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Mechanisms of endogenous retrovirus control in the host cell

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I

I hereby certify that I have written this thesis independently and that I have not used other than the cited sources.This thesis has not been submitted for any other degree or purposes.

1 INTRODUCTION 9

2 AIMS 10

3 LITERATURE REVIEW 11

3.1 EPIGENETICS 11
3.1.1 DNA methylation 12
3.1.2 Establishment of the methylation pattern in vertebrates 12
3.1.3 Histone modifications and chromatin function 16
3.2 RETROVIRUSES 17
3.2.1 The retroviral life cycle 18
3.2.2 ERVs and other retroelements 20
3.2.3 Silencing and regulation of retroviruses and retroelements 22
3.3 HERVS AND THEIR DOMESTIFICATION 26
3.3.1 ERVs in placenta 27
3.3.2 Human syncytins 28
3.4 PERVS AND XENOTRANSPLANTATION 30
3.4.1 History of xenotransplantation 30
3.4.2 Problems and risks of xenotransplantation 31
3.4.3 Humanization of pigs 32
3.4.4 Zoonotic infection 33
3.4.5 PERVs 35

4 MATERIALS AND METHODS 38

4.1 CELL CULTURES 38
4.2 HUMAN TISSUE SAMPLES AND DNA SAMPLES 38
4.3 PIG TISSUE SAMPLES 39
4.4 PLASMIDS 39
4.5 BISULFITE CONVERSION OF GENOMIC DNA FOR METHYLATION ANALYSIS 41
4.6 METHYL-SPECIFIC QUANTITATIVE PCR (MS QPCR) 41
4.7 BISULFITE SEQUENCING 42
4.8 TREATMENT OF HELA AND ST-IOWA CELLS WITH AZAC AND TSA 44
4.9 SSSI METHYL-ACCEPTING ASSAY 44
4.10 RNA EXTRACTION AND REVERSE TRANSCRIPTION 44
4.11 CDNA PCR AMPLIFICATION 44
4.12 *IN VITRO* DNA METHYLATION 47
4.13 DNA TRANSFECTION AND REPORTER EXPRESSION ASSAYS 47
4.14 TRANSFECTION, VIRUS PRODUCTION AND INFECTION 47
4.15 FLOW CYTOMETRY ANALYSIS 48

5 RESULTS 49

5.1 ROLE OF CPG METHYLATION IN THE REGULATION OF SYNCYTIN-1 AND -2 EXPRESSION 49
5.1.1 CpG methylation of ERVWE1 and ERVFRDE1 in human tissues and cell lines 49
5.1.2 Stability of CpG methylation of the ERVWE1 5' LTR in non-placental cells 51
5.1.3 CpG methylation suppresses the transcriptional activity of ERVWE1 LTR in vitro 53
5.1.4 Quantitative analysis of the ERVWE1 methylation in tumors 54
5.1.5 Analysis of the ERVWE1 methylation and expression in tumors 55
5.1.6 Analysis of the ERVFRDE1 methylation in the testes and testicular tumors 58
5.2 CPG METHYLATION AND THE EXPRESSION OF PERV 59
5.2.1 CpG methylation decreases the transcriptional activity of PERV LTRs. 59
5.2.2 The majority of PERV LTRs in porcine tissues are methylated. 60

5.2.3 Quantitative analysis of hypomethylated PERV LTRs in porcine tissues and cell lines *62*
5.2.4 Methylation pattern of individual PERV proviruses *65*
5.2.5 CpG methylation stability of the PERV LTRs *66*
5.2.6 Methylation of PERV proviruses in human cells *69*
5.3 PERV-A RECEPTORS 70
5.3.1 Comparison of PERV-A receptor sequences *70*
5.3.2 Functionality of human and rodent PARs *71*
5.3.3 Quantification of RatPAR receptor RNA *72*
6 DISCUSSION 74
7 CONCLUSIONS 86
8 SUMMARY 89
9 SOUHRN 89
10 REFERENCES 90

ABBREVIATIONS

α1,3-gal	α1,3-galactose
α1,3-GT	α1,3-galactosyltransferase
AHXR	acute humoral xenograft rejection
ACXR	acute cellular xenograft rejection
Aza-C	5-azacytidin
BSE	bovine spongiform encephalopathy
BPTF	bromodomain and PHD finger transcription factor
CHD1	chromodomain 1
DMEM	Dulbecco's modified Eagle Medium
DAF	decay accelerating factor
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
env	envelope glycoprotein
ERV	endogenous retrovirus
ES	embryonal stem
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Gag	group specific antigen
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GALV	gibbon ape leukaemia virus
GCMa	glial cell missing a
H	histone
hASCT2	human Na+ dependent neutral aminoacid transporter 2
HAR	hyperacute rejection
HIV	human immunodeficiency virus
HDAC	histon deacetylase
HP1	heteroprotein 1

HERV	human endogenous retrovirus
HMTase	histon-methyltransferase
HuPAR	human PERV-A receptor
IAP	intracisternal A-type particles
ICF	immunodeficiency, centromeric instability, facial anomalies syndrome
K	lysine
L1	LINE 1
LINE	long interspersed nuclear elements
LTR	long terminal repeat
MBD	methyl-CpG binding domain
MFSD2	major facilitator superfamily domain containing 2
MHC	major histocompatibility complex
MeCP2	methyl CpG binding protein 2
MLV	murine leukaemia virus
MMLV	Moloney murine leukemia virus
MS qPCR	methyl-specific quantitative PCR
muPAR	murine PERV-A receptor
NURF	nucleosome remodelling factor
ORF	open reading frame
PAR	PERV-A receptor
PBMC	peripheral blood mononuclear cells
PBS	primer binding sequence
PERV	porcine endogenous retrovirus
PHD	plant homeodomain
Pol	polymerase
R	repeat sequence
ratPAR	rat PERV-A receptor
RBS	repressor binding sequence
RT	reverse transcriptase

RPII	RNA polymerase 2A
SARS	severe acute respiratory syndrome
SINE	short interspersed nucleasr elements
SU	surface unit
TM	transmembrane glycoprotein
TSA	trichostatin-A
U3	unique 3’ end sequence
U5	unique 5’ sequence

1 Introduction

Endogenous retroviruses (ERVs) are retroviruses which infected the host germ cells, integrated into the host genome and are inherited by the descendants together with other host genes. First ERVs were discovered in the late 1960s and early 1970s in mouse and chicken (reviewed in Weiss, 2006). Since then, ERVs have been found in all researched vertebrates. In mammals, ERVs form about one tenth of the genome and in human, retroviral sequences represent 8% of the genome (International Human Genome Sequencing Consortium, 2001; Mouse Genome Sequencing Consortium, 2002). The lack of selection pressure led to accumulation of mutations and deletions in the proviral genomes. For example, the human genome does not contain any complete replication competent ERV; however, some open reading frames (ORFs) remained untouched. In contrast, in pigs a recently integrated replication active family was detected (Patience et al, 2001). Thanks to long co-evolution, the host organisms became adapted to the ERVs and *vice versa*, ERVs are not pathogenic under normal circumstances. Sometimes the co-evolution led to symbiosis and the host employs the viral genes and proteins or the regulatory sequences. This is the case of syncytin genes involved in the placenta development (Mi et al., 2000; Blond et al., 2000). However, their expression must be tightly regulated because ERV expression in inappropriate tissues was shown to be connected with neurodegenerative or autoimmune diseases or cancer development. Various mechanisms including DNA methylation, histone modifications and other epigenetic mechanism are involved in ERV silencing.

Replication competent ERVs may be transmitted both vertically and horizontally. Transmission of viruses to new non-adapted species is usually accompanied by pathogenic symptoms in the new host. The best known example of retrovirus zoonosis is the transmission of low-pathogenic simian immunodeficient virus SIV to human, where its human derivative HIV causes AIDS. Recently, substantial attention has been paid to possible transmission of porcine ERVs (PERVs) to human because pigs are considered to be convenient donors for xenotransplantation. Infection of human cells *in vitro* was demonstrated (Patience et al., 1997); however, no transmission to patients treated with porcine material has been observed. It is still unclear what are the mechanisms ensuring human resistance to PERV despite the close contact of human and pig tissues.

In my work, I studied the regulation of ERVs by DNA methylation, particularly its involvement in the regulation of human syncytins expression in placenta and tumors and in the silencing of PERV expression in pig tissues. Additionally, I analyzed the resistance to PERVs at the level of viral entry.

2 Aims

ERVs form a substantial part of the mammalian genome; however, it is not yet clear what benefits and drawbacks they represent for the host organism. Both these aspects are subjects of my work. Even though some ERVs are essential for the host, their strict regulation is crucial for the maintenance of genome integrity and for the protection against oncogenic and fusogenic properties of ERVs and other pathogenic manifestations. The main topic of my theses is involvement of DNA methylation in regulation of human endogenous retroviruses (HERV) and porcine endoenous retroviruses (PERV).

Recently, HERV proteins *syncytin-1* and *syncytin-2* were shown to be involved in human placenta development. Their fusogenic and immunosuppressive properties are inevitable for placenta correct function a fetus protection. However, in other tissues the same features could induce tissue impairment. In the first experiments we examined whether DNA methylation of 5'LTRs of ERVWE1 and ERVFRDE1 bearing *syncytin-1* and *syncytin-2*, respectively, is connected with their decreased expression. (1.1) We aimed to determine the ERVWE1 and ERVFRDE1 DNA methylation in placenta tissue with physiologic expression of *syncytins* and in other human tissues where *syncytins* were not detected.

Increased expression of HERVs, including ERVWE1 and ERVFRDE1, was observed in various tumors. Their immunosuppressivity and fusogenicity could influence cancer development and prognoses. (1.2) We examined the presence of ERVWE1 RNA in various tumors with special attention to the testis, where weak expression was observed also in healthy tissue. We have focused on the efficiency of ERVWE1 RNA splicing in the screened tissues because splicing is another regulatory step in retroviral expression and could be important in the regulation of ERV proteins as well.

Cell transformation is often accompanied by changes in the DNA methylation pattern. We assumed that expression of syncytins should be preceded by demethylation of their regulatory sequences and we compared ERVWE1 and ERVFRDE1 5'LTRs in tumors and non-tumor tissues with particular orientation to testicular tumors.

Another group of ERVs that have recently been in the center of attention are PERVs because of their possible threat for human in the cases where pigs were used as donors of organs for xenotransplantation. The close contact of pig and human tissues in the xenotransplanted patient could be ideal for zoonotic infection and evolvement of new human

viruses. It was shown that some PERVs are able to infect human cells *in vitro* and that in contrast with most animal cells, human cells possess functional receptors. However, no transmission was detected in patients treated with porcine material. This discrepancy is probably connected with immune protection of the human organism as well as with differences of virus production in the tissues and in cell culture. We intended to examine whether DNA methylation of PERVs plays a role in the determination of the transmission status of porcine cells. (2.1) We have verified the PERV 5'LTR sensitivity to DNA methylation *in vitro* and (2.2) analyzed the PERV LTR methylation in a number of porcine tissues from various pig breeds and in transmitting and non-transmitting porcine cell lines. We aimed to identify either tissue or pig with globally hypomethylated PERVs and increased PERV expression or to identify particular highly expressed hypomethylated PERV provirus.

Mammalian cells infected with retrovirus usually recognize the retroviral sequence, silence its expression and gradually methylate the provirus. The high permissiveness of human cells to PERVs suggests that they are not able to induce efficient PERV silencing. (2.3) We aimed to resolve the progression of PERV LTR methylation in infected human cells.

Human cell permissiveness to PERVs is largely determined by the presence of functional receptors. To date, only receptors for PERV-A have been identified. Despite that PAR homologs were detected in all screened animals, their cells are not permissive to PERV-A. The mouse homolog was identified as non-functional as PERV-A receptor. (3) We aimed to identify the reason for mouse and rat resistance to PERV-A entry.

3 Literature review

3.1 Epigenetics

Epigenetic mechanisms chemically modify chromatin without changing the nucleotide sequence and in this way regulate gene expression. Epigenetic modifications are heritable and remain through mitosis as well as meiosis. The major epigenetic modification of the DNA molecule is cytosine methylation. Other epigenetic mechanisms consist in various modifications of the histone tails such as acetylation, methylation, phosphorylation, ubiquitylation or sumoylation. These modifications are interconnected and together remodel the chromatin into more or less open and transcriptionally active form.

3.1.1 DNA methylation

DNA methylation is a covalent modification of DNA catalyzed by DNA methyltransferase enzymes (DNMTs). The DNA methylation is essential for the cell differentiation, genomic imprinting, inactivation of the proviral sequences and the transposable elements and for the inactivation of the sexual chromosome in the homogametic sex. High methylation usually leads to transcriptional silencing. Methylation is involved in gene expression regulation in animals, plants as well as in fungi. In prokaryotes the methylation was also discovered; however, distinct sequences are methylated there and its function differs as well.

The level of methylation significantly varies in different animal genomes. In vertebrates the genomic DNA methylation is found throughout the genome predominantly within the CpG dinucleotides. In contrast, several well-studied model systems such as *Saccharomyces cerevisiae* or *Caenorhabditis elegans* have no recognizable *Dnmt*-like genes and are devoid of DNA methylation. *Drosophila melanogaster* has a DNMT which induces methylation of the minority of cytosines. Unlike in vertebrates these are part of the CpT and CpA dinucleotides. In fungi that have genomic 5-methylcytosine, only repetitive DNA sequences are methylated (Antequera et al., 1984). The most frequent pattern in invertebrate animals is the mosaic methylation, comprising domains of heavily methylated DNA interspersed with domains that are methylation free (Simmen et al., 1999). The highest levels of DNA methylation among all eukaryotes have been observed in plants, with up to 50% of cytosine being methylated in some species (Montero et al, 1992).

In vertebrates approximately 60% to 90% of CpG dinucleotides are modified. The exceptions are CpG islands, CpG-enriched sequences that frequently coincide with gene promoter regions and are generally unmethylated. Methylation commonly suppresses the transcriptional activity by recruiting methylation-dependent repressors. Among these repressors belong proteins with the methyl-CpG binding domain (MBDs), MeCp2 (reviewed by Bogdanovic and Veenstra, 2009) and several structurally unrelated methyl-CpG-binding zinc-finger proteins of the Kaiso family (Kaiso/ZBTB33, ZBTB4 and ZBTB38). (Prokhortchouk et al., 2001). Most of these proteins associate with histone deacetylase activity and establish silent chromatin.

3.1.2 Establishment of the methylation pattern in vertebrates

Although stable and inheritable in somatic cells, global DNA methylation patterns are dynamic during the mammalian life cycle. Global remodeling of DNA methylation occurs

twice in mammals, during gametogenesis and preimplantation development (Morgan et al., 2005). The first erasure of DNA methylation marks takes place during gametogenesis, when also the imprinted marks are reset, which is followed by a wave of remethylation that is needed for establishment of the parental imprints. The second demethylation event takes place during preimplantation development and does not affect the imprinted regions (Mann and Bartolomei, 2002).

DNA demethylation is an active process. It is mechanistically linked to the appearance of single-stranded DNA breaks and the activation of the base excision repair pathway. The genome-wide DNA demethylation is interconnected with chromatin changes. The histone chaperones, which are implicated in histone exchange, accumulate in primordial germ cell nuclei undergoing reprogramming. Therefore, it seems that the mechanism of histone replacement is critical for these chromatin rearrangements to occur (Hajkova et al., 2008, 2010)

The fast demethylation after fertilisation in not common for all vertebrates. For example, in the Xenopus paternal genome chromatin structure changes without active demathylation were observed (Stancheva et al., 2002)

The global demethylation is followed by the de-novo methylation. The DNA methylation mark is set by three DNMT family members: DNMT1, DNMT3a, and DNMT3b. DNMT3a and DNMT3b fall in the group of *de novo* methyltransferases, enzymes that are able to methylate previously unmethylated CpG sequences, while DNMT1 functions as a maintenance methylase, copying the preexisting methylation marks onto the new strand during replication (reviewed by Jeltsch, 2006; Bogdanovic and Veenstra, 2009). Although generally thought of as a maintenance methylase, DNMT1 has also been shown to function as a *de novo* DNMT (Pradhan et al., 1999). In addition, two non-canonical family members, DNMT2 and DNMT3L, have been discovered (Okano et al. 1998 9592134; Aapola et al., 2000). The loss of DNMT1 proved to be lethal with the majority of embryos not passing midgestation, although the embryonal stem (ES) cells remained viable and proliferative (Li et al., 1992). The DNMT1-depleted mouse fibroblasts showed reactivation of placental and germ line markers pointing out the role of DNMT1 for tissue-specific gene expression and embryonic development (Jackson-Grusby et al., 2001).

DNMT3a and DNMT3b targeting in mice revealed that both *de novo* DNMTs are essential for early mouse development (Okano et al. 1999). Although the expression patterns of DNMT3a and DNMT3b are largely overlapping, the functions that they carry out do not seem to be completely redundant since both knockouts turned out to be lethal. DNMT3a-

depleted mice appeared normal after birth but died at four weeks of age. On the other hand, no DNMT3b knockouts were recovered at birth. The double knockout induced a more severe phenotype since the affected embryos showed developmental defects at E8.5 and died shortly after gastrulation.

DNMT2 appears to be dispensable for *de novo* DNA methylation in mouse ES cells (Okano et al. 1998), while *in vitro* experiments detected only a weak methyltransferase activity (Hermann et al. 2003). DNMT2 was found to function as a tRNA methyltransferase that specifically methylates cytosine 38 in the anticodon loop (Goll et al. 2006).

DNMT3L is a catalytically inactive DNMT which is known to associate with both DNMT3a and DNMT3b to establish regions of maternal imprinting (Hata et al. 2002). Furthermore, DNMT3L is able to recruit histone deacetylases through its plant homeodomain (PHD) zinc-finger-like motif and possibly directs repression onto newly established imprints (Aapola et al. 2002; Deplus et al. 2002, reviewed by Bogdanovic and Veenstra, 2009).

The establishment of the methylation pattern is not yet clear. One possibility is that *de novo* DNA methylation in early mammalian development is an indiscriminate process potentially affecting all CpGs. Compatible with the default model is the apparent absence of intrinsically unmethylatable DNA sequences in mammalian genomes. However, not all regions of the genome are equally accessible to DNMTs. DNMT3B in particular is known to be required for *de novo* methylation of specific genomic regions, as mice or human patients with DNMT3B mutations are deficient in methylation of pericentromeric repetitive DNA sequences and at CpG islands on the inactive X chromosome. DNMT3B may therefore be adapted to methylate regions of silent chromatin (reviewed by Bird et al., 2002).

Another hypothesis to explain global methylation is that the DNA methylation machinery is preferentially attracted by certain DNA sequences in the mammalian genome. The presence of high levels of methylation in DNA outside such a DNA methylation center could be explained by spreading into the surrounding DNA. A hypothetical trigger for DNA methylation is DNA sequence repetition, which can promote *de novo* methylation in filamentous fungi and plants under certain circumstances (reviewed in Martienssen and Colot 2001). The most suggestive evidence in mammals concerns manipulation of transgene copy number at a single locus in the mouse genome using cre-lox technology (Garrick et al. 1998). High levels of transgene repetition were found to cause significant transgene silencing and concomitant methylation. As the copy number was reduced at the locus, the level of methylation decreased and the efficiency of expression increased.

Several lines of evidence suggest that DNA methylation does not intervene to silence active promoters, but affects genes that are already silent. *De novo* methylation of proviral sequences in embryo cells depends on DNMT3A and DNMT3B (Okano et al. 1999), but initial retroviral shutdown occurs as usual even when both these *de novo* methyltransferases are absent (Pannell et al. 2000). Clearly, *de novo* methylation is not required for silencing in the first instance; reinforcing the view that methylation is a secondary event.

The new cell-specific methylation pattern is established after blastula implantation (Fig.I). The methylation level is quickly increasing in the embryoblast while it is supressed in trophoblast (Santos et al, 2002).

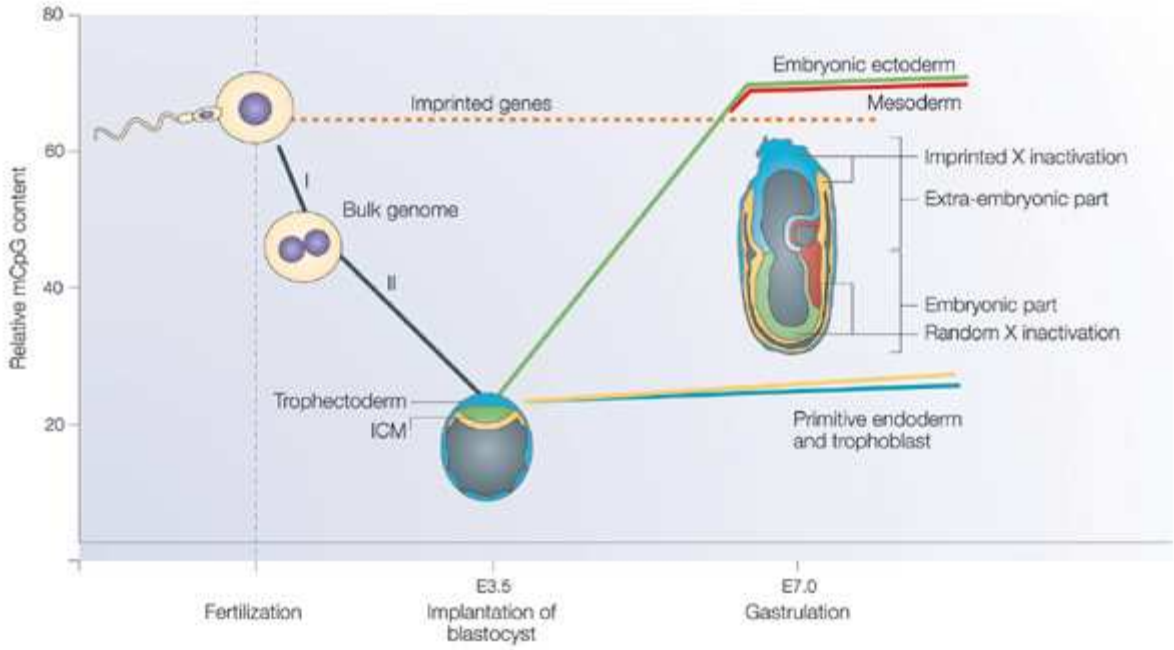


Figure I - DNA-methylation reprogramming during early mouse development (adapted from review by Li, 2002). *The methylation status of the bulk mouse genome, which consists of repeats and unique genes but excludes most CpG islands and imprinted regions, undergoes dynamic changes during early development. After fertilization, the bulk genome undergoes demethylation through an active demethylation phase (I), followed by a passive demethylation phase (II). The methylation level of a blastocyst reaches the lowest point at embryonic day (E)3.5. After implantation, the bulk genome becomes hypermethylated in the embryonic ectoderm (green) and mesoderm (red) through active de novomethylation, whereas the genome of extra-embryonic cells, such as the primitive endoderm (yellow) and trophoblast (blue), remains hypomethylated. The parental methylation imprints in imprinted genes (orange) escape demethylation and de novo methylation. Interestingly, X inactivation is imprinted in the primitive endoderm (yellow) and the trophectoderm-derived cells (blue), whereas it is random in the embryonic tissues. ICM, inner cell mass.*

3.1.3 Histone modifications and chromatin function

DNA methylation is closely connected with modifications of? chromatin structure. Chromatin is generally organized into silent heterochromatin and active euchromatin containing most of the genes. Nucleosomes are the basic units of chromatin consisting of 147 bp of DNA wrapped around a histone octamer. Two copies of each of the following core histones are present in a nucleosome: H2A, H2B, H3 and H4. All of them have a globular C-terminal domain and an unstructured N-terminal tail (Luger et al., 1997). Interestingly, a variety of modifications are associated with these tails. Histone modifications include methylation of arginine, methylation, acetylation, ubiquitination and sumoylation of lysines, and phosphorylation of serine and threonine. Lysine acetylation leads to transcriptional activity. Lysine methylation may have different effects in dependence on particular methylated lysine and on the number of methyl groups. For example, trimethylation of H3K9 or H3K27 is a repressive modification, while trimethylation of H3K4 is associated with active chromatin. Different combinations of modifications lead to different levels of transcription. Most of the acetylated residues reside in the N-terminal tails of histones except for H3K56, which resides in the core domain (reviewed by Kouzarides et al., 2007). Histone modifications are also connected with the alternative splicing regulation (Luco et al., 2010).

In many cases, chromatin modifications serve as recognition sites for the recruitment of effector molecules. Several distinct binding modules have been identified in various nuclear proteins, coupling a particular histone modification with cognate effector proteins. Thus, the composition of modifications on a given histone can either recruit or occlude a set of proteins. Effector proteins may alter chromatin structure by binding two or more nucleosomes as found with heteroprtein 1 (HP1) and Polycomb group proteins. Effector proteins can also act as adaptors to attract additional chromatin-modifying enzymes or remodeling complexes to augment the chromatin alteration initiated by the modification. Such an example can be found in HP1 binding to trimethylated H3K9 (Jacobs and Khorasanizadeh, 2002) and DNMT1 (Smallwood et a., 2007). These initial interactions can recruit SUV39H1 and/or DNMT1 and further promote H3K9 methylation, HP1 binding, and DNA methylation, which may in turn result in further transcriptional gene silencing or chromatin repression (Fucs et al., 2003). Also promoters marked by trimethylation of H3K27me3 frequently become DNA methylated during differentiation (Mohn et al., 2008). On the contrary, methylation of H3K4 negatively correlates with the DNA methylation. The interaction is mediated by the DNMT3L, which specifically binds the non-methylated H3K4 and by its carboxyterminal domain interacts with the DNMT3a (Jia et al., 2007). Reversely, the PHD domain of bromodomain and PHD

finger transcription factor (BPTF), a component of the nucleosome remodelling factor (NURF) chromatin remodeling complex, recognizes trimethylated H3K4 and brings the remodeler with it (Wysocka et al., 2006). Some other effector proteins possess enzymatic activities themselves, as exemplified by chromodomain 1 (CHD1) remodeling ATPase, which binds to trimethylated H3K4 and introduces active structure remodeling. Similar effector proteins have been identified for DNA methylation. A series of methyl CpG-binding proteins, such as MBDs and MeCP2, have demonstrated the ability to interpret DNA methylation marks in different biological contexts (reviewed by Bird, 2006). Specifically, it has been demonstrated that interpretation of DNA methylation marks by MBDs and MeCP2 has additional assurance *via* recruitment of histone deacetylases (HDACs) for gene silencing (Fuks et al., 2000).

Most of these epigenetic regulators and many more functionally diverse factors were confirmed in an extensive study with small interference RNA library targeting 200 predicted genes, including potential activators, silencers, chromatin remodelers, and ancillary factors. Interestingly, the study indicates little functional redundancy as combinatorial knockdown of factors was not required for reactivation (Poleshko et al., 2010).

3.2 Retroviruses

Retroviruses are RNA viruses that are replicated in the host cell via the enzyme reverse transcriptase to produce DNA from its RNA genome. The DNA is then incorporated into the host genome by the enzyme integrase. The virus thereafter replicates as part of the host cell DNA. Retroviruses are enveloped viruses that belong to the viral family *Retroviridae*. The outer envelope is formed by a phospholipid membrane obtained from the host cell. The envelope glycoprotein (E*nv*) is anchored in the lipid membrane. It consists of the transmembrane glycoprotein (TM) essential for the membrane fusion and the non-covalently bound surface unit (SU) responsible for the receptor binding. SU is highly variable and largely decides on the viral host specificity. *Gag* (group-specific antigen) proteins form the capsid containing an RNA dimer in complex with the nucleocapsid protein, proteins with the enzymatic activity such as reverse transcriptase (RT), RnaseH, integrase, protease, tRNA molecules which serve as primers for the reverse transcription and some cell proteins.

The retroviral genome is formed by an RNA dimer with a 5’ methylguanosine cap. In the proviral DNA sequence both ends are formed by the long terminal repeats (LTR) consisting of the unique 3’ end sequence (U3), repeat sequence (R) and unique 5’ sequence (U5) (Fig. II). The RNA genome has the R and U5 on the 5’ end and the U3 and R on the

3’end. The U3 contains most of the regulatory sequences such as the enhancers and promoters. The polyA signal is in most mammal retroviruses and in all lentiviruses encoded by the R sequence, in most avian retroviruses by the U3 sequence. The U5 is followed by the leader sequence with the encapsidation signal PBS (primer-binding sequence) and at least three genes; *gag*, *pol* and *env*. Apart from these basic genes retroviruses also encode some additional regulatory proteins. (reviewed in Coffin et al., 1997)

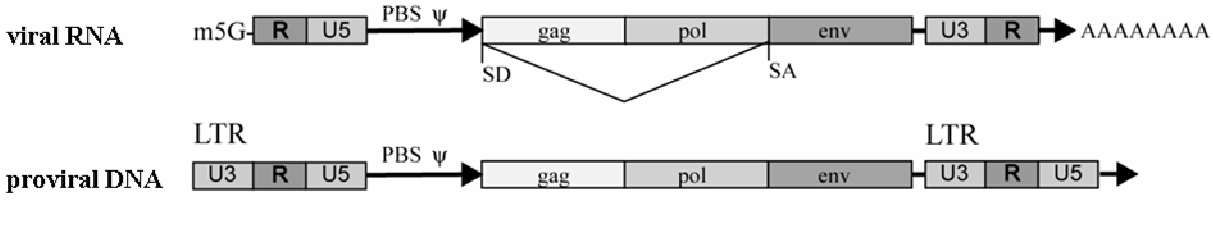


Figure II - Structure of retroviral genome. *R- short repetition, U5 – 5’ unique sequence, U3 – 3’ unique sequence, Ψ – encapsidation signal, SD/SA – splice donor/acceptor sites*

3.2.1 The retroviral life cycle

Entry of the virus to the cell is the first step of the retroviral life cycle and the presence of a specific receptor on the cell surface largely decides about the cell permissiveness. The SU domain is crucial for receptor binding whereas TM is responsible for the fusion. A wide variety of surface molecules are used as receptors and the receptor molecule is specific for each retrovirus. Sometimes a co-receptor is needed for the entry. Receptor-independent infection was observed as well (Wensel et al., 2003).

After entry the capsid disassembles in the cytoplasm and the reverse transcription can be initiated. RT is a multifunction enzyme with an RNA-dependent DNA polymerase activity, RNase activity degrading the RNA in RNA-DNA hybrids, DNA-dependent DNA polymerase activity and 5’-specific RNase activity. As a primer for RT serve either tRNA or DNA. Thanks to the intramolecular jumps the resulting viral DNA is longer than the original RNA. U5 on 3’ end and U3 on 5’ end are added and in this way the LTRs are created. Reverse transcription is rather inaccurate and causes high mutability of retroviruses. Therefore, retroviruses rapidly evolve resistance against antiviral drugs like in the case of HIV therapy. Another result is the presence of many non-active mutated retroviruses in the host genome.

The viral DNA is integrated into the host DNA with the help of the viral integrase. The integrated viral DNA is termed provirus. The sequence specificity is an object of intensive research. Different retroviruses exhibit different preferences for different sequences or structures. For example, HIV integrates mainly into the GC-rich sequences where the majority

of the genes are present (Elleder et al., 2002), the murine leukemia virus (MLV) prefers active promoters (Wu et al., 2003) and the avian sarcoma virus is *in vivo* detected particularly within or close to the genes broadly expressed in multiple tissues (Plachy et al., 2010). Generally, retroviruses prefer the regions with open chromatin.

The provirus transcription is catalyzed by the host RNA polymerase II and is regulated by the 5' LTR which contains the binding sites for the host transcription factors and the transcriptional apparatus. More complex viruses such as HIV bind also their own transcriptional factors. The regulatory sequences together with the Env are decisive for the viral tropism. The enhancer must be effective and specific for the host transcription factors. Part of the viral RNA remains non-spliced and serves as the genomic RNA and the capsid proteins and enzymes are translated from it. From part of the RNA *gag* and *pol* genes are excised and the Env protein is expressed. Env is synthesized on the rough endoplasmatic reticulum and cleaved by the cellular proteases into TM and SU proteins, which are exposed on the cell surface.

Polyprotein precursor Gag is expressed on free ribosomes. During the translation in 5 to 20 % a frameshift occurs before the end of *gag*. In this way the stop codon it skipped, the *pol* gene is translated as well and the polyprotein Gag-Pol is synthesized. The rate of different proteins is thus regulated. According to the type of retrovirus the viral particle is assembled. C-type viruses and lentiviruses appear to assemble the internal structures of their particles concurrently with envelopment at the plasma membrane. D-type and B-type viruses assemble immature particles in the cytoplasm prior to envelopment at the plasma membrane. Spumaviruses also assemble immature proteins in the cytoplasm but do not undergo an obvious maturation step after budding. Some ERVs form IAP particles (intracisternal A-type particles), which are formed similarly as the C type retroviruses except that they bud exclusively into internal membranes. The phospholipid membrane is acquired during budding. In the complete viral particle the RNAs dimerise and the polyproteins are cleaved by the viral protease into functional enzymes (Coffin et al., 1997).

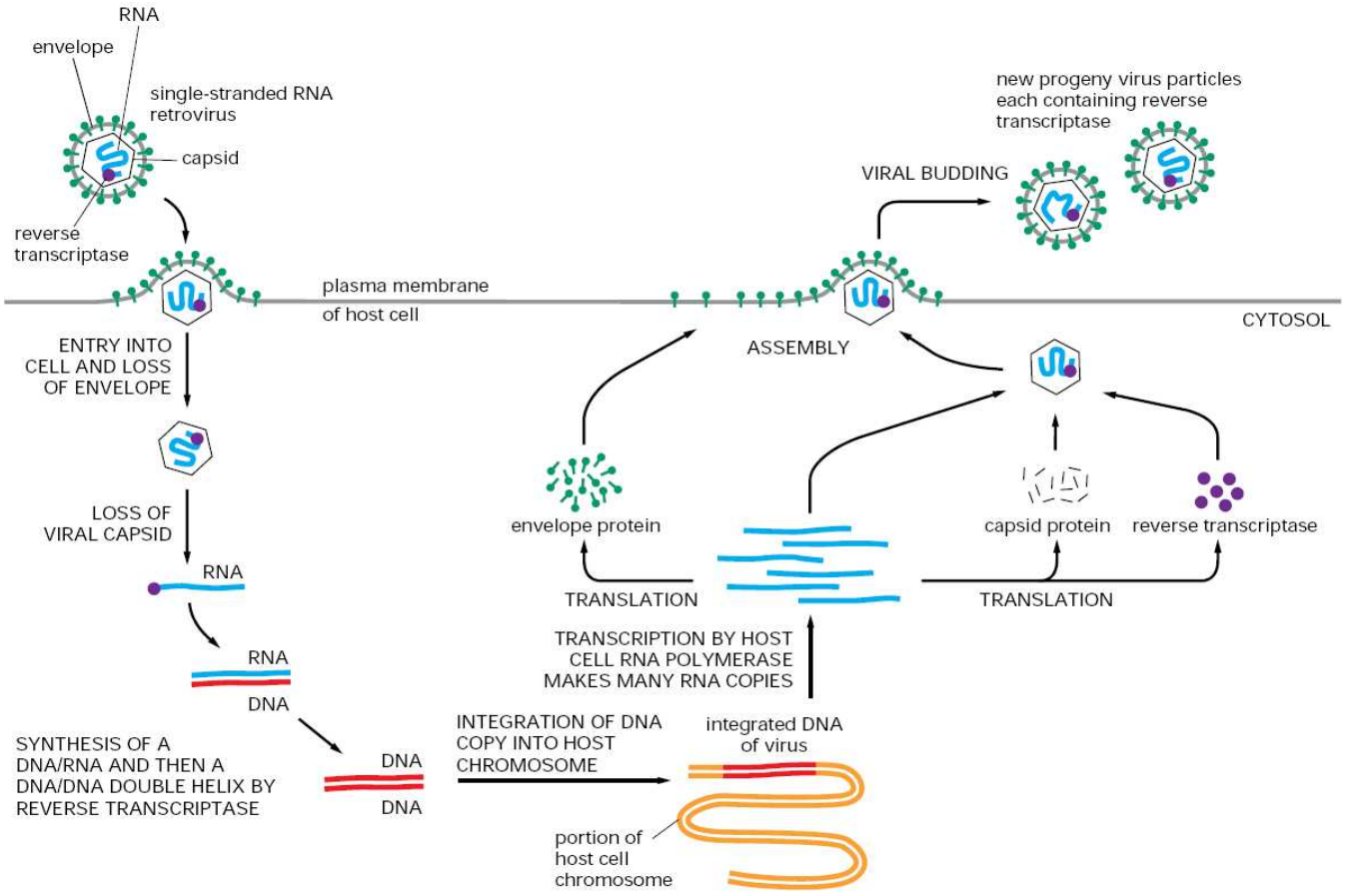


Figure III - The life cycle of a retrovirus. Reproduced from Alberts et al., 1998.

3.2.2 ERVs and other retroelements

Retroelements are genomic sequences duplicating via RNA intermediates that are reverse-transcribed and inserted at new genomic locations. They are present in all eukaryotes. In mammals retroelements and sequences derived from them form nearly half of the genome while the coding sequence forms only about 5 percent (*International Human Genome Sequencing Consortium, 2001; Mouse Genome Sequencing Consortium, 2002*). We can distinguish autonomous retroelements, which encode their own RT, and non-autonomous retroelements, which prosper from the RT of the autonomous ones. There are two major classes of autonomous retroelements, non-LTR retroelements long interspersed nuclear elements (LINE), which are in mammals represented mainly by the LINE-1 family (L1), and ERVs, and three major classes of non-autonomous retroelements, short interspersed nucleasr elements (SINE) represented mainly by Alu elements, SVA and processed pseudogenes (Fig. IV) (reviewed by Goodier and Kazazian, 2008).

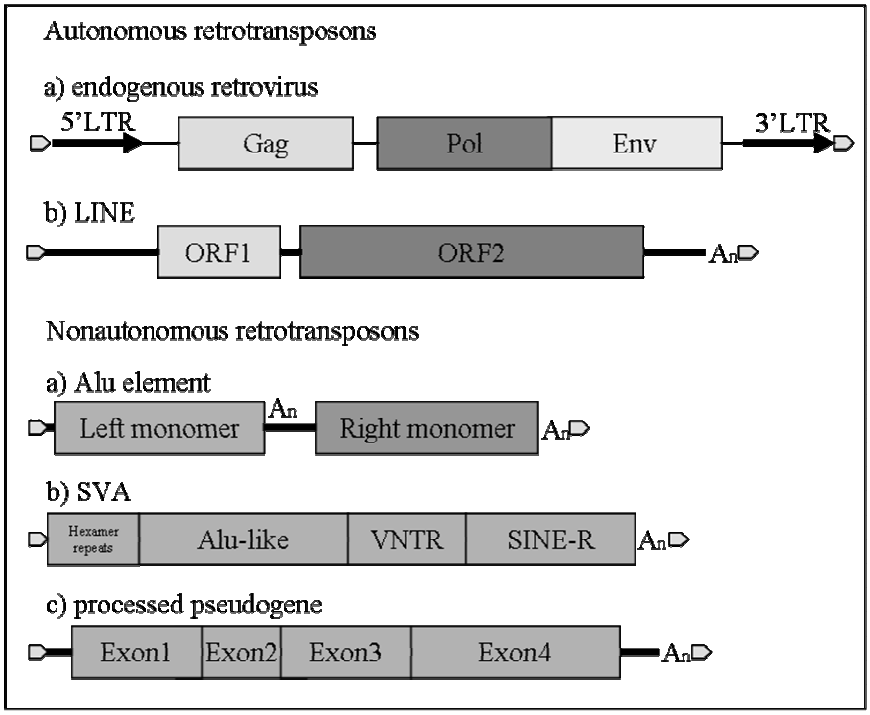


Figure IV - Retroelement classes.

ERVs have evolved from the exogenous retroviruses that infected the germ cells or the embryonic cells in early developmental stage. Most of the ERVs are not infectious and their genes are defective thanks to accumulation of random mutations. The most common retroviral element is a solo LTR, which is a remnant after homologous recombination with the second LTR.

Autonomous non-LTR retrotransposons are 4 to 6 kb long and usually contain two ORFs. ORF1 encodes an RNA-binding protein and ORF2 encodes a protein with endonuclease and reverse transcriptase activities. Integration preferences have been shown also for some non-LTR retrotransposons. For example, R1 and R2 retroelements of drosophila or bombix mori, respectively, integrate into particular ribosomal genes (Jakubczak et al., 1990). Mammalian L1 integrates preferentially in TTTT/AA consensus site, which is cleaved by its endonuclease. L1 reverse transcriptase exhibits strong *cis*-preference, which ensures preferential retrotransposition of its own RNA. L1s have a broad impact on the mammalian genome diversity; beside retrotransposing themselves, they are also involved in expansion of Alu elements, which account for 10% of human genome, SVA elements, and processed pseudogenes comprising about 0.5% of the human genome.

Alu elements are the most successful retrotransposons considering their copy number per human genome is going beyond million. They evolved approximately 65 million years ago from 7SL RNA – component of signal recognition particle (SRP) (Ullu and Tschudi,

1984). Similarly as SRP, Alu RNA is able to bind ribosome and with high probability becomes the substrate for nascent L1 reverse transcriptase. Alu activity depends mainly on their primary sequence and on their RNA ability to interact with SRP to form RNA protein complexes (Bennett et al., 2008). They have massively expanded in many primate genomes. One new Alu insertion is estimated to occur for every 20 live human births (Cordaux et al., 2006). This activity makes L1-mediated Alu retrotransposition a significant mutagenesis factor with impact on human genome complexity as well as a cause of heritable diseases such as hemophilia, cystic fibrosis, Duchenne muscular dystrophy and many others (reviewed by Belancio el al., 2009). In mouse and rat genome, homologous B1 sequences have been discovered. They form about 2.5% or 1.65 % of the genome, respectively (*Rat Genome Sequencing Project Consortium, 2004*). Different SINEs have been discovered in all analyzed eukaryotes. In 2003 a new class SINE3 derived from 5SrRNA have been revealed in the *Danio rerio* genome (Kapitonov and Jurka, 2003).

SVA elements are much less abundant L1-dependent retrotransposons. According to a number of recent *de novo* disease-causing mutations, SVAs are rather active retrotransposons (Ostertag et al, 2003). Their ability to use the L1 retrotransposition machinery is probably connected with the presence of Alu-like sequence.

Another class of sequences copied with the help of L1 are the processed pseudogenes. Processed pseudogenes have the structure of cDNA from a cellular mRNA. They lack the untranscribed part of promoter and introns and they end with a poly(A) tail. In most cases, these pseudogenes are not functional, primarily because of the uncomplete promoters and secondarily because of mutation accumulation in the lack of selection pressure. However, on rare occasions, the element had integrated downstream in the vicinity of an active promoter and such event resulted in a new expression pattern. Several human genes emerged in this way, for example PGK2 and PDHA2 are expressed functional pseudogenes derived from *PGK1* and *PDHA1* genes or protein-coding *RPS27* pseudogene evolved from ribosomal gene *RPS27* (Balasubramanian et al., 2009). Pseudogenes can also be involved in regulation of the original gene (Piehler et al. 2008).

3.2.3 Silencing and regulation of retroviruses and retroelements

Protection of the organism against retroviral infection and against retroelement amplification has many mechanisms in common. The defense against retroviral infection can be multileveled, beginning with restriction of the entry into the cell, continuing with transcription silencing, invalid RNA splicing, block of the RNA export or block at the level of

polyprotein maturation. The retrovirus integrates into host DNA as a provirus and, therefore, its expression is tightly dependent on cellular regulation mechanisms. Cells enabling the viral replication are termed permissive, cells obstructing the retroviral cycle at any level are termed non-permissive.

One cell defence mechanism is represented by the factor APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G), which deaminates the cytosines of the nascent DNA strand of the retroviral genome. By deamination, cytosines are converted to uracils, which leads to mutation or degradation of the DNA containing uraciles (reviewed by Goff, 2003). Viruses can protect themselves against this mechanism by proteins such as Vif in HIV that prevents integration of APOBEC3G into virus particles and inhibits its expression (reviewed by Mangeat & Trono, 2005, Sierra et al., 2005). APOBEC3G may serve as anti-viral factor via a deamination-independent mechanism (Jonsson et al., 2007).

The retroelement amplification is restricted mostly to the transcription level. Retroviral and retroelement transcriptional silencing is associated mainly with DNA methylation and complement chromatin modifications. RNA interference is also involved.

Retroviral gene silencing is achieved foremost by its LTR methylation. The methylation pattern is not set at one distinct moment, but the genome of the silenced provirus is subjected to DNA methylation for weeks and the density of methylated CpG dinucleotides gradually increases (He et al., 2005). It implies that the DNA methylation machinery will be more probably involved in the decrease of the stability of expression and gradual silencing than in the early silencing events following integration. The dependence of expression on the methylation status has been demonstrated on numerous retroviruses. For example, the 5'LTR Moloney murine leukemia virus (MMLV) is heavily methylated in murine cells and the provirus is silenced. DNA methylation is not the primer silencing mechanism in this case. In embryonic cells the methylation independent transcription suppression is induced by the repressor binding sequence (RBS) present in LTR. This suppression is in next steps confirmed by the DNA methylation (Kempler, 1993). Also HIV transcription is not primarily suppressed by DNA methylation. HIV-1 methylation does not directly correlate with its expression suppression *in vivo* (Pion et al., 2003); however, it is crucial for its long-term silencing and stability of HIV-1 latent reservoir. The non-methylated proviruses are easily reactivable (Blazkova et al., 2009). The sensitivity of retroviruses to methylation varies in different host cells. Non-permissive mammalian cells completely suppressed expression of partially methylated RSV, while its suppression in permissive bird fibroblasts occurred with significant delay and heavy LTR methylation was necessary.

Total increase of promoter DNA methylation reduces transcription; however, some CpGs are more important for the transcription decrease. For example, methylation of one CpG in HIV LTR near to the NF-κB binding sequence disables binding of this transcription factor and leads to significant silencing (Bednarik et al., 1991). In Prague RSV strain, methylation of a single HpaII site CCGG was sufficient for major expression decrease (Hejnar et al. 1999)

Methylation of the inserted provirus can influence the methylation status of surrounding DNA. In our laboratory we performed the methylation analysis of RSV and host genome flanking sequences. Unmethylated active provirus induced demethylation of the originally hypermethylated integration site. However, this state was only temporal and retroviral silencing was accompanied by permanent hypermethylation of the whole DNA region. Its suppression was not reversible either by DNMT or HDAC inhibitors (Hejnar et al., 2003).

Often, weak methylation can be reversed by the demethylating agent 5-azacytidine (Aza-C). With DNA methylation increase, the inhibitor of HDAC trichostatin-A (TSA) becomes necessary for the activation (Lorincz et al. 2001). According to McInerney et al. (2000) long-term silencing of MMLV promoter cannot be reversed even by combination of AzaC and TSA, which means that additional factors are in play. Retroviral reactivation may be in future used for treatment of HIV-1 positive patients to eradicate the latent proviruses. Also for reactivation of heavily DNA methylated HIV the HDAC inhibitor was necessary (Blazkova et al., 2009). All these examples illustrate cooperation between histone modifications and DNA methylation.

Particular histone modifications are connected with retroelement silencing. Mouse genome analysis shows specific enrichment of methylation of H3K9, H3K27, H3K20 within all murine repetitive sequences in differentiated cells (Martens et al, 2005).

For each histone methylation in different developmental stages a special histone-methyltransferase (HMTase) is required. For example, for methylation of H3K9 six HMTases are known, knockout of each is lethal at different embryonal stage. From the point of view of ERV regulation the most interesting HMTase is ESET crucial in days E3.5-E5.5, when ERVs become inactivated (Dodge et al., 2004). Its inevitability for silencing of both, endogenous and introduced retroviruses in mouse has been confirmed by Matsui (2010,) in double ESET knockout. In contrast, H4K20 HMTases knockouts had comparable retroelement expression as wild-type animals (reviewed by Rowe and Trono, 2011). Association of heterochromatin protein 1 was described in silenced proviruses (Poleshko et al., 2008). Several studies described an important role of the SWI/SNF complex in retrovirus expression (reviewed in Iba et al., 2003).

Finally, retroelement silencing is often connected with its localization in a specific nuclear compartment. Interestingly, retroelements may be directly involved in formation of the silent compartment. Recently, L1 has been shown to participate in inactivation of the X-chromosome by two diverse ways. Either the L1 are suppressed and create a silent nuclear compartment into which genes become recruited, or they are transcribed and drive the antisense transcription of the nearby genes, which leads to RNA interference (Chow et al., 2010).

Some retrotransposons with chromodomain like Ty3/gypsy retrotransposons integrate preferentially into silent compartmens (Gao et al., 2008,). Others, such as mammalian ERVs, prefer integration near to active genes; however, due to negative selection, most ERVs remain outside of genes or in antisense orientation.

It is not clear how the retroviral and other retrelement sequences are recognized. After methylation erasure methylation is re-established by Dnmts. In mouse prospermatogonia Dnmt3a mainly methylates short interspersed repeat SineB1. Both Dnmt3a and Dnmt3b were involved in the methylation of IAP and L1. Only Dnmt3b was required for the methylation of the satellite repeats. Severe hypomethylation in Dnmt3L-deficient prospermatogonia indicates the critical function and broad specificity of this factor in *de novo* methylation (Kato et al, 2007). Different contribution of different Dnmts on retroelement silencing suggests that each class is recognized by a distinct process. In SINE retroelement recognition overrepresentation of approximately 8 bp spaced CpG may be in play as the Dnmt3a-Dmnt3L dimerized complex recognizes specific histone code binding the DNA by its two catalytic sites in the distance of 8-10 bp (Ooi et al., 2007; Ferguson-Smith and Greally). RNAi may also be involved in repetitive element recognition and silencing like in the case of the afore mentioned L1 elements or yeast retroelements Ty (Jiang, 2002).

Retroviral sequences may be recognized thanks to LTR because repetitions often serve as Dnmt targets. One of the mechanisms described in embryonic carcinoma and embryonic stem cells involves TRIM28, which recognizes the primer binding site of MMLV Pro^{tRNA} and triggers dimethylation of the H3K9. Dimethylated lysine is recognized by heterochromatin protein 1, which induces heterochromatinization of the region and retrovirus inhibition (Ellis et al., 2007, Wolf & Goff, 2007).

In silencing of retroviral sequence immediately or soon after retrovirus integration may be involved protein Daxx, which represses gene expression by recruitment of histone deacetylases 1 and 2 (Hollenbach et al., 2002) and interacts with the ASLV integrase (Greger

et al., 2005). It is not clear whether Daxx protein also influences the expression of non-ASLV retroviruses.

Retroviral sequences can be protected from methylation silencing, which is of special importance for the use of retroviral vectors. Various antimethylation and insulation strategies have been applied to increase the provirus expression stability. For example, one protective element was provided from the investigation of the chicken β-globin locus. The DNA sequence at the 5' end of the chicken β -globin locus can function as an insulator (Chung et al., 1997, Zhao & Dean, 2004). Another promising protective sequence is the core element of the CpG island, Sp1 site. Its insertion into RSV LTR has been shown to be highly protective (Machon et al., 1998; Senigl et al. 2008, Hejnar et al, 2001).

3.3 HERVs and their domestification

HERVs have been discovered for the first time in human placenta in 1973 (Kalter et al., 1973a). Retrovirus-resembling particles were observed by electron microscope budding on the basal membrane of syncytiotrophoblast. Similar particles have been observed in placenta of other primates and later in different cell lines (Kaltler et al, 1973b).

HERVs are classified according to the tRNA binding the PBS sequence and dubbed with the corresponding amino acid abbreviation. Wide and thorough analysis of retroelements and their phylogenetic relationship was enabled by completing the human genome sequence (*International Human Genome Sequencing Consortium, 2001*). It seems that all the HERVs are defective; however, some have retained intact some ORF or LTR and these can be beneficial for human organism. On the other hand, they are also involved in carcinogenesis or various autoimmune diseases. In every respect, HERVs were crucial in human evolution.

Many HERV LTRs are used as strong promoters for human genes (Buzdin et al., 2006). Among others endogenous retroviral sequences are required for the tissue-specific expression of a human salivary amylase gene (Ting et al., 1992), ERV3 regulated krüppel-like H-plk involved in human ontogenesis (Kato et al., 1990), alcohol dehydrogenase C1 with U3 ERV9 region (Chen et al., 2002), proapoptotic p63 isoform drived by ERV9 LTR (Beyer et al., 2011) and many others (reviewed in Cohen et al. 2009).

Retroviral sequences can also provide poly-A signals such as in the case of *HHLA2* and *HHLA3* human genes (Mager et al. 1999) or enable alternative splicing (Kowalsky et al, 1999). Also haptoglobin-derived gene or placenta-expressed PLT have modified splicing due to retroviral sequences (Hatada et al., 2003; Goodchild et al., 1992*)*

Some tissues express originally retroviral protein, especially placenta and tumor tissues. DNA hypomethylation across the genome is common for these tissues, which render at least some HERVs and other retroelements transcriptionally active. The presence of HERVs in diverse tumors could be used for diagnosis as well as for immunoterapy (Staufer et al., 2004). The most pronounced changes in methylation levels in the same study were observed for members belonging to younger families such as HERV-H, HERV-W, and HERV-K. Their upregulation was observed in melanoma, breast cancer, leukemia, testicular tumor and many others (reviewed by Romanish et al, 2010). The strongest evidence for direct involvement of HERV proteins in malignancy comes from work on small accessory HERV-K proteins, rec and np9. The rec protein is a product of alternative splicing of *env*, and is a functional homolog of the HIV Rev and HTLV1 Rex proteins. Mice over-expressing rec develop features similar to human germ cell tumors (Galli et al. , 2005).

3.3.1 ERVs in placenta

Out of all tissues, HERVs are most active in placenta, which is probably connected with its low DNA methylation. So far, protein expression of retroviral families ERV1, HERV-F, ERV3, HERV-W and HERV-FRD has been detected. HRES1, ERV1, 9, HERV-H, E and K retroviral RNA was detected as well (reviewed by Muir et al., 2004). HERV-W and HERV-FRD were proved to be essential for placenta development.

ERVs are probably involved in several placenta functions such as induction of trophoblast cell fusion and their differentiation into syncytiotrophoblast, suppression of maternal immune reaction against the embryo, protection against the exogenous retrovirus, cytotrophoblast cell replication regulation, induction of trophoblast invasiveness. Mostly the *Env* glycoproteins are involved in the placenta development. Both, the fusion and the immunosuppression are facilitated by their transmembrane domain. In exogenous retroviruses the immunosuppressor domain serves for suppression of the NK cells immune reaction and induces the monocyte lysis and cytokine expression in Th2 lymphocytes (Harris et al., 1987). Another way of immune suppression performed by exogenous retroviruses is for example replacement of major major histocompatibility (MHC) I glycoproteins by their homologs to protect against the NK cells. Immunosupressive properties were shown also for ERVs. For example, HERV-H expressed in cancer cells posses the immunosupressive domain which might help to protect the tumor (Mangeney et al., 2001). The immunosupression in placenta is probably ensured by several HERVs, principally by the HERV-FRD (Mangeney et al., 2007).

Best proved is the retroviral *Env* role in the trophoblast syncytialization. The first discovered viral protein involved in placenta development was syncytin 1, the *Env* coded by a HERV from the W family (Mi et al., 2000; Blond et al., 2000). Retroviral family HERV-W is present only in genomes of old world monkeys, great apes and human. Phylogenetic analysis shows that HERV-W integrated into the primate genome over 25 million years ago (Huh et al., 2003). In human genome 654 copies with HERV-W origin have been identified up to now. Out of these 343 are short solo LTRs issued from provirus recombination. Seventy seven retrotransposed by the retroviral machinery contain at least some internal coding sequences. The rest of HERV-W retroelements have probably replicated by the L1 replication machinary (Pavlicek et al., 2002). Locus ERVWE1 encoding syncytin-1 is highly conserved among people as well as among primates (Mallet et al., 2004), which suggests its necessity in placenta development. This was rather surprising as other placental mammals lack the HERV-W family and therefore syncytin-1. Further research revealed that other *env*s are also involved in human placenta development. ERV-3 was formerly known to support trophoblast differentiation (Lin, 1999). Next, the fusogenic activity of HERV-FRD *Env* and its placenta-specific expression has been proved. It was dubbed syncytin-2 (Blaise et al., 2003). In other mammals syncytins from yet other ERV families have been identified. First, murine *Envs* with placenta-specific expression and fusogenic activity syncytins A and B have been described, both conserved in all tested *Muridae*. None of these is related to human syncytins. The critical role of syncytin A was further confirmed by preparation of mouse knockout (Dupressoir et al., 2005; Dupressoir et al, 2009). Syncytins were later discovered in rabbit (Heidmann et al, 2009) and in sheep (Dunlap et al., 2006) as well. It can be assumed that every mammal, except for mammals with the most primitive placentas, has at least one *Env* enabling its placenta development and correct function.

3.3.2 Human syncytins

As mentioned above, two conserved placenta-specific *Envs* with fusogenic activity have been discovered, Syncytin-1 from the HERV-W family and Syncytin-2 from HERV-FRD.

Syncytin-1 uses as receptor commonly expressed surface protein hASCT2 (human Na+ dependent neutral aminoacid transporter 2), which also serves as retroviral D receptor (Blond et al., 2000). Receptor mRNA expression has been demonstrated in placenta by Northern blot. The presence of receptor protein has been proved on the basal membrane of syncytiotrophoblast (Kudo and Boyd, 1990), where it colocalizes with syncytin-1.

Syncytin-2 utilizes as receptor Major Facilitator Superfamily Domain Containing 2 (MFSD2) belonging to a large family of presumptive carbohydrate transporters with 10-12 membrane-spanning domains. It has been highly conserved in evolution. In contrast with hASCT2, MFSD2 has placenta-specific expression, which was demonstrated by quantitative RT-PCR analysis.

Placental dysfunctions are often accompanied with aberrant syncytin-1 expression. For example, pre-eclampsia and HELLP syndrome, serious obstetric complications, are connected with decrease of syncytin-1 mRNA (Knerr et al., 2002). Another study demonstrates incorrect localization of syncytin-1 on the apical membrane of microvilli instead on basal membrane in pre-eclamptic patients (Lee et al., 2001). Recent study shows that syncytin-2 levels were more importantly impaired than syncytin-1 and presents syncytins mRNA low expression level as a marker of pre-eclampsia severity (Vargas et al., 2011). Reduced syncytin-1 expression also correlated with insufficient or late syncytiotrophoblast differentiation in Down's syndrome pregnancies (Frendo et al., 2001).

On the contrary, upregulation of syncytins in non-placental tissues is suspected of involvement in autoimmune diseases and tumor progression. Albeit in both cases syncytins are not the only HERVs with increased expression and many other factors are in play. Syncytin upregulation has been demonstrated in lesions of multiple sclerosis patients, which could be connected with proinflammatory properties of the *Env* (Antony et al., 2004; Komurian-Pradel, 1999). Further, in patients experiencing their first manifestations of schizophrenia or schizoaffective disorder *pol* transcripts of HERV-W were observed in cerebrospinal fluids (Karlsson, 2001). Syncytin increased expression was also detected in various cancers such as breast cancer (Bjerregaard et al., 2006), colorectal cancer (Larsen et al., 2009), endometrial carcinoma (Strick et al., 2007) or in leukemia and lymphoma cells (Sun et al., 2010). Cell-to-cell fusion in tumors may contribute to aneuploidy and promote gradual development of malignancies (Duelli and Lazebnik, 2003).

Expression of syncytins must, therefore, be tightly controlled in order to avoid pathogenic fusions or inflammation in non-placental tissues. Many factors are involved in the regulation of syncytin expression. Especially regulation of syncytin-1 has been studied in detail. The promoter region and transcription initiation site of syncytin-1 were localized within the 5′ LTR of ERVWE1 provirus by deletion analysis and reporter assay for the basal promoter activity (Cheng et al., 2003). Mutagenesis of this promoter region together with DnaseI footprint analysis revealed that the CCAAT motif and the octamer protein binding site are critical for transcriptional regulation of syncytin-1. Another study (Shen et al., 2002)

showed that a transcription factor called Glial Cell Missing a (GCMa) binds to two GCMa binding sites upstream of the 5′ LTR and enhances syncytin-1 expression in BeWo and JEG3 choriocarcinoma cells. The human choriocarcinoma cell lines have been widely used as a relevant model of trophoblast differentiation. Conversely, mutation of the ecdysone receptor response element slightly increases basal promoter activity, suggesting that this nuclear hormone receptor is a negative regulator of the syncytin-1 gene (Cheng et al., 2003). Similarly, a correlation between the decreased level of oxygen in placenta and insufficient expression of syncytin-1 was also detected in BeWo cells.

Considering the retroviral origin of syncytins the influence of DNA methylation on its transcription is highly probable. In this study we compare syncytin-1 and -2 DNA methylation of 5' LTR in different tissues and cell lines, the stability of 5′ LTR methylation pattern and the sensitivity of 5′ LTR promoter activity to *in vitro* methylation.

3.4 PERVs and xenotransplantation

PERV particles were for the first time observed by electron microscope in 1971 in pig kidney cell line PK15 (Armstrong et al., 1971). The main reason for studying PERV is the possibility of the use of porcine cells, tissues or organs for xenotransplantation. In this context it is important to ask whether PERVs would pose any public health risk when transplanted into a human host. The question became even more pressing when in 1997 Patience et al. described infection of human cells by PERVs *in vitro*.

3.4.1 History of xenotransplantation

Use of xenotransplantation for replacement of non-functional organs was probably considered since the beginning of medicine history. The earliest reports of transplantation concern human-to-human transplantation; however, using animal organs was more frequent in history. This had two reasons: first, it was easier to obtain animal organs, second, nothing was known about the immunological interspecies barriers and the major problem was to stop bleeding and restore circulation (reviewed by Deschamps, 2005). The oldest reported case of xenotransplantation was done in Iran. It concerned replacement of an osteomyelitic fragment of scull with a dog one. Xenotransplantation experiments were performed during the whole history including blood transfusion, skin transplantation, pancreas transplantation to diabetic patient and many others with no major success.

Significant improvement followed discovery of the first immunosuppressive drug Imuran. In 1964 doctor Reemtsma transplanted a chimpanzee kidney to a 23-yr-old woman.

She died 9 months later from acute electrolyte imbalance. This is the longest survival ever recorded for the xenotransplantation of an organ. In 1984, a very promising xenotransplantation of baboon heart to 12 day old baby Fae was done. The immune system was not yet matured and cyclosporine A was available. However, the child survived only for 20 days (Bailey et al., 1985)

The advantages of xenotransplantation success are evident, because it would help to solve the organ shortage. After all the successes with allotransplatations, trade with human organs is becoming a serious problem. In 1985, the Ethics Committee prohibited buying and selling organs and tissues. However, no major improvement was achieved. In 1992 pig livers were used at least to prolong the waiting time for the donor of liver (Makowka et al., 1994). In 1995, a trial was accomplished to treat AIDS with baboon bone marrow (with HIV-resistant blood cells); however, the cells survived only for a brief time (Ildstad, 1996).

In 1960s porcine skin became popular for burn treatment and it is currently the most widely used xenograft (Bromberg et al., 1965). Pig skin was long-term used in the Prague Burns Centre at University Hospital Vinohrady. From 1973 to 2005 porcine grafts were used as a temporary cover, which improved the burn healing significantly (Broz et al., 1999). This treatment is not used there anymore because of the risks potentially connected with the use of porcine material. From this point of view use of acellular xenodermis in combination with human allogeneic keratinocytes is of interest. (Matouskova et al., 2006)

In 1997, in London in the laboratory of R. Weiss it was shown that PERVs are able to infect human cells *in vitro*. This discovery led to a vast debate and partial moratorium of xenotransplantation experiments until the safety of this technique would be proven (Bach and Fineberg, 1998; Vogel, 1998) The use of primates as donors was banned in 1999 because of the known risk of cross-species infections (e.g. HIV) (Butler, 1999).

3.4.2 Problems and risks of xenotransplantation

The main problems of xenotransplantation were already mentioned above: the graft or organ rejection and the risk of infection by animal pathogens – zoonotic infection.

The acceptor immune system responds to the transplanted graft by distinct types of rejection. The immunologic response after allotransplantation is reduced by careful donor selection and life-long immunosuppression. However, the response toward xenograft is more complex and it is not yet known how to overcome it. Several types of rejection occur after the xenotranplantation: hyperacute rejection (HAR), acute humoral xenograft rejection (AHXR),

acute cellular xenograft rejection (ACXR) and long-term chronic rejection (reviewed by Cozzi et al., 2006)

HAR damages graft within minutes to hours before any graft function can be detected (Pino-Chavez, 2001). It involves both immunological and non-immunological factors. It is initiated by xenoreactive natural antibodies, which activate the complement cascade, mediate complement deposition and endothelial cell activation that results in platelet activation, coagulation and disruption of vascular endothelium. Out of all antibodies the most important seem to be the antibodies against the α1,3-galactose (α1,3-gal) epitope (Sandrin et al. 1993), which is added by α1,3-galactosyltransferase (α1,3-GT) expressed in all mammals except for old world monkeys, apes and human. Anti- α1,3-gal antibodies then activate the complement pathway (Yin et al., 2004).

Acute humoral rejection starts more than 24 hours after transplantation. It has very similar properties; however, the initiation of graft function is observed. Ultimately, it results in vascular thrombosis and edema. Deposits of antibody and complement are hallmark features, causing endothelial cell activation, swelling or disruption (Pino-Chavez, 2001). Anti-α1,3-gal antibodies are important in the establishment of AHXR as well; however non-αGal antibodies may also play a role as α1,3-GT -/- pigs still induce AHXR (Kuwaki et al., 2005). The ACXR doesn't directly damage the graft; however, it may elicit anti-xenograft humoral immune response. Because of the problems with HAR and AHXR, little is known about the chronic rejection.

3.4.3 Humanization of pigs

Pigs are considered to be the best source of organs for xenotransplantation for many reasons. They are easy to breed and people are highly experienced in pig breeding. The size of porcine organs is quite similar to human. Pigs are evolutionarily distant from human, which decreases the probability of sharing pathogens. Also, as I have mentioned, there is already some experience with pig-to-human xenotransplantation. The low cost of pig breeding in comparison with e.g. apes should also be taken into account.

However, organ rejection is a severe problem in the use of pig tissues and organs. Therefore, there is an effort to develop pigs with lowered ability to activate the human immune system when used for transplantation. Pigs with modified genes crucial for rejection would be much a better source of organs. To prepare these, a cloning technique had to be evolved and adapted to pigs. Cloning consists in nuclear transfer from cultivated somatic cells to an enucleated oocyte. The cultivated cells can be appropriately modified according to

needs. In 1995 the first cloned mammal Dolly the sheep was born (Campbell et al., 1996). It took five more years until the first cloned piglet was born (Pennisi and Normile, 2000).

To avoid the HAR the complement pathway has to be suppressed. One strategy to inhibit this immunologic pathway is introducing human complement inhibitors. Possible interesting genes are human decay accelerating factor CD55 (DAF), membrane cofactor factor CD46, or membrane inhibitor of reactive lysis CD 59. The first transgenic pig expressed human CD55 (Cozzi et al., 1997) and clearly demonstrated that this strategy could overcome HAR in pig-to-primate heart (Schmoeckel et al., 1998), kidney, and liver transplants. Graft survival of days to weeks was achieved depending on the degree of immunosuppression. Subsequently, pigs expressing either human CD46 or the combination of CD55 and CD59 were produced, with similar results. More significant immunosuppression led to median graft survival of 76 days (range 56–113 days) with CD46 transgenic pig-to-baboon heart xenografts. However, high immunosupression was accompanied by a high frequency of recipient cytomegalovirus infection (Reviewed in Gock et al., 2010).

The main activator of the complement pathway is the α1,3-gal. Therefore, the key aim was preparation of knockout pigs without the gene for α1,3-GT. This was achieved for the first time in 2002 by Phelps et al. In 2008, ten baboons underwent xenotransplantation with organs from miniature swine with transgenic human DAF or from α1,3-GT knockout pigs. The graft survival was 32 to 179 days. The baboons still had to be immunosuppressed; however, with lower levels of immunosuppressives than had to be used after transplantation of organs from non-modified pig. The graft survival was also prolonged.

Considering the safety of xenotransplantation, breeding of pigs with minimal numbers of PERVs or without a few selected potentially risky PERVs is debated. Another strategy is introduction of PERV suppressing genes such as human *APOBEC3* or PERV-specific siRNA. These topics will be discussed later.

3.4.4 Zoonotic infection

A close contact of porcine and recipient tissue in immunosuppressed patients could serve as an ideal environment for adaptation of porcine pathogens to the human organism. The probability of infection of a xenotransplanted patient by animal pathogens (zoonotic infection) is increased by several factors (reviewed by Fishman and Patience, 2004): the xenograft serves as a permissive reservoir in which donor organisms bypass host defenses without a need for a 'vector' to achieve disease transmission. Nothing is known about the

behavior of organisms from the donor species in immunosuppressed humans. Novel clinical syndromes resulting from infection with such pathogens are not distinguishable. Donor-derived pathogens may not cause disease in the native host species but may cause disease in a new host ('xenotropic organisms') and, also, they may acquire new characteristics by genetic recombination or mutation. Donor-recipient incompatibility of MHC antigens may reduce the efficacy of the host's immune response to infection within the xenograft.

Recent human epidemics of viral infection have been traced to animal-derived strains that have been adapted to human hosts. These include hantavirus (mice), severe acute respiratory syndrome (SARS) owing to a new coronavirus possibly associated with civets, BSE (bovine spongiform encephalopathy), and the most known HIV that evolved from primate viruses SIV, which caused the whole-world pandemics. In each case, the epidemiology was defined after the recognition of a new clinical syndrome and the development of new, rapid, molecular assays for the causative agent.

All these examples of recent zoonotic infection show their impact on the entire population, not only on the affected individual who was in close contact with the infected animal. This experience implies the importance of the discussions about the possible animal-to-human disease transmission during xenotransplantation.

A number of potential viral pathogens have been identified including porcine herpesviruses (cytomegalovirus, lymphotropic herpesviruses), circovirus types 1 and 2, porcine reproductive and respiratory syndrome virus, porcine encephalomyocarditis virus, swine influenza viruses, African swine fever virus, hepatitis E-like virus, pseudorabies virus, parvovirus, polyomaviruses of swine and also PERVs (reviewed by Scobie and Takeuchi, 2009)

It is possible to avoid the presence of most of these pathogens by breeding pigs in specific pathogen-free conditions. However, pigs used for xenotransplantation still have to be properly tested for the presence of all possibly dangerous pathogens. Samples of tissues and serum from donors and recipients should be archived, as it has been mandated by FDA guidelines for future use in tracking unsuspected or novel pathogens in clinical trials of xenotransplantation. Although exogenous viruses can be removed from the transplantation source by breeding pigs in specific pathogen-free environments, such techniques cannot eliminate PERV present in the pig germ line DNA.

3.4.5 PERVs

The genome of domestic pig contains more than one hundred PERV copies (Patience et al., 2001). About half of them belong to the youngest PERV group γ1 with many functional full-length copies. Apart from this recent family four more γ and four βPERV families were identified. Three subgroups of β PERVs are related to MMTV and β3is related to HERV-K. PERVs known to be infectious belong to the gammaretrovirus genus and gammaretroviruses, such as gibbon ape leukemia virus (GALV) and MLV, can cause cancer, leukemia or neurodegeneration. The γ1 PERVs evolved approximately 7.6 to 3.4 million years ago, which correlates with the time of separation between pigs and their closest relatives, American-born peccaries. Within the γ1 group, there are three subgroups of infectious gamma retrovirus families PERV-A, -B and -C, which utilize different cellular receptors. PERV-A, -B, and -C vary in the *env* sequence within the SU domain (Le Tissier et al., 1997). Analyses showed that PERV-A and PERV-B have wider host ranges, including several human cell lines, compared with PERV-C e*nv*, which infected only pig cells. Cell lines derived from various small mammal species, including mink, rat, mouse, and rabbit were infected by PERV-B while only human cell lines and mink cell line were infected by PERV-A. In contrast, no vector transduction was observed in nonhuman primate cell lines, casting doubt on the utility of nonhuman primates as models for PERV zoonosis (Takeuchi et al., 1998; Patience et al, 1997). To date, only PERV-A receptors have been identified. Two paralog human PERV-A receptors have been cloned and their homologs were found in all complete genome sequences (Ericsson et al., 2003). However, most cell lines are not permissive to PERV-A either due to mutation in crucial amino acids or due to low receptor expression (Mattiuzzo et al., 2007).

Short after the discovery that PERVs are able to infect human cells an extensive study of patients treated with living pig tissue was performed. Blood samples were collected from 160 individuals of age from 2 to 77 years, who underwent extracorporeal splenic, kidney or liver perfusion, bioartificial liver perfusion or treatment with pancreatic islet cells or skin xenograft. Neither immunologic analyses nor PCR confirmed PERV infection in any of the patients. However, the presence of donor cells in the recipient was observed in 23 patients for up to 8.5 years (Paradis et al., 1999). Several analogous studies have been performed later with similar results (Di Nicuolo et al., 2005, 2010; Wang et al., 2006, Hermida-Prieto et al., 2007)

In order to evaluate the potential risk posed by the transmission of PERVs an animal model would be valuable. Although the infection of different species cells was successful, no

productive infection of any PERV-inoculated animal occurred. In an attempt to establish a small animal model, naive guinea pigs, non-immunosuppressed rats, rats immunosuppressed by cyclosporin-A and immunosuppressed rats treated with cobra venom factor were inoculated with PERVs produced from porcine kidney PK-15 cells, infected human 293T kidney cells and mitogen-stimulated porcine PBMCs. Animals were also inoculated with PERV-producing PK-15 and 293T cells. No antibodies against PERV and no provirus integration were observed in any of the treated animals (Specke et al., 2001). In 2006 mice transgenic for human PERV-A receptor 2 (HuPAR-2) was created. After inoculation with infectious PERV supernatants, viral DNA and RNA were detected at multiple time points, indicating productive replication (Martina et al., 2006). Transient transmission was also detected in thymectomized fetal lambs after pig islet xenotransplantation (Popp et al., 2007), but so far no symptoms were detected.

The greatest threat comes from PERV-A/C recombinants (Wood et al., 2004). PERV-A and PERV-B have been shown to infect human cells with low titers. A recombinant PERV-A14/220 with half of the SU region derived from PERV-A and the remaining sequence derived from PERV-C is approximately 500-fold more infectious than PERV-A. Chimeric *env* analysis suggests that the enhanced infectivity for human cells is probably caused by the novel juxtaposition of *env* gene sequences of PERV-A14/220, perhaps by stabilization of the *Env* glycoprotein or increased receptor binding (Harrison et al. 2004). PERV-A14/220 is only one example of many recombination events generating new PERVs. Phylogenetic analyses between 16 full-length sequences revealed that such recombination events generating more active PERV-A appear to occur in pigs rather frequently. It also indicated that PERV-A *env* is more prone to recombination with heterogeneous backbone genomes than PERV-B *env* (Bartosch et al., 2004). In this context, pigs lacking non-human-tropic PERV-C would be more suitable as donor animals for clinical xenotransplantation and even replication-deficient expressed PERVs could generate human tropic viruses by recombination.

There is a high level of PERVγ1 insertion polymorphism, and individual PERV copies are heterologously distributed in unrelated pigs. So far all tested animals were positive for PERV-A and -B, but the minority of pigs are PERV-C negative. The recombinant PERV A/C were not detected in the germ line. The expression of PERV RNA varies as well. Generally it is very low and viral protein expression is not observed in some animals at all (Dieckhoff et al., 2009).

Various restriction factors for protection against retroviruses are known. PERVs are restricted by human APOBEC3G (Jonsson et al., 2007), their release can be inhibited by

tetherins (Mattiuzzo et al., 2010); on the contrary, PERVs are insensitive to restriction by divergent TRIM5*α* molecules (Wood et al., 2009). PERV production can also be reduced by introduction of small interfering RNAs (Dieckhoff et al., 2008), intracellularly expressed single domain antibodies directed against PERV *Gag* (Dekker et al., 2003) or sugar-modifying enzymes to remodel PERV *Env* glycoprotein (Miyagawa et al., 2006)

In our study we will show that PERVs in porcine tissues are heavily DNA methylated, which leads to expression silence.

4 Materials and methods

4.1 Cell cultures

HeLa, PK15 and ST-IOWA cells were grown in F-12 and MEM-D mixed 1:1 (Sigma) supplemented with 1% NaHCO3, 10% fetal calf serum, penicillin-streptomycin mix (100 µg/ml each) and 2.5 µg/ml amphotericin B. Choriocarcinoma BeWo cells and JEG-3 cells were grown in the same medium supplemented with 10% fetal calf serum and 300 µg/ml glutamine. Primary human skin fibroblasts were derived from skin obtained after breast reduction surgery from the Clinic of Plastic Surgery, Faculty Hospital Královské Vinohrady, Prague. They were cultured in Eagle's H-MEM, supplemented with 1% NaHCO3, a mix of nonessential amino acids, 0.12 g/l natrium pyruvate, 10% calf serum, penicillin-streptomycin mix (100 µg/ml each) and 2.5 µg/ml amphotericin B. Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (FBS, BioSera). Quail QT6 cells, murine MDTF (*Mus dunni* tail fibroblast), rat NRK, HSN cells and XC were grown in DMEM supplemented with 10% FBS.

4.2 Human tissue samples and DNA samples

Samples of placental tissues with a prevalence of chorionic villi were obtained from Gynecologic Clinic, Faculty Hospital Královské Vinohrady, Prague. Breast cancer cells from invasive ductal carcinomas were obtained from the Department of Gynecology and Obstetrics of the Third Faculty of Medicine, Charles University in Prague. Human ethics approval was granted by the Committee for Ethics, Handling the Recombinant DNA and Clinical Research at the Institute of Molecular Genetics, and informed consents were obtained from the patients. DNA samples of peripheral blood mononuclear cells (PBMC) from ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome patients were a kind gift from Keith Robertson, Epigenetic Gene Regulation and Cancer Section, NCI, NIH, Bethesda.

All samples of healthy testicular tissues and testicular tumors were obtained from the Department of Urology of the Third Faculty of Medicine, Charles University in Prague. Biopsies taken from patients with hormone-sensitive prostate cancer treated by bilateral orchiectomy represented healthy testicular tissue (T1, T2) with prevalence of spermatogenic epithelium from which the tunica albuginea was discarded. Histological inspection showed normal testicular tissue with decreased spermatogenesis because of the advanced age of these patients. Seven testicular tumors were included to this study; T, T3, T4, T7 and T10 are

histologically at least partially seminomas, T contains large fraction of embryonic carcinoma and yolk sack carcinoma, T3 big component of granulomatous multinuclear cells; T6 is a mixed germinal tumor with embryonic carcinoma and teratoma components, T9 is a testicular lymphoma. Histologically normal testicular tissue surrounding the tumors was obtained by macrodissection. Samples of suppository uterine carcinoma, endometrial carcinoma, and ovarian teratoma were obtained from the Department of Pathology, Third Faculty of Medicine, Charles University in Prague. Human ethics approval was granted by the Committee for Ethics, Handling Recombinant DNA and Clinical Research at the Institute of Molecular Genetics, and informed consents were obtained from the patients.

4.3 Pig tissue samples

The samples of pig skin, pancreas, lung, heart, brain, kidney, ovary, liver and muscle were taken from three, testis from another four six-month-old large white pigs coming with the veterinary certificate to the slaughterhouse from commercial breeds in the Czech Republic. Small samples were taken immediately after slaughtering the animals and frozen in liquid nitrogen. Samples of term pig placenta were obtained from two minipigs from the Institute of Animal Physiology and Genetics, Liběchov, Czech Republic and frozen on dry ice. Samples of PBMC were kindly provided by Linda Scobie, Department of Biological and Biomedical Sciences, Glasgow Caledonian University

4.4 Plasmids

Reporter vector pLTR-W-luc was obtained by amplification of the whole 5′ LTR of syncytin-1 from human genomic DNA using the Expand Long Template PCR System (Roche) according to manufacturer's instructions with following primers: forward 5′-TTCAACATCCATTCCAACACCACC- 3′ (nucleotides −202 to −179 upstream to the LTR) and reverse 5′-CTGAGTCTTAAGTCCGGTGGCAC-3′ (nucleotides 913 to 936 from the beginning of the LTR). In order to discriminate between the LTR of syncytin-1 and LTRs of other HERV-Ws present in the human genome, the forward primer is complementary to the unique sequence immediately flanking the 5′ LTR of ERVWE1. PCR cycles were as follows: 5 min 95°C; 20 cycles, each consisting of 1 min 95°C, 2 min 60°C, 1 min 72°C; 19 cycles, each cycle consisting of 1 min 95°C, 2 min 60°C, 1 min 72°C plus a 20 s ramp per cycle; finally 8 min 72°C. The blunted PCR fragment 1138 bp in length was cloned into the SmaI site of pBluescript (Stratagene). To generate pLTRluc, we cleaved the resulting plasmid with NotI, blunted the NotI ends and digested again with HindIII. This DNA fragment was used to

replace the HindIII–SmaI promoter region of the luciferase reporter vector pGL3-promoter (Promega). In this way, the luciferase reporter gene in resulting pLTRluc was driven by the 5′ LTR of the human syncytin-1 gene.

Reporter vector pLTR-A-luc was prepared from plasmid 5'MAMBA1. This plasmid was prepared by amplifying half of MAMBA1 provirus (accession number EU789636.1) with primers for3 5'-AGTGTCTGAGAATTGCTTGGACC-3' and rev1 5'-CAAATGCCTTCTGGTGCTCA-3' from porcine chromosomal DNA and cloning into pGEM-T-easy vector. PCR was performed with long distance polymerase (Roche) according to the manufacturer's recommendations in 35 cycles of 95°C for 15 s, 61°C for 30 s, and 68°C for 8 s. The LTR was excised from the MAMBA1-5' by PvuI and HindIII. The PvuI site was blunted by T4 polymerase. The PvuI-HindIII fragment was then used to replace the SmaI – HindIII promoter region of the luciferase reporter vector pGL3-promoter (Promega, Madison, WI). Vector pLTR-B-luc contains LTR of PERV60 which was excised from pCR PERV60 (Bartosch et al., 2002) by KpnI and HindII. The KpnI site was blunted by T4 polymerase. The KpnI-HindIII fragment was then used to replace the SmaI – HindIII promoter region of pGL3-promoter. Resulting pLTR-A-luc and pLTR-B-luc plasmids were prepared using the QIAGEN Plasmid Midi column (Qiagen, Hilden, Germany).

Part of plasmids used for receptor analyses and cell infection were previously described: MLV-based retroviral vectors pCNCG carrying the *eGFP* gene (Neil et al., 2001), pCFCR with unique EcoRI site (Ylinen et al., 2005), MLV *gagpol* expression plasmid CMV (Towers et al., 2000), G protein of vesicular stomatitis virus (VSV-G) expression plasmid pMDG (Naldini et al., 1996), replication competent PERV-A 14/220 plasmid. PERV3a is a chimeric construct between A14/220 and PERV60 with LTRs of PERV60 origin (Bartosch et al., 2004).

HuPAR-2 was tagged at the C-terminus with influenza virus HA-tag by PCR of the construct pcDNA3/huPAR-2 (Ericsson et al., 2003) using KOD HiFi polymerase (Novagen) and the primers G3 5'-GATTGATGAATTCACCACCATGGCAGCACCCACG-3'and G4 5'-GATCTTGCGGCCGCTCAAGCGTATTCTGGAACATCGTATGGGTAAAGCTTGGG GCCACAGGGGTCTACACAGTCCTTTCTGCTTTG-3' and introduced into pcDNA3 using EcoRI and NotI restriction sites. These primers introduced the Kozak sequence at the ATG of the receptor and the HA-tag in the C-terminus downstream of a HindIII restriction site. Using EcoRI and NotI restriction sites, the HA-tagged receptor was introduced again into pcDNA3. In this way the resulting plasmid pcDNA3/huPAR-2HA contains two HindIII restriction sites,

one in pcDNA3 and the other introduced in frame upstream of the HA-tag using the G4 primer. HA-tagged *huPAR-1* and *muPAR* genes were obtained by PCR of constructs pcDNA3/huPAR-1 and pcDNA3/muPAR [14] with the primer pairs G3;G5 5'-GAAGGTAAGCTTGGAGTCACAGGGGTC-3‘ (*huPAR-1*) and G6 5‘-GATTGATGAATTCACCACCATGGCAGCACCTCCG-3‘ and G7 5‘-GAAGGTAAGCTTGAGGCCACACTGGTC-3‘ (*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. All the HA-tagged receptors were also subcloned into the retroviral vector pCFCR using EcoRI and NotI restriction sites.

The plasmid bearing HA-tagged C-terminal ratPAR was prepared by amplification using KOD HiFi polymerase and primers M1 5‘-GATTGATGAATTCACCACCATGGCAGCACC-3‘ and M3 5‘-GCAGGTAAGCTTAGGGCCACACTGATC-3‘ with PCR conditions: 95°C, 30 s, 52°C 30 s, 72°C 90 s. Primers were designed to anneal to the rat homolog of *huPAR-1* [Genbank: XM_343272]. The M1 primer introduced the Kozak sequence in front of the ATG of the receptor ratPAR. M3 introduced the HindIII restriction site. The resulting product was introduced into pcDNA/huPAR-2HA. This product was then subcloned into pCFCR.

4.5 Bisulfite conversion of genomic DNA for methylation analysis

DNA samples for bisulfite analysis were isolated by phenol-chloroform extraction. Bisulfite treatment of DNA was performed with EZ DNA Methylation™ Kit by Zymoresearch and Qiagen EpiTect bisulfite kit according to manufacturer's instructions**.**

4.6 Methyl-specific quantitative PCR (MS qPCR)

Fifty ng of converted DNA was used for quantitative PCR based on the MESA GREEN qPCR MasterMix Plus for SYBR Assay Kit (Eurogentec) and Chromo4 system (Bio-Rad) for real-time PCR detection. We performed quantifications of the hypomethylated ERVWE1 together with the reference DNA sequence human RPII and of the hypomethylated PERVs together with the reference DNA sequence porcine ELF2. All measurements were carried out in triplicates. The negative controls included water or converted DNA negative for the analyzed sequence as a template. The specificity of the amplified PCR products was confirmed by melting curve analysis and by sequencing the PCR products. The number of hypomethylated copies per cell was obtained by normalization to the reference sequence using the following formula: $2^{-\Delta Ct} = 2^{-(Ct\ sample\ -\ Ct\ refrence)}$ (Livak and Schmittgen, 2001). The

volume of the reaction mixture was 25 µl with 300 nM final concentration of each primer. PCR reactions were supplemented with 0.9 M betain and 0.9% dimethyl sulfoxide (DMSO). To increase the ability of primers to distinguish between CpG and TpG, we have localized the crucial complementary thymines in the reverse primer at the very 3'end. To achieve high specificity, all bases distinguishing the non-methylated CpGs were on the lock nucleic acid backbone (LNA) (Gustafson, 2008). LNA bases are marked with + in the primer sequence. The specificity for the hypomethylated LTRs was confirmed by sequencing of representative clones. Following primers were employed in appropriate conditions:

primer	sequence	PCR conditions:
umW/F	5'-GGATGAGGGTAAAATGTTTGGAGATAT-3'	95°C 5 min, 40x: 95°C 15 s, 60°C 1 min, plate read
umW/R	5'-TTACCAACTC+AAATACCTAAATTTATATCCC+A-3'	
qbisRPII/F	5'-GGGAGTAGTGTGAGAAGAAGGGTATATAT-3'	95°C 5 min, 40x: 95°C 15 s, 60°C 1 min, 72°C 30 s +3 s/cycle) , plate read
qbisRPII/R	5'-CAACATAATAATCTCAAAAACCCCCTTCATAA-3'	
qbisPuniv/F	5'-TATT+TRGGGTCGTAGTTTTTTATTTTTG+T-3'	95°C 5 min, 40x: 95°C 20 s, 56°C 1,5 min, 72°C 1 min (+3 s/cycle) , plate read
qbisPuniv/R	5'-AACAAACAAAAAAATC+AAAC+AAYC+AC+A-3'	
qbisELF/F	5'-GAAGGGAGTTGGGATAAGGTGGAGTAAATTT-3'	95°C 5 min, 40x: 95°C 20 s, 64°C 1 min, 72°C 30 s +2 s/cycle) , plate read
qbisELF/R	5'-CCCCAAATACCTCAATTCCCACTATACCATA-3'	

4.7 Bisulfite sequencing

Fifty ng of converted DNA was amplified by Taq polymerase (Takara) according to the manufacturer's recommendation. The supplied buffer was supplemented with 0.9 M betain and 0.9% DMSO and for some reactions with MgCl. The volume of the reaction mixture was 25 µl with 300 nM final concentration of each primer. Most PCR products were obtained by the semi-nested PCR using the primer marked with number 1 as first and the primer marked with number 2 as second. The third primer was used in both rounds of PCR. The second round of PCR started with 1 µl of the first round PCR product, and the PCR conditions were identical. For DNA amplification the following primers were used in appropriate conditions:

primer	sequence	PCR conditions:
bisFRD/F1	5'-GTAAGTAGTTTTATTAGGAGG-3'	F1-R: 9x: 95°C 20s, 55°C 1 min (-0.5°C/cycle), 72°C 33 s; 35x: 95°C 20s, 50°C 1min, 72°C 33s; repeated with F2-R
bisFRD/F2	5'-TTTTAGTTTAGGAATGTTAGG-3'	

bisFRD/R	5'-AATCACTACACCATTTAAAAA-3'	
bisW/F1	5'-TTGGATAGTGAATATAGATA-3'	F1-R: 30x: 95°C 1 min, 52°C 2 min, 72°C 1 min; repeated with F2-R ; 5mM MgCl
bisW/F2	5'-AGATATAGTAATTATTTTGT-3'	
bisW/R	5'-CAAAAAAAAACTACTATTAC-3'	
bisM1/F1	5'-GTTTGAGAATTGTTTGGATT-3'	F1-R: 14x: 95°C 30s, 61°C 30 s (-0.5°C/cycle), 72°C 1 min; 36x: 95°C 30s, 54°C 30 s, 72°C 1 min; repeted with F2-R; 3.4mM MgCl
bisM1/F2	5'-AAGAGGAGAAGTTAATTGTT-3'	
bisM1/R	5'-CACCAAACTAAAAAACCAA-3'	
bisM7/F1	5'-GTAGTGTTTGAGAATTGTTTGGATT-3'	F1-R: 14x: 95°C 30s, 61°C 30 s (-0.5°C/cycle), 72°C 1 min; 36x: 95°C 30s, 54°C 30 s, 72°C 1 min; repeted with F2-R; 3.4 mM MgCl
bisM7/F2	5'-GGAAAAATAGGTTTTTGAGTATGTGT-3'	
bisM7/R	5'-CAAAAAAAATCCCTTTACCTCCAAA-3'	
bis6SH/F	5'-AGAGGGTGTTTATATTTTTGTTAAGT-3'	F-R1: 30x: 95°C 30 s, 56°C 30 s, 72°C 1 min; repeated with F-R2; 5 mM MgCl
bis6SH/R1	5'-CAAACAACAAAAAAATTTTTATTCCAAA-3'	
bis6SH/R2	5'-AACAACTTTTATAAAATTCACAACAAAA-3'	
bisRW/F1	5'-TTTTAAATAATTGAAAGGATGAAA-3'	F-R1: 30x: 95°C 30 s, 63°C 30 s, 72°C 1 min; repeated with F-R2; 5 mM MgCl
bisRW/F2	5'-TTTTGAGTATATGTTTTTAGGT-3'	
bisRW/R	5'-AAATATAAAACCAACAAAAAAAAC-3'	
bis14/220/F1	5'-TAGGTAAAAGATTAGGTTTTTTGTTG-3'	F1-R: 10x: 95°C 20s, 57°C 1min (-0.5°C/cycle), 72°C 30s; 30x: 95°C 20s, 52°C 1min, 72°C 30s; repeated with F2-R; 3.4 mM MgCl
bis14/220/F2	5'-GGGAGTTTTTAATTGTTTGTTTAGT-3'	
bis14/220/R	5'-ACTAAAAACAAACACTCAAAACAA-3'	
bis3a/F1	5'-GTTGTTAGTAAATAGGTAGAAGGTT-3'	F1-R: 10x: 95°C 20s, 57°C 1min (-0.5°C/cycle), 72°C 30s; 30x: 95°C 20s, 52°C 1min, 72°C 30s; repeated with F2-R; 3.4 mM MgCl
bis3a/F2	5'-TTTGGATTTTGTAAAATTGATTGGT-3'	
bis3a/R	5'-AAAAATCCCTTTACCTCCAAATC-3'	
bisPuniv/F	5'-GGTTTTGTTGTGAATTTTATAAAAGTTGTTT-3'	10x: 95°C 20s, 57°C 1min (-0.5°C/cycle), 72°C 30s; 30x: 95°C 20s, 52°C 1min, 72°C 30s; 3 mM MgCl
bisPuniv/R	5'-CTTAATACAAACAACAAAAAAATTTTTATTCC-3'	

PCR products were subsequently cloned using the pGEM-T vector cloning system (Promega). Individual PCR clones were sequenced by GATC-biotech with universal pUC/M13 reverse primer. Only PCR clones with at least 95% conversion of Cs outside CpGs were taken into account.. Results were analyzed using ClustalX software and http://cpg.nw.cz and http://quma.cdb.riken.jp/ websites.

4.8 Treatment of HeLa and ST-IOWA cells with azaC and TSA

HeLa and ST-IOWA cells were grown up to approximately 70% confluence and then cultivated at indicated concentrations of azaC (Sigma) and TSA (Sigma). After 48 h, the treated cells were harvested for DNA analysis.

4.9 SssI methyl-accepting assay

We have used the slightly modified protocol of Wu et al. (1993). Analyzed samples of genomic DNA were digested to completion overnight with 5 U HindIII per μg of DNA. DNA concentration was determined spectrophotometrically and then verified on 1% agarose gel. Three hundred nanograms of digested DNA was incubated with 4 U of SssI methylase (CpG methylase, New England Biolabs), 2 μM S-adenosyl-L-[methyl- 3H]methionine ([3H]SAM, 81 Ci/mmol, Amersham) and 1.5 μM non-radioactive S-adenosylmethionine (SAM, New England Biolabs) in the buffer supplied by the manufacturer at 37°C for 17 h. The reactions were stopped by adding 0.4 μl 32 mM SAM and spotted onto glass microfiber filters (Whatman). The filters were dried, washed twice in 10 ml of 5% trichloride acetate acid for 10 min and twice in 10 ml of 70% ethanol for 10 min, dried again and placed in a scintillation vial containing 4 ml of scintillation mixture. The scintillation was counted for 1 min, and the results were normalized to the value of a control mock-demethylated sample.

4.10 RNA extraction and reverse transcription

Total RNA was extracted from cultured cells or from tissues with TriReagent (Sigma) according to the manufacturer's protocol. The tissue samples were first homogenized with TissueRuptor (Qiagen). RNA samples were treated with DNaseI (Roche) before RT-PCR to remove any contaminant DNA for 15 min in the M-MLV reverse transcriptase buffer (Promega). One microgram of RNA was reversely transcribed into cDNA using random haxanucleotides or oligo(dT)12–18 primer and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol. RNA for quantification and cloning of ratPAR was extracted using an RNeasy kit (Qiagen) and incubated with 5 U of RNase-free DNase (Promega) for 30 min at 37°C. Two micrograms of RNA were reversely transcribed into cDNA using random hexanucleotides and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol.

4.11 cDNA PCR amplification

Non-quantitative PCR of ERVWE1 was used for comparison of samples from HeLa cells treated with AzaC and TSA. The used primers were complementary to ERVWE1 *env*

sequence syncytin-1 amplifying both, spliced and non-spliced RNA: forward, 5′-AGGAGCTTCGAAACACTGGA-3′ and reverse, 5′-GTGAGCTAAGTTGCAAGCCC-3′ (Kudo et al., 2003); β-actin, forward 5′-CA CCATGTACCCTGGCATTG-3′ and reverse 5′-GCCGGACTCGTCATACTCCT-3′ (Okahara et al., 2004). The expected sizes of the PCR products were 494 bp for syncytin-1 and 190 bp for β-actin. cDNA samples were used for PCR amplification as follows: 90 s 95°C, 30 cycles each consisting of 15 s 95°C, 45 s 60°C, 50 s 72°C, and finally 2 min 72°C for syncytin-1, and 90 s 95°C, 30 cycles each consisting of 15 s 95°C, 15 s 55°C, 20 s 72°C, and finally 2 min 72°C for β-actin. The amount of template cDNA was determined experimentally so that the reference gene gives about the same amount of PCR product. PCR products were separated on 1% agarose gel. As a negative control for the absence of exogenous DNA contamination, reactions run in the absence of the reverse transcriptase revealed no amplified product.

Splice-specific quantitative PCR was used for quantification of ERVWE1 transcripts which were normalized to RPII housekeeping gene transcripts. One microliter of cDNA was used for the quantitative PCR based on the MESA GREEN qPCR MasterMix Plus for SYBR Assay Kit (Eurogentec) and Chromo4 system for real-time PCR detection (Bio-Rad). The volume of the reaction mixture was 20 μl with 400 nM final concentration of each primer. ERVWE1 non-spliced and spliced transcripts were quantified with the same forward primer (5´-ACATTTTGGCAACCACGAAC-3´) and selective reverse primers, non-spliced reverse (5´-AAAGTGGAAGCTGGCTTGAG-3´) and spliced reverse (5´-GGCCATGGGGATTTATGATT-3´). ERVFRDE1 non-spliced and spliced transcripts were quantified with the same forward primer (5´-CAAGTCAAGGGCTGAACAGG-3´) and selective reverse primers, non-spliced reverse (5´-CAGAGCCACTGTGGTTGAGA-3´) and spliced reverse (5´-TGTATTCCGGAGCTGAGGTT-3´). Primers used for the *RPII* gene, *RPII* forward primer (5´-GCACCACGTCCAATGCACT-3´) and *RPII* reverse primer (5´-GTGCGGCTGCTTCCATAA-3´), were localized in different exons. External standards were constructed by PCR using BeWo cDNA and transcript-specific primer sets. Resulting PCR fragments of ERVWE1 were cloned into the pGEM-TEasy (Promega) and verified by sequencing. Ten-fold serial dilutions of external standard plasmids containing the genomic or spliced cDNA of ERVWE1, ranging from 10 to 10^6 copies per reaction, were used for construction of the calibration curves. Ten-fold serial dilutions of plasmid containing the fragment of the human *RPII* gene, ranging from 10 to 10^6 copies per reaction, were used for the construction of RPII calibration curve. Cycling conditions for ERVWE1 and RPII were 5 min at 95 ^{o}C, 45 cycles of 15 s at 95 ^{o}C, 20 s at 55 ^{o}C, 30 s at 60 ^{o}C. The negative controls included water as a template. All quantitative RT-PCRs were performed in triplicate. The specificity of the PCR products amplified was confirmed by melting curve analysis and by

sequencing the PCR products. To assess the amount of contaminating exogenous DNA, either genomic or plasmid, we included reactions run in the absence of the reverse transcriptase as negative controls. The background values of these negative controls were subtracted from the results of respective reactions with reverse transcriptase.

Quantitative PCR of PERV transcripts was performed with primers PERV_RNA forward 5'-AGTCCTCTACCCCTGCGTGG-3' and reverse 5'-CTCCAAGTCGGTTCTCGGGTGT-3' universal to amplify 16 different PERVs from all subgroups. Transcripts were normalized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* housekeeping gene transcripts with primers GAPDH forward 5'-CGTCAAGCTCATTTCCTGGTACG-3' and reverse 5'-GGGGTCTGGGATGGAAACTGGAAG-3'. QPCR was performed similarly as described before. The volume of the reaction mixture was 25 μl with 300 nM final concentration of each primer. Plasmid with PERV-14/220 was used as external standard for PERV transcripts. The external standards for GAPDH were constructed by PCR using ST-IOWA cDNA and transcript-specific primer sets. Resulting PCR fragments were cloned into the pGEM-TEasy (Promega) and verified by sequencing. Calibration curves were prepared by serial dilutions as described above. Cycling conditions for PERV and GAPDH were 5 min at 95 ^{o}C, 40 cycles of 15 s at 95 ^{o}C, 30 s at 60 ^{o}C, 30 s at 72^{o}C. Controls and background values subtraction were performed as described above.

Quantitative PCR of ratPAR was performed with 2.5 μl of the RT reaction using Quantitect Probe PCR Mix (Qiagen) 0.4 μM of each primers Q1 5'-TCAAGGTGTCTCCCATCAATTTC-3' and Q2 5'-CGTCAACACCCAAAAGAATGTG-3', 0.2 μM of Fam-Tamra labelled probes PR 5'-CTGAGCGTTTCTCTG-3' (Sigma). The amount of RNA between each sample was normalized using the housekeeping gene 18S rRNA, primers Q3 5'-TCGAGGCCCTGTAATTGGAA-3' and Q4 5'-CCCTCCAATGGATCCTCGTT-3' and probe P18 5'-AGTCCACTTTAAATCCTT-3'.The assay was performed in duplicate using the ABI PRISM 7000. Thermocycling conditions were: 50°C, 2 min; 95°C, 15 min; 40 cycles of 95°C, 15 s and 60°C, 1 min. The numbers of copies of each product were calculated from standard curves obtained by serial dilution of the plasmid pCFCR/ratPAR. Part of the 18S mRNA gene was amplified using primers ZF;ZR from human total RNA and cloned into TOPO BLUNT 2 (Invitrogen) following the manufacturer's instructions.

4.12 *In vitro* DNA methylation

The reporter vectors with LTR-driven luciferase gene was methylated *in vitro* by incubating 10 μg of plasmid DNA with 10 U of Sss1 methylase (CpG methyltransferase, New England Biolabs) in the recommended buffer containing 160 μM SAM for 2 h at 37°C. To confirm that the methylation was complete, we digested the methylated plasmid by methyl-sensitive restriction enzyme HpaII and compared the reaction product with the HpaII digest of the non-methylated plasmid DNA.

4.13 DNA transfection and reporter expression assays

The methylated and mock-methylated LTR luciferase constructs were transfected into BeWo HeLa cells or 293T cells. The cells were grown on 35 mm Petri dishes to 50% or 70% confluence, respectively, and 1 μg of plasmid DNA was applied using the FuGene-6 lipofection reagent (Roche) at a DNA/reagent mixture ratio of 1:3 in OPTIMEM (Invitrogen). Equal amount of plasmid pCMVβgal (Stratagene) bearing the CMV promoter-driven β-galactosidase (β-gal) was cotransfected together with pLTRluc, and β-gal activity was measured to normalize the transfection efficiency. OPTI-MEM was replaced with standard cultivation medium 24 h post-transfection. Cell lysates were prepared 48 h after transfection using lysis buffer containing 150mM NaCl, 20mM Tris pH 7.5, 1% Triton X-100 and 2mM EDTA. Luciferase activity was measured in the mix of 10 μl of lysate and 30 μl of luciferin solution (Promega) using luminometer DLReady™ Promega, detection system Berthold. To measure the β-gal activity, the mixture of 30 μl of cell lysate, 200 μl of 100 mM phosphate buffer, 3 μl of 100 mM magnesium buffer, 1 μl of β-mercaptoethanol and 66 μl of 0.4% o-nitrophenyl β-D-galactopyranoside (Sigma) solution was prepared. This mixture was incubated at 37°C for 10–20 min, and then the reaction was stopped by adding 500 μl of 1 M Na2CO3. The β-gal activity was measured spectrophotometrically at the wavelength 420 nm (Beckham DU 640).

4.14 Transfection, virus production and infection

Viral particles carrying the receptor genes were produced by co-transfection of 3.5 μg of three plasmids, CMVi for MLV Gag-Pol, MDG for VSV-G and MLV vector genome pCFCR carrying the receptor gene (ratio 1:1:1.5) on confluent 293T cells in 100 mm-dish using 18 μl of FuGene- 6 reagent. Cells were washed 24 hours later and at 48 and 72 hours the supernatant containing viral particles was harvested and passed through a 0.45 μm filter (Millipore). Replication-competent PERV-A 14/220 expressing the reporter gene EGFP,

EGFP(PERV-A), was produced as follows. A similar three-plasmid transfection reaction on 293T cells was performed using pCNCG instead of pCFCR in order to produce MLV/EGFP particles. The virus-containing supernatant was used to transduce 293T cells. The stable EGFP-expressing 293T cells were then transfected using FuGene-6 with the replication competent PERV-A 14/220 plasmid. The titer of EGFP(PERV-A) viral particles was assessed by infection of 1 × 105 293T seeded in a 6-well plate using serial dilutions of the supernatant. After two months the titer was stable at 2×10^5 EGFP 293T transducing units/mL. The receptor transduction and EGFP(PERV-A) infection were performed as follows: 5×10^4 target cells were seeded in a 12-well plate and the day after, 500 μl of virus-containing supernatant was added. Receptor or EGFP expression was verified 48 hours post transduction/infection by flow cytometry analysis. PERV3a virus was prepared in 293T cells transfected with PERV3a plasmid using FuGene-6. The supernatant was collected and filtrated 48 h later and used for infection.

4.15 Flow cytometry analysis

Cells transfected or transduced with HA-tagged PAR were detached with PBS-5 mM EDTA and blocked by incubation for 30 min in PBS-10% FBS on ice. The cells were washed twice in PBS, resuspended in PBS-2% FBS containing 1:100 dilution of mouse monoclonal antibody HA.11 (Covance) or 1 μg of mouse monoclonal antihuman CD71 antibody (Santa Cruz) and incubated for 1 hour at 4°C. After two washes with PBS-2% FBS, the cells were incubated with 1:200 dilution of the secondary antibody anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugate (Jackson Immunoresearch) in PBS-2% FBS for 45 min at 4°C. Cells were washed three times and resuspended in PBS. To assess EGFP(PERV-A) infection efficiency, 48 hours post-infection cells were harvested and resuspended in PBS. All the samples were processed in a FACScan cytometer (Becton-Dickinson) and analyzsed using CellQuest software.

5 Results

5.1 Role of CpG methylation in the regulation of syncytin-1 and -2 expression

5.1.1 CpG methylation of ERVWE1 and ERVFRDE1 in human tissues and cell lines

As transcription of vertebrate genes, ERV and other retroelements is often regulated by CpG methylation, we have compared the level of methylation within the ERVWE1 and ERVFRDE1 regulatory region in the placenta, in other human tissues and in cultured cells. Using the technique of genomic sequencing after bisulfite treatment, we assayed the CpG methylation pattern of five CpG dinucleotides within the 5′ U3 region of the ERVWE1 provirus bearing the *syncytin-1* gene and eight CpG dinucleotides within the whole 5’ LTR of ERVFRDE1 bearing the *syncytin-2* gene (Fig. 1a). In order to distinguish 5′ LTR from the 3′ LTR, as well as other members of the HERV families, we have designed at least one primer for the semi-nested PCR into the unique sequence adjacent to the 5′ LTR (Fig. 1a). The nucleotide sequence of PCR products confirms that we have selectively amplified the 5′ LTR of ERVWE1 and ERVFRDE1. In all samples apart from placenta and choriocarcinoma cell lines, ERVWE1 promoter was found to be methylated. ERVFRDE1 was found to be heavily methylated only in fibroblasts; however, its methylation was analyzed in fewer tissues. In term placenta samples, all ERVFRDE1 5’ LTR sequences exhibited a low methylation level, whereas the obtained ERVWE1 sequences were either heavily methylated or almost unmethylated (Fig. 1b). The bimodal methylation pattern of ERVWE1 is probably connected with the methylation progress during the placenta development. Further, we analyzed two choriocarcinoma cell lines BeWo and JEG3 with high expression of both *syncytins*. In BeWo cells we analyzed both HERV 5’LTRs, in JEG3 only ERVWE1 5’ LTR was examined. Both 5’ LTRs were found to be completely demethylated in choriocarcinoma cell lines (Fig. 1c). A decreased level of methylation of the ERVFRDE1 was observed in one sample of testis (Fig. 1e). Among a representative number of sequences obtained from skin fibroblasts we have not found any unmethylated clone, with most of them being fully methylated in both HERVs (Fig. 1d). Furthermore, we analyzed the methylation pattern of the ERVWE1 U3 region in HeLa cells and PBMCs from methylation-deficient ICF syndrome patients. Although these cells display a decrease in the overall genome methylation level due to their transformed character or a lack in Dnmt3b activity, we have not found any decrease in methylation density within the ERVWE1 5′LTR U3 region, and most of the clones were fully methylated (Fig. 1f,g).

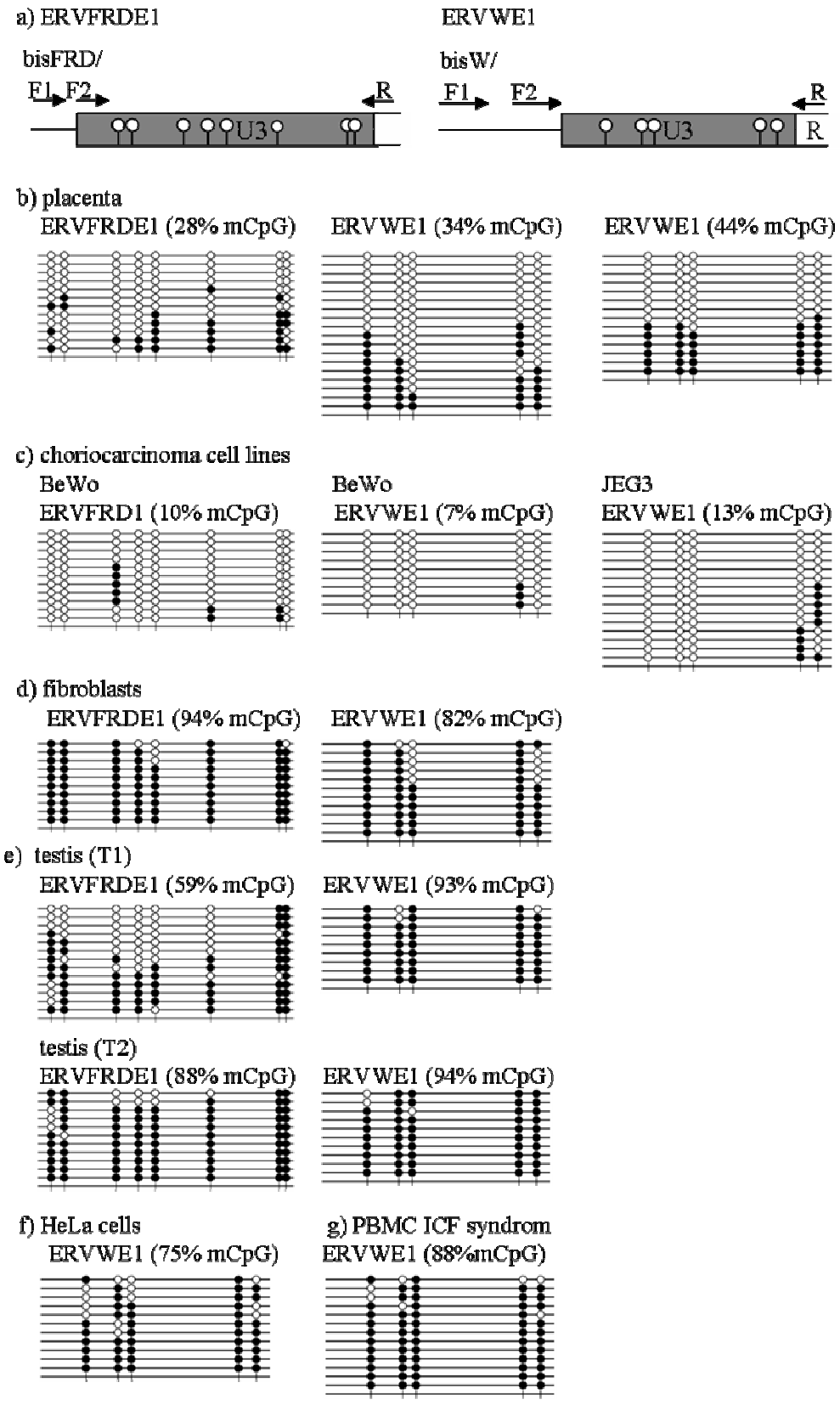


Figure 1 - CpG methylation status of the U3 region of ERVWE1 and ERVFRDE1 5′ LTR. *(a) Schematic representation U3 regions of the examined ERVWE1 and ERVFRDE1 5′ LTRs provirus. Arrows show location of PCR primers. Lollipops represent CpG within the U3 regions. (b) CpG methylation status of 5′ U3 ERVWE1 and 5′ U3 ERVFRDE1 regions detected by bisulfite sequencing in placenta tissue samples (c) CpG methylation status of 5′*

U3 ERVFRDE1 in BeWo and 5′ U3 ERVWE1 in JEG3 and BeWo choriocarcinoma cell lines (d) CpG methylation status of the same sequences in fibroblasts (e) in testis and (f) 5'U3 ERVWE1 methylatin status in cervical carcinoma HeLa cell line and (g) PBMC from patients with ICF syndrome. In parentheses is depicted the percentage of methylated CpGs from all CpG dinucleotides. Methylated CpG dinucleotides are depicted by solid circles; unmethylated CpG sites are indicated by open circles.

5.1.2 Stability of CpG methylation of the ERVWE1 5' LTR in non-placental cells

If CpG methylation ensures transcriptional suppression of ERVWE1 and prevents improper cell-to-cell fusion in somatic tissues, it should be very stable and resistant to accidental fluctuations. We therefore analyzed the stability of the CpG methylation level within the U3 region of ERVWE1 5′ LTR in HeLa cells treated with azaC and trichostatine-A (TSA), inhibitors of DNA methylation and histone acetylation, respectively. We applied two different concentrations of azaC alone or together with TSA. The higher concentrations of both agents severely decreased the proliferation of HeLa cells, indicating that we have reached the maximum of tractable genome demethylation. The effectiveness of whole-genome demethylation was checked by the methyl-accepting assay using the *Sss*I methyltransferase. Low concentration of azaC alone had no effect on the U3 5′ LTR methylation level, although we detected a substantial decrease in total genome methylation. Higher concentration of azaC alone and particularly in combination with TSA resulted in further decrease in total CpG methylation, but only a slight decrease in methylation of the analyzed U3 region. A small fraction of analyzed sequences were found to be demethylated, but the majority of them remained untouched (Fig. 2a). Furthermore, it seems that the third CpG dinucleotide is less resistant than others to the azaC/TSA-induced CpG demethylation. Furthermore, we analyzed whether the mild demethylation of HeLa cells releases the block in the transcription of ERVWE1. RNA from azaC- and TSA-treated HeLa cells and from BeWo cells, as a positive control, was isolated and subverted to RT-PCR. After 30 cycles of PCR, we did not detect any ERVWE1 transcript in HeLa cells (Fig. 2b). The increase in PCR cycle number to 35 led to the detection of a very low amount of ERVWE1 transcript in mock-demethylated and in AzaC- and TSA-demethylated HeLa cells (data not shown). This probably represents an accidental background transcriptional activity of ERVWE1, which, however, does not correlate with the decrease of CpG methylation.

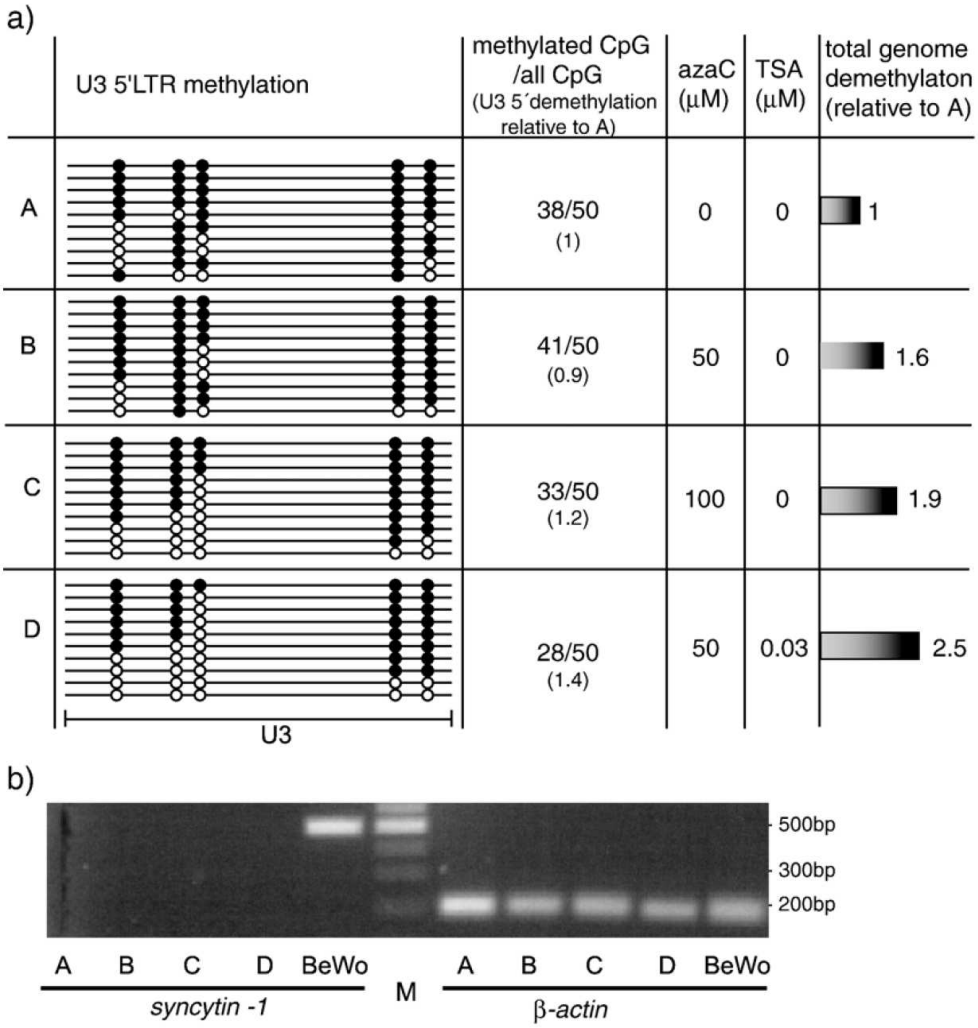


Figure 2 – Impact of the DNMT and histone deacetylase inhibitors, azaC and TSA, on the CpG methylation and the expression of ERVWE1 in HeLa cells. *(a) Methylation pattern of the ERVWE1 5′ U3 region in HeLa cells demethylated with increased concentrations of azaC and/or TSA. Micromolar concentrations of azaC and TSA are indicated on the right. Numeric representations of CpG methylation show numbers of methylated CpG/numbers of all CpG. The relative decrease of CpG methylation in comparison with mock-treated HeLa cells is given in parentheses. The relative extents of total genome demethylation as estimated by the increase of methyl acceptance are given on the right side of the figure. Methylated CpG sites are indicated by solid circles, unmethylated CpG sites are indicated by open circles. (b) Expression of ERVWE1 was determined by RT-PCR with ERVWE1 env-specific primers in HeLa cells demethylated with increasing concentrations of azaC and TSA as indicated below. BeWo cells were used as a positive control of ERVWE1 expression; β-actin mRNA levels were used as an internal loading control. M, 100 bp ladder with indicated fragment sizes.*

5.1.3 CpG methylation suppresses the transcriptional activity of ERVWE1 LTR *in vitro*

The tight negative correlation between CpG methylation in the U3 region of ERVWE1 LTR and transcriptional activity suggests that methylation could be the mechanism of transcriptional suppression. In order to assess the negative influence of U3 CpG methylation, we have fused together the complete ERVWE1 5′ LTR and the luciferase reporter gene. In this way, we have created a new reporter vector pLTRluc. We methylated all CpG sites in pLTRluc *in vitro* with the CpG-specific *Sss*I methylase and measured the promoter activity after transient transfection of methylated and mock-methylated pLTRluc DNA into HeLa and BeWo cells. Both HeLa and BeWo cell lines displayed high luciferase activity after transfection by the mock-methylated reporter vector; *in-vitro*-methylated pLTRluc vector, however, induced only weak luciferase activity in both cell lines (Fig. 3). We conclude that CpG methylation suppresses transcription of ERVWE1 and the non-methylated U3 region of ERVWE1 is transcriptionally active in HeLa cells in the absence of placental-specific transcription factors.

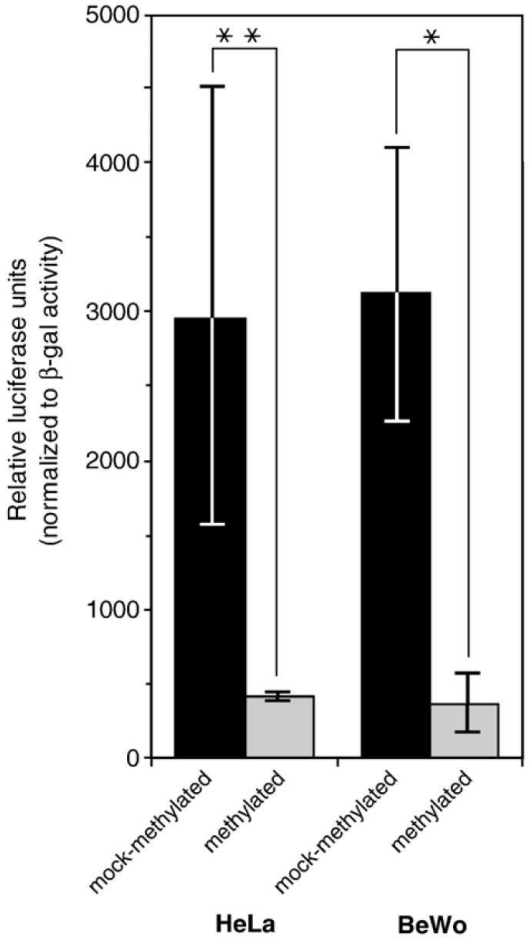


Figure 3 – Effect of CpG methylation on the transcriptional activity of ERVWE1 5′ LTR in HeLa and BeWo cells. *The complete ERVWE1 5′ LTR was fused with the luciferase reporter into pLTRluc. This vector was in-vitro-methylated with SssI methyltransferase, and 1 μg of plasmid DNA was transfected transiently into HeLa and BeWo cells grown on 35 mm Petri dishes. The transcriptional activity of mock-methylated (black columns) and methylated (grey columns) constructs was measured as luciferase activity two days after transfection. Error bars correspond to SE calculated from four parallels in HeLa and from six parallels in BeWo. *P < 0.05; **P < 0.01*

5.1.4 Quantitative analysis of the ERVWE1 methylation in tumors

Upregulation of *syncytins* in non-placental tissues is suspected of involvement in tumor progression (Bjerregaard et al., 2006; Larsen et al., 2009; Strick et al., 2007; Sun et al., 2010). ERVWE1 demethylation in tissues with aberrant *syncytin-1* expression would confirm the importance of DNA methylation in its transcriptional suppression. Using MSqPCR we quantitatively analyzed the number of hypomethylated copies of ERVWE1 in various tumors in comparison with the healthy tissues (Fig. 4b). We analyzed samples from six patients with different testicular tumors, one with cancer of cervical carcinoma, one with endometrial carcinoma and one with breast cancer. The quantitave analysis was performed with bisulfite-treated DNA samples with a primer specific for the non-methylated CpGs within the U3 region of 5'LTR (Fig. 4a). Results were normalized to a reference sequence encoding the RNA polymerase 2A (RPII) with primers qbisRPII. Primers complementary to the reference gene do not pair with any CpG dinucleotides to amplify the converted sequence independently of its methylation status. MSqPCR revealed a clear increase of the number of hypomethylated ERVWE1 in several testicular seminomas, whereas in all other tumors including non-seminoma testicular tumors and in healthy controls, the number of hypomethylated copies was negligible. From patients T, T3 and T7 at least one sample of seminoma and one healthy testicular tissue was collected. From patient T6 teratoma mixed with embryonic carcinoma and healthy control was collected. From patient T4 we obtained two biopsies of seminoma and one with diagnosed carcinoma *in situ*. From patients SU, EM1, OV and BR we collected samples of cervical carcinoma, endometrial carcinoma, ovarian teratoma, and breast carcinoma. A healthy control sample was not available. We have demonstrated that ERVWE1 LTRs obtained from seminomas are significantly less methylated than the healthy control and other tumors. An increased number of hypomethylated copies was also detected in the carcinoma *in situ* sample T4 c*is* from a seminoma patient. The presence of hypomethylated copies in this sample suggests that demethylation occurs already in the early forms of carcinonoma. The ERVWE1 RNA expression was significantly increased in all seminomas including the carcinoma *in situ* in contrast with all other tissues and tumors (Fig. 5a).

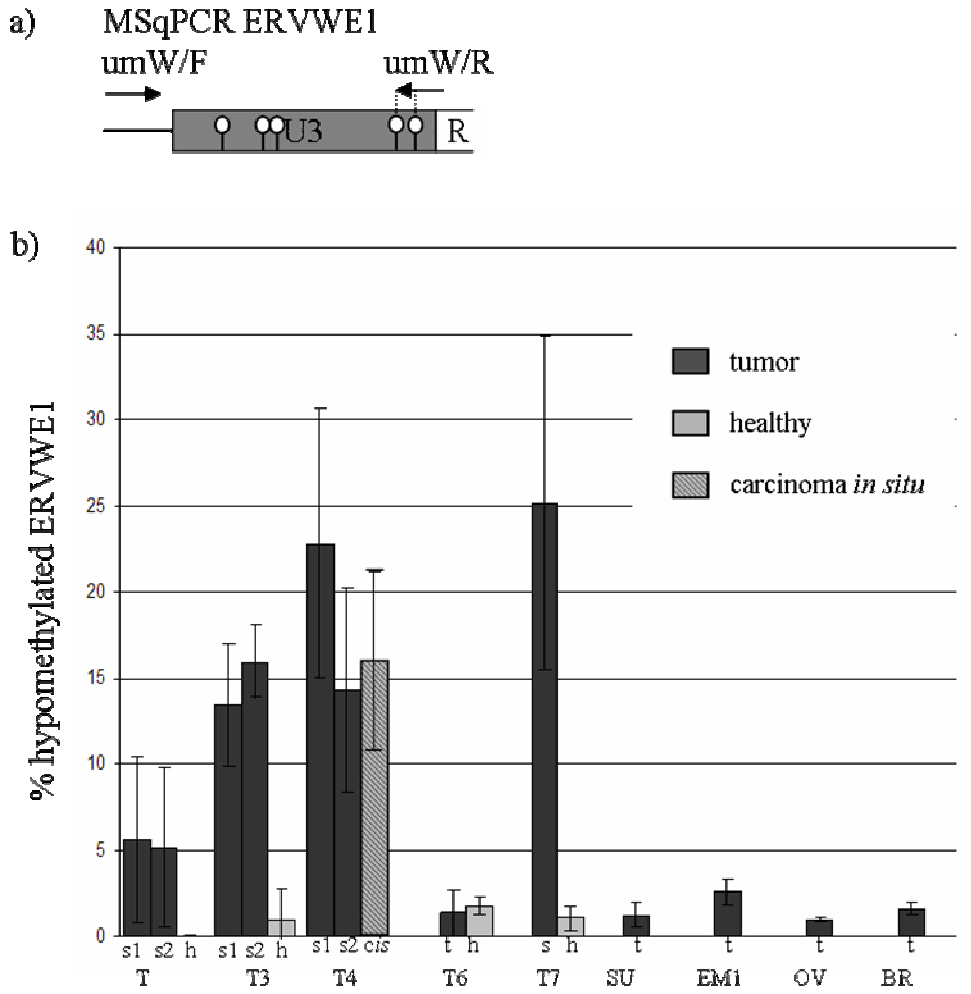


Figure 4 - Quantitative analysis of 5' U3 ERVWE1 methylation in the tumor. *(a) Schematic representation of the 5' LTR U3 region of the examined ERVWE1 provirus. Arrows show location of PCR primers. Lollipops represent CpG within the U3 regions. Vertical lines joining the arrow with U3 box mark the CpG dinucleotides decisive for amplification of the converted non-methylated sequence. (b) The numbers of hypomethylated 5' U3 ERVWE1 in various tumors and healthy controls were estimated by MS qPCR and normalized to reference sequence RPII. Results are shown as average percentage of the hypomethylated 5' U3 ERVWE1 in BeWo cells from three triplicates. Tumor samples are marked with t, healthy controls with h, seminomas with s, carcinoma in situ with cis. No healthy controls were available for SU, EM1, OV and BR. Error bars correspond to SE calculated from triplicates.*

5.1.5 Analysis of the ERVWE1 methylation and expression in tumors

We have performed the bisulfite sequencing of ERVWE1 to confirm the MS-qPCR results and the splice-specific real-time RT-PCR to analyze the RNA expression in all collected testicular samples. The bisulfite sequencing confirmed the heavy methylation of ERVWE1 5'LTR in non-seminoma testis. High methylation was detected in healthy tissues (Fig. 5b, c) as well as in T6 sample of teratoma mixed with embryonic carcinoma and T9 sample of lymphoma (Fig. 5b). We have also confirmed strong methylation of breast carcinoma (Fig. 5d). In seminomas the ERVWE1 methylation decrease was confirmed (Fig

5c). The methylation level of all samples was slightly lower than the MS-qPCR results suggest. The methylation decrease in most seminoma samples was even more significant. Only the low methylation in the carcinoma *in situ* of patient T4 was not confirmed by this technique. Certain differences are in agreement with the different possibilities of both methods. Whereas with MS-qPCR we are able to quantitatively analyze the methylation of two 5' LTR CpGs, with bisulfite sequencing we are able to qualitatively analyze all five U3 CpGs, although, however, only in a few representative copies in the DNA sample.

Further, we examined the ERVWE1 RNA expression. Since the *syncytin-1* protein is translated only from a correctly spliced RNA, we analyzed the expression of both, spliced and genomic RNA. In all examined seminomas the expression levels of both non-spliced and spliced transcripts were increased compared to normal tissues and other tumors (Fig. 5a). The total expression varied from 39% to over 600% of the expression level of RPII used as a reference gene. The control non-tumor testicular samples collected from the seminoma patients T7 and T10 express 2% and 4% of RPII, respectively, which is ten times less than the lowest expression in seminoma samples. The ERVWE1 expression in lymphoma T9 and embryonic carcinoma mixed with teratoma T6 was similar in the tumor sample and in control and varied from 1 to 3% of RPII expression (Fig 5a). Control samples from non-tumor patients T2, T5 and T11 varied from 2% to 16% of RPII. Further, we show that the splicing was efficient in all seminomas with the ratio of spliced over non-spliced transcripts varying between 1.7 and 3.9, while in the majority of other samples the spliced RNA was undetectable and in the sample with highest ratio the expression of spliced and non-spliced RNA was equal (Fig. 5a). These results suggest splicing efficacy to be another control step in *syncytin-1* expression. The ERVWE1 expression was variable in biopsies taken from different parts of the same tumor, which is consistent with the histological heterogeneity of the tumors. The increase of ERVWE1 RNA expression in different samples does not correlate with the level of LTR demethylation. While the most substantial demethylation was observed in patient T10, the RNA expression raised least and vice-versa, in samples with major expression we measured only 12% methylation decline. This is in agreement with the involvement of other factors in *syncytin-1* regulation. Demethylation is a necessary but not sufficient prerequisite for its expression. We conclude that spliced ERVWE1 RNA is present specifically in the testicular seminomas and that methylation together with inefficient ERVWE1 splicing prevents improper *syncytin-1* expression in most tissues including many tumors, but not in seminomas.

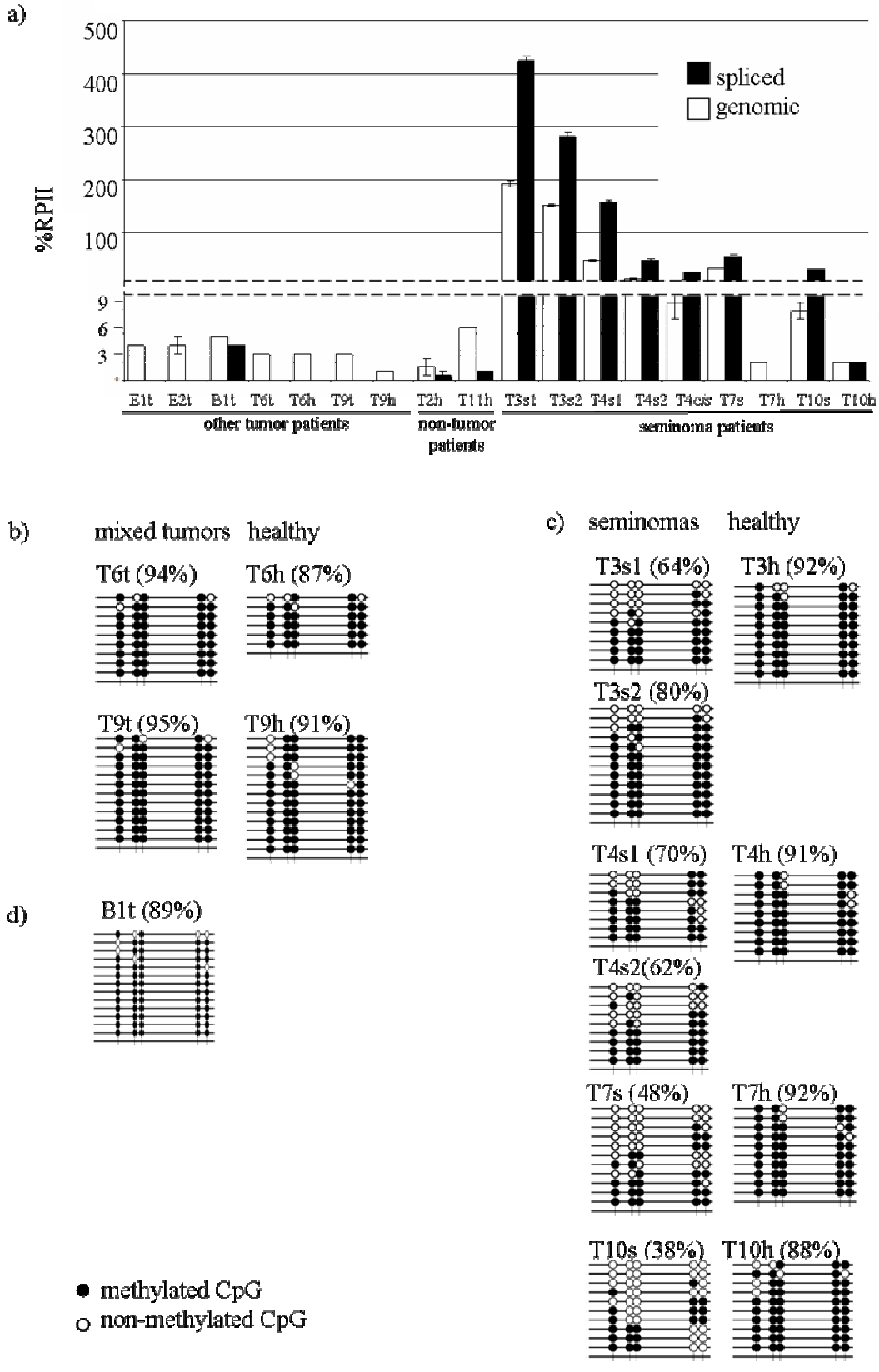


Figure 5 - Transcription, splicing and CpG methylation of ERVWE1 in tumors. *Tumor samples are compared with healthy control from the same patient when possible. Tumor samples are marked with t, healthy controls with h, seminoms with s, carcinoma in situ with cis. (a) The levels of genomic (open columns) and spliced (black columns) transcripts of ERVWE1 in samples from two patients with endometrial carcinoma E1, E2, one patient with*

breast carcinoma B1, one with mix of embryonic carcinoma and teratoma T6, one with lymphoma T9, two samples of healthy testes T2,T11, and nine samples from four patients with seminomas T3, T4, T7, and T10 were estimated by qPCR and are shown as the average percentage of the RNA polymerase II expression ± SD from three triplicates. The y axis is interrupted at the level of 10% of RPII and continues on larger scale. The CpG methylation status of the 5'LTR U3 ERVWE1 region of (b) two non-seminoma testicular tumors T6 and T9 with healthy controls, (c) six seminoma samples from four patients T3, T4, T7, T10 with healthy controls, and (d) breast carcinoma was examined by the bisulfite sequencing technique. Methylated CpG sites are indicated by solid circles, unmethylated CpG sites are indicated by open circles. Numbers in parentheses depict the percentage of methylated CpG dinucleotides.

5.1.6 Analysis of the ERVFRDE1 methylation in the testes and testicular tumors

Syncytin-2 expression in tumors would be of special interest due to its immunosuppressive nature. In the study by Gimenez et al. (2010) almost complete demethylation in a testicular tumor was shown. We were interested whether we could observe differences between tumors similarly as in the case of ERVWE1 promoter. We have analyzed four seminomas T, T3, T4 and T7 (Fig. 6a) and one teratoma combined with embryonic carcinoma T6 (Fig. 6b). The tumor tissues were compared with healthy tissues from the same patients. We have detected a decrease of methylation in all tumors including the non-seminoma. Only in one seminoma biopsy T4s2 collected from the T4 patient the observed methylation was higher than in the control tissue. This pattern strikingly contrasts with the completely demethylated second biopsy T4s1. The methylation in tumor samples varied from 11 to 84%. The content of methylated CpG of ERVFRD1 in the control testicular tissue varied from 72 to 100%. Moreover, we detected 5'LTR U3 ERVFRDE1 of a non-tumor patient with merely 59% of methylated CpG (Fig. 1e). However, the two CpG on the 3'end of the U3 region were mostly methylated in all healthy controls. This observation is in agreement with the results in the study by Gimenez et al. (2010). The high variability of samples suggests that many factors can easily influence the LTR methylation pattern. The methylation of two 3' CpGs seems to be more stable than the rest. Despite a clear decrease of methylation in the tumor in comparison with the healthy tissue from the same patient the direct connection between ERVFRDE1 demethylation and tumor development is disputable, as low methylation was observed in the non-tumor testes, too. Neither the demethylation of 3' CpGs shows clear correlation with tumor development, although demethylated 3' CpGs were observed only in seminoma samples.

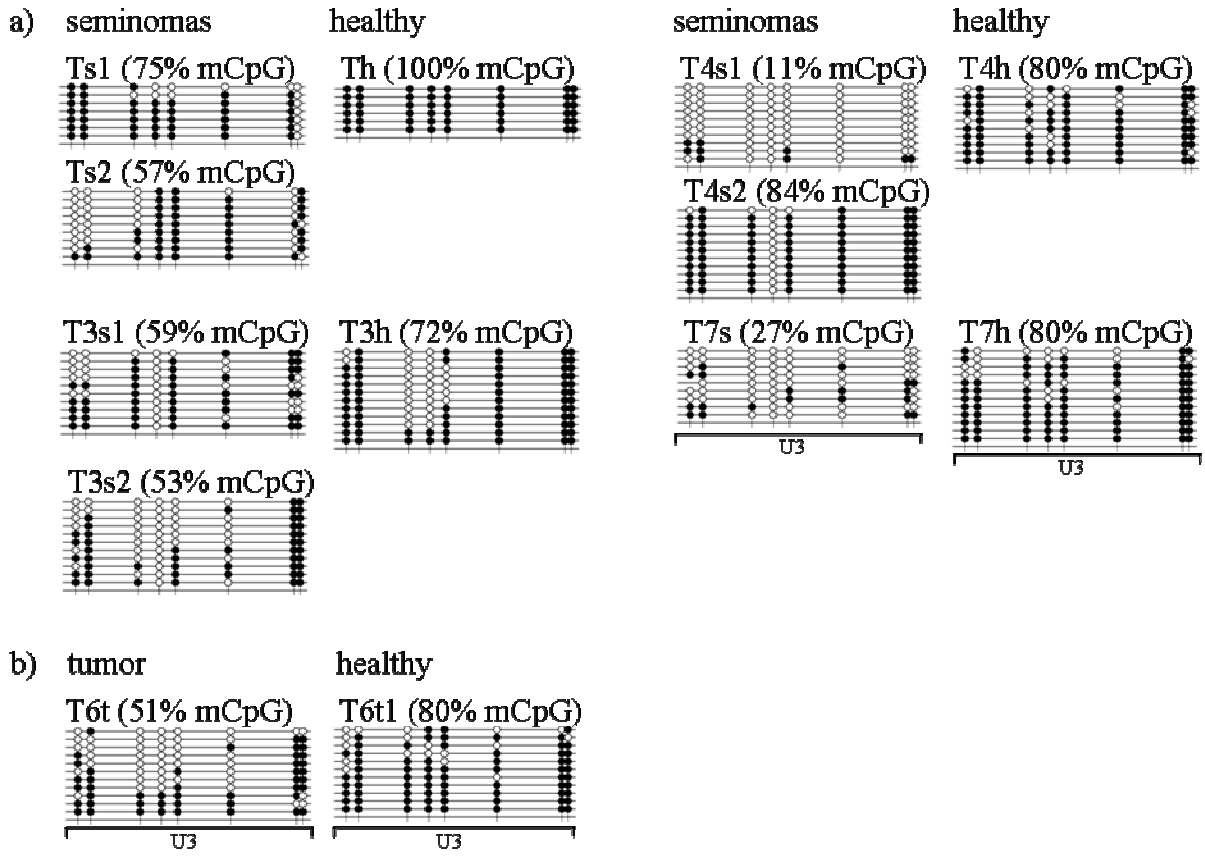


Figure 6 - The CpG methylation status of the 5'LTR U3 ERVFRDE1 region *of (a) seven seminoma samples from four patients T, T3, T4 and T7 with healthy controls and (b) one non-seminoma testicular tumor T6 with healthy controls was examined by the bisulfite sequencing technique. Methylated CpG sites are indicated by solid circles, unmethylated CpG sites are indicated by open circles, and numbers in parentheses depict the percentage of methylated CpG dinucleotides.*

5.2 CpG methylation and the expression of PERV

5.2.1 CpG methylation decreases the transcriptional activity of PERV LTRs.

We were interested whether DNA methylation plays a role in PERV regulation. Primarily, we examined the sensitivity of PERV 5'LTR regulatory sequences to DNA methylation. In order to assess their sensitivity we performed an experiment with *in vitro* methylated reporter constructs similarly as we tested the ERVWE1 5' LTR. We have prepared constructs with firefly luciferase gene *luc* drived by two different PERV LTRs: plasmid pLTR-A-luc and pLTR-B-luc with LTRs from PERV MAMBA1 and PERV-60, respectively. These plasmids were methylated *in vitro* by the bacterial CpG-specific *Sss*I DNA methylase and transiently transfected into human kidney 293T cells. The luciferase activity induced by the methylated plasmids was more than five times lower than in cell transfected by non-methylated plasmids. The luciferase activity in cells transfected with pLTR-A-luc was about twice lower than in cells with pLTR-B-luc. The decrease of activity after

methylation was similar with both plasmids (Fig. 7). We conclude that PERV-A and PERV-B LTR-driven transcription is sensitive to CpG methylation *in vitro*.

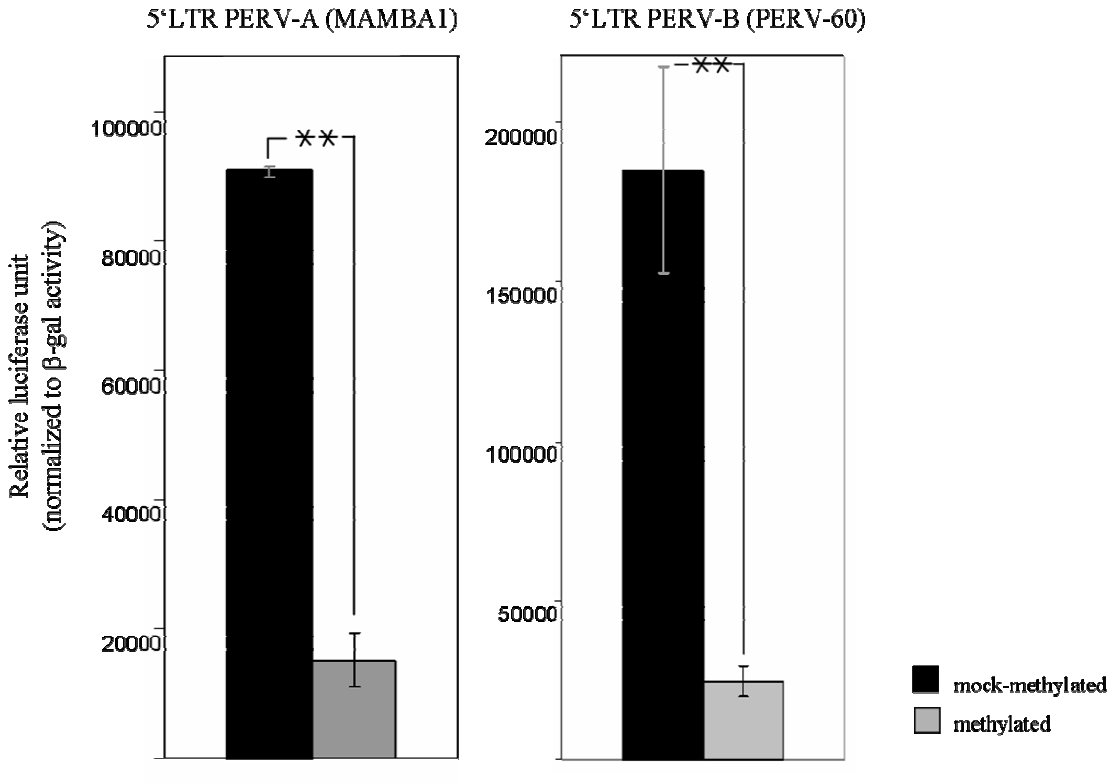


Figure 7 - Effect of CpG methylation on the transcriptional activity of PERV-A and PERV-B 5′ LTR in 293T cells. *The complete PERV 5′ LTRs were fused with the luciferase reporter into pLTR-A-luc and pLTR-B-luc. These vectors were in-vitro-methylated with SssI methyltransferase, and 1 μg of plasmid DNA was transfected together with pCMVβ-gal transiently into 293T cells grown on 35 mm Petri dishes. The transcriptional activity of mock-methylated (black columns) and methylated (grey columns) constructs was measured as luciferase activity two days after transfection and normalized to β-gal activity. Error bars correspond to SE calculated from three parallels. **P < 0.01*

5.2.2 The majority of PERV LTRs in porcine tissues are methylated.

We have performed the global analysis of PERV LTR CpG methylation by bisulfite sequencing. Using primers universal for most PERVs, we have analyzed methylation of three CpGs from the U3 region and seven CpGs from the R region in 11 different porcine tissues. In order to obtain bisulfite sequences from most of the PERVγ1 proviruses, we have designed primers complementary to 16 different PERVγ1 LTR sequences from all three subgroups (accesory numbers AJ279056.1, AY570980.1, AF435967.1, AJ133817.1, AF435966.1, AJ293656.1, AY099323.1, AJ279057.1, AJ293657.1, AJ133816.1, AJ133818.1, AY099324.1, HQ540593.1, AF038600.1, AF038599.1) avoiding the CpG sites within the primers. We have used these primers to amplify bisulfite-treated DNA from 11 porcine tissues from one or two individual pigs. The obtained PERV LTR

methylation pattern is ambiguous. The scheme (Fig. 8a) presents CpG dinucleotides common for all 16 PERV sequences depicted as white lollipops; CpGs symbolized by grey lollipops are missing in at least one sequence out of the 16 PERVs. This variability of reference sequences made it impossible in some cases to distinguish the converteded non-methylated CpGs from the TpG dinucleotides. The majority of PERV LTRs were prevalently methylated in all tissues apart from one sample of placenta and decreased methylation was observed in the sample of lung (Fig 8b). Interestingly, this contrasts with lower LTR methylation in porcine cell line PK15 expressing a high level of PERV RNA and infectious PERV particles. The high level of methylation was confirmed in the sample of pig kidney despite that the PK15 cell line is derived from kidney epithelium. Heavy methylation of the cell line ST-IOWA derived from fetal swine testis is similar as in our sample of testis. The hypomethylation of PERVs in the placenta sample was anticipated. We can assume total lower methylation and expression of ERVs in porcine placenta similarly as in other mammals. The decrease of methylation in lung sample was surprising. However, in this case none of the LTR sequences was completely demethylated; rather few CpGs were demethylated in each of the obtained sequences, which may not have been sufficient for the PERV expression.

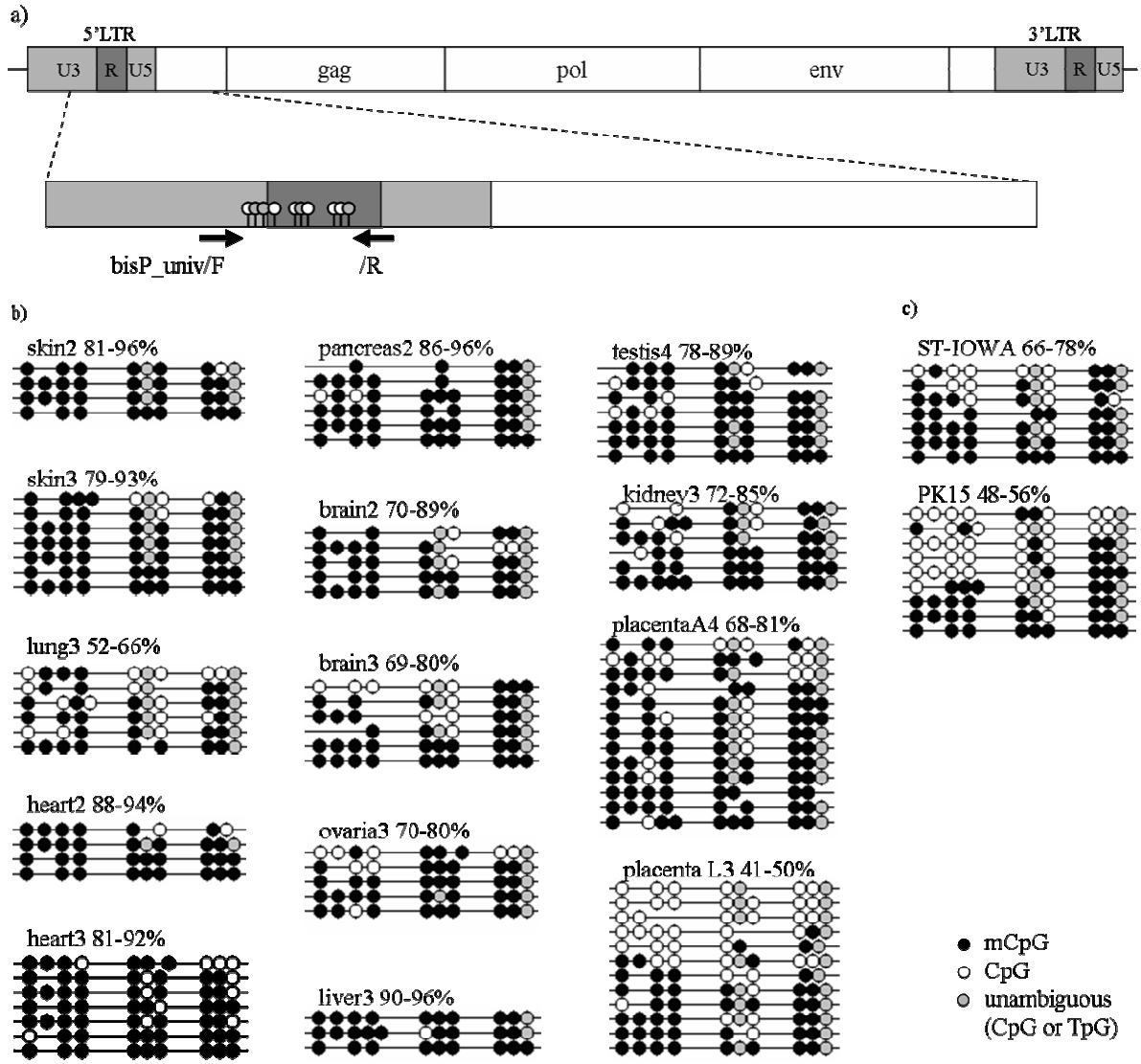


Figure 8 - CpG methylation status of the U3/R region of PERVs. *(a) Schematic representation of complete PERV provirus and of its 5' LTR region and leader sequence. Arrows show location of PCR primers. White lollipops represent CpGs common for all PERV sequences; grey lollipops represent CpGs present in part of the PERV sequences. (b) CpG methylation status of the U3/R region of PERV LTR detected by bisulfite sequencing in pig tissue samples, (c) CpG methylation status of the U3/R region of PERV LTR in porcine cell lines. The total number of CpGs per sequence varies from 8 to 10 CpGs. Numbers depict the percentage of methylated CpGs from all CpG dinucleotides. The lowest number is the percentage of methylated CpGs from all CpGs including the unambiguous, the highest number is the percentage from all CpGs excluding the unambiguous CpGs. Methylated CpG dinucleotides are depicted by solid circles, unmethylated CpG sites are indicated by open circles, unambiguous CpGs are indicated by grey circles.*

5.2.3 Quantitative analysis of hypomethylated PERV LTRs in porcine tissues and cell lines

Bisulfite sequencing allows us to detect the methylation status of the majority of LTRs. However, the porcine genome contains several tens of PERVs and expression of one or a few demethylated proviruses could be crucial for the PERV transmission while the majority of

methylated PERVs may not be important. Therefore, we decided to compare the hypomethylated PERVγ1 LTR copies in porcine tissues quantitatively and find out whether we could detect significant differences between them. We used the MS qPCR for this purpose. We designed primers complementary to 16 different PERVγ1 sequences binding the region rich in CpG dinucleotides (Fig. 9a). Primers were designed to be complementary to the non-methylated CpGs converted to TpGs. To increase the ability of primers to distinguish between CpG and TpG we localized the crucial complementary adenosines and thymines in the forward and reverse primer, respectively, at the very 3'end when possible. All bases distinguishing the non-methylated CpGs were on the LNA backbone. With these primers, we have quantitatively amplified the hypomethylated PERV LTRs. Results were normalized to a reference sequence coding the elongation factor of RNA polymerase 2 (ELF2) with qbisELF primers. Primers complementary to the reference gene do not pair with any CpG dinucleotides to amplify the converted sequence independently of its methylation status. We succeeded to amplify the hypomethylated proviruses from every examined tissue from all pigs and from the cell lines PK15 and ST-IOWA (Fig. 9b). According to the results of MS qPCR the cell line PK15 contains the highest number of the hypomethylated PERVs. One sample of placenta contains about half of the hypomethylated PERVs detected in PK15. In other tissues the number of hypomethylated copies of PERVs was three times to ten times lower than in the cell line PK15. We did not detect any significant differences between tissues common for all pigs; neither did any examined pig display a different methylation level of PERVs in its tissues. The decrease of PERV LTR methylation in PK15 detected by this technique is more significant than what suggested the bisulfite sequencing.

In representative tissues, we analyzed the PERV RNA expression by quantitative RT-PCR (Fig. 9c). The measured PERV RNA varied from less than 0.1 to 2.5% of GAPDH RNA. In PK15, PERV expression reached 28% of GAPDH. Due to the poor quality of RNA we were not able to measure the PERV expression in the sample of placenta L3 with low methylation. We conclude that the decreased PERV methylation correlates with increased PERV expression. PERVs in porcine tissues are extensively methylated and silenced.

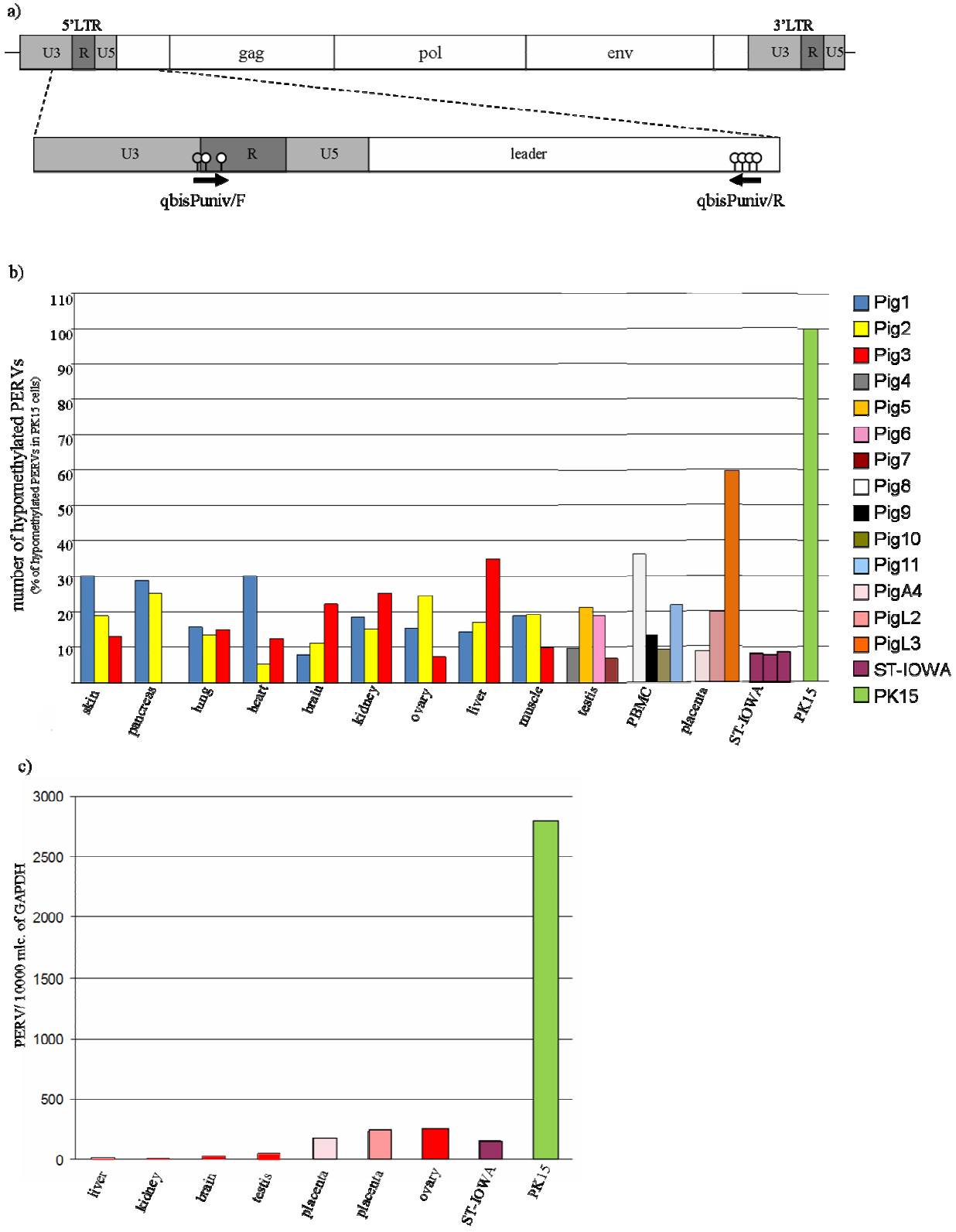


Figure 9 - DNA methylation quantitative analysis of 5' LTR and leader sequence of PERV proviruses in pig tissues and cell lines *a) Schematic representation of complete PERV provirus and of its 5' LTR region and leader sequence. Arrows show location of PCR primers. White lollipops represent CpGs common for all PERV sequences; grey lollipops represent CpGs present in part of the PERV sequences. Only CpGs within the location of PCR primers are depicted in the scheme. b) The numbers of hypomethylated PERV 5' LTRs*

per genome in 12 pig tissues and two cell lines were estimated by MS qPCR and normalized to the number of ELF2 DNA copies estimated by qPCR. Results are shown as average percentage of the number of hypomethylated PERV 5' LTRs per genome in PK15 cell line from three triplicates. Tissues were collected from two to four pigs. c) The levels of transcript of PERVs in seven pig tissues and two pig cell lines were estimated by qPCR and are shown as the average number of PERV RNA molecule per 10000 RNA molecules of GAPDH from three triplicates. Each color represents individual pig or cell line.

The differences between the data obtained from bisulfite sequencing and the MS qPCR may have several causes. First, the results obtained from sequencing demonstrate methylation of the whole PCR fragment, whereas MS qPCR results depend only on the methylation status of the CpG within the primers. Second, we sequenced the R region of LTR whereas the MS qPCR analysis targeted the sequence on the border of U3 and R regions and the leader sequence. Third, via MS qPCR we are able to amplify and quantify specifically the demethylated sequences despite their minor representation in the sample, whereas use of the bisulfite sequencing enables identification of only the prevailing methylation pattern.

5.2.4 Methylation pattern of individual PERV proviruses

Identification of particular transcriptional active PERV copies would be helpful in selection of pigs with low PERV expression. High homology between individual PERV proviruses and insertion polymorphism in individual pigs disables identification of active proviruses according to the expressed PERV RNA. Determination of the methylation status of selected provirus can be used as confirmation or disconfirmation of proviral activity estimate. We analyzed the methylation status of four proviruses; two PERVs from subgroup A termed MAMBA1 and MAMBA7 and two from subgroup C termed 6SH and RW. These proviruses were selected on the basis of the estimate that these PERVs might be transcriptionally active (based on unpublished results of Hector et al. and Buzdin et al.). PERV-C 6SH was found to be hypomethylated in about half of the analyzed sequences (Fig 10c), whereas the other PERVs were fully methylated (Fig 10a, b, c). Sequences of PERVs MAMBA-1 and -7 are obtained from porcine skin samples, sequences of PERVs 6SH and RW are obtained from blood cells. MAMBA1, MAMBA7, PERV-C 6SH and PERV-C RW sequences originate from four, one, four and three animals, respectively. All sequences were amplified with reverse primers specific to the internal PERV sequence and at least one forward primer specific to flanking sequences to ensure the amplification of provirus from one integration site. The analyzed part of the regulatory sequence was selected according to the available knowledge of the provirus sequence and in order to avoid the CpG positions. The bimodal methylation pattern of PERV-C 6SH provirus reminds methylation of ERVWE1 promoter. It

may be a result of imprinting or we may have detected patterns of more blood cell types with different PERV methylation. Interestingly, the hypomethylated pattern is restricted to the PERV sequence. The CpG within the flanking sequence is methylated in most sequences in all animals. We conclude that the PERV-C 6SH is probably transcriptionally active.

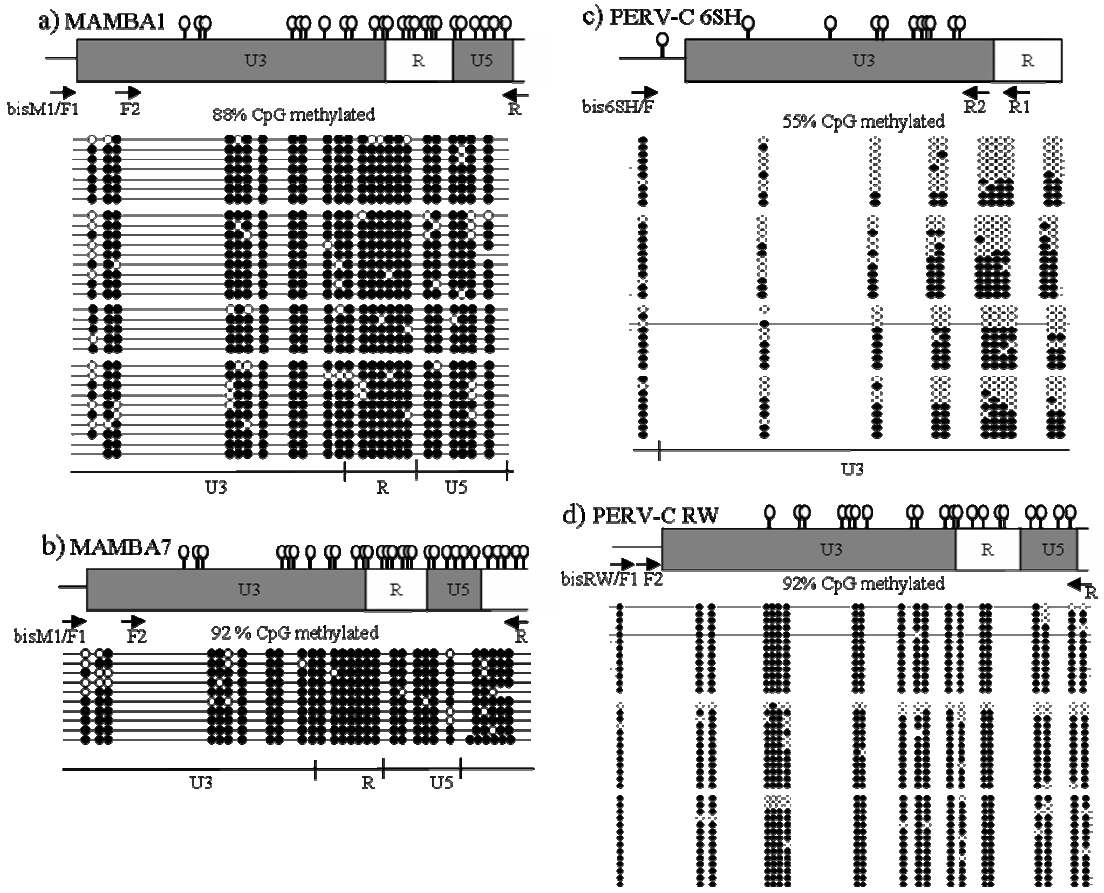


Figure 10 - CpG methylation status of the 5' LTR region of four individual PERV proviruses. *Schematic representations of the examined regions are shown for each provirus. Arrows show location of PCR primers. Lollipops represent all CpGs within the examined regions. The CpG methylation status of regulatory sequences of (a) PERV-A MAMBA1 in skin samples from four animals, (b) PER-A MAMBA-7 in skin sample from one animal, (c) PERV-C 6SH in blood sample from four animals and (d) PERV-C RW in blood samples from three animals was examined by the bisulfite sequencing technique. Methylated CpG dinucleotides are depicted by solid circles; unmethylated CpG sites are indicated by open circles.*

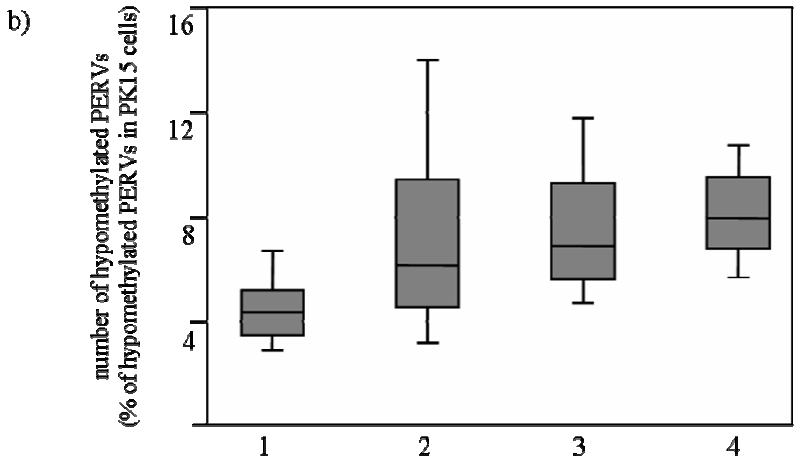
5.2.5 CpG methylation stability of the PERV LTRs

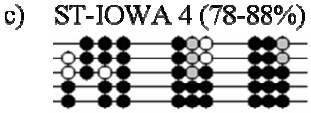
In clinical transplantation, ischemia-reperfusion injury induces oxidative stress. Demethylation of cytosines is one form of DNA damage caused by these conditions (Parker et al., 2008 19104428). We therefore analyzed the stability of the CpG methylation level within the PERV LTR in ST-IOWA cells. We treated them with inhibitors of DNA methylation and histone deacetylation, azaC and TSA, respectively. We applied azaC alone or together with TSA in two different concentrations (Fig. 11a). By MS qPCR we detected only minor

demethylation with significant difference only between non-treated cells and the treated ones independently on the agent concentrations and combinations (Fig. 11b). However, the number of demethylated PERV LTRs was more than ten times lower than in PK-15 cells. We did not detect any decrease of methylation by bisulfite sequencing (Fig. 11c). All LTR sequences obtained from the ST-IOWA cells treated with combination of the highest concentrations of azaC and TSA were nearly completely methylated. Furthermore, we analyzed whether the treatment influenced the expression. RNA from azaC- and TSA-treated ST-IOWA cells was isolated and quantified by RT-PCR (Fig. 11d). The obtained values were related to the expression of GAPDH. PERV RNA expression of the non-treated cells was 1.6% of the level of GAPDH expression. Surprisingly, we have observed decreased PERV expression in the treated cells. Considering the very low PERV expression in the non-treated cells we can assume that the decrease of expression is a consequence of a misbalance induced in the cell by the inhibitors rather than an effect specific for the PERVs. Possible increase of GAPDH would influence the results.

a)

	1	2	3	4
AzaC (μM)	0	100	50	100
TSA (μM)	0	0	0,03	0,03





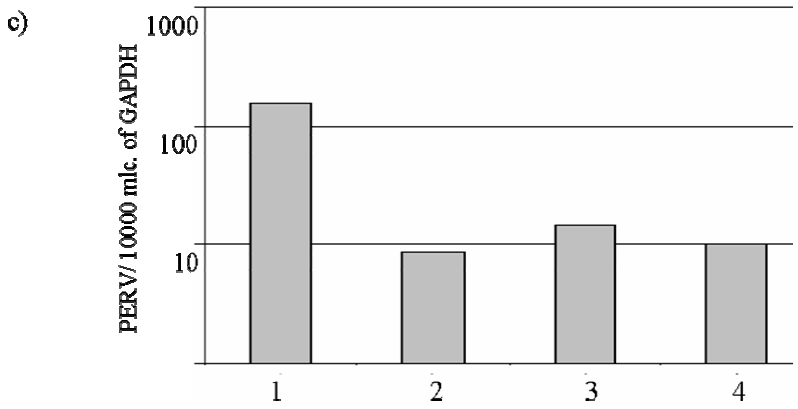


Figure 11 - Impact of the DNMT and histone deacetylase inhibitors azaC and TSA on the CpG methylation and the expression of PERVs ST-IOWA cells. *(a) Depicted concentrations of AzaC and TSA and their combinations were used for treatment of four cultures of ST-IOWA cells numbered 1, 2, 3 and 4. (b) The numbers of hypomethylated PERV 5' LTRs per genome in ST-IOWA samples from cell cultures 1-4 treated with increasing concentrations of AzaC and TSA were estimated by MS qPCR and normalized to the number of ELF2 DNA copies estimated by qPCR. Results are shown as average percentage of the number of hypomethylated PERV 5' LTRs per genome in PK15 cell line from three triplicates. Horozontal line represents average values, error bars represent 95% confidence intervals, boxes represent upper and lower quartiles. According to ANOVA test ST-IOWA 1 differs from other samples at the level of probability $p < 0.05$. (c) The levels of transcript of PERVs in ST-IOWA samples from cell cultures 1, 2, 3 and 4 were estimated by qPCR and are shown as the average number of PERV RNA molecule per 10000 RNA molecules of GAPDH from three triplicates. (d) CpG methylation status of U3/R region in ST-IOWA sample 4 detected by bisulfite sequencing. Converted DNA was amplified with bisP_univ primers (Fig. 8a).*

5.2.6 Methylation of PERV proviruses in human cells

Host cells often protect themselves against retroviral infection by silencing of the newly integrated proviruses by methylation. We were interested whether human cells are able to silence in this way PERV proviruses in case of infection. To examine this, we infected the highly sensitive human cell line 293T with PERV-14/220 or PERV-3a. Infected cells were collected after different intervals from two days up to two months. We have observed a very slow slight increase of 5‘LTR methylation (Fig 12). In the long-term-infected 293T cells used as a source of PERV-14/220 virus we have not detected any methylated PERV LTR (Fig 12b). Low methylation of proviruses in this cell line could be connected with high PERV expression and frequent new integrations into the host DNA. These results suggest that at least at the transcription level human cells have very poor protection against PERVs. An interesting phenomenon of changing the repetition number within the U3 region was observed in the replicating PERV-3a (Fig. 12a). Despite that the virus used for infection was collected from cells transfected with one plasmid isolate bearing the PERV-3a with three repetitions within the LTR, in the infected cells we detected LTRs with one, two or three repetitions. Repetitions are marked by gray rectangles. These repetitions have significant influence on the efficiency of LTR as promoter (Sheef et al., 2001; Denner et al., 2003). Fast changes of repetition numbers demonstrate the inaccuracy of reverse transcriptase.

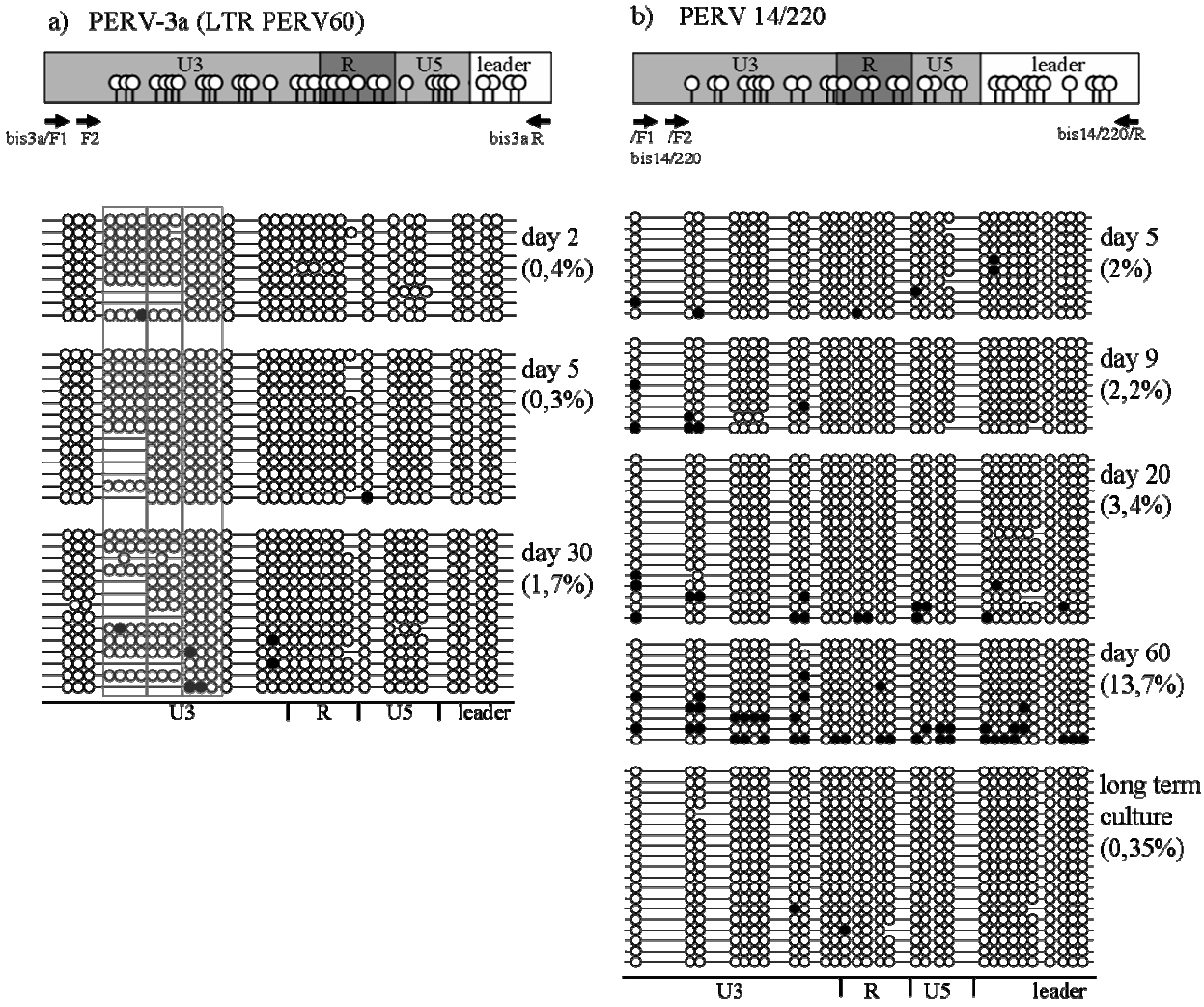


Figure 12 - Progression of CpG methylation of the 5' LTR and leader sequences of PERVs after infection to human 293T cells. *Upper schemes represent 5' LTR regions and leader sequences of the examined PERVs. Arrows show location of PCR primers. Lollipops represent CpGs within the depicted regions. Lower plots represent the CpG methylation status of indicated regions. Numbers on the right side indicate the time of cell collection after infection and in parentheses the percentage of methylated CpGs. (a) PERV3a-infected cells were collected in three terms and (b) PERV14/220-infected cells in four terms. Last plot represents the examined region in 293T cells used. Gray rectangles indicate position of U3 repetitions. Methylated CpG dinucleotides are depicted by solid circles, unmethylated CpG sites are indicated by open circles.*

5.3 PERV-A receptors

5.3.1 Comparison of PERV-A receptor sequences

The majority of tested cells including mouse and rat cell lines have been shown to be resistant to PERV-A infection (Takeuchi et al., 1998; Wilson et al., 2000). The host range of gammaretroviruses is often determined by the functionality of their receptor genes (Tailor et al., 2000). Transfection of cDNA for human PERV-A receptors huPAR-1 and 2, but not their murine homolog, muPAR, conferred PERV-A infectivity in otherwise resistant rabbit and

murine cell lines (Ericsson et al., 2003). Based on these results we hypothesized that the PERV-A resistance of mouse and rat cells may be due to defective mutations for PERV-A receptor function in muPAR and the rat homolog, ratPAR, and that such mutations may be shared in these two rodent species. We set out our initial experiments to test this hypothesis and first cloned a cDNA for rat PAR from PERV-A-resistant NRK cells. Its predicted amino acid sequence is almost identical (only 2 a.a. difference in 450 a.a.) to that in the rat genome database [GenBank: XM_343272] and differs from the muPAR sequence by 9.6% (Table 1). MuPAR and ratPAR are similarly distant from huPAR-1 and -2, about 20% mismatch and share 43 rodent-specific mutation (a.a. present in the mouse and rat but different from human) in 450 a.a.

Table 1: Amino acids identities

			RatPAR
		MuPAR	90.4%
	HuPAR-1	81.1%	79.3%
HuPAR-2	86.1%	79.6%	79.0%

5.3.2 Functionality of human and rodent PARs

Next, we tested the receptor function of rodent PARs in comparison with human PARs. In this assay, all receptors were expressed as C-terminal HA-tagged forms using an MLV-based retroviral vector. This allowed stable PAR expression in various target cells and quantification of their surface expression by immunostaining with an anti-HA antibody. Human 293T, murine MDTF, rat NRK and quail QT6 cells were transduced to express various PARs, so that 50 to 70% of the cells expressed PAR on their surface. PERV-A infection of cells with or without various PARs was tested using high-titer PERV-A containing an MLV vector genome encoding EGFP [EGFP(PERV-A)] (Bartosch et al., 2004) (Fig 13). The overexpression of any PAR in human 293T cells did not increase the infection efficiency, suggesting that endogenous huPAR expression supports maximal PERV infection in these cells. Despite no PERV-A infection being recorded in MDTF, NRK and QT6 cells without exogenous PAR, these resistant cell lines became susceptible to PERV-A infection upon expression of huPAR molecules (Fig. 13). This result suggests that PERV-A infection is blocked at the entry level and that expression of a functional receptor can overcome this block. MuPAR, unlike huPARs, could not rescue PERV infection when expressed in resistant cell lines (Fig. 13). This result, consistent with the previous report (Ericsson et al., 2003), confirmed that muPAR expressed on the cell surface is defective in PERV-A receptor function.

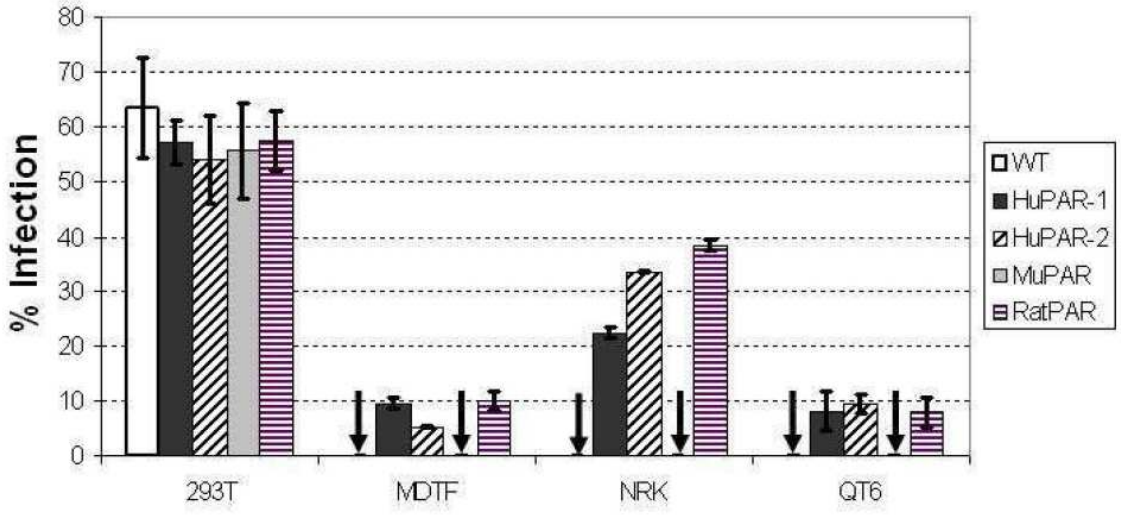


Figure 13 - PERV-A receptor function of HuPARs and their rodent homologs. *The different cell lines were transduced with the same amount of retroviral vector encoding the HA-tagged receptor genes. Transduced cells were then infected with EGFP(PERV-A). Forty-eight hours post-infection cells were analyzed by flow cytometry and the efficiency of infection was determined as percentage of EGFP-positive cells. The histograms represent the average ± SE from three independent experiments. The arrows indicate an infection below detectable levels.*

5.3.3 Quantification of RatPAR receptor RNA

RatPAR, like huPARs and unlike muPAR, allowed PERV-A infection in all the resistant cell lines, including rat NRK cells from which it was derived (Fig. 14). It was suspected that the ratPAR expression level is critical for sensitivity to PERV-A entry. Due to the unavailability of an anti-PAR antibody, it was not possible to investigate endogenous protein expression. Therefore, the amount of ratPAR mRNA was measured by real time RT-PCR in three rat cell lines, NRK, HSN, and XC, before and after exogenous expression of ratPAR. PERV-A infectivity of these cultures is plotted against the ratPAR mRNA level in Fig. 14. Rat cells became PERV-A sensitive when the level of ratPAR mRNA was increased 40–500 fold by exogenously expressing ratPAR. The endogenous expression level of ratPAR therefore appears to be too low to support PERV-A infection, whereas exogenous ratPAR was overexpressed to the level high enough to allow PERV-A entry into rat cells. To demonstrate the dependence of PERV infection on the rat-PAR expression level, we produced QT6 cell clones with various expression levels of C-terminal HA-tagged ratPAR. PERV-A infection efficiency was dependent on the ratPAR expression level as measured by anti-HA surface staining. Overall, the mechanism of resistance to PERV-A entry differs between two rodent species, mouse and rat, and the molecular basis of muPAR defect was further investigated (Mattiuzzo et al., 2007).

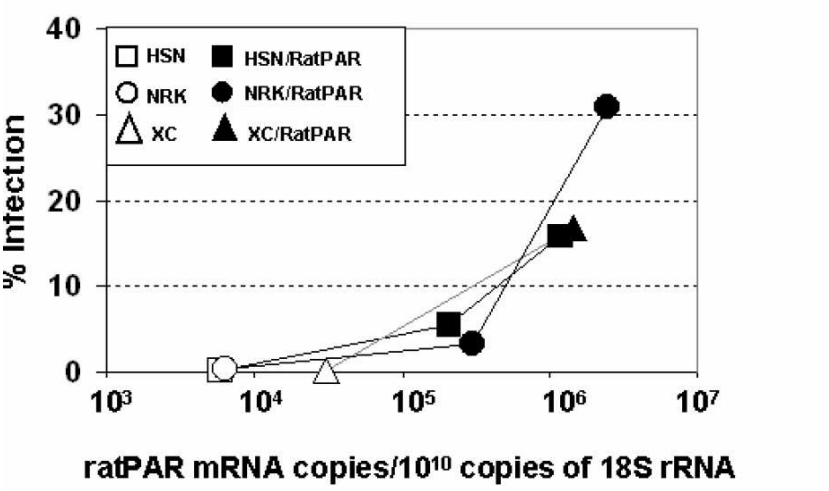


Figure 14 Quantification of RatPAR receptor RNA. *NRK, HSN and XC rat cells were transduced with a retroviral vector encoding the ratPAR gene. Two independent transductions were performed in NRK and HSN cells. The RNA from transduced and untransduced rat cells were extracted. The amount of ratPAR was determined by real-time RT-PCR and normalized to equalized copies of 18S rRNA. The results were correlated with the efficiency of EGFP(PERV-A) infection. All the samples were run in duplicate and the experiment repeated at least two times.*

6 Discussion

In the first part of our study, we focused on the regulation of HERVs involved in human placenta development, ERVWE1 from family HERV-W bearing the gene *syncytin-1* and ERVWFRD from family HERV-FRD bearing the gene *syncytin-2*. In the second part we focused on the regulation of a recent family of PERVs γ1. In both cases, we were interested mainly in the regulation by DNA methylation. We have shown that methylation is involved in the regulation and silencing of all examined ERVs; however, the differences in the expression cannot be explained purely by DNA methylation and other factors are needed for their activity. For example, chromatin modification and correct mRNA splicing are other factors necessary for Env protein production. Expression of *syncytin-1* and *syncytin-2* is strictly placenta-specific and their expression in other tissues is not physiologic. In contrast, PERVs neither fulfil any particular role in the host cells nor induce any visible pathology in pigs. Low expression of PERVs is detectable in various porcine tissues, suggesting that PERV expression in general is not harmful for the porcine organism and silencing is important mainly for keeping the genome integrity and moderation of insertional genotoxicity. However, silencing of some particular PERV loci may be crucial, similarly as silencing of particular HERVs. A certain variability of the PERVγ1 methylation pattern and expression is probably connected with their recent integration (reviewed in Reiss et al., 2007). In the last part of our study we focused on the resistance of two rodent species mouse and rat to PERV-A. We have demonstrated that both species are resistant to the viral entry; however, the mechanisms of this resistance are diverse. Results concerning the DNA methylation of *syncytin-1* were published in 2006 (Matouskova et al., 2006). Our results concerning PERV methylation have not yet been published. The PERV-A receptor study was published as a part of the study performed by Giada Mattiuzzo (Mattiuzzo et al, 2007)

The regulation of the *syncytin-1* transcription has been so far studied in terms of transcription factors and their binding sites, hormonal levels and oxygen level in the placenta. GCMa is the best candidate for the role of master activator of *syncytin-1* because it interacts with two GCMa-binding sites upstream from the 5' LTR and increases the level of *syncytin-1* transcripts in BeWo and JEG3 choriocarcinoma cells about 4- and 3-fold, respectively (Yu et al., 2002). Furthermore, it is specifically synthesized in the developing placenta (Basyuk et al., 1999). Ecdysone receptor response element was identified as a negative *cis*-regulatory sequence within the *syncytin-1* 5' LTR Cheng et al. (2003). Regulation of *syncytin-2* has not

yet been studied in great detail. In the ERVFRDE1 5'LTR, several regulatory elements such as two Sp1 sites, GATA, E2, CAAT box, NF-B or HSE were identified (Gimenez et al., 2009).

Provided that HERVs as well as other transposable elements are transcriptionally suppressed by methylation, as recently shown e.g. in the HERV-K family (Lavie et al 2005), and that placenta, where the *syncytins* are expressed, contains hypomethylated tissues derived from trophoblast and primitive endoderm (Li, 2002 for the review), we analyzed the CpG methylation pattern of *syncytin* regulatory sequences and its influence on *syncytin* expression. In accordance with our assumption, we have detected hypomethylated or completely unmethylated *syncytin-1* promoter in the placenta, although only in part of the obtained sequences. The obtained sequences of ERVFRDE1 LTR were all found to be completely or partially demethylated (Fig. 1b). The bimodal methylation pattern could have reflected an imprinted regulation of ERVWE1 (Smallwood et al., 2003). Methylation of one allele in a parent-of-origin manner is often related to genes involved in placenta morphogenesis (Reik et al., 2001). However, later analyses of trophoblast in different stages of pregnancy revealed that the methylation increases from complete demethylation in the first trimester placenta to the highest methylation at term, which argues against the proposed imprinting hypothesis (Gimenez et al., 2009). The apparently increased frequency of hypermethylated sequences from the first trimester to term is consistent with the decrease in ERVWE1 *env* expression observed between 37 and 40 weeks of gestation (Chen et al., 2006). In contrast, ERVFRDE1 is unmethylated throughout the whole gestation and modulation of its expression is apparently dependent on the transcriptional factors. In choriocarcinoma-derived BeWo and JEG3 cell lines, we found complete or nearly complete demethylation of promoter sequences of *syncytin-1*. Methylation of *syncytin-2* was analyzed only in the BeWo cell line and all obtained clones were completely or almost completely demethylated as well (Fig. 1c). These results are consistent with the expression of *syncytins* in both cell lines. In HeLa cells containing heavily methylated *syncytin-1* LTR, the expression was silenced. In all tested tissues we detected heavily methylated ERVWE1 LTR, which is in consistence with its restricted expression (Mi et al, 2000). The importance and stability of *syncytin-1* methylation also confirms the methylation of the promoter in blood cells from ICF patients who have decreased overall methylation thanks to the mutated DNMT3b (Hansen et al., 1999) (Fig. 1g).

Syncytin-2, which is also restricted to the placenta (Parceval et al., 2003), was found to be methylated in fibroblasts (Fig. 1d) but not completely methylated (59%) in the testis (Fig. 1e). Half of the sequences obtained from this sample were completely demethylated apart

from two CpG at the 3'end. These two CpGs were preferentially methylated also in biopsies of healthy testicular tissue from patients with testicular cancer and in some tumor samples. These results are consistent with the results of Gimenez et al. (2009), who also described precise and accurate methylation of these CpGs close to the TATA box. Similar impairment of the transcriptional induction by disturbing the attachment of the TATA binding protein was observed for the RANKL gene promoter (Kitazawa et al, 2007). It is tempting to speculate that low methylation of *syncytin-2* in the testes enables its expression there and plays some role in the testicular immune privilege. However, this partial demethylation was identified by Gimenez also in blood cells. Therefore, we can assume that the precise CpG methylation and transcription factors are sufficient to regulate the expression of *syncytin-2*.

Treatment of HeLa cells with high doses of DNA methylation and histone deacetylase inhibitors AzaC and TSA affected the *syncytin-1* methylation just slightly, and it had no effect on the *syncytin-1* expression (Fig. 2). This indicates that the hypermethylation of the *syncytin-1* promoter is very stable because such treatment efficiently demethylates and transcriptionally activates most sequences examined (reviewed by Karpf et al, 2002). This resistance of *syncytin-1* methylation to the DNMT inhibitor strongly supports its role in transcriptional suppression.

It would be interesting to compare these results with the resistance of *syncytin-2* as its methylation seems to be considerably more variable even in healthy tissues. Different stability of methylation of 3' CpG dinucleotides would imply their importance. The variable methylation pattern of *syncytin-2* in similar samples could be connected with its easy modification by diverse factors influencing epigenetic marks such as age, environmental factors, diet, hormonal levels or injury (reviewed by Rodríguez-Rodero, 2010).

A similar experiment to analyze the methylation stability was also performed with the porcine cell line ST-IOWA. Neither here have we succeeded to decrease the level of methylation of retroviral promoters (Fig. 11b, c). The stability of PERV methylation is of special importance for xenotransplantation because the xenografts undergo stressful conditions of ischemic and reperfusion injuries. During the reperfusion, oxidative damage occurs by generation of oxygen and hydroxyl free radicals. Overproduction of these reactive oxygen species is a common underlying mechanism damaging various cellular components, including proteins, lipids, and DNA. Free radicals are known to cause extensive damage to the cell membranes in transplanted organs (Kosieradzki et al., 2003) that may then form acceptor surfaces for the alternative pathway of complement activation and deposition (Thurman et al., 2005). It has been shown that progressive oxidation of the methyl group of methylated

cytosines could result in permanent aberrant demethylation of 5-methyl cytosines in the DNA of transplanted organs (Parker et al, 2008). Here we show that the methylation of PERV sequences is resistant to 48-hour-long treatment with inhibitors of DNA methylation and deacetylation in concentrations up to 100 μM AzaC together with 0.03 μM TSA. For comparison, considerable transgene reactivation was observed in pig fibroblast cells treated with 0.5 μM AzaC for 48 h and the CMV methylation levels were decreased markedly after AzaC, TSA or combined treatment (Kong et al., 2011). This experiment suggests the PERV DNA methylation stability in stress conditions. By quantitative RT-PCR we have detected decreased PERV RNA levels after the inhibitory treatment (Fig. 11c). This unexpected effect is probably connected with a complex effect of these inhibitors on the cell and the toxicity of AzaC and TSA rather than specific for their silencing effect on the PERV expression. A complex effect of the treatment was shown on the pig fibroblasts whose viability was markedly decreased after 48 h treatment with 2 μM AzaC (Kong et al., 2011).

Further, we focused on *syncytin* methylation in cancer cells. A global disorder of DNA methylation is often observed in various cancer cells and simultaneously, HERV expression increase and demethylation was detected (Gimenez et al., 2010). Our results show high methylation and very low expression of ERVWE1 in all analyzed tumors except for seminomas despite that other laboratories showed *syncytin-1* expression in many other tumors as well (Fig. 4, 5). For example, we identified fully methylated promoter and negligible expression in breast cancer in contrast to the study by Bjerregaard et al. (2006). In addition, we have demonstrated a similar methylation pattern and expression in one sample of endometrial carcinoma or lymphoma in contrast with the studies by Strick et al. and Sun et al. (2007; 2010). This discrepancy could be explained by a high variability of tumor tissues. The question is whether *syncytin-1* has any importance for cancer development or whether activation of various HERVs in the tumor is a pure coincidence. One theory suggests that fusion induced by retroviral *Env* is a critical event in cancer development. Most cells made by accidental fusion are likely to be abnormal. This is supported by what is known about hybrids made by treating cells with inactivated viruses or fusogenic chemicals *in vitro*, which essentially recapitulates accidental fusion occurring in the body. The abnormalities of these hybrids include an unstable genome, unstable gene expression and properties not found together in a normal cell, which are features shared with cancer cells. Accidental cell fusion can contribute to cancer development in two ways: by destabilizing the genome and by changing gene expression (reviewed by Duelli and Lazebnik, 2007). This model argues that cells can become cancerous by first becoming tetraploid and then undergoing a period of

chromosomal instability resulting in aneuploidy (reviewed by Ganem et al., 2007). Breast and endometrial cancers ploidy falls into two groups, nearly diploid and triploid to tetraploid (Pradhan et al., 2006; Kronenwett et al., 2004). It would be interesting to test whether *syncytin-1* is predominantly expressed in the aneuploid cancers, as the hypothesis predicts. Interestingly, testicular seminomas, where we have detected hypomethylated *syncytin-1*, are prevalently aneuploid tumors (Hittmair et al., 1995). We have shown that *syncytin-1* promoter is completely methylated in all tested testicular cancers, such as lymphoma and mixed embryonic carcinoma with teratoma, apart from seminomas.

The germ cells neoplasms are conventionally devided into two major cathegories: Seminomatous and non-seminomatous germ cell tumors. Seminomatous tumors are composed of cells that ressemble primordial germ cells or early gonocytes. Non-seminomatous germ cell tumors include trophoblastic tumors, embryonal carcinomas, teratomas, choriocarcinomas and other rare trophoblastic tumors and yolk sac tumors (reviewed by Winter and Albers, 2011). However, it is not clear from which stem cells the different tumors are derived.

We hypothesize that *syncytin-1* methylation is increasing during the germ cell differentiation. That would explain the low methylation of seminomas and higher methylation of the non-seminomas. Complete demethylation of *syncytin* in choriocarcinoma-derived BeWo cells is in accordance with this theory because choriocarcinoma is also derived from early progenitor stem cells. To show that *syncytin-1* methylation occurs during germ cell differentiation it would be interesting to analyze an identical seminomatous tumor arising in the ovary where it is called dysgerminoma.

The decreased methylation in seminomas and high methylation in other types of tumors and in healthy tissues was confirmed by two techniques, methyl-specific qPCR and bisulfite sequencing. MS qPCR allows detection of minor methylation patterns. In contrast with bisulfite sequencing, it enables comparison of methylation of a larger number of samples. It can be used for comparison of samples but not for identification of the exact methylation pattern. This result depends on the methylation status of CpG dinucleotides within the primers. The technique could lead to confusing conclusions in case only some particular CpG dinucleotides within the analyzed region are crucial for the silencing. To ensure maximum specificity of the PCR, we used LNA primers. LNAs are nucleic acid analogs that contain a 2′-*O*, 4′-*C* methylene bridge within the ribose ring that imparts a rigid conformational structure enhancing thermal stability and improving bp discrimination. LNAs can be substituted into DNA oligonucleotides at selective sites to enhance hybridization performance and have been used in applications in which mismatch discrimination is critical,

such as single nucleotide polymorphism genotyping using allele-specific PCR and fluorogenic probes (Ugozzoli and Hamby, 2004). Despite that LNAs have demonstrated superior performance in many molecular applications; they are not yet widely used for DNA methylation analyses. MS qPCR is not sufficiently precise to identify the number of hypomethylated copies per cell; however, we demonstrate the use of MS qPCR in combination with LNA primers as a potent method for quantitative comparison of methylation of a particular sequence in different samples. The results obtained via MS qPCR were in most samples similar to bisulfite sequencing results. Only sample T4 c*is* identified as carcinoma *in situ* was identified as hypomethylated, whereas bisulfite sequencing revealed complete methylation. In this sample, the advantage to detect the minor methylation pattern could play a role. This result suggests that already in the early stages of tumor development the non-methylated *syncytin-1* is present.

RNA splicing analysis revealed the lack of spliced ERVWE1 RNA in all tissues apart from placenta and seminomas (Fig. 5a). We suggest RNA splicing to be an additional mechanism of *env* expression regulation. Detection of low levels of *syncytin-1* in cells with strongly methylated promoter suggests that *syncytin-1* epigenetic suppression of *syncytins* is not absolute and additional mechanisms, e.g. splicing and processing of ERVWE1 mRNA, must be involved in their control. Splicing of genomic ERVWE1 mRNA was already shown to be inefficient in the testes as only the non-spliced form could be seen on the Northern blot (Mi et al., 2000). Smallwood et al. (2003) demonstrated the increasing level of non-spliced ERVWE1 mRNA in term placenta and suggested that the relative amounts of spliced and non-spliced mRNAs could regulate *syncytin-1* expression during pregnancy. The regulatory potential of retroviral splicing generally results from the need of balance between genomic mRNA and spliced subgenomic mRNA(s). HIV-1 translates its own trans-activator, Tat, from a spliced mRNA transcript and downregulation of HIV-1 splicing is observed during the provirus persistence in memory cells (McLaren et al., 2008). Another example of specific splicing control is the suppression of *env* splicing of RSV in mammalian cells (Berberich et al., 1990). It was suggested that mammalian cells lack chicken-specific *trans* factor(s) binding to *cis* regulatory sequences, which could explain the suppression of *env* splicing (reviewed by Arrigo and Beemon, 1998; Mc Nally, 2008), and the non-permissiveness of these cells for RSV replication (reviewed by Svoboda et al., 2000). In replication-defective HERVs, the balance of non-spliced and spliced transcripts is not important any more, but the *cis* splice signals can be adopted for cell-specific control of splicing and expression of retroviral glycoproteins. Cell-specific splicing in ERVs remains to be studied systematically, but in the

light of our recent findings it might be a useful indicator of the biological activity of HERVs with intact *env* genes, which do not fulfill the criteria for *sensu stricto syncytins*. Blaise et al. (2005) identified a new four-member family of HERVs, HERVP(b), with one full-length *syncytin-2*-like *env* gene fusogenic in HeLa cells. Its expression was observed in many healthy tissues without any significant specificity for the placenta. Out of six full-length *env* genes of the young HERV-K(HML-2) family, the *env* encoded by HERVK108 was also shown fusogenic in human embryonic kidney cells as well as in mouse, hamster, and cat cells (Dewannieux et al., 2005). Similarly, the rest of 16 intact *env* ORFs, which did not score yet in cell fusion assays, might be proven as biologically relevant in the future. Description of their splicing in various tissues could identify particular cell types where these retroviral glycoproteins play any role in cell functioning.

We have shown that in tissues and cell lines with low *syncytin-1* expression the detected expressed ERVWE1 RNAs were all or almost all unspliced (Fig. 5a). This result suggests either a lack of some splicing factor in these cells or that the splicing efficiency is connected with the transcription level. Splicing and transcription are closely connected processes. RNA polymerase II and transcription activators associate with splicing factors and slow elongation through the intron-exon boundary, or even stalled RNA polymerase II gives more time to recognize the splicing signals, which is particularly important for the suboptimal signals used for alternative exons inclusion (De la Mata et al., 2003; Luco et al., 2010, reviewed by Bentley, 2005). The rate of spliced to non-spliced RNA is similar in all samples with increased *syncytin* expression despite the variability of expression from 39% to over 600% of the expression level of RPII. This contradicts the hypothesis that the efficient splicing credits only to the efficient transcription and supports the presence of a splicing factor in these seminomas. It would be interesting to analyze splicing of other HERVs in these samples. For further evaluation of the role of *syncytin-1* in seminomas, a proof of the presence of the function glycoprotein on the cell surface is essential. However, the glycosylation of the protein makes the immunodetection difficult and we did not obtain sufficiently unambiguous results using any of the tested antibodies

The methylation of ERVFRD 5'LTR U3 in the testis is more variable than ERVWE1 (Fig. 6). In most samples the CpGs at 3' end are methylated in both healthy and tumor samples. We cannot observe any difference between seminomas and the non-seminoma tumors either in overall methylation or in the methylation of 3' end CpGs. Even the healthy controls are considerably variable, only the 3' CpGs are prevalently methylated in all heathy controls. These results suggest, similarly as the results from non-tumor patients, that

ERVFRDE1 U3 is easily modified. It should be taken in account that neither the healthy controls are from healthy patients and that the presence of tumor in the testis may induce an immune reaction or change the hormonal balance in the whole organ, which could influence the LTR methylation. Gimenez et al. (2010) also showed a clear decrease of ERVFRDE1 LTR methylation in testicular tumor and its rather low methylation in healthy testis. However, they demonstrated methylation of only one patient, which is not adequate considering the variability of the tumors. It would be interesting to study the ERVFRDE1 expression in tumors in more detail because its immunosupressive domain could be beneficial for the tumor protection and may influence their prognoses.

To confirm the causality of high methylation and low expression we performed the test of LTR sensitivity to methylation *in vitro*. Similar experiments were used to confirm the sensitivity of *syncytin-1* 5'LTR as well as the PERV 5'LTRs. All tested LTRs show significant sensitivity to methylation. Mock-methylated ERVWE1 5'LTR induced similar luciferase activity in both tested cell lines, BeWo and HeLa (Fig. 3). The LTR is functional in HeLa cells despite that they do not naturally express any *syncytin-1*. This suggests that no placental-specific transcription factors are necessary for the basal *syncytin-1* promoter activity. High luciferase expression in HeLa cells could also be an effect of transient transfection, which enables expression from many copies in one cell. Further, transient transfection does not reflect the chromosomal context of the analyzed sequence. The chromatin plasmid modifications partially differ from chromatin of chromosomes. Therefore, the transcription factor demands may differ as well. Stable transfection would better imitate the chromosomal gene structure; however, the methylation of stably transfected plasmid would probably change during the selection and therefore it cannot be used to test the sensitivity to DNA methylation. Further, we cannot distinguish the influence of the methylation of the whole plasmid and the luciferase gene from the influence of 5'LTR methylation. However, the gene bodies of the expressed genes are often methylated, and this dispersed CpG methylation may have a positive effect on their activity (reviewed by Suzuki and Bird, 2008). Results by Gimenez et al. (2009) confirm our experiment and show that both, ERVWE1 LTR extended with enhancer sequences and shortened version, are sensitive to CpG methylation *in vitro* as well.

The sensitivity of PERV LTR was examined using two different plasmids with luciferase driven by LTR from PERV-A and by LTR from PERV-B. The measured luciferase activity in cells transfected with PERV-A LTR was about twice lower than the luciferase activity driven by PERV-B LTR (Fig. 7). According to the analysis of PERV LTRs, the

transcriptional activities of the PERV-A, PERV-B, and PERV-C LTRs relative to each other differ in 293T cells. The increase in activity depends largely on the 39-base-long directly repeated sequence in U3 of PERV LTR. Our construct with PERV-B LTR contains three and half repetitions while the construct with PERV-A LTR contains no repetition. Although Wilson et al (2003) showed that a significant increase of activity can be observed only in LTRs with more than four repeats, it is credible that the difference in the activity of promoter with zero and three and a half would be significant as well. Our results confirm that 293T cells contain the transcription factors necessary to interact with the elements found within the repeat sequences. The estimated promoter strength is incomparable with *syncytin-1* LTR because the experiments were not performed simultaneously and the β-gal activity was not measured after the same interval. We can assume that at least most PERV LTRs are sensitive to CpG methylation. This result is consistent with Park et al. (2010), who showed the influence of *Sss*I methylase-mediated methylation on the activity of four different LTR elements.

Having demonstrated that DNA methylation plays a role in PERV silencing, we compared PERV methylation in different porcine tissues. Our approach enables analysis of the vast majority of PERV proviruses from all three subgroups. Low methylation of PERVs in some tissue would suggest higher PERV activity or their easy reactivation and potential risk for the xenotransplantation. We have performed the comparison by bisulfite sequencing and by MSq PCR similarly as we have analyzed the methylation of *syncytin-1* (Fig. 8b, 9b). Both techniques show that the level of PERV methylation is similar in all tissues except for one sample of placenta, where we have detected higher numbers of hypomethylated PERVs. Further, we have demonstrated PERV hypomethylation in the cell line PK15. According to bisulfite sequencing, the PERVs in PK15 cell line were partially demethylated in about half of the analyzed sequences and the methylation was slightly higher than in the placenta sample L3. The quantitative analysis shows PK15 to contain about three times to ten times more demethylated PERVs than most tissues and nearly twice more than the placenta. The relative differences in the outcome of these two approaches may result from different parts of the analyzed regulatory sequences. For comparison, we analyzed the boundary of U3 and R regions and the R region of PERV LTRs by bisulfite sequencing and the U3/R boundary of 5' LTRs and the leader sequence by MS qPCR (Fig. 8a, 9a). The selection of the analyzed regions of LTR was notably limited by strict criteria for the primers. First, to obtain information about the methylation pattern of the majority of PERVs, all primers had to be complementary to all PERV subgroups and therefore had to be designed in a highly conserved

region. Second, for bisulfite sequencing we had to avoid all CpG dinucleotides, while for MS qPCR we had to include as many CpG dinucleotides as possible, preferentially at the 3' end of the primer. These limitations in the choice of LTR regions to be analyzed could lead to confusing results if methylation of some CpGs was of higher importance than others. However, bisulfite sequencing of LTR and leader sequence of particular PERV integrations showed uniform methylation in the whole sequence, suggesting that no CpGs have higher impact (Fig. 10).

Low methylation of PERVs in PK-15 is consistent with high expression of PERVs in this cell line. Surprisingly, we did not detect any fully methylated sequence from PK-15. This could mean that partial demethylation is sufficient for PERV expression. We have demonstrated high PERV RNA expression in PK-15 by RT-PCR (Fig. 9c). PK-15 cells were the first where PERV expression was shown and the expression on PERV was sufficient to infect human 293T (Patience, 1997). However, high expression of this cell line cannot be explained merely by methylation decrease. Specific transcription factors are necessary for the full LTR activity. Transcription factor-binding site search has identified potential protein binding sites, including binding sites for SOX5, Ets-1, Evi1, GATA, v-Myb or CEBP. Within the U3 direct repeats, protein binding sites for NF-Y and GATA were identified. The necessary transcription factors are absent for example in IOWA cells (Wilson et al., 2003).

Interestingly, in samples from kidney from which the PK-15 cell line is derived we have detected the fully methylated PERV LTRs. Two further pig kidney cell lines (IB-RS-2 and SK6) also express viral particles, while no viral particles could be seen in preparations made from primary or secondary cultures of pig kidney cells (Armstrong et al., 1971). We have demonstrated that long-term cultivated kidney cell lines have changed their methylation pattern in contrast with primary kidney cells.

The placenta samples are highly variable because pigs have diffuse epitheliochorial placenta, which in contrast with human discoid hemochorial placenta forms thin extended layers that are not easily separable. It is probable that each sample represents a different layer of placenta (Fig. 8b, 9b). As in other mammals, we expected placenta to be hypomethylated and to express more ERVs that other tissues (Li, 2002; Kalter et al., 1975). It would be interesting to find out whether pigs use some envelope glycoproteins of ERVs as *syncytin*. However, pigs have a primitive type of noninvasive placenta and neither syncytium nor binucleated cells as were observed in other ungulates do occur. Syncytialization is apparently not part of their placenta development. Due to the noninvasive type of placenta the env-mediated immunoprotection of the fetus is not critical, either. However, binucleated cells

were detected in very close taxon peccaries. The originaly observed syncytial epithelium was not confirmed (Santos et al, 2006). Differences in PERV expression in the placenta accompanied by placenta structure differences between these two taxons could be interesting from the evolutionary point of view.

In other tissues we have not detected any sample with markedly increased number of hypomethylated PERVs confirmed by both techniques (Fig. 8b, 9b). This is consistent with rather low PERV expression. However, the low PERV expression is probably not only due to methylation silencing because few hypomethylated LTRs were detected in most tissues. The lack of transcription factors and the presence of inhibitors may represent another silencing mechanism. This is in consistence with low activity of transiently transfected PERV LTRs in porcine cells in contrast with cells from other animal species. Perhaps this reflects the evolutionary pressure to select for LTRs with reduced transcriptional activity once a retrovirus becomes an endogenous gene (Wilson et al., 2003). The similar methylation of PERV sequences in various tissues reflects similar total genome methylation in these tissues (Yang et al., 2011). Our methylation analysis suggests that none of the tested organs or tissues is prone to produce PERV particles more than the other. In would be interesting to extend our methylation and PERV RNA expression analysis to genetically modified pigs which will be used as organ donors. Further, similar analysis of organs xenotransplanted to primates should be performed.

Although genome-wide methylation analysis cannot be used for detection of a particular active provirus, it can be used for confirmation of the transcriptional activity. Out of four tested proviruses from subgroups A and C we have shown one to have hypomethylated LTR (Fig. 10). The PERV-C 6SH provirus was predicted as active after wide PERV-C integration site analysis and comparison of its distribution in pigs of different breeds. *In vitro* co-culture analysis was also carried out to assess the ability of pigs to transmit PERV to human and/or porcine cells. By looking at the prevalence of PERV-C loci in transmitting animals, active loci could be identified (Hector et al., to be submitted). Demethylation of 5'LTR in about half of obtained sequences suggests that this locus may indeed be capable of expressing significant levels of PERV-C. The locus RW was predicted by the same methods as the locus 6SH; however, the high methylation level suggests that expression of this provirus at any significant level is unlikely.

Foreign species often defend themselves against retroviral infection by silencing the integrated virus by DNA methylation. We have shown that human 293T cells are unable to effectively methylate any of the two PERVs we used for their infection, neither PERV-3A nor

the high-titer PERV14/220 (Fig. 12). After two months PERV14/220 proviruses contained 14% of methylated CpGs while the PERV-3A less than 2%. In contrast, RSV or MMLV LTRs become silenced by methylation within weeks (Senigl, 2008, Stewart et al., 1982, He et al., 2005). Senigl et al. showed slower progression of RSV silencing in the 293T cell line than in the other tested cell line NIL2. It is possible that 293T methylate retroviral sequences generally less efficiently than other cells. Other human cell lines should be tested to verify the PERV resistance to methylation. It is not clear how cells distinguish the retroviral sequences to methylate them. High content of CpG islands in the retroviral genome may have influence on its protection against methylation (Park et al., 2010). We have shown inefficient methylation of PERV; however, other protective mechanisms of human cells were described. For example, the human APOBEC3G protein reduces the PERV transmission to nearly undetectable levels (Jonsson et al, 2007). Human tetherin and its porcine homolog are able to inhibit the release of PERVs by an order of magnitude (Mattiuzzo et al., 2010). Interestingly, none of these protective mechanisms are potent in the 293T cells.

Despite the slow increase of PERV14/220 methylation the long-term culture of infected 293T cells remains completely unmethylated (Fig. 12b). This proviral protection could be ensured by a chromosomal positional effect enabling some clones to be fully protected against methylation. However, the PERV infection of 293T is productive, and presumably many different integration sites were analyzed by bisulfite sequencing. We hypothesize that the high PERV titer results in frequent new integrations, and therefore, most analyzed proviruses may represent newly integrated non-methylated proviruses despite the long time of cultivation.

During the passages of PERV-3a we have observed fluctuation of the number of 39-bp repeat boxes (Fig. 12a). This fluctuation during cell cultivation has been observed before. It was shown that this increase of repeat number correlates with PERV expression (Sheef et al., 2001; Denner et al., 2003). Multimerization of enhancer repeats was described not only for exogenous retroviruses, but also for endogenous viruses. (Wolgamot and Miller, 1999). These data indicate that recombinant PERVs generated during the infection of human cells can adapt and subsequently replicate with greater efficiency.

In contrast with human cells, rodent cells are resistant to PERV-A infection. The resistance to PERV-A is at the level of viral entry; however, the mechanism differs between mouse and rat cells: the murine homolog of PAR (muPAR) is defective in PERV-A receptor function, whereas the rat cell encodes a fully functional PAR protein. RatPAR can rescue PERV-A infection in non-permissive cell lines, including the resistant rat cell lines from

which it has been cloned (Fig. 13, 14). The PERV-A infection of rat cells upon overexpression of ratPAR is reminiscent of the results from a previous study which shows that overexpression of amphotropic MLV and GALV receptors from Chinese hamster cells and FeLV-C receptor from MDTF cells supports viral infection in the cell lines of their origin (Tailor et al., 2000). This type of resistance to viral infection can be explained by subthreshold levels of receptor expression or stoichiometrically limited masking or interference mechanisms (Eiden et al., 1994; Miller and Miller 1993; 1992). The mechanism which determines the threshold level of ratPAR expression for PERV-A infection is currently unclear. However, our results suggest that other component(s) on the cell surface may be responsible for a successful interaction between virus and receptor, as has been previously proposed for other gammaretroviruses (Pizzato et al.,1999; Chung et al., 1999; Wang et al., 1991).

Although we cannot exclude the possibility that these changes are a stochastic evolutionary outcome, it is more likely that certain selective pressure, at least partly, caused these changes. It is tempting to speculate that severe epidemics of PERV-A-like viruses may have selected 'PERV-A-resistant' rodents by two independent mechanisms.

In this study we have been investigating different ERVs from various points of view. ERVs present indispensable parts of the genome as well as potentially dangerous elements. The resulting effect on health condition of the host depends on the exact regulation of their expression. We have shown methylation to be an important silencing mechanism regulating HERV as well as PERV. It seems that methylation is partially responsible for low PERV expression in the tissues and notably reduces the risk of zoonotic transmission during xenotransplantation. However, the risk remains because in contrast with mouse and rat cells, some human cells are permissive to porcine retroviruses thanks to functional receptors and lack of antiviral protective molecules. Furthermore, human cells silence incorporated PERVs inefficiently. We can conclude that we have furthered the understanding of co-existence of ERVs and their hosts as well as the interaction of ERVs with a potential new host.

7 Conclusions

ERV silencing is largely mediated by DNA methylation. The expression of ERVWE1 and ERVFRDE1 encoding proteins *syncytin-1* and *-2* was shown to be restricted to the placenta, where they are essential for its correct development. We have demonstrated that 5’ LTRs of

both HERVs are hypomethylated in the term placenta. ERVWE1 5’ LTR displays a bimodal methylation pattern, whereas ERVFRDE1 5’ LTR is demethylated in all obtained sequences. ERVWE1 5’LTR is completely methylated in all other analyzed tissues. Methylation of ERVFRDE1 5'LTR in non-placental tissues is remarkably more variable and only two CpGs near to the TATA box are methylated consistently. DNA demethylation was shown to be a necessary prerequisite for expression of both syncytins.

Resistance of ERVWE1 5'LTR methylation to DNMT and HDAC inhibitors AzaC and TSA further stress out the importance of syncytin-1 suppression by DNA methylation. The sensitivity of ERVWE1 5'LTR to CpG methylation was confirmed *in vitro* as well.

Expression of syncytins in inappropriate tissues connected with some pathogenic effect would be the best proof of the relevance of their DNA methylation-mediated suppression. We have shown a significant increase of ERVWE1 RNA expression in testicular seminomas in comparison with healthy controls, whereas in other examined tumors the RNA levels were negligible. Furthermore, we have shown that only in seminomas ERVWE1 is efficiently spliced and *syncytin-1* can be expressed. Similarly, efficient splicing was observed in placenta and chorioma cell lines. The increased transcription corresponds with hypomethylation of ERVWE1 5'LTRs. The decrease of ERVFRDE1 5'LTR methylation in tumors was not remarkable.

Silencing of PERVs was studied in connection with their possible transmission from pig tissue to a xenotransplanted patient. PERV transmission to human cells was observed *in vitro*; however, transmission to a patient treated with porcine material was not detected. We have shown that the PERV expression largely correlates with the number of hypomethylated PERV LTRs. By a quantitative approach we estimated the number of hypomethylated PERVs in all tissues to be several times lowers than in the PERV-transmitting cell line PK15 except for one sample of placenta with more than half of hypomethylated PERVs in comparison with PK15. We have not detected any pig with generally decreased PERV methylation. We succeeded in identifying one hypomethylated PERV-C provirus detected in PERV-transmitting porcine blood cells. We can conclude that the methylation status plays a substantial role in determination of the transmission status of the cells and that PERV LTRs are mostly strongly methylated in the porcine tissues.

We have demonstrated that the high permissiveness of human 293T cells is partially caused by their inefficiency in silencing of the integrated PERVs.

Finally, we have shown two different reasons for rodent cell resistance to PERV-A entry. The mouse receptor is deficient due to amino acid mutations, whereas the rat receptor is functional; however, its expression in all the examined rat cell lines is insufficient.

8 Summary

In my thesis I am dealing with human endogenous retroviruses (HERVs), which are involved in placenta development, and with porcine endogenous retroviruses (PERVs) in the context of the risk of their transmission to a patient xenotransplanted with a pig organ.

We have shown DNA methylation to be an important silencing mechanism regulating HERV as well as PERV. (1) Whereas in placenta the demethylation of HERVs ERVWE1 and ERVFRDE1 is crucial for its correct function, in the testis it is connected with seminoma development. (2) It seems that methylation is partially responsible for low PERV expression in tissues and notably reduces the risk of zoonotic transmission during xenotransplantation. (3) However, the risk remains because in contrast with mouse and rat cells, some human cells are permissive to porcine retroviruses *in vitro* thanks to functional receptors and their inability to efficiently silence the integrated PERVs.

9 Souhrn

Ve své práci se zabývám lidskými endogenními retroviry (HERV), které se účastní vývoje placenty a prasečími endogenními retroviry (PERV) v souvislosti s nebezpečím jejich přenosu na člověka při transplantaci prasečích orgánů.

Ukázali jsme, že methylace DNA je důležitý umlčující mechanismus regulující expresi HERV i PERV. (1) V placentě je demethylace dvou HERV, ERVWE1 a ERVFRDE1, nezbytná pro její správný vývoj, zatímco ve varlatech je spojená se vznikem seminomů. (2) Methylace PERV je zřejmě zodpovědná za jejich nízkou expresi v tkáních, čímž značně snižuje nebezpečí zoonotického přenosu při xenotransplantacích. (3) Určité riziko však zůstává, protože na rozdíl od myších a potkaních buněk jsou lidské buňky *in vitro* vysoce permisivní vůči PERV, a to především díky funkčnímu receptoru a neschopnosti umlčet integrovaný PERV.

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