Xenotransplantation: Reduction of the risk of transmission of porcine endogenous retroviruses (PERV)

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Abstract

Xenotransplantation using porcine cells, tissues or organs may reduce the widening gap between demand and supply of human donor organs. However, this may be hampered by the presence of porcine endogenous retroviruses (PERVs), which belong to the γ-retroviruses family and are integrated in the porcine genome and were shown to infect human *cells in vitro.* Three subtypes of PERVs were identified, PERV-A and PERV-B are human tropic and pose a direct risk and are ubiquitous while PERV-C infect only pig cells and is not present in all pig strains. To date, there are no records of *in vivo* transmission of PERVs to human or primates following porcine material xenotransplantation. Nevertheless, many of the γ-retroviruses are pathogenic and able to induce tumors and immunodeficiency, which can't be excluded for PERVs. While breeding animals under SPF conditions may eliminate most of pathogens and exogenous viruses, PERVs are difficult to eradicate from the porcine genome and different strategies must be used to prevent their possible transspecies transmission to human.

The present study is a contribution to the evaluation of the risks imposed by xenotransplantation as well as to the prevention of PERV transmission to human.

Although PERV-C is not human-tropic, the high infectious PERV-A/C which results from the recombination between PERV-A and PERV-C raised a major concern for xenotransplantation. For this reason choosing PERV-C-free strains with low PERV expression is relevant. The first part of this work addresses the prevalence and the expression of PERV in Göttingen minipigs, which are used for numerous biomedical investigations and are well characterized. PERV-A, -B and -C were found in all animals tested and their expression was low. Infection of human 293 cells was not observed even after mitogen treatment of the pig peripheral blood mononuclear cells (PBMCs).

RNA interference was used in order to reduce the expression of PERVs in pigs and transgenic pigs expressing PERV-*pol*-specific shRNA were generated in our group. In the second part of this study the long term effects of PERV specific RNA interference and reduction of PERV expression in these pigs was investigated. Over 3 years, the expression of shRNA was persistent and PERV expression was consistently reduced in the pig PBMCs. We also investigated the expression of PERV and shRNA in different organs of the pigs. Moreover, new triple-shRNA expressing vectors were produced using high efficient siRNAs. The use of these vectors showed a significant improvement in reducing the PERV expression *in vitro*.

The third part focuses on the use of zinc finger nucleases (ZFN) to knockout PERV genes. ZFN specific for the PERV-*pol* gene were used, and expression of ZFN proteins in porcine kidney cells was demonstrated by different methods. However, the high rate of ZFN expression and the high copy number of PERV proviruses seemed to have cytotoxic effects. ZFN activity couldn't be detected since a surveyor nuclease assay widely used for detection of ZFN induced gene disruption couldn't be applied in this case.

In the last part vaccine strategies to prevent PERV transmission were investigated. Neutralizing antibodies directed against the recombinant viral envelope proteins gp70 and p15E were produced in hamsters. The neutralization effect of the immunized sera was measured using a qRT-PCR based neutralization assay.

To summarize, this work contributed into improving the reduction of PERV expression by RNAi, and addressed for the first time the possibility of the use of ZFNs to knock out a gene with high copy number such as PERV.

Keywords: Xenotransplantation, PERV, prevalence, RNAi, zinc finger nuclease, neutralizing antibodies.

Zusammenfassung

Die Xenotransplantation mit porzinen Zellen, Gewebe oder Organe könnte die immer größer werdende Lücke zwischen Nachfrage und Angebot der menschlichen Spenderorgane reduzieren. Dies kann jedoch durch die Anwesenheit von porzinen endogenen Retroviren (PERVs) erschwert werden. PERVs gehören zu den γ-Retroviren und sind im Genom aller Schweine integriert und können menschliche Zellen *in vitro* infizieren. Drei Subtypen des PERVs wurden identifiziert, PERV-A und PERV-B sind ubiquitär und besitzen die Fähigkeit humane Zellen *in vitro* zu infizieren. Daher stellen sie ein unmittelbar Risiko für die Xenotransplantation dar. PERV-C infizieren nur Schweinezellen und sind nicht in allen Schweinestämmen vorhanden. Bis dato, wurde kein Beweis über eine *in-vivo*-Übertragung von PERVs auf Menschen oder nichthumanen Primaten nach Xenotransplantation von Schweinmaterial erbracht. Dennoch, viele der γ-Retroviren sind pathogen und können Tumore und Immundefizienzen verursachen und dies kann auch für PERVs nicht ausgeschlossen werden. Mit der Aufzucht der Schweinen unter DPF (designated pathogen free) Bedingungen kann es gelingen, die meisten Krankheitserreger und Viren zu eliminieren, was im Fall von PERVs nicht möglich ist, da PERV Proviren bis 100-mal im porzinen Genom integriert sind. Daher müssen andere Strategien verwendet werden, um eine möglich Übertragung auf den Menschen zu verhindern.

Die vorliegende Studie ist ein Beitrag zur Bewertung der PERV-Risiken durch die Xenotransplantation als auch zur Vorbeugung von PERV Übertragung auf Menschen.

Auch wenn PERV-C nicht humantrop ist, stellt das aus der Rekombination zwischen PERV-A und PERV-C stammende hoch infektiöse PERV-A/C eine besondere Gefahr für die Xenotransplantation dar. Aus diesem Grund sollten ausschließlich PERV C-freie Tiere mit niedrigen PERV Expression benutzt werden. Der erste Teil dieser Arbeit befasst sich mit der Prävalenz und der Expression von PERVs in den gut charakterizierten und für zahlreiche biomedizinische Untersuchungen verwendeten Göttingen Minipigs. PERV-A, -B und -C Proviren wurden bei allen getesteten Tieren gefunden, wobei ihre Expression relative niedrig war. Selbst nach Mitogenstimulierung der Schweine-PBMCs konnte keine Infektion menschlichen 293 Zellen beobachtet werden.

Basierend auf der RNA-Interferenz wurden in unserer Arbeitsgruppe transgene Schweine generiert, die PERV-pol spezifische shRNA exprimieren und eine reduzierte PERV

Expression zeigten. Im zweiten Teil dieser Studie wurde die langfristige Wirkung der PERV spezifischen RNA-Interferenz untersucht und eine Dauerhafte Reduktion der PERV Expression beobachtet. Über 3 Jahre war die Expression von shRNA persistent und die PERV Expression war permanent reduziert in den PBMCs. Die Expression von PERV und shRNA wurde ebenfalls in verschiedenen Organen der Schweine untersucht. Darüber hinaus wurden neue dreifach-shRNA Vektoren mit hoch effizienten siRNAs entwickelt. Die Verwendung dieser Vektoren zeigte eine signifikante Verbesserung bei der Verringerung der PERV Expression *in vitro.*

Der dritte Teil konzentrierte sich auf den Einsatz von Zink Finger Nukleasen (ZFN) zum Knockout von PERV Genen. PERV-pol spezifische ZFN wurden hergestellt und verwendet, und die Expression von ZFN Proteine in der porzine Nierenzelllinie PK15 wurde mittels verschiedenen Methoden nachgewiesen. Allerdings schienen die hohe ZFN Expression und die hohe Anzahl der Kopien der PERV Proviren zytotoxische Effekte zu haben. Außerdem konnte die ZFN Aktivität nicht bewiesen werden, da der Surveyor Nuklease Assay, der oft für die Detektierung von ZFN induzierten Genmutationen verwendet wird, hier nicht angewendet werden konnte.

Im letzten Teil wurden Impfstoffstrategien zur Verhinderung der PERV Übertragung untersucht. Neutralisierende Antikörper, die sich gegen die rekombinanten viralen Hüllproteine gp70 und p15E richten, wurden in Hamstern generiert. Der neutralisierende Effekt der immunisierten Sera wurde mit einem qRT-PCR-basierten Neutralisation Assay gemessen.

Zussamenfassend kann gesagt werden, dass diese Arbeit trug zur Verbesserung der RNAi-basierte Reduzierung der PERV Expression beitrug. Im Rahmen dieser Arbeit wurden zum ersten Mal ZFN verwendet, um ein Gen mit so hoher Kopienanzahl wie PERV zu mutieren.

Schlagwörter: Xenotransplantation, PERV, Prävalenz, Göttingen Minipigs, RNAi, Zins Finger Nuclease, neutralizierende Antikörper.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
Ab	Antibody
Aa	Aminoacid
BfR	*Bundesinstitut für Risikobewertung*
bp	Base pair
CBP	Calmodulin-binding Peptide
CFP	Cyan Fluorescent Protein
Ct	Threshold cycle
CTL	Cytotoxic T-lymphocyte
cyp	Cyclophilin
DPF	Designated pathogen free
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope protein
ERV	Endogenous Retrovirus
FFPR	Fusion peptide proximal region
FLI	*Friedrich-Löffler-Institut*
Gag	Group specific antigen
Gal	Alpha-Gal-Glycosylation
GaLV	*Gibbon-Affen-Leukämievirus*
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
HAR	Hyperacute Rejection
HERV	Humanes endogenous Retrovirus
IFU	Infectious unit
IMF	Immunofluorescence
IN	Integrase
IXA	International Xenotransplantation Association
KoRV	Koala-Retrovirus
LTR	Long Terminal Repeat
MPER	Membrane proximal external region
MuLV	Murine Leukemiavirus
NT	Neutralisation
ORF	Open reading frame
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PERV	Porcine endogenous Retrovirus
Pol	Polymerase
PRR	Prolin rich Region
qRT-PCR	Quantitative Real-time PCR
RKI	Robert Koch-Institut
RNAi	RNA Interference
rpm	Rounds per minute
RT	Reverse Transkriptase
SA	Splice acceptor
SCNT	Somatic cell nuclear transfer
SD	Splice donor
shRNA	Short hairpin RNA
siRNA	Short interfering RNA

SNA	Surveyor Nuclease Assay
SU	Surface unit
TAH	Total Artificial Heart
TM	Transmembrane envelope protein
UV	Ultraviolet light
VAD	Left ventricular assist device
WB	Western Blot
XT	Xenotransplantation
YFP	Yellow Fluorescent Protein
ZFN	Zinc Finger Nuclease

1 Introduction

1.1 Transplantation

Allotransplantation or the transfer of cells, tissues or organs between individuals of the same species presents the optimal solution for patients suffering from organ failure. Since the first successful transplantation of kidney in the fifties [1], this replacement therapy has become an established standard medical discipline throughout the world. However the discrepancy between the worldwide increasing number of patients on the waiting lists and the low human donor potential pose a problem and alternatives for allotransplantation are under intensive investigation such as use of artificial organs, stem cell studies and the use of other species as sources for transplants or xenotransplantation.

1.1.1 Allotransplantation

The history of transplantation is tightly bound to the understanding of the immune system and the immunosuppression. The first allograft kidney transplantation attempt in 1933 failed because of hyperacute rejection of the allograft [2]. Later in the fifties the work group of Dr. Joseph Murray successfully succeeded to transfer a kidney allograft between identical twins avoiding this way an immune response [1]. This was followed by a series of successful transplantations of liver [3], heart [4], bone marrow [5] during the 1960's when control of immune system became feasible. A better understanding of the function of the human immune system in the last decades, the improvement of immunosuppression technologies, introduction of biological in addition to chemical immunosuppression and the discovery of new immunosuppressants such as cyclosporine [6] as well as the establishment of a worldwide network for cooperation permitted a long period of suppression of the immune system and thus prolonged the survival time of allografts in recipients.

Since the start of the transplantation, the number of implants grew up progressively and a total of 6277 organs have been transplanted in Germany alone during the period of January-October 2013 (Eurotransplant International Foundation 2013). These replacement operations concerned mainly kidneys as well as liver, heart, lung and pancreas coming from deceased and living donors (German Organ Transplantation Foundation, DSO). However the number of registered patients on active waiting list in Germany came to 10754 in the end of November

2013, which exceeds far the available transplants and results in death of about 1000 persons every year because of leak of suitable donor organs (Eurotransplant 2006).

1.1.2 Alternatives to allotransplantation

Alternatives aiming to shrink the expanding gap between demand and supply of transplants are extensively under investigation and some of them are already in clinical use. Among these substitutes are the artificial implants devices emerged as promising substitute in case of failure of natural organs. The most popular example is the Ventricular Assist Device (VAD) which serves as substitute for heart. VADs are mechanical blood pumps used usually in case of weakened hearts as support [7]. Usually they present a short-time bridge till heart recovery or during the waiting time for a heart transplant and they helped decreasing mortality to about 50% [8]. In contrary, Total Artificial Hearts (TAH) are devices that replace totally both failing heart ventricles and the valves. These devices are commercially available from different manufacturers like the SynCardia TAH (SynCardia, Tucson, USA) or the AbioCor™ (ABIOMED®, Inc.; Danvers, Mass). Artificial devices which replace other organs like kidney or liver are still not possible because of the complexity of these organs. However high complication rates limiting this therapy were reported, like bleeding [9], neurological events [10] and sepsis [11].

Other opportunities present the regenerative medicine and tissue engineering that aim to generate human tissues and organs from induced pluripotent stem cells (iPSCs) which can be isolated from the patient thus avoiding the immune rejection. These cells can be cultured under different conditions and forced to differentiate into specific cell lineages and tissues and can be used in immunohematologic diseases, neuronal destruction and heart failure [12]. For example generation of autologous chondrocyte implantations (ACI) which present a promising technology for cartilage regeneration [13] or the use of pluripotent mesenchymal stem cells (MSCs) for the improvement of cardiac functions in rat models of dilated cardiomyopathy [14]. Recently studies reported the generation of the first functional three-dimensional vascularized liver generated from human iPSCs [15]. Beside the technical hurdles that hamper the progress in stem cells studies, clinical application of stem cells raises safety concerns such as the tumorigenic potential of undifferentiated iPSCs [16], the risk of contamination by xenogeneic pathogens originated from the *in vitro* culture preparations of the stem cells [12], the immunological rejection of the cells and at last the risk of reactivation

of oncogenes such as c-Myc [17]. Therefore it would be beneficial to investigate other alternatives such as xenotransplantation.

1.1.3 Xenotransplantation

Xenotransplantation is by definition the transfer of living cells, tissues or organs from one species to another. Such transplants are named **xenotransplants** or **xenografts**. Xenotransplantation includes also perfusion of foreign cells by blood of the host. We talk about **concordant xenotransplantation** when transfer occurs between closely related species like hamster-rat, mouse-rat, and rhesus monkey-baboon pairs. In contrary **discordant xenotransplantation** is used for transplantations between unrelated species.

First trials using whole organs xenotransplants

Already at the beginning of the 20th century first unfortunate trials were performed to transfer kidneys from rabbit, rhesus monkey, cheep and chimpanzee to human [18]. The main cause of failure was the hyperacute immune rejection of the xenotransplants. First success of xenotransplantation occurred some 50 years later at the time when the mechanisms of immune response and immune rejection became clearer. A kidney transplant from chimpanzee survived for 9 months in human patient who was treated with strong immunosuppressants [19]. Despite the higher organization of the following trials of transferring sheep's and pig's hearts, baboon's and pig's livers [18] and baboon's heart [20] to human, no satisfactory results were recorded. The last two xenotransplantations of whole organs occurred in Poland and India where hearts of pigs were transferred to human with dismal results [21].

Use of cells and tissues

Unlike whole organs xenotransplants, the use of cells or tissues seems to be less complicated and promising and in the last years many clinical attempts to use cells from pigs were successful. For example embryonic porcine mesencephalic tissues were transplanted in patients with idiopathic Parkinson's disease or Huntington's disease. Porcine cells were tolerated without serious adverse events and patients showed clinical improvement [22, 23]. Furthermore, porcine islets of Langerhans producing insulin were also used in type 1 diabetic patients. Together with porcine Sertolli cells, the porcine islets of Langerhans were protected from the human immune system by using subcutaneous autologous collage-generating devices and thus immunosuppression was not required. These capsules protect the cells and

avoid the diffusion of porcine cells or microorganism in the recipient, but allow transition of insulin, nutrient and gazes [24, 25]. The company Living Cell Technology (LCT, New Zealand) is pioneering this technology. The encapsulated porcine cells are produced under the name DIABECELL® and are currently in a late-stage clinical trial.

Further application of porcine cells and tissues is the extracorporeal perfusion used as urgent help for bridging patient with liver failure and which are waiting for human liver transplants. These bioreactors are charged with genetic modified humanized porcine hepatic cells (hCD55/hCd59) [26, 27].

Use of non-human primates: pros and cons

Non-human primates (NHPs: chimpanzee, gorilla, baboon) are phylogenetically the closest to human and show anatomical, physiological and immunological similarities and thus present the obvious choice as donor animals for xenotransplantation. Despite the fact that the only successful organ transplantation was a primat-to-human transplantation of kidney [19], they were ruled out from xenotransplantation for several reasons: (i) Ethical approaches were raised because of their superior intelligence degree and the physiological and behavioral features similar to humans and which implies that NHPs can suffer like humans [28, 29]. (ii) Most of NHPs species are threatened with extinction and have long gestation periods and low number of offspring. (iii) Concerns were also raised regarding the safety of the process since most of NHPs harbor several pathogens which may be infectious for human considering the similarity of the immune systems among the primates. The most striking example presents the evolutionary studies that suggested a cross-species transmission of the simian immunodeficiency virus (SIV) from chimpanzee (SIVcpz) and sooty mangabey (SIVsm) which generated the human immunodeficiency virus types 1 and 2 (HIV-1 and -2) [30]. Furthermore, baboons harbor several exogenous and endogenous retroviruses such as simian foamy viruses (SFV), simian T-cell lyphotropic virus (STLV), baboon endogenous virus (BaEV) and simian endogenous retrovirus (SERV) which may be transmitted to humans [31].

Pigs and xenotransplantation

For several reasons, pigs (sus scrofa) were chosen as adequate substitutes.

(i) The ethical concerns are less sharp than in case of NHPs since millions of pigs are slaughtered annually for human consumption and the idea of use of pigs for transplantation is more accepted in the public [32].

(ii) Pigs' organs present physiological and anatomical similarities with human and come in different sizes [33-35].

(iii) Pigs have a short reproduction cycle of about 115 days, a short period to reach the reproductive maturity (4-8 months), produce high number of large sized litters (5-12) and most of their organs reach the maximum size suitable for human body within only 6 months [36].

(iv) They can be easily bred with low costs and most importantly in controlled, hygienic environments the so called designated pathogen free conditions (DPF).

(v) Pigs are suitable for genetic engineering and this was shown by several studies which succeeded to generate pig clones from genetically modified porcine cells by somatic cell nuclear transfer (SCNT) [37, 38].

However several hurdles remain to be overcome before porcine organs can be used for xenotransplantation such as the physiological incompatibilities, the immunological concerns and the risk of xenose by transmission of porcine endogenous retroviruses (PERVs) and other microorganisms.

Physiological and endocrinological incompatibility

The use of complex organs like liver or kidney may raise the risk of endocrinological incompatibility because of the multitude of enzymes and hormones produced by these organs which may not be suitable for the function of the human organs and vise versa. For example the porcine renin is not able to perform cleavage of human angiotensin [39] and the human parathormone is not compatible with the porcine kidney. In case of successful xenotransplantation, parathormone induces an increase in the elimination of phosphor from the human body which is lethal [40, 41]. It is also established that the repertoire of proteins produced by the porcine liver may not be sufficient for a good functioning of the human body [42]. It was also shown that human proliferating factors may cause an uncontrolled proliferation of porcine cells because of the lack of antagonist in the porcine organs. Further physiological discrepancies are the difference in body temperatures which is 39°C for pigs as well as differences in the pH values, in life-span, blood pressure and heart rate [43].

Immune rejection

As for allotransplantation, one of the major problems of xenotransplantation is the immune rejection of the xenografts. This problem was already known since the first unsuccessful trials of xenotransplantation [44]. However the progress in understanding the mechanisms of the

immune rejection and the discovery of immunosuppressants solved a big part of the problem. In this context, xenotransplantation has the advantage that whole animals can be humanized and genetically modified to be suitable as organ donors for human [37, 38], while human stem cells studies are still facing many difficulties to reach the phase of whole organ production. Four phases of rejection types were described, the **hyperacute rejection (HAR)** caused by pre-existing antibodies and occurs within minutes after transplantation, the **acute humoral xenografts rejection (AHXR)** which appears after 24 hours, the **cellular rejection** leaded by the cytotoxic T-cells and the **chronic rejection**.

The hyperacute rejection (HAR) happens very quickly after transplantation and leads to a rapid lost of the xenotransplants. HAR is elicited by preformed antibodies that bind to the xenoantigens in the endothelial cells of the xenografts and activate the complement cascade [45]. This results in platelet activation, coagulation and so disrupts the vascular endothelial integrity.

Within few hours the organ will swell and change its color to dark blue and develops edema, hemorrhage and vascular thrombosis followed by onset of necrosis [46]. The main epitope responsible for HAR is the non-reducing trisaccharide group, galactosyl α-(1,3)-galactosyl β-1,4-*N*-acetyl glucosaminyl commonly known as Gal epitope [47] which is ubiquitous in mammals but absent in some primates like human [48]. The human immune system recognizes the antigen from the gut, since it is present in microbes of the intestinal bacterial flora and builds specific Gal-IgG antibodies. Around 1% of the human circulating antibodies pool is specific for the Gal epitope [49, 50]. Furthermore, the complement system plays an important role in the hyperacute rejection of xenotransplants by the activation of the endothelial cells which in their turn induce the formation of a pro-coagulant surrounding leading to rejection.

In order to avoid or delay the HAR several approaches were tested. One of them was the inhibition of the complement activation [51] by administrating cobra venom factor or C1-inhibitor in pig-to-primate models. Both cases succeeded to prevent the onset of HAR [52, 53]. However, the use of somatic cell nuclear transfer allowing the generation of transgenic pigs made it possible to establish genetically modified pigs whose organs can be tolerated by the immune system of human recipient, for example the "humanized" transgene pigs expressing human complement regulators CD55 (decay accelerating factors) [54], CD46 (monocot chemoattractant protein) [55] or CD59[56] which protects the porcine cells from complement-induced lysis. Other transgenic pigs lacking or with reduced Gal epitope were

also produced. Such as the pigs overexpressing the human β-D-mannoside β-1,4-*N*-acetyl-glucosaminyltransferase III (GnT-III) which reduce the Gal epitopes [57] furthermore α1,3-galactosyltransferase gene (GGTA1)-KO pigs subsequently lacking the Gal epitopes were generated [37, 58, 59].

The next immune barrier to be overcome is the **acute humeral xenografts rejection** (AHXR). The AHXR is poorly understood and shows a similar pathological course as the HAR involving swelling, vascular thrombosis and edema [60], however it appears with a time delay of at least 24 hours post transplantation. It involves antibodies and complement activation as well as infiltration of neutrophils and CD8+T cells [61]. In contrary to the HAR, in the case of AHXR the xenotransplants may function for few hours before rejection [62]. Recent studies suggested that the prevention of the AHXR depend on control of the elicited humeral response against non-Gal antigens and the disorders of the coagulation system [63-65].

Several attempts to avoid the AHXR were performed, among them the use of extracorporeal immunoadsorption to reduce the anti-pig antibodies and serum cytotoxicity in recipient for several days [66].

Beside the humeral immune rejection xenotransplants should face the **cellular rejection** which is set on by the recognition of xenogenic MHC molecules by the T cell receptors. This leads to the activation of CD8+ cytotoxic T-lymphocytes (CTLs) and subsequently apoptosis of porcine endothelial cells and rejection of the xenograft. Given the fact that peptide differences between different species is much greater than between individuals of the same species, the cellular response for xenotransplants is greater than to allotransplants and may require more immunosuppression to be overcome [67, 68].

Regarding the **chronic rejection**, the understanding of chronic rejection in xenotransplantation is still very poor, since most of xenografts tested didn't reach this stage yet and were rejected earlier by HAR or AHXR. However recent studies on pig-to-baboon cardiac transplantation where the xenotransplants survived under chronic immunosuppression for 179 days, a chronic xenotransplant vasculopathy developed with features similar to allotransplants manifesting deposition of extracellular matrix in blood vessels, fibrosis, infiltration of lymphocytes and inflammatory cells, diffuse intimal thickening followed by apoptosis and ultimate rejection of the organ [69, 70]. In clinical allotransplantation the use of immunosuppression proved beneficial in this case.

To summarize, based on biotechnological techniques and genetic engineering significant advances have been achieved in understanding the mechanisms of xenografts rejection and

made it possible to overcome the initial hurdles associated with rejection and xenografts survival has been significantly prolonged. Even if not totally satisfying, these results bring hope that long-term survival of xenografts may be achieved not too far in the future.

Biological risks

In allotransplantation

The next major general concern of transplantation medicine is the risk of the transfer of infectious agents. An allograft could harbor several human microorganisms and viruses which may be transferred to the recipient by leak of immunological barriers and due to the immunosuppression treatment which accompany the transplantation usually. A wide range of donor-derived infectious microorganisms such as bacteria, fungus, and viruses were described. Among the striking examples of viruses are the hepatitis B and C, Epstein-Barr virus, HIV and the cytomegalovirus (CMV) [71]. Such infections reduce further the cohort of possible human donors, since some of these pathogens especially the viruses cannot be eradicated.

In xenotransplantation

Xenozoonoses or pathogens that may be transmitted across the species barriers are of major concern and present a challenge for xenotransplantation. If infectious diseases in allograft pose a risk for the individual human recipient, zoonotic diseases may present a threat for individuals as well as for the general population. Microorganisms which are tolerated by one species (pig donor) may be pathogen in another (human recipient) because species lines have been crossed and thus these pathogens become transmissible from human to human [72]. Zoonoses are responsible of more than 60% of the approximately 400 emerging diseases identified since 1940 [73]. For example it is thought that the human immunodeficiency virus (HIV) may have crossed the species barrier from non-human primates to become a global pandemic [30]. Potential xenoses can be divided into non-viral and viral infections.

Non-viral infection: A wide range of pig-derived non-viral infectious microorganisms such as *Mycobacterium avium, Trichinella spiralis, Cryptosporidium parvum, Campylobacter coli, Strepptococcus suis, Erysipelothrix rhusiopathiae, Toxoplasma gondii* and *Brucella suis* were described [74, 75]. Most of these pathogens can be eradicated from the pig herd, by the

possibility of maintaining the pigs under sterile conditions and by monitoring them regularly for infectious agents [76]

Viral infection: Furthermore pigs harbor several infectious viruses which have a pathogenic potential for human. These include for example the Nipah virus [77] Menangle virus [78], and Tioman virus [79], hepatitis E virus (HEV) [80], porcine cytomegalovirus (PCMV) which is able to infect human fibroblasts *in vitro* [81], porcine gammalymphotropic herpesvirus (PLHV) [82] and the influenza virus which can cross the species barrier and infect humans [83]. Analyses of the H1N1 1918/1919 influenza virus, which killed at least 40 million people, revealed that this strain might have originated from the swine influenza virus [84].

The vast majority of these viruses can be eliminated from the pig herd using sensitive detection assays for screening, by maintaining pigs under SPF conditions and using specialized animal husbandry. Although no transmission of potential infectious viruses was detected during the last decade in humans exposed to live porcine cells [85], some viruses are still pose a relevant risk for xenotransplantation especially the viruses whose DNA is integrated into the germline of pigs, such as the porcine endogenous retroviruses (PERVs) which can infect human cells *in vitro* and are widely distributed in the genome of pigs and thus cannot yet be eradicated [86].

1.2 The porcine endogenous retroviruses

1.2.1 Retroviruses

The porcine endogenous retroviruses are part of the gammaretroviruses. The *Retroviridae* are enveloped single-stranded (+) RNA viruses. Viral RNA is reverse transcribed (reverse transcriptase, RT) during the replication cycle and integrated (integrase) as provirus into the genome of the host cell [87]. The discovery of the endogenous retroviruses in the late 1960s followed that of the Mendelian inheritance of RNA tumor viruses' genomes by their hosts. The inheritability of virus-transformed phenotype without viral replication, give rise for the Temin's hyptothesis which postulate the generation of DNA intermediate that can be integrated into the genome of the host cell [88]. This hypothesis was reinforced by the discovery of the viral reverse transcriptase [89].

The family of *retroviridae* comprises two subfamilies the *orthoretroviridae* and *the spuma retroviridae* defined by common taxonomic denominators such as structure, composition and replicative properties (Figure 1 [90]). A taxonomy based on the phylogenetic evolution classifies the *orthoretroviridae* in 7 genera: *Alpharetroviruses, Betaretroviruses, Deltaretroviruses, Gammaretroviruses, Epsilonretroviruses* and the *Lentiviriuses* while the *Spumaviridae* family comprises one genus the *Foamiviruses* (International Committee on Taxonomy of Viruses, ICTV). The first three genera are considered simple retroviruses, while the rest are considered complex and harbor small accessory proteins encoded by alternatively spliced transcripts that are absent in simple retroviruses [91].

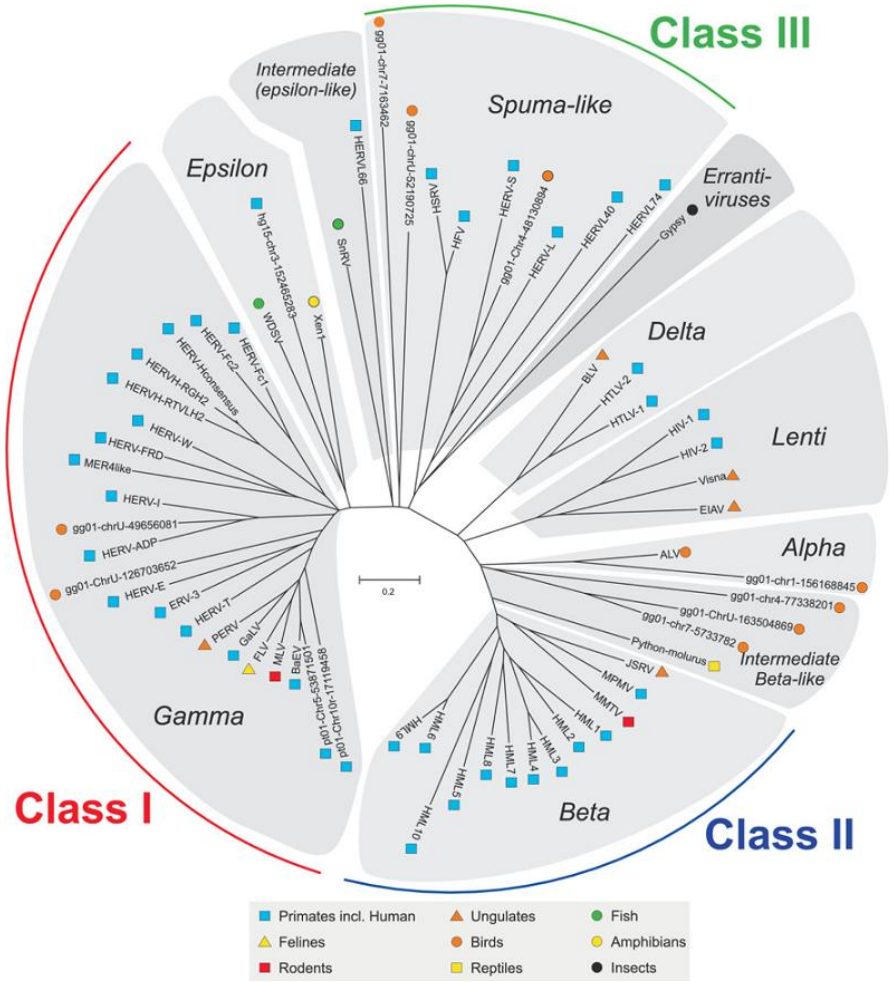


Figure 1. Phylogenetic tree of retroviruses (source: Jern et al., 2005)

Retroviruses can be **exogenous** or **endogenous (ERV),** both contain the whole set of retroviral proteins necessary for replication, encapsulation and budding and can thus be transmitted horizontally from host to host. However ERVs are present as proviral DNA permanently integrated in the germline of all vertebrates and some non-vertebrates. ERVs are remnant of infectious exogenous ancestors, which after being stably integrated in the host genome have accumulated mutations; hence only very

few of them under certain conditions are still capable of expression of complete infectious particles. They are inherited vertically in a Mendelian fashion as normal DNA [92, 93].

About 5-8% of the human genome consists of ERVs (HERVs). A wide range of beneficial functions was described for ERVs, for example the *env* gene of HERVs encodes for the expression of syncytins (Syncitin-1 and -2, respectively HERV-W and HERV-FRD), which is essential for the formation of syncytiotrophoblast early during embryogenesis [94, 95]. Other functions were described such as gene regulation [96, 97], DNA repair and recombination [98], additionally, they are thought to be used by the host as restriction factors to block the infection of pathogenic exogenous retroviruses [99].

As the ERV in other mammals, PERVs are estimated to form a significant part of the porcine genome. The first description of PERV particles was in the 1970's in the supernatant of porcine kidney (PK15) cells and other porcine cell lines such as peripheral blood mononuclear cells (PBMCs) (Figure 2) [100, 101] and showed morphological characteristics of gammaretroviruses [102-105].

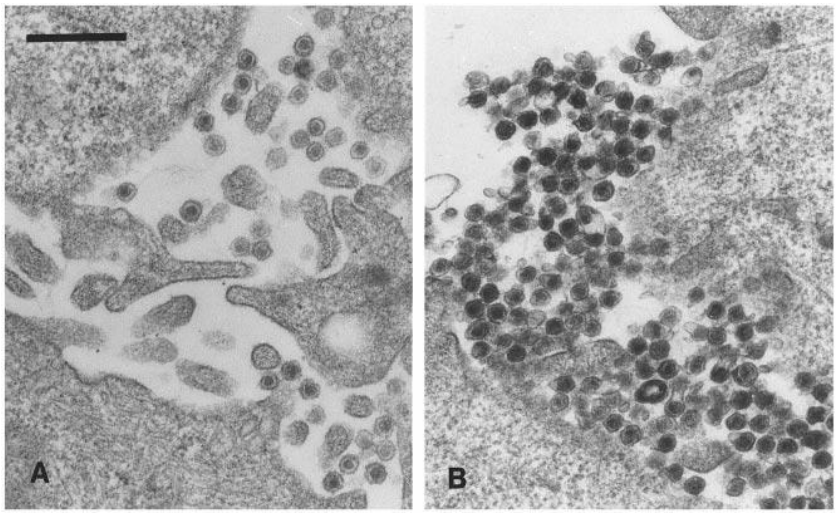


Figure 2. Electron microscopie of the PERV

PERV particles produced from PK15 cells. (A) And PERV infected human 293 cells (B). Bar, 500nm[105].

It was suggested that PERVs probably originated from murine retroviruses [105]. The analysis of the mutations in the long terminal repeats (LTRs) of the PERV sequences revealed that the endogenization may have occurred before 7.6 million years [106]. Since then the copy number of PERVs in the porcine genome didn't stop to increase due to re-infection and intracellular transposition [107, 108] . This number is strain dependent and can vary from 10

to 100 [109, 110]. The replication-competent PERVs are further classified in 3 subtypes according to their *env* sequence, **PERV-A, -B** and **–C** (see below) [110-112]. A modern example of endogenization event presents the koala endogenous retrovirus (KoRV) [113] which is related to murine and gibbon ape leukemia viruses (MuLV and GaLV respectively) [114]. KoRV was found as endogenous and exogenous virus in koalas from the north of Australia, but was totally absent in the animals of the south which could be explained by a wave of infection and endogenization originated from some virus-transmitting rodents from Southeast Asia and spreading southwards [115].

1.2.2 Structure of PERV

All retroviruses such as PERV have a common structure which consist of a virion of about 100-150 nm in diameter (Figure 3). The viral envelope shows protruding transmembrane glycoprotein (TM), p15E in PERV, bound to the surface glycoprotein (SU), gp70. A matrix made up of myristilated proteins (MA) are localized inside the virion just beneath the envelope and bound by link proteins to a protein shell made up by capsid proteins (CA) Retroviral capsid exhibits different structures. The capsid protect a complex of nucleocapsids proteins (NC) and two single-stranded RNA bound to several copies of reverse transcriptases (RT) and integrases (IN), which form together the inner core.

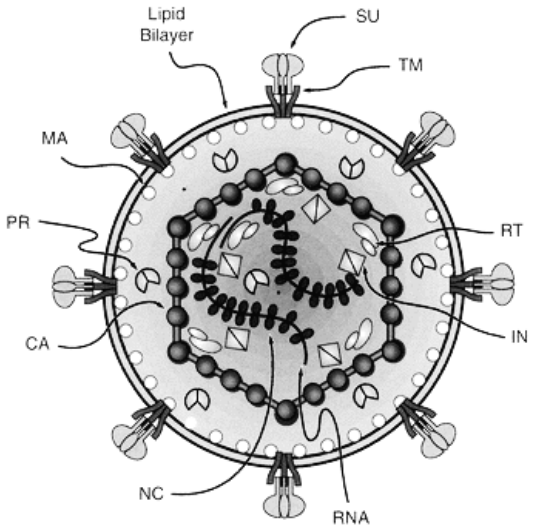


Figure 3. Schematic representation of gammaretroviruses

SU: surface unit, TM: transmembrane protein, RT, reverse transcriptase, IN: Integrase, NC: nucleocapsid, CA: capsid protein, PR: protease, MA: matrix protein.

1.2.3 Genomic organization and function of the env protein

The PERV genome is highly organized with the least information necessary for its own replication and propagation (Figure 4). It consists of two single-stranded RNA of about 8100 base pairs [116], both ends of the integrated proviral DNA are flanked by long terminal repeats (LTRs). LTRs play an essential role in the viral integration and transcription. They are composed of U5 ("unique"), R ("redundant") and U3 elements where enhancers, the promoter and the poly (A) addition signal are located. A primer-binding site (PBS) is located at the end of the U5 element and is important for initiating reverse transcription by annealing to a cellular $tRNA_{Gly}$ [117]. The genome contains also the splice donor and acceptor sites which result in two splice variants. The first comprise the full-length sequence coding for Gag and Pol proteins, and a spliced variant (3 kb) coding for Env. The RNA contains three major open reading frames (ORFs) encoding the Gag, Pol and Env. The *gag* gene encodes for the viral protease, the matrix, the capsid and the nucleocapsid proteins [118], the *pol* gene encodes for the reverse transcriptase and the integrase.

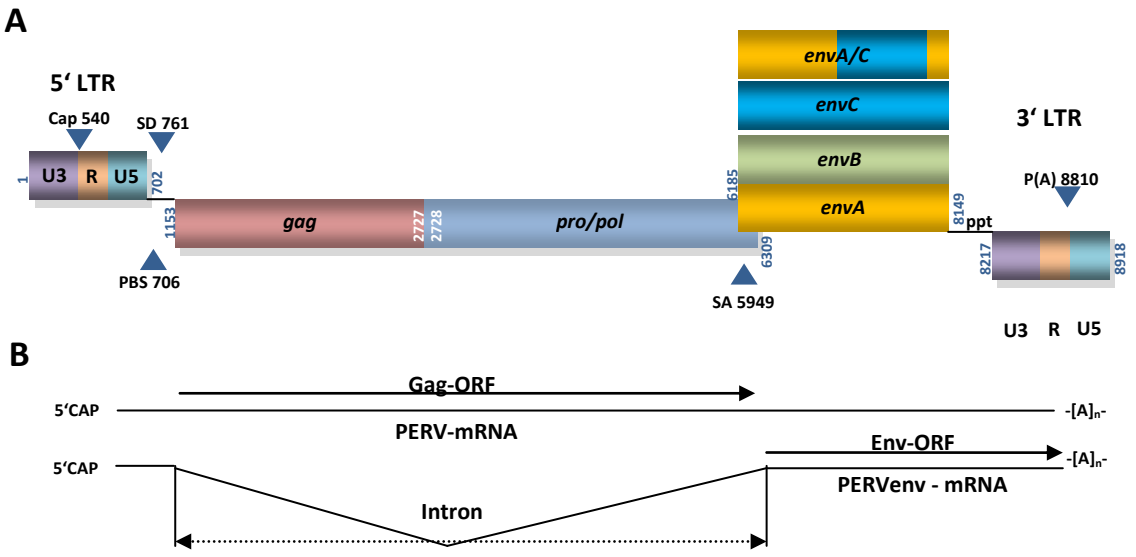


Figure 4. Structure of the proviral PERV

(A) 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; pro/pol, protease/polymerase gene; env, envelope protein gene. Env subtypes of PERVs and the A/C recombinant are presented. (B) open reading frames of PERV and different splice variants are represented [119].

The surface of the virion containing Env spikes plays a main role in the viral attachment and the fusion of viral and cell membranes during the infection process. A bipartite precursor envelope protein is the product of the spliced variant mRNA of the *env* gene [119]. Env are glycoproteins, the precursor Env protein undergoes glycosylation then it is cleaved by a cellular protease into the non-covalently associated SU and TM. The glycosylation of the Env protein is required for infectivity by playing a role in the binding to host receptor [120, 121] and the SU or gp70 elicite a host specific glycosylation and thus determine the tropism of PERV. For example, the PERV-A Env contains 9 potential N-glycosylation sites, env-B has 6 and –C has 8 [122]. There is no sufficient information about the mechanism of PERV infection and the exact role of the glycosylation on the interaction of receptor and viral envelope. However it is known from other retroviruses that immune response involves sometime the modification of the glycosylation of receptor proteins which lead to loss of infectivity of the virus, while some retrovirus change the glycosylation of their Envs for survival [120, 121].

Whereas the TM protein doesn't present sequence variations, the SU protein harbors variable domains based on them the transcription active PERVs were classified into the 3 subtypes PERV-A, -B and –C. PERV-A and –B are permanent in the genome of all pig strains and are able to infect cells from different species like human cells *in vitro* (polytropic), while PERV-C is ecotropic and not ubiquitous and infect only porcine cells [110, 123, 124].

Additionally, a recombinant PERV-A/C was described and which contains the cell fusion domain of PERV-C and the receptor binding site of PERV-A which makes it polytropic [125-127]. The recombinant PERV-A/C is known to reach higher titer than PERV-A. This increase was associated with some substitutions in the variable region [128] and the increase in the length of LTR [129].

1.2.4 The PERV replication

As for most gammaretroviruses, the viral infection is receptor-mediated and requires a multiple transmembrane domain cell-surface receptor. For PERV-A two receptors in human cells were described and named the human PERV-A Receptor (HuPAR1 and HuPAR2) while the PERV-B receptor(s) are still unknown [130]. Both HuPAR contain 11 transmembrane domains and thus a transporter function can be attributed [131-133]. The HuPAR1 was identified as a G-protein coupled receptor for gamma-hydroxybutyrate [132, 134] while the cellular function of HuPAR2 is still unknown [135].

The binding of the gp70 envelope protein to the receptor activates the transmembrane protein (TM), and resulting conformational changes induce the fusion of viral and host cell membrane (Figure 5). This fusion leads to entry of the viral capsid into the cytoplasm, where it undergoes partial disassembly, the viral nucleoprotein complex is liberated and the RNA genome is reverse transcribed into a double-stranded DNA by the viral RT using cellular dNTPs. The double-stranded linear DNA intermediate as well as the Reverse Transcriptase, Matrix proteins and Integrase are assembled together to form a subviral particle termed as pre-integration complex. Since PERVs have no accessory proteins, the pre-integration complex can only be integrated in the genomic DNA during mitosis. After nuclear import the viral DNA becomes integrated into the cell chromosome with the aid of virion integrase and form a stable provirus achieving then the status of a cellular gene, which can be expressed and replicated by cellular enzymes in concert with chromosomal DNA. The transcription of the provirus generates spliced and unspliced mRNAs. The translation products and the progeny RNA are then assembled at the cell membrane, where viral RNA is incorporated into capsids. These capsids are released from the cell by budding of the plasma membrane, which has incorporated also the viral envelope proteins.

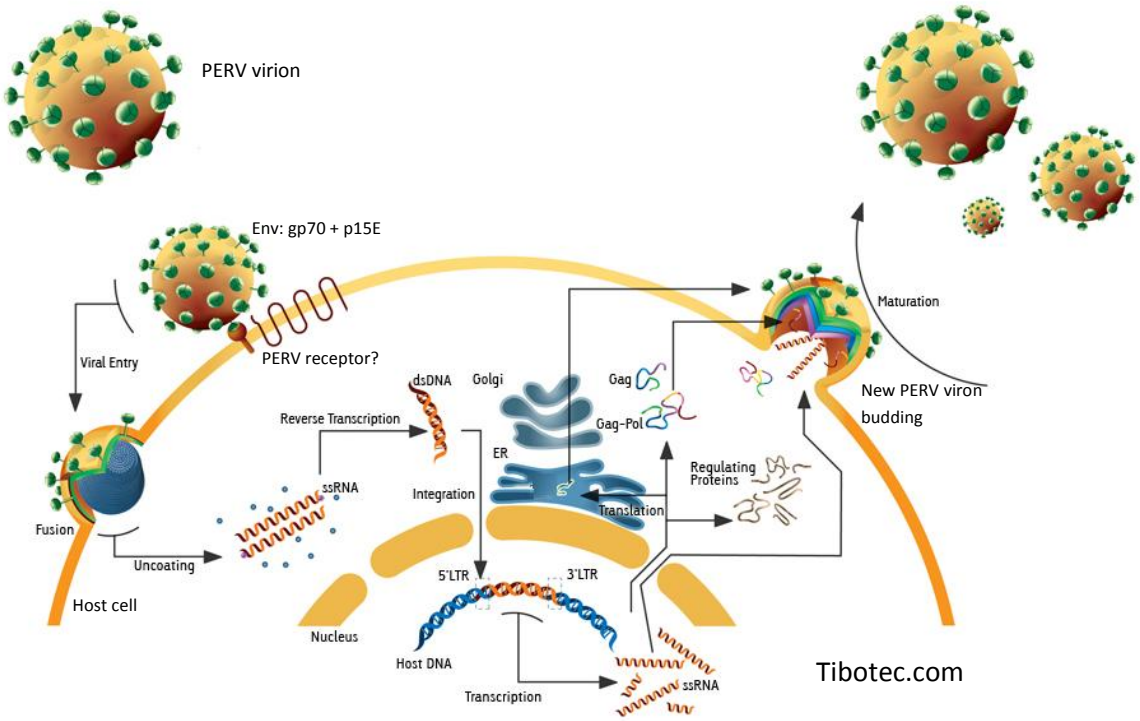


Figure 5. The PERV replication cycle

Budding of viruses is followed by cleavage of the precursor polyproteins by the viral and cellular proteases. If the mature PERV virion had incorporated enough Env in its membrane, then it will be able to infect new cells and begin a new cycle (for a review see [86]).

1.2.5 The pathogenicity of PERV

Integration and titer

PERVs are widely distributed and expressed in all pig strains and hence can't be avoided in xenotransplant [136, 137]. In cell culture PERV have low titer of expression, however, passaging of PERVs through human cell line led to increase of viral titer which is caused by increase of LTR length [129, 138]. These LTR changes were also observed in other gammaretroviruses like MuLV and FeLV and were also associated with increase of pathogenicity [139]. Furthermore PERV integration sites were found in the human genome localized within high gene density regions and CpG-rich island. The integration frequency correlated with the virus titer [140].

No pathogenesis described

No pathogenic symptoms were described in the natural host of PERV *in vivo* or *in vitro*. Furthermore, there is no indication of infections in small animals or non-human primates [105, 136, 141, 142]. The PERV transmission after transplantation of porcine islet cells into SCID mice was the result of pseudotyping of PERV with MuLV, since mice don't express PERV-A receptors [124, 130]

Related retroviruses are pathogenic

It is known that most of retroviruses are pathogenic; however PERV risk is still difficult to evaluate. An animal model would help to understand the pathogenicity of PERV. Recently mice expressing the HuPAR-2 receptor were generated and were found susceptible to PERV infection [143] however, may be also pseudotyping as shown above. Such genetic modified animal models could help understanding PERV risk.

Absence of *in vivo* model of PERV requires study of pathogenicity in related viruses.

Tumor and immunosuppression risk

Infection by most, if not all retroviruses is known to cause severe immunodeficiency in a wide range of species e.g. by FeLV, MuLV, KoRV, the human T-lymphotropic virus (HTLV) [100, 144-148], HIV [149] and simian immunodeficiency virus (SIV). Particularly, a highly conserved domain within the transmembrane protein was associated with immunosuppression and is known as immunosuppressive (ISU)-domain [100]. The ISU domain of PERV is identical to the ISU domain of FeLV, MuLV, GaLV and KoRV and all Isu domains were demonstrated to induce immune suppression [101].

Several PERV-related retroviruses exhibit tumorigenic properties such as MuLV, FeLV, KoRV [150] and GALV [151]:

FeLV: about 60% of infected cats die from opportunistic infections due to immunodeficiency of the infected cat and 5-10% develop leukemia/lymphomas [86]. The tumorigenic effect of FeLV was attributed to the high expression rate and insertion mutagenesis as well as the modification of the LTR [152].

MuLV was demonstrated repeatedly to be able to induce tumors by insertional mutagenesis in the host cells and high similarities between MuLV and PERV integration profile raise concerns about tumorigenic effects of PERV-like MuLV [153].

Although PERV was isolated from transformed pig kidney cell lines [103, 104, 154] and was isolated from lymphoma cells and radiation-induced cells [155], the oncogenic roles of PERV are still unclear. Lately, high expression of PERVs were found in melanomas from Munich miniature swine (MMS) Troll pigs [126].

These results are important for the clinical use of xenotransplantation, since a PERV transspecies transmission may induce an immunodeficiency and tumors [100, 112, 151, 156].

Recombination and complementation risks

Among retroviruses, emergence of new pathogenic viruses due to interspecies recombination and complementation are well known, such as HIV. A possible recombination and complementation events of PERV with human endogenous and exogenous retroviruses may also give rise to new virus [157]. Limited studies suggested that such events are very unlikely to happen (PERV/HERV) [86, 158-161]. In this case it would help to analyze the copy number and location of PERV in the porcine genome and data for some strains are now available [109, 116, 162].

Risk of PERV from genetically modified pigs

Strategies aiming to eliminate the replication-competent proviruses could help reducing the virus titer. However, remaining non-competent proviruses may still undergo recombination and complementation events. Furthermore, some non-competent proviruses may express env proteins containing the immunosuppressive domain and thus induce immunosuppression [86]

In addition, the use of transgenic modified xenotransplants may complicate the investigation of the risk of PERV. Such transgenic porcine material may interfere with the immune system of the recipient and help protecting PERV from the immune response. An example is the use of porcine transplants from C-reactive protein (CRP) expressing pigs. The expression of the

CRPs by the porcine cells help reduce the hyperacute xenograft rejection, however it will help protecting the PERVs produced by these cells [163]. Furthermore, viral particles budding from these cells may contain human CRP which will help those avoiding virolysis by activation of the complement [164]. A similar phenomenon was observed in ST-IWOA cells expressing the CD59 (see above), which produced PERV viral particles containing CD59 [165]

Recent studies made on human recipient which were exposed to porcine xenograft (liver cells, islet cells) revealed no infection despite persistent michrochimerism observed in some patients [105, 166-169]. However further investigation must be done since in these trials no long-term xenotransplantation was performed, furthermore only PBMCs of recipients were tested [163].

1.2.6 Strategies to avoid PERV-induced xenosis

1.2.6.1 RNA interference

Short interfering RNAs (siRNAs) are double-stranded RNAs (19 – 23 bp) duplexes that trigger silencing of target genes in sequence-specific manner [170, 171]. Long dsRNAs are initially recognised by an enzyme of the RNase III family of nucleases, named DICER, and processed into small double-stranded molecules (19-23 nucleotides) termed siRNA. Each of these small double-stranded siRNAs, is formed by a so-called guide strand and a passenger strand (Figure 2.2). The endonuclease Argonaute 2 (Ago 2) catalyzes the unwinding of the siRNA duplex. Once unwound, the guide strand is incorporated into the RNA Interference Specificity Complex (RISC), which is a multi-protein complex with RNase activity, while the passenger strand is released. RISC uses the guide strand to find the mRNA that has a complementary sequence leading to the cleavage of the target mRNA [172] by the endonuclease argonaute.

Based on RNA interference, several siRNAs targeting highly conserved region in PERV sequences were designed and tested [173]. Furthermore cells expressing permanently these siRNAs as small hairpin (sh)RNA were generated. Cells expressing an shRNA targeting the polymerase gene *pol*, which is identical to sequences in PERV-A, PERV-B and PERV-C were used to generate shRNA transgenic pigs by SCNT. The shRNA expression and PERV reduction was monitored over a period of 6 months [174, 175]. The long term expression and

efficiency of shRNA has not yet been investigated. Monitoring of the pigs for shRNA expression and PERV reduction was part of the present work. Furthermore expression of siRNAs in triple-shRNA expressing vectors will be investigated.

1.2.6.2 Knockout with Zink Finger nucleases

The high frequency of proviral PERVs in the porcine genome makes it impossible to knock out all PERV genes by classical methods. However, the recent development of the zinc-finger nucleases (ZFNs) had opened new opportunity to generate a PERV-KO pig.

ZFNs are functional as heterodimer and each arm contains a DNA-binding domain which recognizes specifically a DNA target sequence of 12-18 base pairs and a cleavage domain, the endonuclease FokI which require dimerization to cleave the DNA. Once the DNA-binding sequences bind to their contiguous target sequences separated by 5-7 bps, the 2 subunits of FokI dimerize and cut the DNA inducing though a double stranded break. ZFNs are high specific and recognize a total target sequence of 24-36 bps.

The double strand break induced by the ZFN results in the activation of cellular repair mechanisms such as the error-prone non-homologous end joining (NHEJ) which results in short deletion or insertion at the cleaved site and hence a knockout of the targeted gene.

Gene knockout by ZFNs have been described in a wide variety of species (reviewed in [176]). The use of ZFNs was also reported for pig cells [177, 178] and ZFN-knockout transgenic pigs were successfully generated by SCNT [37, 179, 180].

Most of genes targeted by ZFNs are biallelic. However, targeting of genes with high copy numbers was also reported, for example, disruption of the multiallelic (3 of 4) Cytochrome P450 Oxidoreductase (POR) in human tumor cell lines using single POR specific ZFN [181]. By treating EGFP-transgenic fetal fibroblasts harbouring about 10 copies of the EGFP gene with EGFP-specific ZFN, Watanabe et al. demonstrated a decrease of GFP expression in treated cells where several copies of the gene were disrupted by the ZFN [177]. Another example of ZFN multiple targeting are the Ghost-CCR5 cells, which are a mixed-cell populations generated by retrovirus-mediated gene delivery and have an average of four CCR5 copies per cell [182].

Based on these results, we aimed in this work to disrupt the possible highest number of PERV genes in the pig cells.

1.2.6.3 Vaccine

Although PERV transmission to human was not yet detected, preventive measures to avoid such transmission in the future are reasonable, and vaccination of patients who will profit of porcine xenotransplants will be of great use. In the case of HIV-1 broadly neutralizing antibodies were already isolated from infected patients, which were directed against the conserved TM (gp41) of HIV-1. However, immunization with both HIV-1envelope antigens didn't succeed to induce such antibodies [183, 184]. In contrast to HIV-1, immunization with the ectodomain of the transmembrane protein p15E of PERV [185], FeLV [186, 187] and KoRV [113] were successful. A successful example of vaccine is given by the FeLV. Already two companies, Leucogen (Virbac) and Nobivac (Intervet) produce vaccines against FeLV which protect the cats from infection. These vaccines were based on recombinant unglycosylated FeLV-p45 [188, 189].

Recently, high titres of neutralizing antibodies were measured in sera of hamsters immunized with the p15E and gp70 of PERV, and higher titres were measured in sera of animals immunized with both antigens [190]. The PERV specific sera recognized two epitopes, the E1 located in the fusion peptide proximal region (FPPR) and the E2 in the membrane proximal external region (MPER) of p15E [185]. However there is no animal model allowing the in vivo analysis of these PERV-specific neutralizing antibodies. Therefore, immunization with the FeLV-p15E, which is closely related to PERV-p15E were carried out. About 50% of cats challenged with the FeLV-p15E were protected from further antigenemia [187].

The immunization studies performed on gammaretroviruses may also help in the researches of HIV vaccine since the epitopes recognized by the gammaretrovirus-p15E antigens are localized in positions similar to the 2F5 and 4E10 epitopes of HIV which are recognized by the HIV broadly neutralizing antibodies [86].

1.2.6.4 Prevalence analysis

As mentioned above, use of PERV-C free pigs for xenotransplantation is important in order to avoid PERV-A and PERV-C recombination. Furthermore, low PERV producer pigs are of interest and different strains and herds should be screened to assess the PERV expression. The viral expression can be assessed using a wide variety of methods. The most accurate is the real-time RT PCR mostly used in this work.

1.3 Aim of the study

The present work was partially supported by the Deutsche Forshungsgemeinschaft DFG (De729/4-3) as contribution into establishing the safety of xenotransplantation when porcine cells, tissues and organs of pigs are to be used for human.

In the present study the following strategies to reduce the porcine endogenous retroviruses (PERVs) transmission risk were assessed:

1- The prevalence and expression analysis of PERV in Göttingen minipigs in order to analyse prevalence and expression of PERVs in well characterized pigs.

2- Investigation of the long term effects of PERV specific RNA interference in transgenic pigs and improving the siRNA activity by the production of new triple-shRNA expressing vectors.

3- Reduction of PERV expression by disrupting the PERV proviruses using the Zinc Finger Nucleases technology.

4- Optimizing the anti-PERV vaccine using recombinant envelope proteins of PERV.

2 Materials

2.1 Chemicals

All chemicals were purchased from Sigma Aldrich Chemie GmBH (Germany) or Carl Roth GmBH (Germany) as mentioned in the text.

2.2 List of bacterial strain

Strain	Genotyp
Top10 F'	*E. coli* F´(*lac*Iq, Tn*10*(TetR)) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
***E. coli* K12 ER2925**	*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2*
BL21-CodonPlusTM(DE3)-RP	*E. coli* B F- *omp*T *hsdS*(rB-, mB-) *dcm*+ Tetr gal λ (DE3) *end*A Hte [*arg*U *pro*L Camr]
RosettaTM 2(DE3)pLysS	*E. coli* B F– *omp*T *hsd*SB(rB– , mB–) *gal dcm* (DE3) pLysSpRARE23 (Camr)

2.3 List of plasmids and vector backbones

GenBank	Description	References
pET-22b(+)	bacterial expression vector	Novagen
pCAL-n	Bacterial expression vector	Stratagen
pBluscript II KS	Bacterial cloning vector	oligoengine
pSUPER basic	Bacterial cloning vector	
RNAi-Ready pSIREN-RetroQ	self-inactivating retroviral expression vector designed to express a small hairpin RNA (shRNA)	Clonetech
pLVTHM	Self inactivating lentiviral vector for direct cloning of shRNAs. Mammalian expression	addgene
pCL-VSV-G	VSV-G expressing envelope vector. Mammalian expression	addgene
psPAX2	Lentiviral packaging vector. Mammalian expression	addgene
pZFN	Expression of Zinc Finger Nucleases. Mammalian expression	Sigma-Aldrich

2.4 Plasmid constructs

Construct	Backbone	Description	Reference
pT-3shRNA	pLVTHM	triple shRNA (pol1, pol2 , gag 2) with 3 promoters (H1, 7SK and H1) expressing vector	this work
T-3shRNA	pSIREN-RetroQ	triple shRNA (pol1, pol2 , gag 2) with 3 promoters (H1, 7SK and H1) expressing vector	this work
pLVTHM-pol2	pLVTHM	pol2 shRNA expressing vector	Dr. Dieckhoff
pet-22b(+) gp70 PERV	pET-22b(+)	PERV SU (EcoRI/SalI) expression vector	C. Wurzbacher
pCal-n-p15E PERV	pCAL-n	PERV-TM expression vector	Dr. U. Fiebig
pSuper pol2	pSuper	Pol2 shRNA Oligos BglII/HindIII inserted	Dr. A. Karlas
ZFN1-CFP	pZFN	ZFN1 expression vector with CFP inserted as marker	this work
ZFN2-YFP	pZFN	ZFN2 expression vector with YFP inserted as marker	this work

2.5 Antibodies and sera

Antibody/sera	Dilution	Use	Reference
Rabbit anti-Mouse Immunoglobulins-HRP	1:1000	WB/ELISA	Dako
Anti-Rabbit-Immunoglobulins-HRP	1:2000	WB/ELISA	Dako
Rabbit anti goat-Immunoglobulins-HRP	1:2000/1:3000	WB/ELISA	Dako
Anti-swine-Immunoglobulins-HRP	1:1000	WB	Dako
Goat 16 anti-p15E (PERV)	1:200	WB/ELISA	AG Denner
Ziege 62 anti-gp70 (PERV)	1:200	WB/ELISA	AG Denner
Mouse Anti-β-actin, monoclonal clone AC-74	1:5000	WB	Sigma-Aldrich
Mouse Anti-Flag M2	1:500	WB	Sigma-Aldrich
Rabbit Anti-DDX3	1:1000	WB	Cell Signaling Technology
Anti GFP	1:1000		

2.6 List of enzymes

Name	Manufacturer
AmpliTaq Gold DNA Polymerase	Applied Biosystems
Pfu DNA Polymerase (rekombinant)	Fermentas
KAPA2G DNA-Polymerase	Peqlab
SuperScript™ III Reverse Transkriptase (RT)	Invitrogen
Platinum® Taq DNA Polymerase	Invitrogen

T4 DNA Ligase	Roche oder Fermentas
Shrimp Alkaline Phosphatase	Fermentas
Restriktionsenzyme	NEB or Fermentas
Lysozym	Sigma
Trypsin	Invitrogen
Proteinase K	Qiagen

2.7 DNA and Protein ladders

Standard	Manufacturer
O'GENERULER™ DNA LADDER MIX	Fermentas
1 KB+ DNA LADDER	Invitrogen
PAGERULER™ PRESTAINED PROTEIN LADDER	Fermentas

2.8 Primers and probes

PCR	Primer /Probe	Sequence	Reference
Sequencing and cloning primers			
T7-Promotor		TAATACGACTCACTATAGGG	Standard
T7-Terminator		GCTAGTTATTGCTCAGCGG	
M13	for	GTAAAACGACGGCCAGT	Standard
M13	rev	CAGGAAACAGCTATGAC	
hu7SK	for	ATAAGGATCCCAATAGAATTCACCAATGGAGACTGCAGTATTTAGC	This work
	rev	TATCAATTGGGCTACTCGAGCATTTGAAGACCGAGGTACCCAGGCGGCG CACAAGC	
Detection PCR			
pol	for	ATGTGGATGAGCGTAAGGGAGTAG	[191]
	rev	GTCTGGGGGCTGCCGAACGAT	
gag	for	TCCAGGGCTCATAATTTGTC	[191]
	rev	TGATGGCCATCCAACATCGA	
envA	for	TGGAAAGATTGGCAACAGCG	[119]
	rev	AGTGATGTTAGGCTCAGTGG	
envB	for	TTCTCCTTTGTCAATTCCGG	[119]
	rev	TACTTTATCGGGTCCCACTG	
envC	for	CTGACCTGGATTAGAACTGG	[124]
	rev	ATGTTAGAGGATGGTCCTGG	
PERV-A VRBF	for	CCTACCAGTTATAATCAATTTAATTATGGC	[127]
PERV-C TMR	rev	CTCAAACCACCCTTGAGTAGTTTCC	
porcine cyclophilin	for	TGCTTTCACAGAATAATTCCAGGATTTA	[192]
	rev	GACTTGCCACCAGTGCCATTA	
porcine β-actin	for	CTCGATCATGAAGTGCGACGT	[192]
	rev	GTGATCTCCTTCTGCATCCTGTC	
porcine Gapdh	for	ACATGGCCTCCAAGGAGTAAGA	[192]

	rev	GATCGAGTTGGGGCTGTGACT	
Detection of Integration of pLVTHM			
GFP	for	GATCACGAGACTAGCCTCGAGGT	[175]
	rev	CCAGGATGTTGCCGTCCTC	
Pol2	for	AACGCTGACGTCATCAAC	[175]
	rev	GGACGCTGACAAATTGAC	
Real-time PCR			
pol	for	ATGTGGATGAGCGTAAGGGAGTAG	[191]
	rev	GTCTGGGGGCTGCCGAACGAT	
	probe	FAM-TAGGACCATGGAGGAGACCTGTTGCC-BHQ	
gag	for	TCCAGGGCTCATAATTTGTC	[191]
	rev	TGATGGCCATCCAACATCGA	
	probe	FAM-AGAAGGGACCTTGGCAGACTTTCT-BHQ	
porcine cyclophilin	for	TGCTTTCACAGAATAATTCCAGGATTTA	[192]
	rev	GACTTGCCACCAGTGCCATTA	
	probe	Cy5-TGCCAGGGTGGTGACTTCACACGCC-BHQ2	
envC	for	TGACCTGGATTAGAACTGG	[126]
	rev	ATGTTAGAGGATGGTCCTGG	
	probe	FAM-CTCTAACATAACTTCTGGATCAGACCC-BHQ1	
envCnv	for	CCCCAgCCCAAGGACCAG	[193]
	rev	AAGTTTTGtCCCCgTTTTAGT	
	probe	FAM-CTtTAACATAACTTCTGGATCAGACCC-BHQ1	
env-A	for	GCAATGGAGCTGCATAACTTC	this work
	rev	TGTTGCCAATCTTTCCATCT	
	probe	TGGCCATTTCCAATTCCCATCA	
Env-A(58)			
env-B	for	CGCCTTATAGACAGCTCGAA	this work
	rev	GCCTCTAGGAGCAACACCTC	
	probe	CCCGTATCAGGGTCAATAATCAGCCA	
pol2 shRNA	for	CGGCGGAGTCAATTTGTCAGC	[194]
	rev	GTGCAGGGTCCGAGGT	
	Loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaaggac	
	probe	FAM-TGGATACGACaaggac-BHQ1	
Pol1 shRNA	rev	CGGCGGAGTATCTTACCTCAC	this work
	Loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaagcag	
	probe	FAM- 5’ TGGATACGACaagcag 3’ BHQ1	
gag2 shRNA	rev	CGGCGGTAACCTGAACTGAC	this work
	Loop primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaagctc	
	probe	FAM- 5’ TGGATACGACaagctc 3’ BHQ1	
ZFN primers			
ZFN set 1PCR 1	for	CGAAGGCACTACTGCTGGAA	this work
	rev	CGTTGGTCATCCATCGGTCT	

ZFN set 1 PCR 2	for	CAGGTGACCCTCCTCCAGTA	this work
	rev	CGTTGGTCATCCATCGGTCT	
ZFN set 1 PCR 3	for	CAGGTGACCCTCCTCCAGTA	this work
	rev	CGTTGGTCATCCATCGGTCT	
shRNA oligonucleotides			
shRNA pol2 7SK	S	ACCTCGGACGCTGACAAATTGACTTTCAAGAGAAGTCAATTTGTCAGCGT CCTTTTTGGAAA	
shRNA pol2 7SK	AS	TCGATTTCCAAAAAGGACGCTGACAAATTGACTTCTCTTGAAAGTCAATT TGTCAGCGTCCG	
shRNA pol2 H1	S	GATCCCCGGACGCTGACAAATTGACTTTCAAGAGAAGTCAATTTGTCAG CGTCCTTTTTGGAAC	
shRNA pol2 H1	AS	AATTGTTCCAAAAAGGACGCTGACAAATTGACTTCTCTTGAAAGTCAATT TGTCAGCGTCCGGG	
shRNA pol1 7SK	S	ACCTCGCAGGAGAGAGGTAACATACTCGAAAGTATGTTACCTCTCTCCTG CTTTTTGGAAA	this work
shRNA pol1 7SK	AS	TCGATTTCCAAAAAGCAGGAGAGAGGTAACATACTTTCGAGTATGTTAC CTCTCTCCTGCG	
shRNA pol1 H1	S	GATCCCCGCAGGAGAGAGGTAACATACTCGAAAGTATGTTACCTCTCTC CTGCTTTTTGGAAC	this work
shRNA pol1 H1	AS	AATTGTTCCAAAAAGCAGGAGAGAGGTAACATACTTTCGAGTATGTTAC CTCTCTCCTGCGGG	
shRNA gag2 7SK	S	ACCTCGCTCATAATTTGTCAGTTCAGGTTACGAATAACCTGAACTGACAA ATTATGAGCTTTTTGGAAA	this work
shRNA gag2 7SK	AS	TCGATTTCCAAAAAGCTCATAATTTGTCAGTTCAGGTTATTCGTAACCTG AACTGACAAATTATGAGCG	
shRNA gag2 H1	S	GATCCCCGCTCATAATTTGTCAGTTCAGGTTACGAATAACCTGAACTGAC AAATTATGAGCTTTTTGGAAC	this work
shRNA gag2 H1	AS	AATTGTTCCAAAAAGCTCATAATTTGTCAGTTCAGGTTATTCGTAACCTG AACTGACAAATTATGAGCGGG	

2.9 Eukaryotic cells

Name	Species	Cell type	Source	Medium
HEK 293	Human	Embryonal kidney	ATCC CRL-1573	DMEM
HEK 293 T			Invitrogen	DMEM
HEK 293-PERV-5°			AG Denner	DMEM
PK15	Swine	Kidney cell line	ATCC CCL-33	DMEM
PFFs	Swine	Primary fetal fibroblasts	Prof. Niemann	DMEM
PBMCs	Swine	Peripheral blood mononuclear cells	Prof. Niemann or Ellegard	RPMI

2.10 Animals

Bacterial culture media	
Luria-Bertani (LB) broth	10 g/l (w/v) tryptone, 5 g/l yeast extract, 0,5 g/l NaCl, pH 7.0
LB agar	LB broth, 14 g/l Agar-Agar (Roth)
2YT	10 g/l (w/v) tryptone, 16 g/l yeast extract, 5 g/l NaCl, pH 7.0
Mammalian cells culture media	
DMEM (PK15, 293T)	10% heat-inactivated fetal calf serum (FCS) (PAA, Pasching, Austria), 2 mm l-glutamin, 100 U/ml penicillin, 100 lg/ml, streptomycin, 15 mm HEPES (Biochrom, Berlin, Germany), ad 500 ml DMEM
DMEM (PFF)	Same supplements as DMEM (PK15, 293) + 0.1 mm b-mercaptoethanol, 1% (v/v) non-essential amino acids and a 1% (v/v) vitamin solution (Sigma-Aldrich, Steinheim, Germany)
RPMI	Same supplements as DMEM (PK15, 293)

*Charles River Laboratories International, Inc; Wilmington USA**Hungarian institute of vetenary vaccines;Gödöllö, Hungary

2.11 Culture media

Bacterial culture media	
Luria-Bertani (LB) broth	10 g/l (w/v) tryptone, 5 g/l yeast extract, 0,5 g/l NaCl, pH 7.0
LB agar	LB broth, 14 g/l Agar-Agar (Roth)
2YT	10 g/l (w/v) tryptone, 16 g/l yeast extract, 5 g/l NaCl, pH 7.0
Mammalian cells culture media	
DMEM (PK15, 293T)	10% heat-inactivated fetal calf serum (FCS) (PAA, Pasching, Austria), 2 mm l-glutamin, 100 U/ml penicillin, 100 lg/ml, streptomycin, 15 mm HEPES (Biochrom, Berlin, Germany), ad 500 ml DMEM
DMEM (PFF)	Same supplements as DMEM (PK15, 293) + 0.1 mm b-mercaptoethanol, 1% (v/v) non-essential amino acids and a 1% (v/v) vitamin solution (Sigma-Aldrich, Steinheim, Germany)
RPMI	Same supplements as DMEM (PK15, 293)

2.12 Software and EDV

Name	**Use**	**Manufacturer**
MxPro QPCR Software	Real-time PCR	Stratagene
MS Office 2003	EDV	Microsoft
Magellan™ Data Analysis Software	ELISA	TECAN
Lasergene Software Version 8.0	Sequence analysis	DNSTAR
Blast-n und Blast-p	Sequence analysis	NCBI
ImageJ	Image processing for concentration estimation	Open source
FlowJo	FACS calibur	TreeStar Inc.

3 Methods

3.1 Molecular biology

3.1.1 Isolation of plasmid DNA

Plasmid DNA was isolated from overnight cultures of E. coli in corresponding medium. Cells were pelleted and lysed under alkaline conditions using either the QIAprep Spin Miniprep Kit (Qiagen, Hilden) or the EndoFree Plasmid Maxi Kit (Qiagen, Hilden) according to the manufacturer. Maxi preps are highly purified and endotoxin free and were be used for eukaryotic cell transfections. DNA was eluted with nuclease free water and conserved at -20°C.

3.1.2 Isolation of DNA

Genomic DNA was isolated from eukaryotic cells using the DNEASY BLOOD & TISSUE Kit (Qiagen, Hilden). Cells were pelleted and re-suspended in 200 µl PBS. Cell lysis and DNA purification were performed according to the manufacturer's instructions. In order to isolate genomic DNA from tissues or organs, 25 mg of each sample were placed in 2 ml microcentrifuge tube containing 2 stainless steel beads (7 mm mean diameter, Qiagen, Hilden) and the appropriate volume of lysis buffer ATL. Cells were lysed in a TissuLyser LT (Qiagen, Hilden) by operating the Lyser for 5 min at 30 HZ. Lysed cells were transferred in a new tube and genomic DNA was purified using the DNEASY BLOOD & TISSUE Kit according to the manufacturer.

PCR Products were purified using the MSB Spin Rapace (Invitek, Germany) as recommended by the manufacturer. For the extraction of DNA fragments from gel agarose, gel slices corresponding to the separated bands were cut under UV-light using the scalpel then melted. Further steps of DNA extraction followed using the Invisorb Spin DNA Extraction Kit (Invitek, Germany) according to the manufacturer. DNA was eluted using nuclease free H2O.

3.1.3 Isolation of total RNA and siRNA

RNA isolation from cells was performed using the RNeasy Minikit (Qiagen, Hilden) as described by the manufacturer. The RNA isolation from Tissues and organ samples was performed using combined method using the TRI-Reagent (Sigma-Aldrich) and the RNeasy Mini Kit (Qiagen, Hilden). Briefly 50-100 mg of tissue samples were homogenized in 1 ml

TRI-Reagent using the TissuLyser LT (Qiagen, Hilden). To the supernatant 0.2 ml chloroform (Merck) was added and the mixture was centrifuged (12000 x g, 15 min, 4° C). The upper phase containing the RNA was carefully transferred to a new tube and mixed with chilled 70% ethanol (v/v in DEPC water). The mixture was then transferred to an RNeasy spin column (RNeasy MiniKit, Qiagen, Hilden) and centrifuged. Further washing and elution steps were performed according to the manufacturer.

3.1.4 Quantification of nucleic acids

The concentration and the purity of nucleic acids were determined using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). For each sample 1 µl were measured in comparison to nuclease free water as control. Protein free samples should have an A 260/280 ratio close to 2.0.

3.1.5 Molecular cloning

3.1.5.1 Restriction endonuclease digestion and dephosphorylation of plasmid DNA

For cloning purposes DNA Plasmids or purified PCR products(2,5 µg DNA) were digested using Fast Digest (Thermo Scientific, Darmstadt) enzymes in a 50 µl reaction volume containing the 10 time Fast Digest common buffer and the Fast Digest enzymes (5 units per µg DNA). The reaction mixture was incubated for 1 hour at 37°C. In order to prevent self-ligation and improve ligation efficiency, linearized plasmids were dephosphorylated by adding 1 unit Shrimp Alkaline Phosphatase (Thermo Scientific, Darmstadt) and incubating at 37°C for 30 min. Digested plasmids or PCR products were then checked on agarose gel for appearance and correct size and concentration was estimated using the ImageJ software. DNA extraction was performed as described below.

3.1.5.2 Annealing and ligation of DNA fragment with T4 ligase

Linearized and dephosphorylated DNA vectors were mixed with the DNA inserts at different ratio in a 10 µl reaction volume, containing 1 µl of T4 DNA Ligase and 1 µl of 10x T4 Ligase Buffer (Fermentas). The reaction mixture was then incubated over night at 16°C. The products of the ligation mixture were introduced into competent E. coli cells as described below.

3.1.5.3 Hybridization and cloning of shRNA oligonucleotides

The shRNA oligonucleotides synthesized by Sigma Aldrich (see table) were designed to hybridize forming overhangs, which are suitable for direct cloning into the digested siRNA expression vectors. Synthetic sense- and antisense-shRNA were diluted to a stock concentration of 50 mM. Oligonucleotides were hybridized reaction mixture containing 1 µl of each shRNA and 5 µl T4 Ligase Buffer and dH2O at a final volume of 50 µ. The mixture was heated to 94°C for 2 minutes and cooled down slowly till 21°C. Afterwards the overhangs of the hybridized shRNA oligonucleotides were phosphorylated by adding 1 µl of T4-Polynucleotide kinase and 1 mM dATP and further incubation at 37°C for 30 min. Finally the shRNAs were ligated into the corresponding siRNA expressing vector (see 3.1.5.2). Ligation products were used to transform competent E. coli (Top 10) and the selection of positive clones followed by colony PCR (See 3.1.5.10) using the corresponding primers as well as sequencing.

3.1.5.4 Cloning of ZFN1-CFP and ZFN2-YFP

In order to study the expression of ZFN proteins and locate them in the cells, the fluorescent markers CFP and YFP were inserted in the ZFN plasmids at the 3' side of the ZFN1 and ZFN2 respectively. CFP was amplified from a pCDNA4 V5 HIS-PLUS CFP using the primers TTTTTAGATCTGCCGCCGCCATGGTGAGCAAGGGCGAGGAG and rev TTTTTCTCGAGCGGAACCTTTCCGGACTTGTACAGCT. The PCR product was purified and ligated into the ZFN1 plasmid by using BglII and XhoI. For ZFN2 a mutagenesis PCR was carried out to replace a stop codon by a BamHI restriction site using the primers for caacggcgagatcaacttcggatccctcgagtctagagggcccg and rev cgggccctctagactcgagggatccgaagttgatc tcgccgttg. The YFP sequence was released from a pCMV-Tag 2B-CD63-YFP with XhoI and ApaI. The insert was purified by gel extraction using the Invisorb Spin DNA Extraction Kit (Invitrogen) and then inserted in an XhoI/ApaI digested ZFN2 plasmid.

3.1.5.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for analytical as well as preparative procedures. According to the DNA fragment size a 1-2% gel was prepared by dissolving the Agarose powder in 1x TAE Buffer (50x : 50 mM EDTA, 1 M acetic acid, pH 8,0) and microwave heating for about 1 minute until the mixture became translucent. The solution was then cooled to approximately 55-60°C and Ethidium bromide was added (0.5 µl / ml) and the gel was poured into a casting tray with the corresponding comb. After the gel solidified, it was

submerged in 1x TAE buffer-filled electrophoresis tank; DNA samples were diluted into DNA loading-buffer (Fermentas) and loaded. Electrophoresis was performed by applying a current between 1-10 Volts/cm. The DNA was visualized by UV using the Gel Doc 2000 (BioRad) or the CHEMOCAM Imager 3.2 (INTAS, Germany) and the size of the DNA was determined using the GeneRuler DNA Ladder Mix (Fermentas).

3.1.5.6 Polyacrylamide gels for resolving small DNA fragments

For a better resolution, a 10% polyacrylamide gel was prepared. Gels were poured into a Bio-Rad gel apparatus (Hercules, CA). After polymerization gels were left at 4°C overnight before use. Gels were then fixed into a Bio-Rad electrophoresis chamber filled with 1x TBE buffer. DNA samples were mixed with 6x loading buffer (Fermentas) and loaded into the wells. Electrophoresis was carried out at constant voltage of 110 V for 70-100 min. gels were then stained for 15-20 min in 1x TBE containing 0.5 µg/ml EtBr and visualized by UV using the CHEMOCAM Imager 3.2 (INTAS, Germany).

3.1.5.7 Sequencing

Sequencing of plasmids or PCR products was performed by the FG18 sequencing facility, where an advanced chain-termination method described by Sanger and colleagues was used. The sequencing reaction was performed with ABI BigDye 3.1v cycle sequencing (Applied Biosystems). A sequencing PCR was performed with the DNA samples and the corresponding primers in triplicates. The reaction mixture is described in the table 1. The PCR products were then measured in the sequencing facility using a 370A DNA Sequencing System (Applied Biosystems). The data analysis was performed using the Lasergene 8 software (DNASTAR).

Table 1. Reaction mix and cycle conditions for sequencing

DNA	150 ng
Primer (10 µM)	0.5 µl
BigDye-Mix 3.1	2 µl
5x ABI-buffer	1 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
2 min	initial Denaturation	96°C	1
10 sec	Denaturation	96°C	25
5 sec	Annealing	55°C	
4 min	Elongation	60°C	

3.1.5.8 Polymerase chain reaction (PCR)

Developed by Mullis in the 1980s the polymerase chain reaction is a robust and powerful tool which allows amplifying specific pieces of DNA (usually 100 to 600 bp) more than a billion-fold [195]. In a first step, double-stranded DNA are “melted” by high temperatures The

mixture is then cooled in the presence of sequence-specific primers (denoted as forward and reverse) that "anneal" to their targets. A thermo-stable polymerase (Taqpolymerase that was isolated from *Thermus aquaticus*) allows the extension of these primers by applying an optimal temperature. The Taq polymerase is a DNA dependant DNA polymerase, which adds covalently template directed deoxynucleotide triphosphate (dNTPs) to the 3'OH end of the primers. Perfect reaction efficiency leads to doubling of target-DNA quantity after each cycle, which results into exponential amplification of the specific DNA fragment. Finally the PCR product is abundant enough to be analyzed on an agarose gel with an ethidium bromide stain. The method was extended to analyze mRNA using reverse transcriptase to convert it into complementary DNA (cDNA). This method of analysis is a qualitative tool for detecting the presence or absence of a particular DNA or mRNA.

3.1.5.9 Gradient PCR

A gradient PCR allows the empirical determination of an optimal annealing temperature for the primers. During the PCR, a temperature gradient is built up across the thermoblock of the thermocycler around the calculated melting point (Tm). This allows the most stringent parameters for every primer set to be calculated. The cycling conditions comprised of 7 min polymerase activation at 95°C followed by 36 cycles of denaturation at 95°C for 30 s, annealing at 50-65°C for 30 s and elongation at 72°C for 1 min, a final extension at 72°C for 5 min.

The reaction conditions were as follows:
Reactions were carried out using the Eppendorf Mastercycler gradient which allows the following temperature gradient: 49.8°C. 50.2°C, 51.1°C, 52.5°C, 54.3°C, 56.2°C. 58.3°C, 60.2°C, 62°C, 63.5°C, 64.6°C, 65.1°C.
Finally PCR products were analyzed on 2% agarose gel.

3.1.5.10 Colony PCR

Colony PCRs were used to screen transformed E.coli clones for positive ligation of vectors. The used primers were chosen to flank the ligation/insertion site of the vector (table2). For the PCR AmpliTaq Gold® polymerase (Roche, Mannheim, Germany) was used. Individual bacterial colonies were picked up using a sterile pipette tip and dipped into a reaction tube containing 25 µl of the reaction mix. A PCR was then performed following the cycling conditions in the table below.

Table 2. Reaction mix and cycle conditions of colony PCR.

10 x PCR buffer	2.5 µl
Primer fwd (10 µM)	0.5 µl
Primer rev (10 µM)	0.5 µl
10x PCR Buffer	10 µl
dNTPs (20mM each)	0.5 µl
MgCl2 (25mM)	3 µl
AmpliTaq Gold	0.25 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
2 min	initial Denaturation	95°C	1
15 sec	Denaturation	95°C	32
30 sec	Annealing	55°C	
1 min/kb	Elongation	72°C	
10 min	Final elongation	72°C	1
hold	Chilling	4°C	1

3.1.5.11 PCR diagnostic

PCRs specific for PERV-pol and -gag [191] as well as different PERV-env subtypes (PERV-A, -B, -C, -A/C and -Cnv) were already described and used in this work: PERV-A and –B [112], PERV-C [124], PERV-A/C [127], PERV-C wild boar (WB) and –Cnv [191, 196]. PERV-C and –Cnv PCRs were mostly performed using the Kapa2G robust PCR Kit (Peqlab, Erlangen, Germany) (Table3).

Table 3. Kapa2G robust PCR reaction mix and cycle condition

Template (RNA)	50 ng
Primer fwd + rev (10 µM each)	1 µl
dNTPs (10mM each)	0.5 µl
MgCl2 (25 mM)	0.5 µl
Kapa2G polymerase (5U/µl)	0.1 µl
5 x PCR-buffer A	5 µl
5 x Enhancer	5 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
10 min	initial Denaturation	94°C	1
30 sec	Denaturation	94°C	30
30 sec	Annealing	AET(*)	
30 sec	Elongation	72°C	
5 min	Final elongation	72°C	1

(*)AET, annealing / extension temperature depends on the primers and time depends on the length of the sequence to be amplified.

3.1.5.12 One-step reverse transcriptase PCR (RT-PCR)

In order to quantify the gene expression on mRNA level One-step reverse-transcriptase real-time PCRs were carried out. Total RNA and not cDNA was used as template and reverse-transcription and PCR were carried out in the same reaction tube. In the present work two systems were used, the SuperScript™III RT (Invitrogen, Karlsruhe, Germany) and the SensiFAST™ Probe No-ROX One-Step Kit (Bioline, Luckenwalde, Germany) according to the manufacturer guidelines. Both systems use a mixture of reverse transcriptase and DNA polymerase in corresponding optimized reaction buffers. The first step involved a reverse transcription (45°C, 10 min for SensiFAST or 50°C, 15 min for SuperScript) followed by activation of the DNA polymerase (95°C, 2 min). afterwards cDNA can be amplified by the PCR reaction.

Principles

Real-time RT-PCR is based on the method developed by Mullis [195], which allows distinguishing and measuring of DNA or RNA sequences even with very small quantity [197, 198]. Real-time PCR monitors the amplification progress by measuring fluorescence after each cycle. The cycle at which the fluorescent signal crosses a threshold level (cycle threshold or Ct) above the background is proportional to the original amount of template, thereby enabling quantification. Fluorescence can be tracked using several dyes like Ethidium bromide (EtBr) or SYBR green which quantifies double stranded DNA. In this work we used TaqMan probes, which is more accurate since it quantifies only the probe sequence.

Operating mode

Primers and probes were ordered by Sigma-Aldrich. The TaqMan probe is an 18-22 nt sequence specific DNA based fluorescent reporter probe labelled with a reporter fluorophores (Table 4) at the 5' end and a quencher fluorophore (Black Hole Quencher, BHQ) at the 3' end.

Table 4. Reporter fluorophores of probes

Dye	Extinction [nm]	Emission [nm]	Quencher
6-FAM	494	515	BHQ-1
HEX™ (GAPDH)	535	555	BHQ-1
Cy5® (cyclophilin)	651	674	BHQ-3

As long as the probe is not cleaved by the Taq Polymerase, the long wavelength colored quencher reduces the fluorescence of the short wavelength colored reporter. As the polymerization continues, the polymerase reaches the probe and cleaved it by its 5' exonuclease activity, releasing the reporter away from the quencher. This results in increasing the fluorescence intensity of the reporter dye which can then be measured. The cycle at which the reaction reaches a fluorescent intensity above the background is called the cycle threshold (Ct). Real-time PCR was performed using an Mx4000 Multiplex Quantitative PCR system (Stratagene, Amsterdam, Netherland), and the data acquisition was processed by the Mx4000 software (version 4.20).

Real-time PCRs were performed as duplexes, where a house-keeping (GAPDH or cyclphilin) gene was always co-amplified and measured. These house-keeping (HK genes served as reference genes to ensure that the observed differences in the expression levels of the genes of

interests were not the result of fluctuations in the amount of the template cDNA. Experiments were performed in triplicate which allowed calculating mean values and standard deviations (see results). The reaction conditions were as follows:

Table 5. Reaction mix and cycling conditions for real-time PCRs

SuperSkript

Template (RNA)	50 ng
Primer fwd + rev (10 µM)	1 µl
Probe (10 µM)	0.5 µl
Primer fwd + rev HK(*) (10µM each)	1µl
Probe HK (10 µM)	0.5 µl
SuperSkript Platinum Taq	0.25 µl
2 x reaction Mix	12.5 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
15 min	RT	50°C	1
2 min	initial Denaturation	95°C	1
15 sec	Denaturation	95°C	40-45
2 min	Annealing/ Elongation	AET(**)	
10 min	Final elongation	72°C	1

shRNA real-time PCR (SuperSkript)

Template (RNA)	20 ng
Primer fw (10 µM)	0.5 µl
Primer rev (10 µM)	0.5 µl
Loop primer (0.05 µM)	0.5 µl
Probe (10 µM)	0.5 µl
SuperSkript Platinum Taq	1 µl
2 x Reaction Mix	12.5 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
5 min	Reverse Transcription	25°C	1
5 min		35°C	
5 min		55°C	
5 min	initial Denaturation	95°C	1
20 sec	Denaturation	95°C	
30 sec	Annealing/ Elongation	58°C	

SensiFAST

Template (RNA)	50 ng
Primer fwd + rev (10 µM each)	1.6 µl
Probe (10 µM)	0.2 µl
Primer fwd + rev HK (10µM each)	1.6 µl
Probe HK (10 µM)	0.2 µl
2x SensiFAST Mix	10 µl
Reverse transcriptase	0.2 µl
Ribosafe RNAse inhibitor	0.4 µl
Nuclease free water	ad 25 µl

Time	Step	Temp	cycles
10 min	RT	45°C	1
2 min	initial Denaturation	95°C	1
5 sec	Denaturation	95°C	40-45
2 min	Annealing/ Elongation	AET	
10 min	Final elongation	72°C	1
hold	Chilling	4°C	1

Real-time PCR (control)

Template (DNA)	50 ng
Primer fwd + rev (10 µM each)	1 µl
Probe (10 µM)	0.5µl
Primer fwd + rev HK (10µM each)	1 µl
Probe HK (10 µM)	0.5 µl
10x PCR Buffer	12.5 µl
dNTPs	0.2 µl
MgCl2	0.4 µl
AmpliTaq Gold	0.25 µl
Nuclease free water	ad 25 µl

(*)HK, House-keeping gene; (**)AET, annealing / extension temperature depends on the primers and time depends on the length of the sequence to be amplified.

3.1.5.13 Relative quantification

The gene expression values were mostly done according to Livack and Schmittgen method [199] by normalizing to the house-keeping gene. The $2^{-\Delta\Delta Ct}$ values were calculated as follows:

$$\Delta Ct = Ct\ target\ gene - Ct\ house\text{-}keeping\ gene$$

$$\Delta\Delta Ct = \Delta Ct\ examined\ animals - \Delta Ct\ control\ animals$$

3.1.5.14 Determination of Efficiency

The real-time PCR efficiency is defined as the probability of duplication of one molecule after each PCR cycle. An efficiency of 100% corresponds to a doubling of the DNA amount per PCR cycle. In order to obtain reliable comparison and calculations the amplification efficiency of target and housekeeping genes should be invariable. Evaluations of the efficiency of all used primers were performed. Acquisition of standard curves followed by carrying out real-time PCRs using serial dilutions of the target sequences (usually in plasmids) and housekeeping genes with known concentrations and copy numbers. The Ct values of reference and target genes were plotted against log of copy numbers. The slope (m) was determined using linear regression and efficiency was calculated using the following function:

$$E(\%) = (10^{-1/m} - 1) \times 100$$

3.1.6 Hybridization of oligonucleotide

The complementary shRNA olingonucleotides were resuspended in nuclease free water at the same molar concentration. Equal volumes of both oligos were mixed and heated at 95°C then cooled down to 25°C (-0.3°C/s) using a thermocycler.

3.1.7 Surveyor nuclease Assay

The ZFN activity was detected using the Transgenomic Surveyor mutation detection kit (Transgenomic, Glasgow, UK) which takes advantage of the Non Homologous end joining DNA repair system triggered by the double strand break at the ZFN target site. The Surveyor nuclease is an endonuclease that cleaves both strands of a DNA at sites of base mismatch. In a first step the ZFN target region is amplified by PCR from genomic DNA of ZFN-treated cells and untreated cells. If the ZFNs are active the PCR would results in a pool of mutated and unmutated amplicon which were hybridized by heating and slowly cooling to form hetero- and homoduplexes. Heteroduplexes contain a "bubble" formed at the site of mismatch. After treating this mixture with Surveyor nuclease, DNA heteroduplexes will be cut at the mismatch sites. The cleavage products can be analyzed by agarose gel electrophoresis or polyacrylamide gel electrophoresis.

3.2 Microbiological methods

3.2.1 Competent cells and transformation

The preparation of Competent E. coli cells were performed as described in the Qiagen bench guide. The protocol is based on the method described by Hanahan and colleagues [200]. The composition of buffers used for this purpose was described in the materials section. The Transformation of competent cells with plasmids and ligation products was performed using the heat-shock method [200] which allows a high efficient introduction of DNA plasmid in the competent cells. Briefly, 50 µl of competent cells were thawed on ice and mixed with 5-50 ng of plasmid DNA or 5 µl of a ligation mixture to be transformed and incubated on ice for further 20 minutes. Afterwards the mixture was heat-shocked for 40 seconds at 42°C in a ThermoStat plus (Eppendorf) and immediately cooled on ice for 2 minutes. Cells were then resuspended in 300 µl of TB medium and grown for 40-60 minutes at 37°C in a heated shaker. Finally different amount of cells were plated on LB-agar plates containing the relevant antibiotics and incubated overnight at 37°C.

3.2.2 Growth of E. coli cultures and measuring cell density

Bacteria from starter cultures or glycerol stocks were streaked on selective LB-agar plates. For growth in liquid medium a starter culture was prepared by inoculating 5 ml of selective LB medium by a single colony from a grown selective plate or by 5 µl of a glycerol stock and incubated at 37°C overnight. Large scale bacterial growth was prepared by inoculating 100 µl

to 1 L of selective LB, TB or 2YT medium by 500 µl of starter culture and incubating at 37°C for 8-12 in a heated shaker by 220 rpm (INNOVA 4330, New Brunswick Scientific).

Cell density was monitored photometrically by reading the optical density at 600 nm using a photometer (Biophotometer, Eppendorf). At least 800 µl of bacterial culture were measured and the machine was blanked with the same culture medium.

3.2.3 Storage of E.coli strains

For a short-term storage, agar plates were sealed with parafilm and stored at 4°C for maximum one month. For a long-term storage glycerol stocks were prepared by adding 0.5 ml of bacterial culture to 0.5 ml of autoclave-sterilized glycerol (100%) into a screw-cap vial. The vial was vortexed vigorously and frozen in liquid nitrogen and stored at -80°C.

3.3 Protein chemistry

3.3.1 Preparation of cell lysate

To prepare samples for western blotting, whole cells were lysed to release the proteins of interest using NP-40 lysis buffer. Cells were washed 3 times with cold PBS at 2000 rpm for 5 minutes. Lysis followed by adding 1 µl lysis buffer per 104 cells (150 mM NaCl, 1.0% NP-40, 50 mM Tris, pH 8.0) and incubating on ice for 10 minutes. Lysates were centrifuged at maximum speed for 10 minutes at 4°C to pellet the cells debris and protein containing supernatant was recovered and frozen down at -80°C till used.

3.3.2 Nuclear and cytoplasmic protein extracts preparation

To detect the presence of ZFN proteins in the nucleus of the nucleofected cells by western blots, nuclear and cytoplasmatic lysates were prepared. The separation of nuclear and cytoplasmic proteins was performed using a NE-PER nuclear protein extraction kit which enables the stepwise lysis of cells and extraction of the cytoplasmic part keeping the nucleus intact. A second step of nuclear lysis allowed the extraction of nuclear proteins away from genomic DNA and mRNA. Briefly, cells were trypsinized and washed with PBS. Pellets were then resuspended in 100 µl of cold ice cytoplasmic extraction reagent I (CERI) by vigorous vortexing for 15 sec. After incubation on ice for 10 min, 5.5 µl of CERII were added and mixed by vortex for 5 sec then incubated on ice for 1 min. after centrifugation for 5 min at

maximum speed (16000 x g) at 4°C the supernatant containing the cytoplasmic extract is transferred to a new tube. The insoluble pellet which contains nuclei was resuspended in ice-cold NER reagent and incubated on ice for 40 min with vortexing for 15 sec every 10 min. after centrifugation at maximum speed for 10 min supernatant (nuclear extract) was transferred to a new tube and stored at -80°C until use.

3.3.3 Expression and purification of recombinant proteins

Two antigens were used for PERV immunization; the recombinant p15E protein which represent the ectodomain of the transmembrane protein (TM; p15E) of PERV-A (a.a. 488-596, accession number HQ688786) as well as the recombinant protein corresponding to the envelope protein (SU; gp70) of PERV-A (a.a. 49-487, accession number HQ688786). The recombinant p15E proteins were expressed in the E. coli strain BL21-CodonPlus(DE3)-RP (Stratagene, Amsterdam, Netherland) using a pCal-n-Flag vector (Stratagene, Amsterdam, Netherland), which allow fusion of the calmodulin binding peptide (CBP) affinity tag to the expressed protein. CBP fusion protein can be then purified by passing cell extracts through calmodulin (CaM) affinity resin. Expression and purification of recombinant p15E proteins was described earlier [185]. The PERV-gp70 recombinant protein was expressed using the expression vector pet-22bb(+) (Novagen, San Diego, CA) with a C-terminal 6x His-Tag sequence, which allows purification by affinity chromatography with nitrilotriacetic acid matrix.

IPTG-Induced expression of gp70-recombinant proteins

Overnight starter cultures of the pet-22b(+) transformed bacteria under ampicillin selection were prepared. Starter culture were used to inoculate 1.3 L LB medium (starting OD600 = 0.1) in 3 L Erlenmeyer flasks. A total amount of 4 L was prepared. Flasks were incubated at 37°C in a heated shaker by 220 rpm (INNOVA 4330, New Brunswick Scientific) for approximately 3 h until the culture reached an OD600 of 0.7. the expression of proteins was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG. Roth, Germany) (0.1 µg/ml) since the PERV-gp70 gene is under the control of lac operon. Cells were then pelleted in an Avanti Centrifuge J-25 (Beckmann) at 8000 x g and washed with PBS at 4°C, 8000 x g (Eppendorf). Pellets were stored overnight at -20°C.

Disruption of cells

The cell pellets were thawed for 15 min on ice and resuspended in 5 ml lysis buffer per gram pellet (100 mM NaH2PO4, 10 mMTris-HCl, 6 M GuHCl, pH 8.0 (NaOH)).

3.3.4 Measurement of protein concentration

Protein quantification was performed using the Pierce BCA™ protein Assay kit. The method is based on the reduction of Cu2+ ion to Cu1+ by proteins in an alkaline medium (The Biuret reaction). The amount of the reduced cuprous ion is proportional to the amount of protein present in the solution. The chelation of bicinhoninic acid (BCA) with Cu2+ ions produces a purples-colored complex exhibiting high absorbance at 562 nm. The working reagent was prepared by mixing BCA reagent A with BCA reagent B (50/1). As standard, albumin (BSA) was used with concentrations of 200, 400, 600, 800, 1000 and 1200 µg/ml. afterwards 10 µl of each sample to measure as well as the standards were added into a 96-well plate and mixed with 200 µl working reagent each. After 30 min incubation at 37°C, absorbance was measured on an ELISA Reader (Tecan) at 560 nm with reference 492 nm. Protein concentrations were calculated using the BSA-standard curve.

3.3.5 Tricin-SDS-polyacrylamid-gelelektrophoresis

Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate proteins according to their size. In order to enhance the sharpness of the bands the separation starts in a 4% stacking gel with a wide-meshed polymer followed by a separation in a fine-meshed 20% separating gel (for buffers and gels see materials). Separating gel was poured into a gel cassette until about two centimetres from the bottom of the glass plates. After solidification of the separating gel, the stacking gel was poured on top and the appropriate comb slid was added between the glass plates. After polymerization the gel was fixed in the electrophoresis chamber which was then filled with electrophoresis buffer. Samples were prepared by mixing (3:1) with a sample buffer containing SDS and β-Mercaptoethanol. Proteins are denatured and linearized to the same shape by the anionic detergent SDS which also coats them with negative charges while β- Mercaptoethanol cleaves disulfide bonds and disrupts tertiary and quaternary structures of proteins. Additionally, the samples are further denatured by boiling for 5 min at 98°C. The so denatured proteins migrate in polyacrylamide towards the positive pole when an electric field is applied and the migration distance is proportional to the molecular weight of the protein. The samples were then loaded into the wells of the gel. We used 10 µl of the PageRuler Prestained Protein Ladder (Fermentas) as molecular weight marker. The electrophoresis was run for 15-20 min with a current of 0.03 A / gel until the samples accumulated at the bottom of the stacking gel. Subsequently a constant current of 120 V was applied for 1-2 hours.

Following electrophoresis, the gel was either stained with Coomassie blue to identify protein bands or proteins were transferred to nitrocellulose membranes for immunoblotting.

3.4 Immunological methods

3.4.1 Immunization

Hamsters (Charles River) were immunized with 300 µg of p15E, gp70 or both. In the last case, gp70 and p15E were immunized in different parts of the body. The proteins were emulsified in complete Freund's adjuvant intramuscularly and subcutaneously (i.m. 50µl, s.c. 700µl). The control animals were immunized with adjuvant and PBS. The immune response was boosted by second and third immunizations using incomplete Freund's adjuvant (Figure 1B). IgGs were concentrated using Vivapure Q Mini spin columns (Vivascience). Control animals were immunized with adjuvant only.

3.4.2 Preparation of sera

For sera preparation whole blood was collected in a covered tube and samples were allowed to clot undisturbed for 2 h at RT. In order to loosen the clots from the membrane samples were stirred using glass rod and left overnight at 4°C. Clots were removed by centrifuging at 3000 rpm (Eppendorf), 10 min, 4°C. Supernatant were immediately transferred into a new tube. Decomplementation followed by incubating the sera at 56°C for 30 min. samples were then conserved as aliquots at -80°C.

3.4.3 Western blot analysis

The electrophoretic transfer of proteins from SDS-gels to polyvinyl diflouropyrolidol (PVDF) membrane (0.2 µm, Millipore Immobilon) was performed as described by Tobin et al. (1979). The SDS gel was placed for 10 minutes in a transfer buffer. The PVDF membrane was pre-wetted in methanol then soaked for 10 min in transfer buffer. A blot sandwich was prepared by placing one or two pre-wetted blotting paper sheet on the anode of a Semi-Dry Transfer Cell (BioRad Trans-Blot SD® Semi-Dry Transfer Cell, Biorad, Hercules,USA), followed by the PVDF membrane then the SDS-gel which was covered by a second layer of blotting paper sheet. The cathode was placed on the top and the blot was run at 20 V for 25 min. After the protein transfer the membrane was incubated in the blocking buffer on a shaker for 1 h at RT. The primary antibody was then diluted in blocking buffer and added to the membrane. After 1.5 h incubation at RT, alternatively over night at 4°C, the membrane was washed (5 x 5 min)

in the wash buffer and incubated with the peroxidise-conjugated secondary antibody diluted 1:1000 for 1 h at RT. After five times (5 min each) washing the detection was carried out by the enhanced chemoluminescence (ECL Western Blotting Detection Reagents Kit, Pierce, Rockford, IL, USA) system according to the manofacturer's protocol. The detection solution was mixed 1:1 and given to the membrane for one minute. The chemoluminescence was detected with the chemocam and the time for development varied depending on the intensity of the chemoluminescence.

3.4.4 ELISA

The enzyme linked immunosorbent assay (ELISA) was used for the titration of antigen specific antibodies present in the sera. Nunclon^{TM} Delta 96-well MicroWell® plates (Thermo Scientific) were coated by the corresponding recombinant antigens, p15E (0.625 µg/well) or gp70 (1 µg/µl) overnight at 37°C. Plates were blocked (PBS with 5% (w/v) BSA and 0.05% (v/v) Tween-20) for 1.5 h at 37°C then washed with 300 µl wash buffer (PBS with 0.05% (v/v) Tween-20) for 10 s. A second blocking buffer (PBS with 2.5% (w/v) BSA and 0.05% (v/v) Tween-20) was used for the preparation of sera serial dilution of sera (1:20 – 1:630). Plates were incubated with sera dilutions for 2h at 37°C. Afterwards, plates were washed 3 times for 10 s with 250 µl wash buffer. Plates were then incubated for 1 h at 37°C with the secondary HPRT conjugated antibody diluted in the second blocking buffer. after 7 total washes (300 µl, 30 s each wash) substrate solution (PBS 30 ml with o-phenylendiamin-dihidrochlorid (1 mg/ml) and 200 µl 30% H_2O_2) was added (80 µl / well) for 20 min and the reaction was stopped by adding 80 µl stop solution (2N H_2SO_4). Absorbances were read by ELISA Reader Spectra Classic (TECAN) at 490 nm (reference: 620 nm). Data analysis was done by the Magelan TM (TECAN) software and MS Excel.

3.4.5 Confocal laser scanning microscopy (cLSM) and image analysis

Cells preparation

PK15 cells were seeded into standard bottom 8 well µ-slides (Ibidi, Munich, Germany) and transiently transfected with the following plasmids: (i) ZFN1-CFP and ZFN2-YFP (ii) a Plasmid expressing a CFP-YFP fusion dimer protein as positive control (iii) two plasmids expressing unconjugated CFP and YFP. All plasmids were transfected alone to acquire a triple set of images from the donor, the acceptor and donor/acceptor. We used for each well 0.3 µg plasmid DNA and MetafectenePro (Biontex) as transfection reagent *according* to the

manufacturer's instructions. One day after post transfection cells were washed twice with PBS, and fixed for 20 minutes using 2% paraformaldehyde dissolved in PBS, washed with PBS and mounted in glycerol containing 0.1% *p*-phenylenediamine

Fluorescence measurements and analysis

FRET measurements were conducted as described previously [201]. All images were acquired using a ZEISS 780 confocal inverted microscope and a 63 x oil immersion objective. Images for FRET analysis were obtained using multitrack instrument setting for CFP and FRET channel, excitation for CFP channel at 405 nm and emission peak at 475 nm/27nm bandwidth, excitation for FRET channel at 405 nm and emission peak at 527/48 nm bandwidth. YFP signals were additionally detected in a single track with an excitation at 514 nm and emission peak at 527 nm/55-nm bandwidth. Donor and acceptor bleed-through coefficients were determined by acquiring images containing only a donor or acceptor, respectively. NFRET values were measured with ZEISS ZEN 2010 software.

3.4.6 Neutralization assay

3.4.6.1 Preparation of viral stocks

In order to perform a neutralization assay, viral stocks should be prepared. For this purpose uninfected 293 cells were seeded in T25 cell culture flasks. When cell confluency reached 30%, medium was replaced by virus containing cell-free supernatants produced by already PERV/5° infected cells. The PERV/5° cells were established by repeatedly passaging recombinant PERV-A/C on 293 cells. Cells were then incubated for 3 days with the viruses afterwards they were trypsinized and further cultured in larger flasks for 3 days. After the 10th day post infection cell culture supernatant were harvested five times every 3rd day. Supernatants were centrifuged (2000 x rpm, 5 min) and filtered (0.2 µm), and aliquots of 1 ml were freeze down in liquid nitrogen. Titration of supernatants followed using real-time PCR and the supernatant with ΔCT (Ct PERV – Ct GAPDH) value of 1 to 2 was chosen for neutralization assay with the same concentration.

3.4.6.2 Neutralization assay

One day prior infection, 100 µl of 293 cells (3000 cells/ well) were seeded into 96-well plates. The decomplemented sera (56°C, 30 min) were mixed with viral supernatant dilution (20 µl to 80 µl) and incubated for 30 min at 37°C before adding to the 293 cells. After 72 h incubation, cells were checked for viability by light microscopy, and then heat inactivated (95°C, 30

min). Cell lysis was performed by 3 cycles of heating/freezing (-80°C, 10 min / 95°C, 5 min) followed by an overnight incubation at 56°C in lysis buffer (nuclease free water containing 0.2 mg/ml proteinase K and 10% (v/v) 10 x PCR buffer. Proteinase K was inactivated by heating at 95°C for 30 min. the proviral DNA was quantified by PERV-gag specific duplex real-time PCR using 3 µl of each lysate sample.

The Ct values of the housekeeping gene GAPDH should be equal in all samples, indicating an absence of cell toxicity. Results were calculated as ΔCT values (Ct GAPDH – Ct PERV). Higher ΔCt values corresponded to lower proviral loads and thus a higher neutralization activity of sera.

3.4.7 Epitope Mapping

A 15-mer peptide library were synthesized corresponding to the whole PERV p15E sequence and presenting an overlap of 12 residues were fixed on glass chip (JPT Peptide Technologies, Germany) and protocol was carried out as described [190, 203].

3.5 Cell culture techniques

3.5.1 Preparation of cultures of porcine primary fetal fibroblasts (PFFs)

Porcine ear fibroblasts were isolated from transgenic and control pigs while Primary porcine fetal fibroblasts for SCNT were established from a day 25 post-conception foetus as previously described (Peterson et al., 2009) in the Institute for Farm Animal Genetics, Mariensee, Germany.

3.5.2 Isolation of porcine PBMCs

Porcine peripheral blood mononuclear cells PBMCs were isolated from heparinised blood samples using Ficoll–Paque gradient centrifugation as described by the manufacturer (Amersham Pharmacia, Uppsala, Sweden). Briefly, the separation medium was pre-warmed to RT and 15 ml were preloaded in a 50 ml Falcons. Up to 30 ml of the heparinised blood were loaded carefully to avoid mixing of the blood with the medium. Gentle centrifugation (800 x g, 15 min RT, without braking) resulted into a Ficoll/plasma interphase enriched in PBMCs. Red blood cells and granulocytes were accumulated at the bottom of the tube. The upper phase was gently discarded. The PBMCs interface was collected in a new 50 ml falcon

and washed with PBS (without Mg^{2+} or Ca^{2+}) and pellets were re-suspended in pre-warmed complete RPMI medium.

3.5.3 Cell counting

Viability and yield of cells were determined using a Neubauer cell counting chamber. In order to distinguish live and dead cells, 10 µl of 0.4% (v/v) trypan blue (Sigma-Aldrich Steinheim, Germany) was added to 10 µl of cell suspension. The cell titer was calculated using the following formula:

Z= N x 2 x 102 cells/ml

(Z= cell concentration. N = total cell count/number of counted large squares, 2 = dilution factor)

The viability of cell population could be determined by calculating the percentage of living cells (uncoloured cells)

3.5.4 Cryoconservation of eukaryotic cells

Cells were trypsinized and washed with PBS (200 x g, 5 min, RT), pellets were resuspended in 1 ml freezing medium containing 90% FCS and 10% dimethyl sulfoxide (DMSO) (Invitrogen) at a concentration of 1-5 million cells/ml/cryovial. Cells were frozen gradually (1°C/minute in a Mr. Frosty freezing container (Nalgene, Rochester, USA) and stored at -80°C overnight then transferred in liquid nitrogen the following day.

For cell thawing the cryovials were shacked in 37°C warm water until the complete thawing. Vials were then wiped with 70% (v/v) ethanol, and the content was quickly washed into 10 ml pre-warmed (37°C) complete medium (25 x g, 10 min, RT) and resuspended in new medium.

3.5.5 Eukaryotic cell culture

Fibroblasts and the porcine kidney cells PK15 (ATCC-CCL-33) were cultured in DMEM while PBMCs were cultured in RPMI. All eukaryotic cells were incubated in 5% CO2 at 37°C and 98% relative humidity in a BBD 6220 incubator (Heraeus). RPMI and DMEM were each supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA, Pasching, Austria), 2 mm l-glutamin, 100 U/ml penicillin, 100 µg/ml, streptomycin, 15 mm HEPES (Biochrom, Berlin, Germany). In the case of fibroblasts, 0.1 mm b-mercaptoethanol, 1% (v/v) non-

essential amino acids and a 1% (v/v) vitamin solution (Sigma-Aldrich, Steinheim, Germany) were added.

Adherent cells were trypsinized every 2 to 3 days. Media were removed and cells were washed with pre-warmed PBS. Depending on the flask size an appropriate volume of trypsin/EDTA (Biochrom) was added for 1-3 minutes at 37°C. cells were then resuspended in pre-warmed complete Medium. The splitting ratio varied according to cell density between 1:2 and 1:8. For PK15 cells a cell scrapers were mostly used to detach them.

3.5.6 Infection Assay

One day prior to infection, 5 x 104 HEK 293 cells were seeded in 12-well plates. Cells were then infected with PERV-containing cell-free supernatants of PHA-stimulated or unstimulated PBMCs cultures. Supernatant were filtered using a 0.2 µm filter (Sartorious Stedim Biotech, Göttingen, Germany) and polybrene was added at a final concentration of 7 µg/ml. plates were then spinoculated (1h, 2000 rpm, 37°C). In parallel, porcine PBMCs were coincubated with 293 cells using cell culture inserts with 0.4 µm pore size membrane (Greiner). Different samples were tested with or without phytohemagglutenin L (PHA)-stimulation (Biochrom, Berlin, Germany) at a concentration of 2.4 µg/ml and by adding or not polybrene. After 3 days incubation 293 cells were split every 2 days four times by trypsinization. Afterward cells were harvested, DNA was isolated as described and PERV-specific PCRs were performed. False-positive results caused by remaining porcine genomic DNA from dying PBMCs were investigated by performing control PCRs using porcine GAPDH and beta-actin PCRs. A PERV A/C adapted to human cells and characterized by duplications in the LTR [129] was used as positive control.

3.5.7 FACS and FACS sorting

Fluorescence activated cell sorting (FACS) was used to measure the fluorescence of GFP expressing cells. 1-5 x 106 cells were fixed by incubating in 2% formaldehyde for 20 min then washed 3 times with PBS and resuspended into FACS buffer (PBS with 5% FCS and 0.02% sodium azide). In order to avoid cell aggregation fixed cells were then filtered through Pre Separation Filters (Miltenyi Biotech). Measurements were performed using a FACS calibur (BD Biosciences) and 1000 cells of each sample were measured. Results were analyzed using a FlowJo software (Tree Star Inc., Ashland, Oregon).

The sorting of live cells was performed in the FACS-service facility of the Max Planck Institute for infection biology (Berlin) using a MOFLO HIGH-PERFORMANCE CELL SORTER (Dako).

3.5.8 Transfection (lipofection)

Transfection was optimized and performed using Lipofectamin as described by the manufacturer. For a transfection in 10 cm2 plates cells were seeded at a density of 1 x 106 cells for 24 h so that confluency reached 80% at the time of transfection. Up to 30 µg plasmid were mixed in 1.5 ml serum free DMEM medium and the mixture was added to the transfection reagent (10 µl Lipofectamin / 1.5 ml serum free medium). DNA/transfection reagent mixture was then incubated at RT for 20 min, during this time 7 ml of fresh medium was added to the cells. Afterward 3 ml of the DNA/transfection reagent mixture were added dropwise to the plate, while gently swirling the plate. Four hours later transfection medium was replaced by 10 ml fresh DMEM medium.

3.5.9 Nucleofection

Cells were transfected using the Amaxa Nucleofector II device (Lonza, Basel, Switzerland). For PK15 and 293 cells the Amaxa cell line nucleofector kit V was used and the Amaxa Basic Nucleofector Kit for Primary Mammalian Fibroblasts (cat.nr. VPI-1002 PC) for PFFs. Cells were subcultured 2 days before nucleofection . On the day of nucleofection cells were trypsinized and washed gently with PBS. For each nucleofection 1-2 million cells were resuspended in 100 µl of the pre-mixed nucleofector solution. DNA plasmids were added and the mixture was transferred into a 2 mm electroporation cuvette and nucleofected with program A-023 for 293 cells, T-023 for PK5 and U-023 for PFFs. After nucleofection cells were immediately transferred into 2 ml pre-warmed medium in a 6-well plate.

3.5.10 Production of lentiviral particles and transduction

Porcine Foetal fibroblasts used for SCNT are slowly dividing and hard to transfect primary cells. Cell transduction using lentiviral vectors of 2nd and 3rd generation has become a major safe tool for efficient gene delivery allowing a stable expression of shRNAs. The method consists into delivering the expression plasmid containing the shRNA cassette as a lentiviral particle which infect the cells and integrate the shRNA cassette into the genome of the cell

allowing a stable expression of the shRNA. Lentiviral particles are generated in 293A helper cells by co-transfecting plasmids expressing the genes necessary for the production of the lentiviruses. In this work were used: (i) the PLC-VSV-G plasmid (Nolan Lab) coding for the envelope proteins, (i) the packaging plasmid PSPAX2 (Trono Lab) coding for the structural protein gag and the retrovirus-specific enzyme pol and (iii) the lentiviral expression plasmid pLVTHM (Trono Lab) which allow the integration of the shRNA cassette stably into the genome of the infected cells.

To produce lentiviral particles, 293A cells were transfected as described (see 3.5.10) using a DNA plasmid mixture of 21 µg as follow: pLVTHM (10 µg), PSPAX2 (7.5 µg) and PCL-VSV-G (3.5 µg). After 2-3 days incubation at 37°C the efficiency of transfection was analyzed by fluorescence microscopy. If efficiency was over 70%, the viral supernatant was harvested, spun down for 10 min at 2000 x g and filtered using a 0.45 µm filter. One day prior to infection PFFs were seed in 6 well plates at a density of 0.1-0.2 Mio cells per well. To infect with virus 2-3 ml of Viral supernatant were added with polybrene (7 µg/ml). Plates were then spinoculated at 2300 rpm for 1h at 37°C. Cells were then incubated over night at 37°C and next day medium was replaced by a new one.

3.5.11 Generation of transgene pigs

The generation of transgenic pigs was performed by Dr. Björn Petersen (Federal Research Institute for Animal Health, Mariensee, Germany) from transgenic porcine fetal fibroblasts (PFFs) by somatic nucleus transfer (SCNT) as described [38].

4 Results

4.1 Prevalence and expression of PERVs

Several methods were developed for the investigation of PERVs and PERV infections and reviewed in [86]. Direct methods were used to detect the proviruses in the cells such as PCRs and real-time PCRs [191, 204-207], Southern blotting [116, 156] and fluorescence *in situ* hybridization [127, 208] which allowed the detection of different PERV subtypes, the investigation of the copy numbers and the localization of proviruses within the genomes. PCR and real-time PCR were used also for the estimation of viral expression on mRNA level [209]. The expression of viral proteins was assessed using different immunological methods [203, 209-211]. Further methods were elaborated to detect and quantify the viral particles and the production of infectious viruses by measuring the reverse transcription activity or the infection ability of the viruses [105, 123, 212-214]. In addition, indirect methods based on immune response of the host and the detection of antibodies by ELISA and Western blots were also used (for a review see [86]).

4.1.1 Establishment of new real-time PCRs for detection of PERVs

PCRs specific for PERV-gag and -pol as well as PERV-env (PERV-A, -B, -C and -Cnv subtypes) were established and used for the detection of proviruses and to assess the release and infectivity of viral particles. In addition, reverse transcription (RT) real-time PCRs specific for PERV-gag, -pol, -envC and PERV-envCnv were described and served for the detection and quantification of PERVs on mRNA levels [191, 204-207]. However real-time PCR specific for PERV-envA and -envB have not yet been established. Here we describe the different steps in establishing these two real-time PCRs which, when added to prior described PERV-specific real-time PCRs will provide a diagnostic test and improve the detection of PERVs proviruses and PERVs expression.

New primers and probes were designed based on the alignment of different known sequences of PERV-A as well as for PERV-B, so that primers could amplify a highly conserved region in the *env-A* and *env-B* genes. A gradient PCR was performed at temperatures between 55°C and 65°C to assess the annealing temperature of primers (Figure 6 A). The estimated primers annealing temperature (AT) corresponding to the higher band intensity were 58°C for PERV-A and 56°C for PERV-B. These temperatures are also in the range of AT of porcine cyclophilin (pCyc).

Since real-time PCRs were performed in duplexes with the housekeeping gene porcine cyclophilin, primers were tested for possible interference. Real-time PCRs were performed for PERV-A and PERV-B with the same amount of mRNA separately in different tubes or together in duplexes with the pCyc primers. No significant differences in CT values were observed in both cases, which exclude interference between primers (Figure 6 B).

The efficiencies of the real-time PCRs were then investigated using a fivefold serial dilutions of vectors containing the env-A and env-B sequences with known concentrations ending with 0.2 plasmid copies per reaction. The porcine cyclophilin was co-amplified as internal standard for quantification and quality control of the template DNA (data not shown). For each run, a standard curve was generated with log of the RNA concentration on the X-axis and cycle threshold on the Y-axis. After exclusion of the highest and the lowest concentrations, a line of best fit was generated using 5 concentration data points. The reaction efficiency was calculated using the following equation: $E = 10^{(-1/m)}/2 \times 100$, where (m) represents the slope of the line. The efficiency of the PCR should be close to 100%. The standard curves, which were generated in these runs, produced linear results and R^2 values were >0.99 for both real-time PCRs. R^2 is a statistical parameter that indicates how good is the Y value (Ct) in predicting the value of X (concentration). In general, all efficiencies tested were close to 100% with 100.4% for PERV-A and 98.2% for PERV-B.

The newly established real-time PCRs were then used to measure the expression of PERV-A and PERV-B in different samples of total mRNA from PK15 cells and PBMCs from Göttingen minipigs MP1 and MP2 (Table1). In order to test the specificity of the real-time PCRs, control vectors containing env-B, env-C and env-A/C sequences were also tested (Figure 6C). As expected, PERV-A expression was higher than PERV-B in all samples and PERV expression in Göttingen minipigs was higher than in PK15. PK15 cells showed an expression of 0.29 Mio PERV-A copies and 0.06 Mio PERV-B copies per 50 ng total mRNA. While PERV expression in MP1 for examples was much higher with 2.3 Mio copies PERV-A and 0.94 Mio PERV-B per 50 ng total mRNA. Vector controls revealed a high specificity of both real-time PCRs, specially the PERV-A real-time PCR which was able to discriminate between env-A and env-A/C containing vectors.

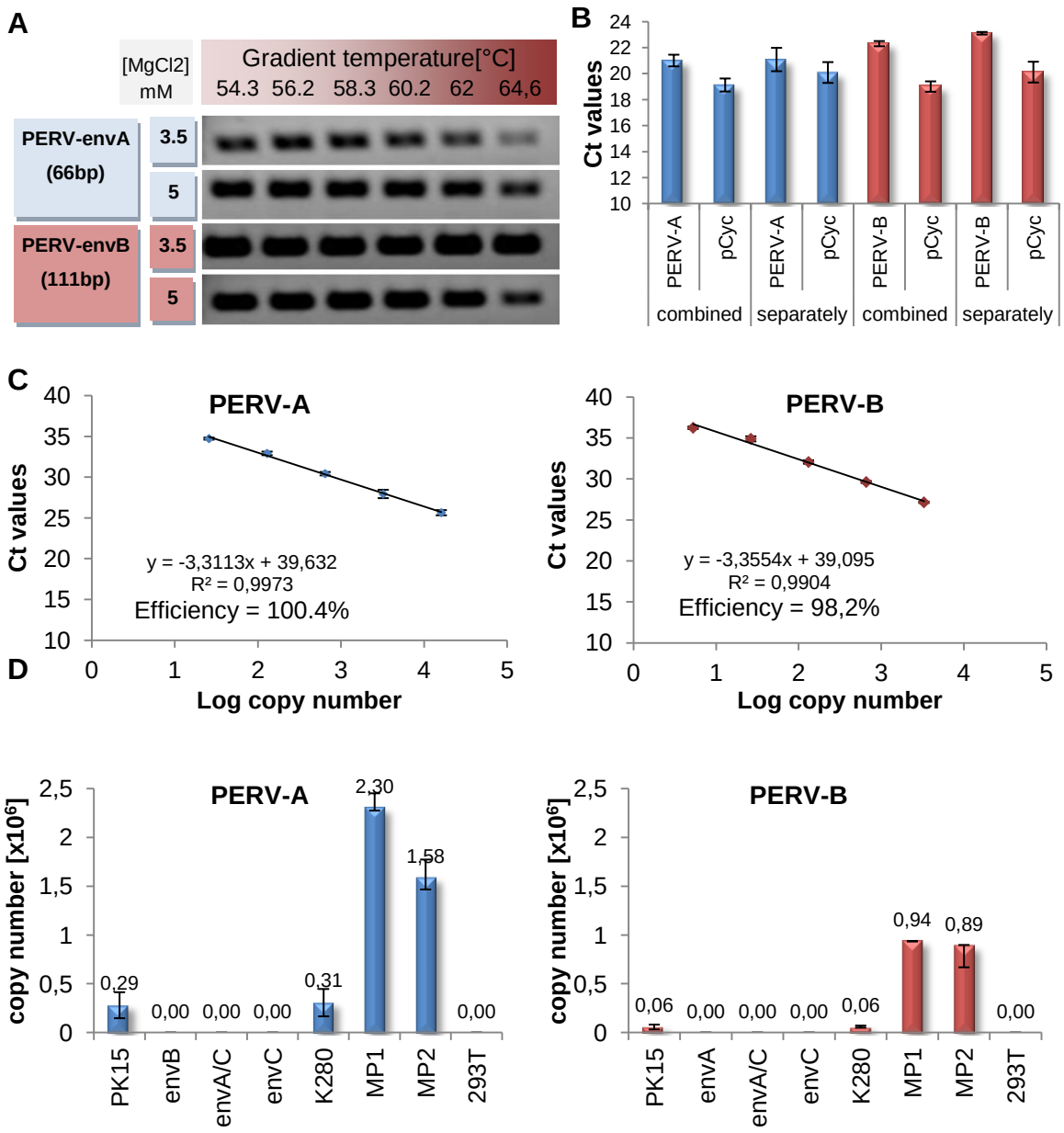


Figure 6. Establishment of PERV-A and PERV-B specific real-time PCR.

(A) Experimental determination of optimal annealing temperature and optimal $MgCl_2$ concentration. Using the gradient function of the Eppendorf Mastercycler, a gradient of 54°C to 64°C and two $MgCl_2$ concentrations were tested, 10 pmol/µl of primers were used. (B) Testing of primer interference of PERV-A and PERV-B combinated with cyclophilin primers for a duplex real-time PCR. (C) Real-time efficiencies for PERV-A and PERV-B. Serial dilutions of PERV-A and PERV-B plasmids with known concentrations and copy numbers were prepared and used. The standard curves generated from the cycle thresholds of each dilutions and the equation of the line of best fit are represented. (D) Test of real-time PCRs using 100 ng of total RNA isolated from PK15, PBMCs of a Landrace German pig (K280) as well as RNA from PBMCs of Göttingen minipigs nr. 1 and 2. RNA from 293T cells was

used as negative control. Specificity of the real-time PCRs was tested using vectors containing env-A, envB and env-C sequences of PERVs.

New real-time PCRs specific for PERV-A and PERV-B subtypes were established and showed high PCR efficiencies comparable to real-time PCRs specific for other PERV subtypes as well as for the porcine cyclophilin housekeeping gene. In addition no Primer interference was observed which allow carrying out these real-time RT-PCRs as duplexes. These results would enable comparative expression studies using of all known PERV subtypes.

4.1.2 Screening of Göttingen minipigs

Göttingen minipigs are used for numerous biomedical researches [215, 216]; however, the prevalence and the expression of different PERV subtypes in these animals were not yet investigated. In this study, screening of Göttingen minipigs was performed using sensitive PCR and real-time PCR methods [122].

Table 6. List of Göttingen minipigs analyzed in detail.

pig number	identification number	gender	age
1	M213540	M	263 d
2	M213553	M	260 d
3	M213582	M	257 d
4	F213225	F	285 d
5	F213454	F	270 d

4.1.2.1 The Animals

In total 15 Göttingen minipigs produced at Ellegaard (Dalmose, Denmark) in the same animal unit were analyzed on DNA level for presence of different PERV subtypes on DNA level. Among them 5 (Table 6) were further investigated at the RNA level for PERV expression. These 5 animals were from 5 different families (families 1, 3, 4, 8 and 9).

The founder colony was produced by entry of animals derived by caesarean section and colostrums deprivation. Animals were kept in full-barrier establishment under strict designated pathogen-free (DPF) conditions according to international standards for animal welfare. Furthermore animals are monitored for absence of a wide range of pathogens twice a year, according to the recommendations of the Federation of European Laboratory Animal Science Associations (FELA-SA). The whole results are published in the Health Monitoring Report (http://www.minipigs.dk/).

4.1.2.2 Experimental design

Blood from 15 animals was transported with sodium citrate on ice (8 h) to the Robert Koch Institute. PBMCs were isolated and genomic DNA was isolated for the prevalence analysis. From PBMCs of 5 Göttingen minipigs which were designated to expression analysis, RNA was isolated from fresh PBMCs as well as from PBMCs stimulated or not with PHA for 3 or 5 days. PERVs expression on mRNA was assessed by carrying out specific real-time PCRs. In order to investigate the release of human-tropic PERV viral particles, an infection assay was carried out by incubating 293 cells with supernatant from PHA-stimulated or unstimulated PBMCs as well as co-incubation of PBMCs and 293 cells (Figure 7).

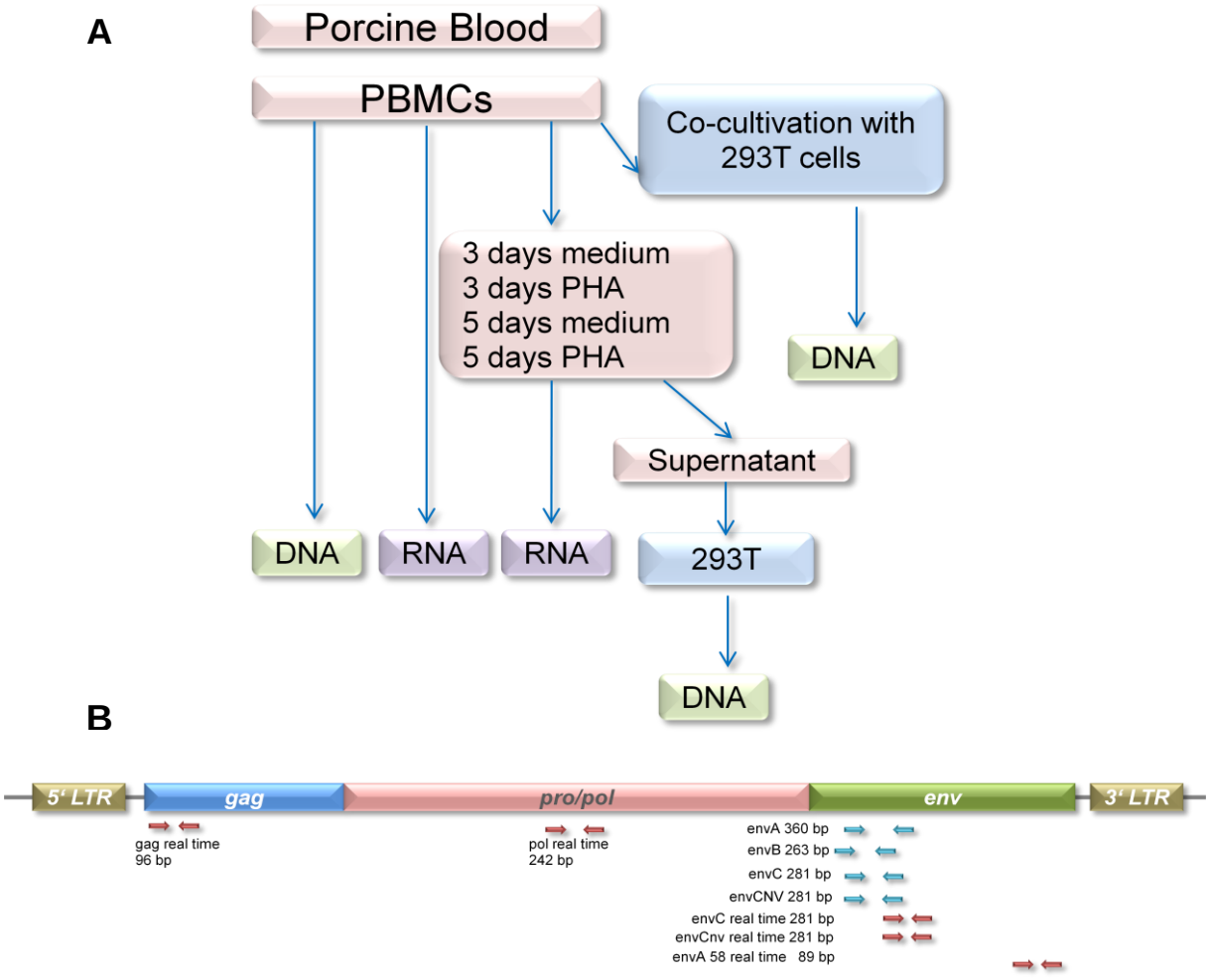


Figure 7. Experimental design of Göttingen minipigs analysis.

(A) PBMCs from different pigs were isolated and DNA and RNA were isolated from fresh PBMCs. PBMCs were incubated with or without PHA for 3 and 5 days and cells RNA was isolated from cells while supernatant was used for incubation with human 293 cells. PBMCs were also co-cultivated with 293 cells using cell culture inserts. **(B)** Schematic presentation of the genome of a PERV provirus and

location of the primers used for the PCRs and real-time PCRs. (LTR, long terminal repeat; gag, group-specific antigen; pro/pol, protease, polymerase; env, envelope proteins). The length of the PCR amplicons is given in bp.

4.1.2.3 Detection of PERVs subtypes in the genome of Göttingen minipigs

In order to investigate the prevalence of PERVs in the genome of the Göttingen minipigs, different PCRs and real-time PCRs were carried out using genomic DNA isolated from PBMCs (Figure 7). Results revealed the presence of PERV-A and PERV-B in all 15 animals (Figure 8A). Same results were observed for the ecotropic viruses PERV-C and PERV-Cnv. In contrast, the recombinant PERV-A/C was absent in the germ line of all investigated animals.

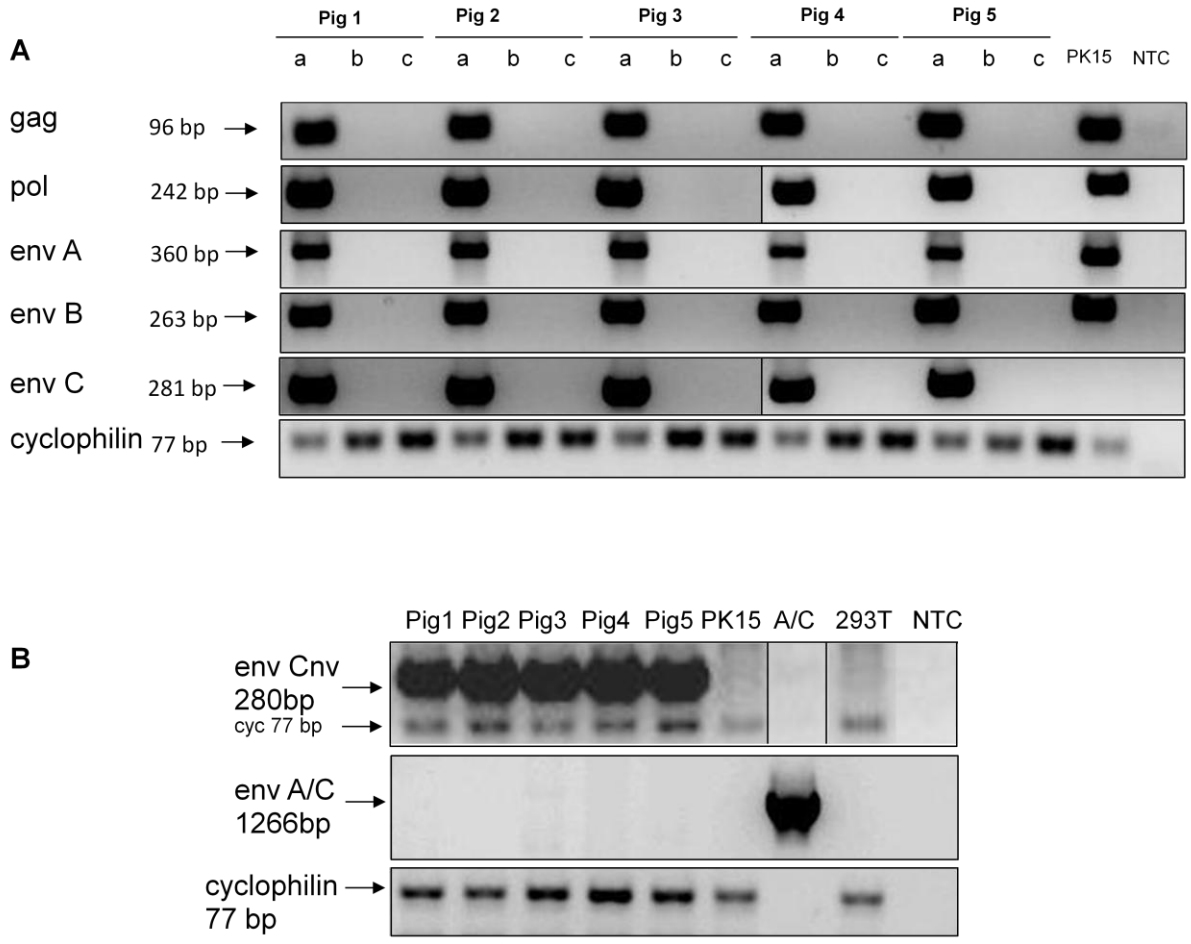


Figure 8. Prevalence of PERV in Göttingen Minipigs

(A) Detection of PERV-A, PERV-B, and PERV-C proviruses in the genome of five Göttingen minipigs by PCR using primers specific for the env genes as well as for gag and pol (Lane a). DNA loading was analyzed by PCR using the primers for cyclophilin. PK15 cells do not harbor PERV-C, NTC, non-template control. In lane b and c, the absence of PERV infection in human 293 cells is

shown. (Lane b) The same PCRs were performed with DNA from human 293 cells after incubation with supernatant from peripheral blood mononuclear cells (PBMCs) stimulated with PHA, and (lane c) with DNA from 293 cells after 5 day co-cultivation with PHA-stimulated PBMCs. (B) Detection of PERV-Cnv proviruses and absence of PERV-A/C proviruses in the genome of five Göttingen minipigs by PCR. As positive control for PERV-A/C, a plasmid was used, as negative controls PK-15 cells, not harbouring PERV-Cnv, and human 293T cells were used. DNA loading was analyzed by PCR using the primers for cyclophilin. PERV, porcine endogenous retroviruses [122].

Real-time PCR were used to quantify the copy number of PERVs. Results showed higher copy number of total PERV and PERV-C provirus in the genome of Göttingen minipigs when compared with the German landrace pigs. However, real-time specific for the PERV-A clone58 is higher in German landrace pigs [119] as shown in Figure 9.

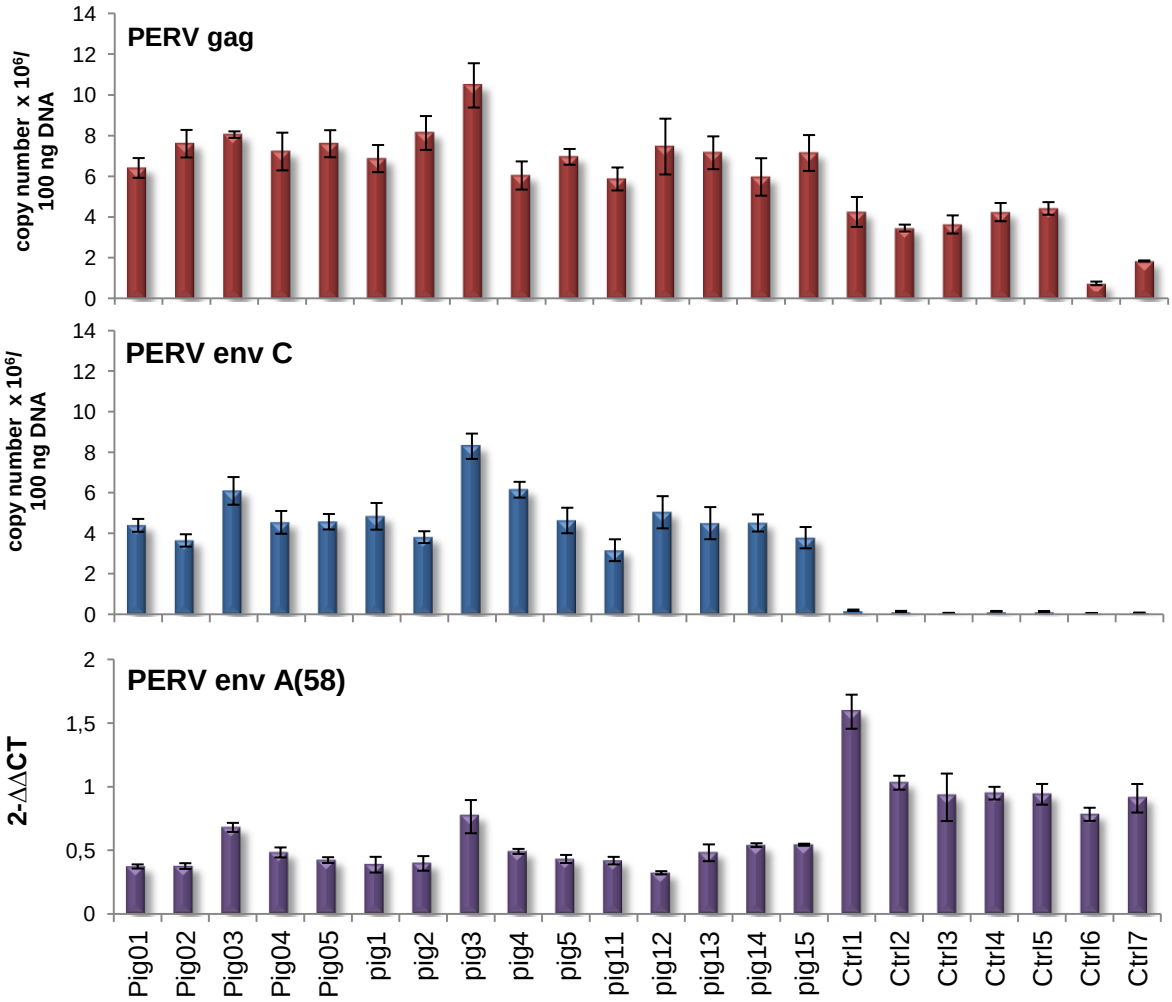


Figure 9. PERV prevalence in Göttingen Minipigs quantified by real-time PCR.

Detection and quantification of PERV proviruses in the genome of 15 Göttingen minipigs (three groups of five animals each, animals 1 to 5 correspond to the animals shown in Figure 8) and seven

German landrace pigs (Ctrl 1-7) by real-time PCR specific for gag, env-C and env-A(58). Results of PERV gag and PERV env-C are given as copy numbers of proviruses. PERV env-A(58) were calculated according to the $2^{-\Delta\Delta Ct}$ method. The standard deviation is based on triplicate measurement.

As expected, the expression of PERVs increased after PHA-stimulation of PBMCs and was higher after 5 days of stimulation then after 3 days stimulation, and always higher compared to PBMCs incubated in medium. These results were in concordance with earlier measurements of PERV expression done for German landrace and Schwäbisch-Hall pigs, however, not as high as the expression described for Yucatan minipigs Table [217, 218].

4.1.2.4 Expression of PERVs in Göttingen minipigs

The analysis of PERV expression on RNA level was performed using real-time PCR specific for PERV-pol, which detect the full-length mRNA of all PERV subtypes and PERV-Cnv, which is able to measure the full-length and spliced PERV-Cnv on mRNA level. The total RNA analyzed was obtained from fresh PBMCs as well as PBMCs stimulated or not with PHA for 3 or 5 days (Figure 10). As expected, the expression of PERV increased after PHA-stimulation of PBMCs and was higher after 5 days of stimulation then after 3 days stimulation, and always higher compared to PBMCs incubated in medium.

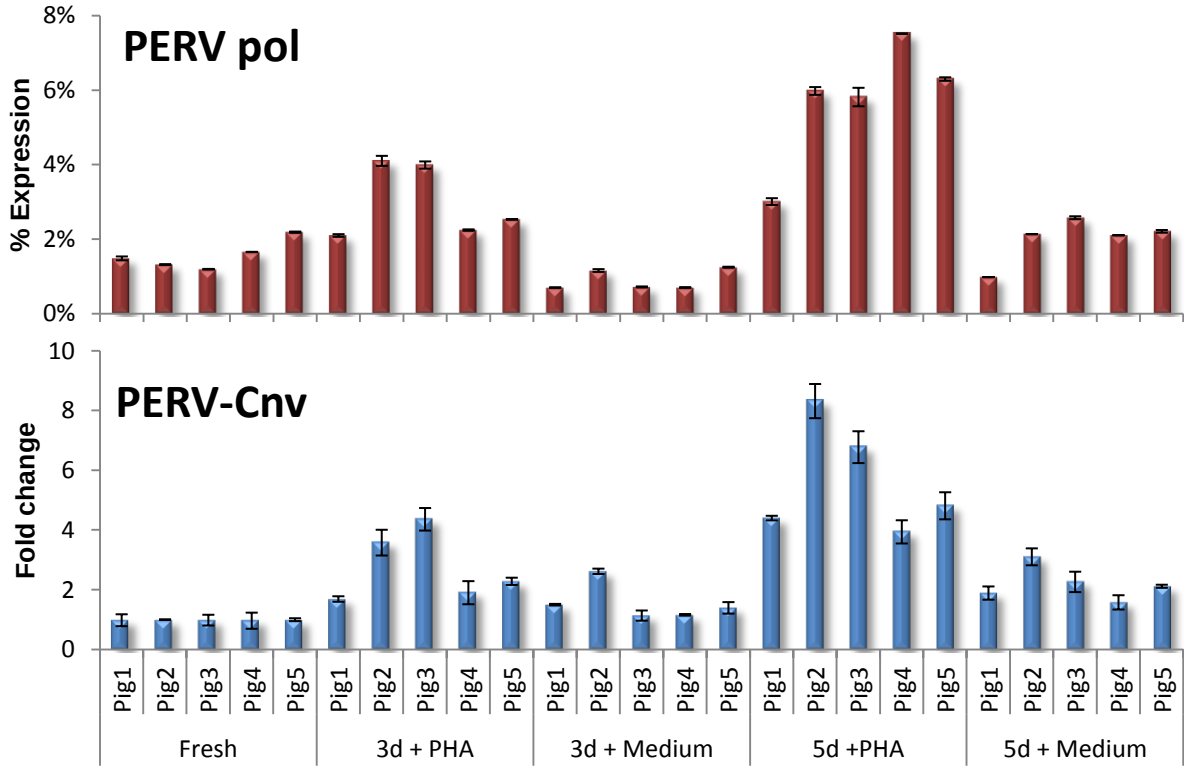


Figure 10. PERV expression in Göttingen minipigs.

Expression of PERV in PBMCs from five Göttingen minipigs (1 to 5). Real-time PCR specific for pol gene allowed measurement of the expression of all PERV subtypes. PERV-Cnv specific real-time PCR was used to measure the expression of only this PERV subtype. RNA was obtained from fresh PBMCs and PBMCs incubated in medium alone or medium with PHA for 3 or 5 days. Values were calculated after the $2^{-\Delta\Delta Ct}$ method using the expression of the housekeeping gene porcine cyclphilin for the normalization. For PERV-pol, values were presented in comparison to PERV pol expression in PK15 cells, which was set 100%. For PERV-Cnv, the expression in freshly isolated PBMCs.

Table 7. Expression of PERVs in different pig breeds.

		Expression (% of PK15)	
Pig breed	n	Freshly isolated	5 days with PHA
Göttingen minipig	5	1.56 ± 0.02	5.72 ± 0.09
German landrace (GL)*	8	0.40 ± 0.10	nd
Schwäbisch-Hall*	9	0.83 ± 0.23	nd
Duroc/GL*	10	0.28 ± 0.09	nd
Large White*	3	3.07 ± 1.16	4.88 ± 1.57
Yucatan micro pig*	1	10.48 ± 5.28	92.58 ± 25.85

*Data from [218]. Nd, not done.

These results were in concordance with earlier measurements of PERV expression done for German landrace and Schwäbisch-Hall pigs, however, not as high as the expression described for Yucatan minipigs Table 7 [217, 218].

4.1.2.5 Absence of virus particle release from PHA-stimulated PBMCs

In order to analyze the release of infectious viral particles, human 293 cells were co-incubated with PBMCs of 5 different pigs or incubated with cell-free supernatant from PBMCs treated or not with PHA for 5 days. To avoid microchimerism, which could be caused by a possible contamination of the 293 cells by porcine cells or DNA, 293 cells were splitted for 3 times after incubation to remove remains of porcine DNA and cell culture inserts with 0.4 µm pore size membrane were used for co-incubation. If released, viral particles may diffuse through the pores and infected the adherent 293 cells. The infection was measured by specific PCR performed with DNA isolated from the treated 293 cells.

To assess the contamination of 293 by porcine DNA, PCR specific for porcine beta-actin and GAPDH were carried out and all results were negative. PCR results revealed no proviral DNA in all samples, 293 with supernatant, co-incubation with PBMCs and each with (Figure 11) and without PHA stimulation (not shown), indicating that no infection with human-tropic PERV viruses occurred. Cultures infected with the PERV-A/C were used as positive control.

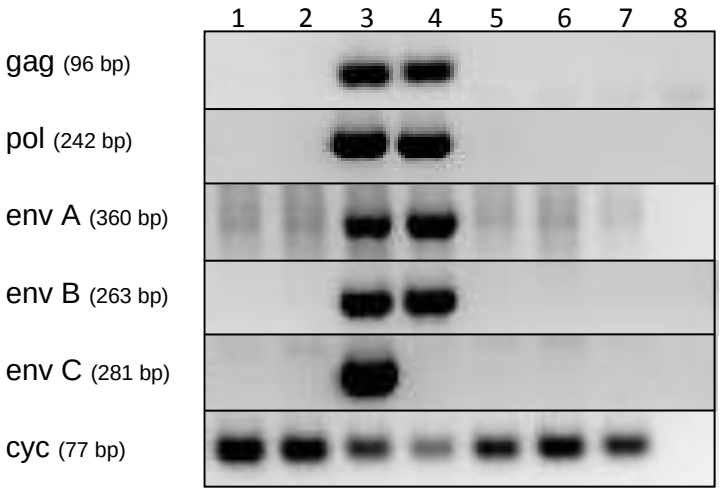


Figure 11. Infection of 293 cells with supernatant of Göttingen minipigs PBMCs.

Detection of PERVs proviruses in DNA of 293 cells was performed by PERV-specific PCRs. PCR using porcine cyclophilin primers was used as control. 1, 2 and 7 correspond to DNA of 293 cells incubated with supernatant of PBMCs from pigs 1, 2 and 3 respectively. 3, PBMCs from pig1. 4, PK15. 5 and 6 293 cells incubated with medium only as negative control. 8, non-template control.

4.2 Reduction of PERV expression with siRNA

In earlier works several synthetic siRNA targeting different parts of the PERVs genome were tested among them the so called pol2 siRNA. The pol2 siRNA target a high conserved region within the PERV-pol gene and is identical in PERV-A, PERV-B and PERV-C. Furthermore, it was proven to be the most effective in reducing the PERV expression of all three virus types when tested in PK15 cells [173, 174]. In following studies stable cell lines were generated which expressed the pol2 as shRNA, using the lentiviral system pLVTHM for transduction of porcine fetal fibroblasts. These cells were then used for the generation of transgenic pigs by SCNT, where the pol2 shRNA was shown to be integrated in the genome and to be expressed in all cells and tissues of the animals. The expression of PERV as well as the pol2 shRNA in these transgenic pol2-pigs was monitored over a maximum of 6 months [126, 175]. However a long term effect of the RNA interference would be necessary for xenotransplantation in human patient. Therefore the expression of the pol2 shRNA and the PERV expression were studied over a period of 3 years, after that animals were sacrificed and organs probes were analyzed in detail for PERV and shRNA expression.

4.2.1 Long-term effects of PERV specific siRNA in transgenic pigs

4.2.1.1 Generation of transgenic pigs

In previous studies pol2 siRNA was selected as most efficient in reducing PERV expression in PK15 cells and was expressed as shRNA under the control of a H1 promoter in the lentiviral vector pLVTHM [126]. Transgenic porcine foetal fibroblasts (P1F10) transduced with pLVTHM-pol2 constructs as well as fibroblasts transduced with empty pLVTHM vectors as control were used for the generation of transgenic pigs using SCNT as described earlier [175].

The generated transgenic pigs and their numbers are presented in Table 8. The control clones 140 and 142 and the shRNA expressing clones 147 and 148 survived, while control clones 136, 137 and 139 died early after birth. Later cells from clone 287 were recloned to generate the animal 287. The integration of the vectors was proved by PCRs specific for *egfp* and the shRNA expression cassette [126]. GFP was expressed in all animals and GFP expression was monitored at different time points for 3 years by analyzing ear fibroblasts cultures isolated from the transgenic animals [194, 196] as well as from PBMCs (Appendix 4) using fluorescence microscopy and FACS analysis.

Table 8. Transgenic animals obtained by somatic cell nuclear transfer

PFF*	Vector	Transgene	Nuclear transfer	Birth date	Clone number
P1F10	pLVTHM GFP		14.11.2007	10.11.03.2008	136.137.139.140.142
P1 F10	pLVTHM GFP Pol2	shRNA Pol2	14.11.2007	12.03.2008	147,148
Recloning†	pLVTHM GFP Pol2	shRNA Pol2	20.11.2008	15.03.2009	287

(*) PFF=porcine primary fetal fibroblasts; (†) clone 287 was recloned from cells of clone 148.

The expression of GFP was higher in cell lines from GFP-control animals compared to cells from animals expressing the pol2 shRNA. Measurements of PERV mRNA expression didn't reveal a significant difference between fibroblasts isolated from pLVTHM-control animals and shRNA transgenic animals [194]. The expression of shRNA had no visible influence on the animals and both groups of animals were healthy during the whole time.

4.2.1.2 Inhibition of PERV expression in PBMCs at different time points

In order to assess long-term efficiency of the shRNA-mediated knockdown of PERV expression, blood samples from shRNA transgenic pigs and control pigs were taken at five different time points. PBMCs were prepared and RNA was isolated from fresh PBMCs as well as from PBMCs incubated with or without PHA stimulation for 5 days (Figure 12).

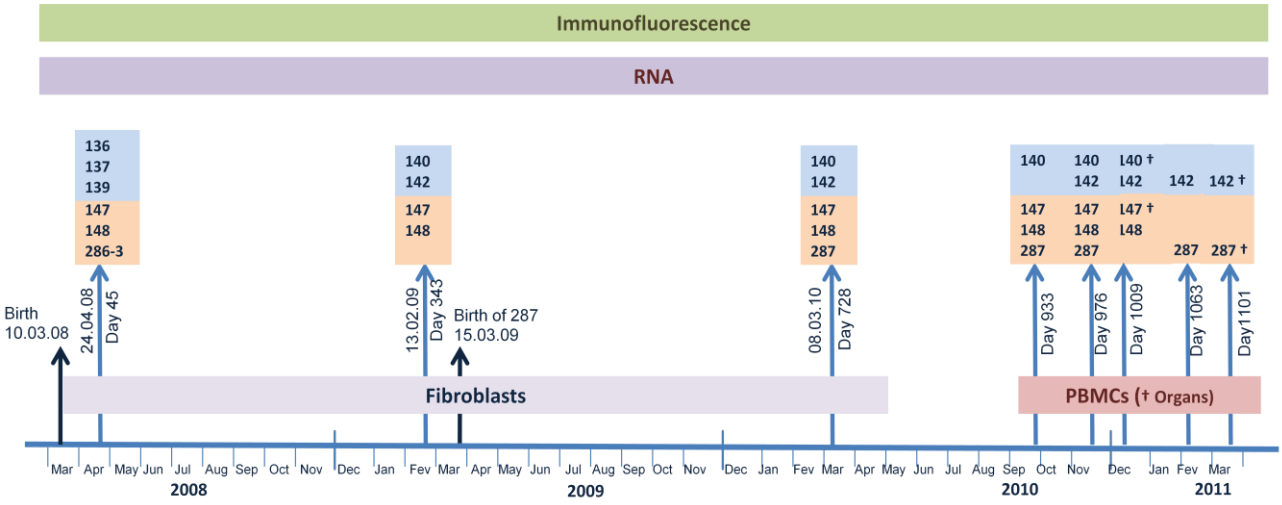


Figure 12. Long-term analysis of the efficacy of RNA interference, experimental design.

Time schedule of the experiment. The control pigs (blue background) were generated from P1F10 fibroblasts transduced with empty vector pLVTHM. The shRNA pigs (orange background) were generated from pLVTHM-pol2 transduced P1F10 cells. Birth and scarification times as well as time

points of taking ear biopsies, PBMCs and organs for PERV and shRNA expression analysis are indicated.

PERV expression was analyzed by real-time PCR. Previous results revealed a fluctuation of the expression of different housekeeping genes in various organs and porcine cyclophilin showed the lowest variation in its expression [175]. Therefore expression of PERV was normalized to the amount of total RNA and copy numbers were calculated using the Ct values and a serial dilution of a plasmid containing the pol sequence of PERV with known concentrations. The expression of PERV was shown to be lower in all shRNA transgenic pigs when compared to control animals (Figure 13A). This reduction was constant during the 3 years of the life span of the animals. These results were also confirmed when PBMCs were stimulated with PHA, which induce the PERV expression. Expression of PERV in PHA stimulated PBMCs of shRNA-animals was lower when compared to PHA-stimulated control PBMCs.

4.2.1.3 Expression of the pol2 shRNA and PERV in different organs

All control and shRNA transgenic animals save clone 148 which was still under long-term observation at the time, were sacrificed and organ samples were obtained. RNA was isolated and PERV mRNA and shRNA expressions were measured. Using the siRNA specific PCR all organs showed a high level of pol2-siRNA expression and organs of clone 147 showed higher level of siRNA expression than animal 287. The spleen showed the highest siRNA expression with 886492 pol2 siRNA copies/ng total RNA in clone 147 and 704020 copies/ng in clone 287. No siRNA expression was observed in the control animals.

Organs were also tested for PERV expression on mRNA level. PERV expression was inhibited in all organs of the shRNA transgenic pig by up to 65% when compared to control animals. And the highest PERV copy number was reserved for spleen with 90659 and 72974 PERV copies/ng of total RNA from vector control animals vs. 28681 and 30000 copies/ng in spleen of the pol2 shRNA animals 147 and 287 respectively. The lowest PERV expression were observed in the heart with 30739 and 31757 copies/ng in vector control animals vs. only 12898 and 1323 copies/ng for shRNA animals (Figure 13B.).

4.2.1.4 PERV protein expression

In order to investigate whether the shRNA could reduce the PERV protein expression Western blot analysis were performed using antisera specific for the transmembrane envelope

protein p15E and the major core protein p27Gag of PERV. Expression of PERV proteins in both control and shRNA transgenic groups was under the detection limit of the assay (data not shown) indicating that in both cases, infection virus particles could not be released.

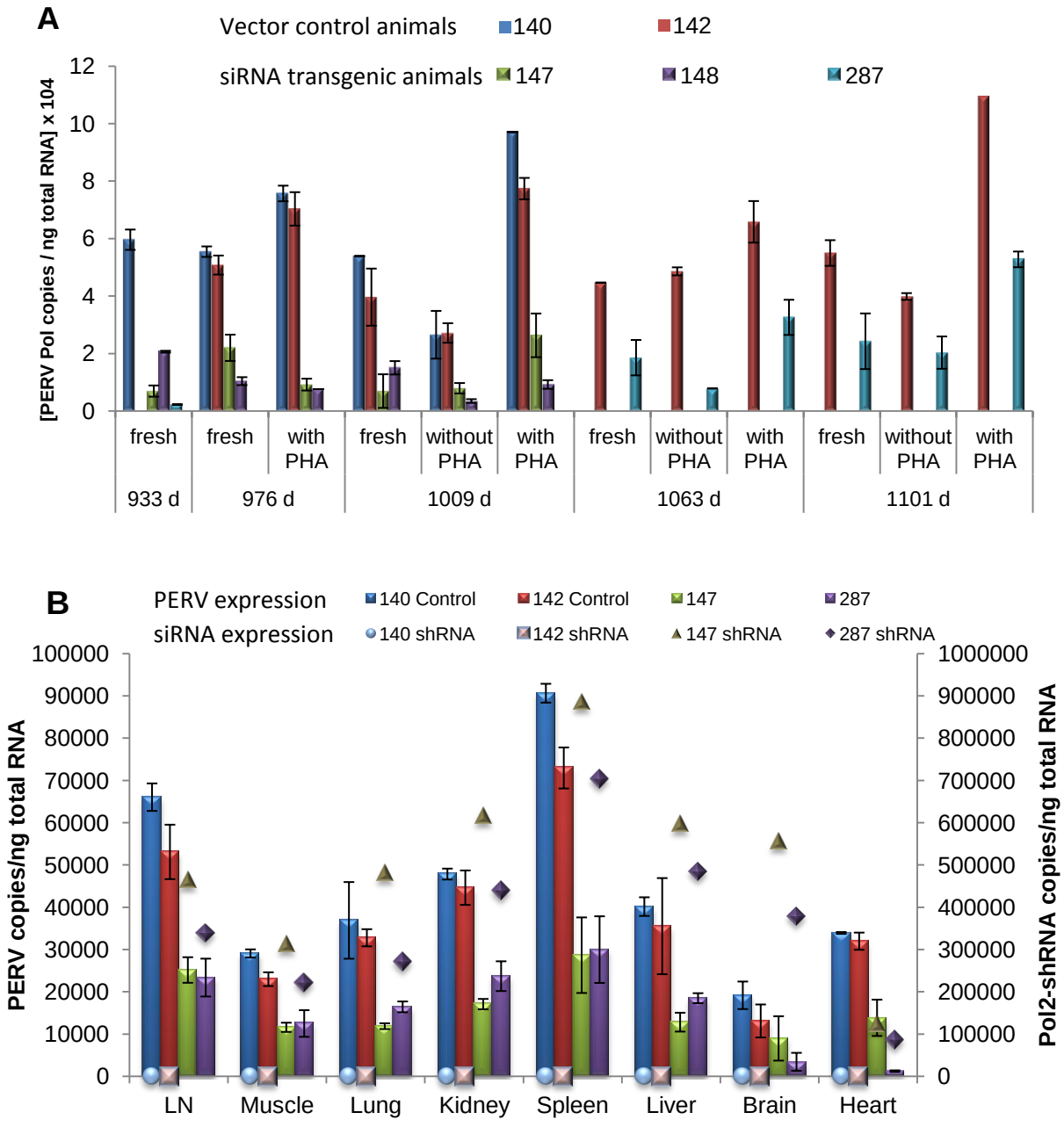


Figure 13. Inhibition of PERV expression in PBMCs of pol2-shRNA transgenic pigs.

(A) PERV expression in fresh and incubated PBMCs with or without PHA stimulation. PBMCs were isolated from control animals and shRNA transgenic animals at 5 different time points. Total RNA was isolated and PERV expression was measured using PERV specific real-time PCR. Values were calculated directly from the Ct values normalized to ng total RNA. All measurements were taken in triplicates and standard deviations are shown. Animals 140 and 147 were sacrificed at day 1009. (B)

Expression of pol2 shRNA (spots) and inhibition of PERV expression (columns) in different organs of the pol2-shRNA transgenic animals 142, 147 and 287 and the vector-control transgenic animals 140 and 142. Pigs were sacrificed and total RNA was isolated from different tissue samples. PERV pol expression and shRNA expression were measured using specific real-time PCR. All tissues of shRNA transgenic animals expressed shRNA but at different levels and showed a reduction of PERV expression when compared to vector-control animals. Standard deviation is based on triplicate measurements.

4.2.2 Generation of triple siRNA transgenic cells

In earlier works done in our and other groups, several siRNA targeting different parts of the PERV genome were designed using different algorithms. These siRNAs were tested and among the most powerful in decreasing the PERV expression in PK15 cells were the pol2 [173], pol1[196] and gag2 siRNAs (group of Dr. L. Scobie, Glasgow). Transfection of PK15 cells with pol2-, pol1- and gag2-shRNA expressing vectors showed a high PERV mRNA reduction till 38%, 10% and 23%, respectively [196]. Furthermore vectors expressing double siRNAs under the control of H1 promoters were generated and tested and pol1/pol2 vectors reduced the PERV expression to 20% when total mRNA of transfected PK15 was tested [196]. In order to enhance the efficacy of siRNAs in reduction the PERV expression, triple-shRNA vectors were produced, which express the 3 most efficient siRNAs under the control of three different polymerase III-dependant promoters and using the pSiren vector which allow a puromycine selection as well as the pLVTHM vector with GFP as selection marker.

4.2.1.5 Preparation of shRNA expression constructs

In a first step the 7SK promoter was PCR amplified from human genomic DNA (see primers in Material section). The designed 7SK primers contained restriction sites so that the PCR amplicon was flanked by BamH1 and EcoRI on the 5’ side and BbsI, XhoI and MfeI on the 3’ end. The 7SK amplicon was then inserted in a H1 containing pSuper-MfeI vector [196], upstream of the H1 promoter by digestion ligation with the BamH1/EcoRI (Figure 14). Synthetic shRNA were designed each as sense and antisense oligopeptides using a 7 nt loop for the pol2 shRNA and a 4 nt loop for pol1 and gag2. Two groups of shRNA were designed: The hybridization of the first group resulted in overhangs which fit into BbsI and XhoI restriction sites specific for the 7SK primers, shRNA of the other group were flanked by BglII/BamHI and EcoRI/MfeI overhangs suitable for insertion downstream the H1 and U6 promoters. ShRNAs were hybridized and added after the 7SK promoter then H1. The double

cassettes (7SK shRNA1-H1 shRNA2) were then fished up by BamHI/MfeI digestion and inserted downstream the U6 into a pSiren vector. Following, shRNAs were inserted downstream the U6 promoter. The triple-shRNA cassette was also cut from the pSiren plasmid and inserted into a pLVTHM replacing the original H1 promoter. In the end for each vector, 5 different combinations of promoters/shRNA were constructed (Table 9) using T1-6 for pSiren vectors and pT1-6 for pLVTHM vectors. T5 and pT5 sequencing showed several mutations and were not used in further experiments.

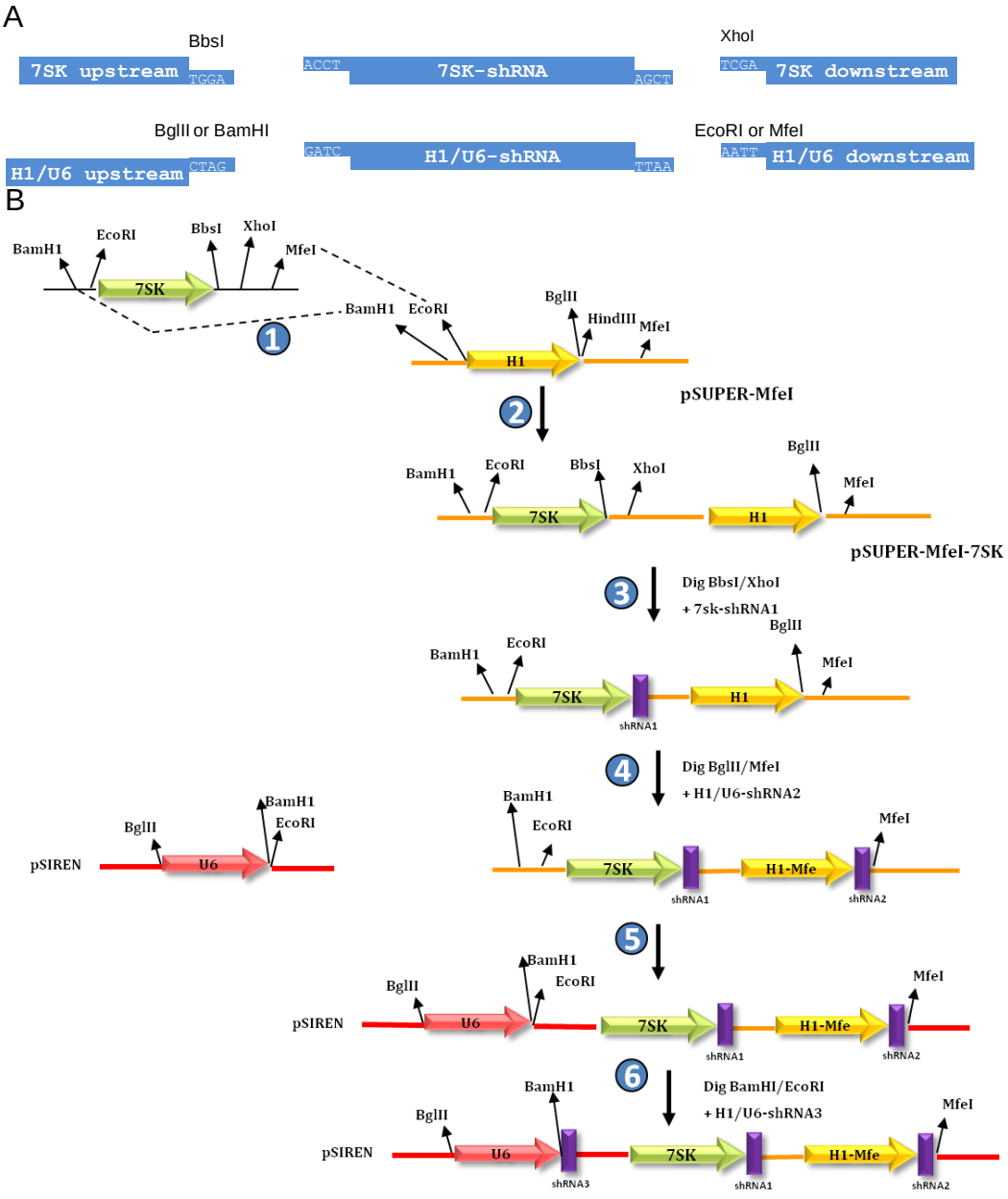


Figure 14. Cloning strategy of triple shRNA vectors.

A Different types of shRNAs with overhangs specific for insertion after 7SK, H1 or U6 promoters. B Different steps of cloning yielding the triple-shRNA cassettes (for more details see text).

Table 9. Triple shRNA constructs based on pSIREN and pLVTHM backbones.

pSIREN puro	pLVTHM GFP	shRNA		
		U6	7SK	H1
T1	**pT1**	pol2	pol1	gag2
T2	**pT2**	pol2	gag2	pol1
T3	**pT3**	gag2	pol2	pol1
T4	**pT4**	pol1	pol2	gag2
T6	**pT6**	gag2	pol1	pol2

All constructs were sequenced and transfection tests with 293 cells were performed after each step in order to assess whether the constructs were functional. The pLVTHM-transfected 293 cells were observed on fluorescence microscope for GFP expression and pSiren-transfected 293 cells were exposed to puromycin selection. The functionality of the promoters was assessed by measuring the pol2 expression by real-time PCR (Data not shown).

4.2.1.6 Establishment of one-step real-time PCR for siRNA quantification

In an earlier work, a real-time PCR for the quantification of pol2 siRNA was established [194, 196]. In the present work we tried to establish new real-time PCR specific for pol1 and gag2 siRNAs. For this purpose we used stem-loop RT primers containing at the 3' side an overhang, which can bind specifically to the 3' ends of the siRNAs (Figure 15A). The real-time PCR consisted into a first step of reverse-transcription of the siRNA using the Super-Script III system (Invitrogen). A heating step led to opening of the stem-loop/siRNA, which served as template for a conventional PCR using a forward primer specific for the siRNA, a reverse primer specific for the stem-loop and a FAM-labelled probe (Figure 15B). To measure the copy numbers of the siRNAs using the described real-time PCRs, serial dilutions of synthetic pol1 and gag2 siRNAs with known concentrations were performed. The newly established real-time PCRs functioned when using synthetic siRNAs (Figure 15C), unfortunately when using the total mRNA isolated from transfected cells, the real-time PCRs couldn't detect the pol1 and gag2 siRNAs, while pol2 siRNA was detected in all tested vectors. In other words, pol2 siRNA was expressed under the control of all 3 promoters used, which is a proof that the promoters were functioning well. However, the predicted sequences of the synthetic siRNA used for pol1 and gag2 might not correspond to the siRNAs which results after the processing of the pol1-and gag2-shRNA with the DICER *in vivo*. Therefore only pol2-specific real-time PCR was used.

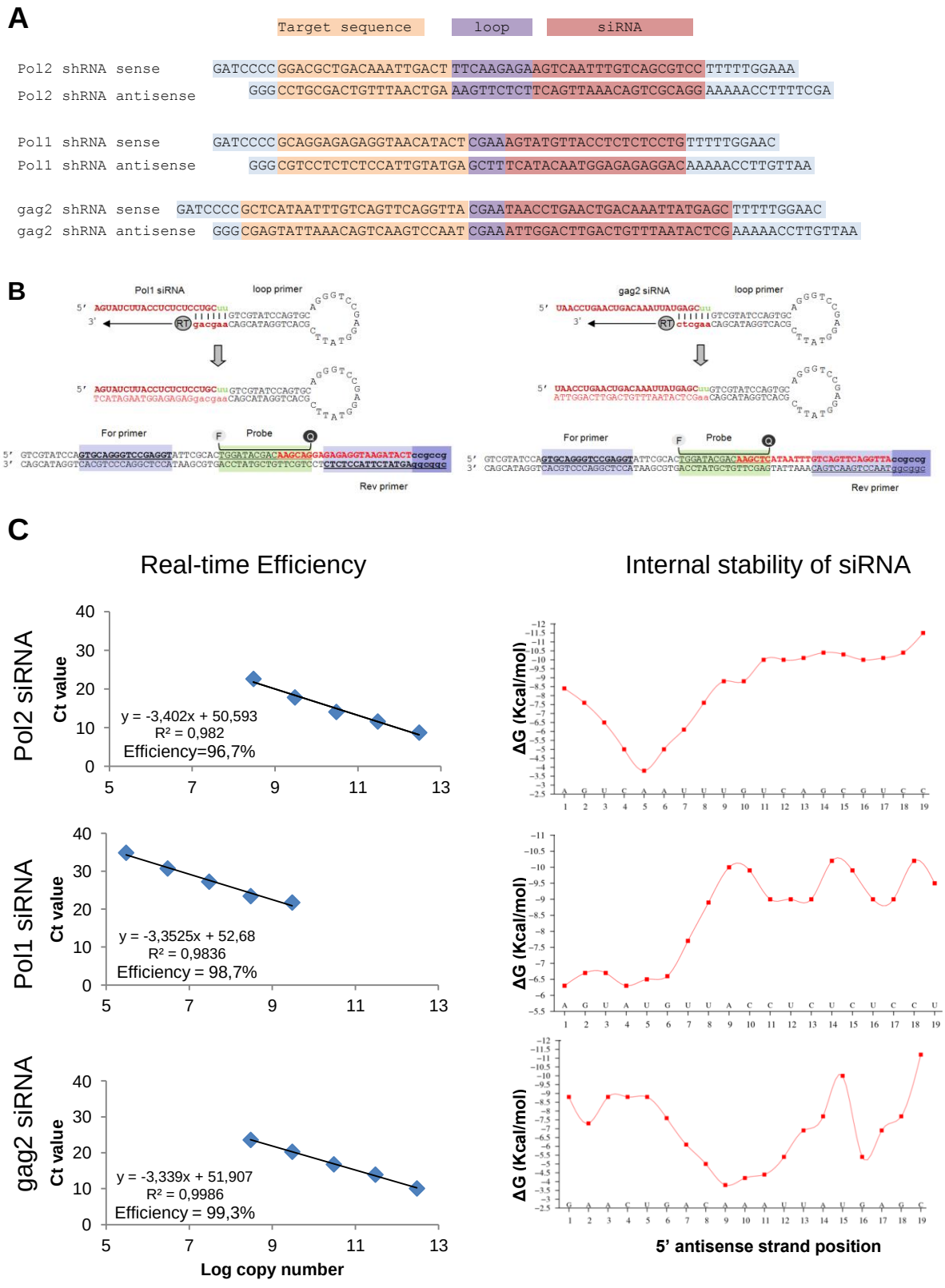


Figure 15. Establishment of shRNA specific real-time PCR.

(A) Design of the shRNAs, here are only the 7SK-shRNA represented. (B) Description of the two-step real-time PCR developed for quantification of the PERV-specific siRNA. In a first step, a loop primer hybridizing to the 3' portion of the siRNA was added and a reverse transcription was

performed. This step is followed by conventional real-time PCR using primers specific for the shRNA and the loop and a FAM-labeled probe. (C) The efficiency of the real-time PCR of each siRNA as well as the characterization of the internal stability profiles of each siRNA for target positions 1-19.

Several Studies showed that asymmetry in the internal stability of the siRNA duplex may play a role into the selection of the guide siRNA strand and the enhancing of the RNA interference [219, 220]. In order to investigate the thermodynamic profiles of the used PERV siRNA, their internal stability was calculated using an Sfold software [221]. After cleavage of the shRNA loop-hairpin, the RISC complex binds to the siRNA duplex and select one of both strand (the guide) to be the functional siRNA while the antisense strand (the passenger) is degraded. It was shown that the strand showing the lowest internal stability on its 5' terminus and in the region of 10-14, binds weakly to its complement and thus survive as guide strand [220]. The profiling of pol2 and pol1 siRNAs showed a decreased internal stability of the 5'-terminus in comparison to the 3'-end; however this was less marked for gag2 siRNA, which on the contrary exhibited a greater instability in the region between 8-13 (Figure 15C).

In order to test the constructs and the efficacy of the RNA interference, PK15 cells were nucleofected as described (see Methods).

4.2.1.7 Reduction of PERV expression in PK15 using T and pT constructs

The first tested constructs were the T vectors with pSiren as backbone. PK15 were transfected and subjected to puromycin selection (0.5, 0.6 or 0.7 µg/ml). Total mRNA was isolated and PERV-pol real-time PCRs were carried out to measure the reduction of PERV expression by the shRNA constructs. A reduction of PERV expression in comparison to mock transfected cells was observed (Figure 16A). This reduction was 68% in T2-PK15, 66% in T3-PK15, and 67% in T6-PK15 cells while Pol2-pSiren reduced the PERV expression of 56%. However, these results were not better than the results described in earlier works when using for example the pLVTHM system. Furthermore, the additive efficacy of siRNA in T vectors didn't show a significant improvement in reduction of PERV expression when compared to the Pol2-pSiren.

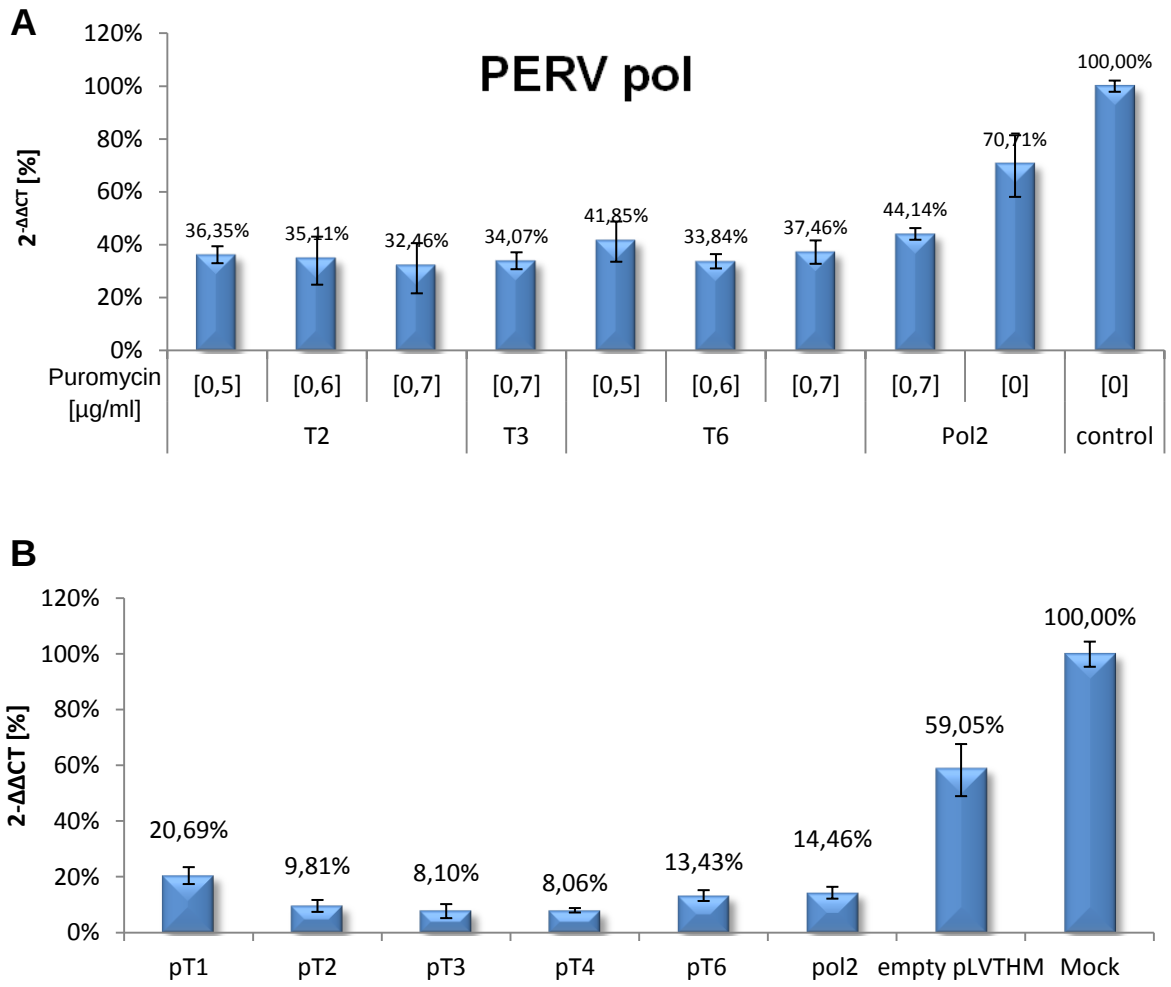


Figure 16. Reduction of PERV expression in PK15 transduced with T or pT vectors.

(A) PK15 cells were transfected with T2, T3 and T6 plasmids as well as Pol2 and Mock controls. Cells were then subjected to puromycin selection using different concentrations. RNA was isolated and pol-specific real time PCR was performed. Results were calculated according to the 2-ΔΔCT method using cyclophilin for normalization, and presented as percentage of the control value fixed to 100%. Standard deviations were calculated for triplicates. **(B)** Analysis of PERV-pol expression in pT-transduced PK15. A pol-specific real-time PCR was performed and gene expression is presented as percentage calculated in relation to mock-transfected cells after normalization against porcine cyclophilin. As control PK15 cells were transfected with an empty pLVTHM plasmid.

Therefore, pT vectors based on the pLVTHM backbone were also tested in PK15 cells. Two days after nucleofection GFP-expressing cells were sorted by a FACS sorter. Total mRNA was isolated from the sorted cells and a PERV-pol real-time PCR was performed (Figure 16B). The PERV expression was significantly reduced in all pT-PK15 cells (<50%). The greatest reduction was observed in pT3- and pT4-PK15 cells, where expression of PERV was reduced to only ca. 8% of the expression in mock-transfected PK15. The Pol2-pLVTHM reduced the expression of PERV to 14,46% which corresponded to the range of values

measured in earlier works [196]. The pT1 vector showed the lowest reduction rate with 20.69% only. This showed that the use of the pLVTHM pT plasmids yielded better results than when pSIREN vectors were used. However the reduction of PERV by triple-shRNA vectors didn't show a significant reduction in comparison to single Pol2-shRNA when total mRNA was tested.

It is well known that the processing of shRNA as well as the whole RNA interference activity take place within the cytoplasm of the cells. By measuring the reduction of PERV expression, total mRNA was used as template. That raised the question about the contribution of the intact nuclear RNA into masking the real efficacy of the shRNAs into reducing the cytoplasmic mRNA. For this reason, total, nuclear and cytoplasmic RNA were isolated as described in the method section and used for pol-specific real-time PCR measurement. In all reactions 100 ng RNA were used as template. Results are presented as percentage of copy number relative to the mock transfected PK15 cells for the 3 different groups (total, nuclear or cytoplasmic RNA) (Figure 16).

A greater reduction in cytoplasmic mRNA of pT-PK15 cells was observed when compared to total and nuclear RNA (Figure 17). While pol2 alone reduced the expression of cytoplasmic PERV mRNA to ~ 14%, better scores were reserved for pT3 and pT4 with only 4% PERV mRNA detected in cytoplasm in comparison to cytoplasm of mock-transfected PK15 cells. The pT2 and pT4 reduced the expression to ~5% and pT1 to only 14%. The empty pLVTHM control vectors showed an insignificant reduction compared to the mock-PK15 in all tested samples. When 2-ΔΔCt values were used standard deviation were calculated using the following equation:

$$\textbf{Positive error: } 2^{-\Delta\Delta Ct} - (1+E)^{-(\Delta\Delta Ct + SD_{\Delta Ct})}$$

$$\textbf{Negative error: } 2^{-\Delta\Delta Ct} - (1+E)^{-(\Delta\Delta Ct - SD_{\Delta Ct})}$$

Were E represents the efficiency of the real-time PCR calculated from a serial dilution of plasmids containing the target sequence with known concentration.

Standard deviations of percentages based on copy number were calculated using the following equation:

$$SD = \frac{100}{Mc^2 \times \sqrt{(St^2 \times Mc^2 + Sc^2 \times Mt^2)}}$$

Where SD: standard deviation, Mc and Sc: Mean and SD of control resp., Mt and Sc mean and SD of sample.

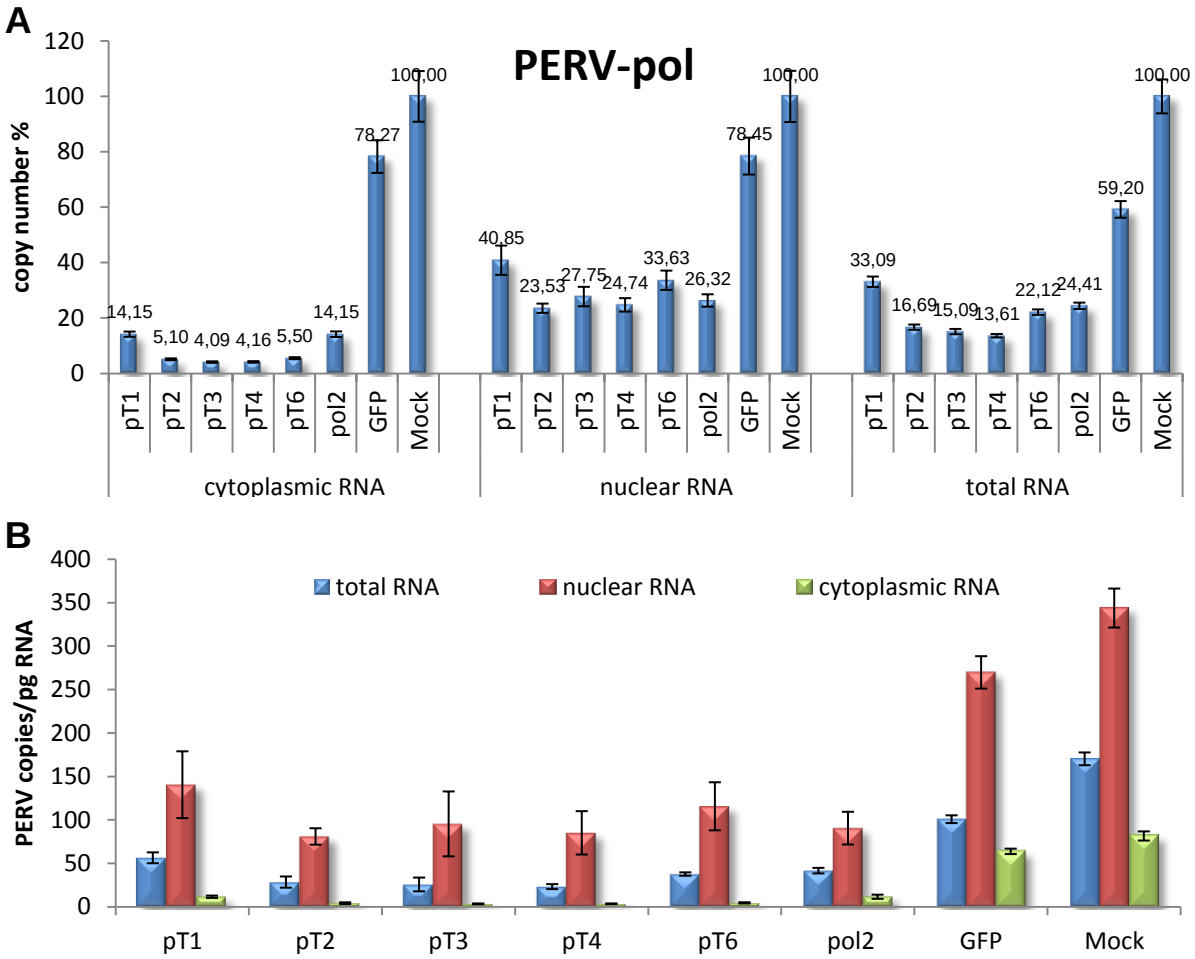


Figure 17. Reduction of the PERV-pol gene expression in cytoplasm and nucleus of pT transduced PK15 cells.

Cells were transfected by different pT vectors and control plasmids (GFP corresponded to an empty pLVTHM vector). Fluorescent cells were selected by FACS sorting and total RNA, nuclear RNA and cytoplasmic RNA were isolated and used for pol-specific real-time PCR. Copy numbers were calculated using a serial dilution of a plasmid containing the pol sequence with known concentrations. (A) Results are presented in percentage relative to the mock-PK15 RNA. (B) Results are presented as absolute copy numbers per pg RNA.

The table 10 shows the same results but as copy number per pg RNA. The ratio of total PERV mRNA to cytoplasmic and nuclear RNA was calculated for each sample. These results helped to see how the PERV mRNA copy numbers tended to shift towards nuclear RNA in pT-transfected vectors revealing thus a reduction of PERV in the cytoplasm of shRNA-cells. For example the calculated (total: nuclear: cytoplasmic RNA) ratio of Mock-PK15 was (2:4:1) while that of pol2-PK15 (4:8:1) and pT3 (8:29:1). These results presented an improvement in the use of shRNA when compared for examples to earlier results where double shRNA in pSiren were used [196].

Table 10. PERV mRNA expression in different RNA fractions.

plasmid	PERV copies/ng RNA			Ration (total:nc:cyt)
	total RNA	nuclear RNA	cytoplasmic RNA	
pT1	56	140	12	5:12:1
pT2	28	81	4	7:19:1
pT3	26	95	3	8:29:1
pT4	23	85	3	7:25:1
pT6	38	116	4	8:26:1
pol2	42	90	12	4:8:1
GFP	101	270	64	2:4:1
Mock	170	344	81	2:4:1

4.2.1.8 Generation of triple-shRNA expressing porcine fetal fibroblasts (PFFs)

In order to produce a new generation of shRNA pigs expressing triple-shRNA, PFFs were transduced with pT2, pT3, pT4 and pT6 as well as empty pLVTHM as control. A pool of shRNA-PFFs as well as GFP expressing cells were sorted by FACS sorter (Figure 18) and sent to the Friedrich-Löffler-Institute (Neustadt, Mariensee) for SCNT.

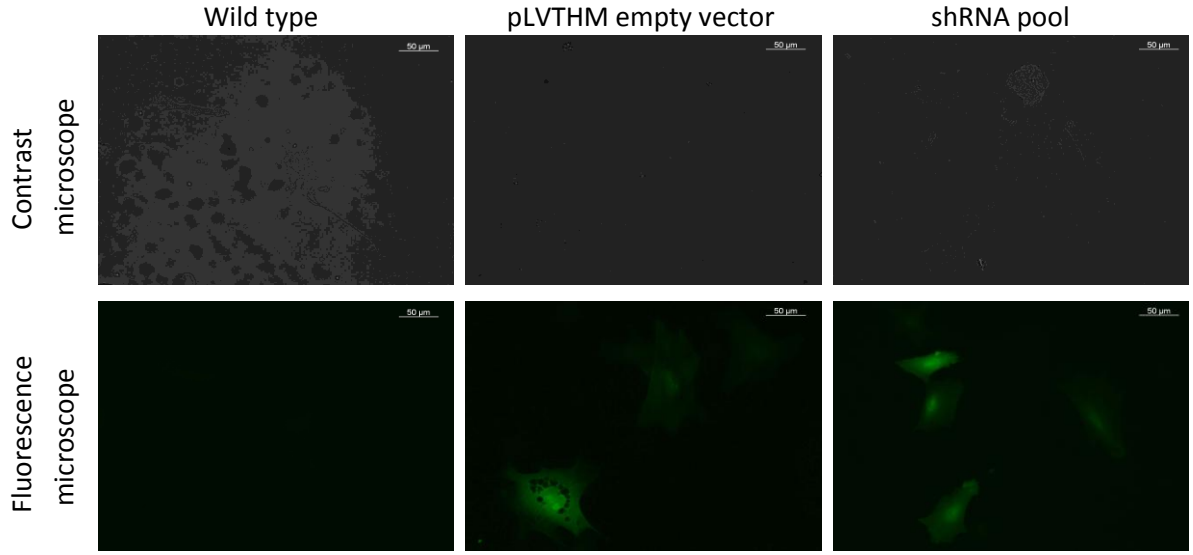


Figure 18. Fluorescence microscopy of shRNA- expressing PFFs.
Fluorescence microscopy of PFFs transfected with empty pLVTHM control vectors or with pol2-plVTHM or different pT plasmids pooled and sorted by FACS.

To summarize, new plasmids expressing three different PERV specific siRNA at the time and under the control of three different promoters were generated. These new constructs were tested on PK15 cells and showed a significant improvement of the PERV expression reduction when compared to single shRNA plasmids. Porcine fetal fibroblasts were stably transfected with the new constructs and can be used for the generation of new transgenic pigs by SCNT.

4.3 Knock-out of PERV by Zinc Finger Nucleases

4.3.1 Design of Zinc Finger Nucleases targeting the PERV gene

Zinc finger nucleases targeting the PERV genes were designed, cloned and validated by Sigma-Aldrich (St. Louis, USA). Among 67 ZFN candidates targeting the *pol* gene of PERV, 10 ZFNs were found to target highly conserved regions of PERV *pol* gene when the sequences were aligned with seven different known PERV sequences (see table in Appendix 2). These 10 chosen candidates were then tested and the 3 most active ZFN pairs were selected and sent as plasmid DNA (see plasmid map in Appendix 5). The full binding sites of the ZFN pairs used are presented in Table 11. The lower case letters corresponds to the cutting site where the double-strand break was created.

Table 11. The three most powerful ZFNs and their target sequences.

ZFN	Target sequence	Position in PERV-A Accession nr. AJ293656
PZFN1/PZFN2 (Set 1)	CGCAAGGACCTTACAgacatACCGCTGACTGGAGAA	3957..3995
PZFN3/PZFN4 (Set 2)	AACATCGTTCGGCAGcccccAGACCGATGGATGAC	4405..4439
PZFN5/PZFN6 (Set 3)	GGCCCCAACCACAGCCAAacaagtGAGAGAGTTTTTGGG	4558..4592

4.3.2 Detection of ZFN expression by Western blot analysis

In order to analyse the expression of ZFN, 2 million PK15 cells per sample were nucleofected with plasmids of ZFN set 1 (0.1 µg, 0.5 µg, 1 µg, 2 µg or 7.5 µg each) and cells were harvested after 12h, 24h and 48 h. ZFN proteins are tagged with an N-terminal 3xFLAG.

To assess the expression of ZFN proteins an anti-flag Western blot assay was performed using total cell lysate and the anti-FLAG M2 antibody. SDS PAGE / Western blot analysis showed high ZFN expression which was proportional to the amount of ZFN plasmids used for nucleofection. However, the strongest expression was detected in cells after 12 h incubation and using 2 µg/ml plasmid. The expression decreased after 24h and 48h (Figure 19).

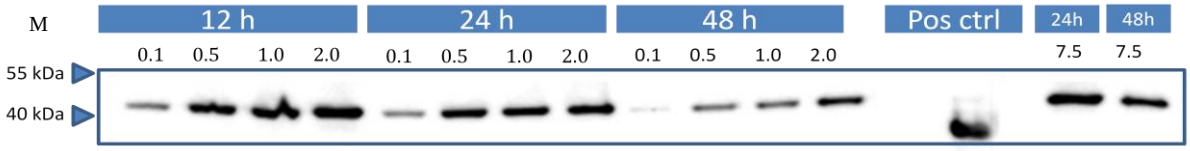


Figure 19. Expression of ZFN proteins in ZFN-nucleofected PK15 cells.

Western blot analysis of the kinetic of ZFN expression in PK15 cells nucleofected with different amounts of ZFN set 1 plasmids. Cell lysate of PK15 cells nucleofected with a plasmid expressing a flag containing protein was used as positive control. The anti-Flag M2 antibody (Sigma-Aldrich) diluted 1:500 was used as antibody. Lane M: size of the bands of the protein marker.

ZFN proteins contain also a nuclear localization signal (NLS) which allows the transportation of ZFN proteins to the nucleus where they can bind to the target DNA. In order to proof the nuclear localization of the left and the right ZFN proteins, cells were nucleofected with both ZFN1 and ZFN2 plasmids of set 1 together or separately. After 48 h incubation, cells were harvested and nuclear and cytoplasmic lysate were prepared using the Pierce NE-PER nuclear protein extraction kit (Figure 20). Bands corresponding to the ZFN proteins detected by anti-Flag antibodies were observed in all extracts but much stronger in the nuclear lysate. Therefore the extracts were also analyzed for cytoplasmic and nuclear contamination with β-actin antibody and the DEAD-box RNA helicase DDX3 antibody (see Materials section) as cytoplasmic and nuclear markers respectively. A negligible contamination of cytoplasmic extract was detected. In the case of ZFN2 lysates this contamination was stronger. Conversely, tracks of β-actin were detected in nuclear lysates, however it is unclear whether this is a contamination since it was shown that actin plays a physiological role in the nucleus [137].

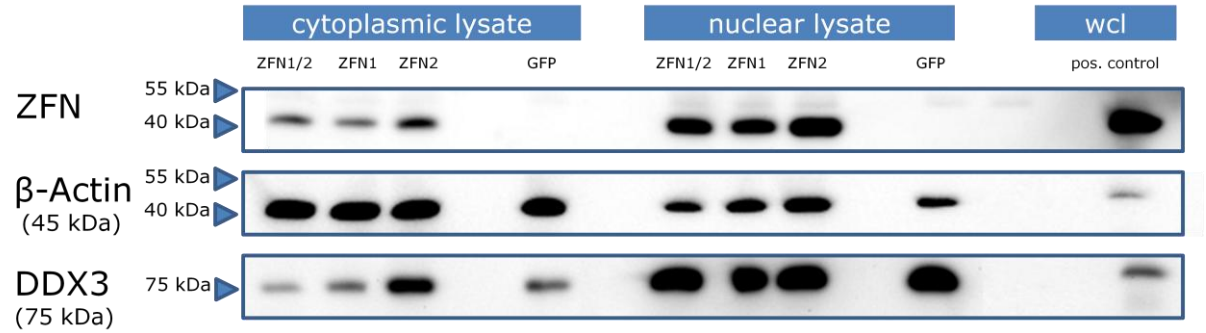


Figure 20. Detection of ZFN proteins in cytoplasmic and nuclear lysates.

PK15 cells were nucleofected with ZFN1 and ZFN2 plasmids together or separately and about 2 million cells per sample were fractionated. Each lane was loaded with nuclear or cytoplasmic extract from about 0.5 million cells. Whole cell lysate (WCL) from pCMV transfected PK15 cells was used as positive control (1 x 10^5 cells). Anti-Flag antibodies were used for detection by SDS-PAGE / Western blot analysis. Cytoplasmic and nuclear segregation was assayed by β-actin and DDX3, respectively.

ZFN proteins were highly expressed in the ZFN transfected cells as shown by the Western blot analysis. This expression reached a peak 2 days post-transfection. Analysis of nuclear and cytoplasmic lysates of ZFN transfected cells revealed the nuclear localization of the ZFN proteins.

4.3.3 Detection of ZFN expression by FRET

In order to investigate the expression of ZFN proteins and their localization in the transfected cells, Förster resonance energy transfer (FRET) imaging was used. PK15 cells were transfected with CFP and YFP linked to ZFN1 and ZFN2 respectively as well as fused YFP-CFP dimer or single plasmids coding for CFP and YFP as controls. In order to analyse the interactions of the fused ZFN and their localization, the cells were fixed 24 h post-transfection and FRET analysis was performed. The normalized FRET values (NFRET) were obtained from a set of three images (donor, acceptor and co-transfected donor and acceptor). In cells transfected with ZFN1-CFP and ZFN2-YFP the expressed proteins were localized predominantly in the nucleus and showed high NFRET values (Figure 21A), indicating that the ZFNs were transported to the nucleus and were localized in close proximity (less than 10 nm), suggesting that ZFN1-CFP and ZFN2-YFP were interacting. We can exclude that the fluorescent proteins CFP and YFP were associated in an unspecific manner, because in order to generate the plasmids pZFN1-CFP and pZFN2-YFP modified SCFP3A and SYFP2 templates were used to improve the brightness of the fluorescent proteins and their monomeric properties [280]. Both fluorescently tagged ZFN proteins strongly co-localize with DAPI staining the nucleus (Figure 21A).

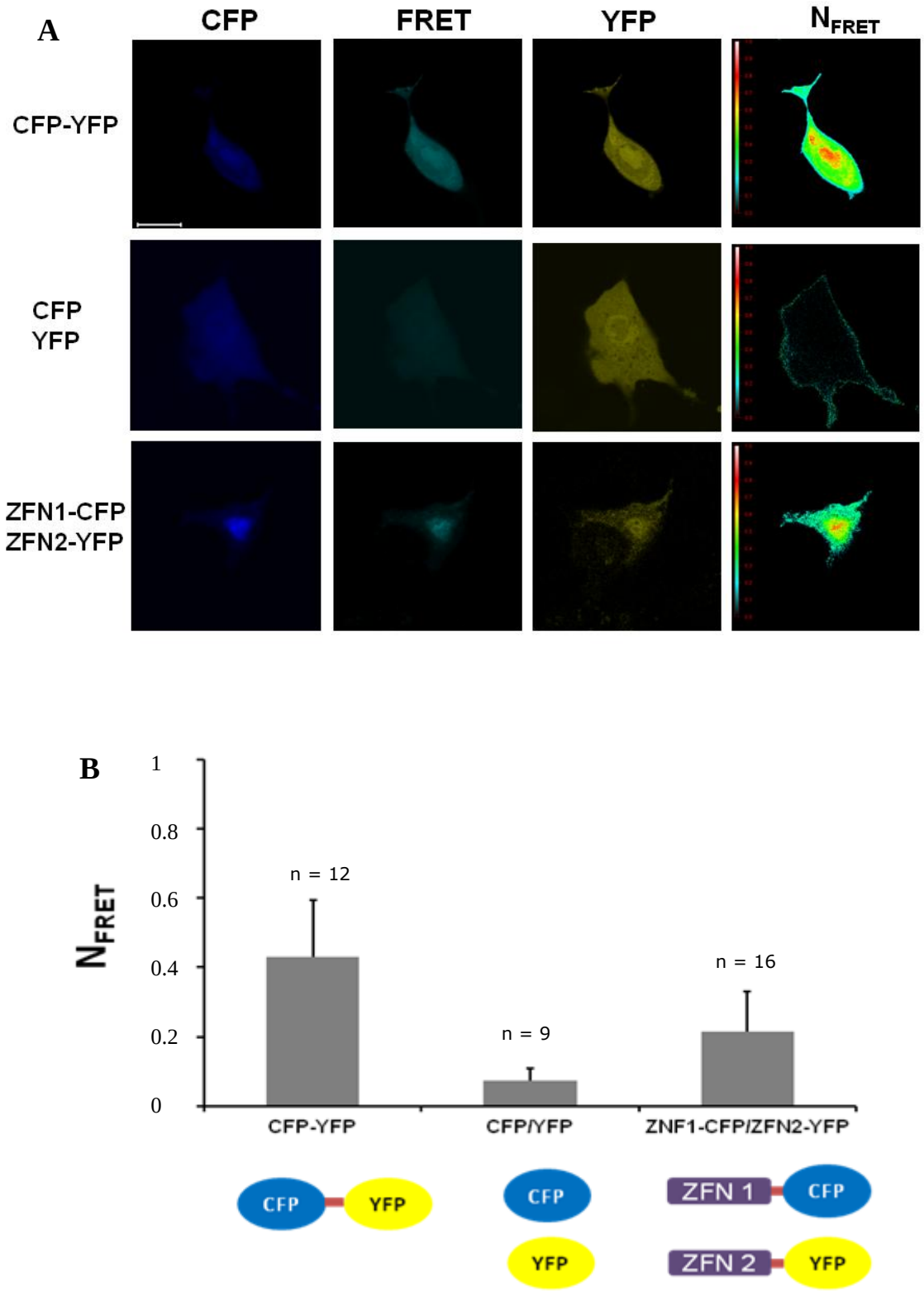


Figure 21. FRET analysis to investigate bimolecular interaction properties of ZFN1-CFP and ZFN2-YFP.

(A) FRET images. PK15 cells were transfected with the CFP-YFP (lane 1), CFP and YFP (lane 2) or ZFN1-CFP / ZFN2-YFP (lane 3) constructs, fixed 24 h after transfection and then imaged in the

following channels: Donor CFP (first column), FRET (second column) and acceptor YFP (third column). The last column displays a corrected and normalized FRET image N_{FRET} calculated from the first 3 channels as described in the methods section. Scale bar = 20 µm. N_{FRET} color lookup bar values range from black (0) to red (10). **(B)** N_{FRET} intensities of 9-16 cells were measured, and the mean N_{FRET} values ±SD are represented.

4.3.4 Expression of ZFN in nucleofected PK15 cells

ZFN plasmids linked with CFP and YFP were also used to assess the cell viability and toxicity of ZFN plasmids. For this purpose, PK15 cells were transfected by nucleofection with 1 µg (not shown), 2 µg or 7.5 µg ZFN1-CFP and ZFN2-YFP together or separately. CFP and YFP plasmids were also used as controls. In the case of single plasmid transfection, the double amount of DNA was used. Cells were then observed daily on a fluorescence microscope and images were taken on days 1 and 5 after nucleofection (Figure 22). Fluorescence microscopy revealed that when nucleofected with both ZFNs, fluorescent cells were disappearing progressively up to 5 days after transfection, while control cells as well as cells transfected with (C or Y)FP ZFN separately were still fluorescent 5 days post-nucleofection, indicating that the cells with both ZFN were dying.

Since the linkage of CFP and YFP at the C-terminus of ZFN proteins near the active center of the FokI nuclease may interfere with the activity of the ZFN, another toxicity test was performed using the original ZFN1 and ZFN2.

PK15 cells were transfected by nucleofection with different amounts of ZFN plasmids (Figure 23A). Additionally, PK15 cells, uninfected 293T cells as well as 293 PERV infected cells were transfected with ZFN plasmids together. As control cells were nucleofected with an empty GFP-pLVTHM vector as control (Figure 23B, C and D). In order to analyse the effect of a single ZFN plasmid DNA, PK15 cells were transfected with ZFN1 and ZFN2 plasmids together or separately (E). Cells were then counted after 1, 3 and 5 days of transfection.

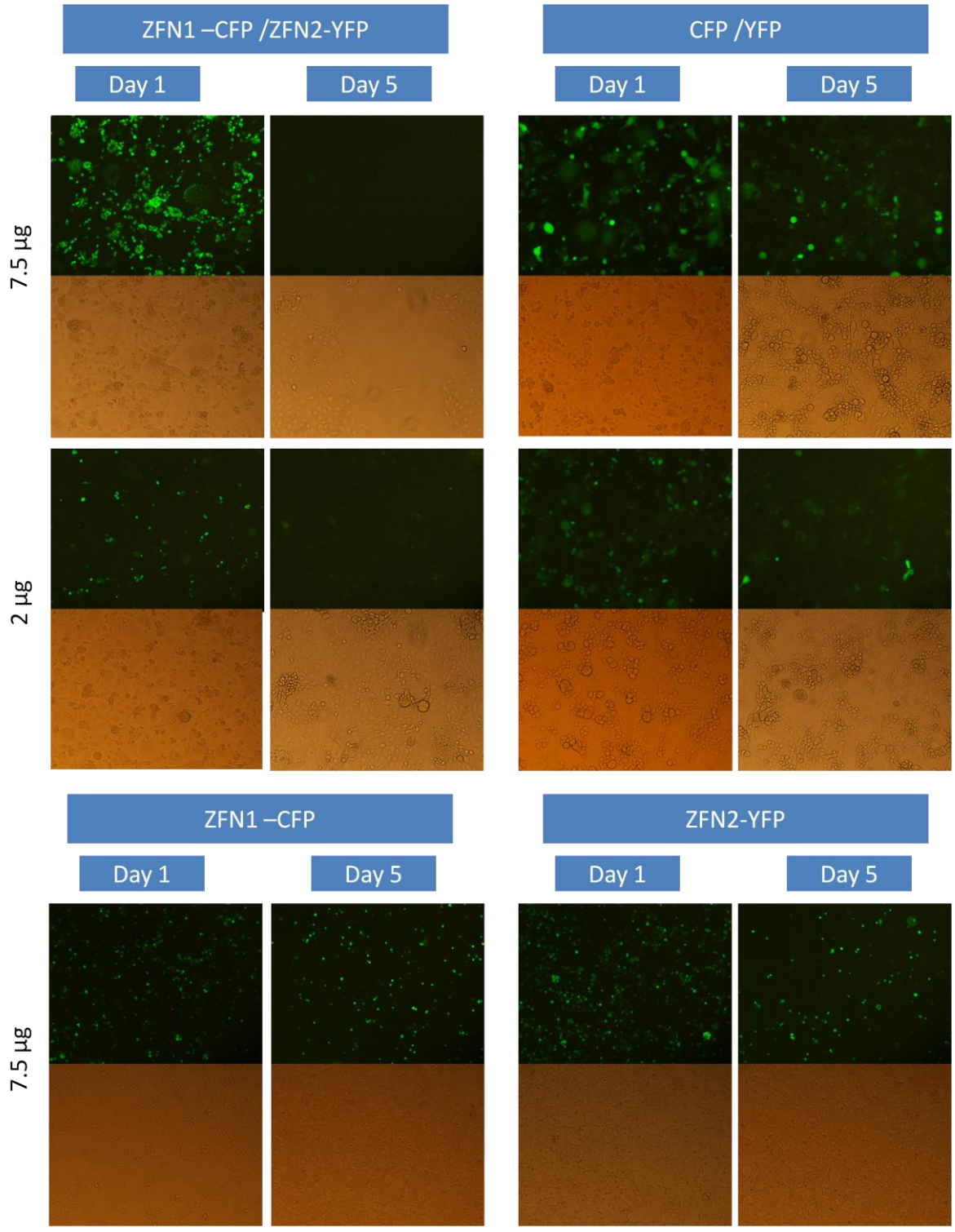


Figure 22. Expression of CFP and ZFP linked ZFN proteins in PK15 cells.

PK15 cells were nucleofected with ZFN1-CFP and ZFN2 YFP together or separately. Imaging by fluorescence microscopy followed 1 day and 5 days after post-nucleofection. PK15 nucleofected with CFP and YFP expressing plasmids were used as control.

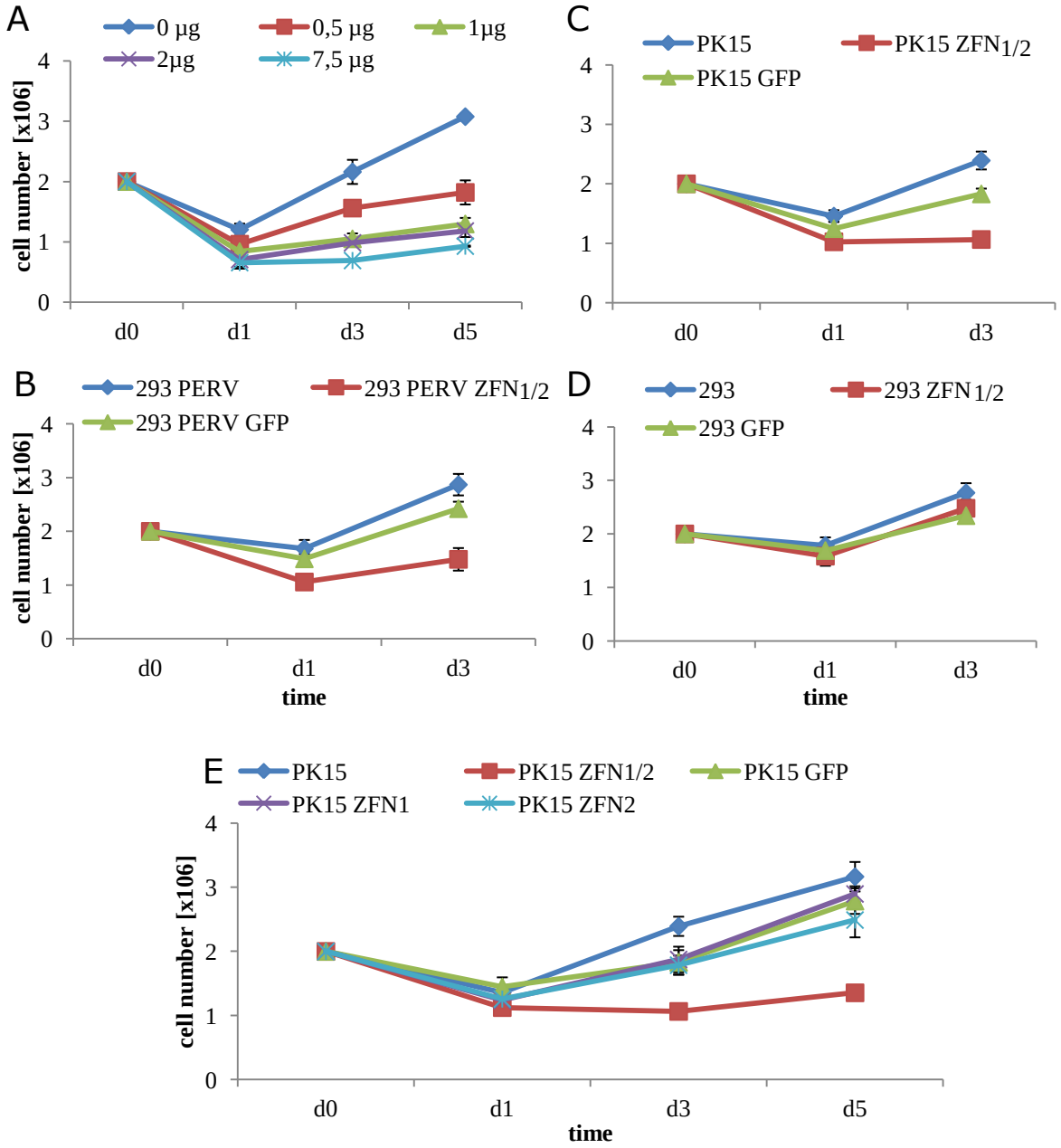


Figure 23. Cell viability after nucleofection with ZFN plasmids.

PK15 cells were nucleofected with different amounts of ZFN1/ZFN2 plasmids at day 0 **(A)** and cells were counted at days 1, 3 and 5 post-nucleofection. New HEK 293 cells were infected for 2 days with supernatant gained from older PERV-infected 293T cells. The newly infected 293 cells were then nucleofected with ZFN1/ZFN2 (2 µg each), pLVTHM-GFP (4µg) or mock **(B)**. PK15 cells **(C)** as well as uninfected 293T **(D)** cells were also nucleofected with the same amounts of plasmids. A double amount of DNA was used in case of transfection with a single plasmid (E).

A common observation for all samples was the reduction of the cell number about 60-70% on day one after nucleofection independently from the plasmid used and the amount of DNA.

However, on day 3 the cell number increased in correlation with the amount and type of plasmid used. The number of PK15 cells increased the faster the fewer amounts of plasmids were used (Figure 23A). PK15 cells as well as PERV-infected 293 cells transfected with both ZFN plasmids showed a significant reduced cell number in comparison to control cells. In contrast, uninfected 293T cells showed no difference in cell count 3 days post-nucleofection of both ZFNs. When ZFN1 and ZFN2 were transfected separately (Figure E) cell viability was not affected and the cell number measured after 5 days was near to control cells, while viability was decreased in cells co-transfected with ZFN1/ZFN2.

4.3.5 PERV expression in cells transduced with ZFN set1

In order to investigate the ZFN activity, total RNA was isolated from PK15 cells and PERV-infected 293 cells transduced with different amounts of ZFN set1. PERV expression was measured using the pol specific real-time PCR. Porcine cyclophilin (PK15) and huGAPDH (293 cells) served as reference. No difference in PERV expression was observed between ZFN cells and untreated cells. Expression of housekeeping genes was the same in all tested samples (data not shown).

Cells transfected with both ZFN1 and ZFN2 plasmids died 2 to 3 days post transfection. Cells transfected with a single ZFN plasmid showed a cell viability which was comparable to that of control cells. This was observed by fluorescence microscopy when cells were transfected with (C or Y)FP ZFN plasmids as well by the direct cell counting of cells transfected with original ZFN plasmids alone or separately.

4.3.6 Surveyor nuclease assay

For the optimization of the surveyor nuclease assay, several parameters were tested for each step of the assay.
In a first trial 1-2 million PK15 cells as well as PERV infected 293 cells were transfected with different amount of plasmid DNA (100 ng, 500 ng, 1 µg, 2µg, 7.5 µg or 10 µg) and in a second experiment a repetition of three nucleofections with 2 days intervals was performed using 100ng, 500 ng or 1µg ZFN1/ZFN2. Afterwards cells were harvested and DNA was isolated as described in the methods section.

In a first step the ZFN target sequence was amplified using 3 different primer pairs (PCR1-3) and using 3 different polymerases: Optimase polymerase (Transgenomic), the Phusion Hot

Start Flex DNA Polymerase (New England Biolabs) and the Expand high Fidelity Plus polymerase (Roche) (Figure 24A).

In a second step PCR products were heated to 95°C for dehybridization and then cooled down slowly for re-annealing. At this stage the wild type sequences and mutated sequence if present will re-anneal building bubbles corresponding to the DNA mismatches, which can be then cut by the surveyor nuclease. 10 µl of each sample were loaded on a 2% agarose gel and the concentration of the PCR amplicon was estimated using ImageJ software. DNA concentration was estimated to be approximately 40 ng/µl for all samples.

In the third step rehybridized amplicons were treated with the Surveyor nuclease. Different combinations of reaction parameters were tested for optimization: different DNA amounts, the enhancer concentration (1 or 2 µl) the $MgCl_2$ concentration (0, 0.5 or 1 µl) the nuclease quantity (0.5, 1 or 2 µl), as well as the nuclease working time (20 min, 40 min, 1h or 2 h). After stopping the reaction with a stop solution, samples were run on 2% agarose gel or 10% polyacrylamide gel. All tested samples revealed the formation of many cleavage products, which can be seen in the figure as a multitude of bands. The band positions are reproducible for the same cell type independently from the reaction conditions. In order to test the nuclease for possible contamination, PCR products were treated directly with nuclease and no cleavage bands were detected (Figure 24C).
In parallel, a G/C control was performed as described by the manufacturer. It consists of two control plasmids with inserts that differ at a single base pair. The amplification of the region containing the mutation with specific primers, followed by dehybridization / rehybridization step and treatment with nuclease, led to cleavage of the heteroduplexes into two bands (416 and 217 bps) (Figure 24D).

4.3.7 Sequencing of ZFN target sequence

In order to investigate the homogeneity of the PERV sequence targeted by the ZFN set 1, a cloning of the PCR1 products was performed using the Topo PCR cloning kit (Life Technologies) as described by the manufacturer and 24 from these clones were sequenced. Sequencing results revealed a high number of point mutations which were distributed all along the tested sequences (see Appendix 3). These mutations could be targeted by the Surveyor nuclease which leads to the high number of bands observed in the assay.

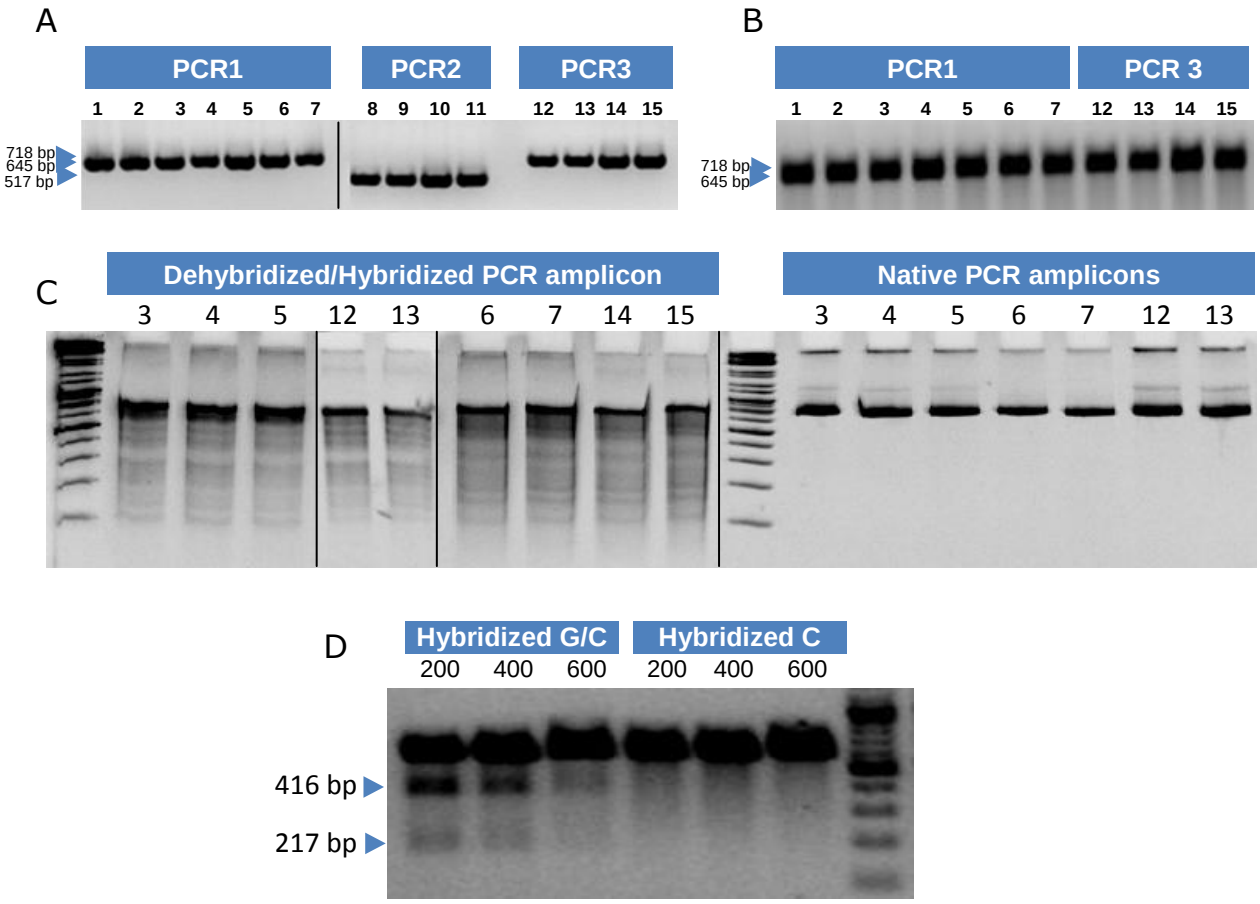


Figure 24. Surveyor nuclease assay

(A) PCR using genomic DNA as template for PCR (PCR1 = 645bp, PCR2 = 517 bp, PCR3 = 718 bp). After running on agarose gel, DNA concentration was estimated using an ImageJ software. Lane 1-5, PK15 cells transfected with 100 ng, 500 ng, 1 µg, 2 µg ZFN and 4 µg pLVTHM resp.; lanes 8 and 12, PK15 transfected with 2 µg ZFN1/2 each, lanes 9 and 13, PK15 transfected with 4 µg pLVTHM. Lanes 6, 10 and 14, PERV-infected 293 cells transfected with 2 µg ZFN1/2 each and lanes 7, 11 and 15 with 4 µg pLVTHM. **(B)** Agarose gel analysis of the rehybridization of PCR amplicons as in A. **(C)** PAGE analysis of dehybridized and hybridized samples after incubation with 1 µl nuclease, 1 µl $MgCl_2$ and 1 µl enhancer for 20 minutes. As control unhybridized samples from PCR reactions were treated with nuclease **(D)** Analysis of the G/C control of the surveyor nuclease assay. Three different amounts of DNA (200, 400, 600 ng) were treated with nuclease as described by the manufacturer and run on gel agarose.

The surveyor nuclease assay is the method of choice for the detection of mutations induced by ZFN treatment. Despite the fact, that ZFN target sequence is similar in all known PERVs, sequencing of the surrounding region of this target site showed a high rate of point mutations which could be a target for the CelI nuclease, which led to the high number of bands observed.

4.4 Generation of neutralizing antibodies against PERVs

During the long exposure of human patients to xenotransplants in the future, production of PERV particles by the remaining porcine lymphocytes may be induced and pose an infection risk for the recipient. Despite the fact that no transmission of PERVs was observed *in vivo* yet, the use of neutralizing antibodies against PERV as vaccines may contribute to the protection from a possible infection. Earlier work described neutralizing antibodies directed against the transmembrane envelope protein p15E and the surface envelope protein gp70 of PERV in goat, rats and hamster [185, 190, 196, 203]. Hamsters showed several advantages when studying the neutralizing antibodies, since unlike rats, they didn't shown pre-existing antibodies against p15E [190, 196]. These studies showed that immunizing hamsters with a mixture of gp70 and p15E resulted into sera with higher neutralizing activity than in animals immunized with the antigens separately. In the present work we aimed to confirm these data, and furthermore to investigate whether the method of application of antigens could influence the neutralization effect, since by mixing p15E and gp70 both molecules may interact together and this could have influence on the production of antibodies.

4.4.1 The antigens

The sequences of the p15E and gp70 recombinant proteins derived from the PERV envA (accession number AJ293656) as depicted (Figure 25). The recombinant proteins were expressed in *E.coli* and purified using affinity chromatography as described [190, 203].

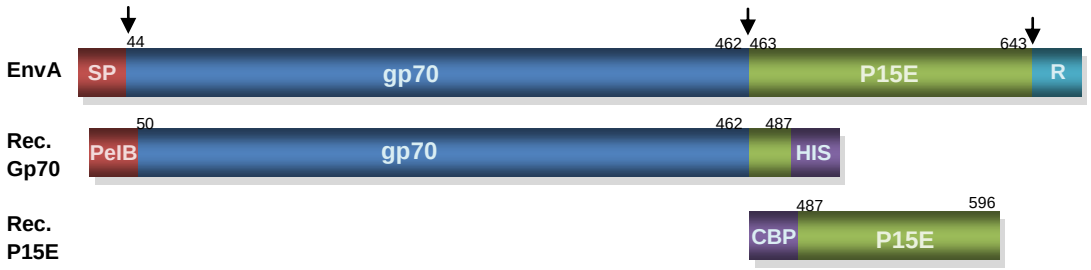


Figure 25. Schematic presentation of the recombinant PERV antigens.

The EnvA polyprotein, the recombinant proteins gp70 and p15E are depicted. SP: signal peptide, arrows: proteinase cleavage sites, PelB: pectate lyase B leader sequence directs the protein to the periplasm of *E.coli*, CBP: calmodulin binding peptide.

Table 12. Cloning and purification of antigens .

	Accession nr.	length	Molecular mass [kDa]	Expression vector	*E.coli* strain	Purification (aff. chromatography)
P15E	HQ688786	a.a. 488-596	16.3	pCal-n[(1)] (Stratagene)	BL21-Codon plus(DE3)-RP (Stratagene)	CBP[(3)]
Gp70	HQ688785	a.a. 50-487	54	Pet22b(+)[(2)] (Novagen)		Ni-NTA (Qiagen)

[(1)][185]; [(2)][222]; [(3)]Calmodulin binding peptide (31a.a.) fused to the P15E sequence and used for purification with affinity chromatography.

4.4.2 Immunization

A total of 16 hamsters were divided into four groups and immunized 4 times with intervals of 3 weeks. The first group was injected with 300 µg of p15E, the second group was given a mixture of p15E and gp70 (300 µg each), to the third group p15E and gp70 were injected separately at different body parts, and the animals of the control group were immunized with adjuvant and PBS. The first immunization proteins or PBS were emulsified in complete Freund's adjuvant with a final volume of 750 µl per animal. For boosting incomplete Freund's adjuvant was used (50 µl i.m. and 700 µl s.c.)

4.4.3 Characterization of binding antibodies by Western blot analysis

Antigen specific antibodies were detected by Elisa and Western blot analysis. Immune sera (IS) from the final bleeding were tested and pre-immune (PI) sera served as control to prove that antibodies were the result of the immunization and were not pre-existing in the animals like in the case of some rats [190]. Antigen specific antibodies were detected in all immune sera (Figure 26A). The repetition of the blots where PI sera were run separately from IS showed that the small bands corresponding to the PI in the first blot (Figure 26A) were only a contamination. In order to quantify the p15E antibodies titer, ELISAs were carried out using p15E antigens. The amounts of p15E specific antibodies observed in sera of animals immunized with p15E were slightly lower than in animals immunized with both antigens. However the difference wasn't significant. In addition, in the groups immunized simultaneously with both antigens, no significant differences were observed in p15E antibodies amounts when p15E and gp70 were applied as a mixture or separately.

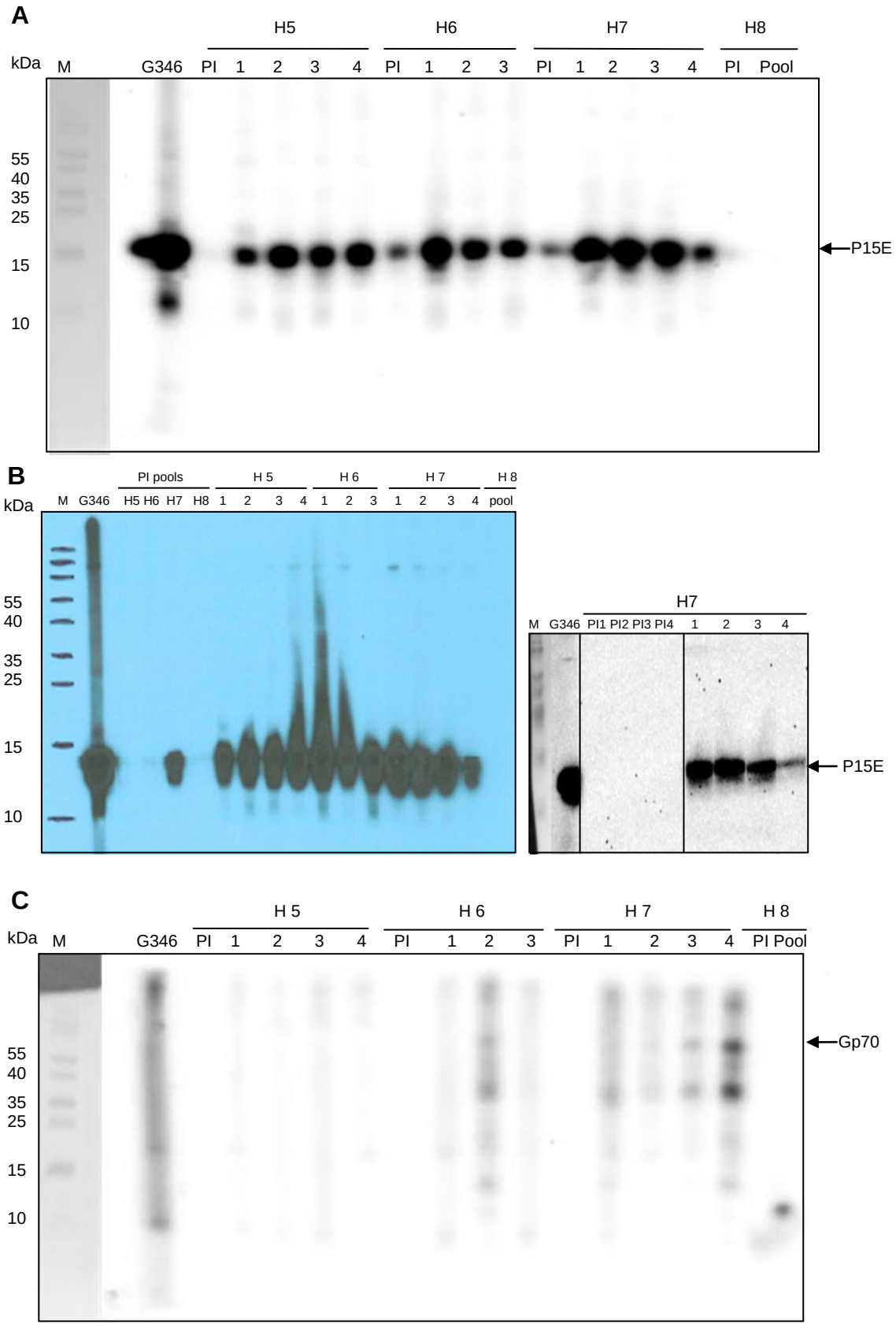


Figure 26. Western Blot analysis of the hamster immune sera.

Western blot analysis using p15E (A,B) and gp70 (C) used for the immunization. Immune sera of the final bleeding of 4 hamster groups were tested. Pre-immune (PI) sera of each group as well as the

immune sera of the control group H8 were pooled. G346 referred to the immune serum of the goat 346. Sera were diluted 1:400 (A,C) or 1:800 (B). Anti-hamster and anti-goat antibodies were diluted 1:2000. Lane M presents position and size (kDa) of protein standards.

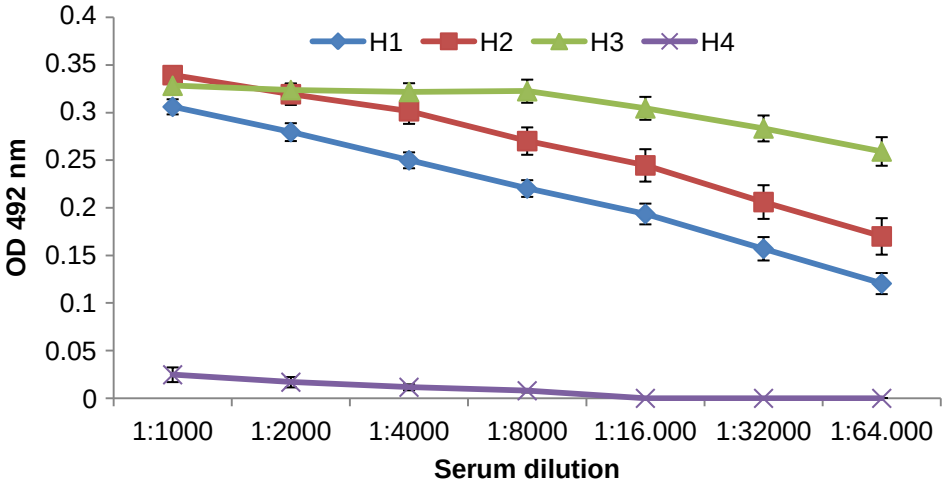


Figure 27. ELISA of the immune sera.

Titration of the sera from immunized hamsters (H1-3) and control animals (H4) using the purified recombinant p15E antigen. The mean values of all animals of each groups as well as the corresponding standard deviations are represented.

4.4.4 Neutralization assay

In order to assess the neutralization capacity and the specificity of the neutralizing antibodies in the immune sera, a neutralization assay was performed as described earlier [203]. The assay is based on the inhibition of PERV proviral integration in human 293 cells after incubation of the virus with neutralizing immune sera. The proviral integration is measured by a quantitative RT-real-time PCR specific for the PERV gag and which recognizes most of the PERV subtypes.

A PERV viral stock with known titer was prepared as described in the Method section, and incubated with 293 cells. Simultaneously, serial dilution of immune sera were prepared and added to the cells. Pre-immune sera were used as control to assess whether the observed neutralizing effect was the result of specific antibodies. Sera from immunized goats (goat 62 and 346) were used as additional controls [203]. If neutralizing antibodies are present in the sera, the proviral integration will be reduced and this would be reflected by higher Ct values of the real-time PCR. For the normalization of the results, a duplex real-time PCR was carried out, which measure additionally the expression of human GAPDH. The use of this

housekeeping gene served also to check the cytotoxicity effects of viruses or sera. The expression of human GAPDH was constant in all samples tested (mean Ct = 24.65 ± 0.7), and therefore ΔCt values were calculated and presented in the figure. Higher ΔCt values correspond to high neutralizing activity.

All immune sera but not pre-immune sera exhibited a neutralizing activity (Figure 28). The titers of neutralizing activity were higher in animals immunized with both antigens than in animals immunized with recombinant p15E alone. However, no significant difference was observed between animals immunized with the p15E/gp70 antigen mixture or animals injected with both antigens but separately.

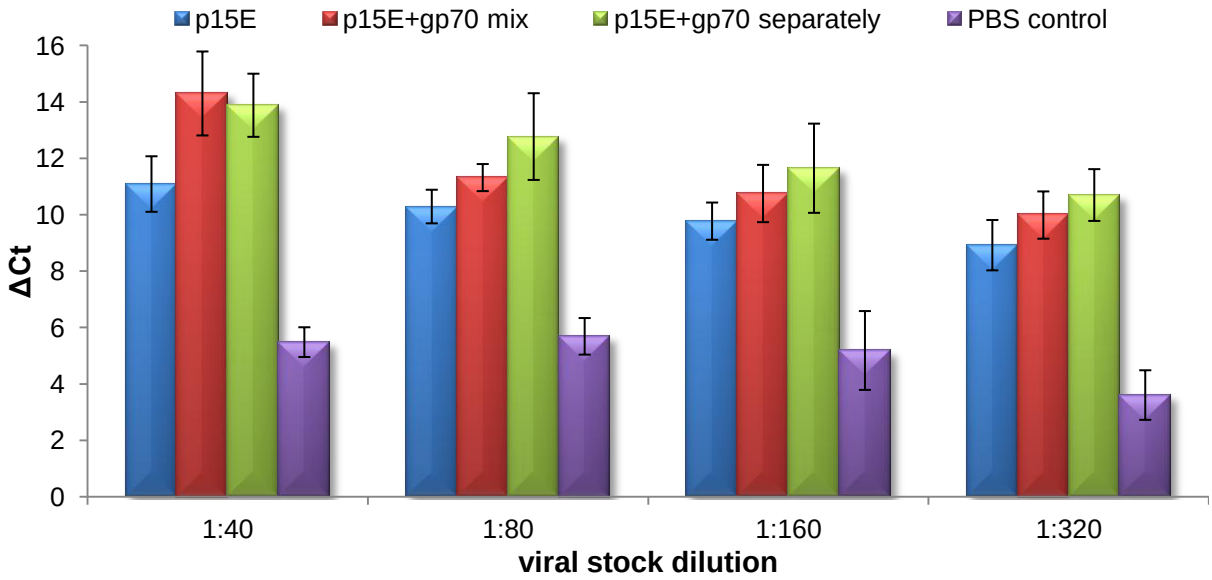


Figure 28. Neutralizing activity of sera.

Sera from all animals immunized with p15E (blue columns), p15E/gp70 mixture (red columns), p15E and gp70 separately (green columns) and control animals were analyzed by neutralization assay. Results are presented as mean values of ΔCt values of each group as well as the corresponding standard deviations.

4.4.5 Epitope mapping

The mapping of the epitopes in p15E which were recognized by the sera was performed as described earlier [190, 203] using 12 a.a. overlapping 15-mer peptides covering the entire p15E of PERV and coated on a glass chip (JPT Peptide Technologies, Germany). Epitope mapping of the serum from animal 6/3 which was immunized by a mixture of p15E and gp70

showed only one epitope localized at the MPER of p15E and corresponds to the E2a SKLRERLERRRR (Figure 29)

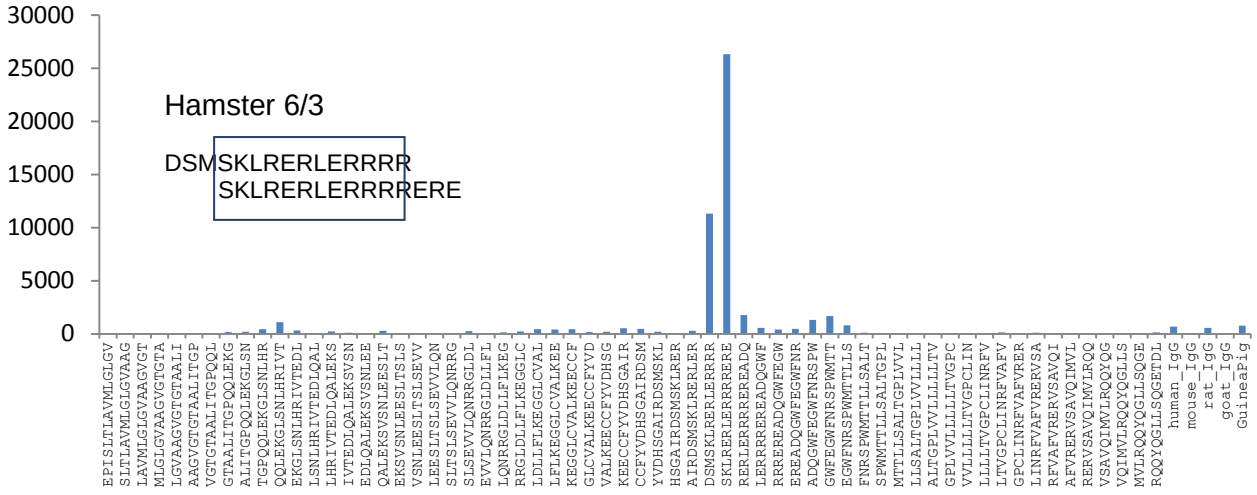


Figure 29. Epitope mapping of immune sera using the glass slide method

Serum from hamster 6/3 were tested using glass slide coated with 15-mer peptides and overlapping by 12 a.a.

5 Discussion

5.1 Improvements of the diagnostic tools

5.1.1 New real-time PCRs suitable for PERVs expression studies

Different methods were developed to measure the copy numbers of proviral PERVs in the genome as well as to quantify the expression of different PERV subtypes on mRNA level. Real-time PCRs specific for PERV-gag, -pol, envA/C, env-C and env-Cnv were described in earlier works [86, 124, 127, 191]. In order to accomplish the list of PERVs real-time PCRs for diagnostic studies, PERV-A and PERV-B specific real-time PCRs were established. Efficiencies of the real-time RT-PCR for PERV-A and PERV-B were very close to the efficiencies of the real-time RT-PCR for PERV-gag and PERV-envC and –Cnv ([185, 191] therefore all cited real-time PCRs could be run in parallel and are comparable. Real-time PCRs run with different env containing plasmids showed that the newly established real-time PCR were very specific. When total mRNA from PK15 cells was tested, PERV-A expression was shown to be higher than expression of PERV-B. In contrast, PERV-A and PERV-B expression was shown not to be very high in PBMCs of Göttingen minipigs. Based on these results, the combination of all PERV real-time PCR is the basis of an effective screening of pigs for PERV expression and helps to classify the tested pigs in low, medium or high producers.

5.1.2 Analysis of the expression of PERV-C in Göttingen minipigs

5.1.2.1 Why should Göttingen minipigs be screened for PERVs?

In the present work, the prevalence and expression of PERVs in the Göttingen minipigs were investigated. Göttingen minipigs are well-characterized pig breed used worldwide for biomedical and toxicological researches [215, 216, 223]. They are microbiologically well characterized and are monitored continuously for absence of different microorganisms. Although their size presents a disadvantage for their use for organ xenotransplantation, their detailed documentation and physiologic parameters render them relevant for islet cell xenotransplantation. For these reason we tried to analyze the PERV prevalence and expression in these pigs systematically using PBMCs from the animals.

The Göttingen minipigs are the result of breeding of three different strains: Minnesota Minipigs, Vietnamese Potbelly Pig and German Landrace. They were generated at the University Göttingen, Germany [224] and are breed at Illegaerd, Denmark in independent, full-barrier specified pathogen-free (spf) facilities and are microbiologically well characterized and screened on a regular basis for the presence of microorganisms (http://www.minipigs.dk/). Unlike other pig strains Göttingen minipigs have small size organs which don't match those of the human body. However, Göttingen minipigs may be important for xenotransplantation when using cells or tissues and considering numerous physiologic advantages like the well-defined health status and strictly managed genetics of the animals. Furthermore, Göttingen minipigs are used for numerous biomedical researches which allowed a detailed documentation of their physiologic parameters [215, 216]; however, the prevalence and the expression of different PERV subtypes in these animals were not yet investigated. In this study, screening of Göttingen minipigs was performed using sensitive PCR and real-time PCR methods. Peripheral blood mononuclear cells (PBMCs) were tested for several reasons: First, PBMCs are easily obtainable from the animals; second, it was described in this work and previous works that PERV expression showed fluctuations when animals of various strains or different organs of the same animal were compared, while PBMCs showed a good correlation when comparing different animals or strains [194, 218].

5.1.2.2 The genome of Göttingen minipigs doesn't harbor recombinant PERV-A/C

Our results revealed that Göttingen minipigs harbor the subtypes A, B, C and Cnv of PERV. However PERV-A/C could be detected neither in the germline nor in PBMCs of all tested animals. Until now the recombinant PERV-A/C couldn't be found in the germ line of pigs. Its presence is restricted only to some organs, which are related to the immune system such as the spleen [125-127, 138, 225, 226]. Furthermore, PERV-A/C viruses were characterized by their high replication titers and their ability to adapt to human cells [128, 129, 226], not to forget that high expression levels of PERV-C in PBMCs was shown to be related to enhanced infectivity of the recombinant PERV-A/C from PBMCs *in vitro* [127]. Collectively, these data make it plausible to exclude PERV-C harbouring pigs from being candidate for xenotransplantation. Unlike the ubiquitous PERV-A and PERV-B detectable in all pigs, PERV-C is not present in all pigs, which allow the selection of PERV-C negative animals. However, it is still unclear whether this aim is reachable. Since in the present study, all of the 15 analyzed Göttingen minipigs were shown to harbor PERV-C and PERV-Cnv.

5.1.2.3 Absence of PERV particles in mitogen-activated PBMCs

The main criterion when evaluating virus safety is the release of human-tropic infectious viral particles. In all five pigs analyzed for production of viruses using highly susceptible human 293 cells, no release of infectious human-tropic viral particles by PBMCs was detected. Furthermore, Western blot analysis revealed no viral protein expression in mitogen stimulated PBMCs (data not shown), although the PERV expression on mRNA levels was higher in comparison to the German landrace pigs. Not to forget that these experiments were limited since the co-incubation of PBMCs and 293 cells method used didn't allow cell-cell contact, which was thought to allow a more effective virus transfer. In order to avoid michrochimerism, further studies should include a co-cultivation of PBMCs with geneticin-resistant 293 cells, which allow selection of 293 cells after cell-cell-contact incubation as described earlier [227].

Göttingen minipigs and their suitability for xenotransplantation

The German landrace pigs analyzed in earlier studies were obtained from animal farms and showed low PERV expression [218]. In contrast, the Göttingen minipigs showed higher PERV expression, however they have the great advantage of being maintained under SPF conditions and for being monitored for the absence of a variety of microorganisms including viruses, bacteria, fungi and parasites (http://www.minipigs.dk). Nonetheless, additional screening for further viruses, like lymphotropic gammaherpesviruses, the cytomegalovirus and the hepatitis E virus, may be necessary to assess whether the use of Göttingen minipigs is appropriate as source animals for xenotransplantation regarding microbiological safety.

Risk of PERV-A/C transmission in miniature swine

Establishment of miniature pigs was started in 1975 in the Massachusetts General Hospital (MGH) by breeding two founder animals and focused on increasing the coefficient of inbreeding and maintaining different swine leukocyte antigen haplotypes within the offspring [228]. Analysis of miniature pigs from the MGH allowed identification of the so-called null pigs, which weren't able to transmit PERV infection to either human or porcine cells [127]. Further genomic analysis of PERV-A/C transmitter minipigs demonstrated that only one provirus of PERV-C was implicated in the formation of PERV-A/C recombinants [229]. Other studies showed that release of human-tropic PERV-A/C by PBMCs of these miniature swines, capable of infecting human 293 cells *in vitro*, was related to the presence of a unique

PERV *gag* restriction fragment length polymorphism [230]. Such genotyping data together with investigations of the gene expression at the mRNA and protein level as well as infection experiments will serve as an important tool in the selection of pigs suitable for xenotransplantation.

To summarize, it was shown that PERV can infect human cells *in vitro*, such as 293 cells [123, 151, 231], however there is still no evidence of *in vivo* PERV transmission during first pre-clinical and clinical xenotransplantations using pig cells (for a comprehensive review see [86]). Preclinical studies also failed to show a transmission, and when animals including non-human primates were inoculated with PERV viruses, even after applying strong immunosuppression for the tested animals in some experiments no virus transmission was observed [232]. Finally, it is important to mention that the donor and transplant screening rules in the context of xenotransplantation will surpass those of donor screening for allotransplantation which was practiced for many years [233], and this may eventually lead to much safer transplants [234].

5.2 Knock down of PERV expression using siRNA

Knock down of gene expression using RNA interference has been established as a powerful method used routinely in molecular biology [170]. Transgenic animals expressing siRNA as short-hairpin RNA from several species were successfully generated. Double stranded RNA trigger silencing of target genes in a sequence-specific manner. These double stranded siRNAs are formed by a guide strand and a passenger strand. The guide strand is chosen by the RISC complex and leads it to the complementary target sequence to be cleaved. PERVs are present in high copy number in the genome of pigs and presents high variability in their env sequence [110, 112]. Therefore classical knock out strategies based on insertion mutagenesis are nearly impossible. However the sequences of the pol and gag genes contain highly conserved regions among different PERV subtypes. This feature was used to design several successful gag and pol-specific siRNA [173, 196].

5.2.1 ShRNA transgenic pigs with reduced PERV expression up to three years

Pol2-shRNA was designed to target a highly conserved region of the polymerase gene *pol* of PERV-A, -B and -C subtypes, and thus should be able to suppress the expression of all types of PERV [173]. Transgenic pigs expressing the pol2 specific shRNA were generated and monitored for PERV and shRNA expression during a period of three years. Results demonstrated a strong and long lasting reduction of PERV expression in ear fibroblasts, PBMCs, as well as in several organs of these animals at the end of their life. The expression of the GFP marker and the pol2 shRNA was also demonstrated to be permanent during the whole period of the study.

The aim of this project was to analyze the long term efficacy of RNA interference in knocking down the PERV genes over a long period of time, which is necessary in cells, tissues and organs to be transplanted in the human body. The main challenges of the present study were first to reduce the already low levels of PERV mRNA expression, second the very low levels of protein translation as well as the absence of viral particles release in pigs. Another problem faced was that the low levels of PERV expression in wild-type animals and the differences observed in the expression between different animals presented a challenge in determining the efficacy of the RNA interference *in vivo*. However a reduction of PERV mRNA level should enhance the safety by preventing the release of infectious particles and reduce complementation or recombination possibilities of the numerous PERV copies in the genome of pigs.

To overcome these challenges, the expression of PERV in PBMCs from transgenic pigs and control pigs was measured in PHA-stimulated cells, which made the differences in expression more visible [101, 235]. It was shown that virus release in miniature pigs depended on the level of mRNA expression and occurred only at a certain expression level. Other pig strains showed the same or higher mRNA expression, but no virus release accompanied this expression [218]. These results imply that virus release depends not only on mRNA expression but also on genetic factors of the pigs and the type of proviruses.

While evaluating the risks of xenotransplantation it is necessary to take into account the viral factors (virus titer and regulation of viral protein expression), the genetic factors of the donor animal as well as of the recipient (restriction factors, innate and adaptive immunity) and the health status of both donor and recipient. However, it was shown in this study that the probability of viral transmission is lower when the release of viral particles is low.

Measurements of the PERV mRNA expression and the shRNA were performed using specific real-time PCRs. As housekeeping genes, porcine GAPDH and cyclophilin were used. Earlier studies showed fluctuations in the expression of these housekeeping genes in different organs, due to this reason, data analysis was mostly carried out based on total RNA. In some cases the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen [199] was used by referring to both housekeeping genes. The measurement of siRNA expression was carried out using a method developed in earlier works [194, 196]. This method is based on using a loop primer which binds to the siRNA and allows reverse-transcription and subsequent PCR-amplification. This method is more accurate than other methods based on Northern blot using phosphor-labeled siRNAs [175] or primers upstream to the shRNA [236].

The efficacy of the pol2 siRNA in reduction of PERV expression was analysed and confirmed in different tissues of transgenic animals. High levels of shRNA and low levels of PERVs were detected in all shRNA-transgenic animals when compared to control-vector transgenic animals. Noteworthy were the fluctuations in shRNA and PERV expression between the different tissues of the same animal. The highest levels of PERV were detected in lymph nodes and spleens, while lowest values were observed in the brain and muscles. These results were in agreement with earlier studies performed on wild-type and transgenic German landrace pigs [175, 218] and Yucatan mini pigs [217]. An explanation of these results could be the composition of cells in different tissues, the methylation status of the PERV promoters and genes [237] as well as the organ specific profile of transcription factors. A comparable

example could be the human endogenous retrovirus (HERV). Studies have shown that the *env* gene of the human endogenous retrovirus (HERV)-W encodes for syncytin, which is involved in human syncytiotrophoblast formation [238]. Syncytins, which originate from different ERVs, were also found in several mammals like mice [239, 240], rabbits, Carnivores [241] and cows [242]. Furthermore, recent studies reported high expression of the PERV-A receptor (HuPAR-2) in the villous trophoblast cells of humans. This expression is enhanced by the overexpression of the transcription factor activator protein (TFAP)-2γ, which is one of the transcription factors involved in the transcription of HuPAR-2 [243]. PERV was not found highly expressed in pig placenta [217] and no syncytin-like proteins were recorded yet, which is in accordance with the absence of syncytiotrophoblast in pigs, where the fetal and maternal tissues are simply apposed and no fetal trophoblast cells fusion occurs [244]. However, these results observed for HERV-W may explain the high PERV expression in some organs. In other words, this difference observed in PERV expression in different organs may be due also to higher susceptibility of their cells to PERV re-infection by the overexpression of PERV receptors. Furthermore, high expression in some organs related to the immune system like the spleen and lymph nodes may be due to an ongoing immune response which may activate the expression of PERVs

5.2.2 Heritability of the shRNA transgene to the offspring

In order to assess the heritability of endogenous shRNA of transgenic pigs, new pol2-shRNA male pigs were need in order to mate them with the females and check the offspring for presence of shRNA and its expression as well as the efficacy of the shRNA in reducing the PERVs of the litter. Male PFFs were transduced with the Pol2-shRNA pLVTHM vector or control empty pLVTHM and were used for SCNT. Afterwards 4 litters were born, one of them dead-born and the other three were tested and unfortunately they all harboured the control empty pLVTHM vectors. This question must be further investigated.

5.2.3 Reduction of PERVs using new triple-siRNA vectors

In earlier works done by our group several PERV-specific siRNAs were tested and showed high interference potential. Significant reduction of PERV expression was observed when the pol2-, pol1- and gag2-siRNAs were used in PK15 cells [173, 196]. Among these siRNAs the pol2 was expressed as shRNA in pLVTHM vectors and transgenic pigs were generated. Pol2 shRNA expression was monitored over three years and PERV reduction persisted [175, 194].

5.2.3.1 Advantages of multiple shRNA

It was shown in the case of other viruses like Hepatitis C [245] and HIV [246-248] that the use of a cocktail of siRNAs yielded better interference effect. Multiple siRNA can prevent the failure of individual siRNA and avoid the emergency resistant strains. Although viral escape was not described for PERV viruses yet, the high copy numbers and diversity of PERV proviruses in the genome of pigs may raise comparable problems.

Furthermore the PERV specific siRNAs used were chosen to target highly conserved region of the known pol and gag genes. Since PERV-*env* genes could present high difference in their sequences, we avoided the use of PERV-env-specific siRNA. For all these reasons a combination of two pol- and a gag-specific siRNAs would have quantitative and qualitative advantages by targeting a high number of PERV copies including different PERV subtypes.

5.2.3.2 Remarks on the design of triple-shRNA vectors

In the first experiments using double-siRNAs directed against PERV, the shRNAs were either placed in tandem downstream a single RNA polymerase II promoter (CAG promoter) [236] or two H1 RNA polymerase III promoters were used one for each shRNA [196]. Several double shRNA were tested by Kaulitz et al. [196], however none of these constructs was able to overcome the potency of the single pol2 or pol1 shRNA. This may be due to the promoter inhibition or interference caused by the leak of enough space between the two shRNA cassettes. It was furthermore demonstrated that co-transfection of two single shRNAs is more efficient than single-shRNA in reduction of PERV expression in PK15 cells [196].

In order to optimize the multiple shRNA plasmids, three different RNA polymerase III promoters were used and the space separating the three shRNA cassettes was taken into consideration for the design of the constructs. Furthermore, two different backbone plasmids were tested, the pSIREN which was already used by Kaulitz [196], and the pLVTHM vector.

Several reports described multiple shRNA vectors, using polymerase III promoters [249-251]. These commonly used promoters are suitable for the siRNA expression since they are able to direct the synthesis of small transcripts such as shRNAs and they require a stretch of 4-5 deoxythymidines as stop codon [252, 253]. The transcription is usually stopped after the second T, yielding an shRNA hairpin loop transcript with a double-thymidine 3'-overhang, which was shown to contribute into the selection of the guide siRNA strand during the RISC processing of the shRNA [254, 255] as well as into the duration of silencing capacity [256]. In

addition it could enhance the resistance of siRNAs to nucleases [170]. Since use of the same repeated promoter could end up into undesired deletions of the shRNA cassettes due to possible recombinations during the transduction of the vectors [257], we decided to use 3 different pol-III promoters the promoter of the human U6 small nuclear RNAs (shRNA) [258], H1 promoter of the human nuclear RNase P and the human 7SK promoter.

It was shown that the use of pol III promoters for shRNA expression might be less advantageous than pol II promoters for gene therapy applications [259]. For example pol III promoters are not capable of spatial and temporal conditional expression as needed for therapeutical applications [260], which is not needed in the case of this study where restriction of shRNA transcription is in contrary unprofitable. Furthermore, pol III promoters are constitutively active in a wide range of cell types, which is advantageous in this case. Another advantage over pol II is the short lengths of pol III promoters, which make it easier to use three promoters in the same vector. On the other hand, pol III promoters are very robust and usually lead to high shRNA expression which may cause cytotoxicity [261]. However after testing the triple-shRNAs only one construct (pT1) exhibited high cell toxicity (data not shown) and was not used for generation of shRNA pigs. The pol III promoters used were the U6, H1 and 7SK promoters.

A cloning strategy was developed in order to insert all H1 and 7SK promoters easily in a pSUPER-Mfe vector. The double-cassettes were then inserted into a pSIREN vector containing a U6 promoter. This cloning strategy allowed moving the triple shRNA cassettes into a pLVTHM vectors. At last, 5 pSIREN based vectors and 5 pLVTHM vectors were constructed and contained different combinations of shRNA/promoters.

5.2.3.3 Study of the internal stability of the used siRNAs

The shRNAs chosen were shown to have high interference potency. Especially the use of the pol1 shRNAs resulted in great reduction of PERV expression in PK15 cells. These results were in correlation with the internal stability of siRNA measured using the SFold software. It was described that asymmetry in internal stability of double-stranded RNA precursor play a role in the selection of the guide siRNA. The strand showing less stability in its 5'end is easier to unwind and thus to be incorporated into the RISC complex [219, 220]. This asymmetry was accentuated in the pol1 siRNA and to a lesser extent in the pol2 siRNA. Gag2-siRNA showed little asymmetry between both ends, however it showed a low internal stability in the 8-12 nt position fulfilling thus the second requirement for guide strand selection.

5.2.3.4 Establishment of pol1 and gag2-shRNA real-time PCR

For the measurement of the siRNA expression, a pol2-specific real-time PCR was already established [194]. In this work, further real-time PCRs specific for pol1 and gag2 were established. These newly established real-time PCRs functioned with high efficiency when synthetic siRNAs were used as templates. However, no detection of pol1 or gag2 siRNAs was possible, whether when total mRNA of shRNA transfected cells was used as a template, or even when shRNA with 4 or 7 nt-loop were tested. But pol2 shRNA was detected in all constructs, which means that all three promoters were active and were able to lead the shRNA expression. Furthermore, the pol2-siRNA expression measured by the pol2-siRNA real-time PCR was shown to be higher in vectors containing single pol2 shRNA than in triple-shRNA, however interference efficiency of triple shRNA was much higher than constructs containing single pol2 shRNA (data not shown). Additionally it is known that a correlation exists between the amount of siRNA and its potency [196, 236]. All this suggested that in triple-shRNA all three siRNAs should be expressed. A possible explanation of the failure of detection could be the unpredictable resulting sequence of the siRNA after processing of the double-stranded shRNA by the DICER complex *in vivo,* and which may not match the tested synthetic siRNA *in vitro*.

5.2.3.5 PERV specific triple-shRNA: a high efficient tool to reduce the PERV expression

The T vectors based on pSIREN backbone were tested in PK15 cells, and positive cells were selected by different concentrations of puromycin. It seemed that the combination promoters/shRNA in T1 was toxic for the cells, since moving the triple-cassette to a pLVTHM vector resulted in the same cytotoxicity. The other T constructs showed low cytotoxicity and a reduction in PERV expression. However this reduction wasn't a significant improvement when compared to the single-shRNA pol2-pSIREN, which may explain earlier results done with double-shRNA using the pSIREN vector [196]. For this reason the triple-shRNA cassettes were inserted into a pLVTHM vector (pT) and tested in PK15 cells by nucleofection and following FACS sorting. The pT vectors showed high efficiency in reducing PERV expression. This expression was reduced to 8% when compared to mock transfected cells, while pol2 single-shRNA reduced the expression to only 15%. These results were comparable to those observed in earlier work when pol2-shRNA was used [196].

However, the measurement of PERV expression was done using total mRNA extracted from PK15 cells. Since the processing of the shRNA as well as the whole RNA interference

process takes place in the cytoplasm of the cells, the nuclear mRNA is protected from the siRNA effects. Thus, measuring the PERV expression using the total mRNA is not accurate and doesn't reflect the real efficiency of the RNA interference. For this reason, cytoplasmic and nuclear RNA were measured separately. Doing so, we could demonstrate that the amount of cytoplasmic PERV mRNA remaining in the cells corresponded to only 4% compared to mock transfected cells, while nuclear mRNA was reduced only to the quarter of its amount in control cells. It is important to mention here that these results were not suitable for the comparison of expression between nuclear and cytoplasmic mRNA, because equal amounts of RNA template were tested, which doesn't correspond to an equal cell number, since no studies were yet done about the nuclear:cytoplasmic RNA ratio in PK15 cells. However, the comparison of PERV mRNA amount in total, nuclear and cytoplasmic mRNA showed that cytoplasmic PERV in pT transfected cells is lower than in cytoplasm of control cells. In other words, using the new triple-shRNA vectors presented an improvement in reducing the cytoplasmic PERV expression when compared to single-shRNA vectors.

5.2.3.6 Generation of triple-shRNA transgenic pigs

Finally, porcine fetal fibroblasts were transfected with the pT vectors as well as pol2 single-shRNA and control empty pLVTHM vectors. The pT1 plasmid was excluded since it showed lower efficacy in reduction of PERV expression in PK15 as well as high cytotoxicity. Since transduction efficiency is very low and the PFFs are vulnerable primary cells which require cell to cell contact for their growth, all shRNA-PFFs were pooled. The pLVTHM control cells as well as the pooled shRNA-cells expressing GFP were selected by FACS sorter and were sent to the Friedrich-Löffler-Institute (Neustadt, Mariensee) for SCNT. Further studies should be done to follow the litter and measure the siRNA and the PERV expression in PBMCs and tissues.

5.3 Knock out of PERV with Zinc Finger Nuclease

In the present study we aimed to knock out a number of PERV genes in porcine cells in order to reduce PERV expression. PERVs revealed to be distributed in the genome of pigs with a high copy number[110], therefore classical knock out methods are difficult to apply in this case. Earlier studies showed an efficient biallelic knockout of the α1,3-galactosyl-transferase (GGTA1) in porcine cells using zinc finger nucleases [37]. Also other studies showed that the ZFN can be applied successfully in porcine cells [262-264]. The *pol* gene of PERV was the region of choice to knock out since previous studies using siRNA targeted against the *pol* gene showed a high reduction of PERV expression [175, 194, 236]. Therefore an alignment of the known PERV *pol* sequences allowed the selection of a high conserved region, which was used to design and select an appropriate ZFN, which could target a large number of PERV copies.

5.3.1 Kinetic of ZFN expression

Kinetic studies of the ZFN expression in ZFN-transfected PK15 cells revealed high expression 12 to 24 h post-transfection followed by a decrease of expression after 48 h. The expression level was dependent on the amount of ZFN plasmid used for transfection. The decrease of ZFN expression after 48 h could be related to the degradation of plasmids in the cells, the silencing of the CMV promoter [265-267] or the death of the cells due to probable toxicity of the ZFNs.

5.3.2 Localisation of ZFN expression

Furthermore, we investigated the localization of the ZFN proteins. The immunoblotting of nuclear and cytoplasmic extract from ZFN-transfected cells using anti-Flag antibodies showed a clear accumulation of the ZFN proteins within the nuclei. The nuclear and cytoplasmic segregation was tested by using β-actin and DDX3 antibodies. The β-actin was present mainly in the cytoplasm, however a relatively lower amount of expression was detected in the nuclear extract. This could be the result of cross contamination but it was most probably nuclear β-actin, which is now known to have important functions within the nucleoplasm [268, 269]. In contrast, DDX3 was shown to be expressed mainly in the nuclear lysate. Only one sample showed a contamination of the cytoplasmic lysate by nuclear extract. The next experiment done with fluorescent marked ZFN confirmed also these results and showed moreover that both ZFN1 and ZFN2 were present predominantly in the nucleus. These results additionally

proved that the ZFN proteins are imported to the nucleus where they can be in contact with their DNA target sites.

This study showed using two methods, FRET and Western blot analysis, that the ZFN1 and ZFN2 proteins are localized in the nucleus. In total 9 to 16 cells were measured and N_{FRET} values were calculated. The co-expressed ZFN1-CFP / ZFN2-YFP accumulated in the nucleus and N_{FRET} values proved that both ZFN arms may interact within the nucleus by binding to their DNA target sequence allowing a dimerization of the Fok1 subunits. Control CFP-YFP dimers showed high N_{FRET} values. In contrast, when unconjugated CFP and YFP were co-transfected, no FRET was observed. However, the overexpression of ZFN proteins, which were highly accumulated in the nucleus, could lead to misinterpretation therefore further studies using for example the two hybrid system might confirm the interaction of both ZFN protein subunits.

5.3.3 ZFN protein cytotoxicity

A general observation in all ZFN nucleofection experiments was the low viability of cells after transfection independent of the nucleofection process. A possible explanation could be the high number of PERV copies in the genome. That is when ZFNs were active they would simultaneously target several sites of the genome which is not comparable to the previously described toxicity due to off-target effects of ZFNs [270, 271].

In order to study the expression of ZFN in PK15 cells as well as their toxicity, transfected PK15 with different amounts of ZFN1-CFP/ZFN2-YFP and control plasmids were observed for 5 days by fluorescence microscopy. The number of cells co-transfected with both ZFN plasmids decreased progressively and green cells disappeared totally after 5 days, while cells transfected with ZFN1-CFP and ZFN2-YFP separately as well as control CFP/YFP were still fluorescent 5 days after transfection, even though fluorescence decreased possibly due to plasmid degradation or CMV promoter silencing. However the used ZFN plasmids were engineered so that the CFP or YFP were placed at the C-terminus of the FokI subunit of the ZFN protein. So an interference of the fluorescent markers with the FokI dimerization and activity couldn't be excluded. In order to shed light on this question, PK15 cells as well as 293 cells and PERV infected 293 cells were nucleofected with ZFN plasmids together or separately. Viable cells were counted 1, 3 and 5 days after transfection. Cell number was reduced to about 60% 1 day after transfection in all samples independently of the plasmids

used as a result of the use of nucleofection for transfection, where the viability was measured to 60-70% (not shown).

The transfection of PK15 cells with an increasing amount of ZFN led to increased cell death proportional to the DNA-plasmid quantity used. Furthermore, results showed that after 3 days the number of cells containing PERV sequences (PK15 or PERV infected 293 cells) was low, while the number of ZFN-nucleofected 293 control cells was comparable to that of mock- or pLVTHM-transfected cells. Additionally when PERV-expressing cells were transfected with ZFN1 and ZFN2 separately, the cell number was comparable to that of control cells. In conclusion, ZFN proteins may be active in the transfected cells only when both ZFN1 and ZFN2 are co-transfected. Nevertheless, this activity might be toxic for cells because of the high copy number of the target PERVs in the genome.

To summarize, the high copy number of PERVs in the genome combined to the high level of ZFN expression in transfected cells, could lead to the ZFN cytotoxicity observed. This overexpression of ZFN proteins in transfected cells is due to the high nucleofection efficiency and to the use of the cytomegalovirus promoter, which is one of the strongest promoters described [136, 272]. To overcome this, the use of a weak or inducible promoter could reduce the ZFN expression to a non-toxic level. An alternative is the use of mRNA for the expression of ZFN since unlike plasmids, mRNAs are quickly degraded in the cells.

5.3.4 Measurement of ZFN activity

PERV expression in ZFN treated cells

There was no reduction in the PERV expression in ZFN treated PK15 cells and PERV infected 293 cells (data not shown). This can be due to the low transduction efficiency or the cytotoxicity of the ZFN proteins.

The Surveyor nuclease assay is not a suitable choice

In order to investigate the activity of ZFNs in the cells, we used the surveyor nuclease assay (SNA) which allows the detection of mutations caused by the ZFN cleavage and DNA repair by the cell. The combination of different reaction parameters constantly generated the same results, where a multitude of bands were observed on agarose or polyacrylamide gels after treatment of rehybridized PCR products with the Surveyor nuclease. A contamination of the nuclease could be excluded by using the C/G control as well as un-rehybridized PCR

amplicons, where two cleavage bands were observed as expected. The presence of a high number of cleavage bands was probably the result of the existence of multiple cleavage sites within the PCR amplicon heteroduplexes treated with the nuclease. This could be due to the presence of various mismatches in the heteroduplexes when different PCR products are rehybridized.

The PCR primers of the SNA were designed so that the approximately 600 bps long amplicons include the ZFN target site. Additionally, the ZFN was designed in a conserved region where all known PERV pol sequences were compared. The presence of different mismatches in the heteroduplexes raised a question about the homogeneity of the PCR amplicons in the porcine cells. In other words, porcine cells may contain diverse unknown PERV sequences and so the amplified region may not be highly conserved, which may interfere with the assay results. For this purpose, cloning of PCR products was performed and sequencing of 24 amplicons revealed the presence of a high number of various mutations.

It is important to note here, that PERV infected 293 cells used in this study were freshly infected by supernatant from older PERV-infected 293 cells, which were in culture for a long period. This might allow the formation of new mutation due to repeated infection and thus, the supernatant may contain different populations of PERV viruses. This was revealed by the profile of cleavage bands of PERV-infected 293 cells, which was different from that of PK15 cells.

Considering all these results, we came to the conclusion that the SNA is not a suitable assay for the measurement and detection of mutations in this case. Further work may be done to assess the activity of ZFN proteins. For examples, by co-transfecting a single PERV plasmid with the ZFN plasmids and testing the PERV mRNA expression or the establishment of control 293 cells infected with a single PERV clone with reduced virus integration number, which can be then used for ZFN nucleofection tests.

5.4 Improvement of the production of neutralizing antibodies against PERVs

Several γ-retroviruses like FeLV, MuLV and KoRV are pathogenic in their infected host. For some of them a trans-species transmission was described, like in the case of KoRV which was able to infect human cells *in vitro* as well as Wistar rats *in vivo*. In rats KoRV was also able to induce a humeral immune response and may be the cause of tumor development [113]. In general retroviruses were shown to induce immunodeficiency which often results in death of infected animals by the emergence of opportunistic infections [100, 144, 273, 274].

Although no PERV transmission was observed from pigs to human yet, PERVs can infect human cells *in vitro*. Furthermore, during a long contact period between porcine cells or tissues and immunosuppressed human recipients an adaptation of PERVs to the human cells cannot be excluded. For this reason it may be reasonable to prepare a PERV-vaccine which could be used as a preventive measure for putative human recipients. On the other hand, the trials for vaccine development may help to understand the general mechanism of neutralization of different retroviruses including HIV-1 by MPER-specific antibodies.

5.4.1 Inducing of p15E-specific antibodies

The immunization of hamsters with recombinant p15E and gp70 induced high titers of neutralizing antibodies. Higher titers of p15E-specific antibodies were observed in the group of animals immunized with both antigens separately. These antibodies strongly recognized the epitope (SKLRERERRR) corresponding to the E1 epitope in the MPER region. The second epitope E2 (FEGWFNR) was recognized by the antibodies but to a less extent (Figure 30). Only one hamster (H6/3) from the group of animals immunized with a mixture of PERV p15E/gp70 and which showed the highest antibodies titers in ELISA was tested by epitope mapping.

It was already shown that immunization with recombinant p15E of PERV induced high titers of neutralizing antibodies recognizing both epitopes in goats, rats, mice or hamsters [113, 203]. Such neutralizing antibodies recognizing similar epitopes were also detected in sera of different animals immunized with the p15E of the feline leukemia virus (FeLV) [186, 187, 275, 276] and the koala retrovirus (KoRV) [113]. These epitopes showed a similarity to the human HIV-1 antibody 4E10, which recognizes the epitope (NWFNIT) in the MPER of the

transmembrane envelope protein gp41 of HIV-1 which is important for vaccine design [184, 277].

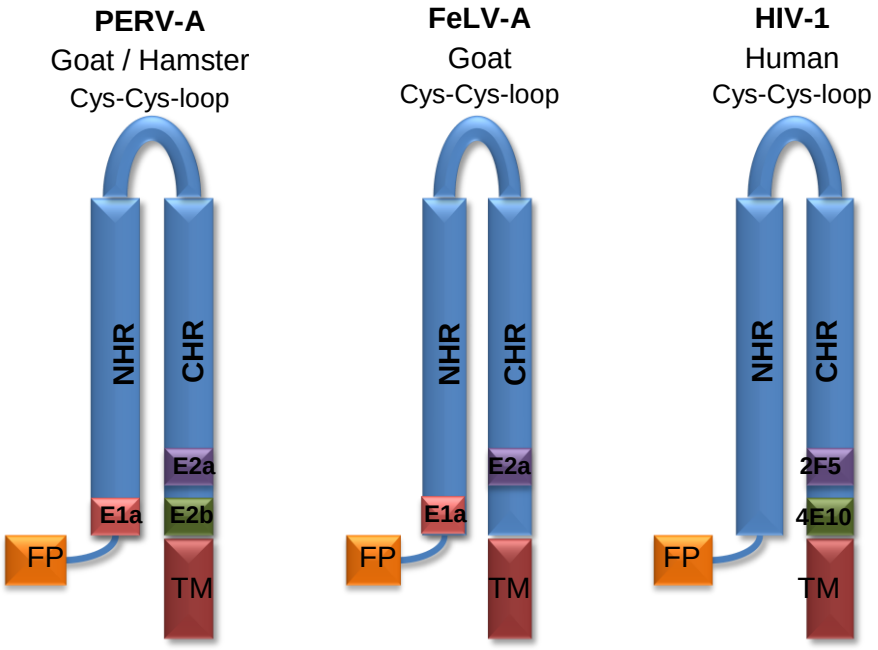


Figure 30. Localisation of epitopes in the transmembrane envelope proteins of PERV, FeLV and HIV-1 recognized by neutralizing antibodies.

This picture shows the localization of the epitopes recognized by neutralizing antibodies in sera of goats and hamsters immunized with the p15E of PERV-A, in goats immunized with p15E of FeLV [86, 186], as well as the position of the epitopes recognized by monoclonal antibodies 2F5 and 4E10 in HIV-1 TM isolated from patients [184]. FP, Fusion peptide. NHR and CHR, N- and C-terminal helical regions, TM, transmembrane protein.

The titer of anti-gp70 was higher in the group immunized with both antigens separately. In contrast, lower titers were observed in the group immunized with a mixture of both antigens.

5.4.2 The neutralizing effect of the PERV p15E immune sera

The neutralizing effect of the immune sera was tested by an neutralization assay similar to a neutralization assay described for HIV-1 [278]. The assay is based on the measurement of proviral DNA in human cells which where incubated in a mixture of immune sera and PERV viruses. The presence of neutralizing antibodies in the sera will hamper the infection of the human cells and thus the copy number of proviral DNA will decrease in comparison to control cells and pre-immune sera. The provirus measurements were carried out by real-time

PCR. Usually the expression of a housekeeping gene like GAPDH is also measured in order to screen for cell viability.

Earlier works showed a neutralizing effect of sera from non-immunized rats [196, 279]. One explanation of this phenomenon is the presence of pre-existing cross-reacting antibodies, which were originally directed against PERV-related retroviruses like MuLV or FeLV or other endogenous retroviruses known to be expressed in numerous species in context of physiological or pathological conditions [190]. For these reasons hamster was the animal of choice for immunization experiments.

Immunization of hamsters with recombinant p15E and gp70 of PERV showed high neutralization effects of sera. This activity is higher in animals immunized simultaneously with both p15E and gp70 [190]. In this work we aimed to investigate the effect of applying both antigens separately in different parts of the body or as a mixture. By mixing gp70 and p15E an interaction between both peptides may occur, which would influence the availability of some epitopes for immunization. Results showed higher neutralization activity in sera of animals immunized with both antigens than in animals immunized with p15E alone, however no significant difference was observed between animals immunized with a mixture of p15E/gp70 or separately.

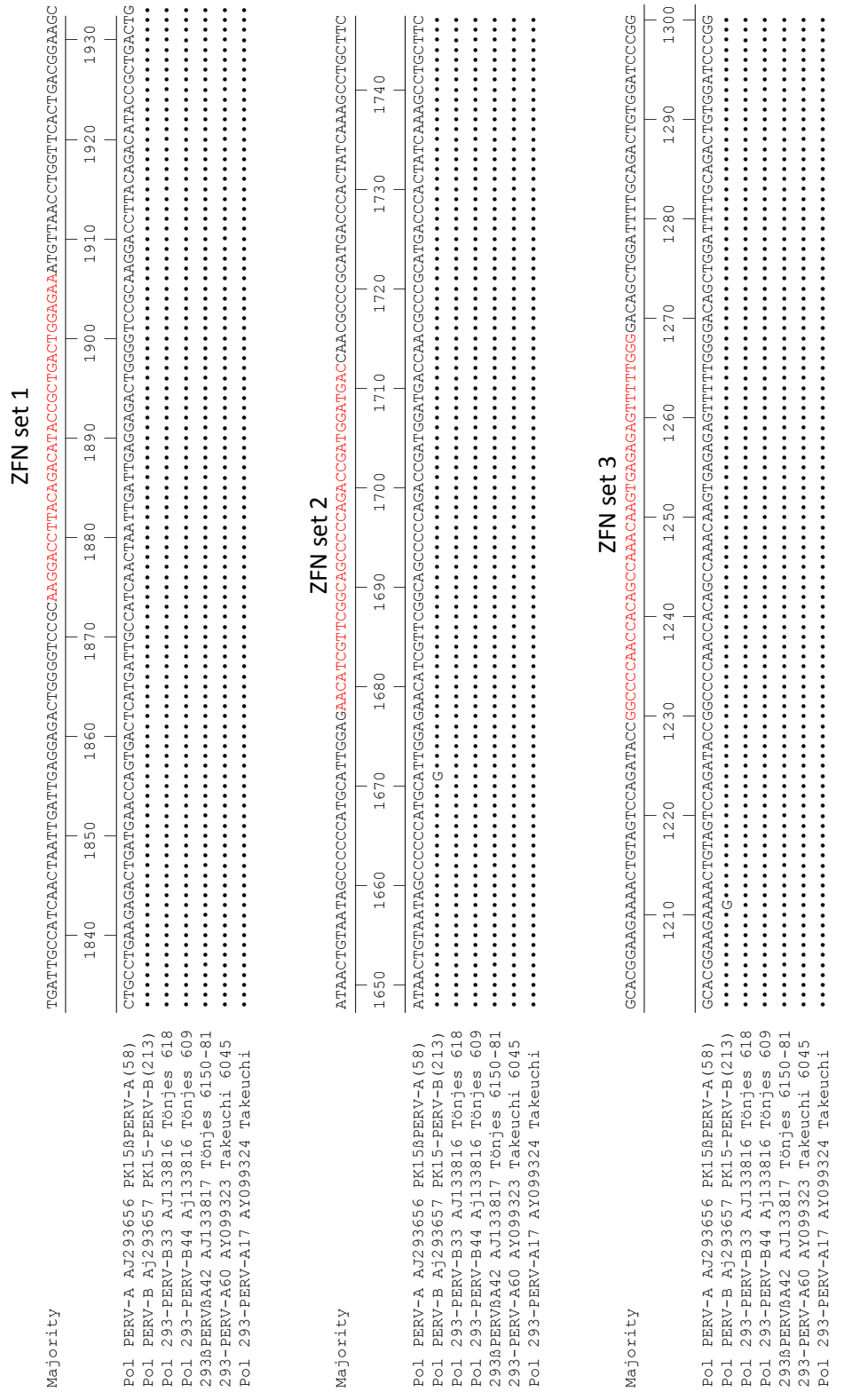
Appendix 1

PERV genes sequences used for the design of siRNAs and Zinc Finger Nucleases

Name	Accession	cell line	mol type	Ref.
Porcine endogenous type C retrovirus proviral gag gene, pol gene and env gene, class A, clone 58	AJ293656.1	PK15	genomic DNA	[119]
Porcine endogenous type C retrovirus proviral gag gene, pol gene and env gene, class B, clone 213	AJ293657.1	PK15	genomic DNA	
Porcine endogenous retrovirus type C proviral gag, pol and env genes and LTR (class B, clone 33)	AJ133816.1	grown in PK15	genomic DNA	
Porcine endogenous retrovirus type C proviral gag, pol and env genes and LTR (class B, clone 43)	AJ133818.1		genomic DNA	
Porcine endogenous retrovirus type C proviral gag, pol and env genes and LTR (class A, clone 42)	AJ133817.1	grown in PK15	genomic DNA	
Porcine endogenous retrovirus A gag-pol polyprotein and env protein genes, complete cds	AY099323.1	293 cells infected with PERV from PK-15 cell supernatant	genomic DNA	[117]
Porcine endogenous retrovirus B gag-pol polyprotein and env protein genes, complete cds	AY099324.1	293 cells infected with PERV from PK-15 cell supernatant		

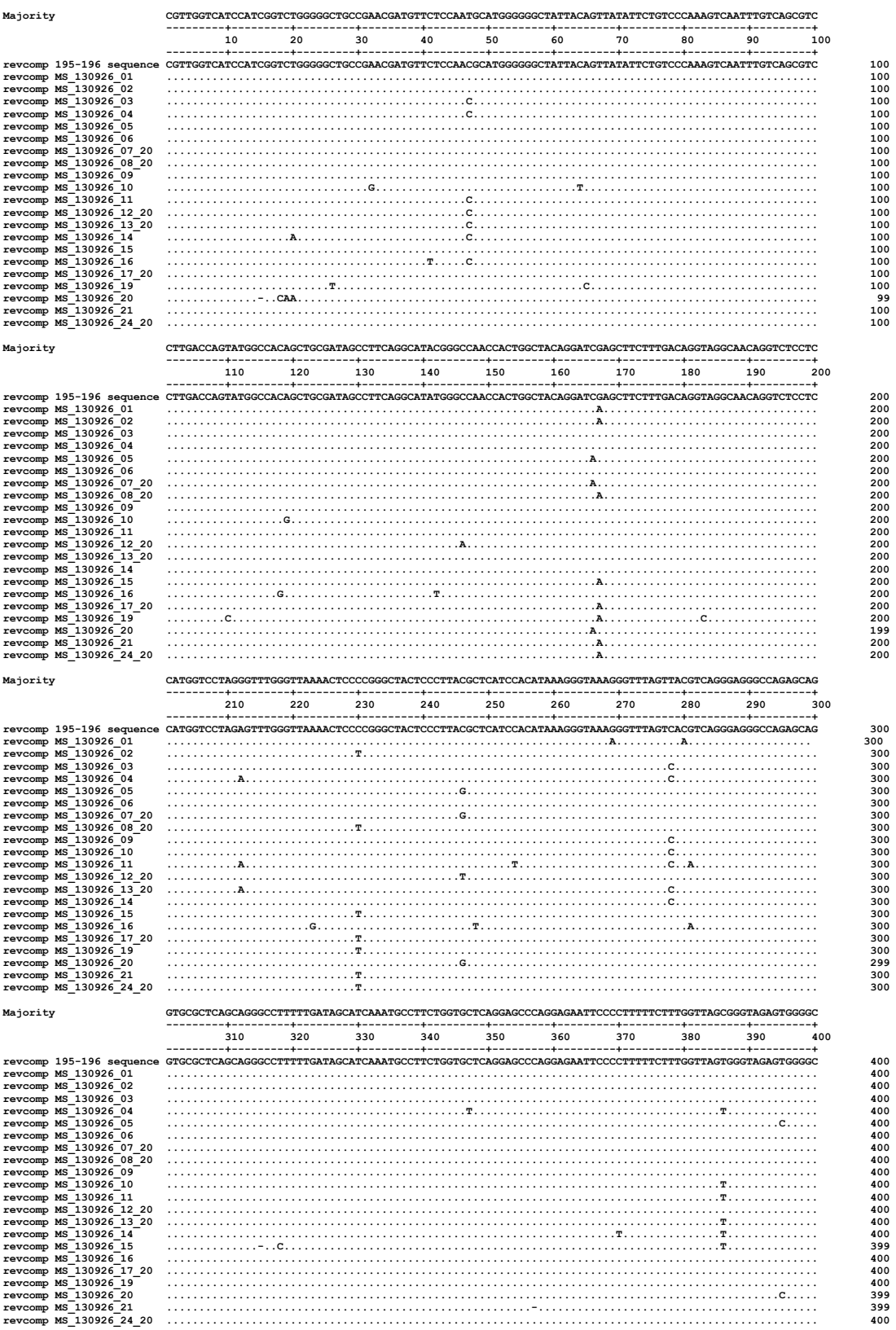
Appendix 2

Alignment of different PERV clone sequences at the ZFN target sequences (red).



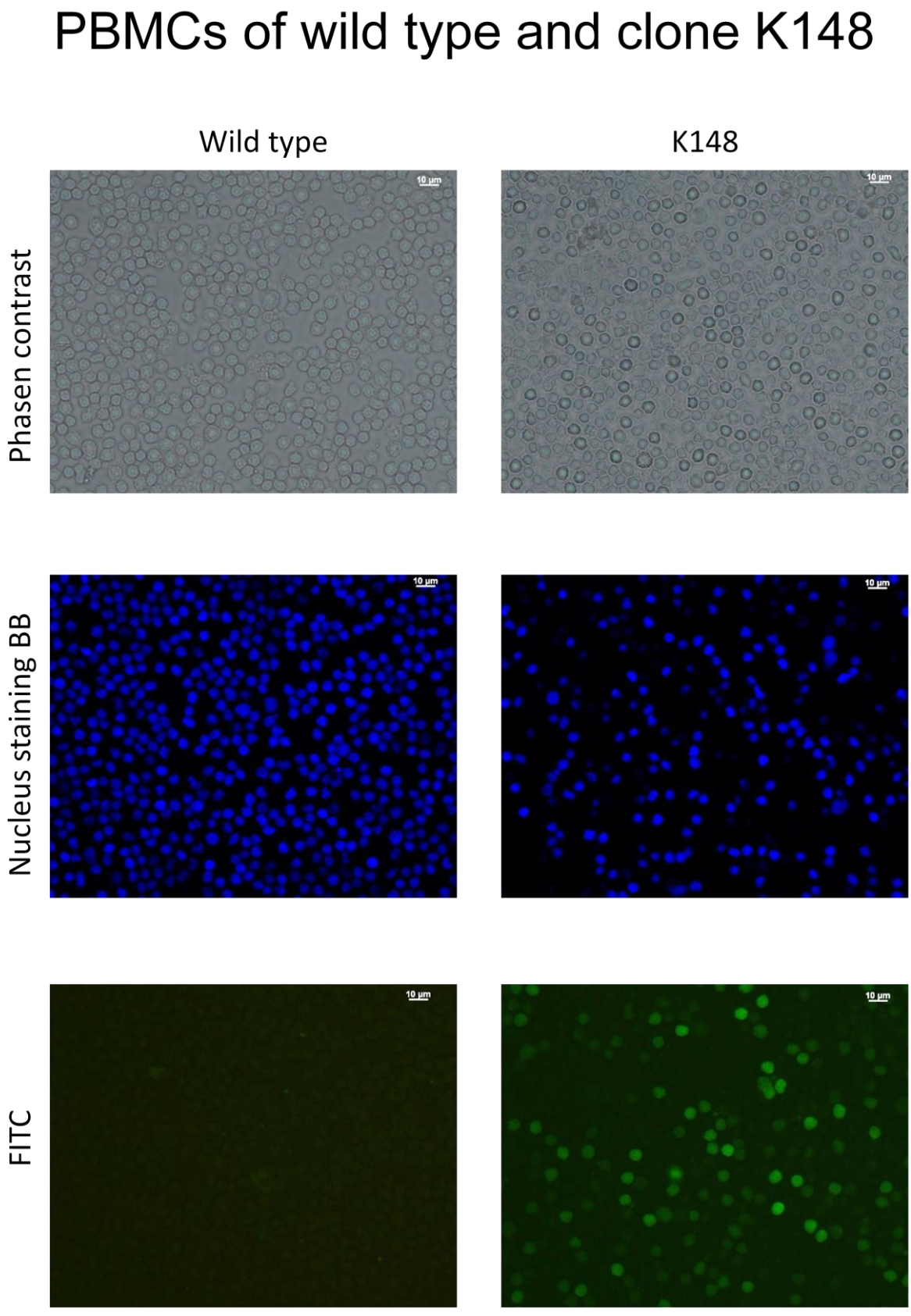
Appendix 3

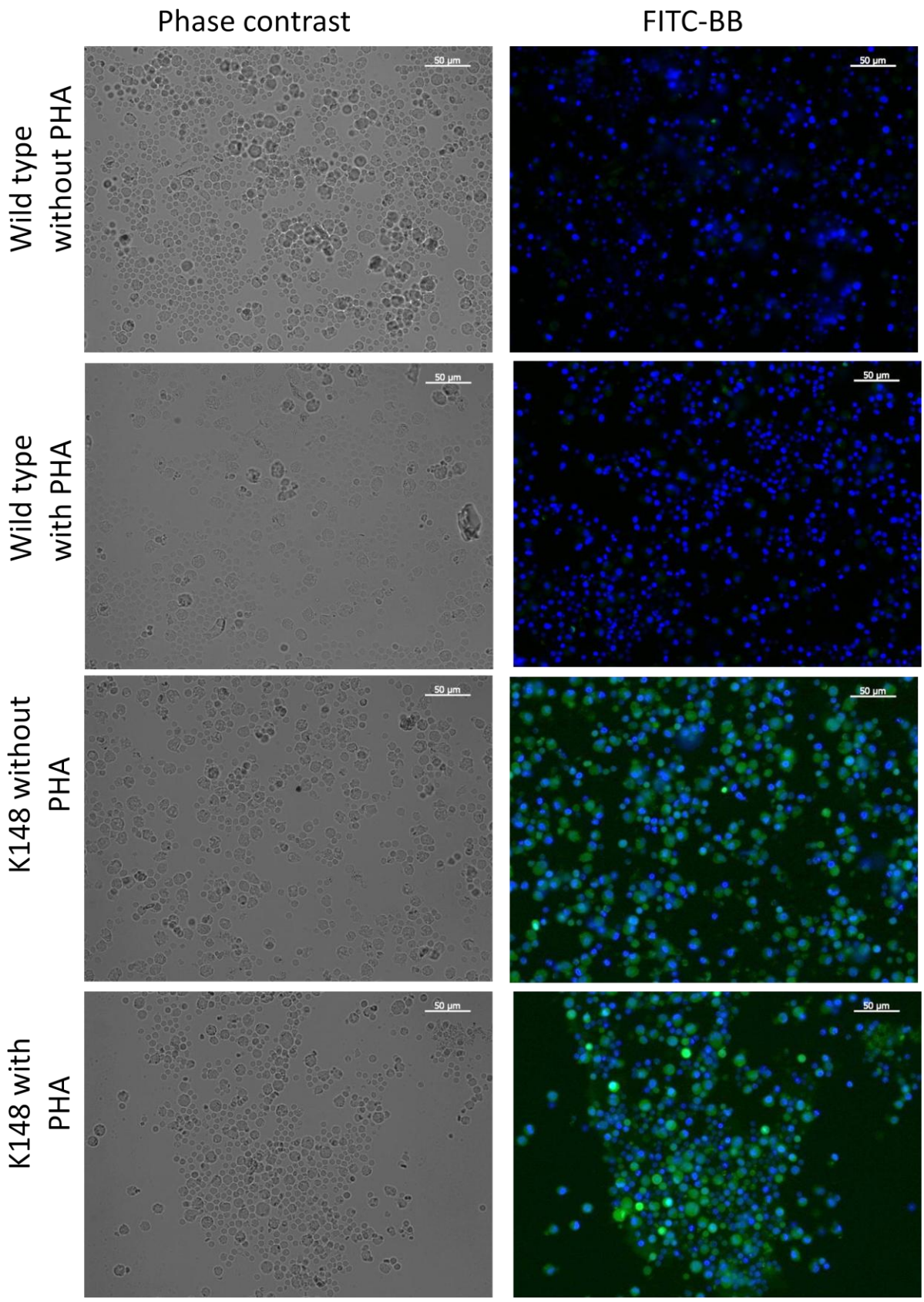
Alignment of different sequenced clones, which were PCR amplified from genomic DNA of PK15 by the ZFN primers PCR1 (see 2.8 in Materials) used for the Surveyor Nuclease Assay.

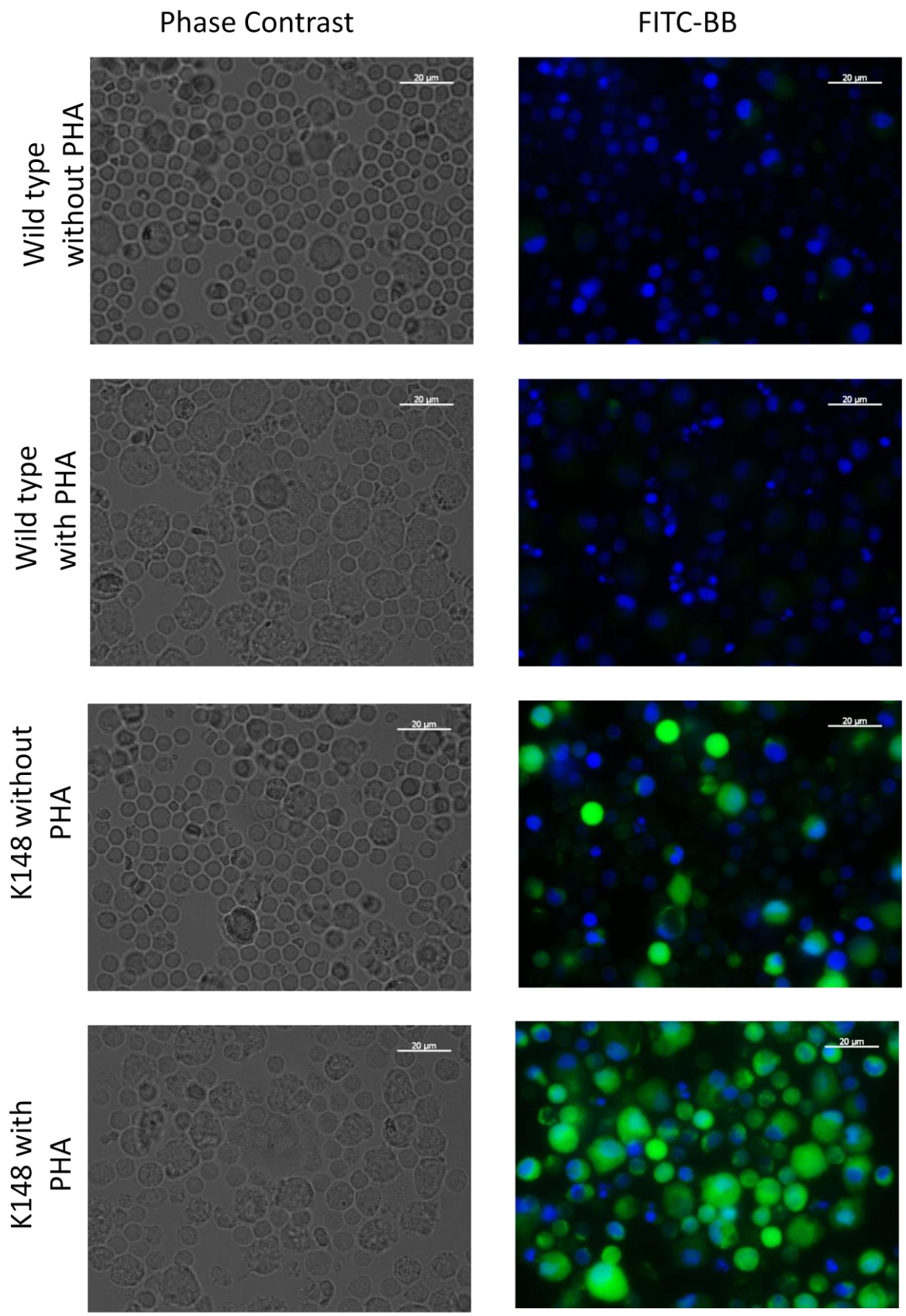


```
Majority                TGCTAAGGTCGCAAACCCCGGGATCCACAGTCTGCAAAATCCAGCTGTCCCCAAAAACTCTCTCACTTGTTTGGCTGTGGTTGGGGCCGGTATCTGGACT
                        ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                                 410       420       430       440       450       460       470       480       490       500
                        ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
revcomp 195-196 sequence TGCTAAGGTCGCAAACCCCGGGATCCACAGTCTGCAAAATCCAGCTGTCCCCAAAAACTCTCTCACTTGTTTGGCTGTGGTTGGGGCCGGTATCTGGACT  500
revcomp MS_130926_01    ..................................................................................................  500
revcomp MS_130926_02    ..................................................................................................  500
revcomp MS_130926_03    ..................................................................................................  500
revcomp MS_130926_04    ..................................................................................................  500
revcomp MS_130926_05    ..............................................................C...................................  500
revcomp MS_130926_06    ..................................................................................C...............  500
revcomp MS_130926_07_20 ................T.................................................................................  500
revcomp MS_130926_08_20 ..................................................................................................  500
revcomp MS_130926_09    ..................................................................................................  500
revcomp MS_130926_10    ..................................................................................................  500
revcomp MS_130926_11    ..................................................................................................  500
revcomp MS_130926_12_20 ..................................................................................................  500
revcomp MS_130926_13_20 ..................................................................................................  500
revcomp MS_130926_14    ...............G..................................C...............................................  500
revcomp MS_130926_15    .....T.....................................................................................  499
revcomp MS_130926_16    ..T.....T..................................................................................  500
revcomp MS_130926_17_20 ..................................................................................................  500
revcomp MS_130926_19    ..................................................................................................  500
revcomp MS_130926_20    ..................................................................................................  499
revcomp MS_130926_21    ..................................................................................................  499
revcomp MS_130926_24_20 ..................................................................................................  500

Majority                ACAGTTTTCTTCCGTGCCTCCGTCAGCCATCGCTGCCCGCCCCGCAAACTGTACCCCAAGTATGTTACCTCTCTCCTGCAAATCTGGGCCTTCTTAGCGG
                        ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
                                 510       520       530       540       550       560       570       580       590       600
                        ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
revcomp 195-196 sequence ACAGTTCTCTTCCGTGCCTCCGTCAGCCATCGCTGCCCGCCCCGCAAACTGTACCCCAAGTATGTTACCTCTCTCCTGCAAATCTGGGCCTTCTTAGCGG  600
revcomp MS_130926_01    ..................................................................................................A.  600
revcomp MS_130926_02    ..................................................G...............................................A.  600
revcomp MS_130926_03    ..................................................................................................  600
revcomp MS_130926_04    .T................................................................................................T....A.  600
revcomp MS_130926_05    ......C...........................................................................................  600
revcomp MS_130926_06    ......C.....................................................G.....................................  600
revcomp MS_130926_07_20 ..................................................................................................  600
revcomp MS_130926_08_20 ..................................................................................................A.  600
revcomp MS_130926_09    ....................................A...T.....................................................  600
revcomp MS_130926_10    ......C.............................C.....................................................  600
revcomp MS_130926_11    ......C.....................................C.....................................C.....  600
revcomp MS_130926_12_20 ..................................................................................................A.  600
revcomp MS_130926_13_20 ......C...........................................................................................  600
revcomp MS_130926_14    ......C...........................................................................................  600
revcomp MS_130926_15    ......C...........................................................................................  599
revcomp MS_130926_16    ..................T.................A.............T.....................................T....T..  600
revcomp MS_130926_17_20 ..................................................................................................A.  600
revcomp MS_130926_19    ..................................................................................................  600
revcomp MS_130926_20    ..................................................................................................A.  599
revcomp MS_130926_21    ..................................................................................................A.  599
revcomp MS_130926_24_20 ..................................................................................................A.  600

Majority                AGGCTCTGTAGCCTAGGTCAGACAATTCCAGCAGTAGTGCCTTCGX
                        ---------+---------+---------+---------+------
                                 610       620       630       640
                        ---------+---------+---------+---------+------
revcomp 195-196 sequence AGGCTCTGTAGCCTAGGTCAGACAATTCCAGCAGTAGTGCCTTCG                                                  645
revcomp MS_130926_01    ...........................................                                                       645
revcomp MS_130926_02    ...........................................                                                       645
revcomp MS_130926_03    ........................-.....T.............A                                                     645
revcomp MS_130926_04    ...........................................                                                       645
revcomp MS_130926_05    ...........................................                                                       645
revcomp MS_130926_06    ......................T....................                                                     645
revcomp MS_130926_07_20 ...........................................                                                       645
revcomp MS_130926_08_20 ...........................................                                                       645
revcomp MS_130926_09    ...........................................                                                       645
revcomp MS_130926_10    ...........................................                                                       645
revcomp MS_130926_11    ..................CC.CC.....-T.............A                                                     645
revcomp MS_130926_12_20 ...........................................                                                       645
revcomp MS_130926_13_20 ...........................................                                                       645
revcomp MS_130926_14    ...........................................                                                       645
revcomp MS_130926_15    ...........................................A                                                      645
revcomp MS_130926_16    ..............T............................                                                          645
revcomp MS_130926_17_20 ...........................................                                                       645
revcomp MS_130926_19    ...........................................                                                       645
revcomp MS_130926_20    ...........................................A                                                      645
revcomp MS_130926_21    ...........................................A                                                      645
revcomp MS_130926_24_20 ...........................................                                                       645
```

Appendix 4

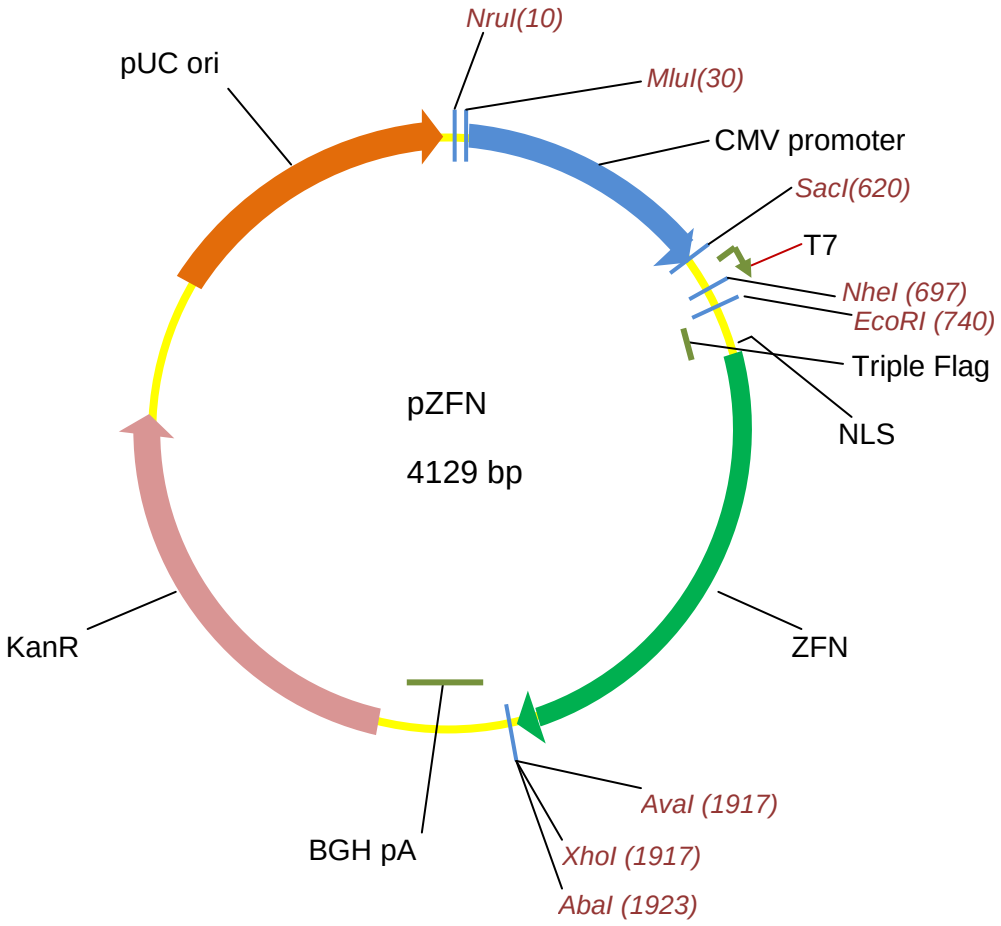

PBMCs of wild type and clone K148
Wild type
K148
Phasen contrast
Nucleus staining BB
FITC
10 µm


Phase contrast
FITC-BB
Wild type without PHA
Wild type with PHA
K148 without PHA
K148 with PHA
50 µm


Phase Contrast
FITC-BB
Wild type without PHA
Wild type with PHA
K148 without PHA
K148 with PHA
20 µm

Appendix 5

pZFN Plasmid, Sigma Aldrich, St Louis, USA



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Curriculum Vitae

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Affidavit

Herewith I declare that the present dissertation has been written independently and with no other sources and aids than quoted.

……..

Mawan Semaan, Berlin, 2014

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