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Infection Barriers to Successful Xenotransplantation Focusing on Porcine Endogenous Retroviruses

Joachim Denner[a] and Ralf R. Tönjes[b]

[a]Robert Koch Institut, Berlin, Germany
[b]Paul Ehrlich Institut, Langen, Germany

Summary

Summary: Xenotransplantation may be a solution to overcome the shortage of organs for the treatment of patients with organ failure, but it may be associated with the transmission of porcine microorganisms and the development of xenozoonoses.

Whereas most microorganisms may be eliminated by pathogen-free breeding of the donor animals, porcine endogenous retroviruses (PERVs) cannot be eliminated, since these are integrated into the genomes of all pigs. Human-tropic PERV-A and -B are present in all pigs and are able to infect human cells. Infection of ecotropic PERV-C is limited to pig cells. PERVs may adapt to host cells by varying the number of LTR-binding transcription factor binding sites. Like all retroviruses, they may induce tumors and/or immunodeficiencies.

To date, all experimental, preclinical, and clinical xenotransplantations using pig cells, tissues, and organs have not shown transmission of PERV. Highly sensitive and specific methods have been developed to analyze the PERV status of donor pigs and to monitor recipients for PERV infection. Strategies have been developed to prevent PERV transmission, including selection of PERV-C-negative, low-producer pigs, generation of an effective vaccine, selection of effective antiretrovirals, and generation of animals transgenic for a PERV-specific short hairpin RNA inhibiting PERV expression by RNA interference.

Introduction

The shortage of allotransplants is the greatest obstacle in organ transplantation. Whereas the number of individuals waiting for an allotransplant is steadily increasing, partially due to a longer life expectance of the human population, there is not a proportional increase in viable donated organs. As of April 2011, more than 111,000 people are on the waiting list for organ transplants in the United States (Organ Procurement and Transplantation Network [http://optn.transplant.hrsa.gov/]). Presently, a large number of patients needing an organ transplant will die while on the waiting list. Health education and organ donation encouragement are suggested as important measures to circumvent this organ shortage; however, different strategies have not resulted in an appreciable increase in donated organs (5).

Xenotransplantation, the transplantation of living cells, tissues, and organs between different species or *ex vivo* transspecies exchange of cells, tissues, and organs, is a suggested solution to the allograft shortage (51, 97). Clinically, the history of xenotransplantation started in 1964, when Reemtsma transplanted a chimpanzee kidney into a human with end-stage renal disease, extending the patient's life a record time of 9 months (265). As xenotransplantation improves into a viable alternative to allotransplantation, a number of medicinal and biological barriers remain (255). Such hurdles include immunological rejection, physiological incompatibilities, and transmission of microorganisms.

Xenotransplantation is also the subject of a broad ethical discussion (4, 311), and clinical xenotransplantation should be performed with authorization by regulatory authorities, based on comprehensive guidelines, e.g., those of the U.S. Food and Drug Administration (FDA) (102) and the European Medicines Agency (EMEA) (99), focusing on safety and benefit-risk ratios. Conditions for

undertaking clinical trials of porcine islet products in type 1 diabetes have been described by the International Xenotransplantation Association (IXA) (133).

Immunological Barriers

The major problem of xenotransplantation is the rejection of porcine cells, tissues, and organs, which occurs in several stages: (i) hyperacute rejection (HAR), (ii) acute vascular rejection (AVR), (iii) cellular rejection, and (iv) chronic rejection. HAR and AVR are mediated mainly by antibodies against the terminal oligosaccharide determinant galactose-alpha-1,3-galactose (Gal) on pig vascular endothelium (52). In contrast, human allotransplantation is associated mainly with cellular and chronic rejection.

HAR is based on preexisting antibodies against Gal-alpha-1,3-Gal residues expressed on pig cells and is now being overcome. Early attempts to reduce the amount of these antibodies by adsorption strategies failed (156). The most successful approach involves the production of (i) transgenic pigs that express human complement regulatory proteins capable of inhibiting the injurious effect of antibody-mediated complement activation on the vascularized pig organ (283, 361) and (ii) pigs with a knocked-out galactose-alpha-1,3-galactosyltransferase gene locus (GalT-KO pigs) (62, 168, 253).

This genetic knockout prevents expression of Gal-alpha-1,3-Gal residues. When hearts from GalT-KO pigs were transplanted heterotopically into baboons by use of an anti-CD154 monoclonal antibody-based regimen, the elimination of the Gal-alpha-1,3-Gal epitope prevented HAR and extended survival of pig hearts in baboons for 2 to 6 months (median, 78 days); the predominant lesion associated with xenotransplant failure was a thrombotic microangiopathy, with resulting ischemic injury (167). Significant prolongation of life-supporting pig-to-baboon renal xenograft survival, for up to 83 days, with normal creatinine levels in baboons, was achieved using GalT-KO donors and a tolerance induction approach (353).

The problem in overcoming AVR is incompletely understood and cannot be controlled yet. However, progress is being made on several fronts, including the development of new immunosuppressive drugs and further attempts to genetically engineer pigs. Although the strength of cellular rejection in xenotransplants remains uncertain, it is expected to be stronger than that observed in allografts. Based on the severity of the T-cell-dependent B-lymphocyte response resulting in antibody formation and the role of antibodies against the transplant in chronic rejection of an allograft, it can also be anticipated that chronic rejection of xenotransplants will be more aggressive than that of allotransplants. However, data for this scenario are not available. Overcoming these barriers will probably require severe and sustained exposure to immunosuppressive drugs or breakthroughs in the development of human immunological tolerance to porcine organs (50, 255, 285, 356).

Physiological Barriers

Extensive research is needed to determine whether animal organs can replace the physiological functions of human organs. Research has been performed in fields such as anatomical design, metabolism, hormonal function, blood viscosity, and coagulation (123, 262, 323). Pig-to-primate kidney transplants function well on several levels but lack compatibility with respect to erythropoietin (EPO) function, since porcine EPO differs to a greater extent from human EPO (123). Human recombinant EPO would need to be substituted in the patients. It can be anticipated that pig-to-human transplants of hearts, kidneys, and lungs will be physiologically feasible.
However, this is not the case for whole-organ liver transplants, where differences between many of the proteins manufactured in the liver (approximately 2,000) may prohibit adequate function. In addition, xenotransplantation of lungs will require more research into their physiology (225). It has become clear that coagulation dysfunction between recipient and donor, as well as inflammation, contributes significantly to different survival times and to the loss of the xenotransplant (262). The induced thrombotic microangiopathy causes ischemic injury to the myocardium during heart transplantations and finally results in consumptive coagulopathy (31, 142).

Risks of Infectious Disease

The infectious disease risks of xenotransplants pose a problem for the recipients of organ transplants and the public at large. The public health risk represents a major concern, and after it was documented that porcine endogenous retroviruses (PERVs) infect human cells in an *in vitro* coculture assay, it was perceived that xenotransplantation might create a new epidemic infectious disease (46, 66, 333, 334, 340). This perception seemed logical, as it was at that time known for just a few years that infection of humans with human immunodeficiency virus (HIV) and the AIDS pandemic were the result of a transspecies transmission of a retrovirus, simian immunodeficiency virus (SIV), to humans. Thus, the same potential was perceived for PERVs, because PERVs are also retroviruses; however, they are not closely related to HIV (see below). A putative spread of infectious diseases via pig-to-human transplants and the inability to assess the risk led the FDA in 1997 to place a hold on ongoing clinical trials involving new drug developments and cellular transplants, such as with porcine islet cells, hepatocytes, and others, pending the development and implementation of monitoring strategies (20, 21). Because PERVs are found in the DNA of all pigs, they cannot be eliminated from all xenotransplants. In contrast, most other infectious diseases of pigs can be excluded by appropriate breeding and safety measures that produce designated or qualified pathogen-free colonies.

Meanwhile, there is evidence from a number of case studies that PERV infection in human recipients of xenotransplants is nonexistent, although most of the human recipients were exposed to porcine tissue for relatively brief periods (for a review, see reference 70). Nevertheless, any clinical trial involving a xenotransplantation product has to be addressed adequately regarding the safety concerns identified over the years.

Pigs as a source for Xenotransplantation

Despite the genetic differences and evolutionary distance between pigs and nonhuman primates, pigs show numerous advantages as donor animals (Table 1). It is still unclear whether the risk of infection is higher with the use of pigs than with the use of nonhuman primates. However, due to the genetic relatedness of receptors and cell metabolism, it seems much easier for microorganisms, including viruses, from a nonhuman primate to infect humans than for microorganisms, including viruses, from pigs. An illuminating example is HIV-1, which is the result of a transspecies transmission from closely related chimpanzees to humans, using the same viral receptors on immune cells (122).

Simple screening methods for porcine microorganisms should be developed, e.g., microarrays, which have been used already for the screening of other retroviruses (291).

Use of transgenic pigs to overcome immunological rejection

In the last few years, numerous transgenic pigs were generated with the goal of diminishing immunological rejection. First of all, HAR may be overcome by two different strategies, i.e., (i) gene knockout to remove the terminal carbohydrate epitopes Gal-alpha-1,3-Gal from the surfaces of pig cells by inactivation of the corresponding enzyme, Gal-alpha-1,3-galactosyltransferase, which adds the terminal Gal residue; and (ii) expression of human complement regulatory proteins on the surfaces of pig cells (for a review, see references 161 and 250) (Table 2).

The human complement regulatory proteins prevent complement-assisted destruction of the transplant. However, when retroviruses bud from the surfaces of cells from transgenic pigs expressing human complement regulator proteins, the viruses acquire numerous cellular proteins, including these human complement regulatory proteins. Expression of these proteins on the virus surface will also prevent the human immune system from eliminating the virus (235, 260, 316), increasing the risk of transmission.

Without the human complement regulator genes on the surface of PERV, anti-Gal-alpha-1,3-Gal antibodies prevent virus infection (207, 208). In addition to the complement regulators, genes involved in adaptive immune responses and coagulation, e.g., the HLA-E/human beta2-microglobulin (338), the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (160), the tumor necrosis factor alpha-

induced protein 3 (A20) (240), thrombomodulin (251), and human heme oxygenase-1 (hHO-1) (252), have also been expressed in transgenic pigs.

Infection barriers

Bacteria, Fungi, and Parasites

Lists of bacteria, fungi, and parasites able to be transmitted to human recipients and able to induce known and unknown zoonoses have been published (70, 109, 237, 286, 329), but the number and specification of the microorganisms which should be eliminated necessarily from the donor animal because of their putative pathogenic impact are still unclear and may also be dependent on the country or continent. In addition, to exclude these microorganisms from pigs generated as source animals for xenotransplantation, sensitive and specific detection methods are required.

Viruses

As in the case of bacteria and other microorganisms, viruses with zoonotic potential for human xenotransplant recipients can be eliminated only when the virus is known and when sensitive detection assays are available (Table 3). Most of the viruses listed (70, 286, 329) can be eliminated easily, but others can be eliminated only with some restrictions. For example, infectious circoviruses can be transmitted from the sow via the placenta and represent a special risk (106, 245, 330), because exclusion by Caesarean section is circumvented in this case. Viruses of special risk include herpesviruses, which are difficult to detect (40, 41, 330), paramyxoviruses such as Hendra virus (331), Nipah virus (43), Menangle virus (27), and Tioman virus (44), as well as PERVs (246), which are integrated into the pig genome (see below).

The exclusion of potential zoonotic pathogens from animal herds under qualified pathogen-free breeding has been discussed widely (308), and comprehensive guidelines on screening for infectious agents, including viruses, have been issued, e.g., by the FDA/U.S. Public Health Service (PHS) (102) and the WHO (233); however, these do not contain a comprehensive list of pathogens to be excluded from pigs used for xenotransplantation. Viruses with the potential to cross the species barrier, including encephalomyocarditis virus (EMCV), hepatitis E virus (HEV), porcine cytomegalovirus (PCMV), and porcine gammalymphotropic herpesvirus (PLHV), should be excluded from the herd (2, 41, 213, 214, 339).

Other viruses to be excluded are swine caliciviruses, including sapoviruses causing gastroenteritis in humans (363), swine torque virus (209), and Reston ebolavirus (13). Although the Reston virus—in contrast to the other filoviruses, Ebola virus and Marburg virus, both inducing fatal hemorrhagic fever—does not induce a disease in humans, its presence in pigs is of concern. All of these viruses must be included in the list of microorganisms that need to be tested for in donor pigs.

Of special interest is the question of whether human viruses can infect and harm the pig xenotransplant. Although the risk of infection of the xenotransplant by human viruses may be low because of missing receptors and an incompatible cell metabolism, some human viruses, such as human cytomegalovirus, human adenovirus, and hepatitis C virus, infect pigs (for a review, see references 215 and 216). It remains unclear whether infection with human viruses will harm the xenotransplant, whether porcine and human viruses may interact (276), and whether screening for human viruses before and after transplantation may be useful.

PERVs

Endogenous retroviruses in eukaryotic species are usually the result of a transspecies transmission of a retrovirus and its integration into the germ line of the new host (72). The origin of PERVs was most likely a murine retrovirus (185). PERVs are present in the genomes of all pigs (182, 246, 248), and some are able to infect human cells *in vitro* (61, 164, 246, 298, 341, 342), therefore representing a special risk for xenotransplantation. There are three types of PERVs, namely, PERV-A, PERV-B, and PERV-C. PERV-A and PERV-B are present in the genomes of all pig strains, at different copy

numbers, and PERV-C is integrated into the genome in many, but not all, pigs (Fig. 1). Whereas PERV-A and PERV-B are polytropic and able to infect human cells and cells of other species, PERV-C is ecotropic and restricted to pig cells. Transspecies transmissions of retroviruses have been shown for many retroviruses, among them HIV-1 and HIV-2, causing the AIDS pandemic, the most disastrous example of a transmission of a zoonotic retrovirus to humans (122). In addition, human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 are other examples of transmission of retroviruses from nonhuman primates to humans (335). Transmission of retroviruses closely related to PERV, such as the transmission of koala retrovirus (KoRV) from still unknown rodents to koalas, has also been described (69, 103, 319, 320). The transmitted retroviruses induce lymphomas (KoRV), leukemias and neurodegenerative diseases (HTLV), and/or immunodeficiencies (HIV, HTLV, and KoRV) in the new host. The severity of the immunodeficiency depends on the virus load of the virus in the infected host.

Biology of PERVs.

Replication-competent viruses encoded by the PERV sequences are morphological type C retroviruses and belong to the genus Gammaretrovirus of the family Retroviridae. Their genome is RNA, and the most important viral enzyme is the reverse transcriptase (RT), transcribing the viral RNA genome into proviral DNA. Viral particles have a diameter of about 100 nm (Fig. 2). They are assembled at and released from the cellular membrane in a budding process and consist of lipid and protein derived from host cells. The precursor molecule of all Gag proteins is cleaved by a viral protease into p27Gag or capsid protein (CA), which is the main structural protein; into p10Gag, the nucleic acid-associated protein; and into the membrane-associated protein MA. The *pol* gene codes for the RT, the integrase (IN), and protease (PR), enzymes required for the transcription of the viral RNA into DNA and for integration of the DNA copy into the cellular genome. The main structural protein is the CA protein p27Gag. It is smaller than the corresponding protein of murine leukemia virus (MuLV), p30Gag (313). The *env* gene codes for a precursor molecule which is cleaved by a cellular furin-like protease into the surface envelope protein gp70 and the transmembrane envelope protein p15E. PERV is closely related to feline leukemia virus (FeLV), MuLV, and the baboon endogenous retrovirus (BaEV). Antibodies against p27Gag of FeLV and p30Gag of MuLV cross-react with p27Gag of PERV and vice versa; however, an antiserum against p24Gag of HIV-1 does not—as expected—cross-react with p27Gag of PERV (313). Antibodies specific for the transmembrane envelope protein p15E of PERV react with p15E of FeLV and KoRV and vice versa (J. Denner et al., unpublished data). The life cycle of PERV begins with the infection of the target cell and ends with the release of progeny viruses (for details, see Table 4 and Fig. 3).

The release of PERV particles by normal pig cells, if any, is very low; the virus production by certain human cells infected with PERV-A and recombinant PERV-A/C (see below) is much higher and increases after adaptation to human cells (82, 128, 341). Using Northern blotting and RT-PCR methods, the expression of PERV-specific mRNA was studied in different pig tissues (3). The presence of a full-length 8-kb mRNA, coding for Gag and Pol, and of a spliced 3-kb mRNA, coding for Env, is the prerequisite for virus production and was seen in PERV-producing cells (154). Expression of both mRNA species was also seen in blood lymphocytes, the spleen, the thymus, lymph nodes, and lungs. Stimulation of blood lymphocytes with the mitogen phytohemagglutinin (PHA) increases the expression of PERV (313, 314, 341, 342).

The amount of released virus depends on the pig strain. Miniature pigs are characterized by very high expression, and German landrace pigs are characterized by a low expression profile (87, 313, 314). In the heart, only very low expression of PERV was observed (3; I. Bittmann, D. Mihica, R. Plesker, and J. Denner, unpublished data). High expression of PERV mRNA was found in melanomas of miniature pigs and was higher than that in normal skin (89). However, the expression in the spleens of these animals was much higher than that in the melanomas. Low expression of PERV mRNA and absence of particle release were observed in pig islet cells from German landrace pigs (144). These results are of clinical importance, since islet cells, hearts, and kidneys are first on the list of potential pig xenotransplantation products for humans.

Expression of mRNA was studied in certain specific-pathogen-free (SPF) pigs by use of a sensitive RT-PCR method (47). According to the data, PERV-A, PERV-B, and PERV-C were expressed in all tissues, with high expression in the kidney, followed by the liver, the lung, and the heart. A low level of expression was observed in the pancreas. The differences in expression were due to the transcriptional activities of the long terminal repeats (LTR) of PERV acting as a promoter, to the

different numbers of U3 repeat boxes in PERV LTR, and to the presence of transcription factors in the cells (82, 281). The expression of PERV-C was much lower than the expression of PERV-A and PERV-B. Comparison of the SPF pigs with conventional pigs indicated that PERV mRNA levels were in the same range, confirming that the use of SPF pigs would not reduce the risk of PERV transmission to human recipients of xenotransplants, as expected. Expression of PERV mRNA was detected in pig hearts, kidneys, and spleens by RT-PCR (246).

In situ hybridization studies of porcine neuronal cells with PERV-A- and PERV-B-specific *env* probes have shown high levels of expression of each variant. By RT-PCR and Northern blot analyses, expression of PERV mRNA was shown in the heart, lung, liver, kidney, ear, lymph node, and spleen, with the highest level of expression in the lung (59).

It is important that the methods used in these investigations do not discriminate between spliced and unspliced (full-length) mRNAs. Env primers used with this method will detect both types of mRNA. To solve this problem, a PCR was developed to discriminate between full-length and spliced mRNAs by using primers upstream of the splice donor and downstream of the splice acceptor (154). Only when spliced *env* mRNA is present is the Env protein produced, and virus particles may be released. Gag and Pol are translated from the unspliced mRNA. However, expression of mRNA, even spliced mRNA, does not automatically mean that virus proteins or particles are produced.

In the first immunofluorescence analyses using a PERV-specific antiserum against p10Gag, no viral proteins were observed in the kidneys, aortas, and hearts of landrace pigs (163). There is evidence that in addition to pig tumor cells and immortalized pig cell lines, several normal pig cells release PERVs and these viruses are able to infect human cells, e.g., PERVs were produced by mitogen-stimulated blood cells as measured by an RT assay and an infection assay (313, 314, 341, 342). Released and pelleted viruses were studied using RT-PCR and PERV-specific primers. It has been shown that all three viruses, PERV-A, PERV-B, and PERV-C, were released from stimulated blood cells in the case of a Yucatan miniature pig expressing high levels of PERV (314). Infection of human 293 kidney cells with blood cell-derived viruses was successful: the transmitted virus was a recombinant of PERV-A and PERV-C, with the receptor binding site and host range of PERV-A and the LTR of PERV-C (342). The release of virus particles from blood cells was transient, with a peak at day 5 (313). Differences in virus production in peripheral blood mononuclear cells (PBMCs) between different pigs and pig strains after mitogen stimulation were observed, allowing classification into high- and low-producer animals. In contrast to German landrace pigs, Yucatan miniature pigs belong to the high producers (87, 313). In Yucatan miniature pigs, expression of the PERV proteins p15E and p27Gag was shown in several organs by immunohistochemistry, with the highest expression in the spleen, kidneys, and lymph nodes (Bittmann et al., unpublished data) (Fig. 4). The expression at the protein level correlated with the expression at the mRNA level. In addition, in the organs with high expression, the *de novo* integration of recombinant PERV-A/C not present in the germ line was observed.

Since mitogen stimulation simulates natural immune responses and stimulation by antigens, the elevated expression of PERV in spleen and lymph nodes may indicate ongoing immune responses. Recently, significant expression of PERV was reported for commercial swine operations in the United States, and an increased incidence of PERV-A/C viremia was described for diseased pigs (242). Since these results are very interesting, they need to be confirmed in other laboratories. As outlined below, screening large numbers of animals by use of ultrasensitive PCR methods requires a clean PCR suite with optimal contamination management guided by good laboratory practice to avoid false-positive results.

Release of PERV-A and PERV-B from primary aorta endothelial cells (PAEC) was observed, and both viruses infected human 293 kidney cells. Virus production was shown for PAEC from German, Russian, and French landrace pigs as well as from Yucatan miniature pigs, Göttingen miniature pigs (196), and NIH miniature swine (261, 290, 347). Since endothelial cells, as part of all solid organ transplants, as well as passenger leukocytes in tissue will be transmitted with solid organs, these results underline the risk arising from the transplantation of vascularized pig organs. Of interest is the question of whether PERV may be released from nonvascularized cellular xenotransplants. Production of PERVs able to infect human kidney cells *in vitro* has been claimed for pig pancreatic islet cells xenotransplanted into SCID mice (332). However, we were not able to confirm these results (143). In addition, it was demonstrated that the reported infection of human cells in SCID mice was due to pseudotyping of PERV genomes with the murine xenotropic endogenous retrovirus MuLV-X and is an

artifact of the murine model (355). Although RT activity was found in serum taken from an hDAF transgenic pig, indicating the presence of retroviral particles, these viruses were noninfectious for human cells (59). However, it is also possible that the RT activity was derived from enzyme released from dead cells.

Recombinant PERVs

Recombinants between PERV-A and PERV-C naturally occurring in miniature swine have been described (14, 89, 198, 236, 343, 347) (Fig. 1). The acquisition of the receptor binding site of PERV-A enables the recombinant viruses to infect human cells. In comparison to that of parental PERV-A, the titer of recombinant PERV-A/C is much higher, and a critical substitution at position 140, localized between the variable regions A and B (VRA and VRB), and another substitution within the proline-rich region of the receptor binding domain were identified (128).

An additional increase in virus titer was observed when a recombinant PERV-A/C derived from pig PBMCs was serially passaged on uninfected human 293 kidney cells *in vitro* in order to simulate the situation during xenotransplantation (82, 342). This increase was due to an increase in the length of the LTR and a multimerization of the nuclear factor Y (NF-Y) transcription factor binding sites (82). Changes in the LTR length were also observed for PERV-A passaged on human 293 cells (82, 280). The changes were based on the multimerization of 37- or 39-bp repeats containing NF-Y binding sites. It has been shown by luciferase reporter assays that multimerization of NF-Y binding sites in the LTR functionally mediates increased transcription rates of PERV (82, 280, 281). Changes in the LTR have been reported for other gammaretroviruses, such as the feline and murine leukemia viruses, to be associated with increased tumorigenicity (11, 12, 83, 304). These data indicate the possibility that PERV can adapt to human cells and subsequently replicate with greater efficiency.

Two different PERV-A/C were found in miniature pigs with melanomas; however, these proviruses were integrated only in the spleen, not in the skin, melanomas, and lymph nodes of the animals (89). *De novo* integrated PERV-A/C not present in the germ line were also found in some organs of clinically healthy Yucatan miniature pigs characterized by extremely high expression of PERV-A, PERV-B, and PERV-C (Bittmann et al., unpublished data).

Although PERV-A/C were shown to replicate and integrate *de novo* in healthy (14, 236, 342, 347) and diseased (89) pigs, a first attempt to infect PERV-C-negative pigs *in vivo* with a cell-free high-titer PERV-A/C failed (158). Neither provirus integration in different organs nor antibody production was observed in the inoculated animal.

The risk of such recombinant PERV-A/C generated in pigs and integrated *de novo* into the genomes of somatic cells for xenotransplantation is obvious (71). However, this risk can easily be eliminated by using pigs not containing PERV-C in their germ line, thus preventing recombination with PERV-A. Another reason not to use PERV-C-containing pigs for breeding is based on the ability of gammaretroviruses, including PERV, to infect cells not harboring the specific receptor by receptor-independent infection (9, 177). In the presence of another retroviral surface envelope protein, e.g., from a human endogenous retrovirus (HERV), and its receptor, PERV-C may infect human cells despite these cells not expressing a PERV-C-specific receptor. A third reason not to use PERV-C-containing pigs is the possibility that mutations in the proline-rich domain of the Env protein of PERV-C may occur and change the tropism of the virus. Mutations in the C-terminal end of the surface envelope protein of PERV-C have been described which finally resulted in binding to and infection of human cells (119).

Distribution of PERVs

The prevalence and distribution of replication-competent PERV within different pig subspecies and transgenic animals used for xenotransplantation are of particular interest. This knowledge allows selection for animals with a low copy number of PERV proviruses. Genomic mapping studies have shown that there are between approximately 10 and 100 proviral PERV loci in the genomes of various pigs (3, 25, 135, 181). Most of these proviruses are defective, and some animals of a miniature swine herd do not release human-tropic PERV (236). In addition, some animals produce PERV-C, which can

recombine with PERV-A and generate a recombinant PERV-A/C (261). Unique full-length proviruses have been discovered by screening genomic libraries, and some of them were generated in bacterial artificial chromosomes (BACs) and were localized chromosomally (Table 5) (227, 228, 267, 268, 289, 290).

Three of these PERVs showed productive infection upon transfection into cells in culture, while one was disabled by two in-frame stop mutations in *pol* in an otherwise intact proviral context. Further analysis of these clones by performing PCR with primers derived from the flanking genomic sequences showed that all proviruses were polymorphic in different individuals and demonstrated slight alterations within their coding sequences. As a consequence, the individual proviruses were present in some pigs and absent in others, and one provirus present in a defined chromosomal position may have had mutations in individual pigs and in different subspecies. Most notably, the polymorphisms in another proviral PERV clone that was located in the swine leukocyte antigen region on pig chromosome 7 revealed an intact *pol* open reading frame (ORF) in some other Large White pigs (228).

Two clones derived from a library of the porcine cell line PK15 were also characterized (164), but they were not localized chromosomally. It is not clear whether these clones are different from the BAC clones or whether the genomic flanking sequences are different due to massive rearrangements during passaging of the cells over decades, as PK15 cells are malignant and transformed (95). For these six PERV clones, their distribution among individual pigs as well as different breeds, i.e., Large White pigs, miniature pigs, Westran pigs, and wild boars, was analyzed (Table 5).

In addition to the endogenous gammaretroviruses, endogenous betaretroviruses have also been detected in the genomes of all pigs (96). These viruses are present in the genomes of all pigs, but they are not well studied; however, it has been shown that they are expressed in some pig tissues (100).

PERV receptors

Interference studies using appropriate *env* sequences in pseudotyped viruses revealed that the different PERV classes use different receptors, thereby explaining the different cell and species tropisms observed in pseudotype assays (318). At the moment, only the receptors for PERV-A, HuPAR-1 and HuPAR-2 (human PERV-A receptors 1 and 2), have been identified (101). The natural function of the receptors is still unknown; however, since they contain 10 or 11 transmembrane domains, a function as a transporter similar to the receptors of many other gammaretroviruses (241) can be suggested. The baboon homologue was functional, whereas the mouse homologue was not able to support PERV infection due to a point mutation (Leu-109-Pro) in the second extracellular loop (204). This agrees with data showing that mouse cells could not be infected *in vitro* (297, 318, 343), and infection experiments using high-titer PERV *in vivo* were negative (143). Reports claiming infection of mice with PERV (48, 64, 332) were based on pseudotyping of PERV with murine endogenous retroviruses (199, 355). Mice transgenic for HuPAR-1 were generated, and it was reported that they could be infected with PERV *in vivo* (200).

Interestingly, in rats, the receptor is functional but normally expressed at a very low level (204) not allowing infection with PERV *in vitro* (81, 297, 343) and *in vivo* (81, 297). Transfection of rat cells with human and rat PAR-1 conferred PERV-A susceptibility (204).

Although cells from nonhuman primates, including chimpanzees, could be infected with high-titer PERV-A/C but not with low-titer PERV (22, 154, 266, 299, 301), virus replication was inefficient (203). In rhesus macaques, cynomolgus macaques, and baboons, the main receptor (PAR-1) was found to be genetically deficient by a mutation at the same position as that reported for mice (Leu-109-Ser), partially explaining the inefficiency of infection. The receptor in African green monkeys does not have this mutation, but nevertheless the replication is quite low (203).

HuPAR-2, the second receptor, also has two regions important for infection. In one region, a single amino acid residue determines binding; however, in both regions, multiple residues influence receptor function for PERV-A entry (193). Whether cellular entry cofactors play a role, as shown for other retroviruses (241), is still unclear. At the viral site in the surface envelope protein gp70, two domains, i.e., VRA and VRB, are necessary for infection, and in addition, sequences in the proline-rich region are also involved in receptor binding (9).

Methods to detect PERV infections

PERV and PERV infections may be detected directly by showing the presence of the provirus in the cells, the expression of viral mRNA or viral proteins, and production of infectious viruses. Indirect detection methods measure the immune response of the infected host as a result of productive infections (Table 6) (75, 130, 312).

In order to measure provirus integration, PCR and real-time PCR methods have been developed (8, 23, 159, 189, 310), as well as Southern blot hybridization methods (3, 61), using PERV-specific primers and probes, respectively. In addition, a melting assay for estimation of the copy number of PERV in pigs has been developed (349). Chromosomal localization of proviruses as well as expression of viral RNA was investigated using fluorescence *in situ* hybridization (FISH) (267, 268, 347). Viral RNA, either mRNA in the infected cell or genomic RNA in released virus particles, was measured by RT-PCR assays (312).

Virus protein expression was studied using specific antisera in an immunoperoxidase assay (IPA) (302), an immunofluorescence assay (61, 107, 163), and a Western blot assay (158, 202, 312) and by immunohistochemistry (Bittmann et al., unpublished data) (Fig. 4). Virus production was analyzed using an assay for RT activity, which measures virus particles containing RT, or by titration, measuring infectious viruses (297, 298, 313).
Virus production and morphology were studied by transmission electron microscopy (89, 192, 300) (Fig. 2) or by scanning electron microscopy (154, 299) (Fig. 2) to characterize the viral particles. Immunogold electron microscopy was used to study the localization of viral proteins in the virus particle, using specific antiviral immune sera against the transmembrane envelope protein and Gag (107, 258, 302).

Indirect methods measure either the humoral or cellular immune response of the infected host. Although no methods for detection of cellular responses against PERVs have been developed until now, numerous methods exist to measure the antibody response (115, 312). First, Western blot assays were performed using cross-reacting antisera against gibbon ape leukemia virus (GaLV) or FeLV as control sera and recombinant Gag protein or infected cells as antigen. Subsequently, PERV-specific antisera were developed (70, 312). Using these methods, sera from healthy blood donors, patients with clinical xenotransplantations, and butchers were examined (70, 244, 312). Butchers in large slaughterhouses were of special interest because of their close blood-blood contact with pigs.

For several retroviruses, including HIV-1, it has been shown that blood-blood contact is the most efficient route of transmission. None of the tested persons showed evidence of PERV infection. However, 4 xenotransplantation patients and 9 healthy controls (blood donors, butchers, and pregnant women) showed an antibody response to p27Gag (244, 312). Since there were no antibodies detected against other viral proteins, the antibodies against Gag do not indicate an infection but most likely represent cross-reactive antibodies. Such cross-reactive antibodies have been described for other retroviruses. For example, cross-reacting antibodies against p24Gag of HIV-1 (279) or against Gag of the human endogenous retrovirus HERV-K (80) have been detected in healthy individuals. Their origin, however, is still unknown. Therefore, for diagnostic purposes, the presence of antibodies specific for at least two viral proteins, one Gag and one Env, should be analyzed.

Monitoring future xenotransplant recipients could include the methods described above. The presence of PERV proviruses or PERV particles, as well as the expression of PERV in infected cells, can be analyzed using molecular biological methods. Immunological methods, including screening assays such as enzyme-linked immunosorbent assay (ELISA) and confirmatory assays such as Western blotting, can be used to search for PERV-specific antibodies (for a review, see reference 75). Since no PERV infections have been observed until now, specificity and detection limits cannot be evaluated, but experience with other retrovirus infections, including HIV-1 infections, clearly demonstrates that retrovirus replication in an infected host is accompanied by antibody production (75). Microchimerism, e.g., the presence of pig cells in transplanted individuals, even for more than 8 years, did not induce an antibody response against PERV (244).

Lessons should be learned from the false-positive reports of the detection of a murine virus, xenotropic murine leukemia virus-related virus (XMRV), in the human population, underscoring the need for accurate and specific testing. XMRV is a murine gammaretrovirus closely related to PERV that was reported to be present in human patients with prostate carcinoma and chronic fatigue syndrome (CFS)

and also in a substantial percentage of clinically healthy individuals (for a review, see reference 73). XMRV was found in patients with prostate carcinoma and CFS by several laboratories in the United States but not by European laboratories. There was no association between XMRV and both diseases, and there is clear evidence that this virus was a contamination with mouse DNA carrying endogenous retroviruses or DNA from human cells infected with XMRV or a direct contamination with XMRV (114, 138, 141, 153, 212, 243, 277, 294, 296). This clearly underscores the requirement for a clean environment for conducting PCR assays, with optimal contamination management according to good laboratory practice, whenever such sensitive PCR methods are used, and it underlines the requirement to use immunological methods, in addition to PCR methods, to measure antibody production in the transplant recipient for the detection of PERV infections.

Screening of the donor pig

Since most of the potential porcine zoonotic microorganisms will be eliminated by careful screening and qualified pathogen-free breeding (287), only the PERVs represent a risk. To select animals with low expression of PERV, sensitive and specific PCR, RT-PCR, and real-time PCR methods have been developed. Since pigs lacking PERV-C in the genome should be selected to prevent PERV-A/C recombinations (79), several methods have been developed to screen for PERV-C in pigs, including PCR, nested PCR, and real-time PCR (159).

Expression of all three PERVs could be measured by real-time PCR using specific primers, and animals with very low expression of PERV can be selected accordingly. A comprehensive characterization of the donor animals is of great importance and has been performed for some herds, e.g., for a designated pathogen-free herd in New Zealand (118). In addition to the herd and the individual animal, the final transplant (islet cells, heart, or kidney) should be investigated carefully. In addition to PCR and RT-PCR, detection of viral proteins and of the release of infectious particles is important for the risk evaluation (also see "Risk assessment").

PERVs and pig tumors

PERVs were first described for transformed pig kidney cell lines (10, 17, 26, 28, 185, 325, 348); later viruses were isolated from lymphoma cells (218, 274, 306, 307) and from radiation-induced leukemia cells (112). However, there is no evidence for an oncogenic property of PERV.

When expression of PERVs in melanomas of selectively bred Munich miniature swine (MMS) Troll pigs was studied, an increased expression in the tumors compared with normal skin was found (89). This breeding herd of MMS Troll is characterized by a high prevalence of melanomas, which histologically resemble various types of cutaneous melanomas in humans. Several genetic factors have been defined in studying inheritance of melanomas and melanocytic nevi in MMS Troll (336). Both the polytropic viruses PERV-A and PERV-B and the ecotropic virus PERV-C are present in the genomes of all melanoma-bearing MMS Troll pigs.

PERV expression was found in cell cultures derived from pulmonary tumor metastases (89). During passaging of these cell cultures *in vitro*, the expression of PERV mRNA and protein increased. RT activity was found in the supernatant, and release of virus particles was shown by electron microscopy. Although PERV expression was elevated in melanomas and melanoma-derived cell cultures, the function of the virus in tumor development is still unclear. At the same time, an increased expression of a human endogenous retrovirus, HERV-K, was found in human primary melanomas and melanoma cell lines (32, 33, 223). Interestingly, in the pigs suffering from a melanoma, PERV expression was much higher in the spleen than in melanoma tissue or other organs. These data correlate with the enhanced expression of PERV in mitogen-stimulated lymphocytes (313, 314) and suggest that immune stimulation in this organ may also activate PERV expression *in vivo* (86). In addition, in the spleen, but not in the tumor or in the skin, *de novo* integrated PERV-A/C were found (see above).

Immunosuppressive properties of PERV

Many viruses have developed strategies to suppress the immune system of the infected host. For example, herpesviruses carry genes in the genome encoding interleukin-10 (IL-10) and decoy cytokine

receptors preventing the action of the natural cytokine (264). The fact that retroviruses are immunosuppressive has been known since the 1960s (36; for a review, see references 65 and 238). Recently, KoRV, which is closely related to PERV, was found to spread among the koala population in Australia. KoRV is highly immunosuppressive, and KoRV infection is associated with fatal opportunistic infections, e.g., by chlamydia (320). By analyzing the mechanism of immunosuppression by retroviruses, it was shown that the transmembrane envelope protein (201) and a highly conserved so-called immunosuppressive domain (isu domain) in this protein are responsible for the immunosuppressive activity. Synthetic peptides corresponding to the isu domain inhibited mitogen-driven proliferation of PBMCs (45, 271). These peptides also modulated cytokine expression in normal PBMCs (125). It was later demonstrated that a peptide corresponding to the isu domain of HIV-1 behaved similarly (76,–,78, 272), suggesting that all retroviruses use their transmembrane envelope protein to inhibit the innate and adaptive immune systems after infection (67). Recently, it was shown that a mutation in the isu domain of a murine retrovirus closely related to PERV abrogated the ability of this virus to replicate in the immunocompetent host; however, it replicated well in immunocompromised mice (282). The virus inhibited the function of NK cells as well as CD8$^+$ cells, indicating that this retrovirus inhibited the innate and adaptive immunity in order to replicate in the infected host. In the case of PERV, it was shown that inactivated purified virus as well as the peptide corresponding to the isu domain of PERV inhibited proliferation of PBMCs and induced IL-10 production in human PBMCs (66, 313; Denner et al., unpublished data).

Absence of PERV transmission in preclinical trials and animal infection experiments.

In contrast to *in vitro* infection studies, animal models allow one to consider the recipient's innate and adaptive immune systems upon infection. They also allow the effects of pharmaceutical immunosuppression required in future clinical xenotransplantations to be studied. In contrast to future human trials, in which PERV infection can be studied primarily in blood cells that are always accessible, the use of animal models also allows for examination of a larger range of tissues and organs.

Despite the knowledge obtained from numerous *in vitro* infection studies that showed that in addition to human cells, a broad range of animal cells can be infected *in vitro* (Table 7), very few animal models have been studied until now (for a review, see reference 79). Three types of experiments were performed: (i) inoculation of virus, (ii) inoculation of virus-producing cells, and (iii) transplantation of living pig cells or organs. Inoculation of rats with PERV or PERV-producing cells (297) or pig islet cells (81) and inoculation of PERV into mink (300) or guinea pigs (297) did not result in infection. Interestingly, a transient infection was observed in guinea pigs (7). Reports on PERV infection of SCID mice (64, 332) and athymic mice (48) represented an artifact of the mouse model: PERV infection of murine cells was the result of pseudotyping with endogenous murine retroviruses (199, 355). These data closely correlate with the absence of a functional PERV receptor on mouse cells (101) and the absence of infection in other mouse models (143, 165).

Since only short-term survival and function of the xenotransplant have been achieved, no animals with long-term functioning xenotransplants can be studied. From this point of view, an unequivocal conclusion about PERV transmission might not be possible. On the other hand, the transplant may release viral DNA during a rejection process; however, this will not infect the recipient. Last but not least, the pig-to-nonhuman-primate transplant models to study cross-species PERV transmission have several limitations, including differences in viral receptors.

As expected, nonhuman primates are of major interest as an animal model for human disease due to the genetic relationship to humans. Immunosuppressed recipients of pig-to-baboon aortic endothelial cell transplants were found—based on PCR—not to be infected with PERV (197). Similar negative results, also obtained using PCR methods, were reported for immunosuppressed baboons and cynomolgus monkeys undergoing solid organ transplantation (187, 295) and for a triple-species xenotransplantation model (344). Numerous other pig-to-nonhuman-primate transplantations have been performed, and no transmission of PERV was observed (Table 8). In addition, in heterotopic thoracic pig-to-baboon cardiac xenotransplantation (15), no PERV transmission was observed using a Western blot assay to detect PERV-specific antibodies (Denner et al., unpublished data).

Negative results were also reported for various nonhuman primate species who received high doses of a PERV-A/C in combination with a triple immunosuppressive treatment (299, 301). In these

experiments, baboons, pig-tailed monkeys, and rhesus macaques were inoculated with viral supernatants from cells producing a PERV-A/C characterized by a long LTR and a high titer. The animals were treated daily with three different conventional xenobiotic immunosuppressive drugs at pharmacologically active dose levels, namely, the calcineurin inhibitor cyclosporine, the inhibitor of mTOR (mammalian target of rapamycin) everolimus (RAD), and methylprednisolone. After more than 300 days of observation, neither provirus integration in different organs nor antibody production was observed in these animals (301).

Whereas most of the preclinical xenotransplantation studies were retrospective investigations, in the first prospective pig-to-nonhuman-primate islet xenotransplantations, the animals were treated with encapsulated pig islet cells and analyzed for transmission of potentially zoonotic porcine viruses, including PERV, porcine cytomegalovirus (PCMV), porcine lymphotropic herpesvirus (PLHV), and porcine circovirus (PCV), using both molecular diagnostic—PCR and RT-PCR—and serologic methods. There was no evidence for transmission of any of these pig viruses into the primate recipients (116).

However, since there is a mutation in the PERV receptor in some nonhuman primates (see above), significantly reducing the efficiency of infection, the predictive value of this model system—at least concerning retrovirus safety—remains unclear.

Absence of PERV transmission in first clinical trials

At present, more than 200 individuals have undergone xenotransplantations of pig cells or tissues, including *ex vivo* perfusion of pig organs or pig cell-based bioreactors (Table 9) (for an extended review, see reference 79). No evidence for virus transmission was obtained; also, neither antibodies against PERV nor provirus integration in blood cells of the patients was observed. In one study, persistent microchimerism was observed in 23 patients, for up to 8.5 years after treatment, without PERV transmission (244). In very rare cases, antibodies binding to p27Gag, the main structural protein of PERV, were found in Western blot analyses (68, 237, 306). Since no antibodies against other viral proteins were detected, an infection was excluded and cross-reactive antibodies were considered the reason for the Gag reactivity (see above).

In another study, 24 patients received porcine fetal neuronal cells for treatment of Parkinson's disease, Huntington's disease, and epilepsy. No evidence of PERV provirus integration in the DNA from PBMCs of all neuronal transplant recipients was found (92, 105, 284). No tests for antibodies against PERV were performed. In addition, no PERV particles were released from cultured fetal porcine neuronal cultures, and there was no transfer of PERV from fetal pig neuronal cells to human cells *in vitro*.

Among the numerous pig cell xenotransplantations and *ex vivo* perfusions using pig organs that have been performed worldwide, bioartificial liver support systems using pig hepatocytes in bioreactors have been studied several times (1, 90, 91, 113, 145, 166, 221, 278, 351). In all systems, no transmission of PERVs was detected.

Transmission of PERV was also studied in patients who received unheated porcine clotting factor VIII (132, 317). Porcine factor VIII has been used to treat severe bleeding episodes in patients with hemophilia who have antibodies against human clotting factors. Viral RNA and RT activity were found in the preparations. These findings may indicate either the presence of viral particles in the preparation or the presence of viral mRNA and RT molecules released from disrupted cells. If there were virus particles, it remains unclear (but seems to be unlikely) whether these particles were still infectious. No antibodies against PERV were found in 88 recipients, suggesting that the risk of PERV transmission was extremely low.

There was no evidence of infection with PERV or other porcine microorganisms in recipients of pig islet transplants for the treatment of diabetes (98, 117, 324).

Some studies were performed without analysis of PERV transmission, e.g., in extracorporeal connection of pig kidneys to dialysis patients (29). In addition, at least two pig organs (heart and liver) were transplanted and rejected, as expected, 24 and 34 h afterwards (60, 190).

Retrotransposition of PERVs

Since it is difficult to exclude all PERV sequences from the pig genome, the question is whether truncated PERVs pose an additional risk in terms of retrotransposition, as was shown for another gammaretrovirus and close relative of PERV, i.e., Moloney murine leukemia virus (MoMuLV) (129, 321). The frequencies for retrotransposition were up to 1.2×10^{-5} and 8×10^{-5} per cell division for PERV and MoMuLV, respectively. The variation in frequency between these two gammaretroviruses is probably a result of different RT and promoter activities (152). Quantitative RT-PCR analyses based on vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped PERV and MoMuLV particles revealed the same amount of viral RNA copies per transfection.

However, C-type RT assay analyses based on the corresponding supernatants revealed an almost 10-fold higher RT activity in the case of MoMuLV. In contrast, reporter gene assays where the luciferase gene was regulated by either the PERV or MoMuLV LTR revealed a 3 to 4 times higher promoter activity for PERV-B (33) in HeLa cells. Taken together, these results correlate closely with the difference in retrotransposition frequency between these two retroviruses. The approximately 10-fold stronger RT activity of MoMuLV is counteracted by a 4-fold higher promoter activity of PERV, leading to a retrotransposition frequency that is up to 7-fold higher for MoMuLV (152).
The frequency of MoMuLV transposition correlates with published retrotransposition values (321, 322). Electron microscopy to identify extracellular particles, as was done for MoMuLV (321, 322), was not performed because immunofluorescence using anti-PERV p10Gag antisera (163) showed no positive staining. In addition, no RT activity was detectable in the supernatant of cells transfected with the PERV provirus used (R. R. Tönjes et al., unpublished data).

Cellular restriction factors interfering with PERV. Viruses depend strongly on a number of factors provided by their host cell due to their limited genetic coding capacity (337). On the other hand, the permissiveness of a host cell is determined by the presence or absence of restriction factors that evolved during host-virus coevolution (120, 121, 126, 127, 345). In the last few years, several restriction factors were characterized as being of particular importance for the replication of retroviruses: TRIM5α, which disrupts the viral capsid (CA) after cell entry; TRIM28, which blocks viral transcription; ZAP (zinc finger antiviral protein), which directs degradation of viral RNAs; tetherin, which traps virions on the surfaces of infected cells; and APOBEC (apolipoprotein B mRNA-editing catalytic polypeptides), which are cytidine deaminases that disrupt viral DNA during synthesis (Table 4 and Fig. 5) (120, 121, 211, 345). Antiviral restriction systems are being studied with the prospect of developing therapeutic agents to regulate the expression of these factors and to enhance antiviral activities.

PERV-A and PERV-A/C are insensitive to restriction by TRIM5α molecules in permissive feline Crandall-Reese feline kidney (CRFK) cells expressing TRIM5α proteins from humans, African green monkeys, rhesus macaques, squirrel monkeys, rabbits, or cattle (346). However, overexpression of either human or porcine tetherin in pig cells significantly reduced PERV production (205).

The mammalian *APOBEC3* (*A3*) genes are part of the *AID/APOBEC* gene family, including *AID*, *APOBEC1* (*A1*), *APOBEC2* (*A2*), and *APOBEC4* (*A4*). The family members share structural and functional domains of zinc-dependent deaminases (reviewed in reference 49). APOBEC proteins deaminate cytidine residues in single-stranded RNA and DNA molecules that are recognized by currently unknown mechanisms. Proteins of the A3 group contain one or two zinc (Z)-coordinating domains and can be classified according to the presence/absence of a Z1, Z2, or Z3 motif (176, 222). In humans, the *A3* locus contains seven *A3* genes; in rodents, there is one, in pigs two, in horses six, and in cats four *A3* genes (24, 147, 175, 222, 234, 365), suggesting an amplification during evolution. HIV-1 mutants lacking the *vif* gene can package APOBEC3G (A3G) into virus particles. Incorporated A3G specifically deaminates cytidine residues to uracil in growing single-stranded DNA during reverse transcription, leading to HIV genome degradation and/or hypermutation (19, 126, 178, 191, 195, 362). More recent studies indicated that deaminase-independent mechanisms might also be involved in the antiviral activity of A3 (18, 139, 140, 146, 206, 224).

The mechanism for how retroviruses counteract or escape the A3s from their own host species is important for understanding virus tropism and host-virus coevolution (270). In the case of HIV-1, the amount of cellular A3G in cells infected with wild-type HIV-1 is dramatically reduced by a Vif-dependent degradation process via the ubiquitination-proteasome pathway (195, 292, 359, 360). In contrast to the well-characterized A3-Vif interaction, little is known about A3-neutralizing strategies

used by retroviruses that do not encode a Vif protein.

Gammaretroviruses such as MuLV or FeLV and PERV appear not to express accessory proteins with a function similar to that of the lentiviral Vif protein or the foamy viral protein Bet (186, 222, 249, 273, 293), both of which inhibit the incorporation of A3 proteins into virions. Despite many studies, the debate on the mechanism of resistance of MuLVs to murine A3 (muA3) has not come up with a generally convincing model (19, 30, 148, 162, 174, 194). However, recent data clearly showed that muA3 is an important *in vivo* restriction factor of the Friend virus complex and the MoMuLV (188, 275, 315) that was used as an internal control. Initial studies showed that porcine A3Z2-Z3 (poA3Z2-Z3) strongly inhibits HIV-1 and weakly restricts MuLV (149).

Furthermore, it was reported that overexpressed poA3Z2-Z3 did not significantly interfere with PERV transmission, and it was concluded that PERV was resistant to its species-specific A3 protein (150). Subsequently, the chromosomal porcine *A3* locus for *poA3Z2* and *poA3Z3* was reanalyzed (93, 94). Data showed that pigs express four different *A3* mRNAs, encoding poA3Z2 and poA3Z3 and, by readthrough transcription and alternative splicing, poA3Z2-Z3 and poA3Z2-Z3 splice variant A (SVA). It was found that PERV was significantly inhibited by various porcine A3s in single-round as well as spreading virus assays. PERV inhibition strongly correlated with a specific cytidine deamination in viral genomes for the trinucleotides 5′TGC (edited nucleotide is underlined), for poA3Z2 as well as poA3Z2-Z3, and 5′CAC, for A3Z3 (93, 94, 179). These results strongly implicate that human and porcine A3s can inhibit PERV replication *in vivo*, thereby reducing the risk of potential infection of human cells by PERV in the course of pig-to-human xenotransplantation.

Risk assessment

PERV does not give any indications of pathology in either its natural host *in vivo*, infected cells of either animal or human lineage *in vitro*, or recipients of PERV inoculations or preclinical or clinical xenotransplantation products *in vivo*. To date, there has been no indication of infections in small animals or nonhuman primates, and hence the effect of PERV *in vivo* cannot be determined. In general, as a retrovirus, PERV might be able to transform infected cells by insertional mutagenesis, as demonstrated extensively for MuLV (184). In addition, immunosuppressive properties of PERV have been shown (66, 313). However, another important but least assessable risk is posed by the possible recombination of PERV with endogenous or exogenous human pathogens, giving rise to absolutely new and unexpected viruses (303). Putative recombination with HERVs does not pose a serious risk due to the absence of infectious sequences, as in the case of HERV-K (326, 327), or the presence of heavily deleted gammaretrovirus-like sequences, as in the case of HERV-R (5). There is no evidence for such recombinations and for copacking of sequences from PERV and HERV (305; Denner et al., unpublished data).

PERV has the capacity to pseudotype MuLV particles (318). Other gammaretroviruses have not been investigated for this effect, as these do not pose a risk to humans (53). However, risks could evolve for xenotransplant patients from gene therapy trials based on MuLV vectors and xenotransplanted pig cells.

To assess the risks of infection and recombination, it is essential to know the exact number and location of all replication-competent PERVs. Part of this information was obtained for some pig strains (61, 135, 226, 228). However, the elimination of replication-competent PERV proviruses would reduce only the release of infectious particles and the probability of recombination between PERV-A and PERV-C. It would reduce the risk of malignant transformation and immunosuppression only partially, since transposition of deleted proviruses can still induce insertional mutagenesis and the immunosuppressive transmembrane protein can be expressed in the absence of other viral genes. In addition, non-replication-competent PERVs can recombine or complement each other to produce infectious particles.

Although it is well known that retroviruses induce tumors, leukemias, and/or immunodeficiencies, it is difficult to evaluate the risk posed by PERVs. The absence of an *in vivo* model of PERV infection in small animals or nonhuman primates requires insight into the pathology induced by related retroviruses. MuLV, FeLV, and KoRV are closely related to PERV. All three gammaretroviruses induce leukemias/lymphomas and/or immunodeficiencies. Similar to the situation with HIV-1 infection, opportunistic infections develop on the basis of the immunodeficiency. Sixty percent of FeLV-infected

cats die from opportunistic infections, whereas only 5 to 10% develop leukemias/lymphomas. Many koalas infected with KoRV suffer from chlamydial infection (319), an opportunistic infection on the background of a severe immunodeficiency that is also common in cats infected with feline immunodeficiency virus (FIV) (232).

Inbred miniature swine actually harbor full-length retroviruses (108). However, some animals of the strains characterized by the haplotype d/d obviously produced only virions of the ecotropic class PERV-C and none of the human-tropic class PERV-A or PERV-B (226, 227, 236). Although ecotropic PERV-C infects only pig cells, not human cells, PERV-C might recombine with PERV-A, thus transferring genes or parts of them to the human-tropic viruses and therefore posing some risk in the course of xenotransplantation.

Risk assessment is based on studies of the number of PERV copies in the genome and on studies of the expression of these proviruses at the levels of RNA and protein. The detection of spliced RNA encoding Env is important (154), but most important is the ability to release infectious virus particles which may be measured in infection assays. As we demonstrated, mitogen stimulation of immune cells increases the release of virus (313, 314). In addition, it should be considered that recombination and complementation may lead to virus release from cells previously unable to release virus.

In conclusion, the virological barriers to xenotransplantation are not as serious as initially perceived (110). The number of human-tropic replication-competent proviruses is limited, and the selection of PERV-C-free pigs seems possible, reducing the risk of recombination. In addition, recombination events with human endogenous retroviruses appear unlikely. As shown below, numerous strategies have been developed, including the use of antiretroviral agents, vaccines based on neutralizing antibodies, and transgenic pigs with downregulated PERV expression (by RNA interference). Since xenotransplantation recipients must be monitored closely for transplant rejection on a routine basis, it is easily possible to monitor patients simultaneously for viral infections.

Unknown Microorganisms

At present, it is possible that a still unknown microorganism is transmitted from pigs to human xenotransplant recipients or from other animals via pigs to humans. Nevertheless, the probability is extremely low, assuming that such transmission would have been observed much earlier due to the close contact between pigs and humans over a long time. Thanks to improvements in virus diagnostics, new, previously unknown viruses have been described for pigs, but most of them are nonpathogenic. Some examples are new paramyxoviruses (27), tiomanviruses (44), and kobuviruses (354, 358). It is almost impossible to develop systematic strategies to detect such unknown viruses and other microorganisms, e.g., by coculture of human and pig cells and screening for cytotoxic effects.

General Strategies to overcome Infection Barriers

Selection of PERV-C-Free Low-Producer Animals

At present, the main tool to prevent transmission of porcine microorganisms, including PERV, is the selection of the donor animals. As shown above, this includes the absence of PERV-C proviruses and low expression of PERV-A and PERV-C. Virus expression can be measured using different methods. RT-PCR and real-time RT-PCR allow measurement of expression at the RNA level, and viral proteins can be analyzed by immunofluorescence, immunohistochemistry, or Western blot analyses of pig tissues. To discriminate between pigs with low and high expression of PERV, a special assay was developed based on the measurement of PERV expression after mitogen stimulation of PBMCs (313, 314). In addition, since recombinants between PERV-A and PERV-C have been described which are characterized by high replication titers (for a review, see reference 71), pigs lacking PERV-C in their genome should be selected to prevent such recombinations. For this purpose, new methods have been developed to screen for PERV-C in pigs, including PCR, nested PCR, and real-time PCR (159).

Vaccination against PERVs

Vaccination of human recipients may be another strategy to prevent PERV transmission, hopefully only a theoretical one. Although successful vaccination against HIV-1 and HIV-2 failed (for a review, see reference 74), effective vaccines exist against gammaretroviruses such as MuLV and FeLV (137, 180, 328). The scientific basis of the difference in efficacy is still unknown. When goats, rats, and mice were immunized with the transmembrane envelope protein p15E of PERV, binding and neutralizing antibodies were induced. These antibodies recognized two epitopes, one in the fusion peptide-proximal region (FPPR) of the transmembrane envelope protein and the other in the membrane-proximal external region (MPER) (104, 157). Interestingly, the epitopes in the MPER of the transmembrane envelope protein of PERV were localized at the same position as the epitopes of antibodies broadly neutralizing HIV-1, originally isolated from HIV-1-infected individuals.

Since PERV does not infect small animals and nonhuman primates, the efficacy of neutralizing antibodies cannot be studied in an animal model. Therefore, infections with related gammaretroviruses, e.g., infection of cats with FeLV, have been used. Immunizing rats and goats with the transmembrane envelope protein of FeLV resulted in binding and neutralizing antibodies recognizing similar epitopes to those after immunization with the transmembrane envelope protein of PERV (171). Similar neutralizing antibodies were also induced in cats (173), and after challenging these animals with infectious FeLV, 50% of the animals were protected from antigenemia (172). Immunization with both the transmembrane and surface envelope proteins resulted in greater immune responses (170) and 100% protection (S. Langhammer and J. Denner, unpublished data).

Transgenic Pigs with PERV Expression Inhibited by RNA Interference

RNA interference is a technology inhibiting expression of genes by the action of gene-specific small interfering RNAs (siRNAs). Efficient siRNAs corresponding to highly conserved regions in the *pol* gene of the PERV genome were selected and were able to inhibit expression of all PERV subtypes in PERV-infected human cells as well as in primary pig cells (155). In addition, a lentiviral vector expressing a corresponding short hairpin RNA (shRNA) was used; transfecting pig fibroblasts with this construct also resulted in downregulation of PERV expression (86). Using this construct and a control construct not containing the PERV-specific shRNA, transgenic pigs were produced by somatic nuclear transfer cloning (88).

The integrated transgene was found integrated in the piglets, the shRNA was found expressed in all tissues investigated, and PERV expression was significantly inhibited compared with that in control animals (88). Similar results were obtained by others (263). Whereas expression of the shRNA was studied previously by an in-solution Northern hybridization analysis (86), meanwhile a real-time RT-PCR was developed to measure expression of the small interfering RNA (M. Semaan, D. Kaulitz, and J. Denner, unpublished data). Although differences in the level of PERV expression were observed in various tissues, as measured by duplex real-time RT-PCR, the expression of PERV was reduced in all organs compared with the expression of PERV in animals carrying a control vector or in comparison to

nontransgenic pigs over a period of more than 3 years (86; M. Semaan, D. Kaulitz, B. Petersen, W. A. Kues, H. Niemann, and J. Denner, unpublished data). At present, the effects of multiple PERV-specific shRNAs on PERV expression are under investigation. This strategy may lead to animals compatible with a safer donor status (less expression of PERV RNA and protein and lower probability of releasing infectious virus particles) of a xenotransplantation product concerning PERV transmission.

Antiretrovirals and PERV Knockout AnimalsSeveral antiretroviral drugs which inhibit replication of HIV-1 and other retroviruses have been tested against PERV, and the RT inhibitors zidovudine (AZT; also known as azidothymidine) and dideoxyinosine (ddI) inhibit PERV replication (257, 259, 302). However, RT inhibitors are expected to induce resistant virus strains shortly after the beginning of treatment, as shown for HIV-1 in treated individuals.

All of the strategies listed here are at the investigational level, and their impact on the virus safety of xenotransplantation is still unclear. At present, the generation of PERV knockout animals appears to be unlikely due to the large number of proviruses, but novel technologies such as the application of zinc finger nucleases for the generation of genetically modified animals may generate pigs without expression of functional PERV.

Outlook

The efficacy and safety of xenotransplantation will be shown—according to the proverb "the proof of the pudding is in the eating"—in the first clinical trials. The ongoing study of pig islet transplantation to diabetic individuals in New Zealand, the first clinical study officially approved by a governmental regulatory authority, in 2009, will be one of the first steps on the way to the clinical application of xenotransplantation (O. Garkavenko, S. Wynyard, D. Nathu, J. Denner, and R. Elliott, submitted for publication).

This way starts with pig islet transplantation because pig-derived insulin has been used for decades for the treatment of diabetes, because in the case of failure the insulin treatment can be continued, and because pig islet transplantation has been shown to have the best efficacy thus far in a pig-to-nonhuman-primate model (287). In analyzing the costs of the treatment of diabetes nowadays, the costs for insulin form a very low contribution; most expenses are generated by secondary health complications such as amputation, heart and kidney diseases, and blindness.

A total of $174 billion was the total cost for the treatment of diagnosed diabetes in the United States in 2007, including $116 billion for direct medical costs and $58 billion for indirect costs (disability, work loss, and premature mortality). A total of 25.8 million children and adults in the United States, or 8.3% of the population, have diabetes. The number of people around the world suffering from diabetes increased from 153 million in the year 1980 to 347 million in the year 2008 (63), and it will affect 380 million people in 2025 (136). A health-economic evaluation suggested that porcine islet cell xenotransplantation may prove to be a cost-effective and possibly cost-saving procedure for type 1 diabetes compared to standard management (16). Pig heart and kidney transplantation may follow, using the experience obtained during the first islet cell trials. Immunological, microbiological, and physiological hurdles may be overcome step by step.

Acknowledgments

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Tables and Figures

Table1 Advantages and disadvantages of different animal sources for xenotransplantation

Parameter	Description or value for pigs (compared to humans)	Advantage to use of pigs	Description or value for nonhuman primates (compared to humans)	
			Apes	Monkeys
Physiology	Similar	Unclear	Nearly identical	Similar
Transplant rejection	Very strong	No, only after genetic modification	Not very strong	Strong
Gal-alpha-1,3-Gal[a]	Yes	No, but irrelevant after genetic modification	No	No
Endangered species	No	Yes	Yes	Yes
Size of organs	Similar	Unclear	Similar	Small
Posture	Horizontal	Unclear	Upright	Upright
Time of gestation (days)	100	Yes	251-289	170-193
No. of progeny	6–10	Yes	1, rarely 2	1, rarely 2
Availability	Unlimited	Yes	None	Low
Costs	Low	Yes	Very high	High
SPF or DPF containment[b]	Possible	Yes	Possible in future at very high costs	Possible in future at high costs
Transgenic to prevent rejection	Available	Yes	None	None
Cloning	Possible	Yes	Possible in far future	Possible in future

[a] Galactosyl-alpha-1,3-galactosyl epitopes, the main reason for hyperacute rejection (HAR).
[b] SPF, specific pathogen free; DPF, designated pathogen free.

Table 2 Genetically modified animals generated for xenotransplantation

Gene knockout pigs to prevent immunological rejection		
Galactose-alpha-1,3-galactosyltransferase knockout (GalT-KO)	Reduced Gal-alpha1,3-Gal (Gal) expression, reduced hyperacute rejection	62, 168, 254
Transgenic pigs to prevent immunological rejection		
hCD46 (human membrane cofactor [hMCP])	Human complement regulation	85, 169, 229, 269
hCD46 + GalT-KO	Human complement regulation plus reduced Gal expression	124
hCD55 (human decay accelerating factor [hDAF])	Human complement regulation	58, 269
hCD55 + endo-beta-galactosidase C	Human complement regulation plus reduced Gal expression	357
hCD59	Human complement regulation	84, 111, 230
hCD55 + hCD59	Human complement regulation	34, 37, 38
hCD46 + hCD55 + hCD59	Human complement regulation	56, 57, 364
H-transferase (alpha1,2-fucosyltransferase)	Reduced Gal expression	54
hCD59 + H-transferase (alpha1,2-fucosyltransferase)	Human complement regulation plus reduced Gal expression	55
hCTLA4-Ig (cytotoxic T lymphocyte-associated antigen)	Inhibits T cell activity	253
hTM (human thrombomodulin)	Activates human anticoagulant protein C	251
hA20 (human A20; tumor necrosis factor alpha-inducible gene)	Controls acute vascular rejection	240
HLA-E (human leukocyte antigen E) + human beta2-microglobulin	Protection from NK cell-mediated cytotoxicity	338
TRAIL (tumor necrosis factor alpha-related apoptosis-inducing ligand)	Reduced posthyperacute cellular rejection	160
GnT-III (beta-D-mannoside beta-1,4-N-acetylglucosaminyltransferase III)	Reduced antigenicity to human natural antibodies	217
hHO-1 (human heme oxygenase 1)	Antiapoptosis	252
Fas ligand (FasL)	Antiapoptosis	42
Transgenic pigs to prevent PERV transmission		
PERV shRNA-treated pigs	Reduced PERV expression	88, 263
Natural pig mutants		
Von Willebrand factor-deficient pigs	Inhibited platelet activation	35
Other transgenic animal models to study prevention of immunological rejection		
Membrane-tethered anticoagulants in mice	Reduced acute humoral rejection	39

Table 3 Viruses representing a risk for xenotransplantation

Virus group and abbreviation	Virus name	Disease in pigs	Transmission to humans	Reference(s)
Herpesviruses				
PLHV-1	Porcine lymphotropic herpesvirus 1	Lymphoproliferative disease	Unknown	96, 109, 220
PLHV-2-3	Porcine lymphotropic herpesvirus 2-3	Unknown	Unknown	41
PCMV	Porcine cytomegalovirus		Unknown	344
Circoviruses				
PCV-1	Circovirus 1	None	Unknown	106
PCV-2	Circovirus 2	PWMS[a]	Unknown	106
Other viruses				
A/H1N1 and other HXNX	Influenza virus	Flu	+	210
PHEV	Porcine hepatitis E virus	Asymptomatic	+	213, 214, 288, 350
Paramyxoviruses	Nipah virus	Respiratory and neurologic syndrome (barking pig syndrome)	Febrile encephalitic and respiratory illnesses	43
	Menangle virus	Reproductive disease		27
	Hendra virus		Febrile encephalitic and respiratory illnesses	331
	Tioman virus	?	?	352
Caliciviruses	Sapoviruses	?	Gastroenteritis	363
Filoviruses	Reston ebolavirus	?	Unknown	14

[a] Postweaning multisystemic wasting syndrome.

Table 4 Life cycle of PERV and cellular restriction factors *a*

Step	Part of the life cycle	Interference of cellular restriction factor
1	Binding of the surface envelope glycoprotein gp70 to the cellular receptor	
2	Changes in the conformation of gp70 and the transmembrane envelope protein p15E, fusion of viral and cellular membranes, receptor-mediated endocytosis, entering of the host cell	
3	Viral RNA enters the cytoplasm as a nucleoprotein complex	TRIM5α acts at the stage of uncoating of the viral core
4	Reverse transcription generates a linear DNA duplex, viral DNA is transported into the nucleus and integrated into host cell chromosomal DNA	APOBEC interacts when retroviral reverse transcriptase copies single-stranded viral RNA into double-stranded DNA
5	Production of viral mRNA and genomic RNA	TRIM28 blocks viral transcription of integrated viral DNA, generating mRNA molecules and new viral genomic RNA molecules
6	Newly synthesized viral proteins and genomic RNA gather to form immature viral particles	ZAP degrades viral RNAs
7	Transport of viral capsid and envelope proteins to the cell membrane, formation of capsids, incorporation of viral RNA, assembly, and budding	
8	Maturation of particles, proteolytic cleavage of precursor proteins, condensation of the capsid	Tetherin blocks release of virions budding on the cell surface

Table 5 Distribution of PERV-A and PERV-B proviruses[a]

Pig chromosome	Location of virus (type, chromosomal location)					
	Large White pig[b,c]	Westran pig[c,d]	Korean pig[e]	Wild boar[c]	Yucatan minipig[c]	d/d minipig[c]
1	A, 1q2.1	A, 1q1.2		A, 1q2.3	A, 1q2.3	
	A, 1q2.3	B, 1p2.2		A, 1q2.4	A, 1q2.4	
	A, 1q2.4					
2	B, 2q2.1	A, 2p1.4	B, 2q2.1 (2q2.2)	ND	ND	ND
3	**A, 3p1.5**	A, 3p1.4		A, 3p1.5		
4	B, 4p1.1			ND	ND	ND
5		A, 5p1.2		ND	ND	ND
		A, 5p1.3				
		A, 5q1.2				
		A, 5q2.1				
		B, 5q1.2				
6		A, 6p1.4		ND	ND	ND
		A, 6q3.5				
7	B, 7p1.1	A, 7p1.3	A, 7p1.3	ND	ND	ND
	B, 7p1.2	B, 7p1.2	B, 7p1.1			
		A, 7q1.5				
8	A, 8p1.2	B, 8p2.2		ND	ND	ND
9	B, 9q2.6	A, 9q2.4	B, 9q2.6	ND	ND	ND
		B, 9q2.4				
10	B, 10p1.2			ND	ND	ND
11	B, 11q1.2	B, 11p1.3	B, 11q1.1 (11q1.2)	ND	ND	ND
	B, 11q1.4					
12		A, 12p1.3		ND	ND	ND
		B, 12q1.1				
13	A, 13q4.2	A, 13q4.1	B, 13q4.2 (13q4.3)	ND	ND	ND
	A, 13q4.3	B, 13q4.1				
	A, 13q4.9					
	B, 13q4.2					
14	B, 14q2.8	B, 14q1.3		ND	ND	ND
15				ND	ND	ND
16		A, 16q2.1		ND	ND	ND
		B, 16q2.1				
17	B, 17q1.2	A, 17q1.4	A, 17q1.2	ND	ND	ND
		B, 17q1.2	B, 17q1.2			
18				ND	ND	ND
X		A, Xp2.1		ND	ND	ND
		B, Xp1.3				
Y	A, Yp1.2	A, Yp1.1		ND	ND	ND
		B, Yq				

[a] The presence of chromosomally assigned PERVs was tested in six different, geographically separate subspecies of *Sus scrofa*. d/d minipigs (column 7) are genetically related to Yucatan minipigs (column 6) but exhibit a defined swine leukocyte antigen haplotype and have been described as nontransmitters of PERV. Westran pigs (column 3) are bred in Australia. The locations of functional proviruses are indicated in bold. ND, not determined.
[b] Data are from the work of Rogel-Gaillard et al. (267, 268).
[c] Data are from the work of Niebert et al. (227).
[d] Data are from the work of Lee et al. (181).
[e] Data are from the work of Jung et al. (151).

Table 6 Sensitive and specific direct and indirect methods to detect PERV*a*

Target	Method(s)	Publication(s) describing or using method(s)
Targets for direct methods		
DNA (virus integration, provirus)	PCR, real-time PCR	8, 23, 159, 189, 310
	Southern blotting	3, 61
	Melting assay	349
	FISH	267, 268, 347
mRNA, viral RNA (genomic RNA, virus expression at the RNA level)	RT-PCR, real-time RT-PCR	312
Viral proteins (virus expression at the protein level)	Immunofluorescence assay	61, 107, 157, 163
	Immunoperoxidase assay (IPA)	302
	Western blot analysis	157, 158, 202, 312
	Immunohistochemistry	Bittmann et al., submitted
	Immunogold electron microscopy	107, 258, 302
Viral particles	Reverse transcriptase assay	297, 298, 313
	Transmission electron microscopy	89, 192, 300
	Scanning electron microscopy	154, 299
Infectious virus	Infection assay	297, 298, 313
Indirect methods		
Detection of antibodies (immune response)	ELISA, Western blot analyses using purified virus, recombinant proteins, or synthetic peptides	70, 75, 115, 130, 244, 312

a See the text for details and further references.

Table 7 Attempts to infect cultured cells of different species with PERV

Type of infection	Species	Reference(s)
Productive infection with replication	Some human cells (e.g., 293 cells), cat	246, 298, 341
Productive infection with absence of replication	Some human cells (e.g., primary PBMCs), mink, nonhuman primates—rhesus monkey (*Macaca mulatta*), baboon (*Papio cynocephalus*), baboon (*Papio hamadryas*), gorilla (*Gorilla gorilla*), and chimpanzee (*Pan troglodytes*)	22, 154, 246, 299, 298, 301, 341
Absence of infection	Mouse	142, 297, 299, 342
	Rat	81, 297, 341
	Rabbit	341
	Cotton rat	297
	Horse	297
	Nonhuman primates—pig-tailed monkey (*Macaca nemestrinas*), African green monkey (*Chlorocebus sabaeus*), and cynomolgus monkey (*Macaca fascicularis*)	22, 299

TABLE 8 Pig-to-nonhuman-primate xenotransplantations [a,b]

Recipient animals (*n*)	No. of animals in study	Xenotransplant	Immunosuppression	Reference
Baboons	15	PAEC (1×10^7) given intravenously; one animal also received a pig heart	CyP at 15-45 mg/kg of body wt	197
Baboons (6), bonnet macaques (6), STZ-treated rhesus macaques (9), STZ-treated capuchins (2)	23	Heart (baboons), skin (bonnet macaques), encapsulated islets (rhesus macaques and capuchins)	CsA, MMF, and Ster for some baboons, FTBI, CsA, and Ster for some macaques	309
Cynomolgus monkeys with human venous patch	6	huCD59-transgenic porcine kidney	CyP, CsA, MPA Ster, C1inh	343
Cynomolgus monkeys	12	Porcine kidney (graftectomy on days 2 to 15)	Cyp, CsA, Ster until day 28	186
STZ-treated cynomolgus monkeys	12	Encapsulated islet	None	116
Baboons	27	hDAF-transgenic heart (14) or hDAF-transgenic kidney (13)	GAS914, CyP, CsA, mycophenolate	219
Baboons	6	hDAF-transgenic liver perfusion		231

[a] Adapted from reference 79 with permission of Wiley-Blackwell.
[b] Abbreviations: C1inh, complement C1 inhibitor; CsA, cyclosporine; CyP, cyclophosphamide; EC, endothelial cells; FTBI, fractionated total body irradiation; hDAF, human decayaccelerating factor; MPA, mycophenolic acid; PAEC, primary aortic endothelial cells; Ster, steroids; STZ, streptozocin (induced diabetes); GAS914, a soluble, polymeric form of a Gal-alpha(1,3)-Gal trisaccharide.

Table 9 Clinical data on xenotransplantations[a,b]

Study group or disease condition	Pig product	Treatment	Test material	Time after Tx	No. of individuals	Reference(s)
Butchers			Serum		44	312
Pig farmers after liver Tx			PBMCs, plasma		14	134
Abattoir workers			PBMCs, plasma		49	134
Hemophilia	HyateC (unheated porcine factor VIII)	Exposure to 1–6 lots in 1992–1996	PBMCs		88	132
Acute liver failure	Bioartificial liver device, 90–100 g wet wt	None	PBMCs	6 h pre- and post-Tx	6	166
	Cryopreserved hepatocytes	Liver Tx after procedure	PBMCs	3 mo to 5 yr posttreatment	28	256
	AMC-BAL		PBMCs, plasma	Before and 0 days to 2 yr after Tx	14	90, 91
	Plasma perfusion through bioreactor followed by liver Tx plus immunosuppression	Exposure for 8–46 h	Plasma	Up to 2-5 yr	8	278
Fulminant hepatic failure	Extracorporeal liver perfusion, transgenic liver	Exposure for 6–10 h	PBMCs		2	183
Chronic glomerulonephritis, renal dialysis	Extracorporeal kidney perfusion		PBMCs	6 h to 36 mo	2	247
Neurological conditions (Parkinson's disease, Huntington's disease, focal epilepsy)	Fetal pig mesencephalon, lateral ganglionic eminence cells	CsA ($n = 11$), graft pretreated with Abs	PBMCs	2-24 mo post-Tx	11 with Parkinson's disease, 12 with Huntington's disease, 1 with focal epilepsy	92
Diabetes	Encapsulated islets	No IS ($n = 1$) or CsA, AZA, and pred ($n = 1$) for kidney Tx		19 mo	2	97
	Porcine fetal islets (4×10^8–2×10^9 cells), porcine c-peptide for up to 450 days post-Tx ($n = 4$)	CsA, pred, and AZA for maintenance	PBMCs, serum for RT-PCR	32–86 mo post-Tx; mitochondrial DNA was detectable for 3 days to 1 yr ($n = 1$) or 3 days ($n = 6$); all had Abs within 1 wk post-Tx	10	131
Acute liver failure	Cryopreserved hepatocytes	Exposure for 2–30 h	PBMCs, serum	Idem	28	244
	Extracorporeal liver perfusion	Exposure for 4.25 h, IS after subsequent liver Tx	Idem	Idem	28	244
Burns	Skin	Exposure for 10 days		36-150 mo	15	131
Chronic glomerulonephritis, renal dialysis	Extracorporeal kidney perfusion	Exposure for 35 or 65 min		32-36 mo	2	2
Various indications	Extracorporeal splenic perfusion	Exposure for 50–60 min		0-102 mo	100	244
Diabetes	Islets			19-93 mo	14	

Figure 1 Structure of proviral PERV. (A) Genes and open reading frames are shown as open boxes. cap, transcriptional start site; PBS, primer binding site; SD, splice donor; SA, splice acceptor; SU/TM, surface/transmembrane envelope protein cleavage site in Env; PPT, polypurine tract, poly(A) addition site; LTR, long terminal repeat; *gag*, group-specific antigen gene; *ppro/pol*, protease/polymerase gene; *env*, envelope protein gene. (B) Schematic presentation of the subtypes of PERV and the recombination events and increase in the length of the LTR during passaging on human cells. Boxes in the LTR indicate sequence repeats.
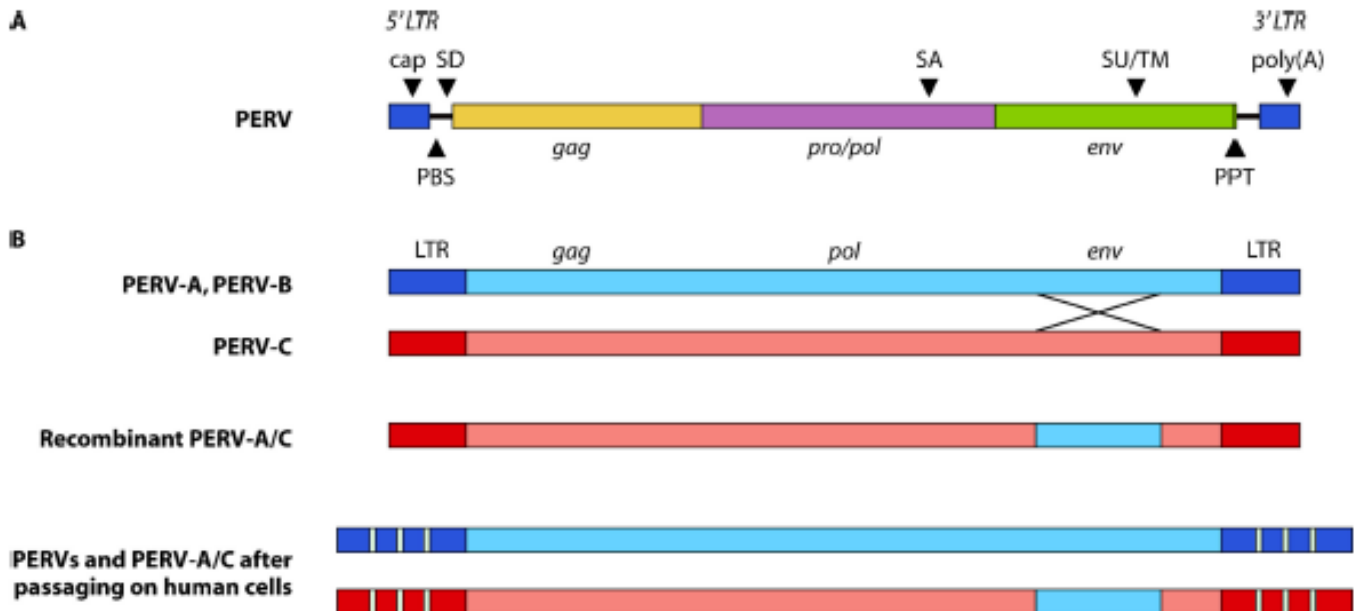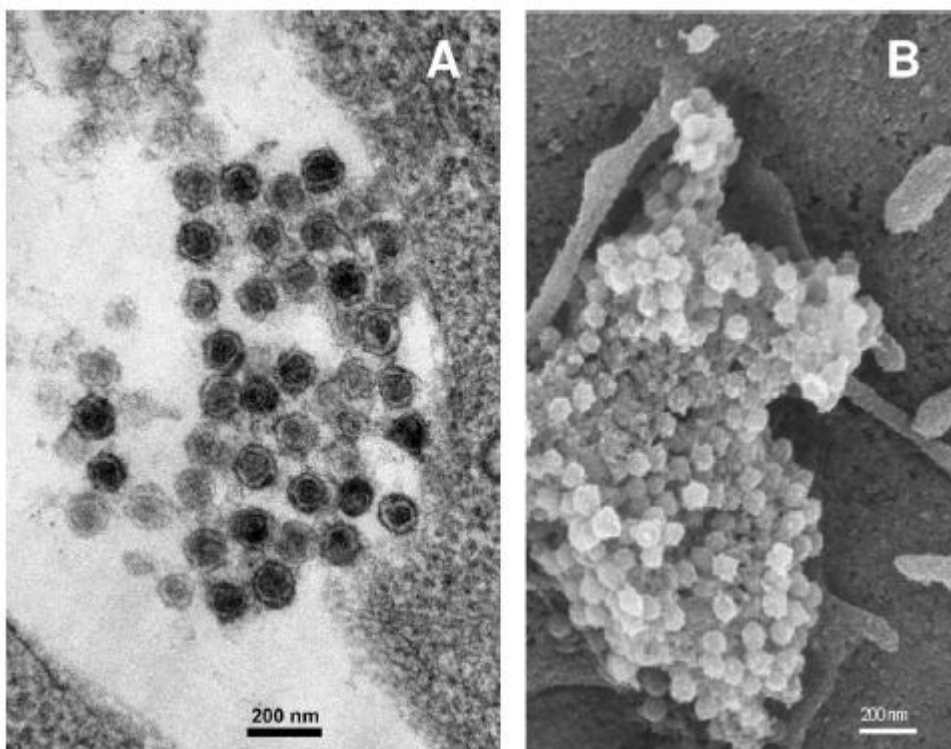

Figure 2 PERVs produced by infected human cells as shown by transmission (A) and scanning (B) electron microscopy. (Courtesy of Klaus Boller, Paul Ehrlich Institute, Langen, Germany.)

[a] See Fig. 3 and 5 and the text for references.

Figure 3 Schematic presentation of the life cycle of PERVs.
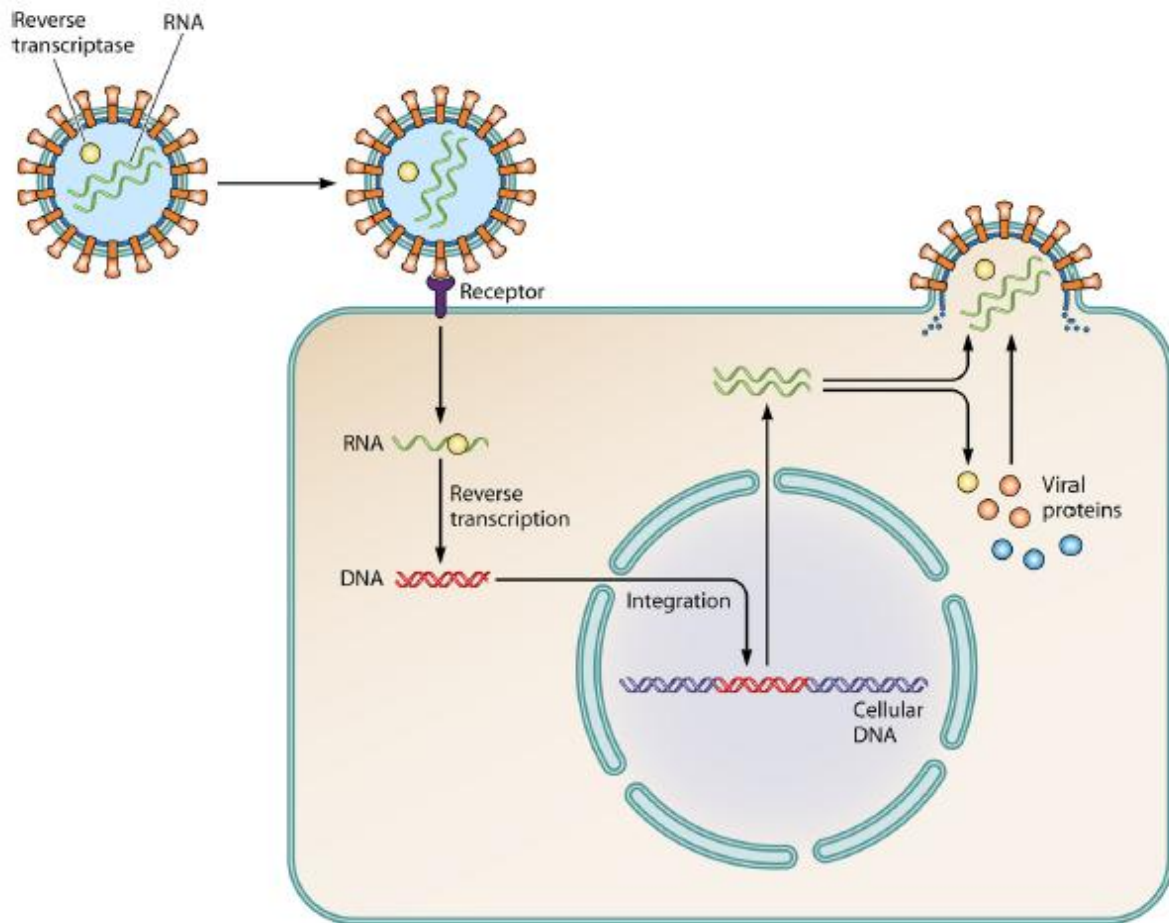For further details, see the text and Table 4.

Figure 4 Immunohistochemical evidence for the expression of PERV in the white pulp of the spleen of a Yucatan miniature swine. The tissue was stained with an antibody specific for the transmembrane envelope protein of PERV.
The immunohistochemical reaction was visualized using the avidin-biotin complex (ABC) method, using aminoethylcarbazole (AEC) as the chromogen. (Courtesy of Joachim Denner and Iris Bittmann, Institute of Pathology, Rotenburg,Germany.)
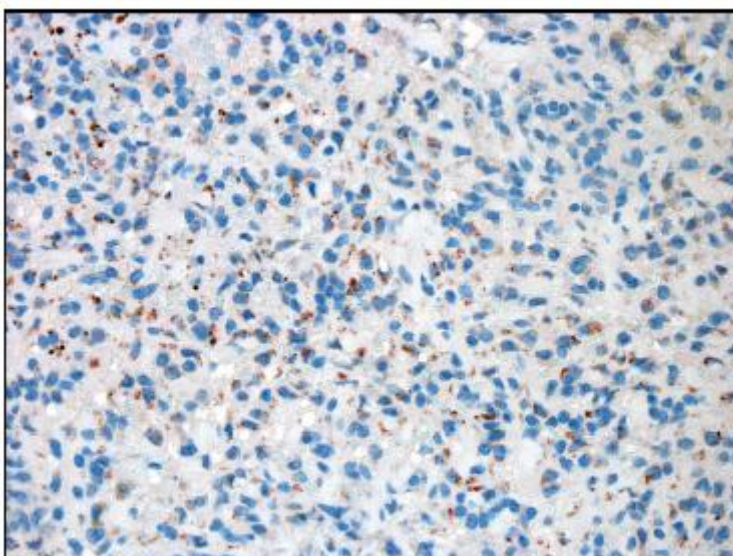
Figure 5 Interference of different cellular restriction factors with replication of PERVs. For further details, see the text and Table 4.