8

Methods and Tools for Detection and Evaluation of the Risks of Porcine Endogenous Retrovirus in Porcine to Human Xenotransplantation

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1. Introduction

Xenotransplantation has been defined by the Public Health Service as any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (a) live cells, tissues, or organs from a nonhuman animal source, or (b) human body fluids, cells, tissues or organs that have had ex vivo contact with live nonhuman animal cells, tissues or organs (PHS 2001). The promise of xenotransplantation is to provide a replenishable, controlled source of cells, tissues, and organs, in order to alleviate the chronic shortage available for human allotransplantation.

Due to comparable organ size, well defined conditions for breeding and husbandry, and a prolonged co-existence with humans for thousands of years, pigs are considered a relatively safe source for clinical xenotransplantation. However, the finding that the porcine germ line harbors genetic loci encoding porcine endogenous retroviruses (PERV), some of which are infectious for human cells, has hampered clinical development of porcine to human xenotransplantation and resulted in renewed scientific interest in PERVs. FDA's Guidance recommends testing porcine xenotransplantation products, when feasible, by co-culturing with a panel of appropriate indicator cells that could facilitate the amplification of all three receptor classes, PERV A, B, or C (FDA 2001).

PERVs are endogenous retroviruses, and members of the genus gammaretrovirus. Endogenous retroviruses are remnants of ancient viral infections, found in the genomes of most, if not all, mammalian species. Integrated into the chromosomal DNA, they are vertically transferred through inheritance.

In the early 1970s, cultured porcine kidney cell lines were reported to release type C retroviral particles, now known as PERVs (Armstrong, Porterfield et al. 1971). After several studies failed to demonstrate a link to cancer in pigs, progress in studying PERV slowed (reviewed in (Wilson 2008)). In the 1990's, as improved immunosuppressive therapies led to an increase in interest and activity in performing porcine to human xenotransplantation clinical trials, there was a renewal of scientific investigation of PERVs to determine whether they might pose a risk to clinical trial participants. One study demonstrated that the pig cell line PK-15 expressed PERVs that could infect human cells (Patience, Takeuchi et al. 1997) ,

and our laboratory showed that mitogenic activation of pig primary peripheral blood mononuclear cells was sufficient to release PERVs that were directly infectious for a human cell line in vitro (Wilson, Wong et al. 1998) . These results and those of others who showed transmission of PERV to human cells in vitro (reviewed in (Wilson 2008) framed the debate for the risks of infectious disease transmission in the context of xenotransplantation.

In this chapter we will incorporate research from our own laboratory, combined with a summary of key studies conducted by different investigators. Our own research has been focused in two major areas: 1) to develop tools and methods to facilitate detection and evaluation of the risks of PERV in porcine xenotransplantation products and recipients; and 2) to identify the cellular and viral determinants of human cell infectivity in order to develop strategies to prevent transmission of PERV to recipients of porcine xenotransplantation products. We will provide a review of the published findings of our own lab and those of others describing detection methods and the biological characteristics of porcine endogenous retroviruses (PERV), including their genomic characteristics, and cellular and viral determinants for human cell tropism. The review of the available data will be in the context using living pig cells, tissues, or organs for human xenotransplantation.

2. Investigation of PERV: Paving a way to prevent infection, supporting use of pigs as source animals for xenotransplantation

2.1 Methods and tools development

Availability of sensitive and specific PERV detection methods has been critically necessary to study the biological characteristic of the virus, for screening animals and xenotransplantation products, when feasible, for studying PERV expression and transmission, and for monitoring porcine xenotransplantation clinical trial participants for evidence of PERV transmission from porcine xenotransplantation products. The challenges to development of PERV detection assays include the following issues:

i. Distinguishing between the replication competent form of the virus and replication-defective genomes and transcripts;
ii. Distinguishing between PERV sequences that are the result of microchimerism (surviving pig cells) vs. transmission of PERV to recipient cells;
iii. The potential for genetic recombination between PERVs and other exogenous or endogenous viruses that may compromise the specificity of detection.

2.1.1 Culture assays

Our laboratory had shown that co-culture of pig PBMCs with human embryonic kidney cells (293-HEK) or swine testis cells (ST Iowa cells) provides a sensitive method to detect replicating PERVs released from primary pig cells (Wilson, Wong et al. 1998). Later, we demonstrated that compared to a panel of other cell lines examined, these are the most sensitive cell lines for detection of PERV (Wilson, Wong et al. 2000). Co-culture of pig PBMC with human 293 HEK and pig ST cells provides a sensitive method for screening primary pig cells and tissues for PERV release (Wilson, Wong et al. 1998; Oldmixon, Wood et al. 2002; Wood, Quinn et al. 2004). In addition, pig aortic endothelial cells and human H1080 cell lines were tested as alternative primary source and target cells, respectively, for co-culture assay to screen pigs, though the data from these cells suggest they may not be as sensitive as 293 HEK (Martin, Kiessig et al. 1998).

2.1.2 Pseudotype assay

A primary determinant of retroviral tropism is the surface envelope glycoprotein (SU). By assembling envelope glycoproteins onto the core and genome of a heterologous retrovirus, the tropism of the resulting virus (called a pseudotype) is typically determined by the envelope. Moloney murine leukemia virus (MoMLV), a closely related gammaretrovirus, can be pseudotyped with many different types of retroviruses, including PERV (Takeuchi, Patience et al. 1998) (Wilson, Wong et al. 2000) and can be used as a tool to study viral tropism and the factors that impact the in vitro host range. In order to study the tropism of PERV, we and others have prepared MLV particles pseudotyped by PERV envelopes and used these in a variety of studies (reviewed in (Wilson 2008), and discussed in more detail here, in section 2.2.3). In this assay, pseudotypes are typically generated through the transient transfection of 293T cells with three plasmids: i) one plasmid encoding the retroviral vector genome carrying the packaging signal, deleted for all structural and enzyme coding genes, and in their place, carrying a reporter gene, such as *lacz*, ii) a plasmid expressing the viral core structural and enzymatic proteins, a region of the genome called *gag-pol*, and iii) a plasmid expressing the envelope gene. After 2-3 days, the supernatant from the transfected cells is collected and applied to the target cells to determine the infectivity titer or tropism conferred by the viral envelope (Figure 1). The studies described in section 2.2.3 use pseudotyped viruses to characterize receptor classes, the in vitro host range, and potential species for animal model.

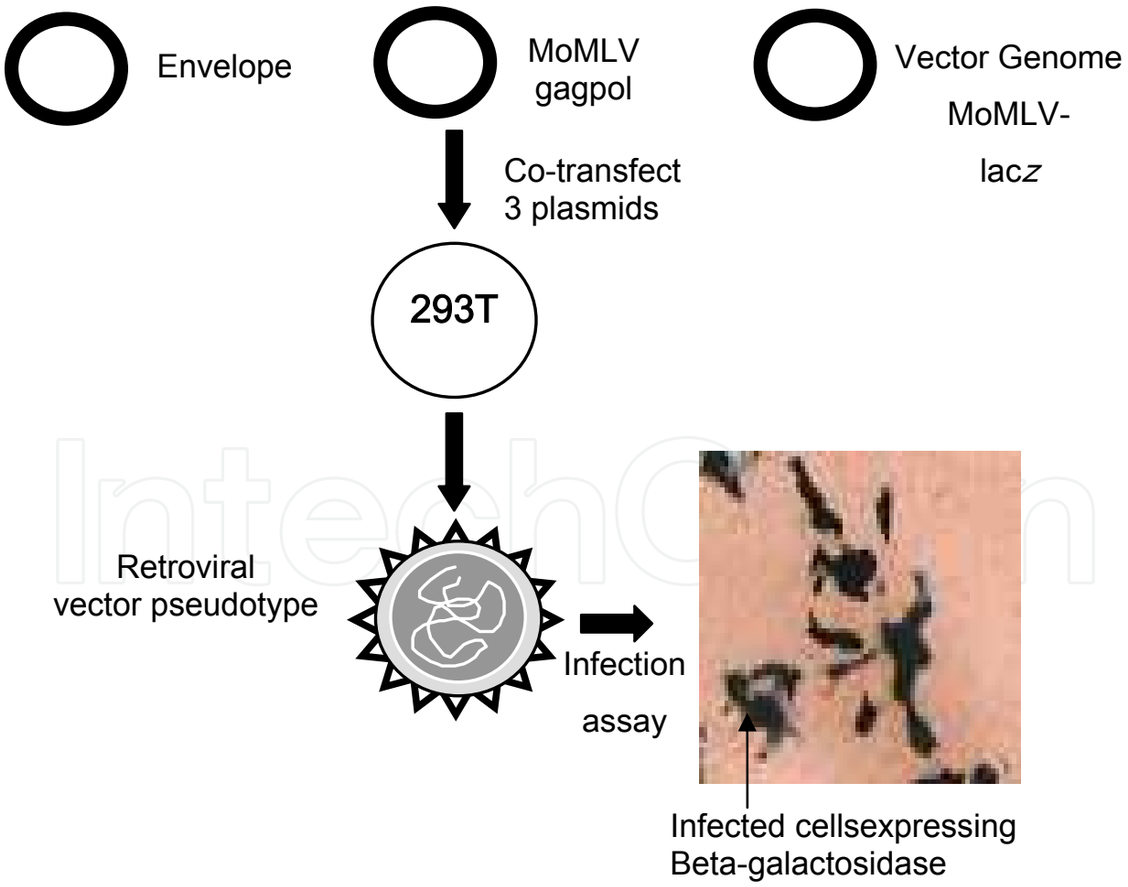

Fig. 1. Typical method used to derive retroviral vector pseudotypes and measure infection properties.

2.1.3 Detection of viral reverse transcriptase activity

Retroviruses carry an RNA-dependent DNA polymerase, also known as reverse transcriptase (RT). Detection of this enzymatic activity provides a unique signature of retroviral infection and therefore, provides a means to monitor the replication of PERV in vitro. The reverse transcriptase (RT) assay is a simple, relatively inexpensive, widely used assay that can detect all retroviruses on the basis of the divalent cation requirement of their RT enzyme, i.e., Mg^{2+} or Mn^{2+}. Basically, in cell or tissue culture when the replication competent viruses exist and continue to expand, the level of the reverse transcriptase activity in the cell culture supernatant increases in correlation to increased virus replication. In the absence of replication, the RT activity may remain low or disappear over time. We and others have used this assay to demonstrate replication of PERVs in a variety of primary cells and established cell lines using an assay based on detecting ^{3}H-TTP incorporation (Wilson, Wong et al. 1998) (Ritzhaupt, Laan et al. 2002). Others have used ELISA-based assays that are available as commercial kits to quantitatively measure RT enzyme (for example, see (Denner, Specke et al. 2008); available from Cavidi AB, Uppsala, Sweden). And a very sensitive measure of reverse transcriptase activity has been developed using PCR-enhanced or PCR-based reverse transcriptase assays (PERT or PBRT; (Irving, Chang et al. 1993; Pyra, Boni et al. 1994), providing a more sensitive and quantitative measure of RT activity, and has also been applied to detecting PERV replication (Patience, Takeuchi et al. 1997; Takefman, Wong et al. 2001).

2.1.4 Quantitative, sensitive detection assays: qPCR and qRT-PCR assays

Sequence analysis of PERV genomes has allowed development of sensitive and specific methods of detection based on PCR amplification. However, the limitation of PCR lies in the inability to differentiate between expression of defective transcripts of the virus and full length replicating virus. However, application of quantitative methods to samples analyzed over multiple time points allows one to determine whether replication is occurring. We have developed quantitative PCR and RT-PCR methods for this purpose (Argaw, Ritzhaupt et al. 2002).

The quantitative PCR (Q-PCR) assay combines sensitivity with specificity and remains a valuable tool to indicate the replication potential of the viruses and for in vivo applications to detect evidence of infection in animals or clinical studies. In this regard, we have developed a real-time quantitative PCR assay based on detection of the PERV *pol* sequence using the *Taq*-Man real-time qPCR technology to quantify PERV RNA or DNA sequences in tissues and cells of human or animal origin (Argaw, Colon-Moran et al. 2004; Levy, Argaw et al. 2007). In this assay, a plasmid construct encoding the PERV-*pol* gene or the in vitro transcribed RNA derived from the plasmid (cRNA) serves as a standard template for amplification of a 178 bp fragment. We have shown that detection of the target sequence is linear over a range from 20 copies to 2 million copies of plasmid DNA and from 100 copies to 1 million copies of the cRNA. This study demonstrated that a real-time (TaqMan-based) quantitative PCR or RT-PCR assay can provide a sensitive, reproducible, and robust method for detecting and quantifying PERV DNA or RNA sequences in samples of human or guinea pig origin spiked with PERV expressing vectors (Argaw, Ritzhaupt et al. 2002) . A similar assay based on SYBR Green I label in combination with primers derived from the PERV *pol*

sequence has been used for estimating copy numbers of PERV integrated in the host genome (Bellier, Dalba et al. 2006) .

It is noteworthy that quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays have higher specificity and could differentiate between the defective transcripts and the full length proviruses by measuring the level of virus expression at different time points. In this regard, the International Xenotransplantation Association in 2009 recommended that qRT-PCR can be used to assess expression levels of human-tropic PERVs in different pig breeds and source animals (Denner, Schuurman et al. 2009).

When using PCR-based methods to study PERV in animals or clinical trial participants that are exposed to living pig cells rather than cell-free virus, it is important to also incorporate a method to distinguish microchimerism from infection. In other words, survival of pig cells that carry many copies of PERV may confound interpretation of a PCR positive result. Therefore, incorporation of analysis for both PERV-specific PCR and for DNA sequences from pig-specific genes that are highly repetitive, such as mitochondrial or centromeric sequences, is necessary to interpret assays based on detection of PERV DNA by PCR (Switzer, Shanmugam et al. 1999; Paradis, Langford et al. 1999; Blusch, Roos et al. 2000).

2.1.5 Immunoassay

An additional method that is useful to monitor for evidence of transmission in vivo in animal models or human clinical trials, is the use of assays to detect anti-PERV immune responses. Immunologic assays provide the advantage of a rapid assay and one that does not require *a priori* knowledge of the site of viral replication in vivo. However, it does not distinguish between development of an immune response due to viral infection and an immune response due to transplanted pig cells expressing viral proteins. A further caveat to consider is the immuno-suppressive regimens applied in many xenotransplantation clinical protocols and the impact the iatrogenic immunosuppression may have on development of an anti-PERV immune response.

Methods to detect an anti-PERV immune response include antibody detection assays such as Western Blot and enzyme-linked immunosorbent assay (ELISA), functional antibody assays such as lymphocyte fusion inhibition and virus neutralization, and assays of cellular immunity such as detection of PERV-specific cytotoxic T-lymphocyte (CTL) activity and lymphocyte proliferation.

Paradis and coworkers used immunological tests to obtain indirect evidence of PERV infection in 160 patients who had been therapeutically treated with living tissues of pigs. In this study, recombinant protein of PERV-B p30 and purified whole virus, isolated from PERV-infected 293 HEK, were used as antigens to screen for anti-PERV antibodies in serum (Paradis, Langford et al. 1999). Similarly, Matthews and coworkers have used Western blotting for the detection of PERV antibodies in xenotransplantation patients (Matthews, Brown et al. 1999). However, the results and interpretation of data from these studies had the inherent problem of lacking a positive control, as no known human sera exist with anti-PERV antibodies, even amongst the patients who have been exposed to pig tissues. To compensate for no known human positive sera, cross-reactive antibodies developed against simian sarcoma-associated virus (SSAV) and other related retroviruses have been used as indirect controls in assays used to detect PERV antibodies by western blot analysis

(Matthews, Brown et al. 1999). We have also used the anti-SSAV antibodies as a control when detecting anti-PERV p30 antibodies in guinea pigs exposed to either cell-free or cell-associated viruses (Argaw, Colon-Moran et al. 2004) .

In addition to assays used to detect antibody, investigators have also developed PERV-specific antibodies for use in antigen detection assays, as well as to identify neutralizing epitopes. The transmembrane (TM) domain of the PERV envelope was used to derive a goat antiserum that is both neutralizing and can be used for immunoassays such as Western blot and ELISA assays (Fiebig, Stephan et al. 2003). Additional studies have reported development of rabbit-based PERV nucleocapsid anti-serum that can be used for Western blot and immunohistochemistry analysis (Krach, Fischer et al. 2000) . Antisera generated to peptides from the capsid protein (p30) or the surface or TM of PERV envelope class B (SU, gp70(B)) showed functionality in several immunological assays, such as immunoblots, immunofluorescence, and immunogold staining used to detect PERV antigens in biological materials (Fischer, Krach et al. 2003) . Anti-sera raised in different animal species (guinea pigs, sheep, and rabbits) directed against PERV antigens were also evaluated for their use in different immunologic assays for PERV antigen detection (Galbraith, Kelly et al. 2000). A monoclonal antibody specific to the PERV *gag* protien was shown useful for detecting infection (Chiang, Chang et al. 2005) . Recently, a monoclonal antibody directed against the PERV-B envelope protein detected a specific peptide sequence in the proline rich region of SU as an epitope for PERV infection (Nakaya, Hoshino et al. 2011) . These monoclonal antibodies may prove useful for screening for PERV antigen, although to date no xenotransplantation studies have used the antibody on porcine or human xenotransplant recipient samples.

2.1.6 Animal models

While PERV infects and replicates in human cells in vitro, the risk to human recipients of xenotransplantaion products is still unknown. However, the observation that the PERV DNA provirus integration pattern in human cells is observed to be similar to murine leukemia viruses, namely a slight bias towards integration in transcriptionally active genes (Moalic, Blanchard et al. 2006), suggests that from a mechanistic point of view, PERV may be tumorigenic in human cells. Ideally, the best way to test whether PERV is tumorigenic through insertional mutagenesis would be to have a permissive animal model. Unfortunately, in spite of intense efforts by multiple investigators, including our own lab, an animal model of PERV infection, replication, and pathogenesis has yet to be proven, although it would provide an invaluable tool to assess PERV-associated pathology, and evaluate potential prophylactic, therapeutic, or diagnostic medical products.

Initial attempts to identify a permissive animal model for PERV were focused on rodents. Based on unpublished data presented at a public advisory committee meeting of the FDA Subcommittee on Xenotransplantation (13 January, 2000 agenda and transcripts available at: http:// www.fda.gov/cber/advisory/ctgt/ctgtmain.htm), we examined whether guinea pigs would provide a permissive animal model for PERV replication. In an effort to develop a mitotically active organ compartment that might be more permissive for viral infection, we induced the following changes in guinea pigs and then exposed them to cell-associated or cell-free PERVs: i) liver damage by hepatotoxic chemicals to stimulate proliferation of cells permissive to retroviral vectors; ii) initial immunization of the animals with PERV to

establish virus-specific antibodies that may enhance infectivity as manifested by other viruses (Olsen 1993; Morens 1994; Yin, Lynch et al. 1999). Despite these measures, we could not detect evidence of replication following exposure of the animals to cell-free or cell-associated PERVs (Argaw, Colon-Moran et al. 2004). Similar results were observed with rats or guinea pigs after PERV was inoculated by either intramuscular or intra-peritoneal routes (Specke, Tacke et al. 2001). No evidence of infection, including lack of detection of anti-PERV antibodies, was shown in immunosuppressed rats transplanted with pig islets (Denner, Specke et al. 2008).

An additional effort to create a permissive small animal model was to introduce the human cDNA expressing the PERV-A receptor, since it was known that the murine ortholog of the PERV-A receptor is reported to be non-functional (Ericsson, Takeuchi et al. 2003) . Exploiting this information, Martina and coworkers, in collaboration with our laboratory, introduced the HuPAR2 cDNA into the germline of mice and generated Hu-PAR2 transgenic mice (Martina, Marcucci et al. 2006). Then the transgenic mice were exposed to PERV, and subsequently qRT-PCR and immunological tests were used to assess viral replication status. Increasingly higher copy numbers of PERV RNA and DNA were detected over the first 2 month period of analysis, although a subsequent decrease in PERV RNA and DNA was observed after 3 months that correlated with detection of neutralizing anti-PERV antibodies (Martina, Marcucci et al. 2006). While not fully explored, the HuPAR-2 transgenic mouse model may provide one model to further study the nature of PERV infection from the perspective of pathogenicity, tissue tropism, and humoral and cellular immunity.

NOD SCID mice transplanted under the kidney capsule with pig pancreatic islets were also evaluated for suitability as a permissive animal model for PERV replication. These studies provided evidence of active viral expression in several tissue compartments suggesting viral transmission (Laan, Lockey et al. 2000) (Deng, Tuch et al. 2000). However, interpretation of these studies changed when a later investigation also performed in immunodeficient NOD/SCID transgenic mice that were transplanted with porcine and human lymphohematopoietic tissues showed evidence of transmission of PERV to the transplanted human cells. The investigators demonstrated that the appearance of PERV positive human cells was most likely due to pseudotyping of PERV C by xenotropic murine leukemia virus, rather than authentic infection by human-tropic PERV (Yang, Wood et al. 2004),. These observations were later confirmed by in vitro studies performed by Martina, et al (Martina, Kurian et al. 2005).

Non-human primates (NHP) were thought by many to provide a good species to evaluate PERV transmission as well as xenotransplant survival by virtue of their phylogenetic closeness to humans, and the use of NHP for evaluation of the effectiveness of porcine xenotransplantation products used in combination with various immunosuppressive therapies to prolong survival in various disease models. Our laboratory demonstrated that NHP cells representing primary and established cell lines from Rhesus, African Green Monkey (AGM) and baboon species were poorly infected, if at all, and that replication is restricted (Ritzhaupt, Laan et al. 2002). These findings were confirmed and extended in a recent study that showed that the ortholog for the PERV-A receptor in rhesus macaque and baboon is non-functional. Similarly, in vivo studies have not found evidence for PERV transmission in NHP transplanted with porcine xenotransplantation products, even when using potent immunosuppression treatments (Moscoso, Hermida-Prieto et al. 2005; Specke,

Plesker et al. 2009). Together, these findings reinforce the conclusion that NHP are not a permissive model for studying the risks of PERV exposure to porcine xenotransplantation recipients.

Studies performed in other species, such as dogs and lambs, have also not shown active viral replication (Edamura, Nasu et al. 2004) (Popp, Mann et al. 2007), leaving the field without a suitable animal model to evaluate the potential for pathogenesis or to provide a means to assess interventions to prevent or treat PERV.

2.2. Genetics and biology of PERV in the context of xenotransplantation

2.2.1 Genome and evolution

Three receptor classes for PERVs have been defined through a combination of sequence alignment and functional receptor studies: PERV A, B and C (Takeuchi, Patience et al. 1998) (Le Tissier, Stoye et al. 1997) (Akiyoshi, Denaro et al. 1998). Analysis of the nucleotide sequence of PERV showed a high nucleotide sequence identity to other gammaretroviral genomes, especially, murine leukemia virus (MLV) and the gibbon ape leukemia virus (GALV), sharing up to 66% identity in the envelope sequence (Le Tissier, Stoye et al. 1997) , and even higher nucleotide identity in the remaining genomic regions, *gag* and *pol* (Akiyoshi, Denaro et al. 1998).

Southern blot and additional genomic analyses have indicated variation in the number, distribution within the genome, and presence of full-length replication-competent genomes, with estimates varying from as low as 3 to as high as 200 copies per genome, depending on the breed and the study (Akiyoshi, Denaro et al. 1998) (Le Tissier, Stoye et al. 1997) (Bosch, Arnauld et al. 2000) (Herring, Quinn et al. 2001) (Rogel-Gaillard, Bourgeaux et al. 1999) (Lee, Webb et al. 2002) (Edamura, Nasu et al. 2004) (Li, Ping et al. 2004) (Garkavenko, Wynyard et al. 2008).

In addition to the known gammaretroviral form of PERV, studies have also identified endogenous sequences in the pig genome representative of the betaretroviral genus (Ericsson, Oldmixon et al. 2001) (Patience, Switzer et al. 2001). Analysis of existing members of the subfamily Suidae and related families suggested that the gammaretroviral sequences representing PERV entered into the subfamily Suidae approximately 3.5 million years ago (Patience, Switzer et al. 2001) (Niebert and Tonjes 2003). The two human tropic envelopes, PERV A and PERV B, have been proposed in one study to predate the pig tropic PERV-C envelope found in members of the genus *Sus* that diverged approximately 1.5 million years ago (Niebert and Tonjes 2005) .

2.2.2 Expression and infectivity of PERV

It has been speculated that there might be tissue- or breed-dependent expression of PERV that might qualify some pig tissues or breeds to be relatively safe for human xenotransplantation. Indeed, the remnants of porcine endogenous retroviruses in genomes of modern-day pigs differ between different breeds of pigs in composition, expression and ability to encode infectious virus. The levels of PERV expression were compared in a large panel of tissues originating from both specific-pathogen free (SPF) and conventional pigs. For all SPF tissues tested, *gag*, *pol*, *env*-A, -B and -C mRNA levels were the same range or

slightly higher than the corresponding tissues of the conventional pigs. Variation of expression of proviruses was also observed between tissues, with the lowest mRNA levels observed in the pancreas and the highest in the kidney (Clemenceau, Lalain et al. 1999). In other efforts elucidating in vivo expression of PERV, a transmission electron microscopy investigation done on transgenic pigs that express human decay accelerating factor, showed no evidence of active virus release despite the detection of viral mRNA in a variety of tissues analyzed. However, detection of reverse transcriptase activity in pig sera screened in this study, suggested the release of virions into the blood stream (Langford, Galbraith et al. 2001). In a more recent study using semi-quantitative duplex RT-PCR, analysis of gag expression was measured for a variety of tissues in twenty Duroc pigs of different ages, ranging from 10-110 days. While the measurements were not precisely quantitative, the results suggested a trend of highest gene expression in liver and lowest in heart, with some trends of age-dependent gene expression levels, although this varied by organ tested and differences were not great (Moon, Kim et al. 2009).

In addition to in vivo based assessment of PERV expression, several in vitro studies have been conducted to analyze the expression and infectious potential of PERV from various cells and tissues of pigs. Replicating PERV particles have been shown to be released in vitro by cultured primary pig blood vessel epithelial cells (Martin, Kiessig et al. 1998), the pig kidney cell line PK-15 (Patience, Takeuchi et al. 1997) and primary pig peripheral blood mononuclear cells (PBMC) (Wilson, Wong et al. 1998) . We also isolated infectious PERV-C from porcine plasma, as well as pig plasma-derived factor VIII, suggesting that in vivo, blood or endothelium constitutively express PERV (Takefman, Wong et al. 2001). We also could culture PERV-C from freshly isolated pig bone marrow cells by co-culture with pig ST cells (McIntyre, Kannan et al. 2003).

For retroviruses, the long terminal repeat (LTR) carries an enhancer, termed U3 that influences transcriptional activity. Therefore, several studies have evaluated the influence of variant sequences in the U3 region on the level of gene expression for PERV. While our own study did not show significant differences in expression in different cell lines tested for LTRs derived from PERV-A, -B, or –C, we did see a slightly lower level of expression with a chimeric LTR called PERV-NIH and all LTRs tested showed lower expression levels in ST-IOWA pig cells compared to other cell lines examined (Wilson, Laeeq et al. 2003). In other studies, it has been shown that the level of PERV expression in permissive cells is associated with the copy number of a 39 base pair (bp) repeat in the PERV LTR U3 region (Scheef, Fischer et al. 2001). A more thorough study of the LTR structures from kidney of NIH mini-pigs has shown that there are four different elements and that one was associated with the highest level of transcriptional activity. The investigators went on to show that the transcriptional activity could be down-regulated in the presence of a methyltransferase suggesting that transcriptional activity is modulated by DNA methylation (Park, Huh et al. 2010) – a mechanism known to also inhibit gene expression of murine leukemia viruses (for examples, see (Jaenisch 1982) (Stewart, Stuhlman et al. 1982)).

In general, it is likely that only a few of the loci present in the pig genome express full-length replication-competent PERV. However, the polymorphic nature of the integrated endogenous viruses makes it difficult, if not impossible, to identify a breed of “PERV-free” pig. The pig genome sequence project currently running (Archibald, Bolund et al. 2010) and genetic knockout technology may provide more information to guide cloning and breeding of pigs with minimal PERV expression as source animals for clinical xenotransplantation.

2.2.3 Factors impacting human cell tropism of PERV

As described previously, the replication competent PERVs that have been identified to date have been classified into one of three receptor classes, referred to as PERV-A, -B, or –C, based on receptor-specificity properties. As shown in Table 1, both PERV-A and PERV-B are able to infect human cells, but use distinct receptors. Two homologous human cDNAs have been identified and shown to function as the receptor for PERV-A, human PERV-A receptors 1 and 2 (HuPAR-1 and -2) (Ericsson, Takeuchi et al. 2003), but neither of these receptors function as a receptor for PERV-B. The PERV-B receptor remains unknown. PERV-C has a more restricted host range, only able to infect pig cells, and its receptor is also unknown. Interestingly, naturally occurring recombinants between PERV-A and PERV-C have been shown to have increased infectivity titer for human cells compared to the prototype PERV-A (Harrison, Takeuchi et al. 2004).

PERV Envelope	Pig (ST-IOWA)	Human (293)	Rabbit (SIRC) expressing HuPAR-2*
PERV-A	+	+	+
PERV-B	+	+	-
PERV-C	+	-	-

*(Ericsson, Oldmixon et al. 2001)

Table 1. Infection properties of PERV receptor classes

Several studies have analyzed the structure of the HuPAR receptors to identify the functional determinants that allow PERV-A infection. Mattiuzzi and coworkers used the murine ortholog and showed that a single amino acid residue at position 109 in the putative extracellular loop was responsible for the inability of the murine receptor to support PERV-A infection (Mattiuzzo, Matouskova et al. 2007). A later study by Marcucci, et al, showed that there is a 10-fold difference in infectivity titer when PERV-A pseudotypes infect cells expressing HuPAR-1 vs. HuPAR-2. This quantitative difference was exploited to further map the regions within HuPAR-2 that confer this 10-fold increase in infectivity titer. While these studies were unable to define a single residue or group of residues, her results pointed to an additional role of the region encompassing amino acids 152-285 in impacting the efficiency of the receptor to mediate PERV-A entry (Marcucci, Argaw et al. 2009). Interestingly, these studies and others have shown that species-specific polymorphisms of the PERV-A receptors have given rise to PERV-A infection-resistant phenotypes for different reasons. For example, as described above, the murine ortholog is non-functional due to a single amino acid difference, and this observation of a single amino acid change impacting function has been observed for the PAR-1 homolog in rhesus macaque, cynomolgus macaque, and baboon (all have serine at amino acid residue 109) (Mattiuzzo and Takeuchi 2010). However, the rat PAR allows PERV-A infection when expressed in non-permissive cell lines, although rat cells do not support PERV-A entry. Mattiuzzo, and coworkers, have data suggesting that the endogenous receptor expression in rat cells is too low to allow PERV-A infection (Mattiuzzo, Matouskova et al. 2007).

The normal cellular function for the human PERV-A receptors is somewhat controversial, in that there are two independent reports of screens that identified very different functions for

the PERV-A receptor: a CNS-specific receptor for gamma-hydroxybutyrate, hypothesized to be a G-protein-linked receptor (Andriamampandry, Taleb et al. 2007; 2008); and a riboflavin transporter, termed RFT1 (Yonezawa, Masuda et al. 2008). While neither study has been followed with additional publications to confirm or deny each of the reported claims, the latter report makes more sense, when the tissue-specific pattern of PERV-A receptor expression and identified gammaretroviral receptors, in general, are taken into account: 1) The PERV-A receptor mRNA has been shown to be widely expressed (Ericsson, Takeuchi et al. 2003), similar to what has been shown for RFT1 (Yonezawa, Masuda et al. 2008); 2) Both RFT1 and PAR have been predicted to have ten transmembrane domains; 3) All receptors identified to date for gammaretroviruses are Class 2A carrier-type facilitator transporters (Hein, Prassolov et al. 2003).

While detailed understanding of the cellular factors required for human cell infection is critical, an equally important area of study is that of cellular restriction factors. Only in relatively recent years, primarily driven by HIV research, have cellular restriction factors been identified (Neil and Bieniasz 2009). While a number of restriction factors have been identified that restrict HIV or other retroviruses from infecting certain cell types, not all necessarily have been shown to also restrict PERV infection. For example, TRIM5α molecules have been shown to restrict a variety of lentiviruses and some gammaretroviruses in a species-specific manner. However, none of the primate or non-primate-derived TRIM-5α, including those from human, African green monkey, squirrel monkey, rhesus macaque, cattle, and rabbit, inhibited infection by PERV-A or a naturally occurring recombinant, PERV-A/C (Wood, Webb et al. 2009). In contrast, there is limited evidence to suggest that two other known restriction factors may have species-specific restriction of PERV replication. Mattiuzzo, et al, demonstrated that the level of processed *gag* in the cell lysate and supernatant was inversely correlated with expression levels of the late-acting restriction factor called tetherin (Mattiuzzo and Takeuchi 2010). These observations suggest that certain non-human primate cells may restrict PERV replication due to a late stage defect in *gag* processing and virus particle release – observations that confirm and extend observations from our earlier study on restriction to PERV replication in non-human primate cells (Ritzhaupt, Laan et al. 2002). Another restriction factor identified initially in studies with HIV is called APOBEC – it is packaged into viral particles and inhibits viral replication by editing cytosine residues through its deaminase activity (reviewed in (Goila-Gaur and Strebel 2008)). Recently, the porcine APOBEC proteins were identified and analyzed for their ability to restrict PERV replication and it was shown that they were packaged into the PERV virions and edited certain cytosine residues, suggesting that this may be a mechanism by which porcine cells restrict this endogenous retrovirus (Dorrschuck, Fischer et al. 2011). Although some have hypothesized that human APOBEC may restrict PERV replication to account for the observed lack of transmission in human xenotransplantation clinical trial participants (see section 2.2.4), a detailed study to determine whether human APOBEC might also restrict PERV has not been performed, to date.

In addition to trying to understand the cellular determinants of human cell tropism of PERV, efforts have also been directed towards looking at the viral determinants of human cell infection. In general, the primary determinant of cell tropism is the viral envelope. Our laboratory has used a variety of approaches to identify the region of the PERV-A envelope required for human cell infection: binding assays, pseudotype infectivity assays, and

generation of recombinant and site-directed variants of envelope to assess structure-function relationships. Using a fusion protein based on the rabbit immunoglobulin heavy chain, we showed several years ago that PERV envelope binding requires a region beyond the prototypical receptor binding domain (RBD) for gammaretroviruses. As shown in Figure 2, the RBD that we mapped using this approach encompassed not just the variable regions A and B (VRA and VRB) as has been shown for other related viruses, but also requires the proline-rich region (PRR) (Gemeniano, Mpanju et al. 2006). Using the pseudotype assay described in section 2.1.5, we and others have tried to identify more precisely the regions of the envelope required for human cell infection. Harrison et al used a naturally occurring recombinant of PERV-A and PERV-C with high titer on human cells compared to parental PERV-A and showed two regions that correlated with increased infection on human cells: amino acid residue 140 that lies between VRA and VRB and additional sequences within the PRR (Harrison, Takeuchi et al. 2004). Our laboratory has also shown that sequences within the C-terminal region of the SU influence human cell infectivity (Gemeniano, Mpanju et al. 2006; Argaw, Figueroa et al. 2008).

We have also shown that PERV-C RBD can bind human cells specifically, in a dose-dependent manner (Gemeniano, Mpanju et al. 2006). More recently, we demonstrated that relatively few amino acid changes in the PERV-C envelope can allow infection of human cells (Argaw, Figueroa et al. 2008). Together, these results suggest that human cells may carry PERV-C receptors that are non-functional for wild-type PERV-C infection, but allow binding, and that just a few amino acid changes within the PERV-C envelope allow entry. Our lab has further shown that the molecule that binds PERV-C and allows mutant PERV-C to infect human cells is distinct from the human PERV-A receptor (Gemeniano, Mpanju et al. 2006; Argaw, Figueroa et al. 2008). These observations suggest that with long-term survival of porcine xenotransplantation products that do not carry PERV-A or other human-tropic forms of PERV naturally, that over time, mutant PERV-C envelopes could be selected to allow infection of human cells.

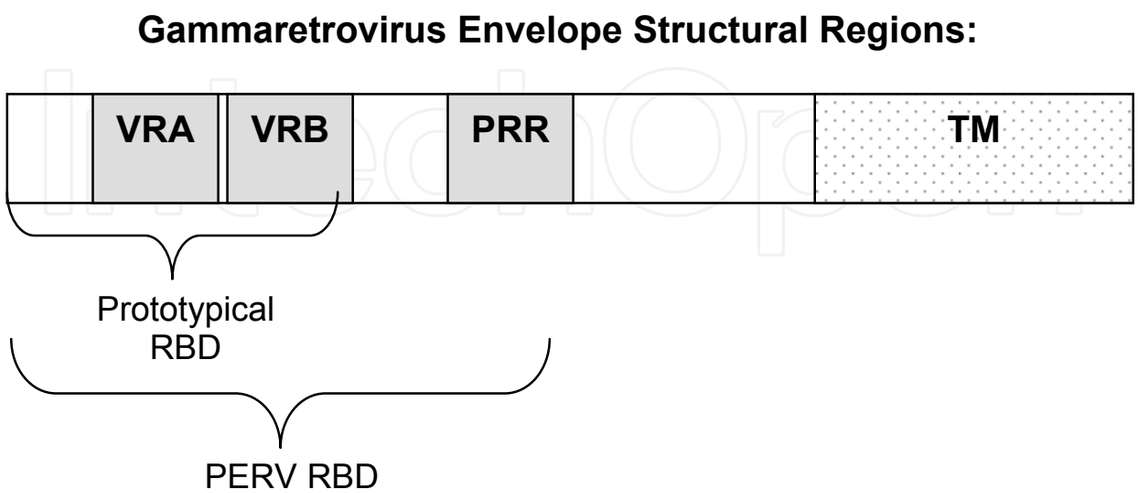

Fig. 2. Receptor Binding Domain for gammaretroviruses and PERV

2.2.4 Human clinical studies

In vitro infection of human cells by PERV, suggesting a risk of cross-species transmission, prompted investigators to perform retrospective studies to determine whether subjects who had previously participated in clinical xenotransplantation trials may have evidence of PERV transmission. Subjects in several different types of xenotransplantation clinical studies were retrospectively analyzed: extracorporeal perfusion through pig cells and organs (Patience, Patton et al. 1998); encapsulated pancreatic islets transplants (Heneine, Tibell et al. 1998; Elliott, Escobar et al. 2007) , and engrafted pig tissues and cells (Paradis, Langford et al. 1999). While one can debate the validity of the various methods chosen (see section 2.1), none of these retrospective studies nor more recent reports of prospectively performed studies (Valdes-Gonzalez, Dorantes et al. 2010) or a survey of abattoir workers (Hermida-Prieto, Domenech et al. 2007) have demonstrated evidence of transmission of PERV to humans exposed to pig xenotransplantation products. Our laboratory also participated in analysis of animal and healthcare workers involved in a clinical xenotransplantation protocol, and did not find any evidence of PERV transmission in that setting (Levy, Argaw et al. 2007). Although the lack of evidence of PERV transmission is encouraging for the safety of these approaches, there are several caveats to interpretation of these negative results: 1) in most recipients, the cells remaining from xenotransplants or extra-corporeally prepared tissues are short lived; 2) assays to monitor PERV transmission may not be sufficiently sensitive and specific; 3) we do not know the best tissue to test for PERV infection, and so for a variety of reasons, investigators sample peripheral blood lymphocytes and serum (Patience, Takeuchi et al. 1997) (FDA 2001). To date, no significant evidence of in vitro infection of human PBMC has been obtained, though that does not rule out the ability of PERV to infect these cells in vivo or their precursor cells in the bone marrow. For a more thorough review of the different clinical studies and methods for assessing human samples for transmission of PERV see (Wilson 2008).

2.2.5 Strategies to prevent or Treat PERV infection in humans

While the actual risk of transmission, replication and pathogenic consequences of exposure to porcine xenotransplantation products to human clinical trial recipients is still not well understood, investigators are pursuing strategies to prevent or treat PERV infection in the xenotransplantation context.

A promising approach that is being investigated is to block expression of PERV RNA using small interfering RNAs (siRNA). Proof of concept studies have been performed in vitro to identify candidate siRNAs; (Karlas, Kurth et al. 2004; Miyagawa, Nakatsu et al. 2005; Dieckhoff, Karlas et al. 2007). Two groups have generated transgenic pigs that express the interfering RNAs (Dieckhoff, Petersen et al. 2008; Ramsoondar, Vaught et al. 2009). Should these prevent PERV expression, use of these as source animals may prevent or reduce transmission of PERV to recipients of porcine xenotransplantation products. While initial reports suggest that the inhibition of gene expression is variable and incomplete, and it is not known whether current levels would prove to be sufficient to prevent PERV transmission, it provides a promising avenue of further effort.

An interesting alternative approach is being evaluated by Yamamoto, et al, using either expression of different mannosidase enzymes or siRNA designed to inhibit expression of the gene encoding the porcine dolichyl-phosphate mannosyl-transferase. They have shown that by essentially altering the mannose residues on the N-linked glycan moieties of PERV

envelope, they demonstrate a decrease in PERV infectivity titers on human cells in vitro (Miyagawa, Nakatsu et al. 2006) (Yamamoto, Nakatsu et al. 2010).

In addition to prevention strategies, treatment options are under development. Strategies that are based on using approved anti-HIV drugs have been previously reviewed (Wilson 2008). More recent developments in this field include new monoclonal antibodies with neutralizing activity. Nakaya, et al, have described a monoclonal antibody that potently neutralizes PERV-B, but not PERV-A (Nakaya, Hoshino et al. 2011). Interestingly, the epitope for this neutralizing antibody maps to the proline-rich region, a finding that corresponds to previously described observations that correlate human cell infection with determinants within the PRR (see section 2.2.3). Another group has described two additional neutralizing monoclonal antibodies, one which has been mapped to the C-terminal region of the SU and the other to the TM domain (Chiang, Pan et al. 2007). Unfortunately, the studies are not sufficiently well-described to determine whether either or both of these antibodies recognize and neutralize only PERV-A or if they neutralize both PERV-A and PERV-B, and no follow-up papers have been published on these antibodies since 2007.

3. Conclusion

As highlighted by a recently reported inventory of ongoing human clinical trials in xenotransplantation, there continues to be interest in the clinical application of non-human cells, tissues, and ultimately organs to treat human disease (Sgroi, Buhler et al. 2010). Fortunately, to date, there is no evidence of PERV transmission to any monitored human recipient of a porcine xenotransplantation product. While these data are encouraging, the risks are still unknown should clinical xenotransplantation become successful in reaching the goal of long-term survival of living pig cells, tissues, or organs, in the recipient. The irony of clinical success is that the likelihood of PERV transmission may increase. Therefore, continued investigation into PERV biology and the determinants of human cell infection should be pursued in order to identify avenues to prevent, reduce or treat PERV infection. Until a permissive animal model is developed, gaps in our understanding of the factors affecting in vivo transmission, replication, and potential for pathogenicity cannot be filled. Improved means to detect evidence of PERV infection are also needed.

4. Acknowledgments

The authors wish to thank the many scientific colleagues that have contributed to the work reviewed in this article, and in particular, those additional members of the Wilson laboratory who have provided major contributions to the studies described herein: Winston Colon-Moran, Mariel Figueroa, Malou Gemeniano, Armin Ritzhaupt, Onesmo Mpanju; and our long-time collaborators, Dan Salomon and his colleagues, and Maribeth Eiden, and her colleagues. In addition, we thank Drs. Andrew Byrnes and Keith Wonnacott for their critical reading of the manuscript.

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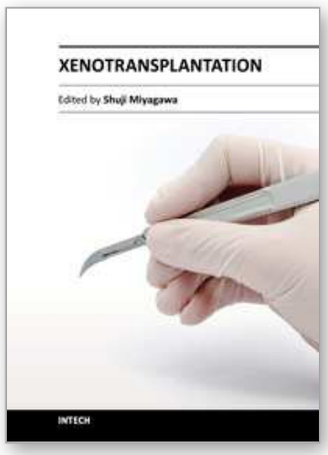

Xenotransplantation

Edited by Prof. Shuji Miyagawa

ISBN 978-953-307-997-4
Hard cover, 126 pages
Publisher InTech
Published online 03, February, 2012
Published in print edition February, 2012

Accompanied by the advent of animal cloning, the technique of nuclear transfer produced alpha1,3-galactosyltransferase-knockout (Gal-KO) pigs in many institutes, including the ones in Japan, at the beginning of 21st Century. In addition, the controversy of the risks of PERV has gradually minimized, because of the fact that there are no cases of PERV infections reported in humans. Furthermore, a large clinical wave for islet allotransplantation resumed the interest of xenotransplantation, especially porcine islet transplantation and some exceptions. Clinical trials were done in many countries so far, such as Sweden, China, Mexico, USA (Inventory of Human Xenotransplantation Practices - IXA and HUG in collaboration with WHO). In addition, a new clinical trial was approved by the government, and resumed the porcine islet transplantation research in New Zealand two years ago.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Takele Argaw and Carolyn A. Wilson (2012). Methods and Tools for Detection and Evaluation of the Risks of Porcine Endogenous Retrovirus in Porcine to Human Xenotransplantation, Xenotransplantation, Prof. Shuji Miyagawa (Ed.), ISBN: 978-953-307-997-4, InTech, Available from: http://www.intechopen.com/books/xenotransplantation/methods-and-tools-for-detection-and-evaluation-of-the-risks-of-porcine-endogenous-retrovirus-in-porc

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