Cell Entry and Exit of Porcine Endogenous Retrovirus A: Receptors and Release Inhibitor

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Declaration

I, Giada Mattiuzzo, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Following the discovery that porcine endogenous retrovirus (PERV) can infect human cells, the potential risk of a zoonotic infection by PERV has been a major obstacle in the xenotransplantation field. The aim of this thesis is to gain a better understanding of PERV biology, so as to help assess and reduce the risk of PERV zoonosis.

PERV subgroup A can enter human cells through two human PERV-A receptors (huPAR-1 and -2). To determine critical regions in the receptor for PERV-A infection, chimeric receptors between huPAR-2 and the non functional murine PAR (muPAR) have been analysed. A single amino acid difference (amino acid 109) was found responsible for the inability of muPAR to mediate PERV-A binding and infection. These results were then applied to the evaluation of PERV infection of non-human primates (NHP). NHP could represent an ideal animal model for assessing the risk of zoonosis following long-term exposure to porcine material. However, PERV does not infect NHP cells with the same efficiency as it does human cells. The data presented in this thesis suggests that in some NHP species the poor infectivity is due to mutation of the same critical amino acid (a.a.109) described for muPAR. However, African green monkey cells express two functional receptors and other mechanisms are likely to be responsible for the low susceptibility to PERV-A infection.

Secondly, I evaluated the effect of a release inhibitor as a possible strategy to reduce PERV dissemination from pig cells. Human tetherin can inhibit retrovirus production from cells. I showed that overexpression of human and newly cloned porcine tetherin in pig cells can reduce the release of PERV. My data suggests that tetherin-expressing transgenic pigs could represent a safer donor in xenotransplantation.

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Abbreviations

a.a.	Amino acid
AGM	African green monkey
AGMPAR	African Green monkey PERV-A receptor
AHRX	Acute humoral xenograft rejection
ALV	Avian leukosis virus
A-MLV	Amphotropic murine leukaemia virus
AP	Alkanine phosphatise
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ASCT	Alanine, serine, cysteine transporter
ASLV	avian sarcoma and leukosis virus
ATP	adenosine triphosphate
BaEV	Baboon endogenous retrovirus
BaPAR	Baboon PERV-A receptor
Bp	Base pair
BSA	Bovine serum albumin
BST2	Bone marrow stromal cell antigen 2
CA	Capsid
CAT-1	cationic amino acid transporter 1
cDNA	Complementary DNA
CNS	Central nervous system
CPR	Complement regulatory protein
cPPT	central polypurine tract
CTD	carboxy terminal domain
CTE	constitutive transport element
cynPAR	Cynomolgus monkey PERV-A receptor
Cyp	Cyclophilin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylene diamine tetraacetate
EGFP	Enhanced green fluorescence protein
EGFP(PERV)	PERV-A14/220 carrying EGFP gene
EIAV	equine infectious anaemia virus
ELISA	Enzyme-linked immunosorbent assay
E-MLV	Ecotropic murine leukaemia virus
Env	Envelope
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
ESCRT	endosomal sorting complex required for transport
Etu	EGFP-transducing unit

FBS	foetal bovine serum
FeLV	feline leukaemia virus
FeLVCR	Feline leukaemia virus C receptor
FITC	Fluorescein isothiocyanate
FIV	feline immunodeficiency virus
Fv	Friend virus
Fv1	Friend virus susceptibility factor 1
GALV	gibbon ape leukaemia virus
GLVR	GALV receptor
HA	(Avian influenza) hemagglutinin
HAR	Hyperacute rejection
HERV	Human endogenous retrovirus
HEV	Hepatitis E virus
HIV	human immunodeficiency virus
HRP	Horseradish peroxidase
HTLV	Human T cell leukaemia virus
HTRC	Human tropic replication competent
huPAR	Human PERV-A receptor
huTHN	Human tetherin
IgG	Immunoglobulin G
IN	Integrase
IFN	Interferon
IOWATHN	Tetherin from porcine ST-IOWA cells
IRES	internal ribosome entry site
i.u.	Infectious unit
JSRV	Jaaksiekte sheep virus
KoRV	Koala retrovirus
LB	Luria-Bertani
Leu	Leucine
LTR	long terminal repeat
MA	Matrix
McERV	*Mus caroli* endogenous virus
MCF	mink cell focus-forming virus
MFSD2	Major facilitator superfamily domain 2
min	Minute
MLV	murine leukaemia virus
MMTV	mouse mammary tumour virus
MOI	multiplicity of infection
Mo-MLV	Moloney murine leukaemia virus
MPMV	Mason-Pfizer monkey virus
mRNA	messenger ribonucleic acid
muPAR	Murine PERV-A receptor
NC	Nucleocapsid

NHP	Non human primate
PAGE	Polyacrylamide gel electrophoresis
PAR	PERV subgroup A receptor
PBMC	Primary blood mononuclear cells
PBS	primer binding site
PBS	phosphate buffered saline
PCMV	Porcine cytomegalovirus
PCR	polymerase chain reaction
PE	Phycoerythrin
PERV	Porcine endogenous retrovirus
PIC	pre-integration complex
PK15THN	Tetherin from PK15 cells
PLHV	Porcine lymphotropic herpes virus
PLLP	Myelin protein plasmolipin
Pit	inorganic phosphate transporter 2
P-MLV	Polytropic murine leukaemia virus
poTHN	Porcine tetherin
PPT	polypurine tract
PR	Protease
Pro	Proline
R	repeat region
ratPAR	Rat PERV-A receptor
RBD	Receptor binding domain
Ref1	Restriction factor 1
RFP	Red fluorescence protein
REV	Reticuloendotheliosis virus
RD114	feline endogenous retrovirus RD114
RDR	RD114 receptor
rhPAR	Rhesus macaque PERV-A receptor
RIPA	Radio immunoprecipitation assay
RNA	ribonucleic acid
RRE	Rev-response element
rRNA	Ribosomal RNA
RSV	Rouse sarcoma virus
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
SA	splice acceptor
SD	splice donor
SDS	Sodium dodecyl sulphate
Ser	Serine
SFFV	Spleen focus-forming virus
shRNA	Small hairpin RNA
siRNA	Small interfering RNA

SIV	simian immunodeficiency virus
SPF	Specific pathogen free
SU	surface unit
THN	Tetherin
THTR	Thiamine transporter
TE	Tris-EDTA
TM	Transmembrane
TRIM	Tripartite motif
tRNA	transfer ribonucleic acid
U	unit
U3	unique 3’ region
U5	unique 5’ region
VSV-G	vesicular stomatis virus G protein
Vpu	(HIV-1) viral protein U
VRA	Variable region A
VRB	Variable region B
v/v	Volume/volume
w/v	Weight/volume
X-MLV	Xenotropic murine leukaemia virus
XMRV	Xenotropic MLV-related virus
XPR	Xenotropic and polytropic MLV receptor
ZAP	Zinc finger antiviral protein
αGAL	Galactose α(1-3)-galactose
Ψ	psi packaging signal

Chapter 1

1 INTRODUCTION

1.1 Xenotransplantation

The term ‘transplantation’ derives from the latin *transplantare* meaning ‘to plant again in another place’ (*trans*=across and *plantare*=to plant), and it is currently used in the medical field to indicate the transfer of cells, tissues and organs from a donor to a recipient. When donor and recipient belong to different species, it is referred as ‘xenotransplantation’ (from the greek *xeno*= foreign). The history of xenotransplantation can be dated back to the beginning of the sixteenth century, when attempts were made to treat human patients by using sheep, lamb and calf blood transfusions or tissue transplantation [reviewed in (Deschamps et al., 2005)]. A few organ transplantations were also conducted at the beginning of the 1900s [reviewed in (Taniguchi and Cooper, 1997)]. Unsurprisingly, all of these experiments were unsuccessful, mainly because the immunological bases of rejection were still unclear. In the second half of the twentieth century, the discovery of immunosuppressive drugs kindled the interest of the scientific community in xenotransplantation. In fact, it became possible to avoid xenograft rejection of an organ belonging to a closely related species. In 1964, Keith Reemtsma transplanted a chimpanzee kidney into a 23 year-old woman. The patient eventually died 9 months later for an acute electrolyte imbalance, but without evidence of rejection (Reemtsma et al., 1964). Furthermore, with the increased rate of success in human-to-human transplantation (namely allotransplantation) due to the arrival of immunosuppressive protocols, the gap between the number of organ transplantations performed every year and the waiting list of candidates is enlarging (Figure 1.1).

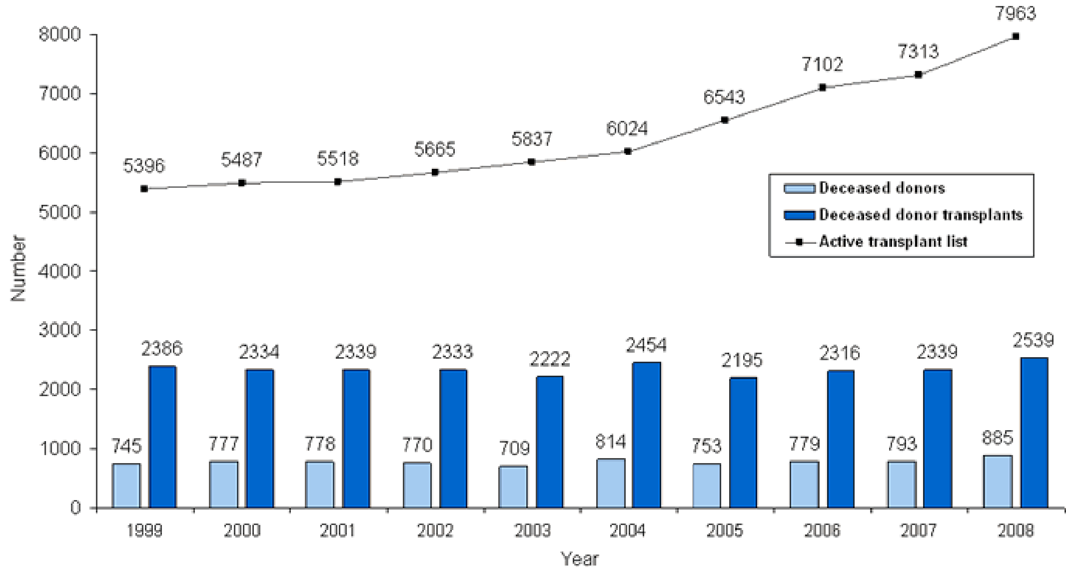


Figure 1.1 UK transplantation statistics.

Numbers of deceased donors and transplants in the United Kingdom between 1997 and 2008 and patients on the active and suspended transplant lists at 31st December 2008, as recorded on 8th June 2009. Statistics prepared by NHS Blood and Transplant from the National Transplant Database maintained on behalf of transplant services in the UK and Republic of Ireland (www.uktransplant.org.uk/ukt/).

Xenotransplantation is a potential approach to alleviate the organ shortage for allotransplantation. Porcine cells and tissues have been employed in the treatment of human diseases with encouraging results: pig foetal neuron cells were grafted in Parkinson's and Huntington's disease patients in the attempt to slow down neurodegeneration (Deacon et al., 1997; Fink et al., 2000); foetal porcine islet-like cell clusters were transplanted to diabetic patients to reverse diabetes (Groth et al., 1994); *ex-vivo* pig-liver perfusions have been used as temporary treatment of hepatic failure (Chari et al., 1994), and porcine skin used as dressing for burns (Reichenbacher, 1975).

There are many advantages of xenotransplantation over allotransplantation:

1) The unlimited graft supply;

2) Surgery can be planned in advance and, therefore, the organs are harvested immediately prior to the operation;

3) Animal donors can be bred in a 'clean'/controlled environment and the organs screened for the presence of infectious agents before implantation.

The US Food and Drug Administration (FDA) has produced guidelines for xenotransplantation regarding donor animal selection, the facilities where to farm them, health surveillance and the screening for pathogens (www.fda.gov/cber/gdlns/xenophs0101.htm). Although the most obvious choice of donor animal would seem to be a closely to humans related species, such as non-human primates (NHP), the animal source recommended by the FDA is swine. Among several reasons, the risk for transmission of infectious disease from pigs to humans is lower than with NHP. Moreover, pigs offer fewer ethical problems since they are currently farmed for food. They have a short gestation (3.5 months) and large litter (6-14 piglets). Maintaining pigs in specific pathogen-free (SPF) conditions is relatively easy and economically affordable. It is also possible to produce genetically modified pigs in the laboratory (Brunetti et al., 2008; Dai et al., 2002; Lai et al., 2002).

Xenotransplantation is not yet a worldwide clinical reality because some obstacles still exist:

1) Some porcine organs may not be physiologically compatible with a human recipient, especially those organs which perform complex biochemical and metabolic functions, such as kidney and liver (Hammer, 2002; Soin et al., 2001).

2) The immune rejection of the xenograft. Three types of reaction have been described [reviewed in (Yang and Sykes, 2007)]. Hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR) are antibody-mediated processes and occurred early after transplantation. If both HAR and AHXR have been prevented, the xenograft may still be subject to cell-mediated rejection.

3) Although farming pigs in SPF conditions can reduce the risk of contamination by most known porcine pathogens, there are still safety issues posed by unknown pathogens and a certain type of viruses

such as porcine endogenous retroviruses, which are part of the pig genome and therefore difficult to eliminate (Patience et al., 1997).

Genetic engineering of the donor pigs can overcome most of the xenotransplantation-related problems described above.

1.1.1 Genetically modified pigs

HAR may occur within minutes after xenotransplantation and it is characterised by the destruction of the vascular endothelium of the donor organ. This process is triggered by natural antibodies present in humans directed against pig antigens in a complement-dependent way. The major antigen in pigs is the disaccharide residue galactose-α(1-3)-galactose (αGal) [reviewed in (Galili, 2001)]. The enzyme α1,3-galactosyltransferase that synthesises αGal is active in most mammalian species (including pigs) but not in humans, apes and Old World monkeys. These species have a high concentration of circulating anti-αGal antibodies in response to continuous antigenic exposure by the gastrointestinal bacterial flora (Galili et al., 1988). Several strategies have been exploited which involve development of transgenic or gene knockout pigs. Animals lacking both alleles of the gene for α1,3-galactosyltransferase have successfully been bred (Kolber-Simonds et al., 2004; Phelps et al., 2003). Their organs have been tested in a pig-to-baboon transplantation, and HAR was avoided. The recipients lived up to 6 months but then developed thrombotic microangiopathy and coagulation disregulation (Kuwaki et al., 2005; Tseng et al., 2005; Yamada et al., 2005). Although these results are encouraging, more has to be done to ultimately avoid xenograft rejection. The future direction of research includes the introduction of further genetic modifications in pigs. To specifically target microvascular thrombosis, transgenic pigs expressing anticoagulant or antiplatelet genes such as CD39, hirudin, or a transmembrane-anchored form of human tissue factor pathway inhibitor, or the knockout of fibrinogen-like protein 2, can be employed [reviewed in (Ekser et al., 2009)]. To inhibit the complement-mediated immune response in the patients, pigs have been genetically modified to express human complement regulatory proteins (CRP) prolonging the xenograft survival from minutes to weeks: decay-

accelerating factor (DAF or CD55), CD59 and membrane cofactor protein, (CD46) (Adams et al., 2001; Bhatti et al., 1999; Cozzi et al., 2000; McCurry et al., 1995).

All these strategies aimed at avoiding the immune response of recipient may increase the risk of the transfer of pathogens between species (section 1.2). Enveloped viruses budding from a host cells acquire and expose proteins present on the cell membrane. The lack of αGal in the donor animal cells will lead to the lack of this strong antigen in the viral particles, diminishing the immune response to the possible viruses harboured in the donor xenograft (Kim et al., 2007; Magre et al., 2004; Quinn et al., 2004). Furthermore, viruses budding from CPR-transgenic pigs would be protected, to some extent, from the complement-mediated lysis. *In vitro* studies conducted to explore this risk were not conclusive. Porcine endogenous retroviruses, budding from a CD59-expressing pig cell line, were neutralised by human serum, although CD59 was incorporated in the viral envelope (Takefman et al., 2002). However, in more recent studies, rhabdoviruses and retroviruses produced through a pig endothelial cell line expressing CD55 (Magre et al., 2004) and PERV produced through cell lines engineered to express CD59 or CD55 (Hazama et al., 2005) were shown to be partially protected by complement-mediated inactivation.
Due to the unpredictable risk posed by immune system-resistant transgenic pigs, more effort should be directed into increasing the safety of using the donor animals.

1.2 Zoonosis in xenotranplantation

Zoonosis (from greek *zoon*=animal and *nosis*=disease) is the transfer of an animal pathogen to a human host.

Infectious disease agents have often co-evolved with their own host species in a harmless relationship. Microbes are adapted to their host and not genetically equipped for infecting a different species efficiently (species barrier). However, occasionally, events of cross-species infection may happen due to physical proximity of different species. In this instance, there are two possible scenarios: the infectious agent will die with, or within, the new host, or adapt and spread in the new species causing epidemic. In the past, the human population has been afflicted (and still is) by several zoonoses. Among the most notorious examples are typhus [reviewed in (Andersson and Andersson, 2000)], plague [reviewed in (Perry and Fetherston, 1997)], Dengue fever [reviewed in (Gubler, 1988)], West Nile virus encephalitis [reviewed in (Kramer et al., 2007)], avian influenza [reviewed in (Alexander and Brown, 2000; de Wit and Fouchier, 2008)], AIDS (Gao et al., 1999; Keele et al., 2006), Nipah virus encephalitis [(Chua et al., 1999; Paton et al., 1999) and reviewed in (Chua, 2003)], new variant of Creutzfeldt-Jakob disease (Will et al., 1996), severe acute respiratory syndrome (SARS) (Drosten et al., 2003) and the recent pandemic swine influenza (Garten et al., 2009; Smith et al., 2009).

A literature survey has revealed that the majority of sources of emerging and reemerging infections in humans are zoonotic pathogens and viruses are greatly overrepresented (Woolhouse and Gowtage-Sequeria, 2005). A possible explanation for this lies in the fast evolution rate of viruses which can therefore adapt to a new host with comparative ease (Drake and Holland, 1999).

In xenotransplantation, the risk of zoonosis is increased because normal host defences such as skin and mucosal surface are bypassed when human and animal tissues are placed in close contact and the patient receiving the xenograft undergoes immunosuppressive treatment. A cross-species infection is not only a danger for the recipient of the xenograft, but can also

lead to the development of a potential new epidemic in the human population. As pig is considered the most suitable candidate as donor species, porcine viruses represent a potential threat. They can be grouped into four categories: 1) exogenous acute viruses; 2) viruses able to establish a persistent infection in the infected cells; 3) endogenous retrovirus; 4) unknown viruses. Regarding the latest, the identification of uncharacterised viruses in pigs, may help to increase the safety of xenotransplantation. New techniques to screen the donor animal for known and unknown pathogens include DNA microarray (Palacios et al., 2007; Wang et al., 2002; Wang et al., 2003) and metagenomic surveys using high-throughput shotgun sequencing technology (Palacios et al., 2008). To improve the safety of an unpredictable risk, patients receiving porcine materials should be monitored in a life-long surveillance program, to promptly detect any unexplained posttransplantation illness (www.fda.gov/cber/gdlns/xenophs0101.htm).

1.2.1 Porcine exogenous viruses

Most known viruses belong to the exogenous acute group. They usually infect a cell, replicate, and kill it on their way out. Pigs are not always a natural reservoir of these viruses, but they may act as a vector to mediate the cross-species infection to humans. Some such examples include influenza virus (Castrucci et al., 1994; Ludwig et al., 1995), Nipah virus (Parashar et al., 2000) and the recently described Reston Ebola virus (Barrette et al., 2009). The porcine exogenous viruses most relevant in xenotransplantation are described below.

1) Swine Influenza viruses belong to the *Orthomyxoviridae* family, Influenza virus A genus. These enveloped viruses have a negative-sense single-stranded RNA genome divided into eight segments (Klenk et al., 2004). Due to the segmented nature of its genome, new strains of Influenza virus may emerge by genetic reassortment of the RNA segments through infection of the same cells by two different strains (Brown et al., 1998; Webster et al., 1995). Zoonotic infection of humans by swine influenza virus was first proven in 1976 by Smith and colleagues (Smith et al., 1976). Reassortment of avian and human influenza viruses in pigs was also detected (Castrucci et al.,

1994; Ludwig et al., 1995). It has been hypothesised that pandemic flu, in 1918, was derived from a swine influenza virus (Taubenberger et al., 1997), but recent genetic analysis could not confirm this hypothesis [reviewed in (Reid et al., 2004)]. Nevertheless, the threat of a reassortment strain of Influenza virus from pigs that could potentially cause a pandemic still exists. Indeed, a pandemic swine flu has recently been described (Garten et al., 2009; Smith et al., 2009).

2) Another porcine virus able to cross the species barrier between pigs and humans is Nipah virus. This enveloped virus is a member of the *Paramyxoviridae* family, whose genome is a linear, negative-sense, single-stranded RNA molecule (Rima et al., 2004). Bats are the natural reservoir of this virus (Chua et al., 2002; Reynes et al., 2005; Sendow et al., 2006). The first outbreak of viral encephalitis due to Nipah virus occurred in Malaysia and Singapore between 1998 and 1999, resulting in 276 cases of encephalitis with 106 deaths (Chua et al., 2000; Chua et al., 1999). During this outbreak, the mode of transmission was mainly pig-to-human with only 8% of the infected individuals not having had direct contact with pigs (Parashar et al., 2000). In the following years, several outbreaks occurred in Asia, with a worsening in mortality, and other modes of transmission such as foodborne and human-to-human were identified (Gurley et al., 2007; Hsu et al., 2004; Luby et al., 2006).

3) The family *Filoviridae* comprises filamentous enveloped viruses whose genome is a linear, negative-sense, single-stranded RNA molecule. Two genera belong to this family, *Marburgvirus* and *Ebolavirus* (Suzuki and Gojobori, 1997). Filoviruses are associated with acute fatal hemorrhagic disease in humans and non-human primates. Reston Ebolavirus has recently been identified in pigs from different farms in the Philippines (Barrette et al., 2009). Although Reston Ebolavirus has not been associated with any diseases in humans (Morikawa et al., 2007), concern arose in that its passage through swine may allow the virus to become more pathogenic. In the xenotransplantation context, the unexpected discovery of Ebola virus in pigs showed how surveillance of the animal donor should be extended to a

wide range of viruses, even those which are not normally associated with disease in pigs.

4) Porcine rotaviruses are associated with gastroenteritis and diarrhoea in the human and swine population. They have a segmented double-stranded RNA genome enclosed in a core made of three protein layers (Holmes et al., 2006). As for the previously described influenza virus, the 11 segments which form the rotavirus genome can rearrange in a superinfected cell, and new strains may emerge [reviewed in (Ramig, 1997)]. Cross-species infection by rotavirus has been documented: human rotavirus has been shown to infect pigs *in vivo* (Ward et al., 1996) and clinical studies have reported human infection by porcine rotavirus (Gabbay et al., 2008; Nguyen and Hildreth, 2000). Furthermore, reassortment between human and porcine rotaviruses does occur (Li et al., 2008; Martella et al., 2008; Mascarenhas et al., 2007; Matthijnssens et al., 2008).

Porcine exogenous viruses described above represent a possible risk in xenotransplantation. However, they cause an acute infection, easily recognised by clinical symptoms and diagnostic tests are available. Therefore, upon surveillance in SPF facilities for these viruses, a possible infection can be contained with ease. Instead, infection by other swine viruses may be not so easily recognisable, and special attention should be focussed on the following viruses.

5) Members of the *Parvoviridae* family are non-enveloped, single-stranded, DNA viruses with a broad host range (Berns et al., 2004). Porcine parvovirus is the aetiological agent in the syndrome of reproductive failure in pigs, which include stillbirths, mummified foetus, early embryonic death and infertility (Dunne et al., 1965; Mengeling and Cutlip, 1976). There are no clinical signs associated with parvovirus. Therefore, animals should be screened regularly for this virus. ELISA-based diagnostic kits are available.

6) Hepatitis E virus (HEV) is the sole member of the genus Hepevirus in the family Hepeviridae (ICTVdB, 2004). This RNA virus is the aetiological agent of enterically transmitted non-A, non-B, acute hepatitis (Balayan et al., 1983).

HEV is involved in many hepatitis epidemics and outbreaks [reviewed in (Panda et al., 2007)]. The overall mortality in humans associated with HEV infection is 0.5-3%, but this can increase up to 15-20% in pregnant women (Mushahwar, 2008). The faecal-oral route (i.e. drinking contaminated water) is the main mode of transmission during an epidemic (Panda et al., 2007). Zoonotic transmission of HEV has been associated with pigs. Swine HEV was first described in 1997 (Meng et al., 1997) and it shared 97% homology with two strains of human HEV genotype 3 (Meng et al., 1998). HEV infection was associated with the ingestion of raw or under-cooked pig meat (Bouwknegt et al., 2007; Deest et al., 2007; Mizuo et al., 2005; Yazaki et al., 2003), and with surgical training using pigs (Colson et al., 2007). In the context of xenotransplantation, the infection of laboratory monkeys, and detection of HEV in pigs bred in specific pathogen-free facilities, is a major concern (Yamamoto et al., 2008).

Other exogenous porcine viruses whose presence has important economic consequences in swine farming, such as porcine reproductive and respiratory virus, classical swine fever virus, swine vesicular virus and foot-and-mouth viruses, present clear clinical features and cross-species infection in human have not be observed with the exception of one case of foot-and-mouth disease in 1966 (Armstrong et al., 1967). This episode highlighted how, although a human infection by these swine viruses is a rare event, it is still a risk that cannot be excluded.

1.2.2 Porcine Herpesviruses

Herpesviruses are enveloped DNA viruses which can persist in the host in a latent phase without causing symptoms for long periods of time (ICTVdB, 2006). Although herpesviruses usually have a narrow host range, cross-species infection and adaptation to the new host have been described (Ehlers et al., 2008). Suid Herpesvirus type 1 (SuHV-1, Pseudorabies virus) is a member of subfamily α-herpesvirinae. Their natural host is swine, but cross-species infections have been described, causing severe neurological symptoms and death of the new host (Glass et al., 1994; Marcaccini et al., 2008). However, infections of humans or primates have not been observed.

Nevertheless, a potential zoonosis or recombination with human herpesviruses cannot be excluded.

Porcine cytomegalovirus (PCMV) is a β-herpesvirus. In preclinical studies in pig-to-baboon transplantation, PMCV replication was enhanced due to immune suppressive protocols, and consumptive coagulopathy was observed in transplanted pig tissues in association with PMCV expression. However, no evidence of PCMV invasion and productive infection has been detected in NHP tissues (Gollackner et al., 2003; Mueller et al., 2004). PCMV can be eliminated by early weaning of the herds bred for xenotransplantation purpose (Mueller et al., 2004).

Porcine lymphotropic herpes viruses (PLHV)-1,-2 and -3 belong to the subfamily γ-herpesvirinae and have high homology with human herpes virus 8 (HHV-8, also known as Kaposi’s sarcoma virus) and Epstein-Barr virus (EBV). Despite PLHV not being activated in pig-to-baboon organ transplantation (Issa et al., 2008; Mueller et al., 2004), the transactivators of HHV-8 and EBV could activate PHLV (Santoni et al., 2006). Furthermore, the same strategy of early weaning proposed for PMCV proved to be ineffective for PHLV (Mueller et al., 2005).

1.3 Retroviruses

Retroviridae are a family of vertebrate viruses, divided into two subfamilies *orthoretroviridae* and *spumaretrovirinae* Only one genus has been described in the *spumaretrovirinae* subfamily, *spumavirus*, whereas six genera belong to the *orthoretroviridae* subfamily: *α-retrovirus, β-retrovirus, γ-retrovirus, δ-retrovirus, ε-retrovirus* and *lentivirus* (Linial et al 2005).

1.3.1 Structure

A retroviral particle's diameter is in the range of 80-120nm, measured by thin-section electron microscopy. The mature virions have a condensed protein core wrapped in a lipid envelope. The viral envelope is derived from the cell plasma membrane and is acquired during budding. The lipid composition of the envelope is rich in sphingomyelin and cholesterol, suggesting that the virus buds from cholesterol-rich rafts in the cell plasma membrane (Aloia et al., 1993; Briggs et al., 2003; Ono and Freed, 2005; Quigley et al., 1971). The envelope is scattered with glycoproteins, called Env. The Env protein is synthesised as a polyprotein precursor, and the leader signal in the N-terminus directs the protein to the ER and then to the Golgi apparatus where the glycosylation process commences. In the Golgi apparatus, cellular proteases cleave Env into two subunits, surface (SU) and transmembrane (TM). During the transport to the cell surface, three SU and three TM oligomerise to form the spike, which will be present on the viral envelope (Eckert and Kim, 2001; Einfeld and Hunter, 1988; Kamps et al., 1991; Wyatt and Sodroski, 1998). The Env proteins interact with the cell surface proteins which mediate the fusion process and the subsequent entry of the virus in the cell. The determinant regions for the receptor usage are located in the N-terminal region of SU in the variable region A and B (VRA and VRB) (Battini et al., 1992; Tailor and Kabat, 1997). In the N-terminus of the TM is located the fusion peptide, responsible for the fusion of the viral envelope with the cell membrane, which occurs following the interaction of SU with the receptor (Hunter, 1997).

Matrix (MA) proteins are associated with the lipid envelope. In most retroviruses, MA proteins are myristylated at their N-terminus and this posttranscriptional modification appears to be essential for retroviral assembly (Bryant and Ratner, 1990; Gottlinger et al., 1989; Rein et al., 1986). Furthermore, MA interacts with Env proteins during budding and it has been suggested for the lentivirus, human immunodeficiency virus (HIV)-1, that this interaction is required for the incorporation of the Env proteins in the virions (Freed and Martin, 1996; Yu et al., 1992).

The condensed core in the retroviral particles is formed by the capsid proteins (CA) and is commonly referred to as the capsid. This structure contains the genomic viral material associated with the nucleocapsid proteins (NC). In all the orthoretroviruses, NC has one or two characteristic motifs ($CX_2CX_4HX_4C$). Mutation of these domains results in the absence of the viral genome in the virions, suggesting a possible role for NC in the packaging of the genomic RNA into the nascent particles (Gorelick et al., 1988; Meric and Goff, 1989). Moreover, NC is important in the reverse transcription process (Meric and Goff., 1989) probably by promoting the annealing of the tRNA primers to the primer binding site, as well as facilitating the strand transfer (section 1.3.3). Indeed, NC has been shown to promote the annealing of complementary RNA sequences (Prats et al., 1988).

MA, CA and NC are present in all the orthoretroviruses. They are encoded by the *gag* gene and synthesised as Gag polyprotein, then cleaved by the viral protease. Additionally, other Gag proteins may be produced, but they vary between retroviruses. For instance, murine leukaemia virus (MLV) *gag* encodes an additional protein p12 (figure 1.2), important for virus assembly and release as well as in the early stages of the infection (Yuan et al., 2002; Yuan et al., 1999).

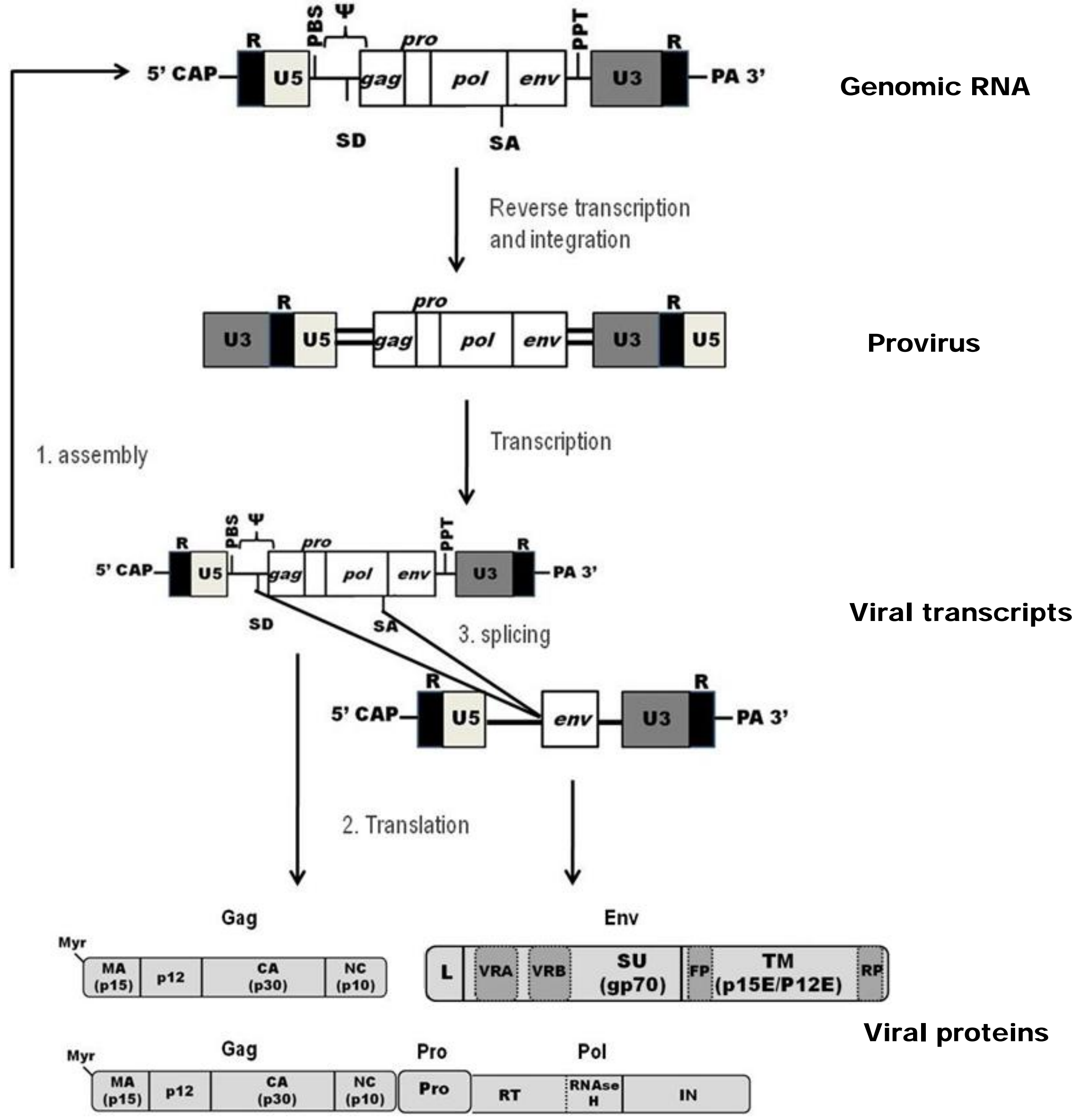


Figure 1.2 Genetic organisation and transcript processing of MLV.

From the top, the genomic RNA has been represented with the following elements highlighted (from left to right): repeat region (R), unique region in 5' end (U5), tRNA primer binding site (PBS), splicing donor site (SD), packaging signal (Ψ), four genes (gag, pro, pol, env), splicing acceptor site (SA), polypurine tract (PPT), unique region in 3'end (U3) and the polyadenylated tail (PA). Following reverse transcription, the viral DNA integrates as a provirus, characterised by the long terminal repeat (U5-R-U3). The full length viral transcript follows three possible routes: assembled in a new virion as genomic RNA (1), translated in the polyproteins Gag and Gag-Pro-Pol (2), spliced and translated in the envelope protein (Env) (3). Gag-Pro-Pol polyprotein is cleaved in the following components (from left to right): matrix (MA), p12, capsid (CA), nucleocapsid (NC), protease (Pro), reverse transcriptase (RT) with the ribonuclease H domain (RNase H), and integrase (IN). The Env is processed by removal of the leader signal (L) and cleavage between the two units: surface (SU), containing the variable region A (VRA) and B (VRB), and transmembrane (TM) containing the fusion peptide (FP) and the R peptide (RP).

1.3.2 Genomic RNA

The genome of the retroviruses consists of two positive-sense, single-stranded, RNA molecules. The size is between 7 to 12 kilobases. The genomic RNA is a dimer held together at the 5' ends. The structure of each molecule resembles a cellular messenger RNA (mRNA). The 5' end is capped and the 3' end is polyadenylated. The genomic RNA inside the virions is the unspliced form of the viral transcripts. At both the extremities of the RNA molecules there are the repeat regions (R) followed by a unique 5' sequence (U5) and a unique 3'sequence (U3). These regions form the long terminal repeats (LTRs) in the integrated provirus. Immediately after the U5, there is the primer binding site (PBS), the region for annealing to a specific cellular transfer RNA (tRNA) which will act as primer for the reverse transcription (section 1.3.3). Retroviruses use different tRNAs; $tRNA^{Lys}$ (HIV-1), $tRNA^{Pro}$ (MLV and PERV subgroup C), $tRNA^{Gly}$ (PERV subgroup A and B), are some examples. The genomic RNA is packaged into a retroviral particle by recognition of the packaging (or encapsidation) sequence, called Ψ, located near the 5' end of the RNA molecules (figure 1.2). At the other end of the molecule there is a region important for reverse transcription, called the polypurine tract (PPT), immediately before the U3 (Vogt, 1997).

The genome of all retroviruses encodes four main genes *gag, pro, pol* and *env*. In all of them, the Env protein is translated from a spliced mRNA which is formed by excision of the *gag, pro* and *pol* genes. The splice donor (SD) site is generally upstream of *gag* and the splice acceptor (SA) site immediately before *env* (Vogt, 1997). Structural proteins are synthesised from the *gag* gene. They are produced as a polyprotein successively cleaved by the viral protease, encoded by *pro*. The cleavage of the precursor Gag and Gag-Pol occurs during or after the assembly of the viral particle, leading to the maturation of an infectious virus. All the retroviruses possess two other enzymes encoded by the *pol* gene: reverse transcriptase (RT) and integrase (IN). RT is a RNA-dependent DNA polymerase. Using the viral genomic RNA as template, RT promotes the synthesis of a new single stranded DNA filament. RT possesses a second domain, a ribonuclease H (RNase H),

which degrades the RNA filament during DNA synthesis (Telesnitsky and Goff, 1997). Once the viral DNA has been synthesised and translocated to the nucleus, IN promotes its integration into the cellular genome. The different steps in the integration process involve the following IN functions: nuclease (cleavage of the 3' ends of the viral DNA), DNA binding and transesterification (3'-OH groups at the viral DNA ends are used to form phosphodiester bonds on the target cellular DNA leading to the joining of the two DNA filaments) (Brown, 1997).

In addition to these four genes, some genera of retroviruses possess other accessory genes. For instance, γ-retroviruses are simple viruses and encode only the four genes described above, while lentiviruses are complex retroviruses and possess additional genes (e.g. HIV-1 encode six accessory genes: *vif*, *vpr*, *tat*, *vpu*, *rev* and *nef*) (Rabson and Graves, 1997; Swanstrom and Willis, 1997).

1.3.3 Life cycle

Viruses need to infect a cell to reproduce (Figure 1.3). Retroviruses enter vertebrate cells by attachment to the cell surface, followed by interaction between the viral glycoprotein Env and specific host surface molecules (section 1.5). This binding causes conformational changes in the Env proteins, leading to the exposure of the fusion peptide at the N-terminus of TM, and its insertion into the cell plasma membrane. As a result, fusion between the viral envelope and the host cell membrane occurs, and the virus core is released into the cytosol [reviewed in (Colman and Lawrence, 2003; Eckert and Kim, 2001)]. The majority of the viral particles, however, enter the cell through endocytosis (Marechal et al., 1998). For lentiviruses such as HIV-1, this route leads to an abortive infection, with the virions being degraded by the proteasome (Fredericksen et al., 2002; Schwartz et al., 1998). Other retroviruses, such as ecotropic and amphotropic MLV (Katen et al., 2001) and foamy virus (Picard-Maureau et al., 2003) can enter cells via the endocytic pathway, in a pH-dependent manner. The preferential route of entry for those retroviruses, which can use both, appears to be cell type specific (Kizhatil and Albritton, 1997).

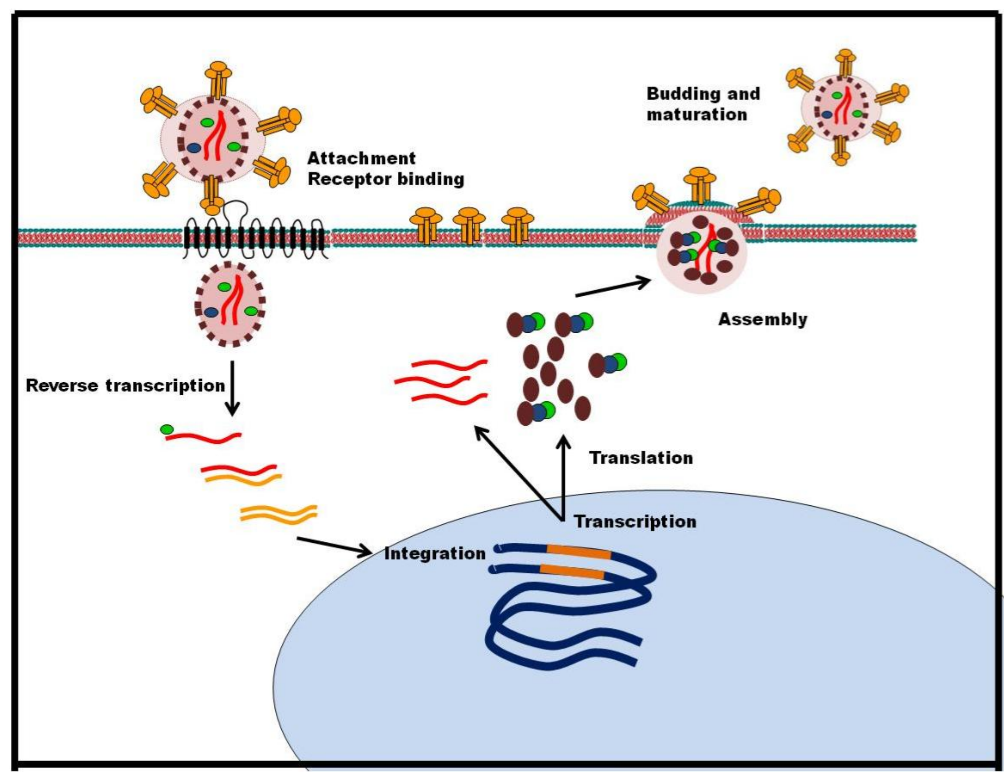


Figure 1.3 Retrovirus life cycle

The retroviral life cycle, schematised here, is described in detail in section 1.3.3.

Reverse transcription takes place in the cytoplasm of the cell and generates a linear, double-stranded DNA using the viral genomic RNA as template (Figure 1.4). The viral RT enzyme starts the synthesis of the minus-strand DNA filament using as primer the 3' end of the tRNA which is annealed at the PBS in the genomic RNA (Figure 1.4 A). The minus strand DNA synthesis continues until RT reaches the 5' end of the viral RNA (Figure 1.4 B). The RNase H domain of the RT digests the RNA filament in the newly synthesised hybrid RNA:DNA (Figure 1.4 C). The transfer of the minus-strand DNA to the opposite end of the genomic RNA is guided by the repeated region, R (Figure 1.4 D). RT resumes the synthesis of the minus-strand DNA. RNAse H digestion removes most of the RNA except the PPT, which is highly resistant to RNase H degradation (Figure 1.4 E). The undigested RNA sequence PPT serves as a primer for the synthesis of the plus-strand DNA (Figure 1.4 F). RT continues copying the minus-strand DNA into a portion of the tRNA used as initial primer, causing its removal (Figure 1.4 G).The plus-strand DNA is transferred to anneal to the PBS site in the minus-strand DNA filament, and the synthesis of both strands completed (Figure 1.4 H and I). The final result is a blunt-ended, linear DNA duplex (Telesnitsky and Goff, 1997).

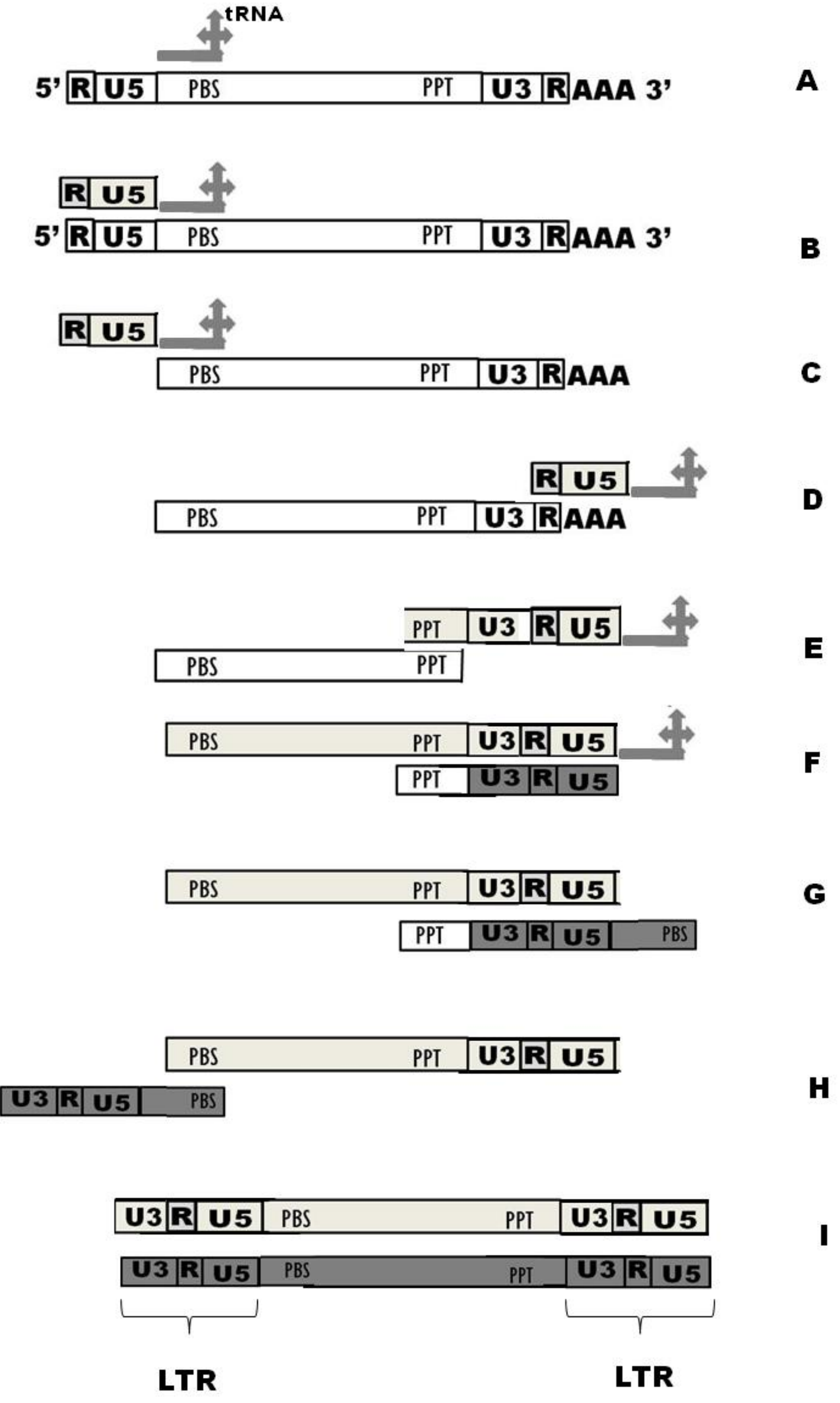


Figure 1.4 Reverse transcritption
The reverse transcription of the retroviral genomic RNA (white) starts with the synthesis of the minus-strand DNA (light grey) at the 3' end of the primer tRNA, annealed at the primer binding site (PBS). The synthesis of the plus-strand DNA (dark grey) commences using the undigested polypurine tract (PPT) as primer. The final product is a linear, double-stranded DNA flanked by two identical long terminal repeats (LTR).

The viral DNA is associated with viral and cellular proteins, forming the preintegration complex (PIC), which is translocated to the nucleus. To gain access to the nucleus, most retroviruses depend on mitosis, where the nuclear membrane is disassembled. However, members of the genus *lentivirus*, such as HIV-1, can pass through an intact nuclear membrane. HIV-1 PIC enters the nucleus via a nuclear localisation signal-mediated, active import through the nuclear pore (Bukrinsky et al., 1992; Gulizia et al., 1994; Heinzinger et al., 1994; von Schwedler et al., 1994). Once in the nucleus, the integrase-DNA complex binds to the DNA and the integrase catalyses the joining of the viral DNA with the host cell DNA. The cellular components of the DNA damage response system mediates the filling in of the nicks and gaps flanking the viral DNA [reviewed in (Smith and Daniel, 2006)]. The site of integration was initially thought to be random. However, a systematic large scale analysis of retroviral integration sites revealed virus-specific preferential integration sites [reviewed in: (Bushman et al., 2005)]. For instance, HIV-1 and simian immunodeficiency virus (SIV) preferentially integrate in transcription units (Crise et al., 2005; Schroder et al., 2002), whereas MLV and porcine endogenous retrovirus (PERV) in the proximity of the transcriptional start sites and near CpG islands, regions abundant in CpG dinucleotide, which are undermethylated and associated with gene regulatory regions (Moalic et al., 2006; Wu et al., 2003).

Transcription of the viral genes can occur efficiently after the integration of the viral DNA and it is mediated by the RNA polymerase II, the enzyme responsible for the synthesis of cellular mRNAs and some small nuclear RNAs. The transcription is promoted by the viral LTR. Direct repeat sequences in the U3 region of the LTR constitute an enhancer element (Laimins et al., 1984; Levinson et al., 1982) which contains a transcription factor-binding site (Speck and Baltimore, 1987). The promoter in the U3 region contains the TATA element upstream of the transcription start site. PERV shares the same structural organisation of other γ-retroviruses, however, the direct repeats (i.e. enhancer) differ from other γ-retroviruses, and appear to bind a smaller number of transcription factors. Another region in the U3 region of the LTR upstream of the repeated elements have been

predicted to contain transcription factor binding sites (Wilson et al., 2003). The cellular transcriptional machinery processes the viral transcripts as for any other cellular mRNAs, capping the 5' end and adding a polyadenylated tail at the 3' end. The full-length, unspliced transcript serves three possible roles: 1) viral genome, packaged into the nascent virions; 2) mRNA for Gag and Gag-Pol translation; 3) mRNA for Env translation, once spliced.
The export to the cytoplasm of unspliced mRNAs requires the action of viral-specific functions. HIV-1 produces an auxiliary protein, Rev (regulatory of virion), which acts as adaptor between the viral RNA and cellular export protein complex [reviewed in (Pollard and Malim, 1998)]. Other retroviruses, including human T- lymphotropic virus (HTLV), mouse mammary tumour virus (MMTV), equine infectious anaemia (EIAV), and feline immunodeficiency virus (FIV) encode Rev-like proteins (Magin et al., 1999; Mertz et al., 2005; Phillips et al., 1992; Rimsky et al., 1988). Simple retroviruses which do not encode accessory proteins rely upon the *cis*-acting element, for the export of the viral RNA. As examples, Mason-Pfizer monkey virus, simian retrovirus type D and Rous sarcoma virus possess a distinct RNA structure, called constitutive transport element (Bray et al., 1994; Ernst et al., 1997; Ogert et al., 1996; Zolotukhin et al., 1994). The Moloney MLV (MoMLV) packaging signal appears to be involved in the nuclear export of full-length RNA (Smagulova et al., 2005).

Ribosomes bind to the viral RNA and start the translation with the start codon for Gag, skipping upstream start and stop codons, probably by internal ribosome entry site (IRES). Evidences for the presence of IRES in the RNA of MLV (Berlioz and Darlix, 1995; Deffaud and Darlix, 2000a), avian reticuloendotheliosis virus type A (Lopez-Lastra et al., 1997), Rous sarcoma virus (Deffaud and Darlix, 2000b) and HIV (Brasey et al., 2003; Buck et al., 2001) have been described. Two polyproteins are synthesised, Gag and Gag-Pro-Pol from the same unspliced viral RNA. β-retroviruses, such as members of the avian sarcoma-leukosis virus group, differ from other retroviruses in that the Pro domain is contained in both precursors because Pro-coding sequence is placed in the gag reading frame (Bennett et al., 1991; Schwartz et al., 1983). The regulation of the translation of the Gag-Pro-

Pol precursor allows the production of more Gag proteins than the enzymes encoded by *pro* and *pol*. Typically, 10 to 20 Gag molecules are made per molecule of Gag-Pro-Pol (Hatfield et al., 1992). Two mechanisms are exploited by retroviruses to generate the Gag-Pro-Pol precursor, both aimed to bypass the termination codon at the 3' end of *gag*. Most retroviruses (α-, β-retrovirus and lentivirus) use a ribosomal frameshift by 'slipping' backwards of one nucleotide (-1) and changing the open reading frame (ORF). In other retroviruses, such as the β-retrovirus mouse mammary tumour virus (MMTV) and δ-retrovirus human T-leukaemia virus (HTLV-1), *pro* lies in a different reading frame from *gag* and *pol* and therefore two (-1) frameshifts, one to create Gag-Pro and a second one for the Gag-Pro-Pol precursor, are required [(Hatfield et al., 1992) and reviewed in (Jacks, 1990)]. Contrastingly, γ-retroviruses read through the termination codon. For instance, in MLV the stop codon is occasionally misread as a glutamine (Yoshinaka et al., 1985).

Electron microscopy has showed at least two pathways for retroviral assembly. For lentiviruses, α- and γ-retroviruses, the viral proteins are transported to the plasma membrane where the assembly takes place. Instead, β- and δ–retroviral cores are assembled in the cytoplasm, and migrate to the plasma membrane where they bud from the cell. The cleavage of the polyprotein precursor by the viral protease occurs during and after the release of the virions (Swanstrom and Willis, 1997). The retrovirus budding has not been fully characterised yet. A region in the Gag precursor, late domain (L), has been described as critical for the release of the virions. The role of the L sequence appears to be the hijacking of the cellular machinery responsible for the budding of cargo-laden vesicles into the multivesicular bodies, such as the class E vesicular protein sorting machinery, which assembles into discrete complexes termed the endosomal sorting complex, required for transport (ESCRT)-I, -II, -III [reviewed in (Demirov and Freed, 2004)].

1.3.4 Retroviral pathogenesis

Retroviruses are associated with a wide variety of diseases including tumours, immunodeficiencies and neurological disorders. The first retrovirus

was originally discovered in 1908 as transmissible oncogenic agents of erythro-myeloblastic leukaemia in chickens, later identified as the avian leukosis virus (ALV). Since then, many other tumourigenic retroviruses were described (Rosenberg and Jolicoeur, 1997). One possible mechanism of retrovirus-mediated oncogenesis is the expression of viral oncogenes. The first transforming gene identified was the *src* oncogene in Rous sarcoma virus, which highly resembles the cellular *src* gene in chicken DNA (Stehelin et al., 1976). Most of the viruses which carry oncogenes are replication-defective because some, or all, of their genes were lost during the acquisition of the cellular oncogene. Other retroviruses induce tumours by provirus insertional mutagenesis. The viral integration site for these viruses has been mapped to the proximity of a cellular oncogene (called proto-oncogene). The promoter and enhancer elements in the proviral LTRs can increase the transcription of the proto-oncogene, leading to neoplasia (Hayward et al., 1981; Neel et al., 1981). Some retroviruses cause tumorigenesis via an Env-mediated mechanism, by interacting with cellular proteins and altering the transcriptional profile of the cells. A different mechanism to induce oncogenesis is represented by HTLV. HTLV-1 regulatory protein Tax, which potently increases transcription of viral gene from the LTR, also stimulates transcription of many cellular genes by binding cellular transcriptional factors, and may be involved in the leukaemogenesis [reviewed in (Maeda et al., 2008)].

HIV-1 is the aetiological agent of the acquired immunodeficiency syndrome (AIDS). Other animal retroviruses which, as well as HIV, target immune system cells can induce immunodeficiencies (Aziz et al., 1989; Daniel et al., 1985; Kanki et al., 1985; Letvin et al., 1985; Marx et al., 1984; Pedersen et al., 1987).

Neurotropic retroviruses are associated with degeneration of neural function in the central nervous system (CNS). Retrovirus-induced spongiform encephalopathies, usually present without an inflammatory response, are characterised by neuronal loss and proliferation and hypertrophy of the glial cells (Andrews and Gardner, 1974; Swarz et al., 1981; Zachary et al., 1986).

In humans, infection of CNS cells by HTLV-1 and HIV-1 causes neurological disease associated with an inflammatory response. Similar encephalopathies have been described in animals such as cats infected with a T-lymphotropic feline virus, later renamed feline immunodeficiency virus (FIV) (Pedersen et al., 1987).

Retroviruses have been associated with other pathologies. As example, anaemia is induced in horses by the lentivirus EIAV [reviewed in (Montelaro et al., 1993)] and in cats by feline leukaemia virus C (Abkowitz, 1991).

1.3.5 Endogenous retroviruses

Integration of a provirus in the genome of germ line cells may imply that the retrovirus can be transmitted vertically to the offspring according to Mendelian genetics, and become an endogenous retrovirus (Boeke and Stoye, 1997). It has been estimated that 8 to 10% of the human and mouse genome are constituted by retroviral sequences (Gifford and Tristem, 2003). Members of every genera of the family *Retroviridae* have been identified in vertebrates, with the exception of the *deltaretrovirus* genus (Gifford and Tristem, 2003; Gifford et al., 2008; Katzourakis et al., 2007). Most of the provirus sequences are transcriptionally silent or carry deletions or point mutations which render the retrovirus unable to replicate. However, some of the viral open reading frames are still active and full-length replication competent viruses have been observed in some species such as chicken, mouse and pigs [reviewed in (Best et al., 1997; Weiss, 2006)]. Several speculations arose regarding whether the expression of viral gene products might provide a selective advantage to the host. In support of this theory, there are some examples:

1) protection from retroviral infection. Expression of Env proteins could prevent infection by retroviruses in the same interference group (retroviruses which uses the same receptor) by saturating, or down-regulating, the cellular receptors. For instance, murine locus Friend virus-4 (*Fv-4*) encodes an ecotropic MLV Env protein. Its expression conferred resistance *in vitro* and *in vivo* to ecotropic, but not amphotropic, MLV (Ikeda et al., 1985; Limjoco et al., 1993). A different mechanism to prevent retrovirus infection has been

described for the murine locus *Fv-1* [section 1.6.1; (Yan et al., 2009)]. Sequence analysis of the *Fv-1* locus showed that it derives from the CA protein of a murine endogenous retrovirus (Benit et al., 1997). In sheep, the defective CA protein of the endogenous Jaaksiekte sheep retrovirus (enJSRV) can block the exit of exogenous JSRV (Mura et al., 2004).

2) placental function. Syncytiotrophoblast formation is one of the initial steps in the placenta development, and involves fusion of the cells of the trophoblast (the outer layer of the blastocyst). A possible involvement of endogenous Env in syncytiotrophoblast genesis has been suggested by the discovery of syncytin. This gene shows 100% amino acid identity with the Env protein of human endogenous retrovirus (HERV)-W, and is highly expressed in the placenta. Expression of syncytin in COS cells, and cells expressing the RD114 receptor (ASCT-2), resulted in the formation of large cell syncytia (Blond et al., 2000; Mi et al., 2000). Another fusogenic gene derived from an endogenous retrovirus (HERV-FRD) *env* gene has been identified and called syncytin-2 (Blaise et al., 2003). The cellular counterpart, the receptor major facilitator superfamily domain containing 2 (MFSD2), has a placental-specific expression and the proposed function is a carbohydrate transporter probably acting at the level of the syncytiotrophoblast (Esnault et al., 2008). Evidence to support the possible contribution of the endogenous retrovirus Env in the host physiology emerged from use of syncytin-A knock-out mice. Syncytin-A and –B are two fully-coding mouse ERV *env* genes, which display fusogenic property when expressed in transfected cells and are highly expressed in the placenta (Dupressoir et al., 2005). Homozygous null embryos for syncytin-A gene died *in utero* due to defective development of the placenta, indicating a critical role for syncytin-A (Dupressoir et al., 2009). Similar, but less conclusive, results were obtained by *in vivo* knock-down of the Env of enJSRV (Dunlap et al., 2006).

Contrastingly, endogenous retroviruses have been implicated in the development of diseases.

1) cancer. An unambiguous causative association between ERVs and cancer has been proved only in mice. Mouse mammary tumour virus (MMTV) has

been discovered in the milk of female mice as a filterable agent responsible for breast cancer (Boeke and Stoye, 1997). Recombination between infecting MLV and endogenous retroviruses leading to the generation of recombinant mink cell focus forming (MCF) viruses has been described as a hallmark in the development of leukaemia in AKR mice (Fan, 1997). Mobility and amplification of ERVs in murine tumour cells have been observed in murine neuroblastoma and melanoma (Pothlichet et al., 2006a; Pothlichet et al., 2006b). In humans, the evidence for tumorigenesis induced by HERV is only circumstantial [reviewed in (Ruprecht et al., 2008)].

2) autoimmune disease. A role for ERV in autoimmune disease has been proposed on the basis of several pieces of evidence. Antibodies reactive against HERV proteins have been found in patients affected by systemic lupus erythematosis (Bengtsson et al., 1996; Li et al., 1996; Perl et al., 1995) and multiple sclerosis (Jolivet-Reynaud et al., 1999). Endogenous retroviral gene expression has been detected in several human autoimmune diseases (Conrad et al., 1997; Perron et al., 1997). However, there are no clear results which establish the causative role of ERV in inflammatory diseases [reviewed in (Voisset et al., 2008)].

Insight into the process of endogenisation has been offered by the discovery of the koala retrovirus (KoRV), detected in the wild and captive koalas as a full-length replication-competent provirus. Sequence analysis showed a 78% nucleotide similarity between the whole KoRV genome and the GALV's, an exogenous gammaretrovirus associated with leukaemia and lymphoma in captive gibbon colonies (Hanger et al., 2000). A positive association was found between an increased level of KoRV RNA in the plasma of koalas and development of leukaemia or lymphoma (Tarlinton et al., 2005). As a endogenous virus, KoRV provirus sequences were detected in the germ line of the koalas analysed. However, the number and position of the provirus insertions vary between unrelated animals. Furthermore, while KoRV has been observed in all the koalas in North-East Australia, in the Southern mainland and islands there is a mixed prevalence of the virus with no animal infected in an isolated population (Kangaroo Island). These observations

suggest that KoRV endogenisation is not fixed, and in the regions with a mixed prevalence it is likely that KoRV behave both as exogenous and endogenous virus (Tarlinton et al., 2006). To investigate the process of endogenisation, KoRV was compared with the closely related exogenous GALV. Critical motif differences between the 17 KoRV and 4 GALV sequences were identified which correlated with a reduced titre of the endogenous virus. These observations lead to the speculation that part of the process of retrovirus endogenisation involves the attenuation of the viral infectivity (Oliveira et al., 2007).

1.4 Porcine endogenous retrovirus

Four groups of β-retroviral and 10 groups of γ-retroviral sequences have been characterised by PCR using degenerated primers (Klymiuk et al., 2002; Patience et al., 2001). β-retroviral sequences were identified in the genomic DNA from animals of the *Suidae* and *Tayassuidae* families, indicating that some β-retroviral sequences entered the porcine lineage before the separation between the *Suidae* and *Tayassuidae* families approximately 20 million years ago (Ericsson et al., 2001; Patience et al., 2001). Instead, γ–retroviral sequences were found only in the members of the *Suidae* family (Patience et al., 2001). In the pig genome the number of full length PERV copies is estimated to be between 50 and 200 (Akiyoshi et al., 1998; Le Tissier et al., 1997; Patience et al., 1997), but less than 10 are replication competent (Niebert et al., 2002). PERV sequences in different swine breeds revealed a heterogenous distribution among breeds and PERV copy number and chromosomal distribution also varied between individuals (Bosch et al., 2000; Edamura et al., 2004; Herring et al., 2001; Lee et al., 2002; Li et al., 2004; Rogel-Gaillard et al., 1999). This is of importance in xenotransplantation because it suggests that careful breeding could allow the elimination of replication-competent PERV and most problematic sequences, such as active PERV-C (section 1.4.2) from the animal donor.

1.4.1 Replication-competent PERVs and host range

Only the endogenous retroviruses representing the γ1 group (Patience et al., 2001) have been observed spontaneously budding from porcine cells (Armstrong et al., 1971; Lieber et al., 1975; Todaro et al., 1974). Sequence analysis indicated closest homology with gibbon ape leukaemia virus (GALV), KoRV, MLV, FeLV and BaEV among retroviruses (Akiyoshi et al., 1998; Czauderna et al., 2000; Hanger et al., 2000; Patience et al., 2001). *In vitro* analysis of the host range of PERV showed that these viral particles can infect human cells (Patience et al., 1997; Takeuchi et al., 1998). Infectious human tropic particles could be also produced from porcine primary peripheral blood mononuclear cells (PBMC), upon mitogenic stimulation *in vitro* (Wilson et al., 1998). Three groups of PERVs (A, B and C) have been

described with high homology in the *gag* and *pol* genes. The major difference between PERV groups lies in the surface unit (SU) of Env, specifically in the regions VRA and VRB, which are responsible for receptor recognition (Akiyoshi et al., 1998; Le Tissier et al., 1997). All three PERV groups can infect porcine cells. PERV-A and PERV-B are human-tropic and their host range includes mink, cat and dog (PERV-A) and mink, mouse, rat and rabbit (PERV-B). PERV-C infection appears to be limited to pigs (Takeuchi et al., 1998; Wilson et al., 2000). Initially, PERV infection of non-human primates cells was not detected (Martin et al., 1999; Takeuchi et al., 1998). However, MLV core particles pseudotyped with PERV-A Env were shown to infect baboon cells (Blusch et al., 2000a) and PERV transmission to baboon B-lymphocytic cell lines was also detected, by PCR assay, after cocultivation with lethally irradiated PK15 cells (Templin et al., 2000).

1.4.2 PERV recombination

Human-tropic PERVs isolated from NIH mini-pigs were shown to be recombinants between PERV-C and human-tropic PERV-A (Oldmixon et al., 2002; Wilson et al., 2000). One of these recombinants, PERV-NIH, had a higher titre than PERV produced from PK15 cells (Wilson et al., 2000) Another isolate, PERV-A14/220, was shown to infect human cells with a significantly higher titre than other human-tropic PERV-A and PERV-B (Bartosch et al., 2004; Ericsson et al., 2003; Harrison et al., 2004). The main determinant for the increased titre has been mapped to the chimeric envelopes (Harrison et al., 2004). Both PERV-NIH and PERV-A14/220 Envs have the receptor binding domain (RBD) in the SU derived from PERV-A (hence, the human tropism) and the TM region from PERV-C (Wilson et al., 2000; Bartosch et al., 2004; Harrison et al., 2004). In addition, PERV-A14/220 has a higher reverse transcriptase activity than PERV-A, suggesting that also *pol* contributes to the higher titre of this isolate (Wood et al., 2009). The origin of these recombinations is not totally clear. PERV-A/C recombinant sequences were not found in the minipig genome, suggesting that the recombination was an event which occured *de novo* in each pig (Scobie et al., 2004; Wood et al., 2004). However, more recently, two different sequences of recombinant PERV-A/C were described in the

genome of four mini-pigs (Martin et al., 2006). It has been suggested that PERV-C sequences drive the recombination process (Wood et al., 2004). Therefore, the use of PERV-C-free pigs in xenotransplantation was advocated to reduce the risk of PERV infection [(Hector et al., 2007) and reviewed in (Denner, 2007)]. In the context of xenotransplantation, another risk associated with PERV recombination could be represented by the emergence of a novel retrovirus by recombination of PERV with HERVs. The likelihood of HERV sequence packaging in PERV particles has been studied and found to be very low, but it cannot be excluded (Suling et al., 2003).

1.4.3 Diagnostic methods for PERV detection

Before PERV was shown to be able to infect human cells (Patience et al., 1997), porcine materials have been used to treat human diseases (Chari et al., 1994; Deacon et al., 1997; Fink et al., 2000; Groth et al., 1994; Reichenbacher, 1975). Retrospective studies have been conducted to assess a possible PERV infection (Clemenceau et al., 2001; Cunningham et al., 2001; Elliott et al., 2000; Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998). In the most extensive study (Paradis et al., 1999), PBMC and serum samples from 160 patients who underwent through different procedures (extracorporeal liver, splenic or kidney perfusion, pancreatic islet cells transplantation, skin xenograft) were collected. The exposure time to the porcine materials varied between 15 minutes to 460 days. Testing was carried out by real-time PCR on the DNA extracted from PBMCs using primers specific for PERV *gag* and *pol* sequences: 81% of the samples were PERV DNA-negative. The PERV DNA-positive samples were assessed by PCR for the presence of pig centromeric or mitochondrial DNA, and all were found positive, indicating microchimerism (i.e. presence of circulating porcine cells in the xenotransplant recipient, but not necessarily PERV infection of patient’s cells). No PERV RNA was found in the serum or saliva. Similar results using similar PCR-based methods were obtained in all the other studies, showing no evidence of PERV infection in patients exposed to the porcine materials (Clémenceau et al., 2001; Cunningham et al., 2001; Elliott et al., 2000; Heneine et al., 1998; Patience et al., 1998). Due

to of the importance of follow up of the xenotransplantation patient, new improved PCR-based methods of detection have been developed to enhance the effectiveness and reliability of monitoring procedures (Blusch et al., 2000b; Lovatt et al., 1999; Shah et al., 2003; Switzer et al., 1999). Immunological methods have also been developed and improved during recent years, mainly based on the detection of the virus in the cells or tissues, either by western blotting or immunostaining (Bartosch et al., 2002; Chiang et al., 2005; Fischer et al., 2003; Galbraith et al., 2000; Matthews et al., 1999; Tacke et al., 2001; Xu et al., 2003). Indirect diagnostic tools employed to detect viral infection have been the analysis of the presence of anti-PERV antibodies in the patients exposed to pig cells and tissues. No seroconversion was observed (Heneine et al., 1998; Patience et al., 1998; Paradis et al., 1999).

1.4.4 Strategies to reduce the risk of PERV transmission

The presence of αGal antigens on the surface of pig cells constitutes a major immunological obstacle in xenotransplantation (section 1.1.1). However, enveloped viruses produced from porcine cells are likely to acquire αGal antigens on their envelope and be readily neutralised by the human immune system (Rother et al., 1995; Takeuchi et al., 1997; Takeuchi et al., 1996). Indeed, it has been showed that natural xenoreactive antibodies can block PERV infection (McKane et al., 2004; McKane et al., 2003). The protection, offered by anti-αGal antibodies against PERV, would not be present when using transgenic pigs developed for xenotransplantation (section 1.1.1). PERV particles produced from transgenic porcine cells expressing complement regulatory proteins manifested a reduced susceptibility to neutralisation by the human immune system (Hazama et al., 2005; Magre et al., 2004; Okura et al., 2008; Takefman et al., 2002). PERV budding from αGal knock-out pig cells were found resistant to complement-mediated inactivation (Quinn et al., 2004). Novel strategies have to be developed to minimise the risk of PERV zoonosis in the context of xenotransplantation.

1) A group of mini pigs have been shown not to produce human-tropic replication-competent (HTRC) PERV (Oldmixon et al., 2002; Wood et al.,

2004; Scobie et al., 2004). The non-transmitting phenotypes have been associated with the lack of critical PERV *loci*. Specifically, PERV-C *locus* is potentially associated with the ability to produce HTRC PERV-A/C or infectious PERV-C (Hector et al., 2007). Pigs lacking these specific *loci* could represent a safer source of xenografts (Garkavenko et al., 2008a; Garkavenko et al., 2008b).

2) Small interfering RNAs (siRNAs) represent a highly conserved mechanism of posttranscriptional gene silencing based on sequence-specific degradation of a target mRNA [(Caplen et al., 2001; Fire et al., 1998; Hammond et al., 2000; Zamore et al., 2000) and reviewed in (Matzke et al., 2001)]. Transfection of siRNA directed against PERV sequences (*pol* and *gag* genes) was able to suppress the viral expression in PERV-infected human cells (Karlas et al., 2004) and in porcine endothelial cells (Miyagawa et al., 2005). From their initial work, Karlas and co-workers identified the most effective siRNAs sequence (pol2) able to achieve 90% PERV suppression when expressed under the control of the polymerase III H1-RNA gene promoter (Karlas et al., 2004). The long-term effectiveness of siRNA-Pol2 was verified in the PERV-producing pig cell line PK15, as well as primary porcine cells by expressing the siRNA in a HIV-based lentiviral vector (Dieckhoff et al., 2007a). The final step was the production of transgenic pigs carrying the PERV-specific siRNA (Dieckhoff et al., 2008). Recently, another group have produced transgenic pigs that express siRNA against PERV *gag* and *pol* sequences (Ramsoondar et al., 2009). Both studies produced a healthy transgenic litter (7 and 3 piglets, respectively) in which the presence of the siRNA was detected. In many porcine tissues the mRNA level of PERV genes was reduced. However, the suppression of PERV particle production could not be assessed. In fact, the amount of PERV proteins in the non-transgenic pigs was not detectable and, therefore, it was impossible to compare PERV production between transgenic and non-transgenic animals (Dieckhoff et al., 2008; Ramsoondar et al., 2009).

3) In addition to conventional antibodies, members of the *Camelidae* family (camels, llama and dromedaries) produce heavy-chain-only antibodies as

natural, functional single chain antibodies (Hamers-Casterman et al., 1993). An antibody specific to p15 matrix protein was derived from a llama immunised with PERV-B Gag protein following screening of a single-chain antibody phagemid library. Intracellularly expressed antibody reduced the production of PERV-A and PERV-B Gag precursor more than 90% in selected single-clone PK15 cells (Dekker et al., 2003). In a similar way to the production of siRNA-transgenic pigs, xenotranplantation animal donors could be genetically modified to express this antibody to reduce PERV production.

4) Engineering pigs to express human APOBEC3G could result in an animal donor less likely to transmit PERV. APOBEC proteins have been described as host restriction factors able to block viral infection (section 1.6.2). PERV transmission to human embryonic 293 cells was reduced from human APOBEC3G-transduced PK15 cells (Jonsson et al., 2007), and from HEK 293 cells cotransfected with human or porcine APOBEC3 and PERV B genome (Dorrschuck et al., 2008).

5) An alternative strategy for preventing PERV transmission involved remodelling of the pig cell surface glycoproteins by the transfer of N-glycan processing transferases into porcine cells. The expression of three enzymes (α-1,2 mannosidase Ib, α3-D-mannoside β-1,2-N-acetylglucosaminyl transferase I, and α-mannosidase II) in pig endothelial cells was effective in reducing PERV infection of to human HEK 293 cells (Miyagawa et al., 2006). No reduction in PERV RNA level in the supernatant of porcine cells was detected. The high-mannose type N-linked sugars have been shown to be important for PERV infectivity, however their role is still unclear (Hazama et al., 2003; Miyagawa et al., 2006).

6) Neutralising antibodies against PERV were produced by inoculation of the ectodomain of the transmembrane envelope protein p15E (amino acid 488-597) into a goat (Fiebig et al., 2003). Antibodies present in the goat anti-serum were able to neutralise PERV infection *in vitro*. These results showed that an anti-PERV vaccine could be a potentially successful strategy. However, effective vaccines against another γ-retrovirus, FeLV, have been produced (Hines et al., 1991; Poulet et al., 2003; Sparkes, 2003; Tartaglia et

al., 1993; Tizard and Bass, 1991; Weijer et al., 1993; York and York, 1991), but none was able to provide a complete protection (Hofmann-Lehmann et al., 2007; Jarrett and Ganiere, 1996; Marciani et al., 1991).

7) Antiviral chemotherapy is probably the most direct system to prevent PERV transmission. Reverse transcriptase and protease inhibitors developed for HIV-1 were tested to assess their efficacy against PERV. Only two nucleoside analog RT inhibitors zidovudine (AZT) and dideoxyinosine were found effective at concentrations achievable *in vivo*, (Powell et al., 2000; Qari et al., 2001; Stephan et al., 2001; Wilhelm et al., 2002). More recently, acyclic nucleoside phosphonates were indicated as promising compounds to reduce PERV replication (Shi et al., 2007).

1.5 γ-RETROVIRUS RECEPTORS

From a biochemical prospective, a receptor is an intrinsic membrane protein with both extracellular and intracellular domains. It possess a binding site on the extracellular domain which specifically recognises the signal molecule, the ligand. The interaction between ligand and receptor alters the tertiary or quaternary structure of the receptor, including the intracellular domain. These structural changes activate a downstream signalling pathway that can alter the biochemistry of the cell (Alberts et al., 2002). In contrast, a viral receptor, which is a molecule in the cell membrane, induces conformational changes in its ligand, the viral envelope glycoprotein.

Viruses have exploited a wide variety of molecules to use as receptors, such as different families of proteins, carbohydrates and lipids [reviewed in (Haywood, 1994)]. The viral receptor mediates the binding of the virus to the cell surface and leads to its entry into the cell. In most cases, one molecule mediates both binding and fusion, such as for the γ-retroviruses. However, some other viruses, such as HIV-1, need a coreceptor (Choe et al., 1996). In addition, the initial step of viral infection consists of the adsorption of the virus to the cell surface, which can be receptor-independent, and mediated by many different cell surface molecules such as heparan sulphate proteoglycans, lectins (e.g. DC-SIGN and DC-SIGNR), integrins and glycolipids (Fortin et al., 1997; Jinno-Oue et al., 2001; Lee et al., 2001; Pohlmann et al., 2001; Saphire et al., 1999). Although other intracellular mechanisms may be present to restrict viral replication in a cell (section 1.6), the pattern of expression of the receptor on the surface of different cells defines the virus host range. For instance, murine leukaemia viruses (MLV) have been classified into four different host-range subgroups according to the distribution of their specific receptors among species. The receptor for ecotropic viruses is restricted to murine or rat cells, whereas the receptor for viruses with a xenotropic host range can be present on the cells of many different species but never on mice. Amphotropic and polytropic viruses use receptors found both on rodent and other species cells, but they do not interfere with one another, indicating that the receptors are distinct molecules (Rein, 1982; Rein and Schultz, 1984).

For the γ-retroviruses, the process of fusion is not necessarily mediated directly by the cognate receptor. In some examples, the conformational changes in the Env glycoproteins that occur following the binding to the receptor enable the same, and other, Env molecules to trigger fusion (Anderson et al., 2000; Barnett and Cunningham, 2001; Barnett et al., 2001; Lavillette et al., 2001; Lavillette and Kabat, 2004; Lavillette et al., 2002b; Lavillette et al., 2000).

Members of the genus γ-retrovirus use a small number of quite related receptors. This fact has been explained by different authors in opposite ways: a convergent [reviewed in (Overbaugh et al., 2001)] or divergent evolution [reviewed in (Tailor et al., 2003)]. In both these reviews, the authors use *ad hoc* examples to support their hypothesis. For instance, Overbaugh and coauthors emphasise that multiple γ-retroviruses use the phosphate transport proteins, Pit1 and Pit2, as receptor. Whereas, Tailor and coauthors use other examples to support their hypothesis, such as that the only avian retrovirus known to use a receptor with multiple transmembrane domains, avian reticuloendotheliosis viruses, is closely related to mammalian endogenous retrovirus (Barbacid et al., 1979). However, although Tailor and coauthors strongly support the divergent evolution, they admit a limited degree of convergent evolution in receptor choice.

There are many similarities between γ-retrovirus receptors, and they will be discussed in the following sections.

1.5.1 Receptor cloning

The first retroviral receptor that was molecularly cloned was the receptor for ecotropic MLV (E-MLV) (Battini et al., 1995; Battini et al., 1992), the cationic amino acid transporter-1 (CAT-1) (Albritton et al., 1989). The strategy used consisted of gene transfer of a cDNA library derived from the E-MLV permissive murine cell line NIH 3T3 into the resistant human cell line EJ, employing retroviral vectors which express a drug-resistant gene. EJ clones which have acquired susceptibility to E-MLV infection were selected by addition to the media of the drug. One year later, the receptor for gibbon ape leukaemia virus (GALV), GLVR-1 or Pit-1, was cloned using the same

technique (O'Hara et al., 1990). This receptor was subsequently determined to also function as a receptor for wooly monkey virus, feline leukaemia virus B (FeLV-B) (Takeuchi et al., 1992), MLV10A1 (Miller and Miller, 1994; Wilson et al., 1995) and KoEV (Oliveira et al., 2006). Similarly, the receptor for amphotropic MLV (A-MLV), Ram-1 or Pit-2, was cloned through screening of a rat cDNA library introduced into a hamster cell line (Miller et al., 1994). The same protein, called GLVR-2 was cloned independently by another group using a different technique. Low-stringency hybridisation with a cDNA encoding GLVR-1 was used to isolate related phage clones from human HL-60 and placental cDNA libraries (van Zeijl et al., 1994).

Using the expression cloning technique other γ-retrovirus receptors have been cloned. The human ASCT-2 receptor or RDR, feline endogenous retrovirus RD114 and retrovirus D-type receptor, allows the infection of not only RD114 but also baboon endogenous retrovirus (BaEV) human endogenous retrovirus W (HERV-W), reticuloendotheliosis virus (REV) as well as β-retrovirus simian retrovirus type D (Rasko et al., 1999; Tailor et al., 1999b). In the same year, the human receptor, XPR-1, that facilitates the entry for both xenotropic and polytropic MLV (X- and P-MLV-) has been cloned by three different groups independently (Battini et al., 1999; Tailor et al., 1999a; Yang et al., 1999). XPR-1 is also required for infection by xenotropic MLV-related virus (XMRV) (Dong et al., 2007). The receptor for feline leukaemia virus C (FeLV-C), FeLVCR-1, has been cloned from human and domestic cat cDNA libraries (Quigley et al., 2000; Tailor et al., 1999c). The existence of a homologue to FeLVCR-1, FeLVCR-2, was predicted by search of the NCBI database. FeLVCR-2 was then cloned and tested for supporting FeLV-C infection but was found to be non-functional (Brown et al., 2006). Recently, a FeLV-A and FeLV-C hybrid Env, named FY981, was isolated from a primary FeLV isolate, and when pseudotyped can use FeLVCR-2 for infection (Shalev et al., 2009). Porcine endogenous retrovirus subgroup A (PERV-A) receptors (Ericsson et al., 2003) and the FeLV-A receptor, the feline orthologue of the human thiamine transport protein 1 (THTR1), have been identified by using the same gene transfer approach (Mendoza et al., 2006).

1.5.2 Receptor topology

All γ-retrovirus receptors thus far discovered are multiple transmembrane (TM) proteins. The number of TM domains in these receptor is between 4, as predicted for the *Mus caroli* endogenous retrovirus (McERV) receptor and 14 of CAT-1 (Table 1.1). The morphology has been initially predicted using bioinformatics tools such as hydro prediction software, and has been experimentally proven. Pit-2 was the first γ-retrovirus receptor whose topology was studied in detail (Salaun et al., 2001). A model of Pit-2 topology was obtained from different TM prediction programmes, such as PredictProtein (www.embl-heidelberg.de/predictrpotein/predict_protein.htmL), DAS server (www.biomedi.su.se/-server/DAS) and TMHMM (www.cbs.dtu.dk/services/TMHMM). Extracellular N-glycosylation sites were assessed by creation of mutants and treatment of PNGase F, an enzyme which removes N-linked oligosaccharide chains. The orientation of the receptor extremities were studied by tagging either the C- or N-terminus.

A similar study was conducted on Pit-1. Its topology was initially predicted by Kyte-Doolittle hydropathy plots to be 10 TM domains with both N-terminus and C-terminus being intracellular (Johann et al., 1992). Ten years later, Farrell and coworkers demonstrated, using HA-tagged Pit-1 in immunofluorescence, flow cytometry and glycosylation studies, that Pit-1 topology was different with both N-terminus and C-terminus being extracellular (Farrell et al., 2002).

Similar experiments have also been done to determine the topology of the FeLV-C receptor (Brown et al., 2006). N-glycosylation experiments (Marin et al 2003) have confirmed the ASCT2 topology previously predicted (Tailor et al., 1999b). So far there are no publications concerning the topology of PAR-1 and PAR-2 [apart from a study described in this thesis, and also in (Mattiuzzo et al., 2007)], and THTR1, the receptors for PERV-A and FeLV-A, respectively.

1.5.3 Physiological function

All gammaretrovirus receptors, whose physiological function has been described, are transporters (Table 1.1). A few years after their cloning and sequencing, the physiological function has been hypothesised through the comparison with homologous proteins in simpler organisms and then experimentally proven. The E-MLV receptor, CAT-1 is a cationic amino acid transporter which shares homology with the *Saccharomyces cerevisiae* arginine and histidine permeases (Kim et al., 1991). GLVR-1 and GLVR-2 share about 25% amino acid identity with a phosphate permease from *Neurospora crassa* and are sodium-dependent phosphate transporters in human cells (Kavanaugh et al., 1994; Olah et al., 1994). For other proteins the description of their cellular function preceded the characterisation of their role as viral receptors. Following the isolation of the cDNA for ASCT-2, which conferred RD114 susceptibility to otherwise non permissive murine cells, its amino acid sequence revealed 97% homology to a neutral amino acid transporter (hATB^0) (Kekuda et al., 1996). Indeed ASCT-2 was showed to function as a transporter of neutral amino acids (Rasko et al., 1999; Tailor et al., 1999b). When the feline receptor for FeLV-A was cloned, its sequence showed approximately 93% homology with the human high-affinity thiamine transporter-1 (huTHTR-1) (Mendoza et al., 2006). Similarly, the function of FeLVCR-1 was predicted to be a transporter by homology with members of the major facilitator superfamily of transporters (Quigley et al., 2000; Tailor et al 1999c). From the observation that cats infected with FeLV-C developed anaemia, the physiological function of FeLVCR-1 was hypothesised and then confirmed, as a heme exporter (Quigley et al., 2004). The function of other γ-retrovirus receptors, such as XPR-1, is still unknown, or not confirmed, such as for PAR-1 and PAR-2 (section 3.1).

For several receptors it has been showed that the transporter function is not necessary to support virus entry. This has been experimentally proven with mutated or truncated receptors: murine CAT-1 (Wang et al., 1994), human Pit-2 (Bottger and Pedersen, 2002; Salaun et al., 2004) and ASCT-2 (Tailor et al., 2001). Contrastingly, retroviral infection reduces the transport activity

of the receptor (Olah et al., 1994; Wang et al., 1992; Wilson et al., 1995). The mechanisms proposed are Env protein-mediated confinement of the newly synthesised receptors to the cytosolic compartment (Heard and Danos, 1991; Jobbagy et al., 2000), or Env interaction with cellular proteins necessary for trafficking the receptor to the cell surface (Fujisawa and Masuda, 2007). The cell can compensate by upregulating transporters with the same solute specificity. Indeed, down-modulation of Pit-1 causes up-regulation of Pit-2 and *vice versa* (Chien et al., 1997; Kavanaugh et al., 1994). However, for other receptors, the disruption of the transport function caused by retroviral infection has been implicated in the development of disease, such as the anaemia in cats infected with FeLV-A or FeLV-C (Qiugley et al., 2004; Mendoza et al., 2006).

name	retrovirus	function	topology	References
CAT-1 SLC7A1	**E-MLV**	Cationic amino acids transporter		(Albritton et al., 1989; Kim et al., 1991; Wang et., al 1992)
Pit-1 GLVR-1 SLC20A1	FeLV-B GALV Wooly monkey virus **10A1MLV** KoRV*	Phosphate tranporter		(O'Hara et al., 1990; Takeuchi et al., 1992; Miller and Miller, 1994; Wilson et al., 1995; Olah et al., 1994; Farrell et al., 2002; Oliviera et al., 2006)
Pit-2 GLVR-2 Ram-1 SLC20A2	A-MLV **10A1MLV**	Phosphate tranporter		(Miller and Miller, 1994; Van Zeijl et al., 1994)
XPR-1	**X-MLV** **P-MLV** XMRV	unknown		(Battini et al., 1999; Tailor et al., 1999a; Yang et al., 1999; Dong et al., 2007)
ASCT-1 SLC1A4	**BaEV** **HERV-W**	Neutral amino acid transporter		(Lavillette et al., 2002a; Marin et al., 2000)
ASCT-2 RDR SLC1A5	**RD114** **BaEV** REV **HERV-W**	Neutral amino acid transporter		(Rasko et al, 1999; Tailor et al., 1999b; Blond et al., 2000; Marin et al., 2003)
FeLVCR-1	FeLV-C FeLV FY981	Heme exporter		(Tailor et al 1999c; Quigley et al., 2000; Quigley et al., 2004; Shalev et al., 2009)

FeLVCR-2	FeLV FY981	Not confirmed		(Brown et al., 2006; Shalev et al., 2009)
PAR-1	**PERV-A**	unknown		(Ericsson et al., 2003; Mattiuzzo et al., 2007)
PAR-2	**PERV-A**	unknown		(Ericsson et al., 2003; Mattiuzzo et al., 2007)
THTR-1	FeLV-A FeLV FY981	Thiamine transport		(Mendoza et al., 2006; Shalev et al., 2009)
MFSD2	**HERV-FRD**	Carbohydrate transport (predicted)		(Esnault et al., 2008)
PLLP (TM4SF11)	**McERV**	Voltage-dependent K$^+$channel		(Miller et al., 2008)

Table 1.1 Summary of the known γ-retrovirus receptors
Highlighted in bold are endogenous viruses (second column). *KoRV has been described both as an endogenous and exogenous virus.

1.5.4 Identification of sites critical for infection and binding

Receptors are the main determinants for viral tropism. Polymorphic variation of the receptors confers resistance to retroviral infection between species or homologous proteins within the same species. The difference can be as little as a single amino acid.

A few years after CAT-1 was cloned, a highly related (87%) gene was identified from a human T-cell line (Yoshimoto et al., 1991). Although the human homologue is very similar in sequence and in structure to murine CAT-1, it does not support E-MLV infection. To identify amino acid residues critical for E-MLV infection, human-mouse chimeric receptor molecules were created and tested for their ability to support infection and binding to Env gp70 (Albritton et al., 1993; Yoshimoto et al., 1993). The substitution of one amino acid residue, tyrosine at position 235 in the third extracellular domain in murine CAT-1 with the corresponding human amino acid proline, abrogated E-MLV infection. Conversely, the substitution of amino acids 242

and 244 in the human receptor with the corresponding amino acids in the murine receptor enabled human CAT-1 not only to support infection but also to bind Env gp70.

The identification of the binding site in Pit-1 and Pit-2 was more difficult until the correct topology was described (Farrell et al., 2002; Salaun et al., 2001). The first candidate proposed to be the binding site for both receptors was a nine amino acid sequence called region A at position 550-558 within Pit-1 (Johann et al., 1992; Johann et al., 1993). Region A was predicted by Kyte-Doolittle Hydropathy plots to be in an extracellular domain and mutation in this region abolished GALV and FeLV-B infection (Johann et al., 1992). Later, other regions of the human Pit-1 and Pit-2 have also been found to be important for infection (Chaudry and Eiden, 1997; Leverett et al., 1998; Lundorf et al., 1998; Tailor and Kabat, 1997), but only in 2002 was the role of region A clarified. Cells expressing a mutant Pit-1 protein in region A does not work as a receptor, but retains its ability to bind the virus. Region A influences infection by controlling the orientation of the Pit-1 molecule in the membrane and therefore the accessibility or steric hindrance of the viral binding site. Furthermore, through the construction of chimeric receptors between Pit-1 and Pit-2, a second region (region B) required for both viral entry and binding was identified (Farrell et al., 2002). The A-MLV binding site in Pit-2 has been identified in the first extracellular domain of the protein by studying chimeric Pit-1/Pit-2 receptors in a infection and binding assay (Feldman et al., 2004).

The same approach has been conducted for ASCT-2 protein, in which a sequence of 21 amino acids in the second extracellular loop plays a critical role in determining receptor function for RD114, BaEV, HERV-W and SRV (Marin et al., 2003a).

Attempts to identify the binding site in the FeLV-C receptor could not use chimeric human/mouse FLVCR-1 proteins because the murine homologue overexpressed in murine cell line works as a receptor, although the mouse cells are not permissive to FeLV-C infection (Tailor et al., 2000). The isolation of FLVCR-2, a closely related protein (52% amino acid identity) which did not support FeLV-C infection, enabled the generation of specific mutants to study

the binding site (Brown et al., 2006). A single mutation (N463E) in the sixth extracellular domain in FLVCR-2 rendered this protein functional as FeLV-C receptor. The opposite mutation or the substitution of the whole ECL6 in FLVR-1 does not abrogate the infectivity. This allowed the identification of another region critical for FeLV-C infection, ECL1. However, neither ECL1 nor ECL6 were recognised as a FeLV-C binding site; their role could be influencing membrane topology with a mechanism similar to Pit-1 region A (Brown et al., 2006).

Critical determinants in XPR-1 for supporting X-MLV and P-MLV infection were discovered using chimeric constructs between human XPR-1 and the non-functional hamster homologue protein (Van Hoeven and Miller, 2005). Two entry determinants are present on XPR-1. One is located in the predicted ECL4 and can be used by both X-MLV and P-MLV. The second determinant is present in the ECL3 and can be only utilised by X-MLV. The identification of two different sites in the same receptor explained the phenomenon of non-reciprocal interference patterns where the infection by one virus (X-MLV) blocks infection by a second virus (P-MLV), but the infection of the second virus only slightly inhibits infection by the first virus (Chesebro and Wehrly, 1985; Miller and Wolgamot, 1997).

1.5.5 Receptor-mediated barriers to infection

Functional receptors do not always meditate a successful infection. Chinese hamster ovary (CHO) cells are resistant to infection by GALV and A-MLV (Miller and Miller, 1992, 1993). Overexpression of Pit-1 and Pit-2 cDNA isolated from CHO cells conferred susceptibility to GALV and A-MLV infection to CHO cells, indicating that these cells code for a functional receptor (Tailor et al., 2000). Similarly, delivery of FeLVCR-1 cloned from *Mus dunni* tail fibroblast (MDTF) cells renders these, otherwise resistant, cells susceptible to FeLV-C infection (Tailor et al., 2000). The hypothesis proposed was that functional receptors were expressed on the surface of these cells at a sub-threshold level and could be additionally inhibited by some masking factors (Tailor et al., 2000). One of these additional mechanisms to reduce, or prevent, retroviral infection is the secretion of

inhibitors in the media (Miller and Miller, 1992, 1993). Secreted endogenous retrovirus ENV have been identified as such an inhibitor (Ikeda et al., 1985; Jung et al., 2002; McDougall et al., 1994; Wu et al., 2005). Another important masking factor has been associated to the N-glycosylation of the receptors. Cells resistant to retroviral infection became permissive following treatment with tunicamycin, a drug which prevent N-glycosylation of proteins (Lavillette et al., 2002a; Marin et al., 2000; Yan et al., 2008). A possible explanation was that the masking of the receptor, due to the endogenous retrovirus Env products described above, was prevented by misfolding of the viral glycoproteins in the presence of tunicamycin (Lavillette et al., 2002). In fact, processing and folding of Env glycoproteins requires N-linked glycosylation (Li et al., 1997; Polonoff et al., 1982). Although this is plausible, it does not completely explain the departure from resistance through abrogation of N-glycosylation. In the absence of tunicamycin, mutation of the N-glycosylation site in the non-functional receptors hamster ASCT-1 and hamster, rat or *Mus dunni* CAT-1 enables these proteins to support RD114 interference group viruses and E-MLV infection, respectively (Eiden et al., 1994; Kubo et al., 2004; Marin et al., 2003a; Yoshii et al., 2008). The proposed mechanism of restriction was that a heavy glycosylation of the receptors close to the binding site would prevent the interaction with the virus (Eiden et al., 1994; Kubo et al., 2004). Treatment of cells with liposomes composed of phosphatidyl serine, a phospholipid component of the cell membrane, mimicked the tunicamycin effect without removing N-linked glycosylic chains (Coil and Miller, 2005). RD114 and MoMLV infect murine NIH3T3 and hamster CHO cells upon treatment with phosphatidyl serine, indicating that fully glycosylated receptors can still support virus entry (Coil and Miller, 2005).

1.6 Host restriction factors

In the continuous fight between the host and retrovirus, eukaryotic cells have evolved several proteins (called restriction factors) that interfere with the life cycle of retroviruses. Contrastingly, viruses evolved to evade these mechanisms, either by mutating the targets of the restriction, or developing countermeasures that antagonise the host cell factors.

Mammalian host factors are the best described. They inhibit different steps in retroviral replication [reviewed in (Wolf and Goff, 2008), and summarised in figure 1.5]. These molecules could be employed to generate safer animal donors for xenotransplantation. Indeed, human and porcine APOBEC3 (section 1.6.3) have been described to be active against PERV, and the creation of APOBEC3-expressing transgenic pigs has been proposed to reduce the risk of PERV transmission (Dorrschuck et al., 2008; Jonsson et al., 2007).

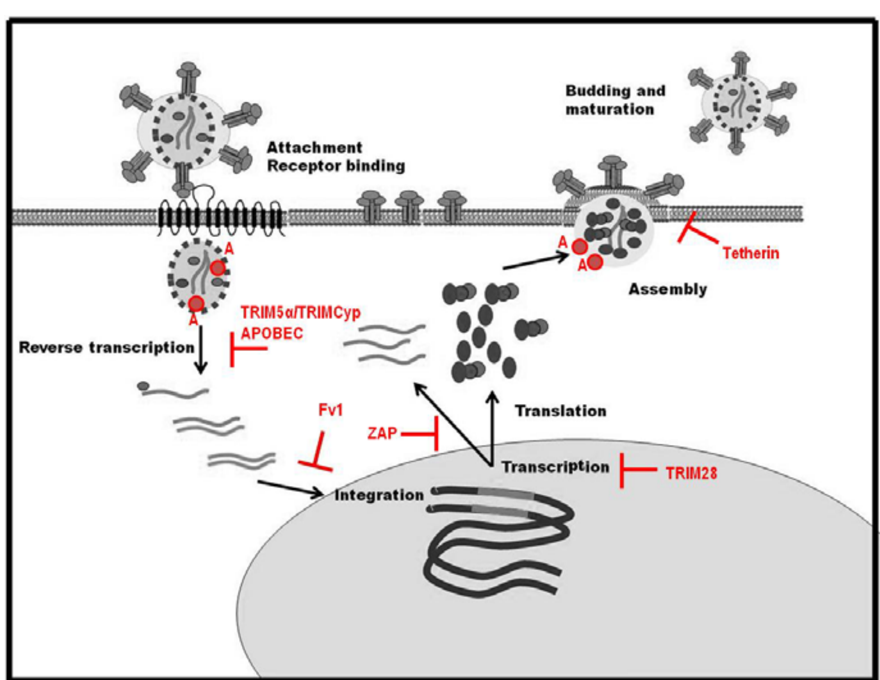


Figure 1.5 Summary of restriction factors acting in different steps of the retroviral life cycle.

Retroviral life cycle is depicted in this model. The restriction factors described in the text are highlighted in red. A, APOBEC, which is incorporated in the virions in the producing cells, but acts at the reverse transcription stage after infection of a new target cells.

1.6.1 Early post entry restriction factors: Fv1 and TRIM protein family

A genetic locus, *Fv1* (Friend virus susceptibility gene 1), in mice was identified as responsible for resistance to infection by murine leukaemia virus (Odaka and Matsukura, 1969; Pincus et al., 1971) and the corresponding gene was cloned (Best et al., 1996). Fv1 interacts with the incoming virus core (Bassin et al., 1978) and prevents the nuclear translocation of the preintegration complex (Jolicoeur and Baltimore, 1976). This interaction, and the consequent restriction, can be abrogated by saturation of the Fv1 protein with replication defective viruses (Bassin et al., 1978). The viral determinant of the interaction with Fv1 lies at amino acid 110 of the CA protein (Kozak and Chakraborti, 1996).

An Fv1-like restriction factor, able to block infection by some strains of MLV and called Ref1 (restriction factor 1), was described in other mammalian cells, including human (Towers et al., 2000). Although Ref1 blocks virus replication before reverse transcription, a step earlier than Fv1, the viral determinant for susceptibility to this restriction is the same as Fv1, amino acid 110 in the CA protein, and the restriction was saturable in a similar manner to Fv1 (Besnier et al., 2003; Towers et al., 2002). A Ref1-like restriction phenotype was also described that acted against lentivirus infection, and was called Lv1 (Besnier et al., 2002; Cowan et al., 2002; Hofmann et al., 1999; Munk et al., 2002).
Rhesus macaque cells are permissive to infection by SIV but not HIV-1. The gene responsible for this tropism, Trim5α, was identified by a rhesus macaque cDNA library screen (Stremlau et al., 2004). Later, it was shown that the Ref1 and Lv1 restriction activity was encompassed by the product of the Trim5α gene (Hatziioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004).

The tripartite motif (TRIM) protein family contains members characterised by three domains [(Reddy et al., 1992) and reviewed in (Nisole et al., 2005)]. At the N-terminus of almost all TRIM proteins is the RING (really interesting new gene) module, a cystein-rich zinc finger binding domain, involved in protein-protein interactions (Borden, 1998). Many RING domains have intrinsic

ubiquitin E3 ligase activity (Freemont, 2000). The second domain is B-box, zinc finger motif. Some TRIM proteins have two B-boxes, B1 and B2. Other than facilitating homo-interactions of the coiled-coil region (Cao et al., 1997), their function is still unknown. The third region is a coiled-coil domain, which mediates the homo- or hetero-oligomerisation of the TRIM proteins (Reymond et al., 2001).
TRIM5α is the largest isoform of TRIM5 proteins, and contains at the C-terminus a B30.2 or SPRY domain; this module interacts with retrovirus CA protein and determines the virus specificity of TRIM5α (Nakayama et al., 2005; Perez-Caballero et al., 2005; Sebastian and Luban, 2005; Stremlau et al., 2005; Yap et al., 2005). In New World Owl monkey TRIM5, the B30.2 domain has been replaced by cyclophilin A (CypA), creating the fusion protein TRIMCyp (Sayah et al., 2004). CypA is a peptidyl prolyl isomerase and interacts specifically with HIV-1 CA protein (Luban et al., 1993). Owl monkey TrimCyp emerged by the retrotransposition of the CypA cDNA between TRIM5 exon 7 and 8 (Sayah et al., 2004). Interestingly, a second and independent appearance of TrimCyp, was found in Old World monkeys (Liao et al., 2007; Newman et al., 2008; Wilson et al., 2008). However, in Old World monkey TRIMCyp, the transposition of CypA took place downstream of TRIM5 exon 8, and the restriction phenotype is different. In fact, it is able to block HIV-2 and feline immunodeficiency virus infection, but not HIV-1 (Wilson et al., 2008).

The mechanism of restriction mediated by Fv1 and TRIM5α is not completely understood. For TRIM5α there are several hypotheses. One possibility is that the interaction between the restriction factor and the virus CA protein promotes its rapid, premature disassembly (Stremlau et al., 2006). This is, however, more an observation of effect than an assessment of mechanism. Ubiquitine E3 ligase activity of the RING domain produces polyubiquitination of TRIM5α and rapid turnover via the proteasome (Diaz-Griffero et al., 2006). It can be speculated that together with the proteasomal degradation of TRIM5α, the TRIM5α-CA complex will also be subjected to the turnover, causing block to the infection. However, drug-induced inhibition of proteasome does not rescue the infectivity (Anderson et al., 2006; Wu et al.,

2006). A proteasomal-independent mechanism of CA degradation has been also proposed (Chatterji et al., 2006).

Some other members of the TRIM family also display antiviral activity.

TRIM28 (also known as Kap-1 or Tif1-β) has been identified as a factor required for the primer binding site-mediated restriction of MLV in embryonic carcinoma (EC) and embryonic stem (ES) cells (Wolf and Goff, 2007). Upon infection of EC and ES cells by MLV, the virus can integrate in the genome, but no viral mRNA can be detected (Barklis et al., 1986; Teich et al., 1977). Following differentiation, EC cells become susceptible to a new infection, but the integrated virus cannot be reactivated (Niwa et al., 1983). This mechanism of retrovirus silencing involved two different steps: an initially cell type-specific restriction (Akgun et al., 1991; Flanagan et al., 1989; Tsukiyama et al., 1989), and a subsequent proviral DNA methylation (Niwa et al., 1983). The target for the cell-specific transcriptional silencing is contained within the 18 nucleotides encoding the primer binding site complementary to the proline tRNA, ($(PBS)^{Pro}$) in the MLV genome (Barklis et al., 1986; Feuer et al., 1989; Loh et al., 1988). A single G to A point mutation, known as a B2 mutation, in the PBS, or the substitution with a sequence complementary to a different cellular tRNA, could relieve the repression (Barklis et al., 1986; Grez et al., 1990; Petersen et al., 1991). TRIM28 has been described as a transcriptional co-repressor, acting in association with the Krüppel associated box (KRAB)-containing zinc finger DNA-binding proteins (Friedman et al., 1996; Le Douarin et al., 1996; Schultz et al., 2002; Schultz et al., 2001). Through an electrophoresis mobility shift assay, TRIM28 was identified as a component of the PBS-mediated restriction complex in EC and ES cells (Wolf and Goff, 2007). However, although TRIM28 is necessary for the restriction, it is not sufficient. The zinc finger protein ZFP809, a member of the KRAB-containing zinc finger protein family, has been showed to be the EC and ES cell-specific factor which acts as a bridge between proviral DNA and TRIM28 (Wolf and Goff, 2009).

One of the best characterised, but poorly understood, TRIM proteins is TRIM19 or PML (promyelocytic leukemia protein). It has been associated

with many cellular functions: cell proliferation (Wang et al., 1998), senescence (Pearson et al., 2000), transcriptional and translational regulation (Kentsis et al., 2001), apoptosis (Hofmann and Will, 2003) and signal transduction (Lin et al., 2004). PML has been implicated, but without conclusive results, in the resistance to infection by human herpes simplex virus (Maul et al., 1993), vesicular stomatitis virus, influenza A virus (Chelbi-Alix et al., 1998), HIV-1 (Turelli et al., 2001), human foamy viruses (Regad et al., 2001) and Lassa virus and lymphocytic choriomeningitis virus (Asper et al., 2004).

TRIM22, or Staf-50, is able to downregulate HIV-1 long terminal repeat-directed transcription (Tissot and Mechti, 1995). TRIM45 has an indirect negative effect on viral replication because it inhibits the activities of transcription factors AP-1 and Elk-1 (Wang et al., 2004).

1.6.2 APOBEC proteins

Apolipoprotein B mRNA-editing catalytic (APOBEC) proteins constitute a family of polynucleotide cytidine deaminases which catalyse the deamination of a cytosine (C) into a uracyl (U). APOBEC1 was the first member of this family to be described. It causes the production of a truncated APOB protein by deamination of C6666 and sequential introduction of a stop codon in the APOB mRNA (Teng et al., 1993).

The role of APOBEC proteins in the restriction of retroviral infection was disclosed through the study of the HIV-1 accessory protein, virion infectivity factor (Vif). HIV-1 deficient in Vif cannot replicate in primary human T cells and macrophages, as well as a limited number of cell lines (Fisher et al., 1987; Gabuzda et al., 1992; Sakai et al., 1993). Using a complementary cDNA subtraction screening for transcripts specifically expressed in non-permissive cell, a 1.5 kilobases gene, CEM15, was isolated. Its expression in permissive cells recreates the non-permissive phenotype for Vif-deficient HIV-1 (Sheehy et al., 2002). CEM15 was later identified as a member of the APOBEC family, APOBEC3G (A3G) (Jarmuz et al., 2002). In the absence of Vif, A3G is incorporated into the HIV-1 particles and, during reverse

transcription of viral RNA, deaminates C residues to U residues in the first strand DNA filament (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003). In the plus strand sequence these modification will produce a guanine to adenine (G-to-A) transition which cause inactivation of the provirus as a result of multiple mutation events, known as hypermutation (Yu et al., 2004b). Infectivity can be reduced also in absence of DNA editing (Newman et al., 2005). The mechanism is not completely understood, but it is associated with a reduction in reverse transcription (Holmes et al., 2007; Iwatani et al., 2007; Mbisa et al., 2007).

APOBEC-mediated restriction appears to be involved in the inactivation of endogenous retrovirus in murine and mouse genomes (Esnault et al., 2005; Lee et al., 2008). Porcine endogenous retroviruses and simian foamy virus may also be inhibited by human A3G (Delebecque et al., 2006; Dorrschuck et al., 2008; Jonsson et al., 2007). Other members of the APOBEC3 family act as restriction factors. In particular, APOBEC3B and 3C have a potent antiviral activity against simian immunodeficiency virus, but not HIV-1 (Yu et al., 2004a). APOBEC-induced mutations are not peculiar to retroviruses. Hypermutated genome of hepatitis B virus (HBV) has been reported *in vivo* and in cell lines (Noguchi et al., 2005). *In vitro* experiments showed that not only A3G can generate this mutation but also A3C, A3B and A3F (Suspene et al., 2005). Moreover, murine *APOBEC3* encodes for *Recovery from Friend Virus* gene 3 (*Rfv3*) (Santiago et al., 2008). Rfv3 was identified as a gene responsible for recovery from Friend virus (FV) viremia and for the generation of FV-specific neutralizing antibody (Chesebro and Wehrly, 1979; Doig and Chesebro, 1979).

HIV-1 and SIV evolved a protein, Vif, to counteract the action of APOBECs. HIV-1 Vif counteracts the action of APOBECs by inducing polyubiquitination and proteasomal-mediated degradation (Conticello et al., 2003; Marin et al., 2003b; Mehle et al., 2004; Sheehy et al., 2003; Yu et al., 2003). The interaction between Vif and A3G is species-specific (Bogerd et al., 2004; Mangeat et al., 2004; Schrofelbauer et al., 2004; Xu et al., 2004). Foamy viruses also produce a protein, Bet, which antagonises A3G. However, Bet,

instead of inducing degradation of A3G, prevents its packaging in the nascent viral particles (Russell et al., 2005). Other viruses have produced different mechanisms to counteract APOBEC-mediated restriction. Human T cell leukaemia virus inhibits the incorporation of A3G in the virions through a peptide motif in the C-terminus of the nucleocapsid domain which prevents the binding of A3G (Derse et al., 2007). Avoiding the packaging into the virions is a mechanism of evasion from APOBEC restriction used also by the β-retrovirus Mason-Pfizer monkey virus and murine leukaemia viruses (Doehle et al., 2006; Doehle et al., 2005). In addition, MLV escapes restriction by an alternative mechanism involving MLV protease (Abudu et al., 2006).

1.6.3 ZAP, a zinc finger antiviral protein

The zinc finger antiviral protein (ZAP) was isolated by screening a rat cDNA library for new restriction factors able to block retrovirus infection (Gao et al., 2002). The step of the viral life cycle targeted by ZAP was the production of new viral RNA (Gao et al., 2002). ZAP binds directly to both the viral RNA through four CCCH-type zinc finger motifs (Guo et al., 2004) and to components of the exosome (Guo et al., 2007). Following these observations, the restriction mechanism proposed is that ZAP mediates interaction between exosome and the newly transcribed viral RNA, resulting in the degradation of the latter (Guo et al., 2007). Zap-mediated restriction was also shown for alphavirus (Bick et al., 2003) and filovirus (Muller et al., 2007). The viral determinants for the interaction with ZAP have been mapped for MLV (in the 3'LTR) and for the alphavirus Sindbis virus (Guo et al., 2004). Interestingly, the two regions have no significant homology (Guo et al., 2004). In humans, in addition to the homologue to rat ZAP, namely ZAP(S), a longer isoform, ZAP(L) have been described. It has been showed that ZAP(L) was more effective than ZAP(S) as an antiviral against MLV and alphaviruses such as semiliki forest virus (Kerns et al., 2008).

1.6.4 Tetherin

HIV-1 viral protein U (Vpu) (Cohen et al., 1988; Strebel et al., 1988) enhances the production of virions from cells (Klimkait et al., 1990). Vpu is

necessary for the release of HIV-1 in certain cell types (Geraghty et al., 1994; Sakai et al., 1993), and in interferon-α-treated cells (Neil et al., 2007). Moreover, expression of Vpu in retroviral packaging cell lines could significantly enhance viral particle production (Kobinger et al., 1997). The presence of a dominant acting human cell restriction factor, counteracted by HIV-1 Vpu, was proposed following heterokaryon fusion experiments (Varthakavi et al., 2003). In the absence of Vpu, HIV-1 mature virions, produced in some human cell lines such as HeLa, accumulate on the cell surface and can be released by protease treatment (Neil et al., 2006). A specific restriction factor was identified by comparative microarray analysis of cells which did or did not need Vpu in order to successfully produce viral particles, together with their response to interferon α treatment (Neil et al., 2008). Among less than ten candidates, CD317, also known as BST2 or HM1.24 antigen (Goto et al., 1994), was chosen for its localisation at the cell membrane and differential expression level in various cell lines. Expression of CD317, renamed tetherin by Neil et al., 2008, specifically inhibited the release of single cycle, VSV-G pseudotyped HIV-1 particles in the absence of HIV-1 Vpu by retaining them on the surface of the cell (Neil et al., 2008; Van Damme et al., 2008). Moreover, tetherin co-localised with HIV-1 Gag in the cell surface and in the intracellular compartments. However, when Vpu was co-transfected, this interaction was disrupted and tetherin co-localised with Vpu instead (Neil et al 2008; Van Damme et al 2008). Tetherin is involved in the growth and development of B cells (Ishikawa et al., 1995) and interacts indirectly with the actin cytoskeleton, playing a critical role in its organisation in polarised epithelial cells (Rollason et al., 2009). However, its biochemical function is undetermined. Tetherin topology is considered unusual: an amino-terminal cytosolic tail, a single transmembrane domain, an extracellular coiled-coil region and a predicted carboxyl-terminal glycosyl phosphatidylinositol (GPI) anchor (Kupzig et al., 2003), and it is expressed as a homodimer (Ohtomo et al., 1999). The encounter between HIV-1 and tetherin could potentially take place in the lipid rafts in the cell membrane. In fact, tetherin is associated with the cholesterol rich rafts (Kupzing et al., 2003), which have been implicated in the budding of HIV-1 (Aloia et al., 1988;

Brugger et al., 2006; Nguyen and Hildreth, 2000; Ono and Freed, 2001). A model of the proposed tetherin-mediated restriction is depicted in Figure 1.6. Tetherin will be discussed in more details in section 5.1.

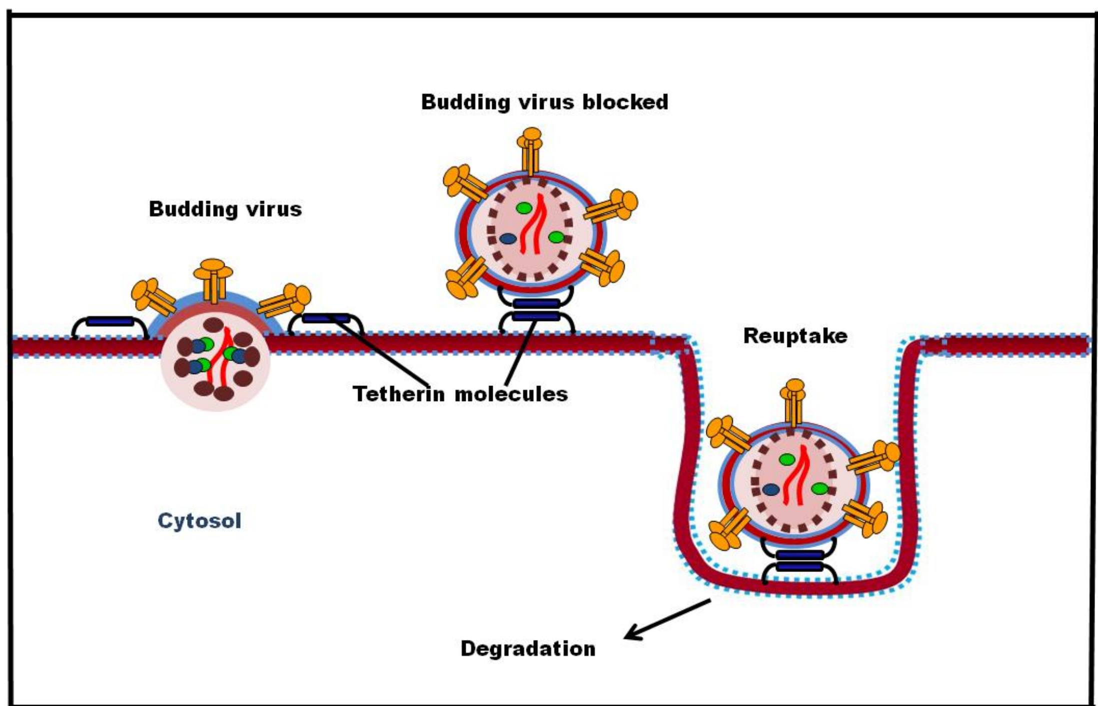


Figure 1.6 Model for tetherin-mediated retrovirus retention.

1.7 Aims

In this thesis the receptors for PERV subgroup A have been characterised. The aim was to identify important regions in the receptor responsible for the virus infection. This could help the development of strategies to prevent PERV transmission to human cells.
In addition, the new knowledge acquired about the PERV-A receptor was employed to evaluate non-human primates as animal models for the study of the risk of PERV infection in the context of xenotransplantation.

As a second aim, a newly described restriction factor, tetherin, was evaluated as a potential molecule for use in the development of transgenic pigs with a reduced risk of PERV transmission, and therefore representing a safer animal donor.

Chapter 2

2 Materials and Methods

2.1 Materials

All buffers and solutions made in house are listed with their formulae in Table 2.1 (section 2.4).

2.2 Molecular biology methods

Plasmid and primer sequences are listed and described in Table 2.2 and Table 2.3, respectively (section 2.4).

2.2.1 Preparation and transformation of chemically competent bacterial cells

10 µL of *Escherichia Coli* DH5α bacteria (Invitrogen) were grown in 5 mL of LB overnight in a shaking incubator at 37°C. The day after, the suspension was diluted 1:100 in LB containing 15mM $MgCl_2$ and incubated at 37°C shaking for 2-3 hours until OD_{600} reached 0.4-0.6. The bacteria were spun down for 15 minutes at 3,500 g in a refrigerated centrifuge (CR4 22, Jouan). The pellet was resuspended in 125 mL of ice-cold solution A (Table 2.1). After centrifugation for 15 minutes at 3500 g at 4°C, the bacteria were resuspended in 12.5 mL of solution A, supplemented with 15% (v/v) glycerol. They were aliquoted and stored at -80°C.

50 µL of competent bacteria were incubated with 100-300 ng of plasmid DNA or with the whole ligation reaction on ice for 30 minutes. After a heat shock of 45 seconds at 42°C, 200-500 µL of LB were added. The bacteria were grown for 1 hour at 37°C, shaking, and spread in a LB agar plate supplemented with the appropriate antibiotic (Table 2.2.2, note).

2.2.2 Plasmid DNA mini preparations

A single bacterial colony was inoculated into 5 mL of LB broth containing the appropriate antibiotic at the concentration indicated (Table 2.2.2, note). The culture was grown overnight in a shaking incubator (200-250 rpm). Bacteria were pelleted, and plasmids extracted using the Qiaprep Spin miniprep kit (Qiagen) or PureLink quick miniprep kit (Invitrogen). Bacterial pellets were resuspended in 250 µL of resuspension buffer and cells were then lysed by incubation for 5 minutes at room temperature with 250 µL of lysis buffer. The mixture was neutralised by addition of 350 µL of neutralisation buffer, and the resulting precipitate was pelletted by centrifugation. The supernatant containing plasmid DNA was applied to an anion-exchange resin column provided with the kit. Purified plasmid DNA was eluted in EB buffer and the concentration determined by spectophotometric analysis using a Nanodrop ND-100 (Nanodrop Technologies). Plasmid DNA preparations were considered of good quality when the absorbance ratio at wavelength 260nm/ 280nm was between 1.8 and 2.

2.2.3 Enzymatic reactions

Restriction enzymes used were obtained from Promega. Reactions were performed according to the manufacturer's instructions in a final volume of 20 µL for single, or 50 µL for a double, enzymatic digestion, in the appropriate buffer, at the temperature required by the enzyme for 1.5-2 hours.

DNA ends were ligated in a 10-20 µl reaction containing 1xT4 buffer, 50 ng of the backbone construct, a ratio of at least 1:3 backbone:insert, DNA and 1µl of T4 DNA ligase [3U/µL] (Promega). Samples incubated either 1 hour at room temperature or 16-24 hours at 4°C.

2.2.4 Agarose gel electrophoresis

DNA samples were separated by agarose gel electrophoresis made by dissolving 1-3 g of agarose (Sigma) in 100 mL of TAE. 0.5 µg/mL ethidium bromide (Sigma) was added to visualize DNA on a UV transilluminator. DNA samples were mixed with 6x loading buffer. 500 ng of 100bp or 1Kb

GeneRuler DNA ladder (Fermentas) were included on each gel to allow size determination of DNA fragments.

2.2.5 DNA purification from agarose gel

DNA bands of the expected size were cut from the agarose gels and purified using Qiaquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

2.2.6 Genomic DNA extraction

Cells were harvested and washed twice in PBS. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. DNA was eluted in 100 μL of AE buffer and the concentration determined by spectophotometric analysis using a Nanodrop ND-100.

2.2.7 RNA extraction

2-10x10^6 cells were harvested, washed twice in PBS, and resuspended in 600 μL of a denaturing guanidine-thiocyanate-containing buffer (buffer RTL) supplemented with 1% (v/v) of β-mercaptoethanol. Cells were vortexed for at least one minute and frozen at -80ºC. The lysate was thawed and 70% (v/v) ethanol was added. Total RNA was extracted using an RNeasy mini kit (Qiagen) following the manufacturer's instructions, and eluted in 50 μL of RNase-free water. RNA concentration was determined by spectophotometric analysis using a Nanodrop ND-100.

2.2.8 Polymerase chain reaction (PCR)

PCR were performed using KOD High Fidelity DNA polymerase (Novagen) or HotStartTaq DNA polymerase (Qiagen) in a Peltier thermal cycler (Dyad) equipped with a heated lid following the recipes, and the amplification parameters, reported below. PCR were conducted in a final volume of 50 μL.

Component	KOD polymerase [2.5 U/µL] (µL)	HotStartTaq [5 U/ µL] (µL)
10x PCR buffer	5	5
dNTPs 2mM (Novagen)*	5	
dNTPs 10mM (Qiagen)*		1
Forward primer 10 µM	2	2.5
Reverse primer 10 µM	2	2.5
$MgCl_2$ 25 mM	2	*(in the PCR buffer at final concentration of 1.5 mM)*
DNA polymerase	1	0.5
Distilled water		

*dNTPs deoxynucleotide triphosphates

Amplification Parameters:

KOD HiFi DNA Polymerase

Step	Time (seconds)	Temperature (ºC)
Initial denaturation	120	98
Three step cycling		
Denaturation	15	98
Primer annealing	2	As reported in Table 2.3
Extension	20	72
Number of cycles: 25		
Final extension	420	72

HotStartTaq DNA Polymerase

Step	Time	Temperature (ºC)
Activation	15 minutes	94
Three step cycling		
Denaturation	30 seconds	94
Primer annealing	30 seconds	As reported in Table 2.3
Extension	0.5-2 minutes	72
Number of cycles: 35-40		
Final extension	7 minutes	72

2.2.9 Reverse transcriptase (RT) reaction

5 µg of total RNA were treated with 2 U of DNase I (New England Biolabs) in a final volume of 50 µL for 30 minutes at 37ºC. 5mM of EDTA was added to protect the RNA from being degraded during the 10 minutes of enzyme inactivation at 75ºC. 20 µL of the reaction were incubated with 1 µg of random primers (Promega) for 5 minutes at 70ºC, and then put on ice. A mixture containing 200 U of Moloney MLV reverse transcriptase (Promega), 1x MoMLV reaction buffer, 25 U of recombinant RNasin ribonuclease inhibitor (Promega), 0.5 mM dNTPs (Qiagen) was added to the RNA-primer mix and incubated for 1 hour at 37ºC. Reverse transcriptase was inactivated by 10 minutes incubation at 65ºC.

For quantitative RT-PCR, cDNA was generated from 1 µg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen) in a final volume of 20 µL. A genomic 'DNA wipe-out' step is included in the kit, and a mixture of random primers and oligo d(T) were used to prime the RT reaction.

2.2.10 Quantitative PCR

Probe-based quantitative PCR was performed using the QuantiTect probe PCR kit (Qiagen) while SYBR Green-based quantitative PCR was conducted using the Quantitect SYBR Green PCR kit (Qiagen). One eighth of the RT reaction or 200 ng of genomic DNA were used per reaction. Each reaction was performed in a final volume of 25 µL in a 96-well plate using the ABI PRISM 7000 or Eppendorf RealPlex 4 real time thermal cycler. Quantitative PCR mix recipes and cycling conditions are reported below. Absolute quantification of gene copies were calculated using plasmids DNA standards as indicated in Table 2.2.1*. Plasmid used to generate standards for 18S rRNA (pCRBluntIITOPO-18S rRNA) was obtained by RT-PCR of RNA from 293T cells using primers CF3;CR3 (Table 2.3.2). The PCR product was cloned into pCR BluntII-TOPO (invitrogen) following the manufacturer's instructions. A 10-fold dilution series of plasmid DNA, range between 10^1 and 10^5 copies, were used to generate the standard curves. For the 18S rRNA gene in the quantitative RT-PCR, the standard curve range was between 10^6 and 10^{10} copies. For SYBR Green-based quantitative PCR, a melting curve

was run at the end of the reaction to confirm the absence of contamination, mispriming or primer-dimer artifacts.

Component	Probe (µL)	SYBR Green (µL)
2x PCR mastermix	12.5	12.5
Forward primer 10 µM	1	0.75
Reverse primer 10 µM	1	0.75
Probe 5 µM	1	
Distilled water		

Probe-based quantitative PCR conditions

Step	Time	Temperature (ºC)
Activation	10 minutes	95
Two step cycling		
Denaturation	15 seconds	94
Primer annealing, extension and fluorescence acquisition	60 seconds	60
Number of cycles: 40		

SYBR Green quantitative conditions

Step	Time	Temperature (ºC)
Activation	10 minutes	95
Three step cycling		
Denaturation	15 seconds	94
Primer annealing	30 seconds	55
Extension and fluorescence acquisition	30 seconds	72
Number of cycles: 40		
Melting curve		

2.2.11 PCR-based mutagenesis

Plasmid mutagenesis was performed by designing two complementary primers containing the mutations of interest. PCR was performed using KOD HiFi DNA Polymerase (Novagen) on 100 ng of plasmid DNA.

2.2.12 Construction of HA-tagged chimeric receptors

HuPAR-2 was tagged at the N-terminus with an influenza virus HA-tag by PCR of the construct pcDNAhuPAR-2 (Ericsson et al., 2003) using KOD HiFi polymerase and the primers GF1 and GR1. GF1 primer introduced the Kozak sequence at the ATG of the receptor downstream of the EcoRI restriction site and the HA-tag in the N-terminus downstream of the ATG start codon. C-terminal HA-tagged huPAR-2 was obtained by PCR using the primers GF2 and GR2. Primer GR2 contained an HA-tag downstream of a HindIII restriction site. Using EcoRI and NotI restriction sites, the HA-tagged receptors were introduced again into pcDNA3. The C-terminal HA-tagged plasmid, pcDNA3/huPAR-2HA, contains two HindIII restriction sites, one in pcDNA3 and the other introduced, in frame, upstream of the HA-tag by the reverse primer. HA-tagged huPAR-1 and muPAR genes were obtained by PCR of constructs pcDNA3/huPAR-1 and pcDNA3/muPAR (Ericsson et al., 2003) with the primer pairs GF2;GR3 (huPAR-1), and GF4;GR4 (muPAR). Using the HindIII restriction site present in the reverse primers, huPAR-1 and muPAR were cloned into pcDNA3/huPAR-2HA upstream of the HA-tag.

An NheI restriction site was introduced into huPAR-2 at the site corresponding to that in muPAR [Genbank: AK008081, nucleotide 805] by PCR-based muatgenesis using primer pairs GF2;MR1 and MF1;GR5, then GF2;GR5, where primers MF1 and MR1 contain the nucleotide change. Primer GR5 includes a HindIII restriction site which allows the cloning of the mutant receptor into pcDNA3/huPAR-2HA. Chimeric receptors H2M a and f were obtained by mix-and-match cloning between huPAR-2 and muPAR using the restriction sites EcoRI and NheI. The other huPAR-2-derived chimeric receptors were produced in a similar way using mutagenesis primers MF2;MR2 (H2M e) and MF3;MR3 (H2M d) in association with the primers GF2;GR5. Similarly, muPAR-derived chimeric receptors were

produced using primers MF4;MR4 (H2M b) and MF5;MR5 (H2M c) in combination with primers GF4;GR4. The mutant huPAR-1 carrying a proline or a serine at position 109 (H1M g or huPAR-1S109) was generated by PCR-mutagenesis using the primers MF3;MR3 or MF9;MR9 in combination with the primers GF2;GR3.

All the HA-tagged receptors were subcloned into the retroviral vector pCFCR (Table 2.2.2) using EcoRI and NotI restriction sites.

The N178A mutation in huPAR-2 was introduced by PCR-mutagenesis using the primers MF6;MR6 in association with the primers GF2;GR5 and the mutant huPAR-2 was cloned into a partially digested pcDNA3/huPAR2HA using EcoRI and HindIII restriction sites.

All the PCRs described above were performed using KOD HiFi polymerase in accordance with manufacturer's instructions. Chimeric receptors were verified by sequencing based on a modification of the Sanger method and analysed using the CEQ 8000 DNA Sequencer (Beckman Coulter).

2.2.13 Generation of soluble myc-tagged PERV-A14/220 SU Env

The soluble surface unit of PERV-A 14/220 Envelope (PERV Env) was cloned into pCAGGS (Niwa et al., 1991) using the restriction sites BglII and NheI and a c-myc tag was introduced at the N-terminus of PERV Env using primers EF1 and ER1 (mycPERV Env). The sequence of human tissue plasminogen activator leader has been introduced in frame upstream to the c-myc tag by PCR of the construct PEE14 (Jeffs et al., 1996) using primers EF2 and ER2, bearing the enzymatic restriction sites KpnI and BglII, respectively.

2.2.14 Receptors cloning

Total RNA extracted from rat or NHP cells was reverse transcribed using Promega MoMLV RT (section 2.2.9). 5 µl of the reaction was used in an RT-PCR using HotStartTaq polymerase and primers as indicated (Table 2.3.2). The PCR product was cloned into pcDNA3 using EcoRI and NotI restriction sites present in the forward and reverse primers, respectively. HA-tagged C-

terminal RatPAR was obtained by PCR using KOD HiFi polymerase and the primers CF1;CR2 which contain the HindIII restriction site, and introduced into partially digested pcDNA/HuPAR-2HA. This product was then subcloned into pCFCR.

A ClaI restriction site was introduced into pcDNA3/huPAR-2HA upstream, in frame, with the HA-tag by PCR-mutagenesis using primers MF7;MR7 in association with primers GF2;MR8 (pcDNA3/huPAR-2ClaIHA). NHP PERV-A receptors were cloned by RT-PCR of total RNA extracted from NHP cells. PCR products were introduced into pcDNA3/huPAR-2ClaIHA using restriction sites EcoRI and ClaI present in the primer sequences. The mutant rhesus macaque PAR-1 containing a leucine at position 109 (rhPAR-1L109) was created by PCR-mutagenesis using the primers MF10;MR10 in combination with the primers CF5;CR5.

All the HA-tagged receptors were also subcloned into the retroviral vector pCFCR using EcoRI and NotI restriction sites

2.2.15 Cloning of porcine tetherin

Total RNA was extracted from pig cells, and cDNA was produced using the QuantiTect Reverse transcription kit (section 2.2.9). 5 µL of the reaction were employed in a RT-PCR using HotStartTaq polymerase and primers CF7;CR7. The PCR product was cloned into a pGEM-Teasy vector in accordance with the manufacturer’s instruction. Porcine tetherin was subcloned into pcDNA3 by enzymatic restriction using EcoRI site. Lentiviral vectors carrying tetherin genes were produced by subcloning them from pCR-huTHN (Neil et al., 2008), pcDNA-PK15THN and pcDNA-IOWATHN into an HIV-based vector pSIN-Dual (Escors et al., 2008), downstream of the SFFV promoter via BamHI and NotI restriction sites.

2.3 Cell culture methods

Cell lines used and growth conditions are listed in Table 2.4 (section 2.4).

2.3.1 Cell transfection

The day prior to transfection, cells were seeded in a 10 cm dish (d10) or a 6-well plate (p6). The number of cells plated varied for each cell line to achieve a density of 80-90% of confluence. 18 µL (d10) or 6 µL (p6) of Fugene-6 transfection reagent (Roche) was diluted in 200 µL (d10) or 100 µL (p6) of Optimem serum-free medium (Gibco). 3 µg (d10) or 1 µg (p6) of plasmid was added to the Fugene-Optimem mixture, incubated at room temperature for 15 minutes and then added to the cells.

2.3.2 Pseudotyped virus production

Pseudotyped viruses were produced by transient three-plasmid transfection (Besnier et al., 2002; Soneoka et al., 1995). A mix containing the following quantities of plasmids was prepared in a total volume of 15 µL of TE buffer: 1.5 µg of retroviral vector, 1 µg of packaging vector plasmid and 1 µg of envelope expression plasmid (Table 2.2.2). 18 µL of Fugene-6 was diluted in 200 µL of Optimem and the plasmid mix added. After an incubation of 15 minutes at room temperature, the mixture was added to 80% confluent 293T cells, seeded in a 10 cm dish the day before. The following day the medium of transfected cells was replaced with 8 mL of fresh DMEM supplemented with 10% (v/v) FBS. Virus-containing supernatant were harvested at 48 and 72 hours post-transfection, filtered through 0.45 µm filters and either used to infect target cells or stored at -80°C.

Pseudotyped viruses described in chapter five were produced from 1x10^{6} 293T cells seeded in a 6-well plate, using 6 µL of Fugene-6 diluted in 100 µL of Optimem. Plasmid mix was composed of 600 ng of retroviral vector, 400 ng of packaging vector plasmid, 400 ng of envelope-expressing plasmid and 200 ng of plasmid encoding the tetherin gene, with or without HIV-1 *vpu* gene.

2.3.3 EGFP(PERV) production

293T cells were seeded in a 10cm dish and, the day after, transfected with 3 µg of pCRPERV-A14/220 encoding full length replication-competent PERV-A14/220. 48 hours later, $1x10^5$ cells were seeded in a 6-well plate and, the day after infected with a VSV-G-pseudotyped MLV vector carrying the EGFP gene, prepared as described (section 2.3.2). Cells were kept in culture. In the supernatant, a mixed population of viruses was expected: PERV-A14/220 wild type and PERV-A14/220 particles carrying the MLV-based retroviral vector encoding the EGFP gene, EGFP(PERV). After two months in culture, titre of EGFP(PERV) (section 2.3.4) was stabilised at $2\pm0.8x10^5$ EGFP transducing units per mL, titered on 293T cells. The PERV-A14/220 [wild type and EGFP(PERV)] titre detected using an anti-PERV CA antibody and colony counting (section 2.3.5) was $3\pm0.1x10^6$ 293T cells infectious unit per mL. The multiplicity of infection (MOI) was calculated as:

$$MOI= \frac{\text{Titre x Volume of infection (ml)}}{\text{Number of cells}}$$

2.3.4 EGFP-pseudotyped virus titration

$5x10^4$ cells were plated in a 12-well plate one day prior to transduction. Serial dilutions of virus-containing supernatant were prepared in DMEM supplemented with 8 µg/mL polybrene (hexadimethrine bromide, Sigma). 500 µL of each dilution was added to the target cells. After 48 hours, cells were trypsinised, fixed in 500 µL of 1% (w/v) paraformaldheyde in PBS and assessed for EGFP expression by flow cytometry. The titre in EGFP transducing unit per mL (Etu/mL) was calculated as average of the data points where less than 30% of the cells were EGFP positive, according to the following formula:

$$\text{Titre (Etu/mL)}= \frac{\text{EGFP-positive cells x number of seeded cells x dilution factor}}{\text{Volume of infection}}$$

2.3.5 PERV titration

Titration of PERV infectious particles in the supernatant of producing cells was performed using a colony formation assay (Bartosch et al., 2002). $3x10^4$ 293T cells were seeded in a 48-well plate the day prior to infection. Serial dilutions of the virus-containing supernatant were prepared in DMEM supplemented with 4 µg/mL polybrene. 200 µL of each dilution was added to the cells. After 72 hours, cells were fixed with an ice-cold 1:1 mixture of methanol and acetone. Cells were washed twice in PBS and blocked for 10 minutes at room temperature with 10% (v/v) FBS in PBS, followed by a hour incubation with anti-PERV capsid antibody (Table 2.5) diluted in washing buffer (2% (v/v) FBS in PBS). Following two washes, a hour incubation with secondary goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) (Jackson Immunoresearch) diluted 1:250 in washing buffer was performed. After two washes in washing buffer, and two more in PBS, AP was detected using NBT/BCIP (Nitro blue tetrazolium chloride/ toluidine salt of 5-Bromo-4-chloro-3-indolyl phosphate) ready-to-use tablets (Roche) according to the manufacturer's instructions. AP-positive colonies were counted using the visible light microscope, and the titre determined as infectious units per mL (i.u./mL):

$$\text{Titre (i.u./mL)} = \frac{\text{Number of AP-positive colonies x dilution factor}}{\text{Volume of infection}}$$

2.3.6 Cell transduction

Retroviral and lentiviral vectors, prepared as described in section 2.3.2, were used to deliver PERV-A receptors and tetherin genes. Target cells were seeded the day prior to transduction in a 12-well plate (p12) or a 6-well plate (p6) at the density of $5x10^4$ (p12) or $1x10^5$ (p6) cells per well. 0.5 mL (p12) or 1 mL (p6) of the 0.45 µm-filtered pseudotyped virus containing supernatant was added to the cells in the presence of 8 µg/mL polybrene. After 48 to 72 hours, cells were employed in the different assays.

2.3.7 Flow cytometry staining

Cells were harvested using 5mM EDTA in PBS, and washed twice in PBS. $5x10^5$ cells per stain were blocked for a hour on ice with 10% (v/v) FBS in PBS. Cells were incubated with primary antibody diluted in washing buffer (2% (v/v) FBS in PBS) for an hour on ice. After two washes, cells were incubated with 1:100 dilution of phycoerythrin (PE)-conjugated secondary anti-mouse IgG antibody (Jackson Immunoresearch) in washing buffer for 45 minutes on ice. The samples were washed twice in washing buffer, resuspended in 500 µL of 1% (w/v) paraformaldehyde in PBS and analysed by flow cytometry using the Becton Dickinson FACSCalibur and Cell Quest software.

When staining both intracellular and extracellular proteins, cells were fixed prior to the immunostaining with 4% (w/v) paraformaldehyde in PBS for 20 minutes at room temperature, and permeabilised with 0.2% (w/v) saponin (Fluka) in PBS for 10 minutes at room temperature. Saponin, at the concentration of 0.2% (w/v), was added to all the buffers during the immunostaining.

2.3.8 PERV envelope binding assay

Cells were detached using 5mM EDTA in PBS, washed twice, and $5x10^5$ cells for each sample were resuspended in 0.5 mL of soluble PERV Env-containing supernatant. Two different soluble PERV Env proteins were used.
In chapter three, c-myc tagged PERV-A14/220 SU Env was produced by transfection of 293T cells with mycPERV Env plasmid (2.2.13) as described (2.3.1). Cells were incubated with soluble mycPERV Env for an hour at 37^oC, and washed twice in washing buffer (2% (v/v) FBS in PBS). Samples were incubated with anti-human myc antibody diluted in washing buffer for an hour on ice. Following two washes, cells were resuspended in washing buffer containing 1:200 dilution of PE-conjugated secondary anti-mouse IgG antibody. After 45 minutes incubation on ice, cells were washed, fixed in 1% (w/v) paraformaldehyde in PBS, and analysed by flow cytometry.
In chapter four, soluble PERV-A360 and PERV-C360 Env were used. The first N-terminal 360 amino acids of PERV-A NIH and PERV-C were

introduced, in frame, upstream of amino acids 96-323 of the rabbit immunoglobulin γ-heavy chain gene (rIgG) in the plasmid pSK100 (Gemeniano et al., 2006). Soluble proteins were produced by transfection of 293T cells. One day post-transfection the medium was replaced with 8 mL of DMEM supplemented with 10% (v/v) FBS and 1x proteinase inhibitor cocktail, Complete mini (Roche). The concentration of PERV Env-rIgG fusion proteins was determined by an enzyme-linked immunosorbent assay (ELISA) (section 2.3.9). $5x10^5$ cells were incubated for a hour on ice with 0.5 mL of transfected 293T cell supernatant containing 200 ng/mL of PERV-A360 or PERV-C360 Env proteins. Cells were washed twice and incubated for 45 minutes on ice in washing buffer containing a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Jackson Immunoresearch). Following two washes, cells were fixed in 1% (w/v) paraformaldehyde in PBS and analysed by flow cytometry.

2.3.9 Enzyme-linked immunosorbent assay (ELISA)

Maxisorp 96-well plate (Nunclon) was coated overnight at 4^oC with 500 ng of monoclonal anti-rabbit IgG (γ-chain specific) clone RG-96 (Sigma) per well diluted in 100 µL of coating buffer (pH 9.6). One day later, wells were washed three times with PBS and blocked with 4% (w/v) bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Two-fold serial dilutions of the PERV Env containing supernatant were added to each well and incubated for an hour at room temperature. Wells were washed three times with 0.1% (v/v) Tween-20 in PBS before adding horseradish peroxidase (HRP)-conjugated polyclonal anti-rabbit IgG (Dako) diluted 1:1000 in 2% (w/v) BSA in PBS. After a hour incubation at room temperature wells were washed 3 times with 0.1% (v/v) Tween-20 in PBS and once in PBS before adding 100 µL of SureBlue TMB (tetramethylbenzidine) substrate (Kirkegaard & Perry Laboratories) per well. The reaction was allowed to proceed for 15 minutes and then stopped with 100 µL of 1M HCl. The optical density of each well was read at wavelength 450nm. The amount of IgG was calculated from a standard curve obtained by two fold serial dilution of rabbit IgG (Dako), range 250-7.8 ng/mL. Samples were run in duplicate.

2.3.10 Immunofluorescence microscopy

The day post transfection (section 2.3.1), 293T cells expressing HA-tagged huPAR-2 were split in three and plated on cover slides and incubated for further a 48 hours. Cells were fixed by incubation with 4% (w/v) paraformaldehyde in PBS for 20 minutes at room temperature. The permeabilised samples were obtained by incubation with 0.1% (w/v) saponin in PBS for 10 minutes at room temperature. For the permeabilized samples, 0.1% (w/v) saponin was added to all the buffers. Slides were washed in PBS and placed on a 30 µL drop of washing buffer (2% (w/v) BSA in PBS) containing a dilution of the primary antibody (Table 2.5) and incubated for a hour at 37°C in a humidified chamber. Cells were then washed three times and placed on a 30 µL drop of washing buffer containing the secondary antibody FITC-conjugated anti-mouse IgG (diluted 1:100) for 45 minutes at 37°C in a humidified chamber. After three washes, the cover slides were mounted in Vecta Shield mounting medium containing propidium iodide (Vector Laboratories). Images were acquired using DM IRE2 confocal microscope (Leica).

Immunostaining of PERV particles attached on the cell surface (section 4.2.9) was performed as described above. However, permeabilisation was conducted by incubating the cells with 0.2% (v/v) Triton X-100 (Sigma) in PBS for 10 minutes at room temperature. The secondary antibody used was FITC-conjugated anti-rabbit IgG, diluted 1:100 in washing buffer.

2.3.11 Western blotting

Cells from a confluent well of a 6-well plate were lysed in 100 µL of radio immunoprecipitation assay (RIPA) buffer supplemented with 1x proteinase inhibitor cocktail, Complete mini, for 30 minutes on ice. Cell debris were removed by centrifugation at 12000g at 4ºC for 8 minutes. 1 mL of the viral particles, containing supernatant of virus-producing cells seeded in the 6-well plate was pelleted by centrifugation for 4 hours at 16000g at 4ºC, and resuspended in 50 µL of 2x Laemmli buffer. After the addition of Laemmli

buffer to 24 µL of the cell lysate, these samples and 24 µL of the concentrated viral particles were boiled for 5 minutes and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Table 2.1). For the glycosylation study (section 3.2.3), 24 µL of cell lysate was incubated with 3 µL of G7 buffer and 3 µL of PNGase F [500U/µL] (New England Biolabs) for 2 hours at 37°C. 10 µL of 4x Laemmli buffer was added and the samples boiled for 5 minutes.

Proteins were transferred onto a methanol-activated polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences) using a semidry blotting system (Amersham Biosciences). The membrane was blocked in 5% (w/v) non-fat dried milk (Oxoid) in PBS and then probed for an hour at room temperature with a primary antibody diluted in 2% (w/v) milk in PBS (Table 2.5), followed by three washes with 0.1% (v/v) Tween-20 in PBS. The membrane was then incubated with an HRP-conjugated secondary antibody diluted in 2% (w/v) milk/PBS (1:3000 for anti-rabbit IgG or 1:10000 for anti-mouse IgG) for 1hr at room temperature. Following three washes with 0.1% (v/v) Tween-20 in PBS, and one in PBS alone, signals were detected by incubation with ECL chemiluminescence reagent (Amersham Biosciences) and exposure to X-ray film (Hyperfilm, Amersham Biosciences).

2.4 Appendix

Table 2.1 Buffers and solutions

1X PBS (phosphate-buffered saline)	1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.4
1x TAE (tris-acetate-EDTA)	40 mM Tris (pH 7.8), 20 mM sodium acetate, 1 mM EDTA
TE (tris-EDTA)	10 mM Tris-Cl, 1mM EDTA, pH 8.0
EB (Qiagen)	10 mM Tris-Cl, pH 8.5
AE (Qiagen)	10 mM Tris-Cl, 0.5mM EDTA, pH 9.0
6x loading buffer	30% (v/v) glycerol, 0.25%;(w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF
Solution A	10 mM $MnCl_2$, 50 mM $CaCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH6.3
ELISA coating buffer	0.16% (w/v) Na_2CO_3, 0.29% (w/v) $NaHCO_3$, 0.02% (w/v) NaN_3, pH 9.6
Luria-Bertani (LB) broth	1% (w/v) bacto-tryptone (BD), 0.5% Bacto yeast extract (BD), 1% NaCl (Sigma), pH 7.0
LB agar	1% (w/v) bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, 1.5% (w/v) bacto-agar (BD), pH 7.0

Western blotting solutions	
RIPA buffer	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Igepal ca-630, 0.5% (w/v) sodium deoxycholic acid, 10% (w/v) SDS, 1% (v/v) Triton X-100
4x Laemmli buffer	200 mM Tris-HCl (pH 6.8), 40% (v/v) glycerol, 8% (w/v) SDS, 0.2% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol
1x running buffer	0.3% (w/v) Trizma (Sigma), 1.9% (w/v) glycine (Sigma), 0.1% (w/v) SDS
1x semi-dry transfer buffer	0.3% (w/v) Trizma, 1.4% (w/v) glycine, 0.1% (w/v) SDS, 20% (v/v) methanol

10% SDS-polyacrylamide gel recipe

components	Resolving (mL)	Stacking (mL)
40% acrylamide mix (Bio-Rad)	12.5	1.25
1.5 M Tris (pH 8.8)	12.5	
1 M Tris (pH 6.8)		1.25
10% SDS	0.5	0.1
10% ammonium persulfate	0.5	0.1
TEMED (tetramethylethylenediamine)	0.02	0.01
Distilled water	24	7.25
Final volume	50	10

Table 2.2 List of the plasmids

Table 2.2.1 Transfection plasmids

Name	Gene	Reference
*pcDNAhuPAR-1	huPAR-1	Ericsson et al., 2003
*pcDNAhuPAR-2	huPAR-2	Ericsson et al., 2003
pcDNAmuPAR	muPAR	Ericsson et al., 2003
N-HA pcDNAhuPAR-2	N-terminal HA tagged huPAR-2	Mattiuzzo et al., 2007; section 2.2.12
C-HA pcDNAhuPAR-2	C-terminal HA tagged huPAR-2	Mattiuzzo et al., 2007; section 2.2.12
pcDNAhuPAR2N178A	C-terminal HA tagged huPAR-2 carrying mutation N178A	Mattiuzzo et al., 2007; section 2.2.12
*pcDNAratPAR	ratPAR	Mattiuzzo et al., 2007
*pCRIIBlunt18S rRNA	nt 101-1932 human rRNA NCBI acc no. M10098.1	Mattiuzzo et al., 2007; section 2.2.10
pCAGGSmycPERV Env	N-terminal myc-tagged PERV-A14/220 SU Env	Mattiuzzo et al., 2007; section 2.2.13
pSKPERV-A360Env	a.a. 1-360 PERV-A NIH Env	Gemeniano et al., 2006
pSKPERV-C360 Env	a.a. 1-360 PERV-C Env	Gemeniano et al., 2006
pCR-huTHN	Human tetherin	Neil et al., 2008
pcDNAPK15THN	Pig tetherin from PK15 cells	Section 2.2.15
*pcDNAIOWATHN	Pig tetherin from ST-IOWA cells	Section 2.2.15
pcDNAVpu	HIV-1 NL4.3 Vpu	Neil et al., 2006

All plasmids carried an ampicillin-resistance gene with the exception of pCRIIBlunt18S rRNA, which encodes kanamycin resistance gene (Table 2.2.2)

*These plasmids were used as quantitative PCR standards

Table 2.2.2 Pseudotyped virus production

Name	drug resistance	Gene	Reference
pCNCG	Ampicillin	Neo^R ;EGFP	(Soneoka et al., 1995)
pCNCR	Ampicillin	Neo^R;RFP	A kind gift from Prof. Greg Towers
pCFCRΔEcoRI	Ampicillin	Fv1;RFP	(Ylinen et al., 2005)
pSIN-DUAL	Ampicillin	$Hygro^R$;	(Escors et al., 2008)
pHRSIN-CSGW	Ampicillin	EGFP	(Demaison et al., 2002)
pCMV intron	Ampicillin	MoMLV GagPol	(Collins et al., 1995)
phCMV-PERV-A14/220	Ampicillin	PERV-A14/220 GagPol	(Wood et al., 2009)
pCMV8.91	Ampicillin	HIV-1 GagPol	(Zufferey et al., 1997)
pMDG	Ampicillin	VSV-G	(Naldini et al., 1996)
FBPERV14/220 SALF	Ampicillin	PERV-A14/220 Env	A kind gift from Dr. Birke Bartosch
phCMV-MLVA	Ampicillin	MLV-A Env	(Sandrin et al., 2002)
pCRPERV14/220	Kanamicin	PERV-A14/220 genome	(Bartosch et al., 2004)

Neo^R geneticin (G-418 sulphate) resistance; $Hygro^R$ Hygromycin B resistance

Antibiotic concentration in LB broth and agar plate was ampicillin 100 µg/mL; kanamycin 50 µg/mL.

Table 2.2.3 pCFCRΔEcoRI-derived constructs

Name	Gene
pCFCR-huPAR2	C-terminal HA-tagged huPAR-2
pCFCR-huPAR1	C-terminal HA-tagged huPAR-2
pCFCR-muPAR	C-terminal HA-tagged muPAR
pCFCR-ratPAR	C-terminal HA-tagged ratPAR
pCFCR-rhPAR-1	C-terminal HA-tagged rhPAR-1
pCFCR-rhPAR-2	C-terminal HA-tagged rhPAR-2
pCFCR-AGMPAR-1	C-terminal HA-tagged AGMPAR-1
pCFCR-AGMPAR-2	C-terminal HA-tagged AGMPAR-2
pCFCR-cynPAR-1	C-terminal HA-tagged cynPAR-1
pCFCR-cynPAR-2	C-terminal HA-tagged cynPAR-2
pCFCR-baPAR-1	C-terminal HA-tagged baPAR-1
pCFCR-baPAR-2	C-terminal HA-tagged baPAR-2
H2M a	huPAR-2 with a.a. 1-161 from muPAR
H2M b	huPAR-2 with a.a.108-110 from muPAR
H2Mc	huPAR-2 with a.a 109 from muPAR
H2M d	muPAR with a.a. 109 from huPAR-2
H2M e	muPAR with a.a. 108-110 from huPAR-2
H2M f	muPAR with a.a. 1-161 from huPAR-2
H1M g	huPAR-1 with a.a. 109 from muPAR
huPAR-1S109	huPAR-1 with a.a.109 from rhPAR-1
rhPAR-1L109	rhPAR-1 with a.a.109 from huPAR-1

Genes are cloned between EcoRI and NotI restriction sites

Table 2.2.4 pSIN-DUAL-derived constructs

Name	gene
pDUAL-huTHN	Human tetherin
pDUAL-PK15THN	PK15 cells-derived tetherin
pDUAL-IOWATHN	ST-IOWA cell-derived tetherin

Genes are cloned between BamHI and NotI restriction sites

Table 2.3 List of the primers

Table 2.3.1 Construction of HA-tagged chimeric receptors

name	Sequence (5' → 3')	T (°C) annealing	Comment
GF1	TTA CAA GAA TTC[d] GCC ACC ATG G[i]TT TAC CCA TAC GAT GTT CCA GAT TAC GCT[h] GCA GCA CCC ACG CTG GGC CGT CTG GTG CTG A	60	N-HA huPAR-2 EcoRI and Kozak sequence
GR1	GAT CCT AAG CGG CCG C[e]TC AGG GGC CAC AGG GGT CTA	60	huPAR-2 NotI
GF2	GAT TGA TGA ATT C[d]AC CAC CAT GG[i]C AGC ACC CAC G	60	huPAR-2 EcoRI and Kozak sequence
GR2	GAT CTT GCG GCC GC[e]T CAA GCG TAT TCT GGA ACA TCG TAT GGG TA[h]A AGC TT[c]G GGG CCA CAG GGG TCT ACA CAG TCC TTT CTG CTT TG	60	C-HA huPAR-2
GR3	GAA GGT AAG CTT[c] GAG GCC ACA CTG GTC	60	huPAR-1 HindIII
GF4	GAT TGA TGA ATT C[d]AC CAC CAT GG[i]C AGC ACC TCC G	56	muPAR EcoRI and Kozak sequence
GR4	GAA GGT AGG CTT[c] GAG GCC ACA CTG GTC	56	muPAR HindIII
GR5	CGT GGC ATC TAG ATT AAG CTT[c] GGG GCC ACA GGG GTC	60	huPAR-2 HindIII
MF1	CCT GTG TGC TAG CCC TAG TGC AA	48	huPAR-2 NheI
MR1	TTG CAC TAG GGC TAG CAC ACA GG	48	huPAR-2 NheI
MF2	GCC CCA GTG GCA GGG AAG CCG TAC TCT GTG GCC TTC CTA	52	H2Me

MR2	TAG GAA GGC CAC AGA GTA CGG CTT CCC TGC CAC TGG GGC	52	H2Me
MF3	GCC CCA GTG GCA GGG CAG CCC CAC TCT GTG GCC TTC CTA	52	H2Md; H1Mg
MR3	TAG GAA GGC CAC AGA GTG GGG CTG CCC TGC CAC TGG GGC	52	H2Md; H1Mg
MF4	GCC CCA GTG GCA GGA CAG CTC CAC TCA GTG GCC TTC CTA	52	H2Mb
MR4	TAG GAA GGC CAC TGA GTG GAG CTG TCC TGC CAC TGG GGC	52	H2Mb
MF5	GCC CCA GTG GCA GGA AAG CTC TAC TCG GTG GCC TTC CTA	52	H2Mc
MR5	TAG GAA GGC CAC CGA GTC GAG CTT TCC TGC CAC TGG GGC	52	H2Mc
MF6	AGA GGT GCC AGC GGT GGG CGC T	52	huPAR-2 N178A
MR6	AGC GCC CAC CGC TGG CAC CTC T	52	huPAR-2 N178A
MF7	GAC CCC TGT GGC CCC ATC GAT TAC CCA TAC GAT GTT	56	huPAR-2ClaIHA
MR7	AAC ATC GTA TGG GTA ATC GAT GGG GCC ACA GGG GTC	60	huPAR-2ClaIHA
MR8	GTT CTT TCC GCC TCA GAA GC	56	pcDNA3
MF9	GCA GGA CAG TCG CAT TCT GTG G	60	huPAR-1S109
MR9	CCA CAG AAT GCG ACT GTC CTG C	60	huPAR-1S109
MF10	GCA GGA CAG TTG CAT TCC GTG	58	rhPAR-1S109
MR10	CAC GGA ATG CAA CTG TCC TGC	58	rhPAR-1S109

[c]HindIII, [d]EcoRI, [e]NotI, [h]influenza virus HA tag, [i]Kozak sequence.

Table 2.3.2 cloning primers

name	Sequence (5'→ 3')	T (°C) annealing	Comment
EF1	AGC TGG AGA TCT[a] GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG[g] CTT GTG ACC AGT CCG AAC TCC CAT AAA CCC TTA TCT CTC ACC	58	PERV Env
ER1	ATG TTC TTA GCT AGC[b] CTA TTC ATC AAG GAT TGC TTT TTC CGG	58	PERV Env
EF2	GCC AGA GGA GGT ACC[f] GCC ACC ATG GAT GCA ATG AAG AGA G	62	mycPERV Env
ER2	GGG TAA GAT CT[a]G GCT CCT CTT CTG AAT CGG GCA TGG ATT TCC TGG CTG GGC	62	mycPERV Env
CF1	GAT TGA TGA ATT C[d]AC CAC CAT GG[i]C AGC ACC	56	ratPAR
CR1	TGA CTG AGC GGC CGC[e] TCA AGG GCC ACA CTG ATC CAC	56	ratPAR
CR2	GCA GGT AAG CTT[c] AGG GCC ACA CTG ATC	56	ratPAR
CF3	TAC CTG GTT GAT CCT GCC AGT A	60	18S rRNA
CR3	TTA CGA CTT TTA CTT CCT CTA GAT AG	60	18S rRNA
CF4	GTC CAG AAT TC[d]A CCA CCA TGG[i] CAG CAC CCA TG	60	rhPAR-2
CR4	CAA GGA TCG AT[l]G GGG CCA CAG G	60	rhPAR-2
CF5	GTT CCA GAA TTC[d] ACC ACC ATG G[i]CA GCA CCC ACA CCC AGC	60	rhPAR-1
CR5	CAA GGA TCG AT[l]G GAG TCA CAG GGG TCT GC	60	rhPAR-1
CF6	GTT CCA GAA TTC[d] ACC ACC ATG	60	AGMPAR-1

	G[l]CA GCA CC ACA CCC GGC		
CF7	ACA CCT CAG GTC AGC AG	50	poTHN
CR7	ATG TCA CCT AGT TTG TAT TCC	50	poTHN

[a]BglII, [b]NheI, [c]HindIII, [d]EcoRI, [e]NotI, [f]KpnI, [g]human c-myc tag, [h]influenza virus HA tag, [i]Kozak sequence, [l]ClaI.

Table 2.3.3 Sequencing primes

Name	**Sequence (5'→3')**	**T (°C) annealing**	**Comment**
T7promoter	CGA CTC ACT ATA GGG AGA CCC	52	pcDNA3
BGH polyA	TTA GGA AAG GAC AGT GGG AGT	52	pcDNA3
Chicken promoter-F	TTC TCC ATC TCC AGC CTC GGG	48	pCAGGS
RabbitpolyA-R	CCC ATA TGT CCT TCC GAG TGA	48	pCAGGS
M13-F	GTT TTC CCA GTC ACG AC	56	pGEM
M13-R	GGA AAC AGC TAT GAC CAT G	58	pGEM

Table 2.3.4 Quantitative PCR primers

name	Sequence (5' → 3')	Comment
QF1	CTG CCT TCC AGG GTC TTC TG	huPAR-1
QR1	TGA GGA CTC TTC CAC CTC TTC CT	huPAR-1
QF2	TCA GGG CCT GAA CTT CAA CTG	huPAR-2
QR2	GCA ATG GCA AAG CCT CTT CT	huPAR-2
QF3	TCA AGG TGT CTC CCA TCA ATT TC	ratPAR
QR3	CGT CAA CAC CCA AAA GAA TGT G	ratPAR
QF4	TCG AGG CCC TGT AAT TGG AA	18S rRNA
QR4	CTT GCC CTC CAA TGG ATC CT	18S rRNA
QF5	AGC CTA CTT GGG ATG ATT GTC AA	PERV gag
QR5	GGC CCC AGG AAC ATT TTT TC	PERV gag
QF6	GTA CCC ACA GGG GGC TTA GGA TC	NHP PAR-1
QR6	CTT GGT GGC TCT TGC AAT GGT G	NHP PAR-1
QF7	GCA GGG CCT GAA CTT CCA TTG	NHP PAR-2
QR8	AAG GCA CCCA TGG GCT GAG AAC	NHP PAR-2
QF9	GTG AGC TGC TTG AGG GAA TC	poTHN
QR9	TTG ACA TTC CTG CTG TGC TC	poTHN
QF10	ACC TGC AAC CAC ACT GTG ATG	huTHN
QR10	CAA GCT CCT CCA CTT TCT TTT GTC	huTHN

Table 2.3.5 Quantitative PCR probes

Name	Sequence (5'→3')	Comment
P1	CCA CCA TCT GTA CCC ACA	huPAR-1
P2	CAG GAG CAG AGG AGG	huPAR-2
P3	CTG AGC GTT TCT CTG	ratPAR
P4	AGT CCA CTT TAA ATC CTT	18S rRNA

All probes are dual-labelled 5'-FAM/3'-TAMRA.

Table 2.4 List of the cell lines

Name	ATTC no.	Description
293T[1]	(DuBridge et al., 1987)	Human embryonic kidney. Epithelial. Express SV40 large T antigen
COS-7	CRL-1651	African green monkey kidney. Fibroblast. Express SV40 large T antigen
FRhK-4	CRL-1688	Rhesus macaque kidney. Epithelial
HeLa	CCL-2	Human cervical carcinoma. Epithelial
HSN	(Currie and Gage, 1973)	Rat fibrosarcoma. Fibroblast
HT1080	CCL-121	Human fibrosarcoma.Epithelial
MDTF	(Lander and Chattopadhyay, 1984)	*Mus dunni* tail fibroblast
MPK[2]	CCL-166	Mini pig kidney. Fibroblast
NIH3T3	CRL-1658	Mouse NIH/Swiss embryonic fibroblast
NRK	CRL-6509	*Rattus norvegicus* Kidney. Epithelial
PK15[2]	CCL-33	*Sus scrofa* kidney. Epithelial
QT6	CRL-1708	Quail fibrosarcoma. Epithelial
RAT2	CRL-1764	*Rattus norvegicus* fibroblast
ST-IOWA[2]	(Quinn et al., 2004)	*Sus scrofa* testis. Fibroblast
VERO	CCL-81	African green monkey kidney. Epithelial
XC	CCL-165	*Rattus norvergicus* epithelial

ATCC American Type Culture Collection

Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with foetal bovine serum (FBS) 10% (v/v) (Biosera) at 37ºC with 5% CO_2.

[1] 293T cells were grown in DMEM supplemented with Glutamax (Gibco) and 15% (v/v) FBS at 37ºC with 10% CO_2

[2] Pig cell lines were grown in DMEM supplemented with 10% (v/v) FBS and 0.1 mM non-essential amino acids (Gibco).

Table 2.5 Primary antibodies

Name	animal source	Dilution	Reference
PERV capsid	Rabbit, anti-serum	1:250 (IM) 1:100 (IF) 1:1000(WB)	Bartosch et al 2004
HA.11	Mouse, monoclonal	1:100 (IF, FC) 1:1000 (WB)	Covance MMS-101R
human c-myc (9E10)	Mouse, monoclonal	1:100 (FC)	Santa Cruz biotechnologies sc-40
human actin AC-40	Mouse, monoclonal	1:1000 (WB)	Sigma, A 4700
Human CD71 3H3077	Mouse, monoclonal	1:100 (IF,FC)	Santa Cruz biotechnologies, sc-70772

IM *in situ* immunostaining; IF immunofluorescence; FC flow cytometry; WB western blotting

Chapter 3

3 Host range determinants of PERV-A receptors

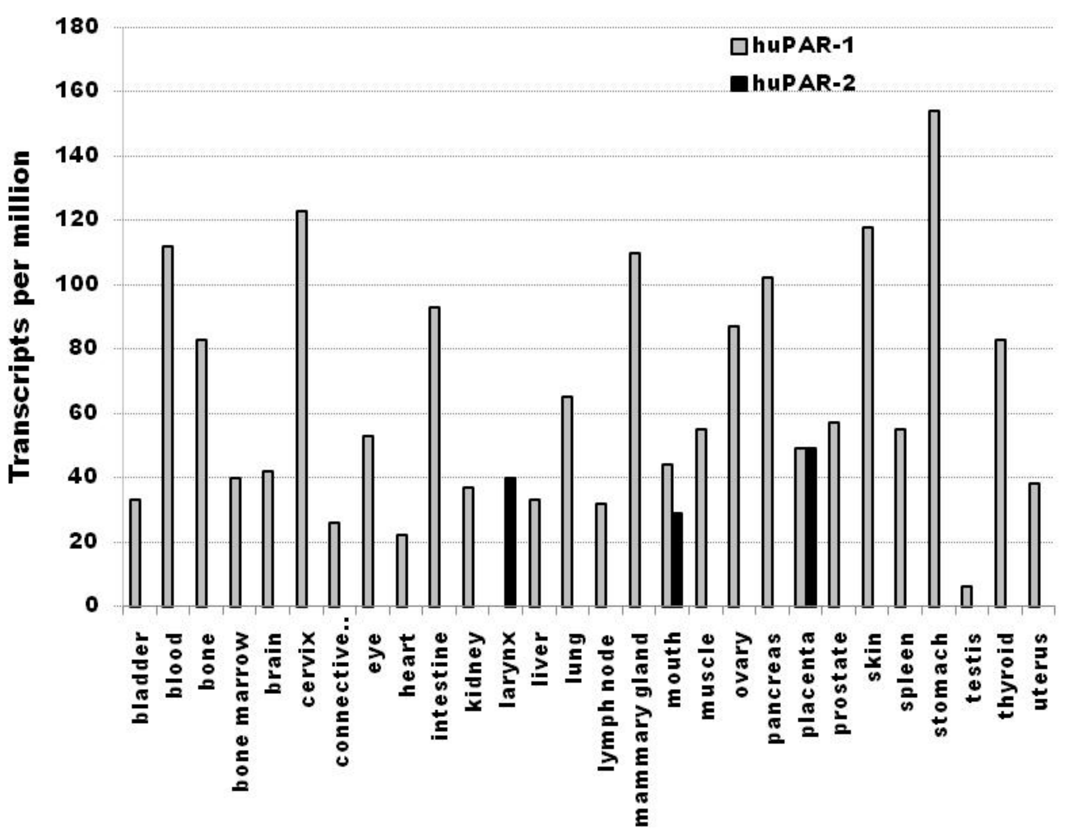
3.1 Introduction

A better understanding of PERV biology is required to predict the possible risk associated with infection in xenotransplantation recipients and to develop therapeutics to prepare for such an eventuality. Three subgroups of replication-competent PERV have been described that differ in their envelope sequence, and therefore host range (section 1.4.1). The studies on PERV entry have followed two lines of investigation: viral envelope and cellular receptor.

By comparison with the sequence of MLV Env, functional regions of PERV Env have been predicted (Le Tissier et al., 1997). These regions are the variable region A (VRA) and B (VRB), which in MLV are responsible for the receptor usage (Battini et al., 1995; Battini et al., 1992), and the proline rich region (PRR) considered important for the correct display of Env proteins on the viral surface and during the fusion process (Lavillette et al., 1998; Weimin Wu et al., 1998). For PERV Env, the characterisation of the determinants for viral infectivity was initially done through the comparison of the high-titre human recombinant PERV-A14/220 isolate with a prototype cell line-derived PERV-A. The PRR in the C-terminal of the SU derived from PERV-C envelope was shown to increase the titre of the recombinant PERV-A/C envelope up to 500 fold if in association with an amino acid mutation (V140I) between the VRA and VRB (Harrison et al., 2004). PRR was shown to be necessary for PERV-A, but not PERV-B, binding to the target cells (Gemeniano et al., 2006; Watanabe et al., 2005). Changing 4 amino acids in the C-terminal region of pig-tropic PERV-C SU with the correspondents in the human tropic PERV-A SU enabled PERV-C Env binding and entry into human cells (Argaw et al., 2008). Furthermore, PERV can infect cells lacking its receptor. PERV-A can infect non permissive rat and mouse cells in presence of soluble GALV receptor binding domain (RBD) (Lavillette and

Kabat, 2004). The proposed mechanism was the activation in *trans* of conformational changes in the receptor which lead to the fusion between the viral and cellular membrane. This alternative pathway for infection was described for MLV (Barnett and Cunningham, 2001; Lavillette et al., 2001) and suggested for FeLV-T, which can infect cells only in presence of FeLV-B SU or a soluble factor called Felix, a RBD domain of an endogenous retrovirus (Anderson et al., 2000; Barnett et al., 2003).

Only the receptor for PERV subgroup A has been identified. PERV-A receptor (PAR) was cloned using a similar approach described previously for other gammaretrovirus receptors (section 1.5.1). A human cDNA library derived from PERV-A permissive cells was delivered via retroviral transduction into resistant rabbit SIRC cells. The retroviral vector contained a drug resistance gene which allowed the selection of the transduced cells (i.e. those which bear a cDNA encoding for a protein able to support PERV-A entry). The cDNA clone selected, named huPAR-1, corresponded to the human sequence FLJ11856, coding for a protein characterised by a domain of unknown function DUF1011. In the GenBank database a second human gene homologous to huPAR-1 (huPAR-2, accession number FLJ10060) and a murine homologue (muPAR, accession number AK008081) were identified. Rabbit SIRC cells, expressing either huPAR-1 or huPAR-2, were sensitive to PERV-A and PERV-A14/220 but not PERV-B or –C. Expression of muPAR did not mediate PERV-A infection. The hydrophobicity profiles of huPAR-1 predicted multiple transmembrane domains (10 or 11) similar to other gammaretrovirus receptors (section 1.5.2). The mRNA for huPARs was found in a wide variety of human tissues by Northern blot analysis (Ericsson et al., 2003). From the NCBI EST profile, huPAR-1 appears to be ubiquitously expressed while huPAR-2 ESTs were detected mainly in the placenta and few other body sites (Figure 3.1).



3.1 EST profile for huPAR-1 and huPAR-2
Approximate gene expression patterns as inferred from EST counts reported in Unigene database (www.ncbi.nlm.niih.gov/unigene) for huPAR-1 (grey, Hs.6459) and huPAR-2 (black, Hs.632247) on 15th December 2009.

As a result of its predicted structure, huPAR-1 was identified as a G protein-coupled receptor (GPR172A or GPCR41). However, all the γ–retrovirus receptors identified at present function as transporters (section 1.5.4). Characterisation of huPAR physiological function has been controversial. In a study aimed at identifying the human homologue of rat γ-hydroxybutyrate (GHB) receptor, two cDNA clones from a human frontal cortex cDNA library were selected. One of these shared the same amino acid sequence of huPAR-1 and the second one was a variant with a frame shift which caused an addition of 42 residues in the C-terminus (Andriamampandry et al., 2007). This paper presents several problems. Negative controls were not always included, the physiological uptake of GHB in different tissues does not correspond to the EST profile and mRNA distribution of huPAR-1, and there is no data showing the expression *in vivo* of the alternative huPAR-1 form with the longer C-tail.

HuPAR-2 has been described as a riboflavin (vitamin B2) transporter (RFT1) (Yonezawa et al., 2008). A rat kidney cDNA library was used to select the cDNA of a multitransmembrane protein of uncharacterised function. The sequence corresponded to the rat protein similar to GPR172B (huPAR-2) (GenBank accession number XM_001075182). Riboflavin was identified as substrate of rat RFT (rRFT) from the screening against 25 compounds. The human homologue (huPAR-2) named hRFT was also shown to be able to increase uptake of radiolabelled riboflavin when expressed in HEK-293 and Caco-2 cells. A major problem in this paper was the lack of mention of huPAR-1, which is as closely related to rRFT as huPAR-2 (section 3.2.5) and ubiquitously expressed (Figure 3.1). By knocking down hRFT in HEK-293 cells, a significant reduction in the riboflavin uptake was observed. However, huPAR-2 expression is low in these cells in comparison with huPAR-1 (section 4.2.4). It would be important to test whether huPAR-1 has the same function as huPAR-2 and measure its expression in huPAR-2 knocked down cells to better interprete the data. Overall, further investigation is needed to assess the physiological function of PARs.

In this chapter, PERV-A entry in human and rodent cells has been investigated. The aim was to identify determinants in PERV-A receptor critical for its interaction with the virus. This could provide information for the development of strategies to characterise and eventually prevent PERV-A infection.
A major part of this chapter has been published (Mattiuzzo et al., 2007).

3.2 Results

3.2.1 PERV-A resistant murine cells express a non-functional receptor

Two human PERV-A receptors (huPAR-1 and huPAR-2) and their murine homologue (muPAR) have been identified (Ericsson et al., 2003). Alignment of the amino acid sequences revealed that huPAR-1 shares 86.1% and 81.1% identity with huPAR-2 and muPAR, respectively. HuPAR-2 and muPAR have 79.6% identical amino acids (Figure 3.2). However, while huPAR-1 and huPAR-2 expression on PERV-A resistant rabbit SIRC cells successfully mediated virus entry, muPAR did not (Ericsson et al., 2003). Furthermore, PERV host range studies showed that murine cells are resistant to PERV-A infection (Takeuchi et al., 1998; Wilson et al., 2000). The role of muPAR in PERV-A resistance in murine cells has been investigated.

Human 293T cells, *mus dunni* MDTF and quail QT6 cells were transduced with a MLV-based retroviral vector carrying huPAR-1, huPAR-2 or muPAR genes. $5x10^4$ transduced cells were infected with EGFP pseudotype rescued by PERV-A14/220 [EGFP(PERV), section 2.3.3]. After 48 hours, cells were analysed by flow cytometry and the efficiency of infection determined by the percentage of EGFP positive cells. PERV-A14/220 pseudotyped virus did not infect murine MDTF or quail QT6 cells. Murine cells bearing a functional receptor (huPAR-1 or huPAR-2) were susceptible to EGFP(PERV) infection, suggesting that PERV-A block in these cells occurred at entry level (Figure 3.3). MuPAR was unable to mediate EGFP(PERV) infection in resistant MDTF and QT6 cells. This result was in agreement with a previous report showing that muPAR does not function as a PERV-A receptor (Ericsson et al., 2003).

```
muPAR           MAAPPLGRLVLTHLLVALFGMGSWAAVNGIWVELPVVVKELPEGWSLPSYLSVLVALGNL 60
ratPAR          MAAPPLGRLVLTHLLVALFGMGSWIAVNGIWVELPVVVKELPEGWSLPSYLSVLVALGNL 60
huPAR-2         MAAPTLGRLVLTHLLVALFGMGSWAAVNGIWVELPVVVKDLPEGWSLPSYLSVVVALGNL 60
huPAR-1         MAAPTPARPVLTHLLVALFGMGSWAAVNGIWVELPVVVKELPEGWSLPSYVSVLVALGNL 60
                ****.  .* *************** *************:*********:**:******

muPAR           GLLLVTLWRRLARGKGEQVPIRVVQGLGIVGTGLLASLWNHVAPVAGKPYSVAFLTLAFV 120
ratPAR          GLLLVTLWRRLAPGKSERIPIQVVQGLSIVGTGLLAPLWSNMALVAGQLHSVAFLTLAFV 120
huPAR-2         GLLVVTLWRQLAPGKGEQVPIQVVQVLSVVGTALLAPLWHHVAPVAGQLHSVAFLTLALV 120
huPAR-1         GLLVVTLWRRLAPGKDEQVPIRVVQVLGMVGTALLASLWHHVAPVAGQLHSVAFLALAFV 120
                ***:*****:** **.*::**:*** *.:***.***.** ::* ***: :*****:**:*

muPAR           LALACCASNVTFLPFLSHLPPPFLRSFFLGQGLSALLPCVLALGQGVGRLECLHVPANRT 180
ratPAR          LALSCCASNVTFLPFLSHLPPPFLRSFFLGQGLSALLPCVLALAQGVGRLECLHVPANGT 180
huPAR-2         LAMACCTSNVTFLPFLSHLPPPFLRSFFLGQGLSALLPCVLALVQGVGRLECPPAPTNGT 180
huPAR-1         LALACCASNVTFLPFLSHLPPRFLRSFFLGQGLSALLPCVLALVQGVGRLECPPAPINGT 180
                **::**:************** ******************** ********  .* * *

muPAR           TGPPIEVSPINFPERFSATTFFWVLTALLGTSAAAFQGLLLLLPSPTSEPT--TGTGLRV 238
ratPAR          TGPPIKVSPINFPERFSAGTFFWVLTALLGTSAAAFQGLLLLLPSPPPEAT--MGTGLRV 238
huPAR-2         SGP-----PLDFPERFPASTFFWALTALLVTSAAAFRGLLLLLPSLPSVTTGGSGPELQL 235
huPAR-1         PGP-----PLDFLERFPASTFFWALTALLVASAAAFQGLLLLLPPPPSVPTGELGSGLQV 235
                .**     *::* ***.* ****.***** :*****:*******.  .. .*   *. *::

muPAR           ETPGTEEEEEEEE-ASPLQEPPGQVAGIVSSPDPKAHQLFSSRSACLLGLLAITNALTNG 297
ratPAR          ETPGTEEEEEEEE-ASPLQEPPGQVASIVSSPDPKAHRLFSSRSACLLGLLAITNALTNG 297
huPAR-2         GSPGAEEEEKEEEEALPLQEPPSQAAGTIPGPDPEAHQLFSAHGAFLLGLMAFTSAVTNG 295
huPAR-1         GAPGAEEEVEESS---PLQEPPSQAAGTTPGPDPKAYQLLSARSACLLGLLAATNALTNG 292
                 :**:*** :*..    ******.*.*.  ..***:*::*:*::.* ****:* *.*:***

muPAR           VLPAVQSFSCLPYGRLAYHLAVVLGSCANPLACFLAMAVLCRSLAGLCGLSLLGMLLGSY 357
ratPAR          VLPAVQSFSCLPYGRLAYHLAVVLGSSANPLACFLAMAVLCRSLAGLYGLCLLGMFFGTY 357
huPAR-2         VLPSVQSFSCLPYGRLAYHLAVVLGSAANPLACFLAMGVLCRSLAGLVGLSLLGMLFGAY 355
huPAR-1         VLPAVQSFSCLPYGRLAYHLAVVLGSAANPLACFLAMGVLCRSLAGLGSLSLLGVFCGGY 352
                ***:**********************.**********.********* .*.***:: * *

muPAR           LMTLAALSPCPPLVGTSAGVVLVVLSWVLCAGTFSYIKVAISSMLHSGGRPALLAAGVAI 417
ratPAR          LMTLAVLSPCPPLVGTSAGVVLVVLSWVLCAGVFSYIKVATSSMLHSGGRPALLAAGVAI 417
huPAR-2         LMALAILSPCPPLVGTTAGVVLVVLSWVLCLCVFSYVKVAASSLLHGGGRPALLAAGVAI 415
huPAR-1         LMALAVLSPCPPLVGTSAGVVLVVLSWVLCLGVFSYVKVAASSLLHGGGRPALLAAGVAI 412
                **:** **********:*************    .***:*** **:**.*************

muPAR           QVGSLLGAVAMFPPTSIYRVFRSGKDCVDQCGL 450
ratPAR          QVGSLLGAIAMFPPTSVYPVFRSGEDCVDQCGP 450
huPAR-2         QVGSLLGAGAMFPPTSIYHVFQSRKDCVDPCGP 448
huPAR-1         QVGSLLGAVAMFPPTSIYHVFHSRKDCADPCDS 445
                ******** *******:* **:* :**.* *.
```

Figure 3.2 PAR amino acid sequences alignment

Amino acid sequences retrieved from Entrez protein database (www.ncbi.nlm.nih.gov/entrez) of huPAR-1 (NP_078807.1), huPAR-2 (NP_060456.3), muPAR (NP_083919) and ratPAR (NP_001103140) were aligned using ClustalW software (Larkin et al., 2007).

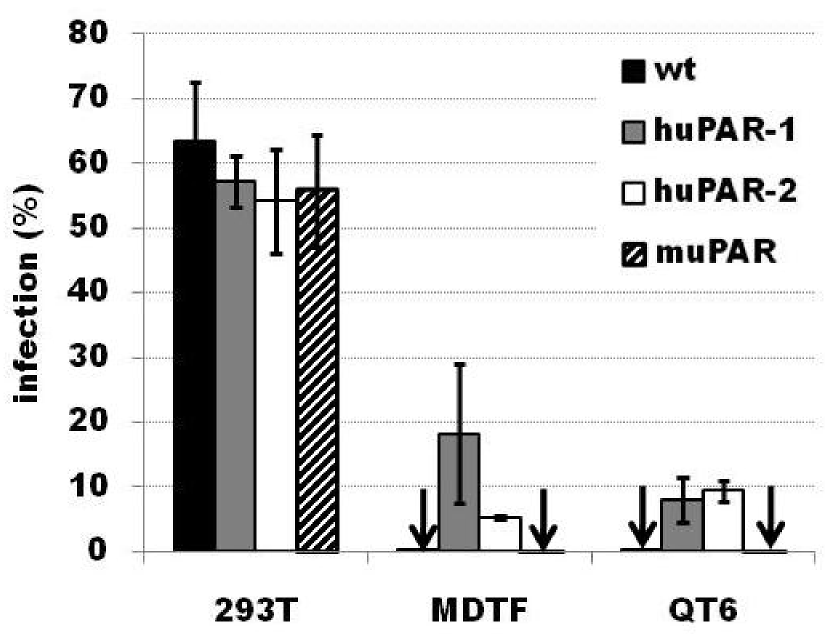


Figure 3.3 HuPAR and muPAR-mediated PERV-A infection

PERV-A permissive human 293T cells and resistant murine MDTF and quail QT6 cells were transduced with a VSV-G pseudotyped MLV-based retroviral vector carrying huPAR-1 (grey), huPAR-2 (white) or muPAR (striped) genes. $5x10^4$ PAR-transduced or parental (black) cells were infected with 500 µL of 293T supernatant containing EGFP(PERV) particles. Infection efficiency was measured as percentage of EGFP positive cells assessed by flow cytometry analysis. Histograms represent the average of two independent experiments (± standard error of the mean). Arrows indicate that the infection was under the detection limit of 0.1%.

3.2.2 Identification of critical amino acids in muPAR for its receptor function

Critical sites in the γ-retrovirus receptor for infection have usually been identified by comparing a functional and a non-functional receptor (section 1.5.2). Following the same approach, human-mouse chimeric receptors were generated and expressed in PERV-A resistant QT6 cells. HuPAR-2 was chosen over huPAR-1 because it could mediate a higher infection efficiency either in QT6 cells (Figure 3.3) or in rabbit SIRC (Ericsson et al., 2003). The NheI restriction site was introduced into huPAR-2 at the same position as in muPAR and used to create chimera H2M a and f in which the first 483 nucleotides were exchanged between the two receptors. C-terminal HA-tagged chimeric constructs were expressed in QT6 cells by transduction with a MLV-based retroviral vector. Receptor expression was assessed by cell surface immunostaining using an anti-HA antibody. Ability to mediate PERV-A entry was tested by infection with EGFP(PERV). H2M f was unable to support PERV-A infection in QT6 cells while the converse muPAR mutant did, indicating that critical sites were included within the first 483 nucleotides (Figure 3.4). Using the alignment of the amino acid sequences, 3 residues shared between huPAR-1 and -2 (QLH) but different in muPAR (KPY) were identified in the N-terminal 161 amino acids. Mutant huPAR-2 containing murine KPY residues (H2M b) were compared against muPAR carrying human QLH amino acids (H2M e) for the ability to support PERV-A infection. Only the chimeric receptor bearing human QLH residues could confer to QT6 cells susceptibility to EGFP(PERV) infection (Figure 3.4). The analysis was narrowed down to identify one single amino acid which if expressed in huPAR-2 (P at position 109, H2M d) could reduce PERV-A infection in H2Md-transduced QT6 cells under the detectable limit. Conversely, when mutant muPAR carrying the substitution P109L (H2M c) was expressed in QT6 cells EGFP(PERV) infection was detected (Figure 3.4). Furthermore to test the importance of the amino acid 109, a chimeric huPAR-1 with mutation L109P was generated (H1M g). QT6 cells expressing H1M g were not susceptible to EGFP(PERV) infection (Figure 3.4). These results suggested that proline 109 in muPAR is responsible for PERV-A resistance in mouse

cells. These observations were confirmed by a recently published report (Marcucci et al., 2009).

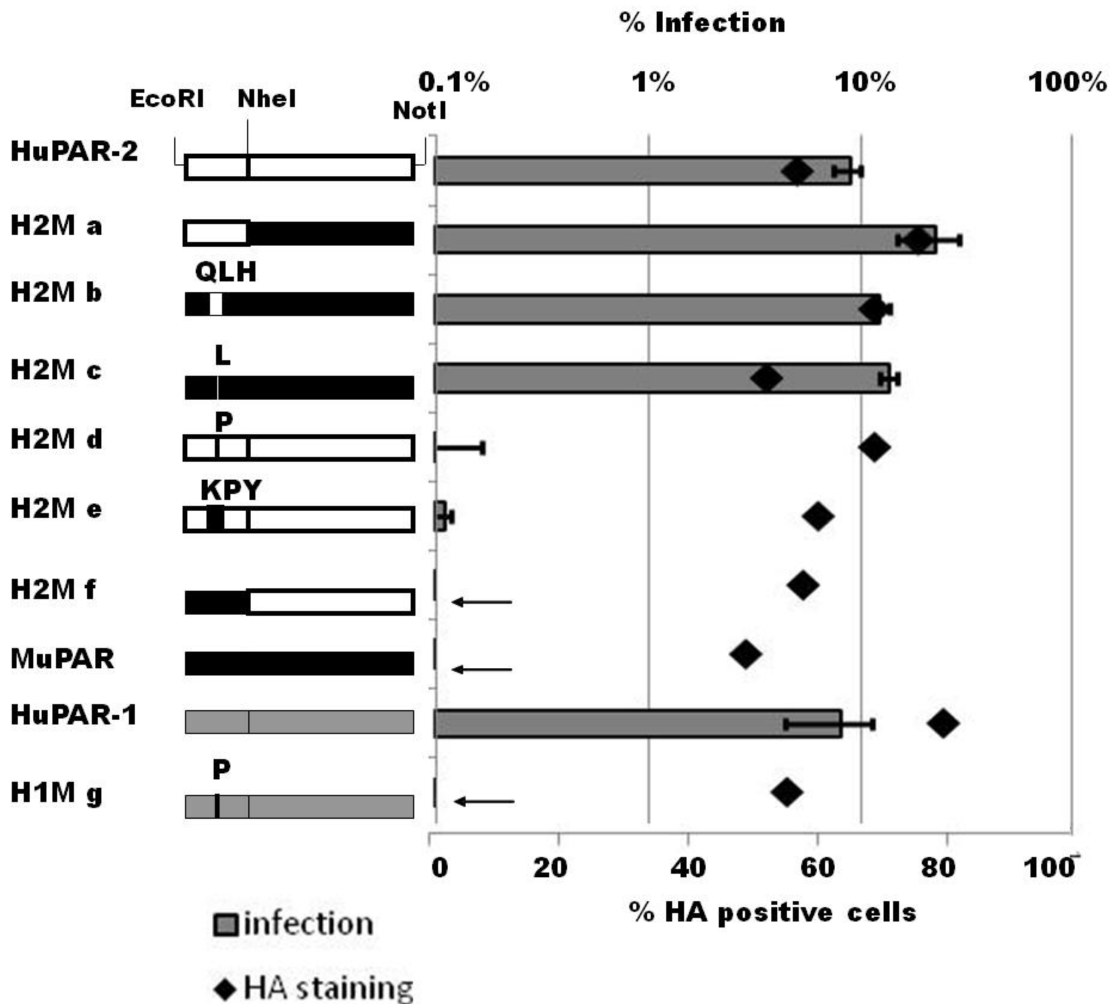


Figure 3.4 Identification of critical residues in PAR for PERV-A infection

Quail QT6 cells were transduced with MLV-based retroviral vector carrying HA-tagged huPAR-2 (white), muPAR (black), huPAR-1 (grey) or chimeric receptor genes. Receptor expression was assessed by immunostaining using an anti-HA antibody and flow cytometry analysis. Representative histograms of HA-staining of PAR-expressing QT6 cells are showed in Figure 3. 6. Percentage of HA positive cells for each receptor is reported (black diamond). $5x10^4$ cells were infected with EGFP(PERV) at MOI on 293T cells of 2. Efficiency of EGFP transduction is expressed as percentage of EGFP positive cells. Histograms represent the average of three independent experiments (± standard error of the mean). Arrows indicate that the infection was under the detectable limit of 0.1%.

3.2.3 Investigation of huPAR-2 topology

To understand the mechanism by which one single amino acid substitution could dramatically change the function of PAR, the localisation of this residue in the receptor structure was investigated. Firstly, the topology of huPAR was predicted. Previous hydrophobicity profile analysis predicted 10 or 11 transmembrane domains (Ericsson et al., 2003). I submitted huPAR-2 amino acid sequence to TMHMM server v2.0 (www.cbs.dtu.dk/services/TMHMM) (Krogh et al., 2001) and the update model showed 11 transmembrane domains, 5 extracellular loops (ECL), an intracellular N-terminus and extracellular C-terminus. To gather evidence supporting this prediction, the orientation of the extremities was assessed.

Two constructs were generated: huPAR-2 HA-tagged either at the N-terminal or at the C-terminal end. The receptors were transfected into 293T cells and their expression analysed at the immunofluorescence microscope or by flow cytometry analysis. Only the C-terminus HA-tagged huPAR-2 was visualised by cell surface staining using an anti-HA antibody. A similar staining was obtained using an anti-human transferrin receptor antibody (anti-CD71), a protein expressed on the cell surface of active proliferating cells. N-terminal tagged huPAR-2 was observed only after saponin treatment, which permeabilises cells allowing the antibodies to stain intracellular target (Figure 3.5 A and B). These results are in agreement with the updated topology prediction of 11 transmembrane domains.

Further evidences supporting the predicted topology were obtained through a glycosylation study. HuPAR-2 has been predicted to contain one N-glycosylation site at amino acid 178 using NetNGlyc 1.0 software (www.cbs.dtu.dk/services/NetNGlyc) (Blom et al., 2004). N-glycosylation sites are usually located in extracellular regions of membrane proteins and asparagine 178 in huPAR-2 is indeed located in the predicted third ECL. Therefore, proving that asparagine 178 is an N-glycosylation site would add strength to the topology proposed. A mutant HA-tagged huPAR-2 bearing the amino acid substitution asparagine 178 to alanine (N178A) was generated and upon transfection of QT6 could support EGFP(PERV) infection with a

similar efficiency than the wild type (Figure 3.6 A). 293T cells were transfected with HA-tagged huPAR-2 wild type or the mutant N178A and cell lysates were processed with PNGase F, an enzyme which removes N-linked oligosaccharide chains. Proteins were separated by SDS-PAGE and immunoblotted using an anti-HA antibody. The western blot analysis showed a shift of the signal in the wild type huPAR-2 treated with PNGase F from 55 KDa to 48 KDa (Figure 3.6 B), indicating that huPAR-2 carries N-linked oligosaccharide chains. Furthermore, the mutant N178A produced a 48 KDa band with or without PNGase F treatment (Figure 3.6 B), confirming that N178 is indeed an N-glycosylation site.

The results obtained in this section, by the analysis of huPAR-2 topology, supported the proposed model described above (Figure 3.6 C). Similar models were obtained by submitting huPAR-1 and muPAR amino sequence to TMHMM server v2.0 and NetNGlyc 1.0 software.

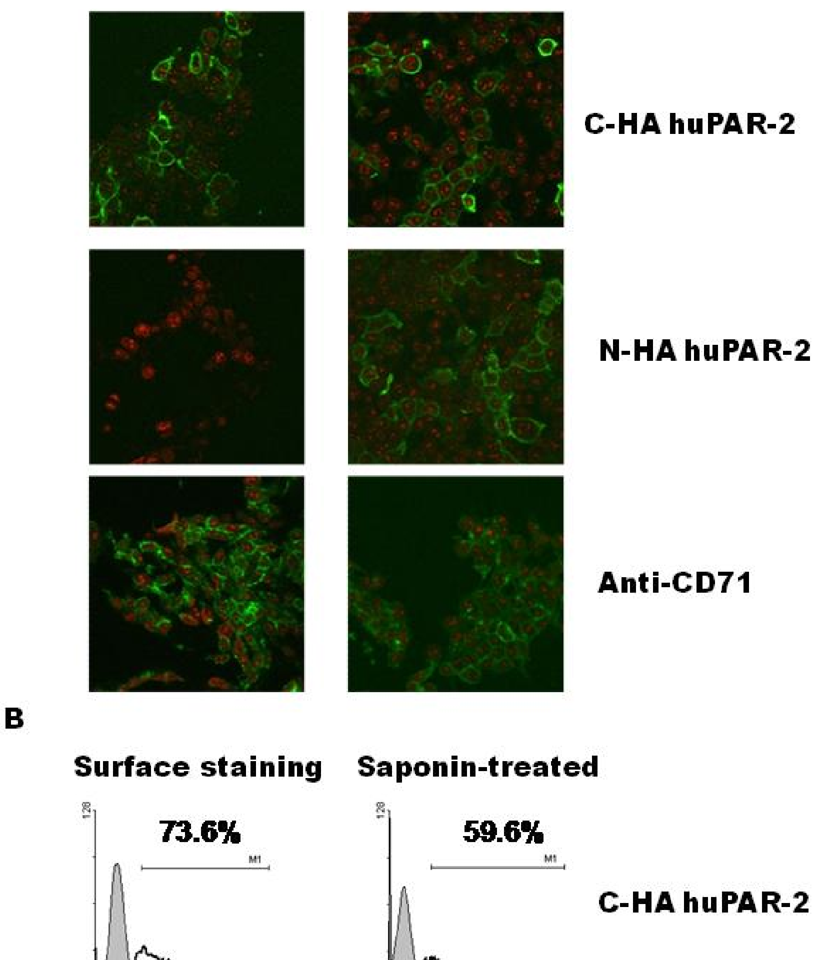
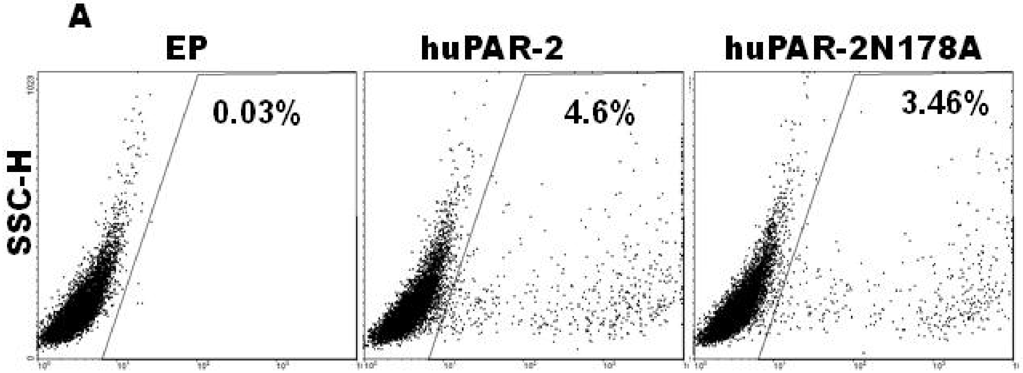
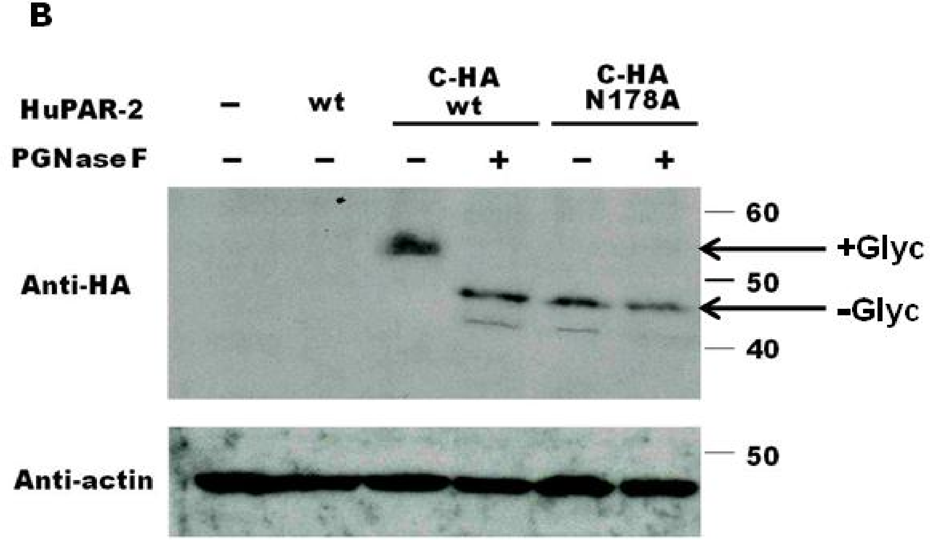

A
Surface staining
Saponin-treated
C-HA huPAR-2
N-HA huPAR-2
Anti-CD71
B
Surface staining
Saponin-treated
73.6%
59.6%
8.7%
56.9%
84.0%
67.1%
Cell number
Fluorescence intensity

Figure 3.5 Cellular localisation of huPAR-2 C- and N-terminal ends

$1x10^6$ 293T cells were seeded and the day after transfected with C- or N-terminal HA-tagged huPAR-2 constructs. 72 hours later, cells were fixed and permeabilised (right column) or not (left column) with saponin. Cells were immunostained with a mouse anti-HA (C-HA huPAR-2 and N-HA huPAR-2) or anti-transferrin receptor (anti-CD71) antibody. **A)** Cells were incubated with an FITC-conjugate anti-mouse IgG antibody (green) and nuclei counterstained with propidium iodide (red), contained in the mounting solution. Images show representative fields acquired using DM IRE2 confocal microscope (oil immersion objective 63X). **B)** Cells were incubated with a PE-conjugated anti-mouse antibody and processed by flow cytometry. Percentage of HA- and CD71-positive cells (bold line) was calculated by comparison to cells treated only with the secondary antibody (grey filled). Histograms showed one representative of two independent experiments.


A
EP
huPAR-2
huPAR-2N178A
0.03%
4.6%
3.46%
SSC-H
EGFP-fluorescence intensity


B
HuPAR-2
–
wt
C-HA wt
C-HA N178A
PGNase F
– – – + – +
Anti-HA
60
50
40
+Glyc
-Glyc
Anti-actin
50

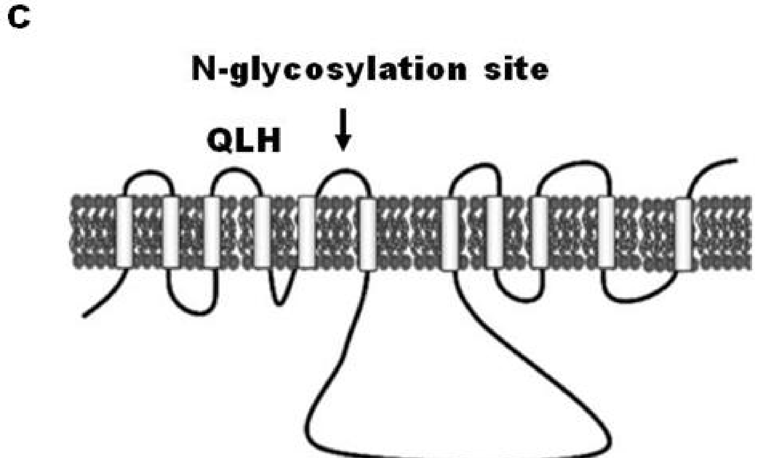

C
N-glycosylation site
QLH

Figure 3.6 N-linked glycosylation of huPAR-2

A) QT6 cells were transfected with pcDNA3 (EP), C-terminal HA tagged huPAR-2 wild type (huPAR-2) or the mutant with asparagines 178 substituted with an alanine (huPAR-2N178A). After 48 hours, $5x10^4$ transfected cells were seeded and the day after infected with EGFP(PERV) at MOI on 293T cells of 2. Three days later cells were examined by flow cytometry. Dot plots showed a representative experiment. **B)** $1x10^6$ 293T cells were seeded and the day after transfected with pcDNA3 (-), untagged huPAR-2 (wild type), C-terminal HA-tagged huPAR-2 (C-HA wild type) or HA-tagged huPAR-2 mutant with the asparagine 178 substituted with an alanine (C-HA N178A). After 48 hours, cells were lysed in RIPA buffer and treated (+) or not (-) with PNGase F. Proteins were separated in a 10% SDS-PAGE and immunoblotted using an anti-HA antibody or an anti-actin antibody as input control. **C)** HuPAR-2 topology model derived by hydrophobicity algorithms (Krogh et al., 2001) and the experiment results (Figure 3.5 and 3.6B).

3.2.4 Proline 109 abrogates PERV-A Env binding to PAR

Amino acid 109 was located in the second ECL according to huPAR-2 topology. To investigate the mechanism responsible for the abrogation of PERV-A infection by proline 109 in muPAR, receptor binding to PERV-A Env was analysed.

Quail QT6 cells were transduced with a MLV-based vector carrying HA-tagged huPAR-1, huPAR-2, muPAR, huPAR-2P109 and muPARL109 genes. Receptor expression was assessed by immunostaining using an anti-HA antibody and flow cytometry analysis. Transduced cells expressed similar levels of receptors as showed by HA staining (Figure 3.7 A). Parental and receptor-transduced cells were incubated with soluble, c-myc tagged PERV-A14/220 (mycPERV) Env protein and immunostained using an anti-myc antibody. No difference was observed between parental QT6 cells incubated in the presence or absence of mycPERV Env (Figure 3.7 B wt). Expression of huPAR-1 and -2, but not muPAR, produced a shift towards higher fluorescence intensity in presence of mycPERV Env, indicating that human receptors can mediate the binding to viral Env. QT6 cells expressing huPAR-2 bearing a proline at amino acid 109 were unable to bind soluble mycPERV Env in this assay. These results suggested that proline 109 in muPAR could be responsible for the lack of infection in mouse cells by altering PERV Env binding to the receptor. However, muPAR with the amino acid substitution P109L did not rescue the binding to mycPERV Env (Figure 3.7 B), even if it supported PERV-A infection (Figure 3.4). This discrepancy between the binding and infection results could be due to a better binding of the trimeric Env present on viral particles than the soluble monomeric form used in this assay. Moreover, other regions in the muPAR molecule could be important for the binding. For example, through the comparison of huPAR-1 and huPAR-2, the region comprised between amino acids 152-285 (from the third ECL till the seventh transmembrane region) was identified as responsible for ten-fold functional superiority of huPAR-2 over huPAR-1 (Marcucci et al., 2009).

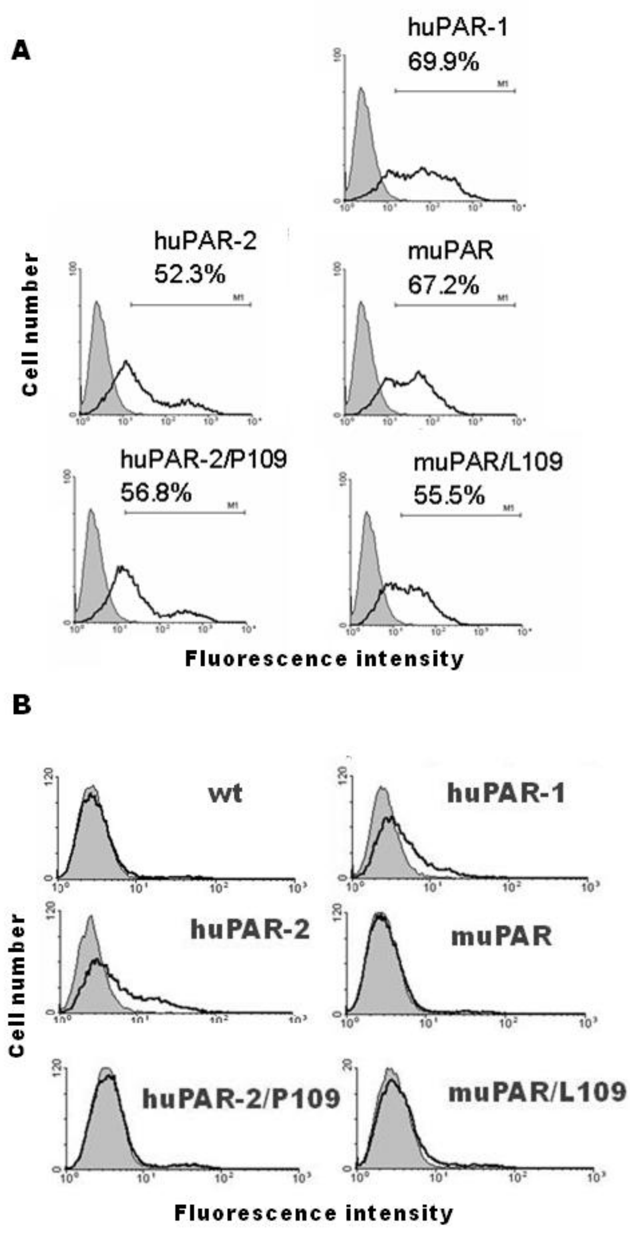


Figure 3.7 Soluble PERV-A binding to PARs

QT6 cells were transduced to express HA-tagged huPAR-1, huPAR-2, muPAR or the chimeric receptors, huPAR-2 with the leucine at amino acid position 109 substituted with a proline (huPAR-2/P109) or muPAR with the converse mutation (muPAR/L109). **A)** Receptor expression was assessed by immunostaining using an anti-HA antibody. Percentage of HA-positive cells (bold line) was calculated by comparison with wild type cells (grey filled). **B)** $5x10^5$ cells were incubated with 1 mL of 293T cells supernatant either untransfected (grey filled) or transfected with N-terminal c-myc tagged soluble PERV-A14/220 SU Env (bold line). Cells were immunostained using an anti-human c-myc antibody and a PE-conjugated anti-mouse IgG secondary antibody. Histograms show a representative result of three independent experiments.

3.2.5 Cloning and characterisation of rat PERV-A receptor

Rat cells are resistant to PERV-A infection (Takeuchi et al., 1998). To investigate whether a non-functional receptor could be responsible for PERV-A resistance, similar to mouse cells, rat PERV-A receptor (ratPAR) was cloned. In the NCBI database a homologue to huPAR was identified (accession number XM_343272). RatPAR has amino acid identities of 90.4% with muPAR, and 79.3% and 79.0% with huPAR-1 and huPAR-2, respectively (alignment shown in Figure 3.2). Specific primers were designed based on the sequence XM_343272 and employed in a PCR using cDNA from PERV-A resistant rat NRK cells as a template. The ratPAR sequence cloned had 2 amino acids different from the sequence deposited in GenBank.

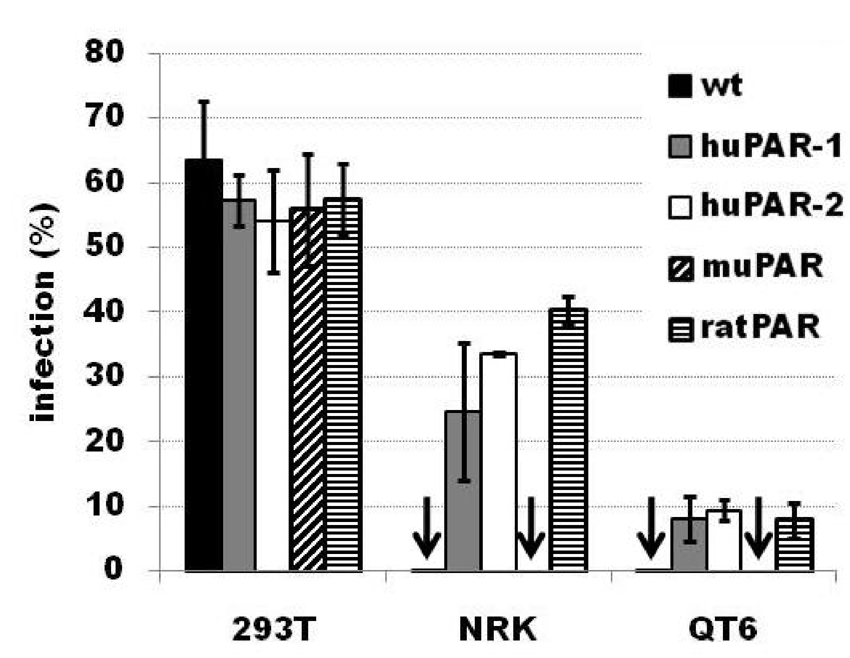
Amino acid sequence alignment showed that ratPAR shared the same ECL2 sequence with huPAR-1 and huPAR-2 (Figure 3.8 A). HA-tagged receptors were delivered by transduction with a retroviral vector into human 293T, rat NRK and quail QT6 cells. The receptors function, to support PERV-A entry, was tested by infection with pseudotyped EGFP(PERV) and monitoring EGFP expression by flow cytometry. PERV-A resistant cells became susceptible upon expression of huPAR-1, huPAR-2 and ratPAR but not muPAR (Figure 3.8 B). RatPAR could also mediate PERV-A infection in QT6 cells as efficiently as huPAR-1 (Figure 3.8 B). Furthermore, QT6 cells expressing similar levels of huPAR-1 and ratPAR receptors (Figure 3.8 C, HA staining), bound with the same efficiency to soluble PERV Env (Figure 3.8 C, mycPERV Env binding). These results showed that ratPAR acted as a functional receptor for PERV-A infection and suggested that a different mechanism was responsible for PERV-A resistance in rats.

A

```
huPAR-2   84-QVLSVVGTALLAPLWHHVAPVAGQLHSVAFLTLALV
huPAR-1   84-QVLGMVGTALLASLWHHVAPVAGQLHSVAFLALAFV
muPAR     84-QGLGIVGTGLLASLWNHVAPVAGKPYSVAFLTLAFV
RatPAR    84-QGLSIVGTGLLAPLWSNMALVAGQLHSVAFLTLAFV
```

ECL2

B



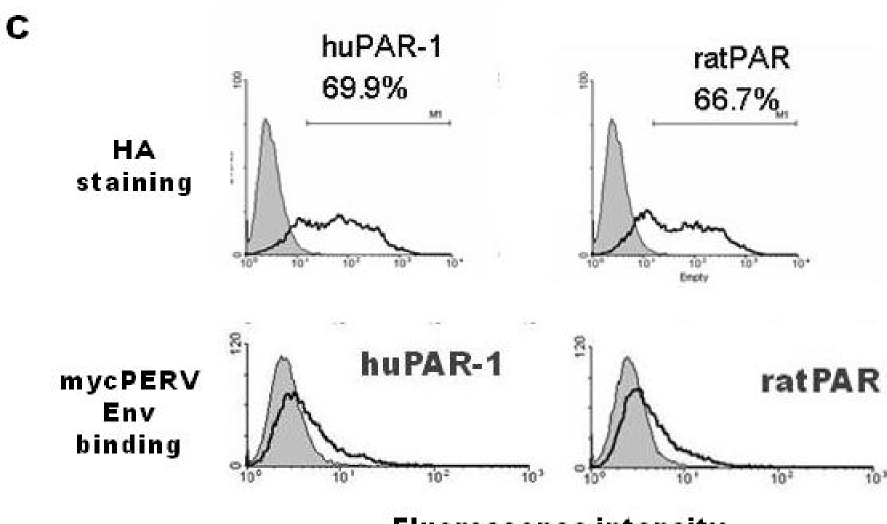


Figure 3.8 Characterisation of rat PERV-A receptor

A) Amino acid sequences of huPAR-2, huPAR-1, muPAR and ratPAR were aligned using ClustalW programme. Partial sequences, containing second extracellular loop (ECL2, underlined) are shown. **B)** Human 293T and PERV-A resistant rat NRK and quail QT6 cells were transduced with an MLV-based retroviral vector carrying huPAR-1 (grey), huPAR-2 (white), muPAR (diagonally striped) or ratPAR (horizontally striped) genes. $5x10^4$ PAR-transduced or parental (black) cells were infected with 500 µL of 293T supernatant containing EGFP(PERV) particles. Infection efficiency was measured as percentage of EGFP positive cells assessed by flow cytometry analysis. Histograms represent the average of two independent experiments (± standard error of the mean). Arrows indicate the infection was under the detection limit of 0.1% **C)** QT6 cells were transduced to express huPAR-1 or ratPAR. Receptor expression was assessed by immunostaining using an anti-HA antibody (top panels). Percentage of HA-positive cells (bold line) was calculated by comparison with wild type cells (grey filled). $5x10^5$ cells were incubated with one mL of 293T cells supernatant either untransfected (grey filled) or transfected with N-terminal c-myc tagged soluble PERV-A14/220 SU Env (bold line) (bottom panels). Cells were immunostained using an anti-human c-myc antibody and a PE-conjugated anti-mouse IgG secondary antibody. Histograms show a representative result of three independent experiments.

3.2.6 Endogenous expression of ratPAR in rat cells

Because rat cells encode a functional receptor, a possible explanation for their resistance to PERV-A infection could be a low level of ratPAR expression. An anti-PAR antibody is not available; therefore the mRNA level of ratPAR was measured and compared to huPAR-1 mRNA from highly susceptible human 293T and HeLa cells.

Total mRNA was extracted from human and rat cells and the copy numbers for huPAR-1 and ratPAR were estimated. On average rat cells expressed a 4 times lower level of ratPAR than huPAR-1 in human cells, with the exception of HSN cells where the difference was up to 25-fold (Figure 3.9). However, to determine whether the difference in the receptor mRNA levels between rat and human cells could be responsible for the lack of PERV-A infection in rat cells, further experiments were required.

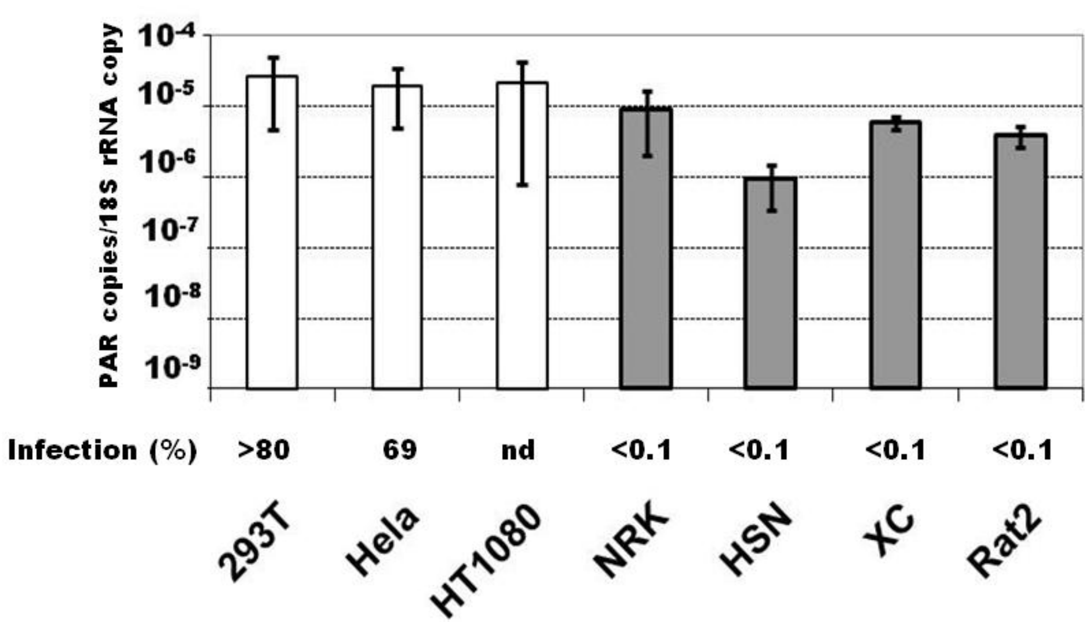


Figure 3.9 Endogenous expression of PERV-A receptors

Total RNA was extracted from human 293T, HeLa and HT1080 cells (white) and rat NRK, HSN, XC and Rat2 cells (grey). 1 µg was reverse described and one eighth was used in a probe-based quatitative RT-PCR using primers QF1, QR1 and probe P1 (for huPAR-1) and QF3,QR3 and probe P3 (for ratPAR) (Table 2.3.4). PAR copy numbers were determined by comparison with a standard curve and normalised to one copy of 18S rRNA. Samples were run in duplicate. Histograms represent the average of three independent experiments (± standard error of the mean). Efficiency of infection was determined by infection of $5x10^4$ cells with EGFP(PERV) at MOI on 293T of 2 and expressed as percentage of EGFP-positive cells. PERV-A infection on HT1080 was not performed. However, HT1080 cells were reported to be permissive to LacZ(PERV-A) transduction (Takeuchi et al., 1998).

3.2.7 Analysis of post-entry restriction factor in rat cells

Before analysing whether rat resistance to PERV-A infection was due to a low expression of ratPAR, other possible mechanisms of retroviral block were investigated. The most well described restriction factors acting against retroviruses post-entry but prior to provirus integration are Fv1 and TRIM5α (section 1.6.1). Their expression in otherwise permissive cells caused a reduction in sensitive virus titre of more than 10-fold (Hartley et al., 1970; Stremlau et al., 2004; Towers et al., 2000). The viral determinant for restriction was mapped in both cases to the CA protein (Besnier et al., 2003; Kozak and Chakraborti, 1996; Perron et al., 2004; Towers et al., 2002).

To analyse whether a similar mechanism occurred with PERV-A in rat cells, VSV-G pseudotyped viruses carrying an MLV-based retroviral vector expressing EGFP were produced by transfection of 293T cells together with packaging plasmid expressing PERV-A14/220 or Moloney MLV (MoMLV) GagPol. MoMLV GagPol was used as control because it has been shown to be resistant to post-entry restriction (Keckesova et al., 2004; Kozak, 1985; Perron et al., 2004). After 48 hours, the pseudotyped viruses were titrated on human 293T and rat NRK cells. On 293T cells no difference was observed in the infection efficiency between pseudotyped viruses bearing PERV-A14/220 GagPol or MoMLV GagPol (Figure 3.10, 293T). In rat NRK cells, PERV-A14/220 GagPol caused a 4-fold reduction in the pseudotyped viruses compared to MoMLV GagPol (Figure 3.10, NRK). Although these results may suggest the possibility of a weak restriction factor present in rat cells, such a small degree of reduction cannot explain the lack of PERV-A infection observed (Figure 3.8 B). Moreover, in rat HSN cells the efficiency of transduction obtained with VSV-G pseudotyped PERV-A14/220 or amphotropic MLV4070 in which *env* gene was substituted with *egfp* gene was similar (Harrison et al., 2004). Taken together these observations suggested that post-entry restriction is not responsible for PERV-A resistance in rat cells.

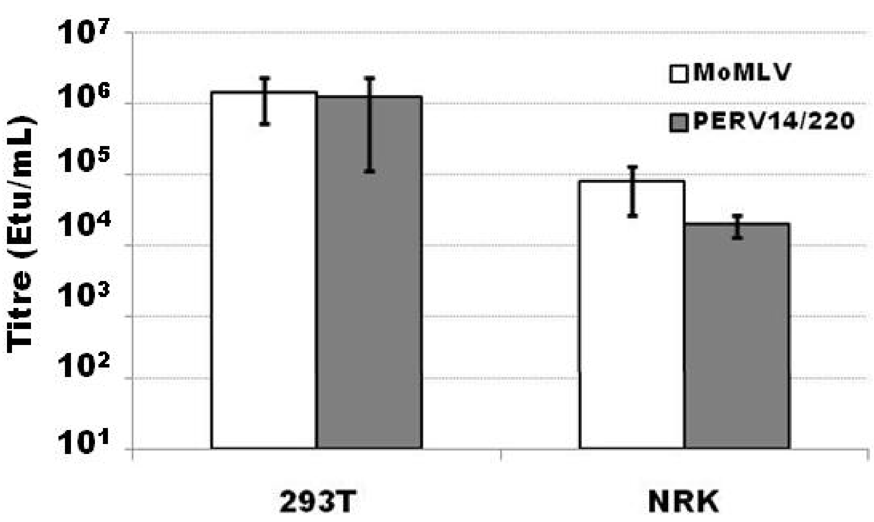


Figure 3.10 Analysis of post-entry block to PERV-A infection in rat cells

VSV-G pseudotyped particles containing MLV-based retroviral vector expressing EGFP, and Moloney MLV (white) or PERV-A14/220 (grey) GagPol were produced by transfection of 293T cells. Viral titres were determined by infection of 293T or NRK cells and monitoring of EGFP expression by flow cytometry. Histograms represent the average of two independent experiments (± standard error of the mean).

3.2.8 Evaluation of N-liked glycosylation in rat cells on PERV-A infection

Removal of N-linked glycosylation by tunicamycin treatment of resistant cells was shown to rescue retroviral infectivity (section 1.5.5). In this section a possible role for N-linked glycosylation in PERV-A resistance in rat cells would be investigated.

Rat NRK, HSN, XC, Rat2 cells were treated overnight with tunicamycin. Human 293T and murine MDTF cells were used as (positive and negative, respectively) controls for PERV-A infection. For each cell line tunicamycin dose was chosen according to the toxicity observed (Figure 3.11). Cells were infected with EGFP(PERV) and 72 hours later analysed using flow cytometry. EGFP transduction of untreated rat cells was under the detection limit. However, upon tunicamycin treatment, a low PERV-A infection could be observed in NRK and Rat2 cells (Figure 3.11). Removal of N-linked glycosylation slightly enhanced PERV-A infectivity in some, but not all, rat cells, suggesting that receptor masking by N-glycosylation could not be the cause of PERV-A resistance in rats.

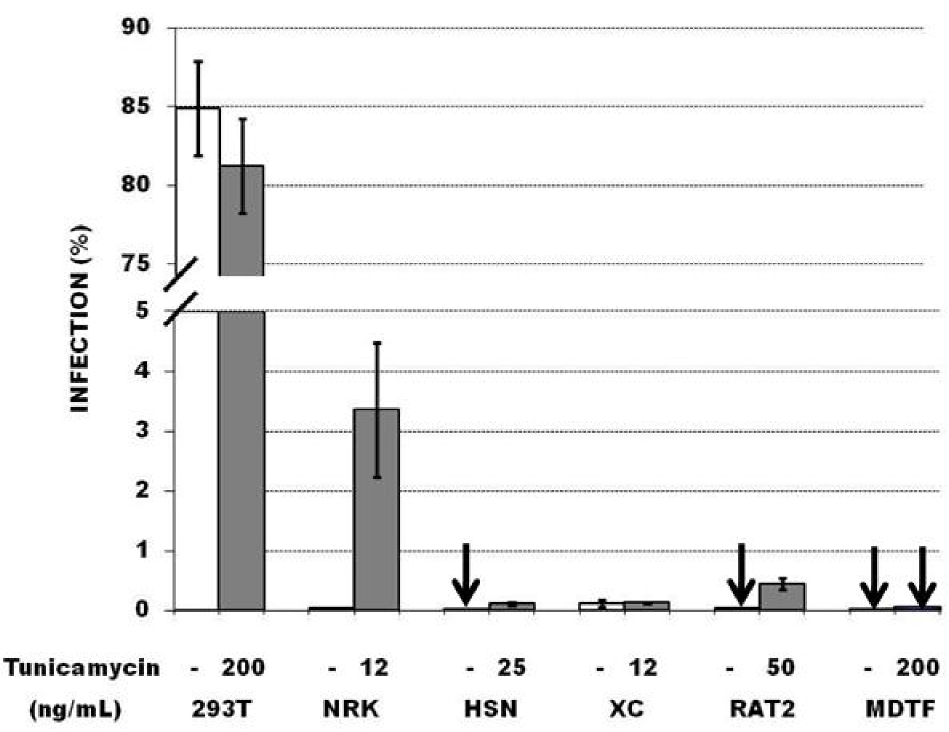
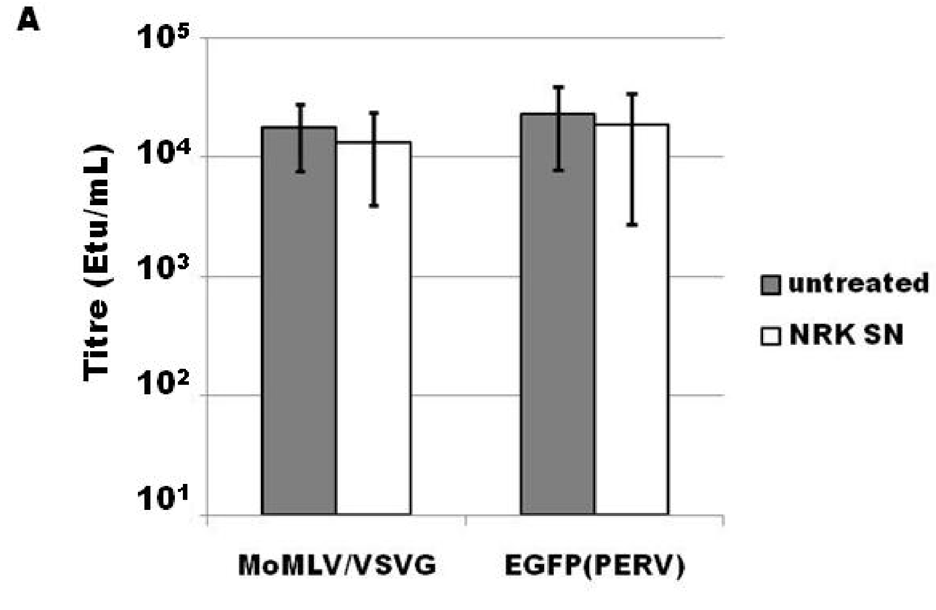


Figure 3.11 Tunicamycin treatment of rat cells

5x10^4 rat NRK, HSN, XC, Rat2, human 293T and murine MDTF cells were treated (grey) or not (white) with tunicamycin at the concentration stated. The day after, cells were infected with 500 µL of 293T supernatant containing EGFP(PERV). PERV-A transduction efficiency was assessed by flow cytometry analysis and expressed as percentage of EGFP cells. Histograms represent the average of two independent experiments (± standard error of the mean). Arrows indicate that the infection was under that the detection limit of 0.1%.

3.2.9 Analysis of inhibitors secreted from rat cells

Another receptor-mediated block to retroviral infection described in literature was the secretion of inhibitors from resistant cells (Miller and Miller, 1992, 1993). To investigate whether a similar mechanism was acting to prevent PERV-A infection in rat cells, huPAR-2 and ratPAR-transduced QT6 cells were grown overnight and then infected with VSV-G pseudotyped MLV carrying EGFP gene or EGFP(PERV) in the presence of conditioned media from rat NRK cells. No clear difference was observed in the EGFP transduction efficiency, indicating that no inhibitors to PERV-A infection were secreted from rat cells (Figure 3.12).



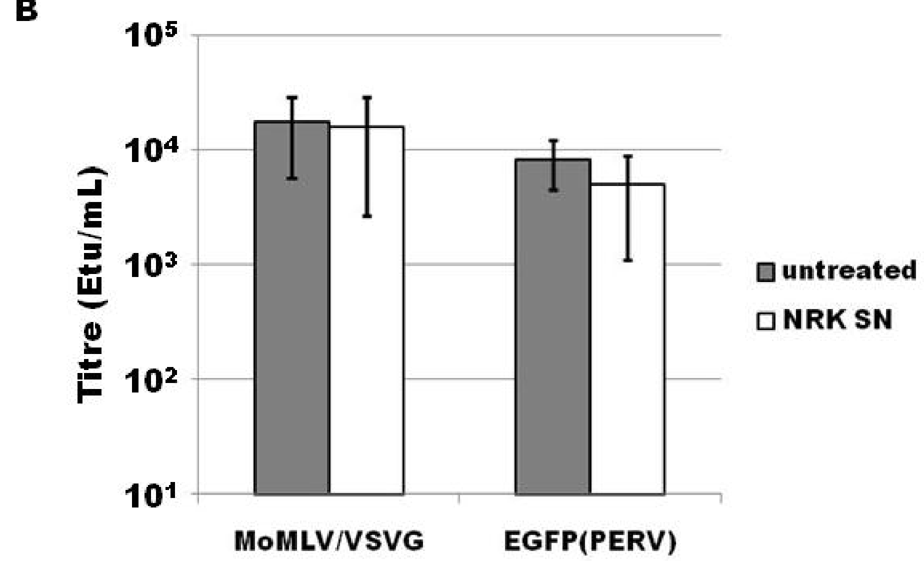


Figure 3.12 PERV-A infection in conditioned media of rat cells

$5x10^4$ huPAR-2 **(A)** and ratPAR **(B)**-transduced QT6 cells were incubated overnight with media alone (grey) or conditioned media from confluent rat NRK cells (diluted 1:1) (white). The day after cells were infected with VSV-G pseudotyped MLV particles carrying a retroviral vector encoding EGFP at MOI on 293T cells of 0.2 or EGFP(PERV) at MOI on 293T cells of 2. Titres were determined by EGFP monitoring by flow cytometry. Histograms represent the average of two independent experiments (± standard error of the mean).

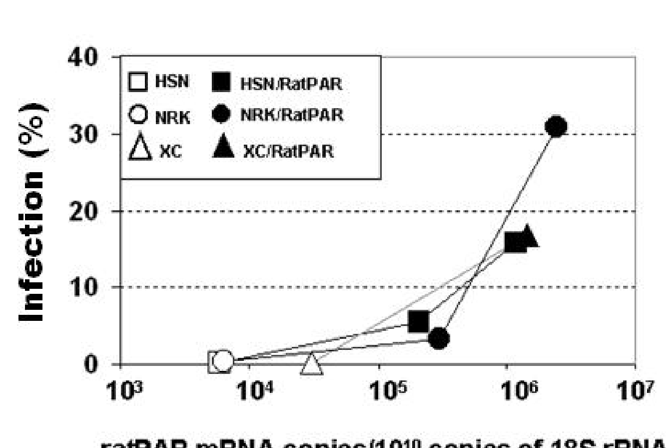
3.2.10 PERV-A infection dependence on ratPAR expression

To understand whether endogenous ratPAR expression in rat cells was too low to support PERV-A infection, a correlation between the amount of exogenous ratPAR and pseudotyped EGFP(PERV) infection was investigated. Rat NRK, HSN and XC cells were transduced with a retroviral vector carrying the ratPAR gene and infected with EGFP(PERV). RatPAR mRNA copy number in RatPAR-transduced cells was measured by probe-based quantitative RT-PCR and plotted against PERV infection efficiency, expressed as EGFP positive cells. All three rat cell lines became susceptible to PERV-A infection when the level of ratPAR mRNA was increased 40 to 500-fold by the exogenously expressing ratPAR (Figure 3.13 A).

To provide evidence for the dependence of PERV-A infection on ratPAR expression level, QT6 cells were transduced with a retroviral vector carrying HA-tagged ratPAR gene. Clonal populations were isolated and the level of ratPAR expression on the cell surface of these cells determined as mean fluorescence intensity of HA staining. RatPAR-transduced QT6 cells expressing different amounts of receptors were infected with EGFP(PERV). Pseudotyped PERV-A infection efficiency was dependent on the ratPAR expression level (Figure 3.13 B).

These results showed that upon overexpression of ratPAR, rat cells become sensitive to PERV-A infection and the efficiency of PERV-A entry correlates with the amount of receptor expressed. This suggested that in rat cells ratPAR is expressed at a subthreshold level and cannot mediate PERV-A infection.

A



B

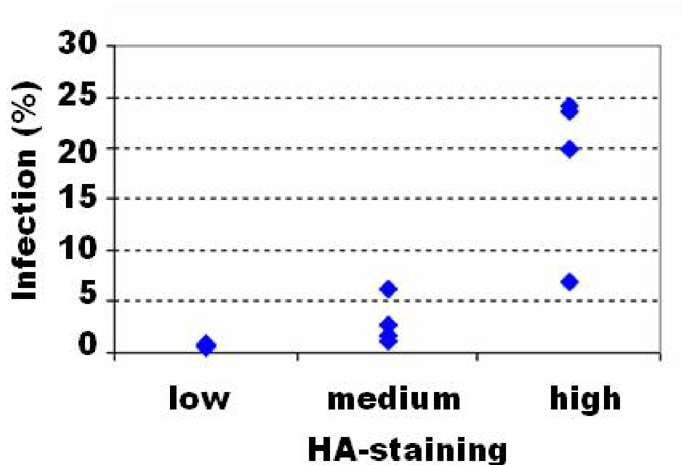


Figure 3.13 PERV-A infection dependence on ratPAR expression

A) Rat NRK (circle) HSN (square) and XC (triangle) cells were transduced with a MLV-based retroviral vector carrying ratPAR gene. Two independent transductions were performed for NRK and HSN cells. Total RNA from ratPAR-transduced (black) or parental (white) cells was extracted and 1 µg were reverse transcribed. One eighth of the final volume was employed in a probe-based quantitative RT-PCR, using primers QF3 and QR3 and probe P3 (table 2.3.4). The amount of ratPAR mRNA was inferred by comparison with standard curve and normalised to 10^{10} copies of 18S rRNA. Samples were run in duplicate. Dots represent the average of two independent experiments. $5x10^4$ ratPAR-transduced and parental cells were seeded and the day after infected with EGFP(PERV) at MOI on 293T cell of 2. After 72

hours, PERV-A infection efficiency was determined by flow cytometry as percentage of EGFP positive cells and plotted against the amount of ratPAR mRNA. Dots represent the average of three independent experiments. **B)** Quail QT6 cells were transduced with a retroviral vector carrying HA-tagged ratPAR gene and single clone populations were isolated. For each individual QT6 clones, the amount of ratPAR on cell surface was determined by immunostaining using anti-HA antibody. The clones were divided according to mean fluorescence intensity (MFI), i.e. ratPAR expression: low (MFI:10-60), medium (60-200) and high (>200). Dots represent the average of the MFI from two independent experiments. Each clone was plotted according to PERV-A infection efficiency, determined by infection of $5x10^4$ cells with EGFP(PERV) at MOI on 293T cells of 2 and expressed as percentage of EGFP positive cells. Data represent the average of two independent infections.

3.3 Discussion

PERV-A can enter human cells through two paralogous proteins, huPAR-1 and huPAR-2 (Ericsson et al., 2003). Rodent cells are resistant to PERV-A infection (Takeuchi et al., 1998). In this chapter, the mechanism of non-permissivity to PERV-A infection in murine and rat cells has been investigated to better understand the molecular mechanism of PERV-A entry.

The murine homologue to huPAR, muPAR, has been identified (Ericsson et al., 2003). Murine MDTF and quail QT6 cells are resistant to PERV-A entry. Upon expression of huPAR-1 and huPAR-2, but not muPAR, these cells become susceptible to virus infection (Figure 3.3), suggesting that PERV-A block occurs at entry level and that muPAR is not a functional receptor for PERV-A.

In other γ-retrovirus receptors, it has been shown that few amino acid changes between homologous receptors in different species determine the ability to mediate virus entry (Albritton et al., 1993; Eiden et al., 1996; Johann et al., 1992; Lundorf et al., 1998; Marin et al., 2003a; Tailor et al., 1993; Yoshimoto et al., 1993). Chimeric receptor of huPAR-2 and muPAR have been generated and tested for their receptor function (Figure 3.4). One single amino acid substitution in huPAR-2 from leucine at position 109 to the murine corresponding proline was able to abrogate PERV-A infection. Conversely, muPAR bearing leucine109 instead of a proline, could mediate PERV-A entry in resistant quail QT6 cells. No other part of the receptor had the same dramatic effect on the function. These data were confirmed by a recently published report (Marcucci et al., 2009).

Because proline 109 in muPAR could abrogate PERV-A infection, its localisation in the structure of the receptor was investigated. HuPAR-1, huPAR-2 and muPAR have the same topology model according to a transmembrane protein prediction software (TMHMM) characterised by 11 transmembrane domains, 5 ECLs, an intracellular N-terminus and extracellular C-terminus (figure 3.6 C). The analysis of the orientation of the extremities (Figure 3.5) and the unique N-glycosylation site (Figure 3.6 B)

provided evidences in support of the predicted topology. From this model amino acid 109 is localised in the ECL2. I hypothesised that this region could be PERV-A binding site and that the change in the structure of the loop due to the pro-to-leu substitution in muPAR could prevent virus binding and hence infection. Supporting data for this hypothesis was obtained by soluble PERV-A SU binding to QT6 cells that were expressing different PAR. Successful binding was observed for functional receptor huPAR-1 and huPAR-2 but not for muPAR. However, in QT6 cells expressing the mutant huPAR-2 with the substitution leu-to-pro at amino acid 109, the binding was abrogated (Figure 3.7 B). However, the exchange of proline 109 with a leucine in muPAR did not rescue the binding to soluble PERV-A SU, even if it did support infection (Figure 3.7 B). A possible explanation is that the trimeric form of Env present on the viral particle surface binds better than the monomeric soluble form used in the assay. Furthermore, other regions in the muPAR could be involved to achieve a binding efficiency which equals to huPAR.

Rat cells as well as mouse cells are resistant to PERV-A infection (Figure 3.3, Figure 3.8 B). However the mechanism appeared different. Firstly, the amino acid sequence of the ECL2 in ratPAR is identical to that of huPAR-1 and huPAR-2, and does not share the proline 109 (Figure 3.8 A). Furthermore, upon overexpression of their own ratPAR, rat cells become susceptible to PERV-A infection, indicating that ratPAR is able to mediate PERV-A entry (Figure 3.8 B). In addition, when QT6 cells express similar amount of huPAR-1 and ratPAR, the efficiency in soluble PERV-A SU Env binding is similar (Figure 3.8 C). As these results indicated that rat cells encode a functional PERV-A receptor, other mechanisms of resistance were investigated.

For other γ-retroviruses it has been shown that overexpression of the receptor rescued viral infection in the resistant cell lines of their origin (Tailor et al., 2000). The mechanism for this resistance was explained as masking, interference or a subthreshold level of expression of the receptor (Eiden et al., 1994; Lavillette et al., 2002a; Marin et al., 2000; Miller and Miller, 1992,

1993; Tailor et al., 2000). These mechanisms were evaluated for PERV-A infection in rat cells. Firstly, the presence of a post-entry restriction factor acting on PERV-A GagPol was examined. In fact, although data presented in this chapter indicated that rat cells are insensitive to PERV-A infection by a receptor-mediated block, overexpression of a functional receptor in rat cells could also cause the entry of a greater amount of virus and saturate a restriction factor acting post-entry. As the best described restriction factor blocking retroviral infection prior integration are Fv1 and TRIM5α and they both target the viral capsid (section 1.6.1), VSV-G pseudotyped retroviral particles containing PERV-A14/220 GagPol were compared in their efficiency of EGFP transduction in rat cells with similar pseudotyped virus bearing the non restricted MoMLV GagPol. The titre obtained with PERV-A 14/220 GagPol was 4 times lower than that obtained with MoMLV GagPol (Figure 3.10). This reduction is lower than that obtained for sensitive N-tropic MLV and HIV-1 with Fv1 and TRIM5α (Stremlau et al., 2004; Towers et al., 2000) and it is not enough to fully block PERV-A infection in rat cells. Therefore, although the presence of a weak restriction factor in rat cells, acting on PERV-A14/220 GagPol, cannot be excluded, it did not explain the mechanism of resistance to PERV-A infection in rat cells.

Likewise, removal of N-linked polysaccharide chains by tunicamycin treatment could partially rescue PERV-A infection in some rat cell lines. However, because the effect was not observed in all the rat cell lines tested and the degree of infection was more than 10-fold lower than in 293T cells (Figure 3.11), the receptor masking by N-linked glycosylation could not be considered the main mechanism of resistance to PERV-A infection. Presence of inhibitory factors in the media of cells was not detected by EGFP(PERV) infection of different cell lines in the presence of conditioned media from rat NRK cell (Figure 3.12).

Endogenous ratPAR mRNA expression in rat cells was measured by quantitative RT-PCR and compared to the level of huPAR-1 mRNA in PERV-A susceptible human cells. On average rat cells express 4 times less receptor than human cells (Figure 3.9). To test whether this difference could

be responsible for the resistance of rat cells to PERV-A infection, the amount of exogenously expressed ratPAR mRNA was correlated to the efficiency of EGFP transduction of the pseudotyped PERV-A14/220. Rat cells became PERV-A sensitive when the amount of ratPAR mRNA was increased of 40 to 500-fold (Figure 3.13 A). Moreover, it was possible to correlate a higher efficiency of PERV-A infection with a higher level of ratPAR expression in QT6 cell clones expressing different amount of HA-tagged receptor (Figure 3.13 B). These results suggested that ratPAR expression in rat cells is under the threshold level which allowed PERV-A infection. However, it is not clear whether this subthreshold level is due to a low transcription (i.e. mRNA expression) or other mechanisms which prevent the display of ratPAR on the cell surface.

In conclusion, two closely related species, rat and mice, have different mechanisms to escape PERV-A infection. Murine cells encode a homologue of huPAR which is defective in PERV-A receptor function. Since one single amino acid exchange in muPAR can rescue PERV-A infection, this indicates a critical region in the receptor, likely involved in the virus binding. In contrast, rat cells express a functional receptor. My results suggest that the level of ratPAR expression is below a threshold level required to support PERV-A entry, but the mechanism for this is not clear. These results could help identifying possible targets for the development of therapeutics that block PERV-A infection, such as neutralising antibodies and peptides mimicking the receptor.

Chapter 4

4 Evaluation of PERV-A receptors in non-human primates

4.1 Introduction

Although PERV infection has not been detected in retrospective analysis of patients treated with porcine cells and tissues (Clemenceau et al., 2001; Cunningham et al., 2001; Elliott et al., 2000; Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998), this risk in xenotransplantation in future cannot be excluded. Retroviruses are responsible for several human and animal pathologies (section 1.3.4) and therefore the possible consequences following PERV infection must be investigated. Small animals as well as non-human-primates (NHP) have been employed as models.

The most common small animal model chosen has been either severe combined immunodeficiency (SCID) or nude mice. In SCID mice, due to a mutation in a DNA-dependent protein kinase, T and B cells are unable to differentiate and therefore both humoral and cellular immune response are not functional. Nude mice have no T cell response. Immunocompromised mice were considered a good model because they mimic the immunosuppression in xenotransplantation patients. Infection by PERV of mouse tissues has been quite controversial. Initial reports showed that PERV produced from pig pancreatic cells transplanted in SCID mice could infect mouse tissues but the virus appeared not to be transcriptionally active in murine cells, suggesting a non-productive infection (Deng et al., 2000; van der Laan et al., 2000). Although microchimaerism was observed, the ratio between PERV-specific sequences and porcine cell sequences in mouse tissues was higher than in pig cells, inferring amplification of PERV genomes by PERV infection of mouse cells. Similar results were obtained in nude mice (Clemenceau et al., 2002; Zhang et al., 2005). However, when microchimaerism was avoided by injection of cell-free viruses, PERV infection of murine cells was not detected *in vitro* or in SCID mice (Irgang et

al., 2005). Following the cloning of human PERV-A receptors, the homologuous muPAR was found not functional in supporting PERV-A infection (Ericsson et al., 2003). The murine model was therefore considered not permissive to PERV-A infection, which is more problematic than PERV-B because PERV-A is present in the pig genome at higher level than PERV-B (Le Tissier et al., 1997) and can recombine with PERV-C to produce high-titre human tropic recombinant PERV (Bartosch et al., 2004; Harrison et al., 2004; Oldmixon et al., 2002; Wilson et al., 2000). However, immunodeficiency mice were still used to test PERV infection of human cells *in vivo*. SCID mice were injected with human peripheral blood leukocytes and porcine cells were transplanted. Human cells were infected by PERV *in vivo* (Kuddus et al., 2004; McKane et al., 2003). The interpretation of these results was complicated by the discovery that PERV particles could be pseudotyped *in vivo* with endogenous xenotropic MLV which can mediate infection (Martina et al., 2005; Yang et al., 2004). A different model which allowed investigation of PERV infection in a fully competent animal was represented by transgenic mice expressing huPAR-2. In this system not only could PERV infection be observed in mouse cells but the immune response could be also evaluated. After infection with cell-free virus (to avoid microchimaerism) PERV DNA, RNA and proteins were detected in several murine tissues and some mice developed anti-PERV antibodies (Martina et al., 2006).

NHP represent an ideal animal model to evaluate the immunological response to pig xenografts because similar to humans, NHPs possess circulating anti-αGal antibody (section 1.1.1). In several studies conducted using transgenic pigs, the animal of choice was mainly baboon (*Papio hamadras*) or cynomolgus monkey (*Macaca fascicularis*) [reviewed in:(Ekser et al., 2009)]. Surveillance for PERV infection of these animals could assess the risk of PERV transmission to xenotransplant recipients. Pig-to-NHP transplantation could offer the opportunity to study long term exposure to the xenograft and to analyse more tissues, two advantages in comparison to the retrospective studies in humans exposed to porcine materials. Studies conducted on NHP injected with cell-free high titre viruses, cells or solid organ transplantation in immunosuppressed animals showed no evidence of

PERV infection [(Elliott et al., 2005; Isaac et al., 2005; Moscoso et al., 2005; Nishitai et al., 2005; Specke et al., 2009) and reviewed in (Denner, 2003)]. The use of NHP to assess the risk of PERV transmission has been debated. Initial studies showed that NHP cell lines were non-permissive for PERV infection (Martin et al., 1999; Patience et al., 1997; Takeuchi et al., 1998; Wilson et al., 2000). Other reports, which used PCR or RT-PCR to detected PERV sequences, suggested that NHP cells are susceptible (Blusch et al., 2000a; Specke et al., 2001; Templin et al., 2000). Using a high titre PERV derived from NIH minipigs and adapted *in vitro* through three passages in human cells it was possible to show that PERV carrying a reporter gene (β-galactosidase) could infect rhesus macaque and African green monkey (AGM) cell lines. In the infected NHP cells, PERV provirus and transcripts were detected but no reverse transcriptase activity was found in the supernatant of these cells, suggesting that PERV infection of NHP cells was not productive (Ritzhaupt et al., 2002). However the mechanism responsible for the poor infectivity and the lack of PERV replication in NHP cells was not explained.

The cloning of PERV-A receptors (Ericsson et al., 2003) and the isolation of the high-titre recombinant PERV-A14/220 (Oldmixon et al., 2002) allowed the re-evaluation of PERV infection in NHP cells. In this chapter, the aim was to clarify the reasons for the low susceptibility of NHP cells to PERV infection and provide information which can be used in the choice of the NHP species to use as animal model to study PERV transmission.

4.2 Results

4.2.1 The low PERV permissivity is mainly caused by reduced entry in NHP cells.

Infection and replication of PERV-A were monitored in different NHP cell lines as shown in figure 4.1.

PERV entry was analysed by infection of $5x10^4$ AGM COS7 and VERO cells and rhesus macaque FRhK4 cells with serial dilution of the EGFP(PERV) supernatant (section 2.3.3). PERV-A titre on COS7 and FRhK4 cells was more than two orders of magnitude lower than that on human 293T cells ($2x10^5$ Etu/mL). The level of infection of AGM VERO cells was under the detection limit (10 Etu/mL) (Figure 4.2 A). To test whether weak PERV-A receptor activity could be responsible for the low infectivity, COS-7, VERO and FRhK4 cells were transduced with a retroviral vector carrying huPAR-2 gene. EGFP(PERV) titre on huPAR-transduced NHP cells increased at least 37 times in comparison with the parental cells (Figure 4.2 A). Although the level of huPAR-2 expression in NHP cells was not assessed, expression of a functional receptor increased the permissivity of NHP cells to PERV infection, suggesting a possible role for NHP PAR in the low level of infectivity.

After EGFP(PERV) infection, wild type and huPAR-transduced cells were kept in culture for the following experiments.

A week post-infection, the level of PERV *gag* gene in the NHP cell genome was investigated. 200 ng of genomic DNA was processed in a SYBR Green-based quantitative PCR reaction using PERV *gag* specific primers (table 2.3.4). PERV Gag copy number in the different NHP cells correlated with the titre determined by EGFP expression. Notably, PERV *gag* was detected in wild type VERO cells, although EGFP expression was under the sensitivity limit of the infection assay (Figure 4.2 B).

1 µg of total RNA, extracted from wild type and huPAR-transduced cells two weeks post-infection, was reverse transcribed and one eighth was processed in SYBR Green-based quantitative RT-PCR. Similar to the results obtained

from the genomic DNA analysis, the level of transcription of PERV Gag correlated with the EGFP(PERV) titre (Figure 4.2 C).

To determine whether PERV can productively infect NHP cells, the presence of infectious viral particles in the supernatant of infected cells was investigated. $1x10^6$ HuPAR-transduced and parental cells were seeded and the day after their supernatant was collected and serial dilutions were used to infect 293T cells. PERV titre was determined 3 days later by colony counting of the infected cells, immunostained with an anti-PERV CA antibody.

No infectious viruses were present in the supernatant of wild-type FRhK4 and VERO cells for up to three weeks post infection. For VERO cells, 5 i.u./mL detected at weeks 2 and 3 were at the borderline of this assay sensitivity, hence not a clear indication of a productive infection. Instead, viral titre in the supernatant of wild type COS7 and huPAR-transduced cells grew in the first two weeks and stabilised at week three (Figure 4.2 D). Interestingly, the difference in the titre produced from COS7 wild-type versus huPAR-transduced cells was similar to the difference in the (EGFP)PERV infection of this cell line. Taken together, these results suggested that once PERV successfully entered the NHP cells, provirus could integrate in the host genome, viral genes be transcribed and infectious particles produced. Therefore, an inefficient cell entry was likely to be responsible for the low susceptibility of NHP cells to PERV-A.

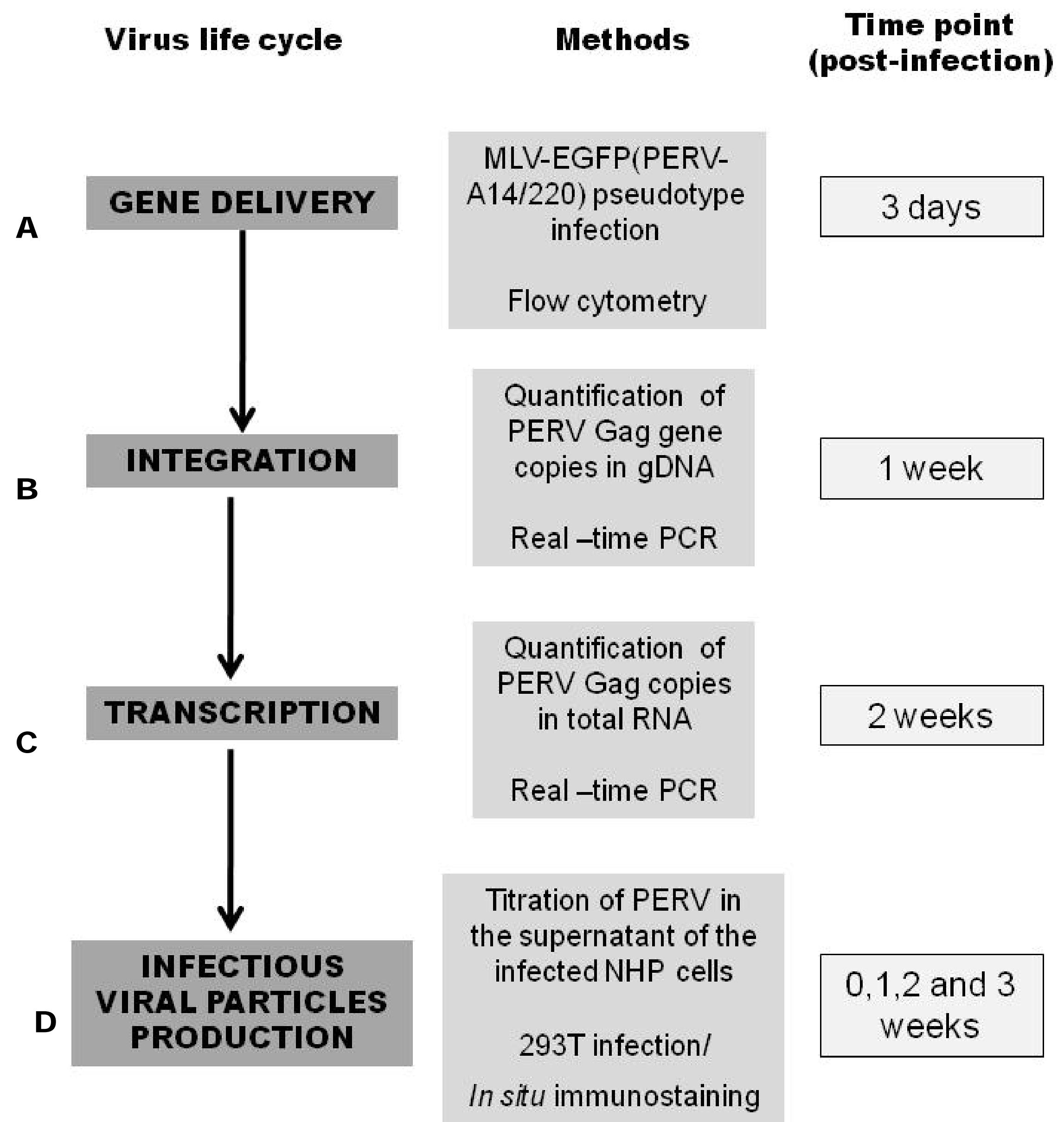


Figure 4.1 Diagram of the analysis of PERV infection in NHP cell
Four critical steps (A-D, results shown in corresponding panels in Figure 4.2) in the virus life cycle (left column) were analysed using the methods described in the middle column performed at specific post-infection time points (right column).

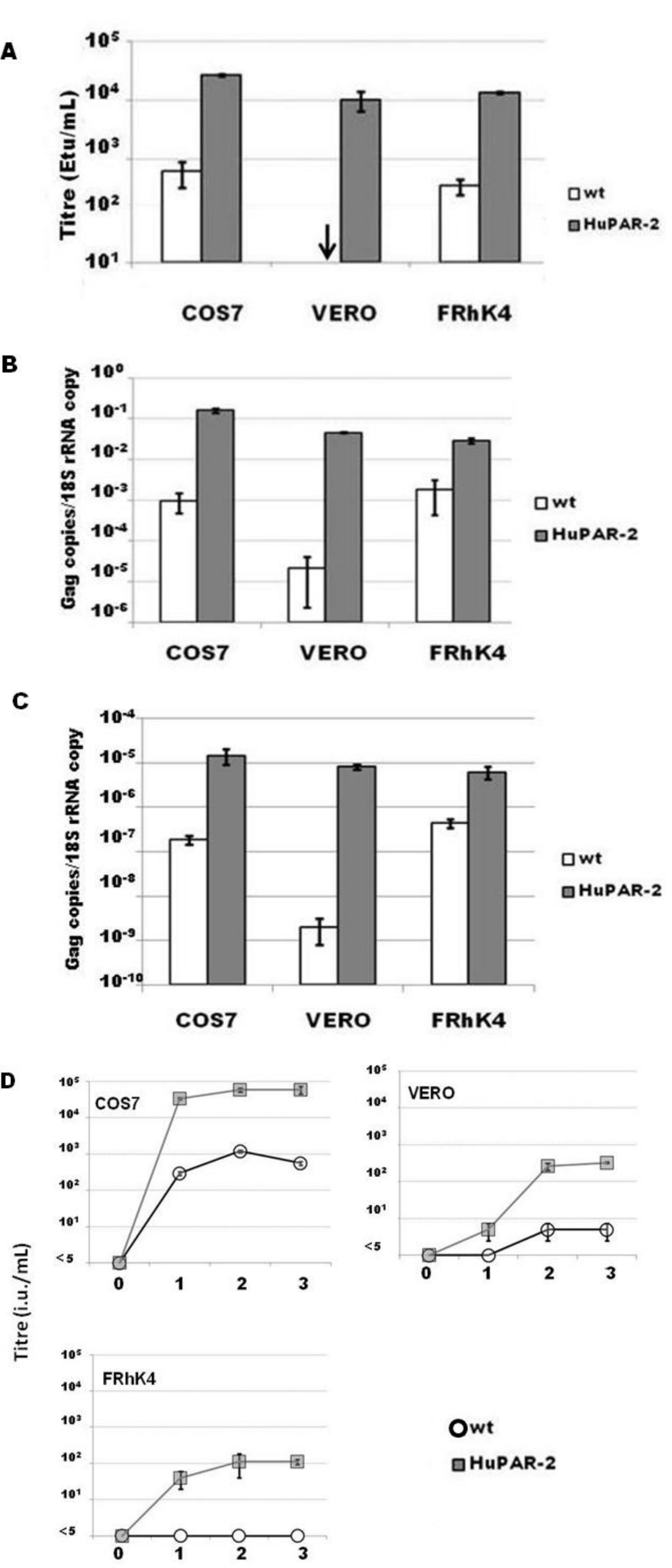

A
B
C
D
Titre (Etu/mL)
COS7
VERO
FRhK4
wt
HuPAR-2
Gag copies/18S rRNA copy
Titre (i.u./mL)
Time (weeks)

Figure 4.2 Monitoring of PERV infection in NHP cells

Rhesus macaque FRhK4 cells and AGM COS7 and VERO cells were transduced with a VSV-G pseudotyped MLV-based vector carrying human PAR-2 gene (huPAR-2). $5x10^4$ huPAR-2 transduced (grey bars) or wild type (white bars) cells were infected with serial dilution of PERV(EGFP) with the highest MOI (on 293T cells) being 2. **A)** 72 hours post infection, efficiency of EGFP delivery was determined by flow cytometry. Titres were calculated as previously described (section 2.3.4). and expressed as EGFP transducing units (Etu)/mL. EGFP(PERV) infection on VERO cells was under the detection limit (<10Etu/mL, arrow). Histograms represent the average of three independent experiments (± standard error of the mean). **B)** One week post-infection, genomic DNA was extracted and 200 ng was employed per reaction in a SYBR Green-based quantitative PCR using PERV *gag* specific primers QF5,QR5 (Table 2.3.4). **C)** Two weeks post-infection, total RNA was extracted and 1 µg was reverse transcribed. One eighth was employed in a SYBR Green-based quantitative RT-PCR using PERV *gag* specific primers. The amount of gag copies was inferred by a standard curve and normalised per 18S rRNA copy. Each sample was run in duplicate. Histograms represent the average of two independent experiments (± standard error of the mean). **D)** At the different time points indicated, $1x10^6$ huPAR2-transduced (grey square) or wild type (white circles) FRhK4, COS7 and VERO cells were seeded. The day after, supernatant was collected and 5 fold dilutions used to infect 293T cells. 72 hours later, cells were immunostained with an anti-PERV CA antibody. Alkaline phosphatase positive colonies were counted at the visible light microscope and titres determined as infectious unit per mL (i.u./mL). Data represent the average of two independent experiments (± standard error of the mean).

4.2.2 Functional analysis of the NHP PERV-A receptors

The NCBI database was searched for homologous sequences to huPAR-1 and huPAR-2 in NHP. Chimpanzee and rhesus macaque PAR-1 and PAR-2 were identified. Baboon PAR-2 (baPAR-2) has been cloned previously (Ericsson et al., 2003). NHP PARs have been cloned by RT-PCR of the RNA extracted from primary cells or cell lines as summarised in table 4.1. Both baPAR-2 and rhesus macaque PAR-1 (rhPAR-1) sequences, cloned from primary baboon PBMC and FRhK4 cells respectively, had one amino acid different (L19F or C309Y) from the sequences deposited in GenBank. However, rhPAR-2 cloned from FRhK4 cells had the same amino acid sequence as XP_001099620.1.

Amino acid sequences were aligned using ClustalW programme. Extracellular domain 2 (ECL2) was located according to the huPAR-2 topology (section 3.2.3). The sequence of ECL2 was well conserved among different species with the exception of a serine instead of a leucine at amino acid 109 in rhPAR-1, cynomologus monkey PAR-1 (cynPAR-1) and baboon PAR-1 (baPAR-1) (Figure 4.3 A). This mutation was of particular interest because it was located at a.a. 109, which has been previously described as critical for PERV-A infection and binding (section 3.2.2 and 3.2.4).

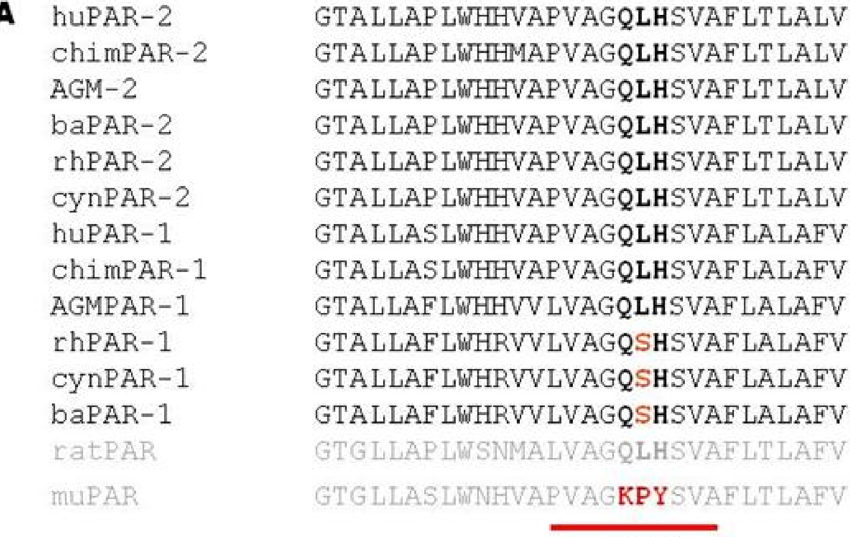
To test the ability of the NHP receptors to support PERV-A entry, NHP PARs were HA-tagged at the C-terminus and subcloned into a MLV-based retroviral vector. PERV-A resistant QT6 cells were transduced using VSV-G pseudotyped MLV particles carrying PAR genes. More than 50% of the cells expressed the receptors as assessed by flow cytometry analysis after immunostaining with an anti-HA antibody (Figure 4.3 B diamond). PAR-expressing cells were infected with EGFP(PERV) and the titre determined by monitoring EGFP signal. The titres have been represented as percentage of the titre obtained on huPAR-1-QT6 cells (Figure 4.3 B bars). All the PARs tested conferred permissivity to PERV-A entry in QT6 cells, with the exceptions of cynPAR-1, baPAR-1, rhPAR-1. These receptors share the serine at a.a. 109, which is different from the other receptors (Figure 4.3 A). To test whether the L109S change was responsible for the inability to support

PERV-A entry, a huPAR mutant carrying a serine instead of leucine in ECL2 (huPAR-1S109) and a rhPAR-1 with the opposite mutation (rhPAR-1L109) were generated. Once expressed in QT6 cells rhPAR-1L109 could efficiently mediated PERV-A entry. Instead, the ability of huPAR-1S109 to function as receptor was reduced up to 85% (Figure 4.3 B). The amino acid substitution in position 109 appeared to have a negative effect on the receptor function.

Receptor	species	NCBI acc no.	cell origin	primers
huPAR-1	*Homo sapiens*	NP_078807.1	Ericsson et al 2003	
chimPAR-1	*Pan troglodytes*	XP_0010990939.1	not cloned	
AGMPAR-1	*Cercopithecus aethiops*	n/a	COS7;VERO	CF6;CR5
baPAR-1	*Papio anubis*	n/a	PMBC	CF6;CR5
rhPAR-1	*Macaca mulatta*	XP_001090939.1	FRhK4	CF5;CR5
cynPAR-1	*Macaca fascicularis*	n/a	primary splenocytes	CF6;CR5
huPAR-2	*Homo sapiens*	NP_060456.3	Ericsson et al 2003	
chimPAR-2	*Pan troglodytes*	XP_523560.1	not cloned	
AGMPAR-2	*Cercopithecus aethiops*	n/a	COS7;VERO	GF2;GR5
baPAR-2	*Papio hamadras*	Q863Y8.1	Ericsson et al 2003	
	Papio anubis		PBMC	CF4;CR4
rhPAR-2	*Macaca mulatta*	XP_001099620.1	FRhK4	CF4;CR4
cynPAR-2	*Macaca fascicularis*	n/a	primary splenocytes	CF4;CR4

Table 4.1 List of NHP PERV-A receptors

PERV-A receptors used in this chapter are listed. NCBI accession number is reported for the deposited protein sequences. For the receptors cloned in this thesis, the cell lines or primary cells from which total RNA have been extracted and the primers used for PAR amplification are indicated.



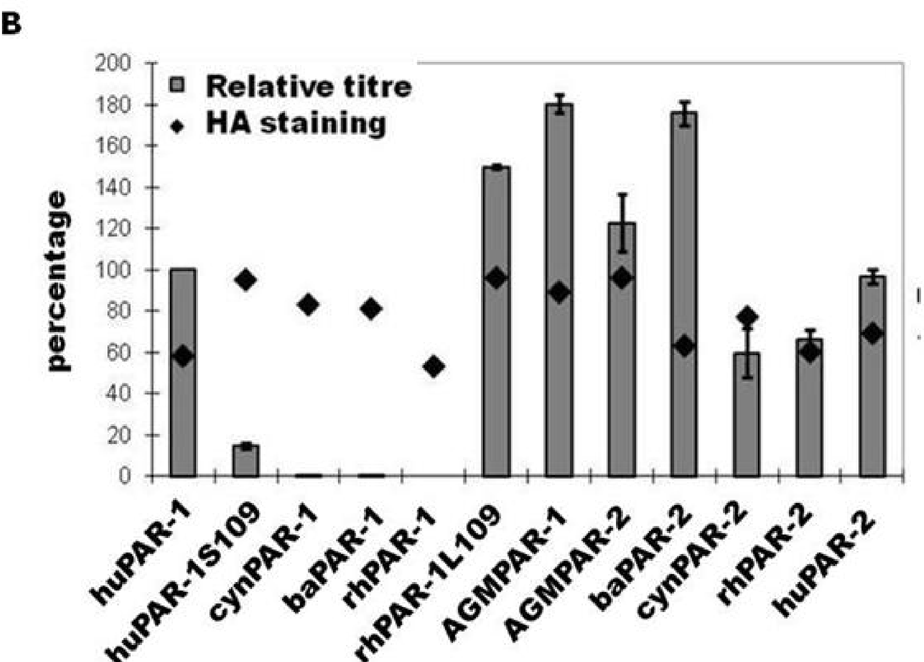


Figure 4.3 Functional analysis of NHP PARs

A) Human (huPAR-1 and huPAR-2), mouse (muPAR), rat (ratPAR), chimpanzee (chimPAR-1 and chimPAR-2), rhesus macaque (rhPAR-1 and rhPAR-2) PAR and baboon PAR-2 (baPAR-2) sequences were present in the NCBI database (Table 4.1). African green monkey (AGMPAR-1 and AGMPAR-2), cynomolgus macaque (cynPAR-1 and cynPAR-2) PAR and baboon PAR-1 (baPAR-1) sequences were obtained by RT-PCR of RNA extracted from NHP cell lines and primary cells using specific primers (Table 4.1). Amino acid sequences were aligned using ClustalW software. Extracellular loop 2 (ECL2) in huPAR-2 as defined in section 3.2.3 is indicated (red line) and the three amino acids different between muPAR and

huPARs are highlighted (red). All the primates and rat PAR sequence share the same three amino acids as huPARs (bold). RhPAR-1, cynPAR-1 and baPAR-1 had one amino acid different (red). **B)** C-terminal HA-tagged PAR sequences were cloned into a MLV-based vector and introduced into quail QT6 cells by transduction of VSV-G pseudotype retroviral particles. Percentage of HA-positive cells was calculated by cell surface staining of the transduced cells (black diamond). $5x10^4$ PAR expressing QT6 cells were seeded and the day after infected with serial dilution of (EGFP)PERV-containing supernatant. The highest MOI as determined on 293T cells was equal to 2. After 72 hours, EGFP expression was monitored and titres inferred. EGFP(PERV) titre on huPAR-1 expressing QT6 cells was $1.7±0.5x10^4$ Etu/mL and arbitrarily chosen as 100% infection. Histograms represent the average of three independent experiments (± standard error of the mean).

4.2.3 Serine 109 abrogates PERV-A binding

The mutation of amino acid 109 from leucine to proline in muPAR (section 3.2.2) or serine in rhPAR-1, cynPAR-1 and baPAR-1 reduced the ability of PERV-A to use these proteins as receptors. MuPAR and huPAR-1P109 binding to soluble PERV-A Env was undetectable. The effect of the serine in ECL2 on the binding was investigated. QT6 cells expressing huPAR-1 or huPAR-1S109 were incubated with soluble PERV-A360 Env (section 2.3.8). The cells were stained using a FITC-conjugated anti-rabbit IgG antibody and processed in flow cytometry (Figure 4.4 A). Incubation with PERV-A360 produced a shift toward higher fluorescence intensity only in cells expressing huPAR-1 but not for those carrying huPAR-1S109. Conversely, rhPAR-1 was unable to bind soluble PERV-A360 but a positive signal was detected for rhPAR-1L109 (Figure 4.4 B). These results suggested that S109 in rhPAR-1 not only negatively affects the receptor function but also the binding to PERV-A Env.

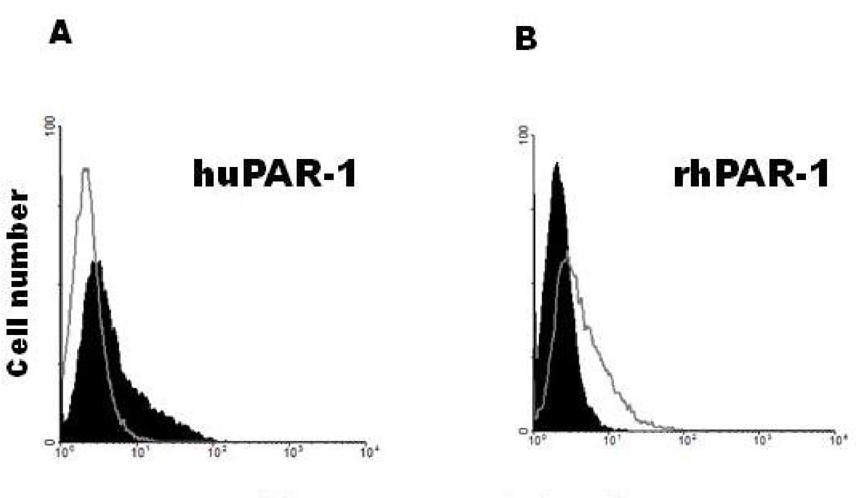


Figure 4.4 PERV-A Env binding of rhPAR-1

$5x10^5$ QT6 cells, stably expressing HA-tagged PARs, were incubated with 100 ng of PERV-A360 Env protein and a secondary FITC-conjugated anti-rabbit IgG antibody. Cells were analysed by flow cytometry. Cells carried the following receptors huPAR-1 (**A**, black filled). huPAR-1S109 (**A**, grey line), rhPAR-1 (**B**, black filled) and rhPAR-1L109 (**B**, grey line). Graphs show a representative result of two independent experiments.

4.2.4 Expression of endogenous PERV-A receptors

AGM cells encode two functional receptors while rhesus macaque cells carry only one, rhPAR-2. To understand the poor permissivity of NHP cells to PERV-A, the expression of PARs in NHP cells were investigated. Although the amount of the receptors displayed on the cell surface would be more informative, due to the lack of an anti-PAR antibody, the mRNA level was measured by quantitative RT-PCR. PERV-A susceptible human cell lines 293T, HT1080, HeLa and primary PBMC were used as a comparison.

PAR-1 mRNA level was found similar in NHP and human cells. Primary cells express about five times less PAR-1 mRNA than the cell lines (Figure 4.5). The amount of PAR-2 mRNA was more variable. All human cells and AGM VERO had a low level of PAR-2, at least 2.5 order of magnitude inferior of PAR-1. Instead, FRhK4 cells, primary baboon PBMC and cynomolgus splenocytes expressed ten times more PAR-2 mRNA than human cells and VERO, with the difference between PAR-1 and PAR-2 mRNA reduced to about 5 fold in primary cells (Figure 4.5).

Rhesus macaque, cynomolgus monkey and baboon express the mRNA for a not functional receptor (PAR-1) at a similar level to human cells. PERV-A infection in these NHP is probably mediated by PAR-2, whose mRNA is expressed at a lower level than PAR-1, hence the poor permissivity to PERV-A. These data showed also that AGM VERO and COS7 cells had two functional receptors and their mRNA was expressed at a similar level to highly permissive human cell lines. Therefore, other causes will be evaluated to understand PERV-A infection in NHP cells.

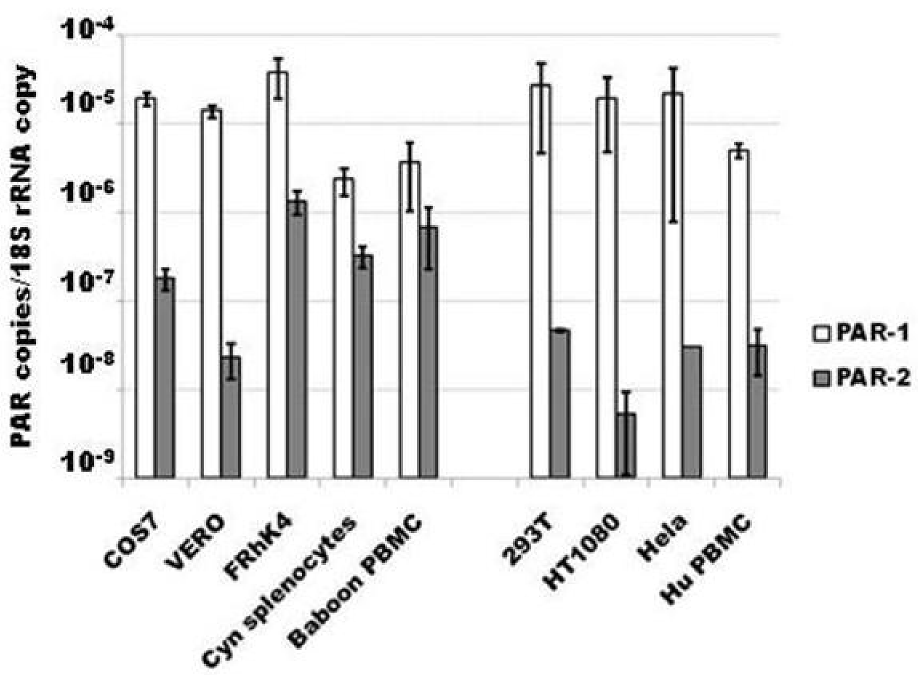


Figure 4.5 Expression of mRNA for endogenous PAR

Total RNA was extracted from NHP and human cell lines and primary cells and 1 µg was reverse transcribed. One eighth of the final volume of the reaction was employed in a SYBR Green-based quantitative RT-PCR. Primers used were QF6,QR6 for NHP PAR-1 (white bars, left) QF1, QR1 for huPAR-1 (white bars, right) QF7,QR7 for NHP PAR-2 (grey bars, left) and QF2,QR2 for human PAR-2 (grey bars, right) (Table 2.3.4). PAR copy number was inferred from standard curves and normalised per 18S rRNA copy. Histograms represent average of at least two independent experiments (± standard error of the mean).

4.2.5 Lack of a post-entry restriction acting on PERV Gag-Pol

Restriction factors acting in a way similar to Fv1 and TRIM5α are the best characterised (section 1.6.1). Their expression in, otherwise permissive, cells have been showed to reduce retroviral titre more than 10-fold (Stremlau et al., 2004; Towers et al., 2000). The viral determinant for restriction was mapped in both cases on the CA protein (Besnier et al., 2003; Kozak and Chakraborti, 1996; Perron et al., 2004; Towers et al., 2002). Their block can be abrogated by virus input saturation (i.e. using a high multiplicity of infection) (Towers et al., 2002).

VSV-G pseudotyped viruses with a MLV-based retroviral vector expressing EGFP were generated by transfection of 293T cells together with packaging plasmid expressing GagPol of either PERV-A14/220 or unrestricted MoMLV (Keckesova et al., 2004; Kozak, 1985; Perron et al., 2004). 48 hours post transfection, the pseudotyped viruses were titrated on human 293T, AGM COS7 and VERO cells. Titre of the MoMLV/VSV-G virus was similar between human and AGM cells. PERV-A14/220 GagPol titre was reduced only 5-fold in AGM cells in comparison to 293T cells (Figure 4.6 A), suggesting that there is no strong restriction factor acting on PERV GagPol.

To further demonstrate that the major determinant of low PERV permissivity in AGM cells is at receptor level and not at a post-entry step, psudotyped viruses with different Env proteins were generated. 293T cells were co-transfected with an MLV-based retroviral vector carrying EGFP gene, a plasmid encoding PERV-A14/220 GagPol and either PERV-A14/220 or MLV-A Env expressing plasmid. Viruses were titrated on 293T, COS7 and VERO cells by monitoring EGFP expression. MLV-A Env was able to rescue the titre of the pseudotyped viruses in AGM cells to a similar level than that seen in human cells, suggesting that the inhibition of PERV infection in AGM cells occurs at cell entry level (Figure 4.6 B).

The presence of a saturable post-entry restriction factor was further investigated. Two different viruses were produced by three plasmid transfection of 293T cells, both have PERV-A14/220 Gag-Pol and PERV-

A14/220 Env. MLV-based retroviral vector encoding EGFP (PERV/EGFP) or pcDNA (PERV) were cotransfected. Human 293T, murine NIH3T3 and AGM COS7 and VERO cells were incubated with different amount of PERV. After 6 hours, cells were infected with a fixed volume of PERV/EGFP supernatant. No difference was registered in the PERV/EGFP titre using different ratio (v/v) of the two viruses (Figure 4.6 C). These data suggested that no restriction factors acting in a Fv1 or Trim5 similar way can be held responsible for the poor susceptibility of NHP cells to PERV-A.

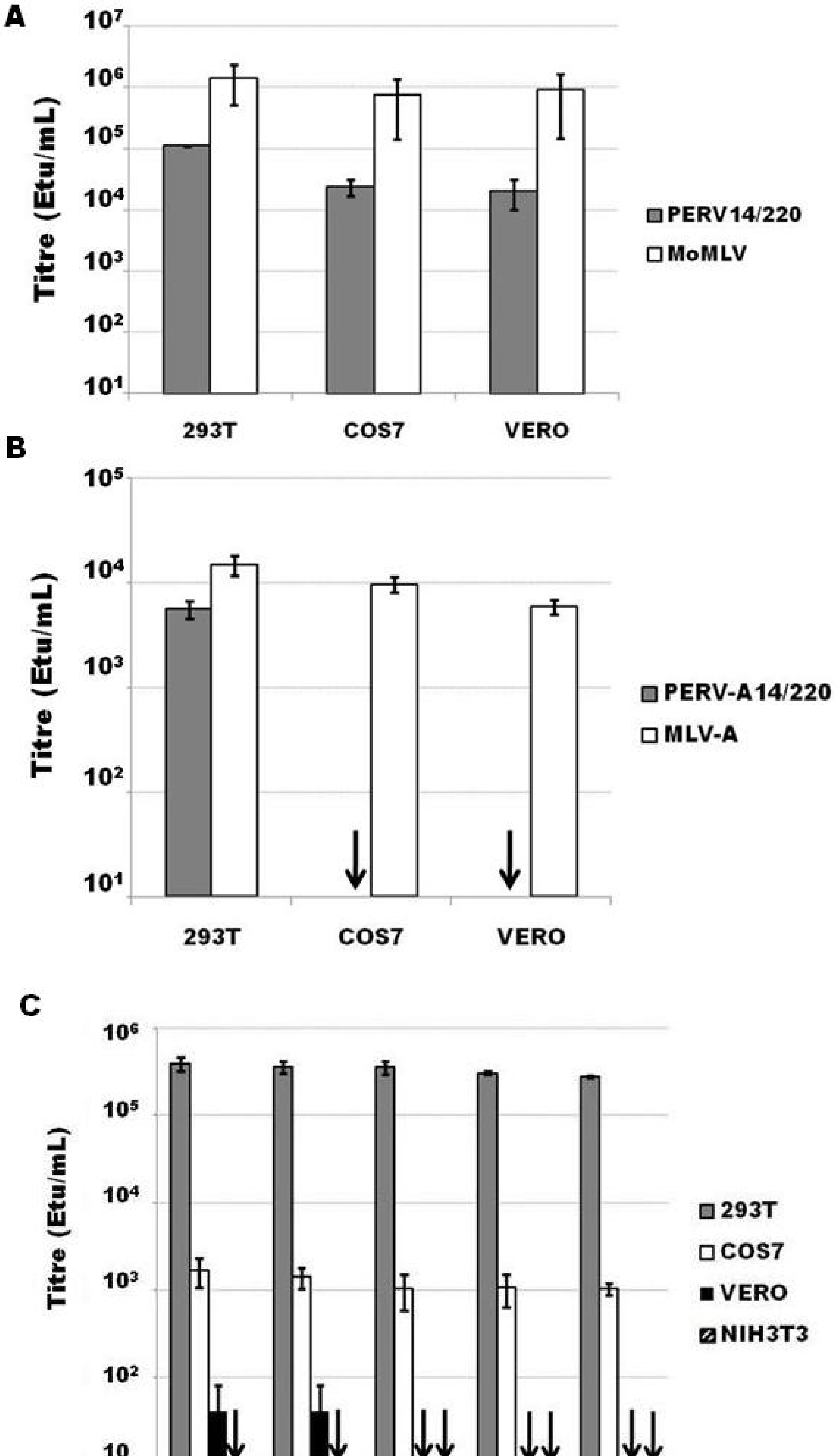

A
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
Titre (Etu/mL)
293T
COS7
VERO
PERV14/220
MoMLV
B
10⁵
10⁴
10³
10²
10¹
Titre (Etu/mL)
293T
COS7
VERO
PERV-A14/220
MLV-A
C
10⁶
10⁵
10⁴
10³
10²
10
Titre (Etu/mL)
2:1
1:1
1:2
1:4
0
Ratio (PERV/EGFP:PERV)
293T
COS7
VERO
NIH3T3

Figure 4.6 Analysis of post-entry block of PERV-A in AGM cells

A) VSV-G pseudotyped viruses carrying MLV-based retroviral vector encoding the EGFP gene were produced by co-transfection of 293T cells with packaging plasmids for PERV-A14/220 GagPol (grey) or MoMLV GagPol (white). Titres on human 293T, AGM COS7 and VERO cells were determined by flow cytometry analysis of EGFP expression. Histograms represent the average of two independent experiments (± standard error of the mean). **B)** Pseudotyped viruses were generated by co-transfection of 293T with MLV-based retroviral vector carrying EGFP gene, PERV-A14/220 GagPol and PERV-A14/220 Env (grey) or MLV-A Env (white). 293T, COS7 and VERO cells were infected with serial dilution of the virus-containing supernatant and titres determined by monitoring EGFP expression. Histograms represent the average of two independent experiments (± standard error of the mean). Arrows indicated a titre under the detectable limit (10 Etu/mL) **C)** Two pseudotyped viruses were produced by three plasmid transfection of 293T cells, both having PERV-A14/220 GagPol and Env. MLV-based vector carrying EGFP gene for the reporter virus (PERV/EGFP) or pcDNA3 for the saturation virus (PERV) were co-transfected. $5x10^4$ 293T (grey), COS7 (white), VERO (black) or NIH3T3 (striped) cells were seeded. Prior to infection, cells were incubated with different amount of saturation virus, with a range between 0 and 500 µL for COS7, VERO and NIH3T3 cells and between 0 and 100 µL for 293T cells. After 6 hours cells were infected with 250 µL (COS7, VERO and NIH3T3) or 50 µL (293T) of the PERV/EGFP-containing supernatant. The fixed amount of reporter virus was chosen to achieve a percentage of infection of about 1% in 293T and COS7 cells. Histograms represent the average of two independent experiments (± standard error of the mean).

4.2.6 Absence of inhibitor secreted from NHP cells

Secretion of inhibitor molecules in the medium has been described as mechanism for MLV-A and GALV inhibition in hamster cells (Miller and Miller, 1992, 1993).

Human 293T cells were incubated overnight with dilutions of the conditioned medium from COS7, VERO or 293T cells and infected with serial dilution of PERV(EGFP) in presence of conditioned medium. After 72 hours, EGFP was monitored by flow cytometry and the titre calculated. No significant effect was observed (Figure 4.7). No factors able to reduce the infection of PERV-A were detected in the supernatant of NHP cells.

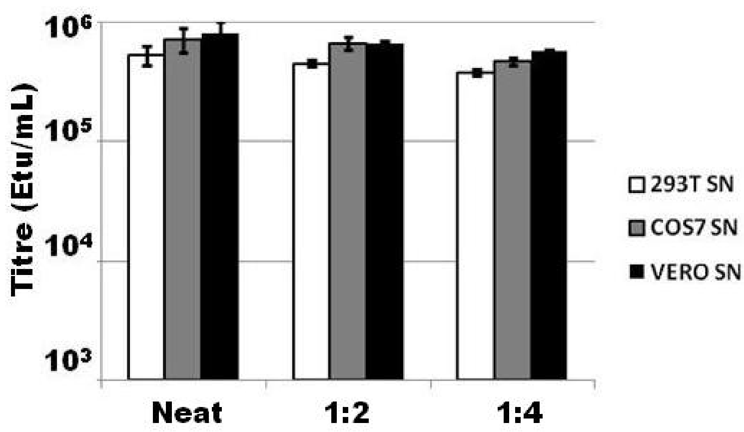


Figure 4.7 PERV-A infection in conditioned media from NHP cells.

$5x10^4$ 293T cells were seeded and incubated overnight with 0.45μm filtered conditioned media (or dilutions as indicated) harvested from confluent 293T (white), COS7 (grey) or VERO (black) cells. The day after, cells were infected with serial dilution of EGFP(PERV) in conditioned media. Titres were determined by flow cytometry analysis. Histograms represent the average of two independent experiments (± standard error of the mean).

4.2.7 Tunicamycin treatment of NHP cells partially rescued PERV-A infection but not binding

Removal of N-linked glycosylation by tunicamycin treatment of the target cells has been showed rescuing retroviral infectivity in certain cell lines (section 1.5.5).

To test whether N-linked glycosylation could play a role in AGM cells low susceptibility to PERV-A infection, 293T, COS7 and VERO cells were infected with serial dilution of EGFP(PERV) after overnight treatment with tunicamycin. Drug concentration was chosen for each cell line according to its toxicity (Figure 4.8 legend). Titres were inferred from EGFP expression. Tunicamycin had no effect on PERV infection of 293T cells. Instead, the viral titre on tunicamycin-treated AGM cells was more than 10 fold higher than untreated cells (Figure 4.8 A). These data suggested that removal of N-linked glycosylation in AGM cells could relieve a possible block to PERV-A infection or compensate an independent inhibition mechanism. To better understand the role of tunicamycin in the improvement of the susceptibility to PERV-A, I analysed the effect this drug had on PERV-A binding on AGM cells.

COS7, VERO and 293T cells were grown overnight in presence of tunicamycin. The day after, $5x10^5$ treated and untreated cells were incubated with soluble PERV-A360 Env or PERV-C360 Env, as negative control. Binding to the cells was detected by staining with a FITC-conjugated anti-rabbit IgG antibody and analysed by flow cytometry (Figure 4.8 B). PERV-A360 Env, but not PERV-C360, successfully bound to 293T cells. As for infectivity, no difference was notable after tunicamycin treatment. No binding was detected to AGM cells either tunicamycin-treated or untreated. These results could suggest that tunicamycin treatment can partially rescue PERV-A infectivity in AGM cells but not the binding. However, the lack of binding when infection was measured could be due to a low sensitivity of this assay.

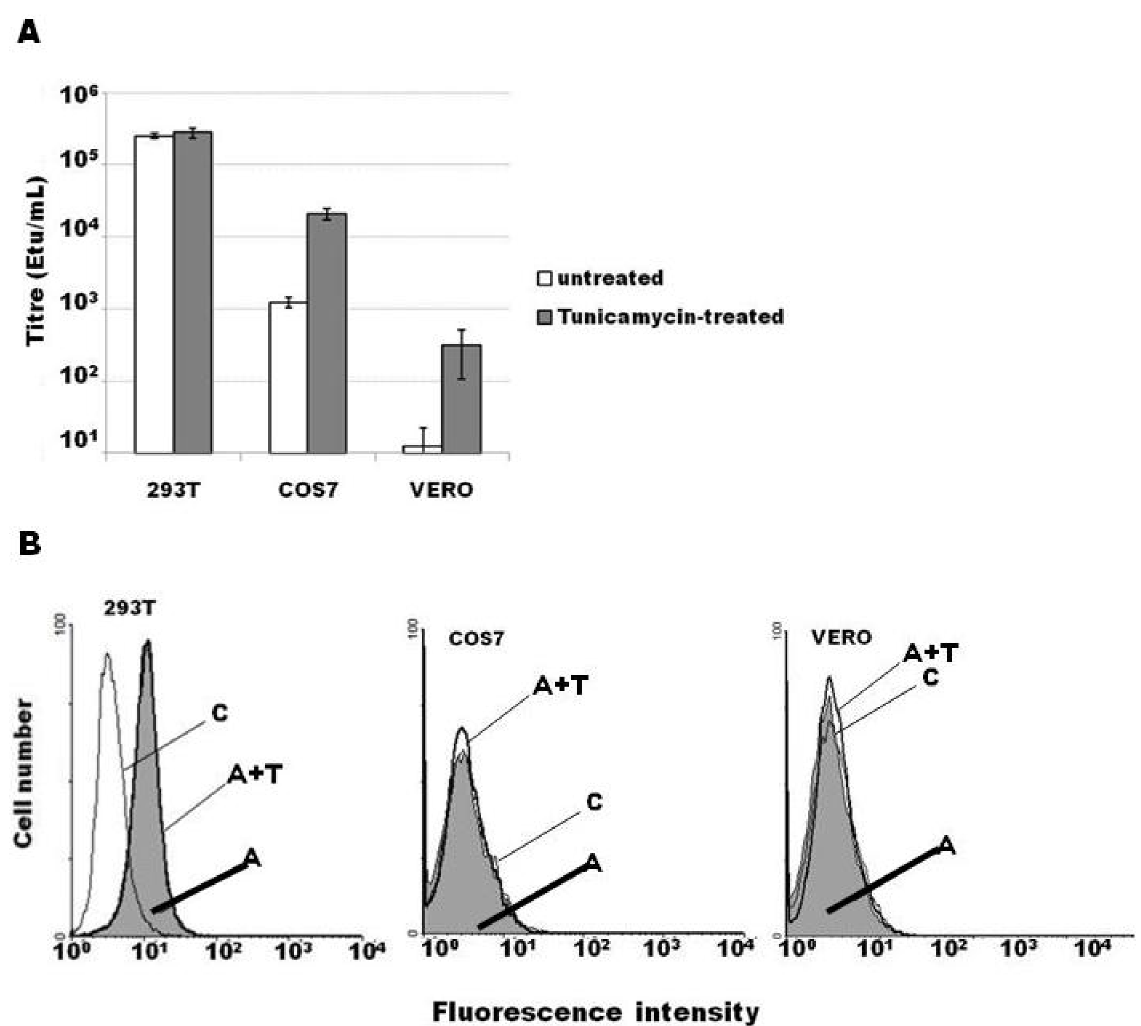


Figure 4.8 Tunicamycin treatment of NHP cells

A) $5x10^4$ cells were treated for 16 hours with tunicamycin at the final concentration of 100 ng/mL (293T cells) or 200 ng/mL (COS7 and VERO cells). Tunicamycin-treated (grey) or untreated (white) cells were infected with serial dilution of EGFP(PERV). After 72 hours, titres were inferred by EGFP expression monitored by flow cytometry. Histograms represent the average of four independent experiments (± standard error of the mean). **B)** $5x10^5$ tunicamycin-treated (+T) and untreated 293T, COS7 and VERO cells were incubated with 100 ng in 0.5 mL of soluble PERV-A360 Env (A) or the same amount of PERV-C360 Env (C), as negative control. PERV Env binding was detected using a FITC-conjugated anti-rabbit IgG antibody and flow cytometry analysis. Graphs show a representative result of three independent experiments.

4.2.8 Analysis of tunicamycin effect on PAR-overexpressing cells

To improve the study of the tunicamycin effect on different PARs and cells, huPAR-1 and AGMPAR-1 were stably overexpressed on COS7 and VERO cells as well as on quail QT6 cells. Receptors were delivered using an MLV-based retroviral vector and cells were transduced between 2 and 3 times until more than 98% of the cells expressed the HA-tagged PARs.

The level of the HA-tagged receptor on the cell surface was determined by staining of the cells with an anti-HA antibody and flow cytometry analysis. The mean fluorescence intensity (MFI) for each cell population was normalised to the MFI of the wild type cells (Figure 4.9 A and B). $5x10^4$ cells were seeded and incubated overnight in presence of tunicamycin. The day after, cells were either infected with EGFP(PERV) or processed for the binding to PERV-A360 Env. Whilst PERV infection of 293T and quail QT6 cells was not affected by tunicamycin treatment, the susceptibility of AGM cells to PERV-A infection was increased regardless the receptor (huPAR-1 or AGMPAR-1) expressed (Figure 4.9 A). This result indicated that tunicamycin partial rescue of permissivity to PERV-A in AGM cells was cell-specific and did not depend on the receptor expressed.

The binding of PERV-A360 Env was quantified by flow cytometry analysis. The MFI obtained by incubating cells with soluble PERV-A360 Env was normalised to the MFI produced using PERV-C360 Env. Binding to AGM cells was measurable only when PARs were overexpressed. Tunicamycin treatment reduced the amount of Env bound to the cells. From analysis using a visible light microscope of the tunicamycin-treated cells and from the morphological gate of the cells during flow cytometry analysis, the reduction in the binding did not appear to be due to toxicity of the tunicamycin treatment.

To further confirm tunicamycin effect on PERV-A infection and binding, single clone populations, expressing different amounts of huPAR-1 or AGMPAR-1, were generated. Quail QT6 and AGM COS7 cells were transduced with a retroviral vector carrying C-terminal tagged huPAR-1 or AGMPAR-1. Single

clones were picked and receptor level on cell surface was measured by staining with an anti-HA antibody and flow cytometry analysis. MFI for each clone was normalised to the MFI of the wild type cells. Cell clones were treated overnight with tunicamycin and processed for PERV-A infection and PERV-A360 Env binding as described above for the PAR-transduced bulk population. Tunicamycin treatment increased susceptibility to PERV-A infection only for COS7 cell clones while QT6 cell clones were unaffected (Figure 4.10 A and B). No difference in the binding to PERV-A360 Env was observed between tunicamycin-treated and untreated cell clones (Figure 4.10 C and D).

Taken together these results showed that PERV-A infection in AGM cells, but not human or quail cells, was increased by tunicamycin treatment, although the mechanism is unclear. Results on soluble PERV-A Env binding to AGM cells suggested that tunicamycin treatment do not enhance virus binding to the receptor.

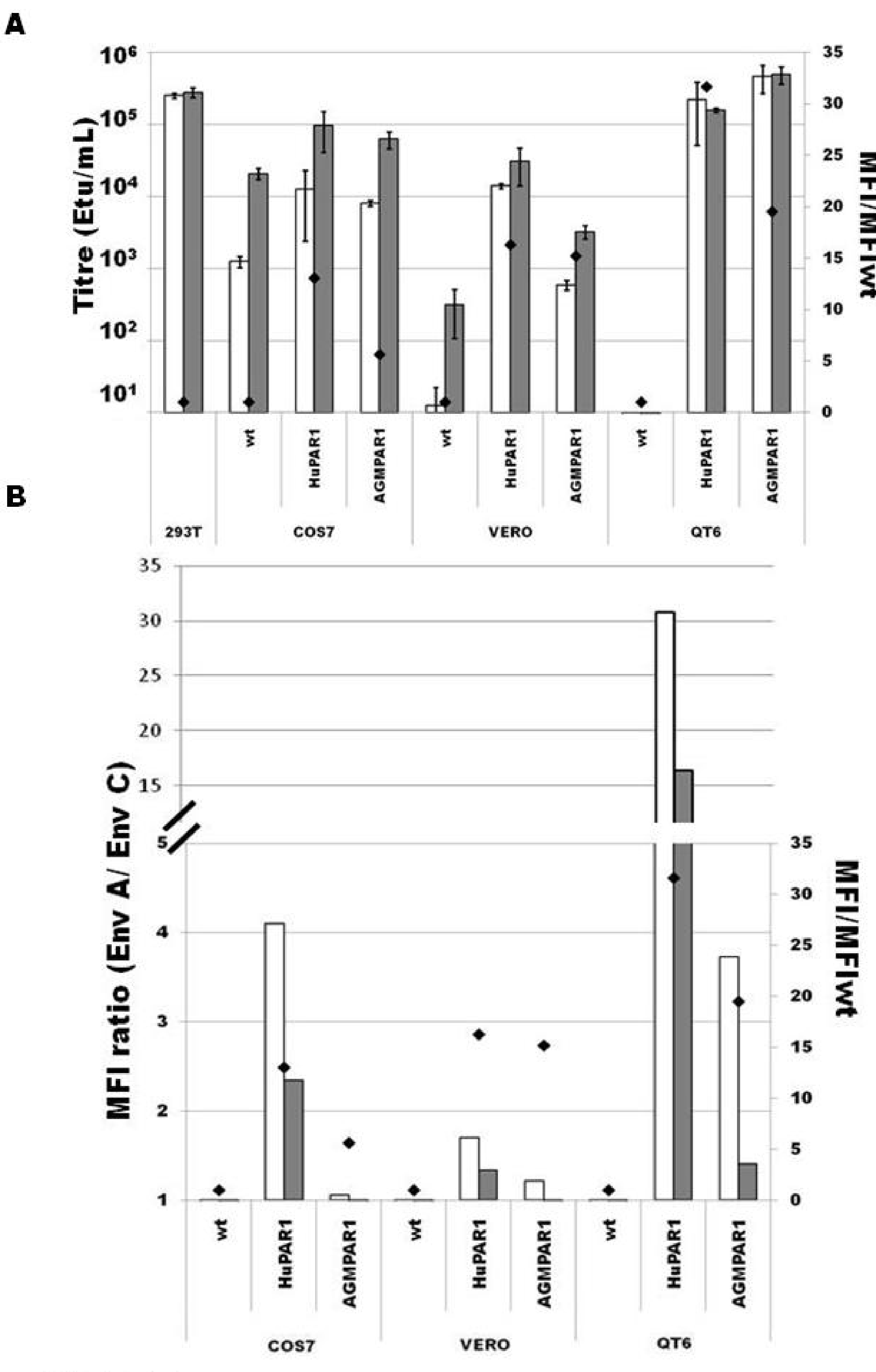

A
B
Titre (Etu/mL)
MFI/MFIwt
293T
COS7
VERO
QT6
wt
HuPAR1
AGMPAR1
MFI ratio (Env A/ Env C)
untreated
Tunicamycin-treated
HA staining

Figure 4.9 Tunicamycin treatment of exogenous PAR-expressing cells

Quail QT6, AGM COS7 and VERO cells were stably transduced with VSV-G pseudotyped MLV-vector carrying HA-tagged huPAR-1 or AGMPAR-1. More than 98% of the cells were positive for anti-HA antibody staining (graphs in Figure 4.12). MFI for each population has been normalised to (i.e. divided by) the MFI of the wild type (HA staining-negative) cells (black diamond). Cells were treated for 16 hours with tunicamycin at the final concentration of 100 ng/mL (293T cells), 200 ng/mL (COS7 and VERO cells) or 25 ng/mL (QT6 cells). **A)** Tunicamycin-treated (grey) or untreated (white) cells were infected with serial dilution of EGFP(PERV). After 72 hours, titres were inferred by EGFP expression monitored by flow cytometry analysis. Histograms represent the average of two independent experiments (± standard error of the mean). **B)** $5x10^5$ tunicamycin-treated (grey) and untreated (white) cells were incubated with 100 ng in 0.5 mL of soluble PERV-A360 Env or PERV-C360 Env. PERV Env binding was detected using a FITC-conjugated anti-rabbit IgG antibody and flow cytometry analysis. Histograms represent the binding measured as MFI obtained with PERV-A360 Env normalised to MFI recorded with PERV-C360 Env.

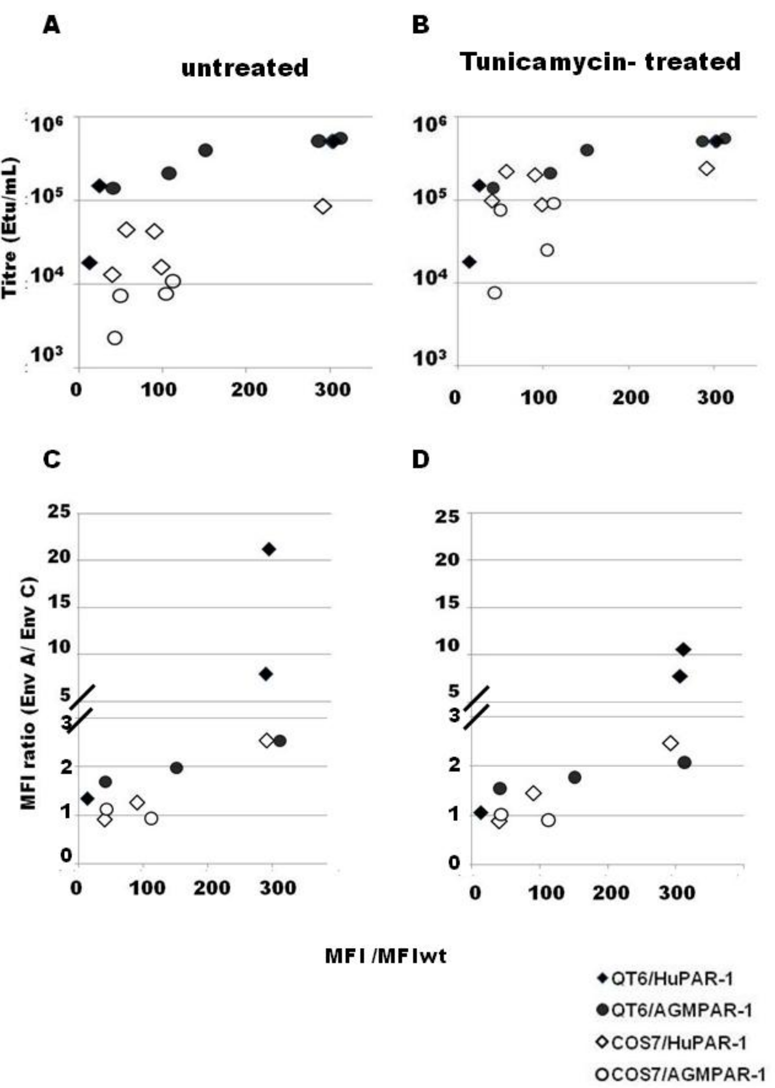


Figure 4.10 Dependence of PERV-A infection and Env binding on PAR expression level

Quail QT6 (black) and AGM COS7 (white) cells were stably transduced with VSV-G pseudotyped MLV-vector carrying HA-tagged huPAR-1 (diamond) or AGMPAR-1 (circle). Single cell clones were grown and the level of the receptor tested by staining with an anti-HA antibody. Clones with different MFI (normalised on the MFI of wild type cells) were selected. $5x10^4$ tunicamycin treated **(B)** or untreated **(A)** cells were seeded and, the day after, infected with serial dilutions of EGFP(PERV). Titres were inferred from EGFP expression and showed in correlation with the HA surface staining. Each dot represents the average of two independent experiments. $5x10^5$ tunicamycin-treated **(D)** or untreated **(C)** cells were incubated with 100 ng in 0.5 mL of PERV-A360 Env or PERV-C360 Env. PERV Env binding was detected by immunostaining with a FITC-conjugated anti-rabbit IgG antibody and showed as MFI Env-A360 normalised on the MFI Env-C360. Dots in the graph represent the average of two independent experiments.

4.2.9 Tunicamycin does not affect PERV-A attachment to the cell surface

Different cell surface molecules other than the receptor could mediated PERV-A adsorption to the cells and facilitate the interaction with the cognate receptor and indirectly influence the infectivity. Tunicamycin treatment prevents N-linked glycosylation of all the cellular proteins. Therefore, I investigated whether tunicamycin-mediated enhancement of PERV-A infection in NHP cells could be due to an increased attachment to deglycosylated proteins other than the receptor.

$2x10^5$ 293T, QT6, COS7 and VERO cells were seeded on a cover slip and treated overnight with tunicamycin. The day after, cells were incubated with $4.5x10^6$ i.u/mL of PERV-A and stained with an anti-PERV CA antibody and visualised by confocal microscopy. Although a quantitative analysis has not been conducted, the representative fields showed that no clear difference in the amount of viruses attached on the cell surface after tunicamycin treatment was observed for AGM cells (Figure 4.11).

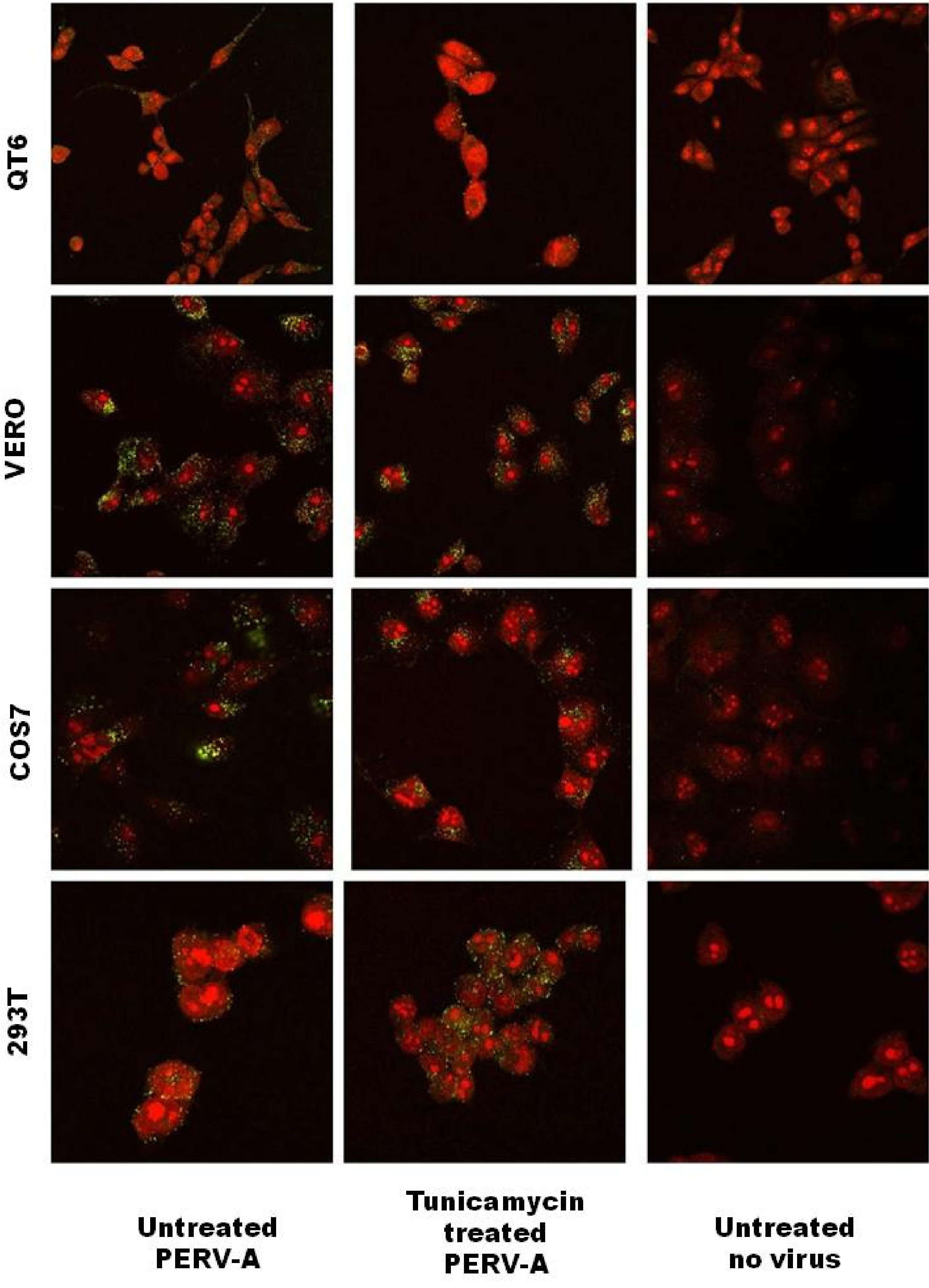

QT6
VERO
COS7
293T
Untreated
PERV-A
Tunicamycin
treated
PERV-A
Untreated
no virus

Figure 4.11 PERV-A attachment to the cell surface

$2X10^5$ cells were seeded on a cover glass and treated or not with different concentration of tunicamycin: 100 ng/mL (293T cells), 200 ng/mL (COS7 and VERO cells) or 25 ng/mL (QT6 cells). The day after, cells were incubated with 1.5 mL of EGFP(PERV), fixed, permeabilised and immunostained with an anti-PERV CA antibody followed by the FITC-conjugated secondary antibody. Cover glasses were mounted on a slide with mounting solution containing propidium iodide to counterstain the nuclei (red). Images show representative fields acquired using DM IRE2 confocal microscope (oil immersion objective 63X). Viral particles appeared as green dot associated to the cells. Cells incubated with media alone, without virus, have been used as negative control.

4.2.10 No evidence of PAR intracellular entrapment in NHP cells

One of the mechanisms proposed for tunicamycin-mediated rescue of retroviral infection was that the lack of N-linked glycosylation could cause misfolding of inhibitory molecule such as an ERV Env (Lavillette et al., 2000). Results presented above suggested that NHP low permissivity to PERV-A infection could not be due to a cellular molecule secreted in the supernatant (section 4.2.6). However, AGM cells could produce a molecule which interacts with the newly synthesised PARs preventing their display on the cell surface. To test this hypothesis, the cell surface level of PAR was compared to their intracellular amount.

QT6, COS7 and VERO cells expressing HA-tagged huPAR-1 or AGMPAR-1 (section 4.2.8) were fixed and immunostained using an anti-HA antibody in presence (intracellular) or absence (extracellular) of saponin, a chemical which permeabilised the cell plasma membrane. Although, a higher level of intracellular receptor was expected, the MFI of saponin-treated cells was lower in all the cells in comparison with those surface-stained (Figure 4.12). This could be due to technical problems. The ratio between HA stainings with or without saponin was calculated after normalisation against MFI of wild type cells (table 4.2). No significant AGM cell-specific difference was observed.

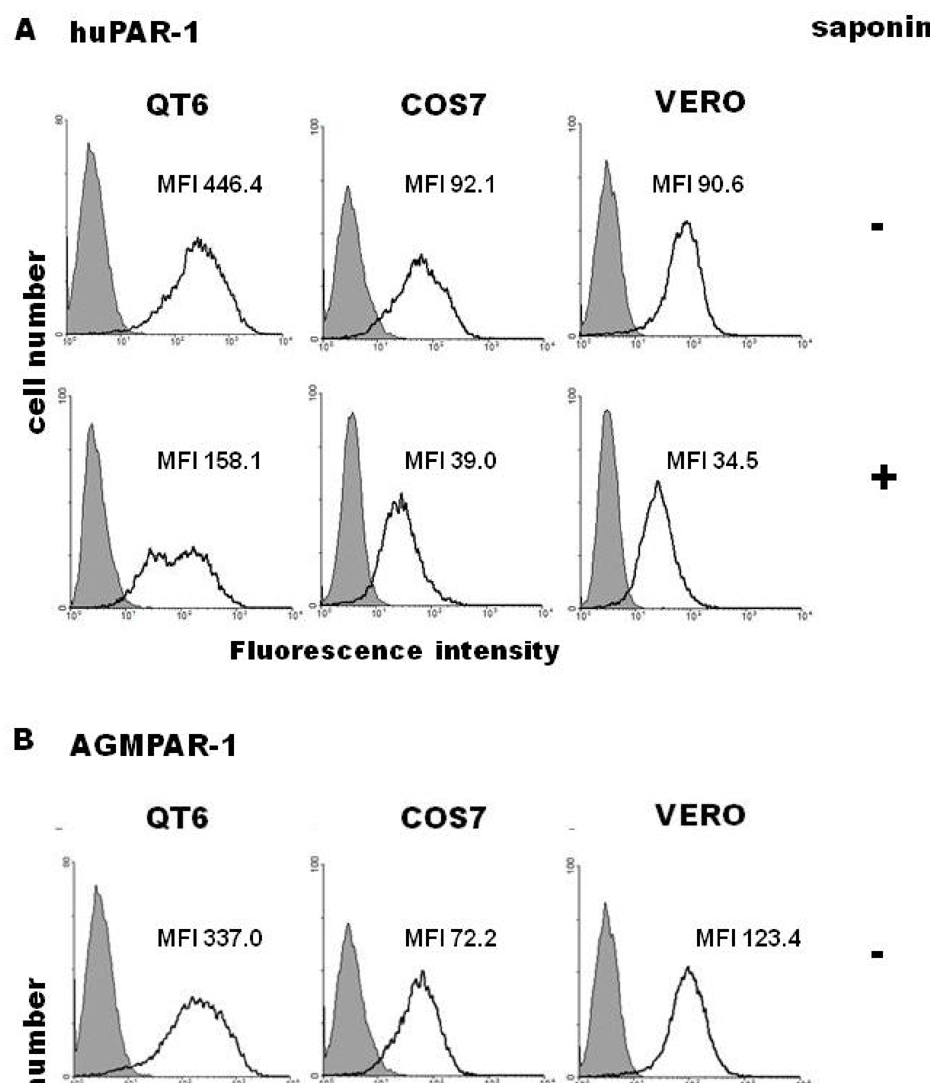


Figure 4.12 Expression of exogenous PAR in the stably transduced cells

Quail QT6, AGM COS7 and VERO cells were stably transduced with VSV-G pseudotyped MLV-vector carrying HA-tagged huPAR-1 **(A)** or AGMPAR-1 **(B)**. Cells were fixed and permeabilised (+) or not (-) with saponin 0.2% (w/v) in PBS. Receptor level was inferred by immunostaining using an anti-HA antibody and a PE-conjugated anti-mouse IgG antibody. Cells were analysed by flow cytometry.

Cells	MFI Ratio
huPAR-1/QT6	2.8
huPAR-1/COS7	2.1
huPAR-1/VERO	2.3
AGMPAR-1/QT6	3.4
AGMPAR-1/COS7	2.6
AGMPAR-1/VERO	1.8

Table 4.2 Ratio of HA staining of saponin-treated or untreated cells

Mean fluorescence intensity (MFI) values were calculated using CellQuest software and normalised on MFI values of the wild type cells. For each sample MFI ratio represented the MFI obtained with a cell surface staining (i.e. no saponin) divided by MFI of saponin-treated cells.

4.3 Discussion

NHP are considered an ideal animal model to evaluate the risk of PERV transmission in the context of xenotransplantation [reviewed in (Denner, 2003)]. Pig-to-NHP transplantation is currently used to test the immunological response to xenograft [reviewed in (Ekser et al., 2009)]. Furthermore, in comparison to retrospective studies on patients treated with porcine materials PERV transmission in NHP could be investigated in a variety of tissues not limited, as in human recipients, to PBMC and serum. NHP could also be treated with drugs to simulate the immunosuppression required in xenotransplantation. However, PERV infection in NHP cells *in vitro* does not occur with the same ease than in certain human cells (Blusch et al., 2000a; Martin et al., 1999; Specke et al., 2001; Takeuchi et al., 1998; Templin et al., 2000; Wilson et al., 2000). Therefore, inferring information applicable to human xenotransplantation from the pig-to-NHP transplantation model could be misleading. Understanding the reason for the poor susceptibility of NHP cells to PERV infection could clarify the suitability and the limitations, and help in the choice, of an ideal animal model.

A previous study of PERV transmission in NHP cells concluded that PERV infection in NHP cells was not productive (Ritzhaupt et al., 2002). In this chapter, I evaluated PERV infection in NHP cells and give indications of possible reasons behind the poor susceptibility of NHP cells to PERV-A.

Using a pseudotyped PERV-A14/220 virus carrying an EGFP reporter gene, different steps in the virus life cycle were evaluated. Upon transduction of NHP cells, EGFP expression was confirmed in AGM COS7 and rhesus macaque FRhK4 cells (Figure 4.2 A). Furthermore PERV *gag* gene was detected in both cellular genomic DNA and RNA (Figure 4.2 B and C). These data agreed with previous studies in which following PERV infection, viral sequences were detected by PCR in the genomic DNA and RNA of infected NHP cells (Blusch et al., 2000a; Ritzhaupt et al., 2002). To understand whether PERV can productively infect NHP cells, the presence of infectious particles in the supernatant of PERV-infected NHP cells was investigated. AGM COS7 cells produced virions able to infect 293T cells up to three weeks

post PERV infection, albeit at low titres (Figure 4.2 D). To further confirm the ability of PERV infected NHP cells to produce infectious viral particles and to understand which step in the virus life cycle was responsible for the low permissivity to PERV, NHP cells were transduceded with the human PERV-A receptor-2 (Ericsson et al., 2003). Indeed, pseudotyped EGFP(PERV) titre on huPAR-2 expressing NHP cells was at least 37 times higher. PERV infection on AGM VERO cells, which was undetectable in the wild type cell, was at similar levels to huPAR2-transduced COS7 and FRhK4 cells (Figure 4.2 A). Infectious virions were produced in all NHP cells, as determined by the infection assay on 293T cells (Figure 4.2 D). These results suggested that low efficiency in PERV entry is the major cause for the low permissivity of NHP cells.

To better evaluate PERV entry in these cells, NHP PARs were analysed. Two PARs in human cells were identified (Ericsson et al., 2003) but only one in mice, rats and pigs. From sequences present in the NCBI database, it was likely that primates have two PARs as humans. While chimpanzee and rhesus macaque homologues to huPAR-1 and huPAR-2 sequences were already deposited in GenBank, PAR sequences for AGM, baboon and cynomolgus monkey was cloned by RT-PCR of RNA extracted from NHP cells. BaPAR-2 has been previously cloned (Ericsson et al., 2003). Protein sequence alignment revealed that rhPAR-1, cynPAR-1 and baPAR-1 have one amino acid difference from huPARs in the second extracellular loop at position 109, where muPAR possesses the critical proline responsible for the PERV-A resistance (section 3.2.2). The ability of the different NHP PARs to support PERV-A entry was tested. PERV-resistant quail QT6 cells were transduced to stably express NHP PARs. The receptors were HA-tagged at the C-terminal to verify their display on the cell surface. PERV-A titre on PAR-expressing QT6 cells was measured and revealed that all NHP PAR receptors were functional except for those containing serine at a.a. 109 (Figure 4.3 B). To confirm the role of this serine in the rhPAR-1 function as receptor, two chimeric receptors were generated: huPAR-1 in which leucine 109 was changed into a serine (huPAR-1S109) and rhPAR-1 with a leucine in position 109 instead of the serine. Infection assay on QT6 cells expressing

these chimeric receptors revealed that rhPAR-1L109 was able to mediate PERV-A entry in QT6 cells (Figure 4.3 B) and binding to a soluble PERV-A Env (Figure 4.4 B). Conversely, PERV-A titre on QT6 cells expressing huPAR-1S109 was strongly reduced (Figure 4.3 B) and the receptor was unable to mediate the binding to PERV-A Env (Figure 4.4 A). These data suggested that a single amino acid substitution in PAR-1 of rhesus macaque, cynomolgus monkey and baboon cells disrupted their function as receptor, leaving these NHP with only one functional receptor (PAR-2). Instead, AGM cells encode two functional receptors, as in human cells.

Low sensitivity to PERV-A infection in AGM cells mirrored the resistance in rat cells. In both cases, cells encode functional receptors. In rat cells a subthreshold level of ratPAR expression was associated to PERV-A resistance, AGM PARs expression in AGM cells was investigated. The lack of an anti-PAR antibody prevented the analysis of the receptor displayed on the cell surface. Therefore PAR mRNA was analysed by quantitative RT-PCR (Figure 4.5). The two functional receptors in AGM COS7 and VERO cells were expressed at a similar level to PARs in human cells. However, the mRNA level of expression could not correlate with the amount of protein expressed on the cell surface. Furthermore, other mechanisms could be preventing PERV-A infection in AGM cells. The reason for the low permissivity of AGM cells to PERV-A were investigated further.

Post-entry restriction factors acting in a Fv-1 or TRIM5α similar way were evaluated by comparison of the infection efficiency on 293T and AGM COS7 and VERO cells of pseudotyped retroviral particles carrying either PERV-A14/220 or MoMLV GagPol. The titre obtained with PERV-A14/220 GagPol in AGM cells was 5 fold lower than that on 293T cells (Figure 4.6 A), while the difference of PERV-A infection between 293T cells and AGM cells is more than 200 times (Figure 4.8 A, untreated). Therefore, although a weak post-entry restriction factor could contribute to the low permissivity of PERV-A in AGM cells, this could not be considered the main mechanism of inhibition. Furthermore, infection with a pseudotyped PERV-A14/220 bearing

MLV-A Env could rescue the titre on AGM cells (Figure 4.6 B), suggesting that the major block to PERV-A infection in AGM cells occurs at entry level.

Infection of 293T cells in conditioned media from AGM cells did not alter PERV-A titre, suggesting that no inhibitor is secreted from these cells (Figure 4.7). Receptor entrapment in cytosolic compartment was also analysed. QT6, COS7 and VERO cells were stably transduced to express huPAR-1 or AGMPAR-1. Cell surface display of the receptor was measured by HA-staining. After permeabilisation, the total amount of HA-tagged receptor in the cells was evaluated. If a mechanism was present in AGM cells which prevent the trafficking to the cell surface, I would expect that the ratio between the cell surface-displayed and total amount of PAR in NHP would be considerably lower than in QT6 cells. My data did not show evidence for reduced surface display of exogenously expressed PAR in NHP cells (Figure 4.12 and Table 4.2) However, it cannot be excluded that endogenously expressed AGM PAR already saturates possible inhibitors of PAR trafficking.

Tunicamycin-treatment of AGM cells prior to PERV infection could enhance the virus permissivity in these cells (Figure 4.8 A) but did not affect binding (Figure 4.9). These results have been confirmed also for PAR-overexpressing AGM cells, but not for quail QT6 cells (Figure 4.9). Tunicamycin-mediated enhacement of PERV infection was similar in AGM cells expressing huPAR-1 and AGMPAR-1, suggesting that the effect is cell-specific (Figure 4.9 and 4.10). In particular, when the same level of PAR-1 (i.e. same MFI) was expressed on the cell surface of QT6 and COS7 cells, titres obtained on QT6 cells were more than 10-fold higher than those on COS7 cells. Tunicamycin treatment drastically reduced this difference (Figure 4.10).

Immunofluorescence analysis of PERV attachment on the cell surface of AGM cells did not show any difference after tunicamycin-treatment (Figure 4.11). This suggested that tunicamycin-mediated enhancement of PERV infection does not involve a change in the N-linked glycosylation of other cell surface molecules, implicated in virus adsorption. Therefore, it is possible that tunicamycin enhances PERV-A infection through removal of N-linked

polysaccharide chains from PAR in AGM cells. This possibility could be tested by the comparison of AGMPAR and an N-glycosylation deficient mutant, lacking the asparagine at position 178 (section 3.2.3) in their ability to mediate PERV-A entry in AGM cells.

In summary, the results from this chapter suggested that poor susceptibility of rhesus macaque, cynomolgus and baboon cells to PERV-A infection is due to a serine in the critical ECL2 of PAR-1 which not only prevent PERV-A entry but also binding. Infection observed in these monkey cells may be mediated by a functional PAR-2. However, the level of expression of its mRNA is lower than PAR-1 in human cells, hence the poor permissivity of these cells to PERV-A. Therefore, rhesus macaque and cynomolgus monkey, which have been the prevalent choice as animal models in pig-to-NHP transplantation [reviewed in (Ekser et al., 2008)], are probably not the most suitable model to predict the risk of PERV transmission in pig-to-human transplantation. Because AGM cells express two functional receptors at similar level than human cells, they could represent a better animal model to evaluate PERV infection. However, no clear explanation was obtained for the poor susceptibility of AGM cells to PERV-A. From the results presented in this chapter, tunicamycin-treatment could increase PERV-A infection in AGM cells, but through an unknown mechanism.

Chapter 5

5 . Inhibition of PERV release from pig cells by tetherin

5.1 Introduction

Genetically modified pigs have been developed to overcome the immunological barrier in xenotransplantation (section 1.1.1). The same strategy has been also employed to reduce the risk of PERV associated zoonosis. Multi-transgenic animals are seen as an optimised source of xenografts, characterised by low probability of PERV infection, absence of hyperacute immune reaction and reduced cellular rejection (Sykes, 2008). Currently, only two groups have adopted this strategy and produced transgenic pigs expressing small interfering RNAs for the inhibition of PERV expression (Dieckhoff et al., 2009; Dieckhoff et al., 2008; Ramsoondar et al., 2009). Both groups found a reduction in PERV gene transcription. However, PERV proteins were not detectable in either non-transgenic controls or in the transgenic animals, which renders the interpretation of their results difficult (Dieckhoff et al., 2008; Ramsoondar et al., 2009). Other molecules have been investigated and could potentially be employed in the development of safer transgenic pigs for use as animal donors in human transplantation: intracellularly expressed antibodies directed against PERV Gag (Dekker et al., 2003), sugar modifying enzymes to remodel PERV envelope glycoprotein (Miyagawa et al., 2006) and the restriction factor human APOBEC3G (Dorrschuck et al., 2008; Jonsson et al., 2007).

In this chapter, another possible candidate molecule whose expression in transgenic animals could reduce the risk posed by PERV has been evaluated. Tetherin (BST-2, CD317, HM1.24) has recently been described as a restriction factor in human cells able to block the release of some groups of enveloped viruses (section 1.6.4). Tetherin (THN) expression varies in different cell types and can be induced by type I interferon (IFN) (Blasius et al., 2006; Neil et al., 2008). Its mechanism of action involves the tethering of

nascent viral particles to the cell membrane of the virus-producing cells (Neil et al., 2008). THN-mediated block to virus production appears to be able to inhibit the production of a broad range of enveloped viruses. In fact, members of other retroviral families, including alpharetrovirus, betaretrovirus, deltaretrovirus, gammaretrovirus, lentivirus and spumavirus (Jouvenet et al., 2009; Neil et al., 2008), filoviruses such as Marburg virus and Ebola virus (Jouvenet et al., 2009; Sakuma et al., 2009), and the arenavirus Lassa virus (Sakuma et al., 2009) are all sensitive to inhibition by human THN. In addition, a proteomic study revealed that CD317 protein levels decreased in the presence of human herpesvirus 8 K5 protein (Bartee et al., 2006), suggesting a possible antagonising role similar to that of HIV-1 Vpu. While THN-mediated block of virus release seems to be part of the innate type I IFN-dependent generally immunity to enveloped viruses, the viral countermeasures to this restriction are species-specific. Among primate lentiviruses only few encode a Vpu protein (Bailes et al., 2003; Bibollet-Ruche et al., 2004; Courgnaud et al., 2003; Courgnaud et al., 2002; Gao et al., 1999). HIV-1 Vpu can antagonise human THN but is ineffective against other primate THNs (Gupta et al., 2009a; Jia et al., 2009; McNatt et al., 2009) or non primate THNs (Goffinet et al., 2009; Gupta et al., 2009a; McNatt et al., 2009). The determinants of susceptibility to HIV-1 Vpu have been mapped to the transmembrane region of THN (Gupta et al., 2009a; Jia et al., 2009; McNatt et al., 2009; Rong et al., 2009). Vpu antagonised THN by removing the restriction factor from the cell membrane via a β–TrCP/E3 ubiquitin ligase complex (Douglas et al., 2009; Mitchell et al., 2009) similar to the mechanism described for the surface down-modulation of CD4 by HIV-1 Vpu (Margottin et al., 1998). Viruses lacking Vpu protein have developed other counteracting systems. In rhesus macaque simian immunodeficiency virus (SIVmac), the accessory protein Nef has assumed the function of overcoming THN-mediated restriction (Jia et al., 2009; Zhang et al., 2009). For other viruses, the anti-THN role is played by the envelope glycoprotein, possibly with a different mechanism from Vpu. Specifically, Ebola glycoprotein antagonised THN-mediated restriction (Kaletsky et al., 2009), and tantalus monkey SIV Env was able to counteract tantalus monkey, rhesus macaque, sooty

mangabees monkey and human THNs (Gupta et al., 2009b). Furthermore, HIV-2 envelope enhances the production of HIV-1 particles in a Vpu-like pattern (Abada et al., 2005; Bour et al., 1996; Bour and Strebel, 1996; Ritter et al., 1996), suggesting its possible role as a countermeasure for THN block.

To understand whether THN can be used to generate transgenic pigs with reduced PERV production, a porcine homologue to human THN has been cloned and characterised. The ability of human and porcine THN to inhibit the release of PERV from virus-producing cells has been investigated. In fact, contrary to previous studies where infection by an exogenous virus in THN-expressing cells was examined, the situation is reverse in pig cells. PERV is already integrated and continuously produced, while THN would been exogenously delivered.

5.2 Results

5.2.1 PERV-producing porcine cells express a homologue of human tetherin

Human THN (huTHN) mRNA sequence (Genbank accession number: NM_004335.2) was submitted as template in the pig EST database using the basic local alignment search tool software [BLAST, (Altschul et al., 1997)]. The first 18 hits share the same sequence (data not shown). Among these, sequence EW580921.2 was arbitrarily chosen to design primers that anneal to the hypothetical transcriptional start and end of the porcine tetherin candidate. As huTHN gene has been reported to be IFN-inducible and silent in certain cell lines (Neil et al., 2008), we used three pig cell lines as a potential cDNA source for pig homologue to huTHN. Total RNA from PK15, MPK and ST-IOWA cells, was extracted and reverse transcribed as previously described (section 2.2.9). The cDNA was employed as template in a PCR using the porcine THN primers CF7 and CR7 (Table 2.3.2). The expected 533bp band was visualised in an agarose gel (Figure 5.1 A) and the PCR products cloned into pGEM T-easy vector. The analysis of the sequences obtained from MPK and ST-IOWA PCR products revealed a perfect match with the EST EW580921.2. However, the PK15 product sequence presented two mismatches at nucleotides 351 and 427, which are non-synonymous substitutions (Figure 5.1 B). Direct sequencing of the PK15 THN cDNA showed that PK15 cells express two types of cDNA: one the same as EW580921.2 and the other with changes at positions 351 and 427 (Figure 5.1, chromatograms).

Human cell lines, 293T and HeLa, express significantly different level of huTHN. As a consequence, while retroviral particles successfully bud from 293T cells, they are withheld on the cell surface of HeLa cells by a THN-mediated mechanism (Neil et al., 2008). To determine the level of porcine THN expressed in pig cells, cDNA from PK15, MPK and ST-IOWA was processed by quantitative RT-PCR and compared to the huTHN mRNA level in 293T and HeLa cells (Figure 5.2). THN mRNA expression in pig cells is

estimated to be 35 times higher than human 293T cells but 5 times lower than HeLa cells. The implication for virus production will be analysed in the following sections.

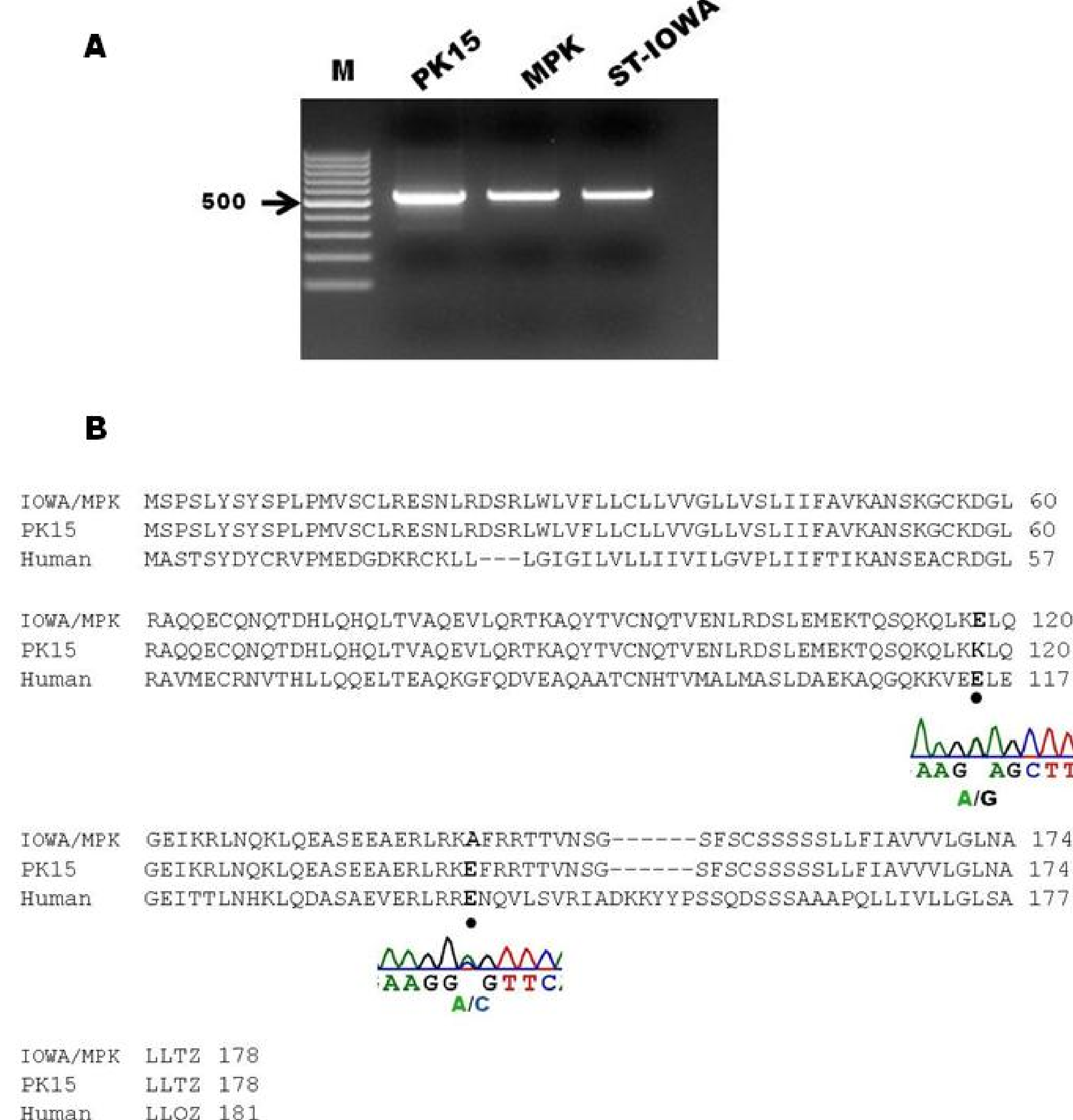


Figure 5.1 Cloning of a porcine homologue of human tetherin

A)1µg of total RNA from pig PK15, MPK and ST-IOWA cell lines was reverse transcribed using QuantiTect Reverse Transcription kit and one quarter of the volume of the resulting cDNA used as template in a RT-PCR using primers CF7 and CR7 (Table 2.3.2). The products were run in a 1.5% agarose gel and visualised by High Performance ultraviolet

transilluminator (UVP). The band sizes were approximately determined by comparison with GeneRuler 100bp DNA ladder (M). **B)** PCR products were gel extracted, cloned into pGEM T-easy vector, and sequenced using primers M13F and M13R (Table 2.3.3). The porcine sequences were aligned against huTHN sequence using ClustalW programme. The two different amino acids between PK15 and MPK/IOWA THN sequences were indicated (•). The nucleotide polymorphisms in PK15THN were visualised in chromatograms obtained by direct sequencing of the PCR product using primers CF7 and CR7.

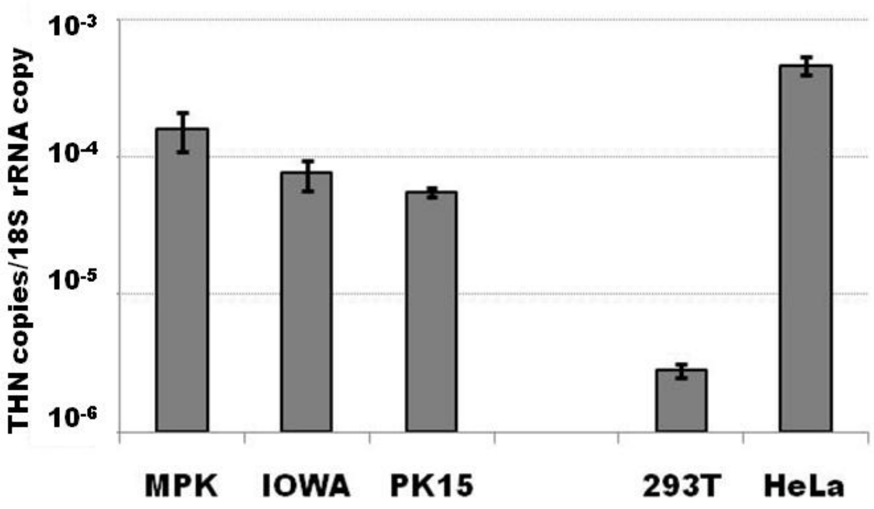


Figure 5.2 Endogenous expression of porcine tetherin in pig cells

1 µg of total RNA from pig PK15, MPK and ST-IOWA, and human 293T and HeLa cell lines was reverse transcribed in a final volume of 20 µl. For each reaction, 2.5 µl of cDNA were added to the QuantiTect SYBR Green PCR mix together with the specific primers: QF9 and QR9 for pig THN, QF10 and QR10 for huTHN, and QF4 and QR4 for 18S rRNA gene. Samples were run in triplicate. The amount of copies for each gene was extrapolated from analysis of the standard curves. Histograms represent porcine and human THN number of copies normalised to one 18S rRNA copy.

5.2.2 Porcine tetherin blocks release of retroviral particles

MPK and PK15 cells produce infectious PERV particles although porcine tetherin is well expressed in these cells. The ability of porcine THN to inhibit viral release similarly to huTHN has been investigated. Due to the two amino acids difference in their sequences, both PK15 tetherin (PK15THN) and MPK/ST-IOWA tetherin (IOWATHN) were analysed.

EGFP-expressing viruses were prepared by transfection of 293T cells with MLV-based retroviral vector CNCG, carrying the reporter EGFP gene, and packaging plasmids encoding PERV-A14/220 GagPol and PERV-A14/220 Env (PERV2a/PERV2a), or Moloney MLV GagPol and amphotropic MLV Env (MoMLV/MLV-A). Viral titres were determined by infection of 293T cells with serial dilutions of the supernatant from the producing cells, followed by monitoring EGFP expression.

Co-transfection of human or porcine THN reduced PERV-A and MLV titres 30 to 80-fold in comparison to those with an empty plasmid (pcDNA3) (Figure 5.3 A). Cell lysate of PERV2a/PERV2a-producing cells and the corresponding supernatant were immunoblotted using a polyclonal rabbit anti-PERV CA antibody. Expression of human and porcine THN did not affect the amount of cell-associated Gag (Figure 5.3 B, cell lysate), but reduced the presence of mature virions (processed capsid protein p30) in the supernatant (Figure 5.3 B, SN). Furthermore, no significant difference in the reduction of released titre could be observed between huTHN, PK15THN and IOWATHN. These data agreed with an observation by Gupta and colleagues in our collaboration showing that huTHN and IOWATHN equally inhibit HIV-based lentiviral vector pseudotyped with VSV-G (Gupta et al., 2009b).

These results confirmed that both porcine THNs we cloned were able to block the release of retroviral particles. The two amino acid mutations in the PK15THN sequence did not affect its restriction function.

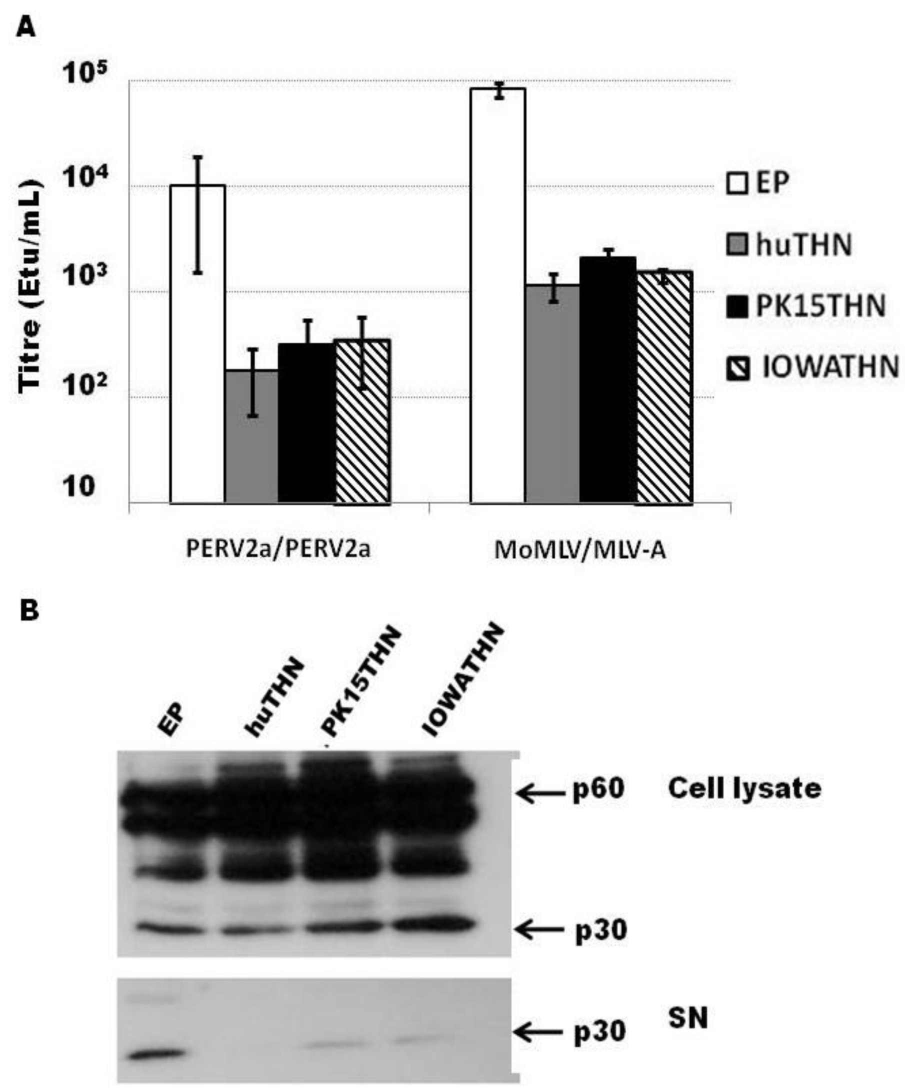


Figure 5.3 Porcine tetherin blocking of PERV and MLV release

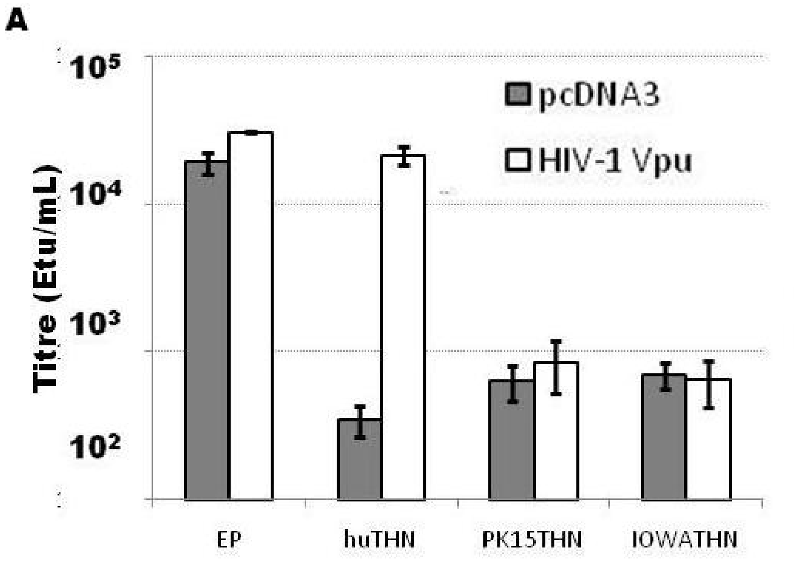
A) EGFP expressing viruses, PERV2a/PERV2a and MoMLV/MLV-A, were produced by three plasmid transfection of $1x10^6$ 293T cells together with 200 ng of huTHN plasmid (grey), PK15THN (black), IOWATHN (striped) or an equal amount of empty plasmid (white). Viral titres were determined by infection of 293T cells with serial dilutions of the supernatant and monitoring EGFP expression by flow cytometry. Histograms represented the average of two independent experiments (± standard error of the mean). **B)** Two days post-transfection, virus-producing cells were harvested and lysed in RIPA buffer. Supernatant from these cells was concentrated by centrifugation and resuspended in Laemmli buffer. A quarter of the total cell lysate and the supernatant were separated in a 10% SDS-PAGE. Proteins were immunoblotted using rabbit polyclonal anti-PERV capsid antibody. In the cell lysates (upper panel) capsid precursor (p60), the intermediate forms and the processed capsid (p30) were visible. In the supernatant (SN, bottom panel) p30 was the main form represented.

5.2.3 Porcine tetherin is insensitive to HIV-Vpu

As eukaryotic cells evolve many factors to block viral infection (section 1. 6), viruses develop countermeasures to overcome them. Due to selective pressure, restriction factors and antagonistic viral proteins are often species-specific, although sometimes some cross-species effect can be observed. HIV-1 Vpu is able to inhibit the restriction posed by THN in human cells (Neil et al., 2008), and its effect on porcine THN has been investigated.

PERV2a/PERV2a, carrying the reporter gene EGFP, has been produced by three plasmid transfection of 293T cells in the presence of either human or porcine THN, or an empty vector, as negative control. By co-transfection of an expression plasmid encoding HIV-1 *vpu* gene, PERV2a/PERV2a titre in the presence of human THN was rescued to the control level (empty plasmid, EP). However, HIV-1 Vpu expression did not rescue the reduction of viral titre caused by porcine THNs, PK15THN and IOWATHN (Figure 5.4 A). Western blot analysis of the producing-cell lysates confirmed that similar amount of Gag was produced in the cells (Figure 5.4 B, cell lysates), but it was differentially released in the supernatant (Figure 5.4 B, SN), consistent with the infection assay (Figure 5.4 A).

These results showed that the block to PERV budding by porcine THN cannot be overcome by HIV-1 Vpu. The same insensitivity of porcine THN to HIV-1 Vpu has also been shown using VSV-G pseudotyped HIV particles as assay virus (Gupta et al., 2009b).



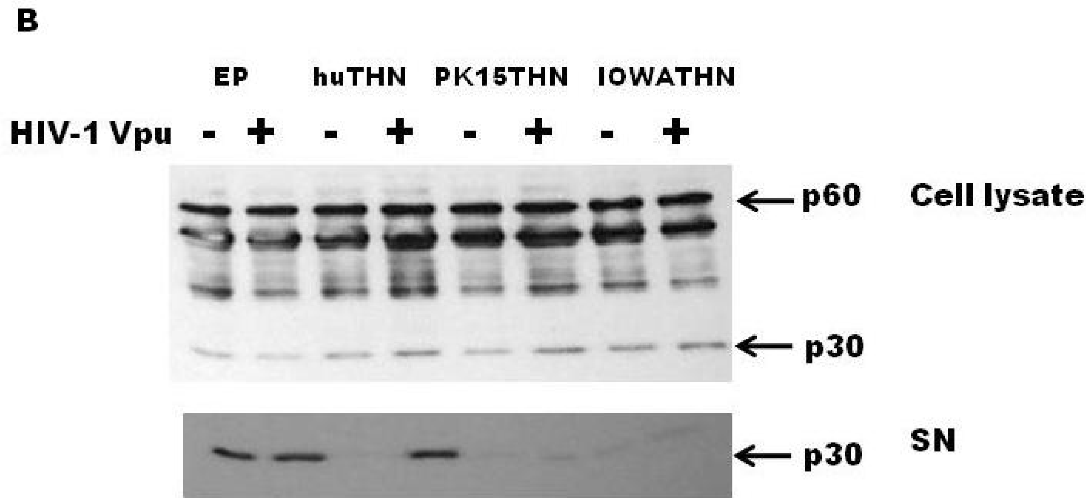


Figure 5.4 Porcine tetherin block is not antagonised by HIV-1 Vpu

A) PERV 2a/PERV2a carrying the reporter gene EGFP was produced by transfection of 293T cells together with 200 ng of huTHN, PK15THN, IOWATHN or equal amount of empty plasmid (EP). 200 ng of an expression plasmid encoding HIV-1 Vpu (white) or the backbone alone (pcDNA3, grey) was also co-transfected. Released virus was titred on 293T cells and monitored by flow cytometry. Histograms represented the average of two experiments and the error bars the standard error of the mean. **B)** PERV2a/PERV2a was produced in the presence of human (huTHN) or porcine (PK15THN and IOWATHN) tetherin and with (+) or without (-) HIV-1 Vpu protein. Producing cells and supernatants (SN) were processed by SDS-PAGE and immunoblotted using an anti-PERV CA antibody. Precursor and intermediate forms of PERV capsid were visualised in the cell lysate (upper panel), while fully processed p30 was the main form present in the supernatant (SN, bottom panel).

5.2.4 No evidence for tetherin countermeasure expressed by PERV-A14/220

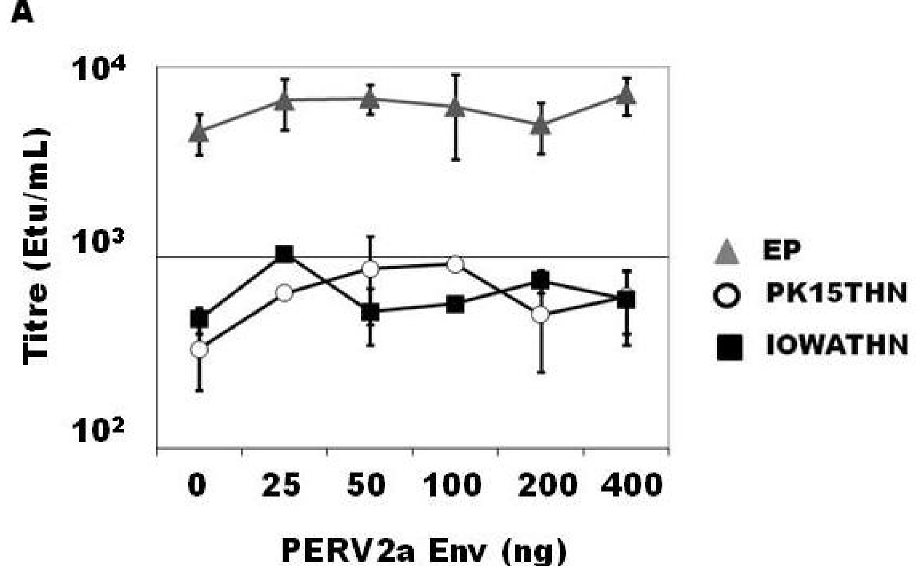
Few lentiviruses encode a Vpu-like protein (Bibollet-Ruche et al., 2004). In other primate lentiviruses, such as rhesus macaque SIV, anti-tetherin function has been assumed by Nef protein (Jia et al., 2009; Zhang et al., 2009). Furthermore, it has recently been shown that envelope glycoprotein of tantalus monkey SIV, can antagonise tantalus, rhesus, sooty mangabees monkey tetherins as well as human tetherin (Gupta et al., 2009b). Similarly, a filovirus, Ebola virus, overcomes human tetherin-mediated restriction with its own envelope glycoprotein (Kaletsky et al., 2009). However, there are no reports on how simple retroviruses such as MLV or PERV can avoid tetherin-mediated restriction.

293T cells were transfected with a retroviral vector expressing the reporter gene EGFP, PERV2a GagPol and VSV-G expressing plasmids, and porcine THN-encoding plasmids. To investigate the ability of PERV-A14/220 (PERV2a) envelope to antagonise THN block, increasing amounts of PERV2a Env plasmid were added to the plasmid mix. After 48 hours, the virus released in the supernatant was titrated on murine, PERV-A-resistant, NIH3T3 cells. These cells were chosen to monitor VSV-G-mediated EGFP transduction specifically, and avoid a contribution in the titre from PERV2a-pseudotyped viral particles. In the absence of PERV2a Env, PK15THN and IOWATHN reduced the viral titre about 10-fold compared to an empty plasmid (EP). The addition of PERV2a Env had no effect on PERV2a/VSV-G titre (Fig 5.5 A, pcDNA3), and did not rescue its reduction caused by porcine THN (Fig 5.5 A). These data suggested that PERV-A Env cannot counteract THN-mediated restriction.

As PERV2a Env alone could not inhibit the restriction posed by THN, I tested whether any other part of the PERV2a genome could. EGFP(MLV) viruses bearing MLV-A Env were produced by transfection of 293T cells together with 200 ng of IOWATHN plasmid. Serial dilutions of the full length PERV2a genome were co-transfected. The viral titre was determined by infection of murine NIH3T3 cells. The PERV2a genome was unable to rescue the 10-fold

reduction of the viral titre caused by IOWATHN (Figure 5.5 B). To corroborate this result, the effect of PERV expression on HIV-1 inhibition by THN was also examined. An HIV-based vector expressing EGFP (pHRSIN-CSGW) was transfected into 293T cells together with packaging plasmids p8.91, encoding HIV-1 GagPol and pMDG, carrying the VSV-G gene. Human or porcine THN was co-transfected in the presence of 200 ng of PERV2a genomic plasmid or an irrelevant construct as negative control (pCNCR). The amount of virus released in the supernatant was titrated on 293T cells. The PERV2a genome was unable to overcome the 20- to 85-fold reduction in EGFP(HIV) titre (Figure 5.5 C).

Taken together these results showed no evidence of an antagonistic effect of PERV2a genome against either human or porcine THN.


A
Titre (Etu/mL)
10⁴
10³
10²
0
25
50
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200
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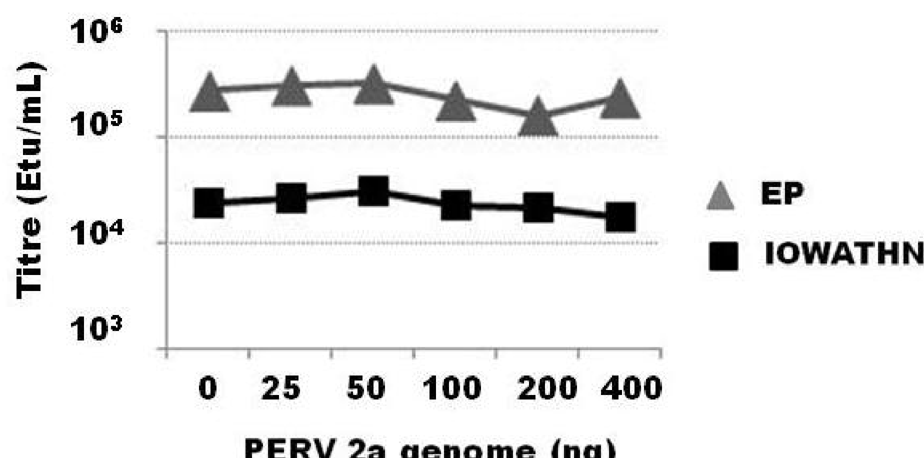
PERV2a Env (ng)
EP
PK15THN
IOWATHN


B
Titre (Etu/mL)
10⁶
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PERV 2a genome (ng)
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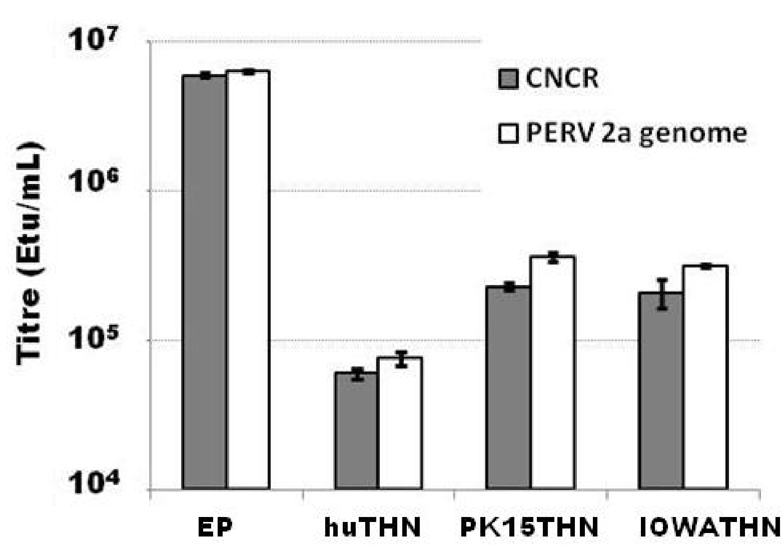

C
Titre (Etu/mL)
10⁷
10⁶
10⁵
10⁴
CNCR
PERV 2a genome
EP
huTHN
PK15THN
IOWATHN

Figure 5.5 PERV-A14/220 Env or genome does not counteract tetherin

A) EGFP encoding MoMLV/MLVA viruses were produced by co-transfection of 293T cells with 200 ng of porcine THN, PK15THN (white circles), IOWATHN (black squares) or an empty plasmid (grey triangles). In addition, a mixture of PERV-A14/220 Env plasmid (PERV2a Env) and empty plasmid pcDNA3, with the total amount of plasmids being 400 ng, was cotransfected. The quantity of PERV2a Env plasmid is indicated in the graph. Viral particles released in the supernatant were titred by EGFP-transduction of NIH3T3 cells and the mean of the titre indicated with the standard error of the mean. **B)** Retroviral particles carrying the EGFP gene with MoMLV core and amphotropic MLV Env were produced in 293T cells in the presence of 200 ng of IOWATHN plasmid (black squares) or empty plasmid (grey triangles). Increasing amounts of full length PERV2a genome was co-transfected together with the empty vector pcDNA3 to equalise the total quantity of plasmids to 400 ng. Titre was determined by transduction of NIH3T3 cells and monitoring for EGFP expression by flow cytometry. **C)** VSV-G pseudotyped HIV particles expressing EGFP were produced by transfection of 293T together with of 200 ng of huTHN, PK15THN, IOWATHN or empty plasmid (EP). 200 ng of PERV2a genome (white) or MLV-based vector carrying RFP gene (CNCR, grey), used as an irrelevant control, were added to the transfection mix. Serial dilutions of the supernatant were employed to transduce 293T cells. Titres were inferred by EGFP expression measured by flow cytometry and the average represented as histograms (± standard error of the mean).

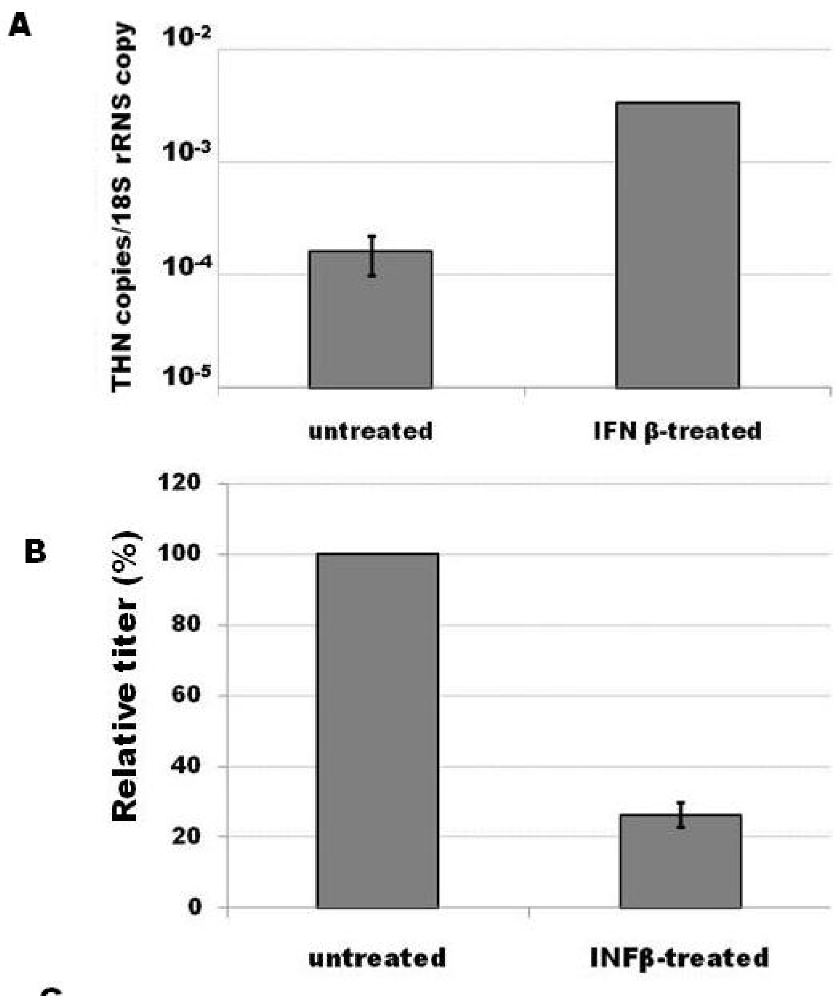
5.2.5 Porcine tetherin is able to reduce the release of PERV particles from pig cells

HuTHN is type I IFN-inducible. Upon treatment of 293T cells with INF-α, the huTHN mRNA amount increases more than 20-fold (Neil et al., 2008). The same dose of INF-α in 293T cells reduces the yield of Vpu-deleted HIV-1 particles released in the supernatant about 10-fold (Neil et al., 2007). To understand whether THN can reduce the amount of PERV particles budding from pig cells, the endogenous THN response to type I IFN was assessed, and the effect of IFN treatment on PERV production in PK15 cells examined.

Pig PK15 cells were treated for 24 hours in the presence of 2000U/mL of type I INF-β. Cells were lysed and the mRNA extracted. The amount of porcine THN was quantified by SYBR Green-based quantitative RT-PCR. IFN-β induced an increase in porcine THN mRNA level 15 times that of untreated cells (Figure 5.6 A).

Serial dilutions of the supernatant from untreated and IFNβ–treated PK15 cells were employed to infect 293T cells. After 72 hours, PERV titre was determined by *in situ* immunostaining of the infected cells using an anti-PERV CA antibody. The PERV titre from IFN-β-treated cells was reduced to 26% that obtained from untreated cells (Figure 5.6 B). The immunoblot of cell lysates and supernatant from untreated and IFN-β-treated PK15 cells showed a similar amount of cell associated PERV Gag (Figure 5.6 C, cell lysates) but a reduction of processed Gag in the supernatant (Figure 5.6 C, SN).

These results showed that similar to huTHN, its porcine homologue is indeed type I IFN-inducible. INF-β treatment of PK15 cells reduced PERV release, possibly via pig THN induction. These data support the hypothesis that overexpressing THN in porcine PERV-producing cells could in fact reduce the release of infectious virus.



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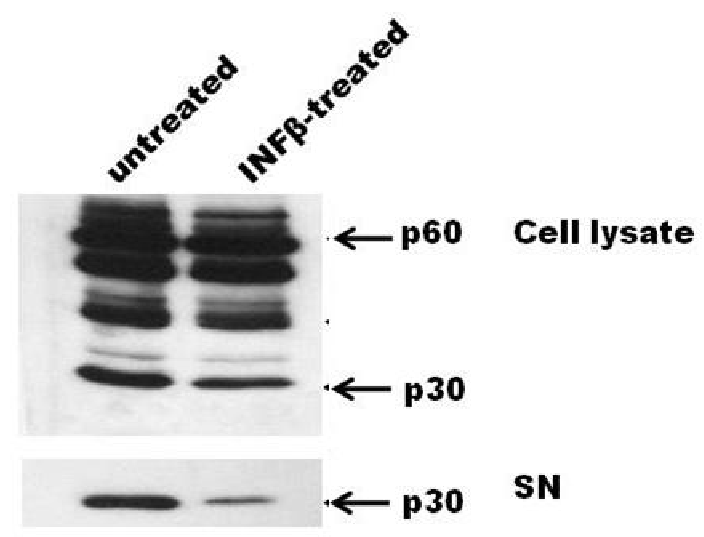

Figure 5.6 Porcine tetherin can reduce PERV particle release from pig cells

$1x10^6$ PK15 cells were seeded in a 6-well plate and 2 hours later 2000U/mL of IFN-β was added to the media. **A)** The day after, cell were lysed and total RNA extracted. 1 μg was reverse transcribed and one eighth was employed as template in a SYBR Green-based quantitative RT-PCR, using primers QF9 and QR9 for porcine THN and QF4 and QR4 for 18S rRNA gene (table 2.3.4). Samples were run in triplicate and the number of porcine THN copies were normalised per copy of 18S rRNA. Histograms represent the average of two independent experiments (± standard error of the mean). **B)** 24 hours after the addition of IFN-β, the supernatant from PK15 cells was used to infect 293T cells and two days later, PERV titre was determined by *in situ* immunostaining of infected 293T cells using rabbit anti-PERV CA antibody. Histograms represent the average of two independent experiments (± standard error of the mean). The reduction in PERV titre in IFNβ-treated cells was found to be significant by t-test (p=0.002) **C)** PK15 cells treated for 24 hours with IFNβ and untreated cells were harvested and lysed in RIPA buffer. Their supernatant was spun down and resuspended in Laemmli buffer. One fourth of the cell lysate and the supernatant was processed by 10% SDS-PAGE and PERV proteins detected using anti-PERV CA antibody. In the cell lysates (upper panel) capsid precursor (p60), the intermediate forms and the processed capsid (p30) were visible. In the supernatant (SN, bottom panel) p30 was the main form represented.

5.2.6 Exogenous expression of tetherin in PK15 cells decreases PERV release

A critical step in the evaluation of THN as a novel strategy to improve the biosafety of porcine donors in xenotransplantation is the ability of exogenously expressed human or porcine THN to inhibit the release of continuously produced PERV particles from pig cells and this needs to be analysed.

Human or pig THN gene was introduced into porcine PK15 cells by HIV-based retroviral particles, carrying also the hygromycin B resistance gene. After 24 hours, hygromycin B was added to the media and the cells were cultivated for the following two weeks to obtain a cell population which was hygromycin B resistant and expressed THN.

Firstly, the expression of the THN in PK15 cells was assessed. Total RNA from transduced and selected cells was processed in a SYBR Green-based quantitative RT-PCR, and the amount of THN mRNA was quantified. Bulk population of PK15THN and IOWATHN-transduced cells expressed, on average, 10-fold more pig THN mRNA than parental cells (Figure 5.7 A). In huTHN-transduced cells, the amount of huTHN was at a similar level to that in HeLa cells (Figure 5.2).

Once the overexpression of THN was assessed, PERV particle release in the supernatant was analysed. The day before infection, THN-expressing PK15 and parental cells were seeded in equal number. Serial dilutions of their supernatant were employed to infect 293T cells, and titre was determined by *in situ* immunostaining of infected cells using an anti-PERV CA antibody. PERV titre from PK15THN and IOWATHN stably expressing cells showed a reduction of 60% in comparison to that from parental cells. Expression of huTHN reduced PERV titre to 23% that of untransduced cells (Figure 5.7 B). These data was supported by western blot analysis of the cell lysates and supernatants from THN-transduced PK15 cells. Whilst the amount of Gag in the cell lysate appeared to be the same between all the samples (Figure 5.7 C, cell lysate), the presence of mature particles in the supernatant of PK15

overexpressing THN was reduced up to 16% that of the parental cells (Figure 5.7 C, SN and band intensity).

These results suggested that overexpression of THN can be employed to reduce the level of viral particles budding from porcine PERV-producing cells.

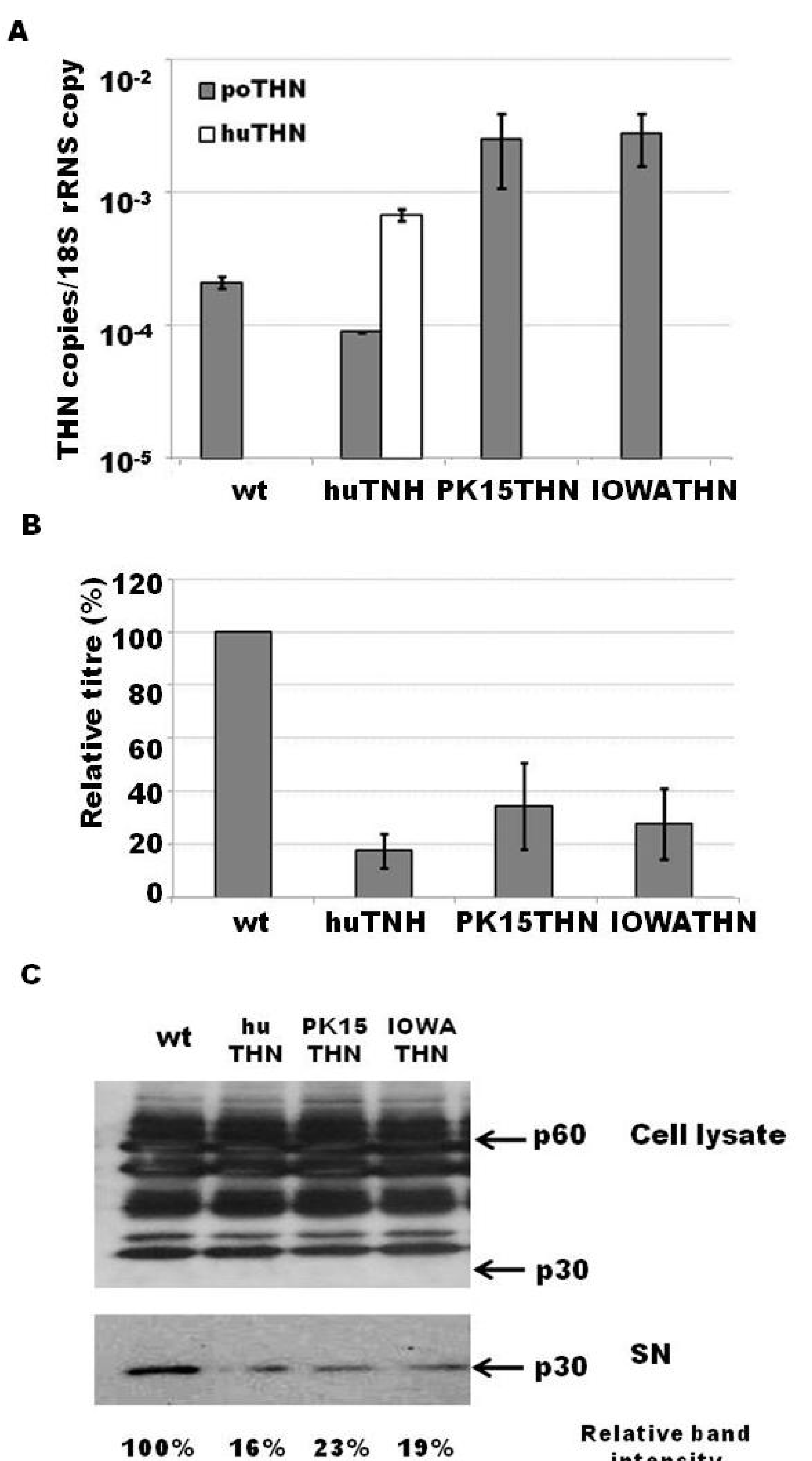

Figure 5.7 Exogenous expression of tetherin in PK15 cells decreases PERV release

PK15 cells were stably transduced by VSV-G pseudotyped HIV-based vector encoding both human or porcine THN and hygromycin B resistance genes. The day following transduction,

Hygromycin B at the concentration of 200 µg/mL was added to the media and the cells cultivated for two weeks. **A)** 1µg of RNA extracted from THN-transduced PK15 or parental cells was reverse transcribed in a reaction volume of 20 µL. SYBR Green-based quantitative RT-PCR was conducted on 2.5 µL of cDNA using primers QF9 and QR9 for porcine THN, QF10 and QR10 for huTHN, and QF4 and QR4 for the 18S rRNA gene. Samples were run in triplicate. The amount of copies for each gene was inferred by standard curves. Histograms represent porcine (poTHN, grey) and human (huTHN, white) THN copies normalised to one 18S rRNA copy. **B)** $1x10^6$ THN-transduced PK15 or parental cells were seeded and the day after, serial dilutions of their supernatant was used to infect 293T cells. Titres were determined by *in situ* immunostaining using an anti-PERV CA antibody. Titres of THN-transduced PK15 were calculated as percentage of the titre from parental cells. Histograms represent the average of two independent experiments (± standard error of the mean). The statistical validity of PERV titre reduction in transduced-PK15 was assessed with t-test. P values were 0.001 for huTHN, 0.028 for PK15THN and 0.013 for IOWATHN. **C)** Supernatant from $1x10^6$ THN-transduced PK15 or parental cells was spun down and resuspended in 2xLaemmli buffer. Cells were lysed in RIPA buffer. One fourth of the cell lysate and the supernatant was separated by SDS-PAGE and immunoblotted using an anti-PERV CA antibody. The chemiluminescence signal was detected by incubation with ECL western blotting reagent and exposed to a X-ray film. The 3 differently processed capsid forms were detected in the cell lysate (upper panel) while in the supernatant (SN) p30 was the most present (bottom panel). Band intensities were determined by analysis of the film with the Kodak 1D programme.

5.3 Discussion

Genetically modified pigs have been generated to overcome the immunological barrier in pig-to-human transplantation (section 1.1.1). The same approach has been employed to reduce the risk of PERV transmission by generating transgenic pigs expressing shRNAs against PERV transcripts (Dieckhoff et al., 2009; Dieckhoff et al., 2008; Ramsoondar et al., 2009). In this chapter a newly described restriction factor, THN, has been investigated as a possible candidate to use in the development of genetically modified pig donors which will enhance biosafety in xenotransplantation.

PERV particles have been observed budding from porcine cell lines and primary cells (section 1.4.1). Initially, I evaluated whether porcine cells encode a homologue to THN and its expression level. By BLAST analysis, a possible candidate was found in the porcine EST database. As THN is differentially expressed in human cell lines (Neil et al., 2008), three pig cell lines were used as source of cDNA to clone porcine THN. The sequences obtained matched with the EST sequence deposited in the NCBI database, with the exception of two non-synonymous nucleotide changes in the PK15 THN (Figure 5.1 B). PK15 cells appeared to possess two heterologous THN alleles. Indeed, by direct sequencing of the cDNA from PK15 cells, the chromatograms showed a polymorphism for both changes (Figure 5.1 B). Since the physiological function of THN remains unclear, speculation on the significance of these polymorphisms is difficult. However, the impact of these two amino acid changes on THN-mediated restriction has been analysed in this chapter (PK15THN).

Both porcine THNs have similar ability to huTHN for reducing PERV and MLV production when co-transfected with the virus-encoding plasmids in 293T cells (Figure 5.3 A). As showed by western blot analysis, THN expression had no impact on viral protein synthesis, but did on the release of the viral particles in the supernatant (Figure 5.3 B). The expression of porcine THN in different cell lines was evaluated by quantitative PCR and compared to that in the human cell lines 293T, which is highly permissive to virus production, and HeLa cells, from which retroviral particles could be efficiently

released only in the presence of factors antagonising huTHN, such as HIV-1 Vpu (Figure 5.2). Porcine THN mRNA was expressed at similar level among pig cells. In comparison to human cells, the three porcine cell lines appeared to have 35 times more mRNA for THN than 293T cells but 5 times less than HeLa cells.

As PERV-producing cells express a discrete level of a porcine THN, it is possible that PERV possesses some countermeasure which acts against this endogenous level of THN and allows PERV production. Therefore, PERV countermeasures for THN were investigated. At least three different viruses, tantalus monkey SIV, HIV-2 and Ebola virus, use their envelope to counteract THN-mediated restriction (Abada et al., 2005; Bour et al., 1996; Bour and Strebel, 1996; Gupta et al., 2009b; Kaletsky et al., 2009; Ritter et al., 1996). The ability to antagonise THN restriction has been evaluated for PERV-A14/220 Env protein. The addition of increasing amount of a plasmid encoding PERV-A14/220 Env did not alter the titre of PERV virus produced from 293T cells in the presence of human or porcine THNs (Figure 5.5 A). The same results were obtained by introducing increasing amount of a plasmid encoding the whole genome of PERV-A14/220 (Figure 5.5 B and C). These data suggested that PERV-A14/220 does not possess an anti-THN function.

Inhibition of PERV particle release by endogenous porcine THN from pig cells was tested. Human THN is type I IFN-inducible (Blasius et al., 2006; Neil et al., 2008). Treatment of PERV-producing PK15 cells with IFN-β caused an upregulation of porcine THN mRNA (Figure 5.6 A) and a reduction of PERV titre (Figure 5.6 B). The decrease of PERV production was associated with a diminished amount of PERV Gag in the supernatant (Figure 5.6 C). These results suggest that IFN-β-induced overexpression of porcine THN can inhibit the release of PERV particles in porcine cells. Whether or not endogenous THN is acting in pig cells to control PERV production under normal physiological condition is unclear.

The final goal was to assess whether exogenously expressed THN could block PERV release from porcine cells. PERV-producing PK15 cells were

stably transduced with a lentiviral vector carrying the human or porcine THN gene and a hygromycin B resistance gene, for selection of transduced cells. The mRNA level of THN was similar to that of huTHN in HeLa cells (Figure 5.7 A and 5.2). PERV titre from THN-overexpressing cells was 23% that obtained from wild type cells (Figure 5.7 B) and this correlated with a reduction of PERV Gag in the supernatant (Figure 5.7 C).

These results showed that by overexpressing THN it was possible to reduce virus release from continuously producing cells. Although these data are encouraging, a complete block of PERV production was not achieved. To improve the system, clones, producing higher amount of THN could be examined. Previous works using shRNAs have showed a similar degree of reduction in PERV production. Reverse transcriptase activity in the supernatant of primary porcine cells was reduced to 25% when shRNA against PERV Pol was expressed (Dieckhoff et al., 2007a) and titre of infectious particles generated from porcine endothelial cell expressing shRNA against PERV proteins was 85% lower than parental cells (Miyagawa et al., 2006). Genetically modified pigs could be generated expressing THN together with other anti-PERV strategies (e.g. shRNAs), which target a different step of the retroviral life cycle and therefore are unlikely to interfere with each other, to decrease the risk of PERV transmission.

Chapter 6

6 Discussion

Porcine cells and tissues have been used to treat human diseases (Chari et al., 1994; Deacon et al., 1997; Fink et al., 2000; Groth et al., 1994; Reichenbacher, 1975) and, at present, two clinical trials are taking place in Russia and New Zealand to treat type I diabetes by injection of porcine, insulin-producing cells (DIABECELL[(R)]) (http://www.lctglobal.com/lct-diabecell-diabetes-treatment.php). Initial studies of pig-to-human transplantation in the 1990s have been interrupted following the discovery that porcine endogenous retroviruses produced from pig cells can infect human cells *in vitro* (Patience et al., 1997). Yet, retrospective studies on patients exposed to porcine materials failed to detect any evidence of PERV transmission. Moreover, no disease has been associated with PERV in pigs, although higher PERV expression was found in melanomas of Munich miniature swine Troll, when compared with normal tissue (Dieckhoff et al., 2007b). The risks of a cross-species infection are unpredictable, as human history exemplified (Kramer et al., 2007; de Wit and Fouchier, 2008; Alexander and Brown, 2000; Gao et al., 1999; Keele et al., 2006; Paton et al., 1999; Chua et al., 1999; Chua, 1999; Smith et al., 2009; Garten et al., 2009; Drosten et al., 2003). Potential pathologies associated with PERV infection could be deduced by similarity with diseases described for other member of the γ-retrovirus genus, which PERV belongs to: development of tumours, leukaemia and neurodegeneration (section 1.3.4). Therefore, during the past 12 years, many studies have been conducted on four main aspects of PERV:

1) Virus biology: replication-competent PERV subgroups and their tropism (Akiyoshi et al., 1998; Le Tissier et al., 1997; Patience et al., 2001; Takeuchi et al., 1998; Wilson et al., 2000), viral determinant for infectivity (Argaw et al., 2008; Gemeniano et al., 2006; Marcucci et al., 2008; Watanabe et al., 2005), recombination (Bartosch et al., 2004; Harrison et al., 2004; Oldmixon et al., 2002; Wilson et al., 2000) and critical PERV *loci* in the pig genome (Herring et al., 2001).

2) Diagnostic tools to monitor PERV expression in both porcine donor and human recipient (Bartosch et al., 2002; Blush et al., 2000; Chiang et al., 2005 Fisher et al., 2003; Galbraith et al., 2000; Lovatt et al., 1999; Matthews et al., 1999; Shah et al., 2003; Switzer et al., 1999; Tacke et al., 2001; Xu et al., 2003).

3) Virus-host interaction. This aspect has been particularly difficult to study due to the lack of evidence of PERV infection in humans and the unavailability of a suitable animal model. The receptors used by PERV subgroup A to infect human cells have been identified (Ericsson et al., 2003) and many animal models evaluated (Clemenceau et al., 2002; Deng et al., 2000; Elliott et al., 2005; Isaac et al., 2005; Moscoso et al., 2005; Nishitai et al., 2005; Ritzhaupt et al., 2002; Specke et al., 2009; Specke et al., 2001; Templin et al., 2000; van der Laan et al., 2000; Zhang et al., 2005).

4) Strategies to prevent PERV infection. Most of these are focussed on the improvement of the safety of the animal donor, like selection of low risk PERV transmission animals (Garkavenko et al., 2008a; Garkavenko et al., 2008b; Hector et al., 2007) or discovery of inhibitory molecules which can be employed for the generation of transgenic pigs (Dekker et al., 2003; Dieckhoff et al., 2009; Dieckhoff et al., 2008; Dorrschuck et al., 2008; Jonsson et al., 2007; Miyagawa et al., 2006; Ramsoondar et al., 2009). Vaccine and anti-viral drugs have also been considered (Fiebig et al., 2003; Powell et al., 2000; Qari et al., 2001; Shi et al., 2007; Stephan et al., 2001; Wilhem et al., 2002).

In this thesis two of these aspects have been explored: virus-host interaction, specifically the study of the cellular receptor used by PERV-A to infect cells, and the evaluation of a new strategy to reduce the risk of PERV transmission.

In chapter three, PERV-A receptor was characterised in order to gain a better understanding of the mechanism of virus entry in human cells. Two functional PERV-A receptors (huPAR-1 and huPAR-2) have been identified in human cells (Ericsson et al., 2003). Critical regions for PERV-A infection in the

receptor have been characterised using chimeric receptors between huPAR-2 and the murine homologue, muPAR, which does not support PERV-A infection (Ericsson et al., 2003 and Figure 3.3). A single amino acid (a.a. 109) was shown to be important not only for PERV-A infection but also for the virus binding (Figures 3.4 and 3.7). These results, together with experimental evidence of the receptor topology (Figures 3.5 and 3.6), suggested that the second extracellular loop, where a.a. 109 is located, is the virus binding site, or at least a part of it. These data may help develop reagents that block PERV entry, such as neutralising antibodies and peptides mimicking the receptor. Furthermore, the characterisation of an important structural domain in the PERV-A receptors, obtained in chapter three, allowed the investigation of the mechanism behind the poor susceptibility to PERV-A of non-human primate (NHP) cells, conducted in chapter four.

As mentioned above, a major problem in the prediction of the consequences of PERV infection is the lack of a suitable animal model. Because NHP are currently employed in xenotransplantation to evaluate the immunological response to porcine xenografts, it would be ideal to monitor PERV transmission at the same time. However, to extrapolate information that may be applied to pig-to-human transplantation, NHP cells should be as permissive to PERV-A as are human cells. Instead, data presented in the literature suggest this not to be the case (Blusch et al., 2000a; Martin et al., 1999; Specke et al., 2001; Takeuchi et al., 1998; Templin et al., 2000; Wilson et al., 2000). It was initially reported that although PERV can infect NHP cells, it cannot replicate in them, because of an unknown mechanism (Ritzhaupt et al., 2002). However, my results suggest that once PERV successfully enters NHP cells, infectious particles could be produced (Figure 4.2). These results were obtained by endowing NHP cells with huPAR-2, suggesting that virus entry is a critical step in the low permissivity of NHP cells. Indeed, rhesus macaque, cynomolgus monkey and baboon PAR-1 receptors were unable to support PERV-A infection (Figure 4.3). By comparison with the data obtained from the analysis of muPAR (Figure 3.4), it was possible to determine that a.a. 109 was the main determinant for the inability of rhPAR-1, cynPAR-1 and baPAR-1 to mediate virus infection. The

low permissivity to PERV-A of cells from these NHP species is probably due to a PAR-2-mediated entry. However, the level of expression of PAR-2 is lower than huPAR-1 in human cells (Figure 4.5). These results suggest that rhesus macaque, baboon and cynomolgus monkey represent a less than ideal model to evaluate the risk of PERV transmission in humans. Yet, they are the most used NHP species in pig-to-NHP transplantation [reviewed in Eckser et al., 2009)] and most PERV transmission studies on NHP animals *in vivo* were conducted on baboons and rhesus monkeys (Moscoso et al., 2005; Nishitai et al., 2005; Simon et al., 2003; Specke et al., 2009; Switzer et al., 2001).

More promising could be the use of African green monkey as a model. Both AGMPAR-1 and AGMPAR-2 are able to support PERV-A infection (Figure 4.3), and they are expressed in at least two AGM cell lines, COS7 and VERO, at a similar level to human cell lines (Figure 4.5). However, AGM cells are poorly infected by PERV-A. It is interesting to note that the resistance to PERV-A by rodents and NHPs is similar. In both cases, some genera in the same order (*mus* or *macaca* and *papio*) have a mutation in the receptor (at the same amino acid) which disrupts its ability to support PERV-A entry, while others (*rattus* or *chlorocebus*) encode functional receptors but are still refractory to PERV-A infection. In chapter four and three, I tried to unveil the mechanism behind the poor (or lack of) susceptibility to PERV-A in AGM and rat cells. In both cases, I couldn't obtain a clear answer. RatPAR expression is lower in rat cells than huPAR-1 in human cells (Figure 3.9), and PERV-A infection depends on PAR expression levels (Figure 3.13), suggesting that ratPAR is expressed at a subthreshold level. However, an explanation for the low expression of ratPAR could not be found. In AGM cells, the expression level of PARs is similar to those in human cells (Figure 4.5). In addition, once the same amount of huPAR-1 and AGMPAR-1 was expressed on the cell surface of PERV-A resistant QT6 cells, the efficiency of EGFP transduction was similar (Figure 4.9 A), suggesting a comparable affinity of the receptors for the virus. Some of the receptor-mediated block mechanisms described for γ–retroviruses have been investigated and none of them could clearly be held responsible for the low permissivity of NHP cells to PERV-A (Figure 4.3-

5-6-7-8). Since tunicamycin treatment could rescue infectivity (Figure 4.8-9-10), its possible mechanism was investigated. My first hypothesis was that a heavy N-glycosylation of the receptor could prevent PERV binding, and that tunicamycin treatment could relieve this block. However, tunicamycin treatment did not increase receptor binding (Figure 4.8-9-10), suggesting that receptor masking by a heavy glycosylation was not the cause of the poor PERV-A susceptibility. Secondly, I looked at PERV attachment to the cell surface of tunicamycin-treated and untreated cells. No difference was observed (Figure 4.11). Therefore, I excluded that tunicamycin treatment was affecting a different molecule, other than the receptor, involved in the adsorption of the virus on the cell surface. Finally, I looked at the ratio between the amount of receptor expressed on the cell surface versus the total amount in the cell, to investigate a possible mechanism of entrapment of the receptor in the cytosol which could be relieved by tunicamycin treatment. There was no evidence of a difference between AGM cells and QT6 cells (Figure 4.12). To conclude, AGM with a functional PAR could potentially be a suitable animal model to evaluate the risk associated with PERV transmission to humans in xenotransplantation. However, further investigations should be conducted to clarify the mechanism behind the poor permissivity of AGM cells to PERV-A and to examine PERV-A infection in primary cell culture.

In chapter five, I evaluated a newly described restriction factor as a possible strategy to prevent PERV transmission. Tetherin (also known as BST2, CD317, HM1.24) has been shown to retain enveloped viruses from different families on the surface of the producing cells, inhibiting virus release (Neil et al., 2008, Jouvenet et al., 2009; Sakuma et al., 2009). This anti-viral mechanism appears to be part of the innate immune system response (Blasius et al. 2006; Neil et al., 2008). To examine whether a similar mechanism was present in pigs, the porcine homologue to huTHN was been cloned (Figure 5.1) and its activity against PERV and another retrovirus (MLV-A) confirmed (Figure 5.3). The efficiency of pig THN in inhibiting viral particles release in the supernatant of the producing cells was similar to that of huTHN (Figure 5.3). A stable expression of either human or porcine THN

in pig PK15 cells could significantly reduce the virus production (Figure 5.7). From these results two main points of discussion emerged.

Firstly, by overexpression of THN in porcine cells, PERV production could only be reduced but not completely blocked. This system could be improved by selection of a clonal population with higher expression of THN and minimal production of PERV. Indeed, similar strategies, already employed to reduce PERV transmission, never achieved a total lack of virus production from porcine cell lines or primary cells. Expression of shRNA directed against PERV Pol led to a 25% reduction in reverse transcriptase activity in the supernatant of shRNA-transduced pig PBMC (Dieckhoff et al., 2007a). Similar results were obtained when antibodies against PERV p15 Matrix were expressed in PK15 cells where, in a clonal population, RT activity was reduced to 7% that from parental cells (Dekker et al., 2003). Suggested improvement to the system by these articles was the expression of multiple shRNAs or antibodies, respectively. However, by combining different strategies which act in different steps of the viral life cycle a better protection could be achieved. In this case, THN could represent an ideal candidate to be employed together with shRNAs, which target viral transcripts before translation. In addition, THN has an advantage over other systems through its ability to target a broad range of enveloped viruses (multiple genera of retroviruses, filoviruses and potentially herpesviruses). Therefore, overexpression of THN in the cells of a pig donor could represent a safer strategy also against unknown enveloped viruses. However, a long term observation of the cells overexpressing THN should be conducted to exclude pathological effects.

Secondly, porcine THN is expressed in the PERV-producing cell lines PK15 and MPK (Figure 5.2). Upregulation of THN mRNA by less than 20-fold by INF-β-treatment induced a reduction of viral particles released in the supernatant (Figure 5.6). Furthermore, from the results presented in Figures 5.4 and 5.5, no countermeasures against THN have been found in the PERV genome. It is puzzling to understand what is happening in pigs *in vivo*. To assess whether THN is somehow controlling PERV release, THN-knock

down pig cells could be examined. If PERV titre increases upon THN-knock down, it would suggest that endogenous porcine THN is acting against PERV *in vivo*. Furthermore, since different human cells express unequal amount of THN (Neil et al., 2008), various porcine primary cells and tissues should be tested for tetherin production. In the scenario that THN was expressed at a low level, speculation could be made that PERV has not developed an anti-THN countermeasure because there was not enough selective pressure.

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