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Published in:
Xenotransplantation

DOI:
[10.1111/xen.12311](https://doi.org/10.1111/xen.12311)

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

[Link to publication in ResearchOnline](#)

Citation for published version (Harvard):
Mourad, NI, Crossan, C, Cruikshank, V, Scobie, L & Gianello, P 2017, 'Characterization of porcine endogenous retrovirus expression in neonatal and adult pig pancreatic islets', *Xenotransplantation*, vol. 24, no. 4, e12311. <https://doi.org/10.1111/xen.12311>

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Characterization of porcine endogenous retrovirus expression in neonatal and adult pig pancreatic islets

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Funding information

Seventh Framework Programme, Grant/Award Number: Grant 601827 "XENOSLET"

Abstract

Background: Pig islets represent an alternative to the current modes of treatment for patients with diabetes. However, the concerns over pathogen transmission including that of PERV limit their immediate, widespread usage in humans. It has been previously demonstrated that PERV copy number and particularly expression levels can vary considerably between individuals and within different tissues of a single animal. In general, expression levels have been found to be particularly low in the pancreas compared to other porcine tissues suggesting a reduced risk associated with the use of this tissue. Data regarding this crucial aspect, however, remain limited and little is known about PERV status of islets themselves, which represent the final product to be transplanted. In addition, comparative analysis of the PERV status of neonatal piglets with adults is important as they are increasingly considered as potential islet donors for xenotransplantation.

Methods: Tissue samples from 51 neonatal piglets (age between 14 and 21 days) and 29 adult pigs were collected from Belgian landrace pigs used for pancreas procurement and islet isolation. Tissue biopsies were used to extract DNA for PERV copy number quantification by qPCR and RNA for PERV expression by qRT-PCR.

Results: As expected, PERV expression demonstrated great variation and was significantly lower in pancreas compared to other tissues. More importantly, PERV RNA expression was found to be specifically enriched in pancreatic islets reaching values similar to those found in other tissues such as liver and kidney. Interestingly, this expression was not coupled with the detection of reverse transcriptase in islet cultures or indeed detection of PERV virus. Lung, spleen, and lymph node consistently showed the highest levels of PERV expression. Comparison of PERV in neonatal and adult pigs showed that copy number did not vary significantly from birth to adulthood. PERV expression on the other hand was significantly lower in neonatal pig islets compared to adult islets and did not increase over the period of culture.

Conclusion: Our study confirms the low level of PERV expression in whole pancreas in a large population of both neonatal and adult pigs (n=80). The level of PERV expression was however higher in the endocrine tissue than in the exocrine cells. There was no correlation between PERV status in donor PBMCs and islet cells, and no evidence of active replication in *in vitro* regardless of PERV expression in islet cells. Moreover,

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neonatal pig islets were found to have significantly lower PERV expression compared to adult islets. Neonatal islets have been suggested as the best choice for xenotransplantation in terms of economic and procurement considerations; the PERV status reported here would also potentially support their use.

KEYWORDS

expression, islets, neonatal pigs, pancreas, pigs, porcine endogenous retrovirus (PERV), xenotransplantation

1 | INTRODUCTION

The use of live porcine cells, tissues, and organs for xenotransplantation is being increasingly investigated by experts in several different fields.¹ In particular, porcine pancreatic islets, which are proposed to solve the problem of the shortage of human islets for the treatment of type I diabetes, are being thoroughly studied to evaluate their compatibility, function and safety for future trials in humans.² In this context, and as with all other xenogeneic tissues, the risk of zoonosis must be carefully evaluated by donor screening, tissue-specific assessment as well as host monitoring and follow-up.^{3,4} Porcine endogenous retrovirus (PERV) has drawn particular concerns as it was shown that porcine PERV-releasing cells can infect human cells *in vitro*,⁵ although no evidence was found for PERV transmission to patients receiving encapsulated pig islets.^{6,7} Given the significant variability of PERV expression between pig breeds, individuals within a same breed⁸ and even different organs/tissues from the same animal,⁹⁻¹² choosing pigs with low genome copy number and low expression levels of PERV is hypothesized to reduce the risk of transmission *in vivo*.¹³ Screening methods often rely on determining genomic DNA (integration) as well as mRNA (expression) copy numbers of PERV-related genes such as *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope) but are sometimes extended to include detection of reverse transcriptase (RT) activity by product-enhanced reverse transcriptase assay (PERT) and viral proteins (replicating virus) in tissues, sera, or culture media.¹⁴ Using one or a combination of these methods, a few studies reported lower PERV expression levels in pancreas compared to other porcine tissues.^{9-11,15} Surprisingly, less is known about PERV status of isolated pancreatic islets, although these are the cells that will constitute the final product to be used in clinical pig-to-human transplantation. A study by Irgang et al. showed no PERV release from isolated landrace pig islets during co-culture experiments.¹⁶ Another study, comparing a donor line with commercial animals, demonstrated that a single animal exhibited a unique pattern with high levels of PERV expression (*pol*-specific primers) in isolated islets compared to other porcine tissues; however, as described, the remaining tissues were highly variable.¹² Another aspect to be considered is the age of islet donor animals. Compared to adult islets, neonatal piglet islets are easier to isolate and to maintain in culture¹⁷ and they offer the possibility of post-transplantation maturation and growth.¹⁸ There is also the option of rapid testing of source material for any pathogens present, while cells are in the culture environment to meet suggested criteria for pathogen evaluation.¹⁹ It

thus seems important to determine PERV status of neonatal pig islets as they are being increasingly considered as the best option to render large-scale pig-to-human islet transplantation feasible. In the present study, we quantified PERV copy number and RNA expression in islets from both neonatal and adult pigs. Our analysis included a number of porcine tissues with particular emphasis on pancreatic tissue to distinguish PERV copy number and expression levels in the exocrine and endocrine compartments of the organ. In addition, PERV expression and reverse transcriptase (RT) activities were examined in the cultured islet cell environment to establish if replication was present.

2 | MATERIAL AND METHODS

2.1 | Animals

All experiments were conducted in accordance with the local ethical committee and carried out in accordance with EU Directive 2010/63/EU for animal experiments. Outbred pigs (Belgian landrace) were provided by Rattlerow-Seghers genetics (Ooigem, Belgium). Piglets were directly delivered to the islet isolation facility in Brussels. Adult pigs were housed in the A. de Marbaix center (Louvain-la-Neuve, Belgium). A total of 51 neonatal piglets (3-8 kg) aged 14-21 days and 29 adult pigs (200-300 kg) aged 1-2 years were used for pancreas procurement and islet isolation.¹⁷

2.2 | Isolation of tissue samples

Tissues from exsanguinated piglets and adult pigs were obtained at the time of pancreatectomy then immediately washed in PBS and stored at -70°C before being sent for nucleic acid isolation and PERV quantification. Sampled tissues included lung, kidney, spleen, mesenteric lymph nodes, liver, and pancreas. Blood samples were taken to prepare peripheral blood mononuclear cells (PBMCs). Briefly, 50 mL heparinized blood were centrifuged at room temperature over Histopaque 1077 from Sigma (Darmstadt, Germany). The opaque interface containing PBMCs was then aspirated, washed in PBS, pelleted, and stored at -70°C. Pancreatic islet isolation from both adults and neonates was carried out as previously described.¹⁷ Collagenase V from Sigma was used for piglet pancreas digestion, and collagenase NB8 from Serva (Heidelberg, Germany) was used for adult pancreas. We obtained 2087±151 islet equivalents (IEQ)/g pancreas (purity >75%) from neonatal piglets and 234±16 IEQ/g pancreas (purity

>90%) from adult pigs. Following islet purification, exocrine tissue samples were taken and stored at -70°C . Islet samples were taken after overnight culture in the case of adult islets and after 8 days in the case of neonatal islets. For some experiments, neonatal islet samples from the first day of culture were taken as described later.

2.3 | Nucleic acid isolation and amplification of PERV

Prior to isolation, 30 mg of each tissue were homogenized using a Rapid Hybrid Ribolyser. RNA was isolated from tissue homogenate and cells using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK), according to manufacturer's instructions. Viral RNA (vRNA) was isolated from serum using the Qiagen viral RNA mini kit (Qiagen, Crawley, UK), according to manufacturer's instructions. To remove any contaminating DNA, RNA samples were DNase treated using Ambion DNA-free (Life Technologies, Paisley, UK). Isolated RNA was quantified by UV spectrophotometry, and integrity was measured using an 18s rRNA assay (Eurogentec, Southampton, UK). Only samples which achieved $\geq 10^8$ 18s rRNA copies per 250 ng RNA were taken forward for PERV copy number analysis. A total of 250 ng of each RNA preparation was tested for the presence of PERV RNA by qRT-PCR amplification as previously described.²⁰ Samples were tested against standards of known PERV quantity to allow the level of PERV expression to be quantified. For each sample, a reaction in the absence of reverse transcriptase was included to control for any DNA contamination. DNA contamination was absent as confirmed by negative values in the qRT-PCR (data not shown). Insulin qRT-PCR amplification was carried out using the following primers and probe; Insulin Forward primer: 5'-TTCGTGAACCAGCACCTGT-3', Insulin Reverse primer: 5'-CTTGGGCGTGTAGAAGAAGC, Insulin Probe 5'-FAM/GAGGCGCTGTACCTGGTG/TAMRA-3', with the following cycling conditions; 50°C for 20 minutes, 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds, 53°C for 15 seconds, and 60°C for 30 seconds. DNA was isolated from tissues and cells using Qiagen Dneasy mini kit (Qiagen, Crawley, UK), according to manufacturer's instructions. Isolated DNA was quantified by UV spectrophotometry, and integrity was measured using an 18s rRNA gene assay (Eurogentec, Southampton, UK). Only samples which achieved $\geq 10^7$ 18s rRNA copies per 250 ng of DNA were taken forward for PERV copy number analysis. PERV genome copy number was carried out by absolute qPCR using SYBR Select reagents (Life Technologies) and primers corresponding to the PERV-*pol* region, as previously described.²¹ Samples were tested against standards of known PERV copy number to allow PERV copy number per genome to be quantified.

2.4 | PERT analysis of cell cultures

Culture media were assayed for PERV using qRT-PCR (as described above) to detect the presence of viral RNA, and SYBR green PCR-enhanced reverse transcriptase assay (SG-PERT), to detect the presence of reverse transcriptase. Prior to qRT-PCR analysis, vRNA was isolated from culture media using the Qiagen mini viral RNA kit (Qiagen, Crawley, UK), according to manufacturer's instructions. SG-PERT analysis was performed as previously described.²²

2.5 | Statistical analysis

Data were analyzed by ANOVA, followed by a Dunnett's test for comparisons of test groups with controls or a Newman-Keuls test for comparisons between test groups. Differences were considered to be statistically significant for P values $< .05$.

3 | RESULTS

3.1 | PERV expression and genomic copy number are low in pancreas

PERV expression showed significant variation between tested tissues. However, tissues with the top 3 highest and top 3 lowest expression levels were consistently the same independent of the age of the animals: Lung, spleen, and lymph node all had more than 2.5×10^4 copies/250 ng RNA while pancreas, liver and kidney all had less than 9×10^3 copies/250 ng RNA. Compared to other tissues, PERV expression was significantly higher in lymph node from adult animals. In piglets, expression was significantly lower in PBMC compared to lung and spleen. Most importantly, PERV expression was the lowest ($P < .001$) in pancreas (183 copies in piglets, 10 copies in adult pigs; Figure 1A). As expected, PERV copy number showed much less variability between tissues, although it was noted that significantly higher copy numbers, compared to pancreas ($P < .05$), were found in lymph node and spleen of both age groups as well as in PBMC of adult pigs (Figure 1B). Differences observed between pancreas and islet PERV copy numbers were not significant when analyzed with one-way ANOVA Dunnett's test.

3.2 | Neonatal pig islets have lower PERV expression compared to adult islets

Further analysis of PERV expression in the two main pancreatic cell populations (endocrine/exocrine) revealed much higher expression levels in islets (endocrine) compared to exocrine tissue (Figure 2). This was the case of both neonatal and adult islets, although the latter showed significantly higher expression compared to neonatal islets ($P < .05$; Figure 2). These data did not reflect the PERV expression noted initially for the pancreas tissue alone (Figure 2).

3.3 | PERV expression in islet culture does not appear to correlate with virus replication

As neonatal islets require to be cultured for 8 days to allow removal of exocrine tissue and maturation of precursor endocrine cells¹⁸, we compared PERV expression at the first (D1) and last (D8) days of culture. Unsurprisingly, PERV expression was low at D1 and much higher at D8 (37.3 ± 10 and 4041 ± 745 copies/250 ng RNA, respectively, $P < .0001$). However, when the comparison was made relative to insulin expression, there was no significant difference between D1 and D8 (0.097 ± 0.02 and 0.052 ± 0.01 , $P > .05$; Figure 3). Compared to other tissues, neonatal islets still ranked among the lowest for PERV

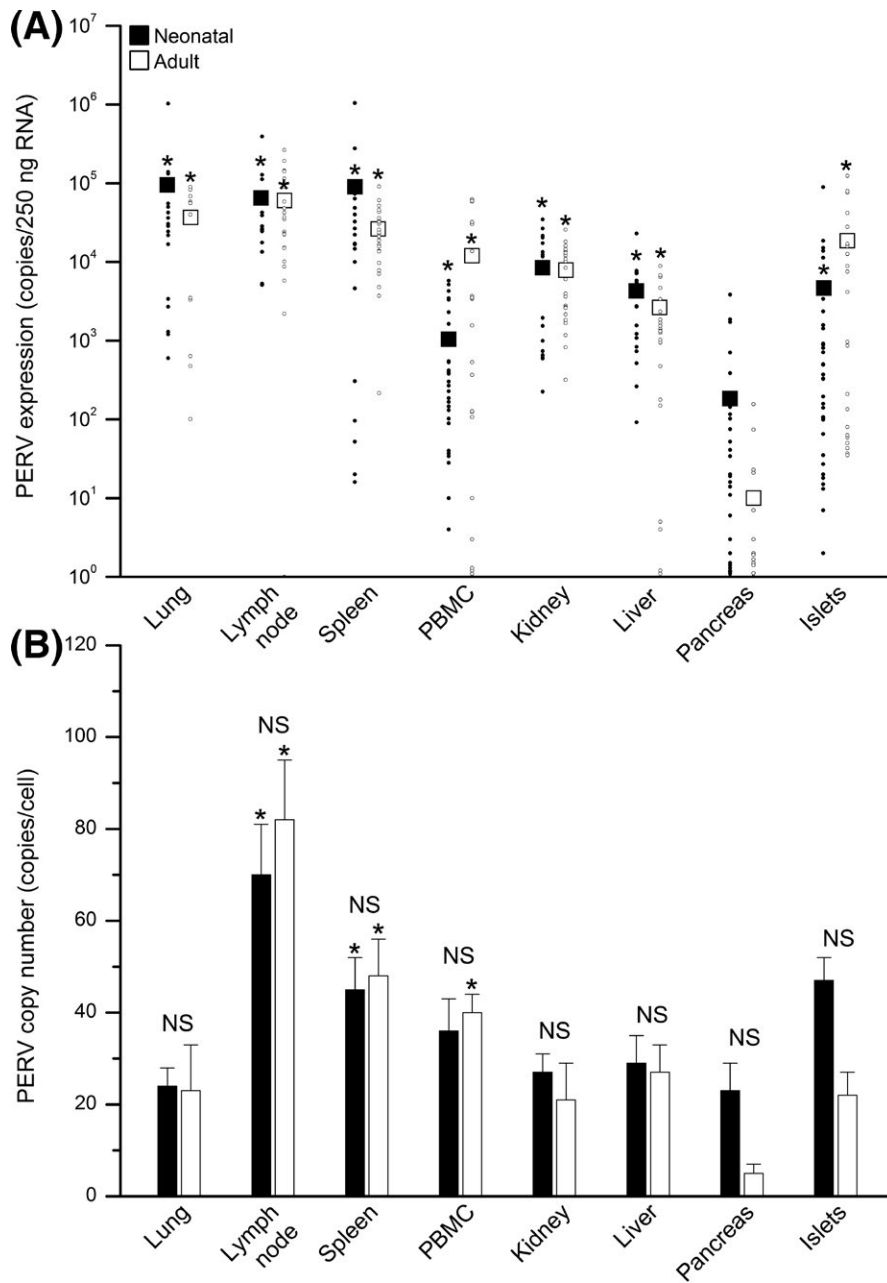


FIGURE 1 PERV-*pol* expression and genomic integration in neonatal and adult porcine tissues. (A) PERV was quantified by qRT-PCR and normalized to 250 ng RNA in the indicated tissues from neonatal (black squares) and adult (white squares) pigs. (B) PERV genomic copy number was quantified by qPCR as indicated. Mean values from $n=51$ neonatal piglets and $n=29$ adult pigs. * $P<.05$ compared to pancreas. NS, no significant difference between neonatal and adult PERV copy number

expression, whereas adult islets had intermediate expression levels (higher than liver, kidney and PBMC but lower than spleen, lung and lymph nodes). Interestingly, we could not detect any RT activity measured by SG-PERT assay²² or PERV expression in culture media supernatants from both neonatal and adult islets independent of PERV expression level in these islets (data not shown).

3.4 | PERV expression in PBMCs is not correlated with PERV expression in pancreatic islets

PBMCs are easily obtainable from live animals, and they are routinely used to evaluate PERV copy number and expression in pigs.²¹ In a panel of 23 neonatal and 21 adult pigs, we could comparatively quantify PERV-*pol* expression in pancreatic islets and in PBMCs from each

animal. Slightly higher expression levels were found in islets compared to PBMCs (Table 1), but these differences did not reach statistical significance ($P>.05$). Interestingly, the correlation between PERV expression in islets and PBMCs was extremely low ($r=0.1$) for neonatal piglets and null ($r=0$) for adults (Figure 4) indicating no comparable relationship between PERV expression levels in these two tissues.

4 | DISCUSSION

Among porcine tissues likely to be used for xenotransplantation, pancreatic islets are one of those already entering clinical trial stage.^{2,23} As most other studies quantified PERV in the whole pancreas and a few have measured islet PERV in intra and/or interindividual comparative

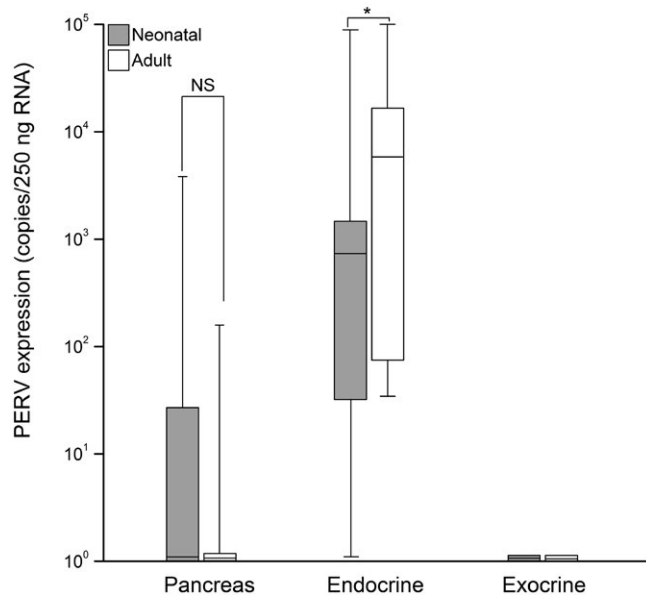


FIGURE 2 PERV expression in endocrine and exocrine pig pancreas. PERV expression was quantified by qRT-PCR and normalized to 250 ng RNA in whole pancreas biopsies, in cultured islets (endocrine) and in exocrine fractions obtained during pancreas digestion and islet purification from neonatal (black columns) and adult (hatched columns) pigs. Data presented as median values and interquartile range from $n=51$ neonatal piglets and $n=29$ adult pigs. The difference between neonatal and adult pancreas was not significant (NS) due to great variability. $*P < .05$ significant difference between neonatal and adult islets

screening, our study focused on this aspect in both neonatal and adult pigs. Our study confirms previous data regarding low PERV expression in pancreas compared to other tissues. However, we report that PERV expression is enriched in pancreatic endocrine islet cells, which were found to carry PERV loads comparable to those measured in liver and kidney in both adult and neonatal pigs. Exocrine pancreas represents 96%–99% of total pancreatic tissue, while islet cells represent only 1%–4%. This would explain the very low PERV expression levels reported by us and others^{9,10} in pancreas extracts and highlights the necessity to measure PERV in isolated islets rather than in whole pancreas. Indeed, the current guidelines on safety for islet xenotransplantation indicate that analysis should be product specific.²⁴

Although neonatal pig islets differ from adult ones with regard to their architecture and the maturity of their cells, they have been shown to be glucose-responsive,^{17,18} logistically and practically easier and much less costly to isolate.¹⁷ We and others have previously compared neonatal and adult islets with respect to their size, insulin content, and secretory function.^{17,25} Before the present study, no PERV screening was carried out in neonatal piglets despite growing evidence of the possibility to use islets from such animals for xenotransplantation. Here, we found PERV expression to be 4-fold lower in islets from 14–21-day-old piglets compared to adult islets ($P < .05$). To make sure that these lower PERV expression levels were not somehow related to the longer culture period (8 days) necessary for neonatal islet maturation and purification, we measured PERV expression in neonatal

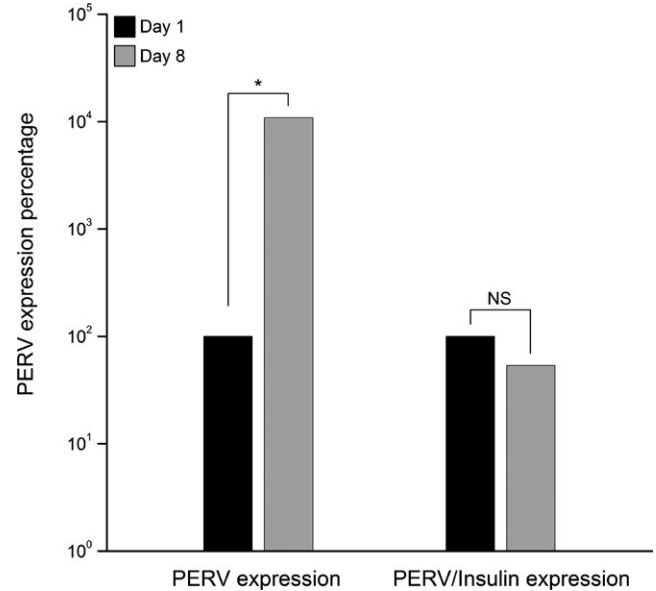


FIGURE 3 Effect of *in vitro* culture on PERV expression in neonatal pig islets. Mean PERV expression at day 1 (black columns) and day 8 (gray columns) following isolation and culture was quantified by qRT-PCR measuring PERV expression alone or relative to insulin (PERV/Insulin expression). Expression at day 1 was taken as 100% for each method. Mean values from $n=19$ islet cultures. NS, no significant difference. $*P < .0001$ significant difference between day 1 and day 8

TABLE 1 PERV expression in neonatal and adult pig islets and PBMCs

	Islets	PBMCs
Neonatal pigs ($n=51$)	2152±958	1268±345
Adult pigs ($n=29$)	20 600±726	10 256±4211

Values are means ± SEM PERV expression/250 ng RNA.

pancreas preparations 24 hours following isolation. However, even lower expression levels were detected at this early stage of culture. We hypothesized that the increase in PERV expression over the 8-day period reflects maturation of pancreatic precursor cells and dramatic loss of exocrine tissue resulting in much higher endocrine-to-exocrine ratio rather than an actual increase of PERV expression. Indeed, when normalized to insulin expression, PERV expression did not significantly change during culture, albeit this would require further investigation if isolated cells were to be cultured over a longer period. In addition, in spite of the presence of PERV expression in the islet cells, we could not detect any RT activity measured by SG-PERT assay²² or PERV expression in culture media supernatants from both neonatal and adult islets. This would suggest that no virus is released or actively replicating, supporting a lack of PERV infectivity in islets isolated from these Belgian Landrace pigs. Indeed, this reflects previous limited data, which describes a lack of PERV release and *in vitro* infection from islet cells.^{16,26}

Among tested tissues, highest PERV expression was detected in lung, lymph node, and spleen in accordance with previously published data from a limited number of adult pigs.^{10,11,27} In our hands, tissues

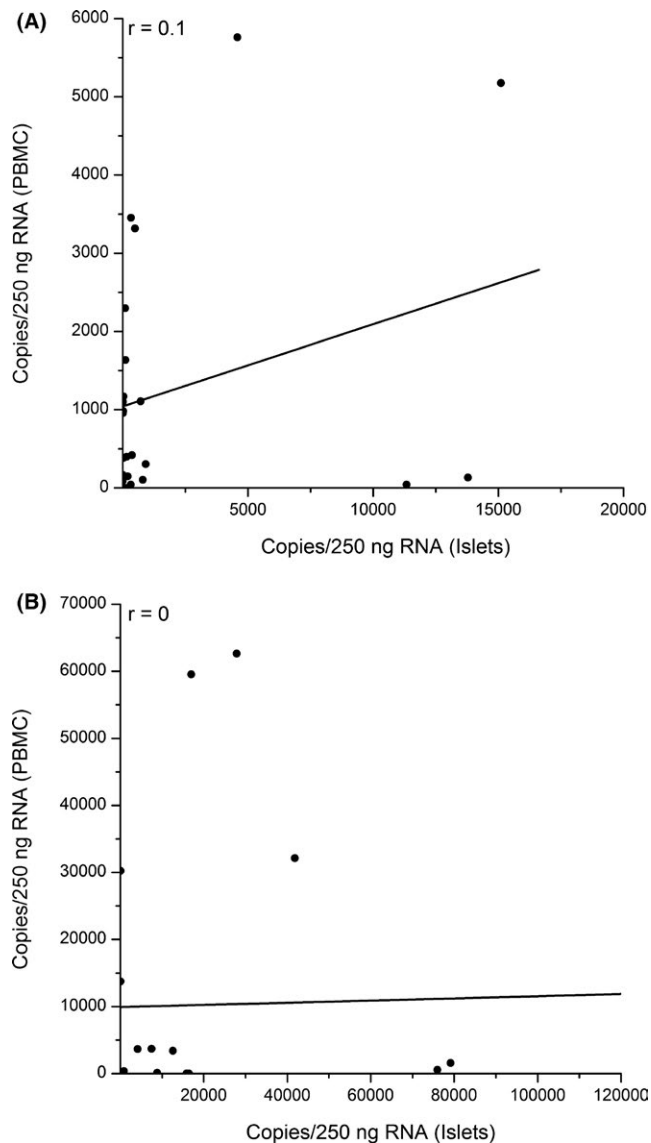


FIGURE 4 No relationship between PERV expression in porcine pancreatic islets and PBMCs. PERV expression was quantified by qRT-PCR in pancreatic islets and PBMCs isolated from neonatal (A) and adult (B) pigs. The line in each graph represents linear regression. Correlation coefficients (r) for each dataset are indicated. Data were analyzed from $n=23$ piglets and $n=21$ adult pigs

with lowest PERV loads were consistently pancreas, liver, and kidney. Two other studies reported high PERV expression in liver^{9,12} and kidney.⁹ However, these were based on only one animal in the study by Guo et al.¹² and nine animals in the study by Cl  menceau et al.⁹ Tucker et al.²⁸ previously reported higher PERV expression in blood samples from animals aged less than 2 months compared to older animals. In our study, neonatal pigs had significantly higher PERV expression in serum and spleen samples and significantly lower in islets and PBMCs compared to adults. We could detect a tendency toward higher PERV loads in piglet samples from the remaining tissues in accordance with data published by Moon et al.¹⁵, but the differences did not reach statistical significance in our hands. As expected, we observed much less variation in PERV copy number. However, lymph node and spleen

from both neonatal and adult samples as well as PBMCs from adults showed slightly significantly higher copy numbers compared to other tissues. There was no significant difference in copy number between neonates and adults.

These higher numbers of PERV integration in tissues of the immune system and blood have been observed in other studies.²⁹⁻³¹ A recent review indicated that these unexpected observations may be due to differences in methodological assessment,³² such as tissue composition, purification methods, target gene, and the use of single copy genes for comparisons. Likewise, the complexity of PERV integration is also problematic when interpreting these data. Our observations demonstrating an increase in lymphoid tissues and blood possibly reflect virus amplification and *de novo* integration, which has been suggested previously.³² If this is the case, then the copy number of PERV within a single animal may be overestimated when examining the PBMC. Interestingly, these integration events apparently occur at a very early stage of life since no further significant increase was observed in adult animals compared to 14-21-day-old piglets. Unfortunately, germline samples were not available from these animals to compare.

Finally, we checked whether we could detect a relationship between PERV expression in pancreatic islets and in PBMCs since the latter are often used for PERV screening and to determine PERV status of donor animals.¹³ We found the correlation between PERV expression in the two tissues to be extremely low. It thus seems difficult to predict PERV loads in a specific tissue based on PERV expression in blood cells. Very recently, an *in vivo* study evaluating the biosafety of agarose-encapsulated porcine islets used a four-checkpoint program system for microbiological evaluation.¹⁹ This program emphasized that screening of the islet product is more likely to detect a pathological agent more reliably than in blood cells, which is supported by the updated IXA consensus statement.⁴ Likewise, PERV analysis may also be more relevant in the islet product with respect to selection criteria given the lack of correlation with PERV in PBMC in this study. In order to minimize the risk of PERV transmission to xenograft recipients, pig-specific and tissue-specific assessment of PERV expression needs to be undertaken.

In conclusion, our study:

1. Confirms the low level of PERV expression in the whole pancreas in both neonatal and adult animals.
2. Shows that this low level is attributable to differences in exocrine vs endocrine tissue. Indeed, islet cells have significantly higher levels of PERV expression compared to pancreatic exocrine cells.
3. Shows that PERV expression in neonatal islets is lower than in their adult counterparts supporting the use of neonatal islets in a xenotransplantation setting.
4. Suggests that it is not possible to predict the level of PERV expression in islet cells based on analysis of PBMC, therefore each islet preparation should be assessed for PERV.

AUTHORS' CONTRIBUTIONS

NM performed islet isolations, analyzed data, and wrote the manuscript. CC and VC performed nucleic acid extractions and PERV

quantification, collected and analyzed data. LS designed research, analyzed data, and critically revised the article. PG designed research, analyzed data, critically revised the article and secured funding.

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How to cite this article: Mourad NI, Crossan C, Cruikshank V, Scobie L, Gianello P. Characterization of porcine endogenous retrovirus expression in neonatal and adult pig pancreatic islets. *Xenotransplantation*. 2017;00:e12311. <https://doi.org/10.1111/xen.12311>