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Distribution and expression of porcine endogenous retroviruses in multi-transgenic pigs generated for xenotransplantation

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Background: Multi-transgenic pigs produced for use in xenotransplantation have to be screened for the presence and expression of porcine endogenous retroviruses (PERV) to select animals with low PERV load. The production of transgenic pigs may also be associated with the integration of the transgene adjacent to or into the locus of a PERV provirus, potentially leading to an enhanced virus expression.

Methods: Non-transgenic animals, single-transgenic, and multitransgenic pigs were screened for the presence of PERV-A, -B, and –C and recombinant PERV-A/C using polymerase chain reaction (PCR). PERV expression was determined by real time reverse transcriptase-PCR. An assay based on the activation of PERV in peripheral blood mononuclear cells by mitogens was used to discriminate between low and high PERV producer animals.

Results: All animals carried PERV-A and -B. A total of 176 from 181 (97.2%) animals carried PERV-C in the germ line and 18 from 64 animals carried PERV-A/C in the genome of lymphoid cells but not in the germ line. The expression of PERV was very low in all animals and not different between transgenic pigs and non-transgenic animals. PERV expression differed between various pig lines. The highest expression was found in mini-pigs and crossing other pig lines with mini-pigs resulted in increased PERV expression in the progeny. However, expression of viral proteins and particle release were not observed in all transgenic animals.

Conclusions: No evidence for elevated PERV expression in (multi-) transgenic pigs was observed. Differences in PERV expression correlated with the genetic background of the animals, not with the specific transgene. Mini-pigs consistently had the highest level of PERV expression and animals with a mini-pig background had a higher level of expression compared with animals without mini-pig background.

Introduction

Xenotransplantation of cells, tissues, and organs from pigs to human patients is a promising solution for the acute shortage of human organs in allotransplantation. For several reasons, pigs are the most favored donor animals for xenotransplants [1]. However, there are three major obstacles to successful xenotransplantation: first, the immunological rejection, second, the physiological incompatibility, and third, the risk of zoonoses [2]. Among pig microorganisms that may be transmitted and are currently known to induce zoonoses, porcine endogenous retroviruses (PERV) are thought to represent the highest risk for human recipients [3]. PERVs are present in the genome of all pigs [4–6] and are released from normal pig cells [7–9], and at least PERV-A, PERV-B, and recombinants between PERV-A and PERV-C (PERV-A/C) have been shown to replicate in human cells in vitro, while PERV-C is restricted to porcine cells [4,5,10–12]. In order to minimize or avoid transmission of PERV, pigs designed for use in xenotransplantation have to be screened for the presence of proviruses as well as for expression of PERV. We have developed an assay that is based on the ability of mitogens to increase PERV expression in peripheral blood mononuclear cells (PBMCs) [8,9]. The use of this assay revealed differences in the expression and release of PERV depending on the pig breed. Differences in PERV expression were also observed between animals of the same breed [8,9].

Recently, recombinant PERV-A/Cs were discovered and de novo integrated proviruses of such recombinants were found in spleen cells of miniature pigs and melanoma bearing Munich miniature pigs [10,13–16], but not in the germ line of these animals. PERV-A/Cs represent a special risk for xenotransplantation because these recombinant viruses may infect human cells and increase their replication competence when passaged on human cells [10,17,18]. The increased titer during passage on human cells was associated with genetic alterations in the PERV-C-derived long terminal repeats of these viruses, increasing the number of nuclear factor-Y transcription factor binding sites [17]. When comparing the replication rate of PERV-A with that of PERV-A/C, replication rate of PERV-A/C was increased and an isoleucine to valine substitution was found at position 140 in the receptor binding domain. Changes in the proline rich region of the envelope protein were also reported [19]. The higher replication capacity of recombinant PERV-A/C may be associated with a higher pathogenicity as it was shown in the case of other retroviruses, e.g., human immunodeficiency virus (HIV) and feline leukemia virus.

Until now, several transgenic pig lines have been produced that express immunomodulatory genes which suppress the hyperacute rejection (HAR) of transplanted porcine tissues and cells (for review see [1]). Usually integration of the foreign gene occurs at random and integration may rarely occur upstream of oncogenes, tumor suppressor genes, and/or proviruses of endogenous retroviruses with the potential for tumor development and/or enhanced expression of endogenous retroviruses. The goal of the present study was to evaluate the presence of PERV variants in pigs carrying one or more transgenes
in comparison with wild-type animals. To reveal whether PERVs are activated in transgenic and multi-transgenic pigs, the expression of PERVs in such animals was compared with the level of PERV expression in non-transgenic animals. We also show the genetic distribution of PERV-C and recombinant PERV-A/C in different pig breeds including wild boars. A stringent screening for PERV-C was performed as the presence of PERV-C in the porcine genome allows recombination with PERV-A that may result in high titer PERV-A/C recombinant viruses [17].

Material and methods

Animals

Blood and tissue samples from female and male nontransgenic pigs including German Landrace (GL), Duroc (Du), Schwäbisch-Hällisch (SH), German Large White (GLW, Deutsches Edelschwein), Mini-pig (MP) as well as transgenic crossbreds (GL/Du, GL/Pietrain, Du/MP, GLW/Du/MP, GL/Du/MP) were obtained from the large animal facilities of the Chair for Molecular Animal Breeding and Biotechnology, LMU Munich, and from the Institute of Farm Animal Genetics (FLI), Mariensee. Transgenic pigs were produced by pronuclear DNA microinjection, by lentiviral vectors, or by somatic nuclear transfer (SCNT) from transfected cells pronuclear (Table 1). For production of transgenic pigs using pronuclear DNA microinjection or lentiviral vectors, fertilized oocytes (zygotes) were obtained by flushing the oviducts of estrus-synchronized and superovulated gilts that had been inseminated 24 and 36 h prior

to slaughter. Embryos were collected and either centrifuged and DNA-microinjected into pronuclei (DNA microinjection) [20] or a lentiviral vector carrying the transgene was injected directly under the zona pellucida (lentiviral transgenesis) [21]. On the same day, embryo transfer was performed endoscopically into the oviduct of estrus synchronized recipient gilts, according to the method described by Besenfelder et al. [22]. Transgenic animal production by SCNT was performed as described recently [23]. In addition, 18 wild boars were hunted at different places near Berlin in 2005 and were included in the analysis.

Isolation and mitogen stimulation of porcine PBMCs

Peripheral blood mononuclear cells were isolated from heparin-treated blood by density gradient separation using Ficoll (PAA Laboratories GmbH, Pasching, Austria). The PBMCs were isolated, washed three times with phosphate-buffered saline, and resuspended in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum (lot 0898K; Biochrom, Berlin, Germany); 106 cells per well of a 24-well plate were stimulated with 80 lg/ml phytohemagglutinin (PHA) (Oxoid, Wesel, Germany) for 5 days at 37 _C. Control cells were incubated in RPMI culture medium alone. Each experiment was performed in triplicate.

Cell lines

Porcine endogenous retroviruses-producing porcine kidney cell line PK-15 and the human PERV-A/ C-infected cell line 293/50 were obtained and cultured as described [17].

Determination of reverse transcriptase activity

Supernatants of non-stimulated and PHA-stimulated porcine PBMCs were collected after 5 days of incubation and stored at)80 _C. Reverse transcriptase (RT) activity was determined using the C-type RT activity Assay (Cavidi, Uppsala, Sweden) according to the manufacturer's protocol.

RNA isolation

TotalRNAwas isolated from whole blood using the TRI-Reagent_ (Sigma, Taufkirchen, Germany) (0.75 ml per 0.25 ml blood). For isolation of total RNA from tissues, samples were homogenized in liquid nitrogen and resuspended in TRI-Reagent_ (Sigma, 1 ml per 50 to 100 mg of tissue). Chloroform (0.2 ml) was added and after centrifugation (12 000 g, 15 min, 4 _C) total RNA was purified from the aqueous phase using the RNeasy kit (Qiagen, Hilden, Germany), including an on-column DNase digestion. For isolation of total RNA from cell cultures, 106 cells were lysed in 650 ll RLT_ (Qiagen) buffer of the RNeasy kit and processed following the manufacturer's instructions. RNA was stored at)80 _C until assayed.

Isolation of DNA from ear biopsies, spleen tissues, whole blood, and PBMCs

DNA was isolated from ear biopsies using a salt chloroform extraction method. Sixty milligram tissue was incubated in 500 ll lysis buffer (160 mm saccharose, 80 mm EDTA, 100 mm Tris-HCl pH 8.0) (Roth, Karlsruhe, Germany) and 20 ll of a proteinase K solution (20 mg/ml, Invitrogen) at 60 _C over night. After addition of 200 ll of a 4.5 m NaCl solution and 700 ll chloroform/isoamyl alcohol, DNA extraction was performed by centrifugation (10 min, 10 000 g, 4 _C). The aqueous phase containing genomic DNA was mixed with 700 ll isopropanol and after centrifugation and washing with 70% (v/v) ethanol, the precipitated DNA was resuspended in 70 ll H2O and stored at)20 _C until assayed. DNA isolation from whole blood or PBMCs was performed using TRI-Reagent BD and TRIReagent, respectively, according to the manufacturer's instructions (Sigma, 0.75 ml per 0.25 ml blood or 1 ml per 106 cells). Wild boar DNA was isolated from spleen tissue using TRI-Reagent.

PERV-specific PCR

Provirus integration was analyzed by PCR, using primers specific for env of all three subtypes, PERV-A, -B [24], and PERV-C [5]. For each reaction 100 ng DNA was used. Temperature conditions were as follows: 94 _C, 15 min; 34 cycles (94 _C, 30 s; annealing temperature, 30 s; 72 _C, 1 min); 72 _C, 5 min. In addition to the PERVC- specific primers (envCfor, envCrev) [5], a new primer pair was designed and applied (envC.2for, envC.2rev). The presence of PERV-A/C recombinants was investigated by PCR using the following primers: PERV-A env variable region B [13], forward (VRBF) and two different PERV-C primers, transmembrane region (TMR) [13] and PERV-C rev [15]. PCR reactions were performed as described above and with the following temperature conditions: 95 _C, 10 min; 40 cycles (95 _C, 30 s; 55 _C, 30 s; 72 _C, 2 min); 72 _C, 10 min.

PERV-C specific real time PCR

Porcine endogenous retroviruses-C specific real time PCR was performed using the MX4000 thermocycler (Stratagene, La Jolla, CA, USA), a 5¢-hexachloro-fluorescein phosphoramidite (HEX)-labeled probe and primers specific for PERV-C (Table 2).

PERV-specific real time RT-PCR

Quantitative real time one-step RT-PCR was performed using the Invitrogen SuperScript III platinium system and the MX4000 thermocycler (Stratagene), a 6-carboxy-fluorescein (FAM)-labeled probe (Sigma) and primers specific for PERV gag (Table 2). PERV expression was normalized to the amount of total RNA as well as to the expression of the house-keeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), b-actin, and hypoxanthine-guanine phosphoribosyltransferase (HPRT). A HEX-labeled probe and primers specific for porcine GAPDH as well as a FAM-labeled probe and primers specific for porcine HPRT (Table 2) were used [25]. 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). Expression was normalized to total RNA, porcine GAPDH, and porcine HPRT, respectively, and related to expression in the PERV-producing porcine kidney cell line, PK-15, and in the human PERV-A/C-infected cell line 293/50. Data were analyzed using the DDCT-method [26]. Integrity of RNA was determined by amplifying GAPDH by one-step RT-PCR. False-positive results due to residual DNA were excluded by parallel experiments omitting the RT.

Results

Genetic distribution of PERV in different pig breeds

Atotal of 181 pigs of different breeds, including GL, Duroc (Du), SH, GLW (Deutsches Edelschwein), MP as well as crossbreds thereof (GL/Du, GL/ Pietrain, Du/MP, GLW/Du/MP, and GL/Du/MP) were analyzed using primers allowing the discrimination between the PERV subtypes A, B, and C. PERV-A and PERV-B proviruses were found in the genome of all animals, only five animals were negative for PERV-C provirus integration. These animals were GL, GL/Pietrain, and Large White (LW)/MP/Du/GL. To exclude false-negative results due to mutations in the primer binding sites, a second primer pair specific for PERV-C was designed (see Materials and methods). Using both primer pairs identical results were obtained. The DNAwas isolated from different sources, such as ear biopsies, whole blood or PBMCs (Table 3). Among the pigs tested were 85 non-transgenic animals. Only one non-transgenic GL animal was PERV-C-negative. Analysis included also 96 transgenic or multitransgenic pigs with different genetic backgrounds. Only four of these were PERV-C-negative, all four had a GL background. All 18 wild boars carried PERV-C proviruses (Table 4). An earlier report had shown absence of PERV-C in a wild boar [27]. There was no correlation between age, weight, or habitat on one hand and PERV-C positivity on the other.

Detection of PERV-A/C in different pig breeds

Two primer pairs were used to detect recombinant PERV-A/C proviruses. In 18 of 164 investigated animals, PERV-A/C was found in DNA derived from PBMCs, but not when tested in parallel in the

DNA derived from ear biopsies (Table 5), indicating that the recombinant provirus in the PBMCs represents a de novo integration of a recombinant virus, not yet present in the germ line. As expected, PERV-A/C was not found in PERV-C free animals.

PERV expression in transgenic and non-transgenic pigs

To study expression of PERV in non-transgenic, transgenic, and multi-transgenic pigs, a PERVspecific one-step RT real time PCR assay was developed allowing to quantify expression of viral full length mRNA. This assay measures quantitatively, expression of PERV-A, PERV-B, and PERV-C. RNA was isolated from whole blood or PBMCs of non-transgenic pigs of the following genetic backgrounds: GL, Du, SH, German white large (GWL), MP as well as Du/GL. Expression of PERV was also studied in PBMCs of transgenic pigs with different hybrid background (MP/Du/ GL, GLW/MP/Du/GL, and GLW/MP/Du) generated by microinjection or lentiviral vectors.

PERV expression was normalized to the amount

of total RNA and compared with PERV expression in the PERV-producing porcine kidney cell line, PK-15, or in the human cell line 293 infected with PERV/50. The level of PERV expression was also normalized to the level of expression of the house-keeping genes, GAPDH and HPRT. PERV expression in PBMCs varied slightly when different pigs of different origins were compared (Table 6). However, there were also differences in PERV expression, when animals of the same litter were compared. Despite subtle differences, PERV expression in primary PBMCs from GL, Du, SH, GWL as well as Du/GL was very low when compared with PERV expression in the cell lines PK-15 (lower than 2% of the expression in these cells). The highest expression was found in a nontransgenic MP (up to 10% of the expression of PERV in PK-15 cells).

PERV expression in different organs of the same animal

Different organs (brain, heart, lung, liver, spleen, kidney, muscle, and pancreas) from three transgenic animals (GL and GL/MP) as well as five nontransgenic GL animals were analyzed to determine organ-specific differences in PERV expression (Fig. 1). Integrity of isolated RNA was confirmed PERV expression in mitogen-treated PBMCs Expression of porcine endogenous retroviruses was measured both in non-stimulated as well as PHAstimulated PBMCs in 32 transgenic and nontransgenic pigs (Table 7). Previous studies had shown that mitogen stimulation increases the level of PERV expression and that this assay discriminates between PERV low and high producers [8,9]. The highest expression was observed in mitogenstimulated PBMCs isolated from non-transgenic MP (93% of the expression of PERV in PK-15 cells). All non-transgenic LW animals showed only low level expression after mitogen stimulation (5%) and were classified as low producer animals. In contrast, expression of PERV in MP/Du/GL and LW/MP/Du animals, transgenic for TNFrelated apoptosis inducing ligand (TRAIL), human decay accelerating factor (hDAF), human leukocyte antigen-E (HLA-E), CD46 and combinations of these transgenes was in the range of 1% to 8% of the expression of PERV in PK-15 cells in the case of non-stimulated, and of 1% to 43% in the case of stimulated PBMCs. Noteworthy is that these animals had some genetic background of MPs (Table 7). There was no correlation between PERV expression and the presence of PERV-A/C proviruses in these animals, the animals with the highest expression of PERV did not carry PERVA/ C (Table 7).

Absence of virus release

As PERV expression at the RNA level reached only a small proportion of the expression ofPERVin PK- 15 and 293/50 cells, which release PERV particles, the question arose, whether viral proteins are expressed and whether virus particles were released from these pigs. Different attempts to detect PERV proteins in normal pig tissues have failed so far. The release of particles was observed only in some mitogen-stimulated PBMCs using a RT activity assay (Table 7). PBMCs from animals #32 (1.72 ± 0.11 mU/ml), #34 (2.76 ± 0.16 mU/ml), and #28 (0.75 ± 0.05 mU/ml, a minipig) released RT activity into the supernatant after stimulation with PHA. No virus release was observed with nonstimulated PBMCs and in most mitogen-stimulated PBMCs. In contrast, RT activities of 12.89 ± 0.20 mU/ml and 26.12±0.21 mU/ml, respectively, were found in the supernatant of the cell lines PK-15 and 293/50, respectively.

Discussion

Here, we screened a large number of transgenic pigs produced for pre-clinical xenotransplantation research for PERV prevalence and expression. Such careful analyses of animals are required to select only pigs with low or no PERV release to minimize the risk of PERV transmission to the patient. PERV are thought to be an important risk factor when porcine cells, tissues, or organs will be transplanted to human recipients, as PERV-A and PERV-B are present in the genome of all pigs and can infect human cells in vitro [28]. Trans-species transmission of retroviruses is a common phenomenon and was reported for HIV-1, resulting in the pandemic, devastating acquired immunodeficiency syndrome, and retroviruses closely related to PERV [for review see [29]. Importantly, PERV-C is not present in all pigs, thus allowing select PERV-C negative animals. Although PERV-C does not infect human cells, it may represent a risk factor due to putative recombinations between PERV-A and PERV-C. PERV-A/C are humantropic and can adapted to high titers when passaged on human cells in vitro [17,18]. As shown in this study, the number of PERV-C-negative animals was low, but by selection or backcrossing, PERV-C free animals could be generated. The number of PERV-C integrants in existing transgenic pig lines is an important issue that needs to be addressed in future studies. Recombinant PERV-A/C have been observed in DNA of cells from lymphoid tissues of several animals; however, there is clear evidence, that these recombinant viruses were not present in the germ line [16].

Differences in PERV expression correlated with the genetic background of the animals, not with the specific transgene (Tables 6 and 7). MPs had consistently the highest level of PERV expression, and animals with a MP background had a higher level of expression compared with animals without MP background. Theoretically, integration of a transgene in the vicinity of a provirus, an oncogene, a tumor suppressor gene, or any other gene may change the expression of these genes. This may result in a higher expression of PERV in tumor development or dead-born
animals, respectively. The present study included only healthy animals and an elevated PERV expression was not observed in transgenic animals compared with the corresponding nontransgenic animals.

Wild boars were included in this study because a previous report had demonstrated the lack of PERV-C and a lower number of proviruses when compared with domestic pigs [27]. In the present
study, all wild animals carried PERV-C, irrespective of age and the spot. Hunted in places very close to Berlin, these animals may have acquired the virus by contact with breeding animals. As only spleen tissue was investigated, but no other sample, it remains unclear whether PERV-C in these animals is endogenous or exogenous.

Our results confirm previous reports on differences in the expression between different pig breeds as well as between animals within a particular breed [8,9,30,31]. The highest expression of PERV was reported in the thymus and lung from MPs [31], and in kidneys and thymus of LW animals [32]. In this study, highest expression was found in the lung from transgenic animals with a GL and MP background (thymus preparations were not available) (FLI Mariensee) (Fig. 1A) and in the spleen of non-transgenic GL animals (LMU Munich) (Fig. 1B). Disseminated or local infections may stimulate PERV expression as activation of retrovirus expression was often described after infections with other viruses [33,34] or Toxoplasma gondii [35]. Such infection may happen in different organs, e.g., in the lung or in the spleen. In addition, both groups lived in different breeding
institutions. The lowest PERV expression was found in pancreatic islet cells throughout all animals (Fig. 1A), confirming previous reports [33, 36]. This could facilitate islet cell transplantation for the treatment of diabetes, which is supposed to be the first application of porcine cells [37].

Specific and sensitive assays were developed in the past years to screen for PERV infection, provirus distribution, and PERV expression, at the mRNA level and the protein level as well [8,9,38]. Here, these methods were applied to screen systematically for PERV distribution and expression in transgenic pigs generated for xenotransplantation research. Animals expressing hDAF and CD46 to overcome the HAR as well as TRAIL and HLA-E to control the cellular immune responses were analyzed for PERV expression (Table 7). Recently, transgenic pigs were generated expressing a PERV-specific short hairpin RNA, where PERV expression was efficiently inhibited by RNA interference [39]. It is anticipated that multi-transgenic animals will minimize the rejection of pig tissues and decrease the risk of PERV transmission. All these strategies will contribute to an optimized source animal characterized by a low probability of PERV release, absence of HAR, and a reduced cellular rejection [40].

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Tables and Figures

Table 1. Production methods of the screened transgenic pigs

Transgene	Production method	Breed/animals	Animal facility	Reference
GFP	Lentiviral transgenesis	German Landrace	Munich	[20]
TRAIL	Pronuclear DNA microinjection	German Landrace Duroc, Berlin Minipig	Munich	[19]
HLA-E/β2m	Pronuclear DNA microinjection	German Landrace Duroc, Berlin Minipig	Munich	[41]
aMHC	Pronuclear DNA microinjection	German Landrace	Munich	a
hDAF (hCD55)	Lentiviral transgenesis	German Landrace Duroc, Berlin Minipig	Munich	b
hDAF (hCD55)	Pronuclear DNA microinjection	German Landrace	Mariensee	[42]
hCD59	Pronuclear DNA microinjection	German Landrace	Mariensee	[42]

aKemter E, Kessler B, Wolf E, unpublished data.
bHofmann, A., Kessler, B., Wolf, E., Pfeifer, A., unpublished data.

Table 2. Primer pairs and probes used for polymerase chain reaction (PCR) and real time PCR

Virus gene detected	Abbreviation, nt number	Sequence	Reference/ accession number
PERV-C	envC, forward	CTGACCTGGATTAGAACTGG	[4]
	envC, reverse	ATGTTAGAGGATGGTCCTGG	
	envC.2, forward: 519–546	GATTAGAACTGGAAGCCCCAAGTGCTCT	EU440732
	envC.2, reverse: 806–777	TCTGATCCAGAAGTTATGTTAGAGGATGGT	
PERV-A/C	PERV-A env VRBF, forward	CCTACCAGTTATAATCAATTTAATTATGGC	[12]
	PERV-C env TMR	CTCAAACCACCCTTGAGTAGTTTCC	
	PERV-C, reverse	TATGTTAGAGGATGGTCCTGGTC	[14]
PERV gag	Forward	TCCAGGGCTCATAATTTGTC	[36]
	Reverse	TGATGGCCATCCAACATCGA	
	Probe	FAM-AGAAGGGACCTTGGCAGACTTTCT-BHQ1	
PERV-C real time	PERV-C, forward: 480–500	CACCTATACCAGCTCTGGACA	EU440732
	PERV-C, reverse: 791–772	ATGTTAGAGGATGGTCCTGG	
	Probe 509–533	HEX-ACCTGACCTGGATTAGAACTGGAAG-BHQ1	
GAPDH	Forward	ACATGGCCTCCAAGGAGTAAGA	a
	Reverse	GATCGAGTTGGGGGCTGTGACT	a
	Probe	HEX-CCACCAACCCCAGCAAGAGCACGC-BHQ1	a
HPRT	Forward	GTGATAGATCCATTCCTATGACTGTAGA	a
	Reverse	TGAGAGATCATCTCCACCAATTACTT	a
	Probe	FAM-ATCGCCCGTTGACTGGTCATTACAGTAGCT-BHQ1	a

aOperon, Cologne, Germany.

Table 3. Presence of PERV-C in pigs of different breeds

Breed/crossbreed	Transgene	DNA isolated from	Number tested	Number of PERV-C-negative
German Landrace (GL)	None	PBMCs	19	1
	GFP	Ear biopsy	1	0
	HLA-E	Ear biopsy	5	0
	TRAIL	Ear biopsy	4	0
	α-MHC	Ear biopsy	1	0
Duroc (Du)	None	PBMCs	12	0
Mini-pig (MP)	None	PBMCs	1	0
Large White (LW)	None	PBMCs	3	0
Du/GL	None	Ear biopsy	22	0
Schwäbisch-Hällisch (SH)	None	PBMCs	10	0
GL/Pietrain	HLA-E	Ear biopsy	1	0
	hDAF	Ear biopsy	1	0
	Lenti DAF	Ear biopsy	5	2
GL/Du	TRAIL	Ear biopsy	4	0
	TRAIL, HLAE	Ear biopsy	3	0
	HLA-E	Ear biopsy	2	0
	hDAF	Ear biopsy	1	0
Du/MP	TRAIL	Ear biopsy	2	0
LW/Du/MP	TRAIL, hDAF	PBMCs	18	0
	TRAIL, HLA-E, hDAF	PBMCs	6	0
	HLA-E, hDAF	PBMCs	5	0
	TRAIL	PBMCs	2	0
	hDAF	PBMCs	14	0
GL/MP/Du	TRAIL, HLAE	Ear biopsy	7	0
	TRAIL, hDAF	Ear biopsy	1	0
	HLA-E	Ear biopsy	3	0
	HLA-E, hDAF	Ear biopsy	1	0
	TRAIL	Ear biopsy	1	0
	hDAF	Ear biopsy	1	0
GL/Pietrain	hDAF	PBMCs	1	1
LW/MP/Du/GL	TRAIL, hDAF	PBMCs	3	1
	hDAF	PBMCs	3	0
Wild boar	None	Spleen cells	18	0
Total			181	5
Total non-transgenic			85	1
Total transgenic			96	4

PBMCs, peripheral blood mononuclear cells; PERV, porcine endogeneous retrovirus.
PERV-A and PERV-B were found in all animals.

Table 4. Presence of PERV-C and PERV-A/C in wild boars

No.	Location[a]	Age (yr)	Gender	Weight (kg)	PERV-C[b]	PERV-A/C
1	A	1–2	Female	50	+	−
2	A	1–2	Female	50	+	−
3	A	<1	Male	18	+	−
4	A	<1	Male	20	+	−
5	A	<1	Male	24	+	−
6	A	<1	Male	23	+	−
7	B	<1	Female	15	+	−
8	C	1–2	Female	55	+	−
9	C	1–2	Female	55	+	−
10	C	1–2	Male	45	+	−
11	C	>2	Female	65	+	−
12	C	<1	Female	15	+	−
13	D	<1	Female	15	+	−
14	D	1–2	Male	45	+	−
15	D	1–2	Male	50	+	−
16	D	1–2	Male	55	+	−
17	D	<1	Female	25	+	−
18	D	>2	Female	70	+	−

PERV, porcine endogeneous retrovirus.
[a]A, B, C, D indicate four different locations near Berlin.
[b]PERV-A and PERV-B were found in all animals.

Table 5. Detection of PERV-A/C in pigs of different breeds

Breed/Crossbreed	Transgene	DNA isolated from	Number tested	Number of PERV-A/C positive
Mini-pig (MP)	None	PBMCs	1	0
Large White (LW)	None	PBMCs	3	0
LW/MP/Du	TRAIL, hDAF	Ear biopsy	6	0
		PBMCs	6	3
	TRAIL, HLA-E, hDAF	PBMCs	5	4
	HLA-E, hDAF	PBMCs	4	2
	TRAIL	Ear biopsy	1	0
		PBMCs	1	0
	hDAF	PBMCs	12	6
		Ear biopsy	2	0
LW/MP/Du/GL	TRAIL, hDAF	PBMCs	2	1
	hDAF	PBMCs	3	2
Wild boar	None	Spleen cells	18	0
Total			64	18
Total non-transgenic			22	0

PERV, porcine endogeneous retrovirus; PBMCs, peripheral blood mononuclear cells; Du, Duroc; GL, German Landrace.

Table 6. Expression of PERV RNA in PBMCs of different non-transgenic Pigs

No.	Breed/crossbreed	% PERV expression of PK-15
1	Du/GL	0.23 ± 0.06
2	Du/GL	0.53 ± 0.15
3	Du/GL	0.14 ± 0.15
4	Du/GL	0.21 ± 0.06
5	Du/GL	0.28 ± 0.08
6	Du/GL	0.30 ± 0.09
7	Du/GL	0.47 ± 0.13
8	Du/GL	0.16 ± 0.04
9	Du/GL	0.22 ± 0.07
10	Du/GL	0.23 ± 0.06
11	GL	0.17 ± 0.05
12	GL	1.21 ± 0.33
13	GL	0.38 ± 0.10
14	GL	0.39 ± 0.10
15	GL	0.26 ± 0.07
16	GL	0.26 ± 0.07
17	GL	0.31 ± 0.08
18	GL	0.21 ± 0.03
19	SH	0.69 ± 0.19
20	SH	0.70 ± 0.19
21	SH	0.16 ± 0.04
22	SH	1.00 ± 0.31
23	SH	1.07 ± 0.30
24	SH	2.09 ± 0.57
25	SH	0.62 ± 0.17
26	SH	0.98 ± 0.27
27	SH	0.22 ± 0.06
28	MP	10.48 ± 5.28
29	LW	3.72 ± 1.37
30	LW	0.17 ± 0.09
31	LW	5.32 ± 2.01

MP, minipig; SH, Schwäbisch-Hällisch; GL, German Landrace; LW, Large White; Du, Duroc; PBMC, peripheral blood mononuclear cell; PERV, porcine endogeneous retrovirus.
Animals in one group always represent one litter.

Table 7. Expression of PERV in pig PBMCs before and after mitogen stimulation

No.	Breed/crossbreed	PERV-A/C	Transgene	% Expression of PK-15		RT activity	
				Non-stimulated	PHA-stimulated	Non-stimulated	PHA-stimulated
32	MP/Du/GL	–	TRAIL, hDAF	4.79 ± 1.3	6.07 ± 1.67	0	1.72 ± 0.11
33	MP/Du/GL	–	HLA-E	1.49 ± 0.46	2.19 ± 0.72	0	0
34	MP/Du/GL	–	HLA-E	3.91 ± 1.10	5.12 ± 1.39	0	2.76 ± 0.16
35	LW/MP/Du	+	hDAF	1.69 ± 0.46	6.45 ± 1.85	0	0
36	LW/MP/Du	+	hDAF	1.84 ± 0.53	7.69 ± 2.40	0	0
37	LW/MP/Du	+	hDAF	1.42 ± 0.40	2.53 ± 0.89	0	0
38	LW/MP/Du	+	TRAIL, hDAF	2.95 ± 0.81	6.09 ± 2.16	0	0
39	LW/MP/Du	+	TRAIL, hDAF	6.94 ± 1.88	7.68 ± 2.61	0	0
40	LW/MP/Du	–	hDAF	0.93 ± 0.30	1.24 ± 0.36	0	0
41	LW/MP/Du	–	TRAIL, hDAF	1.32 ± 0.37	8.79 ± 2.96	0	0
42	LW/MP/Du	+	hDAF	0.63 ± 0.19	1.77 ± 0.51	0	0
43	LW/MP/Du	+	hDAF	2.69 ± 0.73	15.02 ± 4.53	0	0
44	LW/MP/Du	–	HLA-E, hDAF	1.53 ± 0.41	8.61 ± 2.34	0	0
45	LW/MP/Du	–	HLA-E, hDAF	1.16 ± 0.32	2.77 ± 0.76	0	0
46	LW/MP/Du	+	hDAF	4.02 ± 1.09	2.75 ± 0.75	0	0
47	LW/MP/Du	+	HLA-E, hDAF	2.14 ± 0.58	12.13 ± 3.29	0	0
48	LW/MP/Du	–	hDAF	3.33 ± 0.97	7.04 ± 1.91	0	0
49	LW/MP/Du	+	TRAIL, HLA-E, hDAF	3.40 ± 0.92	5.03 ± 1.38	0	0
50	LW/MP/Du	–	TRAIL, HLA-E, hDAF	2.20 ± 0.60	3.02 ± 0.83	0	0
51	LW/MP/Du	+	TRAIL, HLA-E, hDAF	2.25 ± 0.62	3.44 ± 0.93	0	0
52	LW/MP/Du	+	HLA-E, hDAF	1.23 ± 0.33	2.55 ± 0.69	0	0
53	LW/MP/Du	+	TRAIL, HLA-E, hDAF	1.50 ± 0.41	5.23 ± 1.47	0	0
54	LW/MP/Du	–	TRAIL, HLA-E	3.54 ± 1.12	37.20 ± 10.96	0	0
55	LW/MP/Du	–	TRAIL, HLA-E	6.00 ± 1.63	13.23 ± 3.86	0	0
56	LW/MP/Du	–	TRAIL, HLA-E	8.19 ± 2.82	43.90 ± 12.01	0	0
57	LW/MP/Du	–	TRAIL	0.74 ± 0.21	39.45 ± 10.94	0	0
58	LW/MP/Du	–	TRAIL, hDAF	4.54 ± 1.71	18.31 ± 5.60	0	0
59	MP/Du/GL	–	HLA-E, CD46	2.67 ± 0.83	11.09 ± 3.22	0	0
28	MP	–	–	10.48 ± 5.28	92.58 ± 25.85	0	0.75 ± 0.05
29	LW	–	–	3.72 ± 1.37	4.21 ± 1.33	0	0
30	LW	–	–	0.17 ± 0.09	5.02 ± 1.38	0	0
31	LW	–	–	5.32 ± 2.01	5.42 ± 1.99	0	0
PK-15	–	–	–	100.00 ± 8.41		12.89 ± 0.20	
293/50	–	–	–	628.95 ± 32.74		26.12 ± 0.21	

MP, Mini-pig; SH, Schwäbisch-Hällisch; GL, German Landrace; LW, Large White; Du, Duroc; PBMC, peripheral blood mononuclear cells; PERV, porcine endogeneous retrovirus; RT, reverse transcriptase; PHA, phytohemagglutinin.

A

B
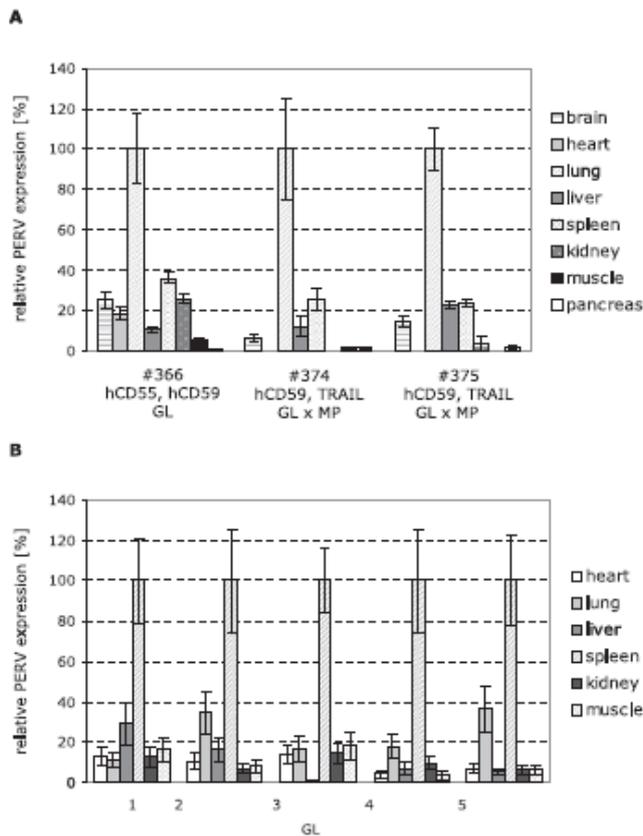
Fig. 1. (A) Expression of porcine endogenous retroviruses (PERV) in different tissues of multi-transgenic (TRAIL, CD55, CD59) German landrace (GL) pigs (#366) and GL pig · mini-pig (MP) animals (#374, #375) as measured by real time polymerase chain reaction. Expression in the lung was set 100%. (B) Expression of PERV in different tissues of fivedifferent non-transgenic GL pigs (1--5), expression in the spleen was set 100%.