell nuclear transfer. After activation, 115 cloned embryos were transferred surgically into the oviducts of one synchronized recipient. Pregnancy was confirmed by ultrasound scanning on days 25 and 35 after embryo transfer. After 116 days of gestation seven piglets were born; one of them was stillborn. None of the piglets showed any malformations. Mean birth weight was 1.3 kg which is similar to that of non-transgenic piglets in our pig facility. Ear biopsies were obtained from six piglets and frozen in liquid nitrogen. Four piglets died soon after birth due to agalactia of the sow. Following repeated application of 2 ml oxytocin (Oxytocin 10 IE/ml; Pharma Partner, Hamburg, Germany) lactation was reinitiated and two piglets survived. They were killed by barbiturate overdose at day 3, and samples of heart, lungs, spleen, liver, kidney, pancreas as well as muscle were taken and frozen in liquid nitrogen for further analysis. Control animals were produced by nuclear transfer using primary fibroblasts from non-transduced porcine fibroblasts P1 F10. At day 89 of gestation, organs of five animals were obtained by Caesarian section, the fetuses had a mean weight of 1.1 kg.

Detection of vector integration

To detect the presence of the transgene, the lentiviral vector pLVTHM-pol2, in the genome of the piglets, PCRs were performed with primers specific for gfp and specific for the shRNA expression cassette (Fig. 1). All of the six tested piglets were positive for gfp (piglet 1 was not tested) and the shRNA expression cassette (all animals were tested positive), respectively (Fig. 2).

Expression of the pol2-shRNA in vivo

For analysis of the expression of the pol2-shRNA in different organs, small RNA molecules were isolated from the organs of piglets no. 6 and 7 and used for Northern solution hybridization with a 32P-labeled short RNA probe corresponding the shRNA sequence. Expression of pol2-shRNA was detected in all organs, including heart, lung, spleen, liver, kidney, muscle, and pancreas (Fig. 3).

Inhibition of PERV expression in different tissues

Total RNA was isolated from different organs of piglets no. 6 and 7 and analyzed using one-step RT real-time PCR. As control, total RNA isolated from the corresponding organs taken from five control, non-transgenic pigs was used. Expression of PERV in different organs of the shRNAtransgenic and the non-transgenic control animals was highest in the spleen and lower expression was found in the liver. Due to the differences in PERV expression related to individual organs, PERV expression was compared in shRNA-transgenic and non-transgenic animals on a per organ basis. The expression levels of PERV in each organ of the control pigs were averaged and set 100%. PERV-expression in the corresponding organs from the shRNA-pol2 transgenic piglets was related to the expression in the control organ. Data were normalized to the amount of total RNA or the expression of the house-keeping genes GAPDH, cyclophilin, and HPRT. In all organs of the pol2-shRNA transgenic piglets no. 6 and 7 PERV expression was significantly inhibited by up to 94% (Fig. 4). Irrespective of the type of normalization (total RNA amount or housekeeping genes GAPDH, cyclophilin, and HPRT) similar expression patterns were observed. Noteworthy, expression of PERV in organs of the control animals

was similar to the expression of PERV in adult animals. Preliminary data showed an expression of 2446 copies of PERV/ng in the hearts from five control animals, 500 and 492 in the hearts of the transgenic piglets 6 and 7, respectively. 4564 copies/ng were found in the control lungs vs. 1337 and 936 in the lungs of the transgenic piglets 6 and 7. In the control liver 1614 copies/ng were detected vs. 166 and 328 in the livers of the transgenic piglets, in the spleens 34068 vs. 1908 and 2419, in the kidneys 2532 vs. 975 and 390, showing the efficacy of the RNAi in vivo.

Undedectibility of PERV protein expression and virus release

When PERV protein expression was studied using Western blot analyses of homogenates from different tissues using antisera specific for different PERV proteins (p15E, p27Gag), no PERV protein expression was observed, neither in the non-transgenic controls nor in the transgenic animals (not shown).

Discussion

Results of the present study demonstrate a strong and long lasting inhibition of PERV mRNA expression in primary porcine fibroblasts using a lentiviral vector system expressing a PERV-specific shRNA and use in somatic cell nuclear transfer. This is the first report demonstrating shRNAmediated reduction of PERV expression in shRNA-transgenic pigs in vivo. The strategy applied here could lead to microbiological safer porcine xenotransplants and is therefore of considerable medical importance. More than fifty copies of porcine endogenous retroviruses are integrated in the porcine genome [6,58,59], rendering conventional gene-knockout approaches not feasible and making RNAi a promising alternative. The RNAi approach has already been used for the in vitro inhibition of HIV [60–62] and other viral pathogens like hepatitis B virus (HBV) [63,64], Coxsackie virus B3 [65], West Nile virus [66], and transmissible gastroenteritis virus [51]. Furthermore, RNAi-mediated reduced HBV expression has been demonstrated in mice in vivo [67,68] by murine hydronamic tail vene injection. Long-term inhibition of HBV was achieved by adeno-associated vector-mediated RNAi [69]. RNAi was also induced by lentiviral gene transfer in transgenic mice with a long-term inhibition of the target gene [70]. In contrast, HIV- 1 escapes from RNAi-mediated inhibition not only through nucleotide substitutions or deletions in the siRNA target sequence, but also through mutations that alter the secondary RNA structure [71]. Resistance and/or escape from the inhibitory effects of RNAi are impossible in the case of PERV, since its genome behaves like a cellular gene.

In future experiments, the number of integrated shRNA vectors as well as the influence on the expression of PERV during life time and the persistence of the shRNA expression in the next generation will be analyzed. The physiological role of endogenous retroviruses is unknown. Our results indicate that the siRNA expression did not compromise the fetal development because the siRNA transgenic piglets were born after a normal gestation period, with a normal birth weight and without malformations. Due to the sequence specificity of the RNAi mechanism and the fact that PERV is a retroviral sequence, off-target effects resulting in gene silencing of vital cellular proteins are highly unlikely. Some RNAi off-target effects have recently been reported when interfering with the expression of cellular target genes [72–75]. Due to the early death of the pol2-shRNA-transgenic piglets caused by agalactia of the sow, long-term observations of the inhibitory effect could not yet be made. In transgenic mice expression of the Neil1 gene, a DNA N-glycosylase, was inhibited by shRNA over an extended period of time in all organs [76].

The detection of the inhibitory effect of the pol2- shRNA on PERV expression in vivo requires a suitable control, especially in the presence of a very low expression of the PERV in the non-transgenic founder pigs. In addition, PERV expression varies in different pig strains [9,31] as well as in different tissues and organs of one individuum [77,78]. Thus detailed analysis of non-transgenic pigs of the same strain provides only limited insight into the RNAi effects. To provide an appropriate control, pigs were produced by somatic cell nuclear transfer using the non-transfected counterparts of the identical primary cell isolate. This allowed us to compare PERV expression between animals and organs isolated from different donors and this comparison revealed a significant reduction of PERV expression in the siRNA transgenic pigs.

Expression data were normalized both to the amount of total RNA and to the expression of different house-keeping genes such as GAPDH, cyclophilin, and HPRT. HPRT is considered to be a suitable reference gene for the analyses of gene expression in different porcine tissues and immune cells [79,80]. Numerous studies on GAPDH expression in humans and rodents showed changes related to different experimental conditions. In addition, the existence of pseudogenes for HPRT [81] and GAPDH [82] complicates the use of these genes as standards in RT-PCR reactions. However, although the expression of all house-keeping genes used here was different in different organs, normalization
of PERV expression according to the expression of each of these house-keeping genes showed a similar inhibitory effect of PERV expression up to 95%.

Based on the low expression of viral RNA also a low expression of PERV proteins was expected. In Western blot assays using sera specific for p15E
and p27Gag of PERV, no protein expression was observed, neither in the non-transgenic controls nor in the transgenic animals. Using the same assays PERV protein expression was also not detected in primary PBMCs from different pig strains such as German landrace, Duroc, Schwäbisch Hällisch, German large white as well as crossbreeds. In PK-15 cells, producing detectable amounts of infectious virus particles, expression of p27Gag was detectable, but still very low and this expression was reduced by RNAi nearly to 100% [36]. Higher expression of p27Gag protein was observed in human 293 cells infected with a high titer strain of PERV, which was only partially inhibited by RNAi [33]. A higher expression of PERV proteins was also observed in pig melanomas and in melanoma cell lines [57].

The absence of viral proteins in non-transgenic and transgenic pigs suggested the absence of PERV release. However, the low sensitivity of the Western blot assay (detection limit: 50 ng for p15E and 100 ng for p27Gag [57] left questions open. Improving the assays using monoclonal antibodies, developing a sensitive immunohistochemistry analysis or using a sensitive assay to detect RT activity in the plasma, such as the PERT (productenhanced reverse transcriptase) assay [83] may
help to compare protein expression and virus release in transgenic and non-transgenic animals
more correctly.

To increase the safety of porcine xenotransplants, multiple shRNAs corresponding to different regions of the PERV genome shall be used. The advantage of the RNAi strategy is, that the
expression of all PERVs will be inhibited and the likelihood of recombinations or complementation
is further decreased. Selection of PERV lowproducer pigs using methods described earlier
[9,31] together with the production of shRNAtransgenic animals may reduce PERV expression in
cells and organs and thus increase the viral safety of porcine xenografts.

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Figures

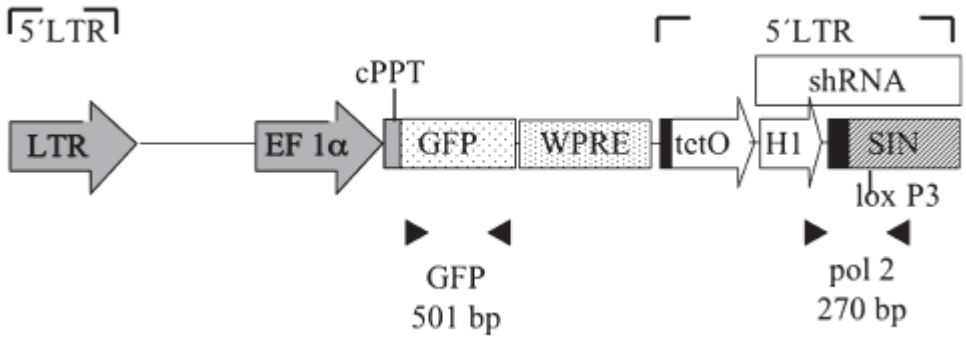

Fig. 1. Localization of the primers specific for GFP and pol2 in the lentiviral vector pLVTHM-pol2 (LTR long terminal repeat, H1 polymerase III H1-RNA gene promotor, shRNA short hairpin RNA, SIN self-inactivating element, cPPT central polypurine tract, GFP green fluorescent protein, WPRE post-transcriptional element of woodchuck hepatitis virus, EF-1alfa elongation factor-1a promoter, tetO tetracycline operator, loxP locus of X-over of P1, recombination site of Cre recombinase for bacteriophage P1, the size of the amplicons is indicated).

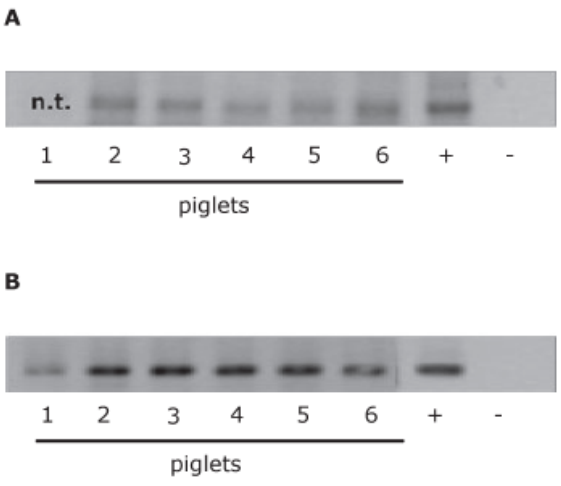

Fig. 2. (A) Integration of the lentiviral vector pLVTHM-pol2 in six piglets demonstrated by PCR analysis using primers specific for GFP, and (B) primers specific for the shRNA expression cassette, positive control (+): vector LVTHMpol2, negative control ()): DNA of non-transduced porcine fibroblasts. N.t. not tested.

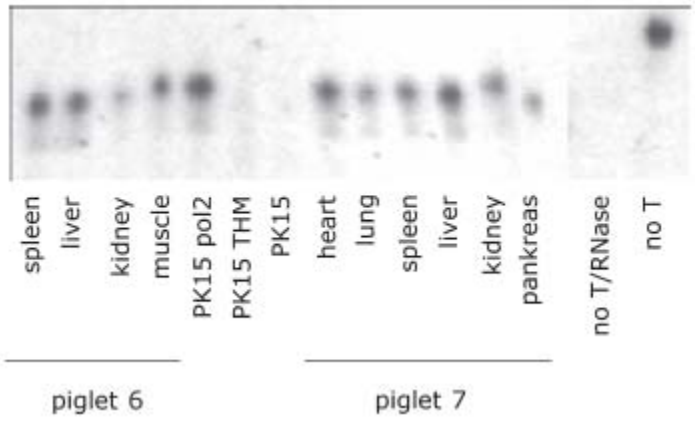

Fig. 3. Expression of the PERV-specific pol2-shRNA in different tissues of piglets no. 6 and 7. As positive control pLVTHM-pol2-transduced PK-15 cells and as negative controls pLVTHM-transduced and non-transduced PK-15 cells were used. Internal negative controls: no target RNA (T)/ RNase (the single stranded probe was degraded by RNase treatment), no target RNA/ no RNase (the full-length probe of 29 nt was not digested).

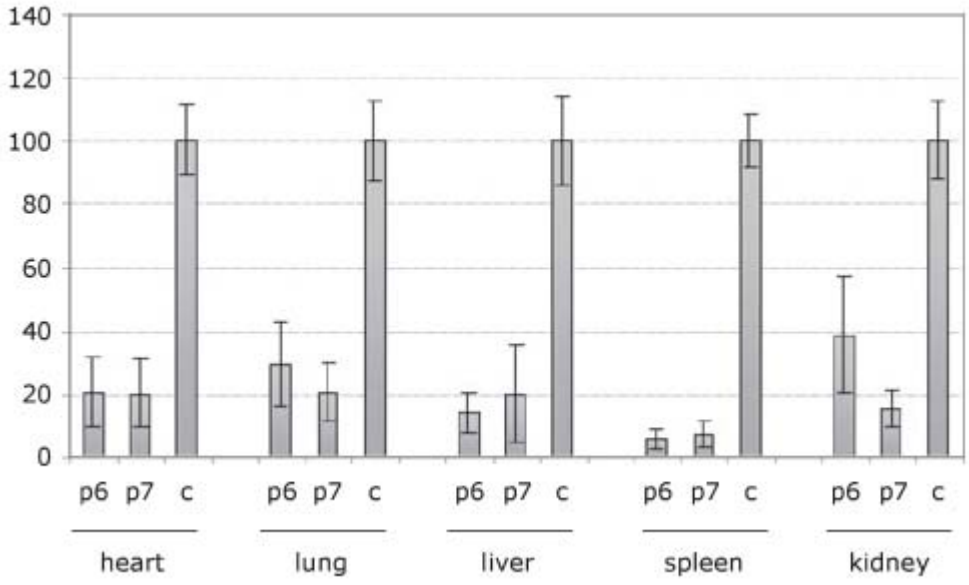

Fig. 4. Inhibition of PERV expression in different tissues of the pol2-shRNA transgenic piglets (p) no. 6 and 7. PERV expression in each organ of control non-transgenic animals (c) measured by one-step RT real-time PCR was set 100% and compared with PERV expression in the corresponding organ of the transgenic animals. In case of control animals the standard deviation is based on the measurement of PERV expression in organs from five different animals, measured in triplicate; in case of transgenic animals the standard deviation is based on the measurement of one organ measured in triplicate in two assays. Expression was normalized to the amount of total RNA, measured by one-step RT real-time PCR.