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From Viruses to Genes: Syncytins

Philippe Pérot, Pierre-Adrien Bolze, and François Mallet

Abstract The content of 5–90 million years old retroviruses and even older retrotransposons of animal genomes and the wide variety of modern retroviruses infecting the same range of species suggest that these elements can be assimilated to shuttle across evolution. A snapshot taken a few decades ago showed us the capture of cellular proto-oncogenes by infectious elements, representing the dark side of the communication between the worlds of viruses and animals. Another snapshot we took more recently shows multiple captures by animal genomes of envelope genes originating from infectious retroviruses, illustrating a phenomenon of convergent evolution. This could be seen as the bright side of these relations as those envelopes were shown to be involved in the earlier steps of human development, i.e. fusion of placental syncytiotrophoblastic layer, therefore they were dubbed Syncytins. Sequencing of more and more animal genomes allowed comparative genomic analyses that revealed how these envelopes have been domesticated in human, mouse, goat, rabbit, etc. More generally, we illustrate in this chapter how close are

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the viral and animal genome worlds and, focusing mainly on the hominoid ERVWE1 locus encoding Syncytin-1, how the different proviruses encoding Syncytins have been domesticated to achieve placental functions. Influence of the chromosomal integration context, the epigenetic control and the splicing strategy upon transcription, and protein maturation processes as well will be discussed in order to illustrate what makes these nowadays genes different from their ancestral infectious counterpart. The price to pay for this beneficial invasion will be illustrated by the possible implications of Syncytin-1 in a wide range of diseases. Last, the apparent stringency of placental regulation will await to be challenged as regard to the evidence of expression in other physiological fusogenic contexts such as myoblasts and osteoclasts.

Keywords Retrovirus • Endogenous retrovirus • Syncytins • Domestication

Abbreviations

ALV	Avian leukosis virus
BaEV	Baboon endogenous virus
BLV	Bovine leukemia virus
cyt	Cytoplasmic tail
en	Endogenous
EnCa	Endometrial carcinoma
Env	Envelope
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
Exo	Exogenous
FcEV	Felis catus endogenous retrovirus
FP	Fusion peptide
GCM	Glial cell missing
GPI	Glycosylphosphatidylinositol
h	Human
HELLP	Hemolysis, elevated liver enzymes and low platelets
HERV	Human endogenous retrovirus
HFV	Human foamy virus
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukemia virus
JSRV	Jaagsiekte sheep retrovirus
KoRV	Koala retrovirus
LTR	Long terminal repeat
m	Mouse
MALR	Mammalian apparent LTR-retrotransposon
MAO	Morpholino antisense oligonucleotide
MLV	Murine leukemia virus

MMTV	Mouse mammary tumor virus
MPMV	Mason-Pfizer monkey virus
MS	Multiple sclerosis
MSRV	Multiple sclerosis associated retrovirus
NO	Nitric oxide
OASIS	Old astrocytes specifically induced substance
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Primer binding site
PCR	Polymerase chain reaction
PcRV	Papio cynocephalus retrovirus
PE	Preeclampsia
RBD	Receptor-binding domain
RD114	A feline endogenous retrovirus
RT	Reverse transcriptase
SERV	Simian endogenous retrovirus
SIV	Simian immunodeficiency virus
SNV	Spleen Necrosis virus
SP	Signal peptide
SRV	Simian retrovirus
SU	Surface unit
TM	Transmembrane unit
tm	Transmembrane domain
URE	Upstream regulatory element
WDS	Walleye dermal sarcoma

1 Introduction

The most convincing clue of filiation between all living things is likely the sharing of nucleic acid molecules. Thus, the RNA world is for biologists quite similar to the primordial soup for astrophysicists: some kind of constructive thought experiment to travel back in time. According to the prevailing theory, primitive cells were possibly very simple membrane structures containing RNA nuons (i.e. any distinct nucleic acid) that have undergone escape and uptake of molecules (Brosius and Gould 1992). Cell division and fusion ensured the dynamic to trying out new nuons, and consequently genetic exchanges became early one core evolutionary force (Brosius 2005). Retroviruses can be seen as RNA shuttles ensuring genetic exchanges from one species genome to another. How old are retroviruses is a difficult issue, but since Howard Temin formulated the initial hypothesis that retroviruses evolved from cellular moveable genetic elements (Temin 1980), knowledge on genomic oncogenes capture and the resulting emergence of infectious transducing retroviruses has made significant steps forward (Pedersen and Sørensen 2010). Another type of capture exists between retroviruses of distant species, consisting in the

swapping of envelopes between species living in the same environment or linked by the food chain. For example, the RD114 infectious endogenous virus comes from two genetic recombinations resulting in two *env*-captures. First, the SERV *env* was captured by the PcRV leading to the BaEV. Second, the acquisition of BaEV *env* by FcEV led to the emergence of RD114 virus (Kim et al. 2004).

The strongest candidates for developmentally regulated cellular fusogens in mammals are Syncytins, a family of single-pass transmembrane envelope proteins, which contribute to cell-cell fusion leading to placental syncytiotrophoblast at least in higher primates, rodents, lagomorphs and sheeps (Pérot et al. 2011). They consist of domesticated endogenous retroviral envelope glycoproteins whose fusion properties depend on the initial recognition of a specific receptor. Syncytins appear to group relatively distinct actors that may exhibit common characteristics leading to membrane fusion and hence are good illustrations of the various evolutionary pathways taken to establish similar but different structures with convergent roles.

During the first 10 years of the twenty-first century, decoding the genomes, notably that of mammals, showed that besides the Syncytins, there were many other ERV envelopes genes for which no function has yet been assigned. For example, deciphering the human genome (International Human Genome Sequencing Consortium 2001) permitted to identify 16 almost intact envelopes ORFs (Blaise et al. 2003, 2005), in addition to the well described two human Syncytins. Beyond these envelope proteins, without unequivocal evidence of infectious agent, retroviral particles were observed in physiological (Lyden et al. 1994) and pathological situations in man (e.g. Boller et al. 1993; Perron et al. 1989), suggesting that endogenous elements can reach a similar complexity level as infectious retroviruses. The degrees of difficulty vary to understand the origin of these retroviral elements whether we consider the simplest level of complexity through a single gene or the ultimate degree of complexity brought with the particles. Outstandingly, the current knowledge on Syncytins embraces at least three levels of complexity. First, in term of architecture, the placenta is probably the more variable organ within mammals (Bernirschke K, Comparative placentation at <http://placentation.ucsd.edu>). Second, Syncytins recognize specific and highly function-divergent and unrelated receptors as observed in human (Blond et al. 2000; Esnault et al. 2008; Lavillette et al. 2002) and rodents (Dupressoir et al. 2005). This illustrates that the proteins involved in cell-cell fusion, such as Syncytins partner receptors, are likely to play pleiotropic roles in other cellular processes like the transport of small molecules, but also the modulation of membrane structures, and that their functions are being achieved through the coupling of these proteins to different upstream and downstream effectors. Third, Syncytins were shown to exhibit other functions than fusion, such as immunomodulation (Mangeney et al. 2007), receptor interference (Blond et al. 2000; Ponferrada et al. 2003) anti-apoptosis (Knerr et al. 2007; Strick et al. 2007) and cellular proliferation (Larsen et al. 2009; Strick et al. 2007), these functions being not shared by all these proteins. Regarding retroviral particles, they could derive either from a single locus or from several loci *via* transcomplementation processes. This is supported by the presence among the hundreds thousand retroviral elements of the mouse genome (Mouse Genome Sequencing Consortium 2002) but

also of the human genome (International Human Genome Sequencing Consortium 2001) of almost complete proviruses and significant number of still intact coding sequences for *gag* and *env* genes (Villesen et al. 2004).

In this chapter we seek to illustrate how one element may move from an infectious virus to become an entity transmitted as a gene. We will show how the study of the placenta allowed to overcome conceptual leaps in understanding the role of HERVs, given that this tissue is historically a privileged place for HERVs expression as well as proteins and particles detection. Then we will describe in detail what are the domestication mechanisms of the Syncytin-1, including the genomic integration context, the control of the transcription and the protein maturation, to see what makes these nowadays genes different from infectious retroviruses. Ultimately, in an attempt to decode the underlying regulatory mechanisms, we will look at the expression and functions of human Syncytins in pathologies and specify how they behave outside of the domestication scene.

2 When Rous Met Mendel

2.1 From Viruses to Genomes

2.1.1 The Retroviral Life Cycle

The rare event that represents the infection of a germ line cell by an exogenous retrovirus leads to the integration into the host genome of a retroviral DNA, or provirus, that becomes part of the genetic heritage of the host. Therefore, this endogenous provirus is transmitted to the next generation in a Mendelian way. The parental infectious retrovirus is a diploid RNA virus whose 8–10 kb compact genome consists of four major genes *gag*, *pro*, *pol* and *env* encoding the proteins required for its replication life cycle, and flanked by 5' R-U5 and 3' U3-R untranslated regions. The *gag* gene encodes matrix, capsid and nucleocapsid proteins necessary for viral RNA encapsidation and particle formation. The *pro* gene encodes a protease required for the cleavage of Gag-Pro-Pol and Gag polypeptidic precursors and also, in the case the Gammaretroviridae genera, for the final Env maturation step leading to fusion competency. The *pol* gene encodes the major viral enzyme machinery, including two enzymes, an RNA-dependent DNA polymerase named reverse transcriptase and an integrase, both sequentially required for the successful conversion of the viral RNA into the proviral integrated DNA form. Last, the *env* gene encodes viral envelope glycoproteins that confer virus infectivity, i.e. receptor recognition *via* the subunit named SU and virus-cell membranes fusion *via* the subunit named TM. In addition, the TM subunit contains motives that is likely to confer to retroviruses immunosuppressive properties. The Fig. 1a shows schematically the replication cycle of a simple infectious retrovirus in order to point out which HERV components, either proteins or regulatory elements involved in transcription initiation

and termination, can fulfil a function by their contribution to a physiological or pathological role. Briefly, following the entry into the cell, the viral RNA is reverse transcribed into DNA by the viral RT using a cell specific tRNA as a primer which hybridizes with the PBS region located at the R-U5 and *gag* junction of the retroviral genome. The resulting double-stranded DNA, which contains at each end a non-coding LTR sequence derived from R-U5 and U3-R viral sequences (see locations in Fig. 1b), is integrated into the genome of the host cell through the action of the viral integrase. The expression of the proviral DNA is then becoming dependent on the host cell machinery that provides the transcription factors required to activate

Fig. 1 From the ancestral infectious retrovirus to the contemporary human endogenous retroviruses family.

(a) Schematic representation of the replication life cycle of an infectious retrovirus, illustrating the functions achieved by the various retroviral elements during the steps of the cycle, and allowing the identification of endogenous retroviral elements which may be involved in a physiological or pathological function. 1 Binding to a cell receptor *via* the viral envelope surface subunit (SU) (Env, ●—), 2 fusion of viral and cell membranes *via* the viral envelope transmembrane subunit (TM), leading to 3 the entry of the capsid in the cytoplasm, 4 the conversion of the viral RNA to cDNA and DNA by the reverse transcriptase (RT, ■), 5 the integration of the provirus flanked by two identical LTRs in the cellular DNA (provirus). 6 Transcription (5'LTR promoter function) controlled by host cell transcription factors and 7 production of genomic and subgenomic (spliced) mRNA 8 transported to the cytoplasm, 9 translation and production of the poly-protein capsid (Gag, ○) and enzymatic machinery (RT, integrase, protease) from the genomic transcript, and envelope, from subgenomic transcript, 10 assembly of the genomic RNA and viral proteins leading to 11 the budding and 12 release of virions which can infect new cells. Identification of elements that may be involved in a function in endogenous retroviruses. (A) Promoter function (U3 region of LTRs) can lead to the synthesis of RNA coding for retroviral proteins (5'LTR) or non-retroviral (solo LTR or 3'LTR). (B) Polyadenylation signal (R region of LTRs). (C) Gag proteins can form particles (intra or extra cellular) able to encapsidate genomic-like RNA that can be reverse transcribed (via RT) and re-integrated. (D) Reverse transcriptase (RT) activity that may contribute to the re-integration of retroviral (RT-HERV and RT-LINE) or cellular (RT-LINE) genes deleted from their introns (pseudogenes). (E) Envelope protein can be expressed at the cytoplasmic membrane (intra and extra cellular portions), interact with a receptor and fuse cell-cell membranes, modulate immunity *via* an immunosuppressive motif. (F) Some HERV loci produce enveloped (or not) particles that can potentially deliver a distant signal in the body; genomes identified so far, however, are all defective for replication. (b) Constitution of a HERV family. The proviral DNA, integrated several millions years ago into the DNA of a germ cell, spread mainly by reinfection and retrotransposition and the different offspring loci went through a mutagenic process (symbolized by a vertical line or a deletion) during evolution. No contemporary copy is infectious as shown by (i) the frequency of *env* gene deletion, (ii) the absence of the U3 region in 5'LTRs on certain entities, (iii) the existence of entities deleted from the majority of their sequences and (iv) solo LTRs. (c) Representation of the chromosomal distribution of genetic entities of the HERV-W family. The position and the number of elements per chromosome are shown; it should be noted on chromosome 7 (arrow) the presence of the ERVWE1 locus containing the unique complete Env open reading frame, i.e. Syncytin-1. (d) Immunohistochemical detection of Syncytin-1 protein (SC-Syn1) at the apical syncytiotrophoblast (ST) microvillus membrane of a 10-week gestation normal placenta. Note that desmoplakin, a protein of the desmosomiale plaque involved in inter-cellular junctions, is absent from the syncytiotrophoblast fused tissue and lines the plasmatic membranes of the cytotrophoblasts (CT). Red blood cells in the maternal blood space (MBS); extravillous cytotrophoblast cells (ECT)

the 5'LTR. The 5'LTR plays the role of promoter and enhancer sequence conferring tissue-specific expression. The distal 3'LTR contains the polyadenylation signal terminating the transcription.

During the evolution, the founding-captured HERV provirus, and latter its son elements, is replicated by mechanisms that essentially rely on transcription, i.e. reinfection and retrotransposition. Due to the general absence of selection pressures, most of the elements contain disruptive mutations, like substitutions, insertions and deletions, in at least one of the structural genes of the provirus. Thus, the preferential loss of the *env* gene of many HERV elements is a common phenomenon that may reflect the unnecessary requirement of this gene once the barrier of species is crossed over (Boeke and Stoye 1997). Nevertheless, open reading frames can persist and lead to protein synthesis or even non-infectious particles. In addition, each family contains numerous solitary LTRs, resulting from the loss of full coding sequences by recombination between two flanking LTRs (Lower et al. 1996). All these mechanisms lead to complex multicopy families each consisting of heterogeneous elements (Fig. 1b). More, all loci of the contemporary HERV families are defective for replication, which means they have lost their infectious properties and are engaged in a vertical mode of transmission exclusively. However, the processes of spread within the genomes has generated, in addition to a significant level of complexity, a generally wide distribution of the sequences as illustrated by the chromosomal location of the elements belonging to the HERV-W family (Blond et al. 1999) (Fig. 1c). Among these HERV-W elements, there is one located on chromosome 7, ERVWE1, that became a bona fide gene (Fig. 1c) (Mallet et al. 2004) and producing a retroviral envelope glycoprotein involved in hominoid placental physiology, as illustrated in Fig. 1d.

2.1.2 Forgotten Territories Seeking an Identity

The definition of a precise nomenclature for animal ERV families is a difficult task in the absence of function or obvious pathology associated with these retroviruses, as opposed to infectious retroviruses. Yet, the development of a systematic nomenclature was tentatively proposed (Blomberg et al. 2009; Mayer et al. 2011). Retroviral classification was historically based on virion morphology observed by electronic microscopy during maturation and assembly of particles (Coffin 1992). Accordingly, retroviruses were designated A-, B-, C- or D-type. The current-usage classification of HERV is based on the PBS sequence located downstream of the 5'LTR, or its similarity to the infectious retroviruses PBS, which is recognized by a specific tRNA. A code based on the letter that refers to the amino acid recognized by the tRNA is applied to become a suffix, e.g. HERV-H exhibits a PBS which is recognized by a histidine (H) tRNA, the HERV-W PBS is homologous to the PBS of the avian retrovirus tryptophan (W) tRNA. However, some names sometimes coexist such as ERV-3 known as HERV-R, or ERV-9 which also share an arginine (R) PBS. This nomenclature can also be misleading, for instance the super-family HERV-K contains 11 phylogenetically distinct sub-groups referred to as HML-1 to HML-11 (Blikstad et al. 2008; Subramanian et al. 2011), what can let think that all the members share

the same PBS, yet HML-5 members are not primed by a lysine (K) tRNA as the name would suggest, but must likely by a methionine (M) or isoleucine (I) tRNA (Gifford and Tristem 2003; Lavie et al. 2004). The International Comity of Taxonomy has now established seven genera of Retroviridae, grouping exogenous and endogenous retroviruses as well, based on sequence homologies of the *pol* region: Alpharetroviruses thus correspond to the avian type C retroviruses (ALV), Betaretroviruses to type B (MMTV, HERV-K) and D (SRV-1) retroviruses, Gammaretroviruses to mammalian type C retroviruses (MLV, HERV-E, HERV-W), Deltaretroviruses to the ancient group composed of HTLV and BLV, Epsilon retroviruses to the WDS viruses family, Lentiviruses contains HIV and SIV and Spumaviruses include HFV and HERV-L (van Regenmortel et al. 2000).

A complete view of the (H)ERV landscape was expected from the publication of several mammalian genomes, including human (International Human Genome Sequencing Consortium 2001) and mouse (Mouse Genome Sequencing Consortium 2002). Although the human and mouse genomes contain essentially different retroviral families, they both display a huge but similar amount of endogenous retroviruses, reaching 8.5 and 9% of their euchromatin, respectively. More precisely, the human genome contains 203,000 copies resulting from about 100 independent infectious events, although only about 40 groups have been studied (Benit et al. 2001; Jurka et al. 2005; Mager and Medstrand 2005; Tempel et al. 2008; Tristem 2000), but it also contains some 240,000 MaLR elements which are considered as the ancestor of infectious retroviruses. It is crucial to appreciate how these hundreds of thousands of retroviral sequences constitute a mass significantly greater than all the human genes, a number currently estimated to range between 20,000 and 25,000 (International Human Genome Sequencing Consortium 2004).

2.2 *Crossing the Boarder: ‘Just leave the woods and you’ll improve your lot’*

Interplay between the primitive virus world and the eukaryotes domain could be observed at the *env* level. Thus, infectious retroviruses appear to have burst, getting out from the genomes of our far ancestors by transcomplementation of cellular retrotransposons with viral envelopes genes (Malik et al. 2000). In turn, endogenous retroviral sequences progressively undergo genetic drift and they spread throughout the genomes as describe previously. As a consequence the separation between endogenous and exogenous retroviruses sometimes is really thin.

2.2.1 KoRV: Ongoing Endogenisation

Koala retrovirus provides a unique opportunity to study the process of ongoing endogenisation as it still appears to be spreading through the koala population (Miyazawa et al. 2011; Tarlinton et al. 2006, 2008). Interestingly, infectious viral

particles are produced by the endogenous form of KoRV and high levels of viraemia have been linked to neoplasia and immunosuppression (Tarlinton et al. 2005). It remains unclear how the host can react when exogenous and endogenous forms of a virus are coexisting within the genome and his environment. Studies on koala might answer this question.

2.2.2 HERV-K: Almost Infectious

In human, the most recent HERV family to have entered the genome, HERV-K, contains tens of almost complete but mutated proviruses that allow the expression of viral proteins which appear able to form retroviral particles. However, due to genetic drift, no complete proviruses able to produce replication-competent and infectious viral particles have been detected. The HERV-K113 locus is the youngest element that belongs to the HERV-K super-family and is still not fixed in the human population as it is only detectable in up to 30% of individuals, depending of ethnicity (Moyes et al. 2005; Turner et al. 2001). HERV-K133 contains intact ORFs for all the viral proteins but does not produce any particles despite *in vitro* potential (Boller et al. 2008; Lee and Bieniasz 2007). Trans-complementation between different HERV-K (HML-2) proviruses could theoretically produce infectious particles, although not demonstrated to date. Interestingly, the infectious potential of HERV-K particles was artificially restored by generating a consensus HERV-K (HML-2) provirus named Phoenix supposed to be the HERV-K family progenitor (Dewannieux et al. 2006). This consensus contained at least 20 amino acid changes on the overall sequence as compared to individual proximal HERV-K loci. By electronic microscopy, this resurrected HERV-K forms viral particles in transfected cells. The budding of its particles is similar to γ -, δ - retroviruses or lentiviruses with no particles preassembling into the cytoplasm.

2.2.3 MSRV: Full Story, Lack of Evidence

MSRV is closely related to the HERV-W family including the Syncytin-1 encoding ERVWE1 locus which is the only W-locus bearing a full-length envelope. The sequencing of ERVWE1 envelope confirmed that the MSRV envelope was not encoded by the ERVWE1 locus (Mallet et al. 2004). It was thus proposed that MSRV particles, if not derived from an as yet uncharacterised exogenous retrovirus, may result from transcomplementation of dispersed HERV-W copies activated simultaneously (Dolei 2005), what appears poorly probable as regards to the HERV-W elements identified in the human genome consensus, unless there is a particular polymorphism uncharacterized to date. An alternative hypothesis would be that point mutations may counterbalance the genetic drift of one or more almost complete HERV-W sequences, reverting them to coding proviruses in multiple sclerosis. In particular, a HERV-W locus located on chromosome Xq22.3 harbors an almost complete ORF for full-length envelope protein but is interrupted by a stop-codon.

The reversion of the stop codon in artificial systems led to the successful expression of a reconstituted full-length HERV-W envelope protein sharing very similar post-translational features with the Syncytin-1 (Roebke et al. 2010). Xq22.3 sequencing from blood of 6 MS patients showed that the stop codon is still present at the germinal level (Bouton and Mallet, unpublished data), even if the presence of punctual somatic mutation within acute demyelinating lesions cannot be definitively excluded. Nevertheless, although this locus lacks a 5'LTR promoter element and thus needs an upstream control element unidentified to date (Nellaker et al. 2006), the transcription of the Xq22.3 truncated envelope has been reported several time in PBMC (Laufer et al. 2009; Nellaker et al. 2006) what is a prerequisite for recombination triggering. So it could not be formally excluded that MSRV/HERV-W genome, associated with particles, may result from very complex recombination events involving several loci on distinct chromosomes (Laufer et al. 2009; Roebke et al. 2010).

2.3 How Functions Were Imagined... Then Found

2.3.1 Hypothesis That Came from the Exosphere

Notwithstanding the often abundant imagination of the scientific community, HERV and transposable elements were first considered as 'junk', 'selfish' or 'parasite' DNA, without any physiological effect. Yet, given the distribution and the nature of these retroviral elements, several functions have gradually been conceived using knowledge gained from retrovirology in decades, and demonstrations have followed that HERV act on their hosts by different mechanisms: (i) HERV may be involved in genomic plasticity during evolution as recombination sites within or between chromosomes (Hughes and Coffin 2001) (ii) they can produce recombination-induced germinal or somatic mutations giving rise to the loss of function of a cellular gene (Blanco et al. 2000; Kamp et al. 2000; Sun et al. 2000) (iii) individual or proviral LTR can modulate the expression of adjacent cellular genes (Cohen et al. 2009; Long et al. 1998; Schulte et al. 1996; Ting et al. 1992) (iv) the expression of HERV proteins with conventional retroviral functions, like fusion, immunomodulation or RNA nuclear export, can influence physiological or pathological conditions of the host (Blond et al. 2000; Boese et al. 2000; Magin et al. 1999; Mangeney et al. 2007).

2.3.2 Barriers to Reach the Inner Knowledge

The analysis of transcriptional expression of HERV is extremely difficult due to the multicopy nature of these families, although locus-specific approaches like microarrays or PCR coupled with sequencing are developed in some laboratories (Flockerzi et al. 2008; Gimenez et al. 2010; Liang et al. 2012; Perot et al. 2012). Indeed, the expression of a family in a given tissue does not generally reflect the expression of

all the elements of this family, but rather results in the activation of a limited number of retroviral copies including the case of a single locus. The major determinants of such differences are related to the particular site of integration, methylation status of the LTR, and the susceptibility to cellular factors and environmental stimuli as well. Expression of HERV in reproductive and embryonic tissues may reflect a contribution to the genetic diversity or the physiological development. In contrast, expression of HERV later in the life of the organism may have adverse consequences. Increased HERV transcripts in cancers and autoimmune diseases confirmed that their activity is altered in pathological conditions. Thus, HERV have frequently been proposed as causative cofactors in such pathologies (Löwer 1999). Nevertheless, it remains complex to demonstrate conclusively whether the expression or re-expression of retroviral sequences is a cause or a consequence of the biological context in which it is detected, e.g. HERV-W and HERV-H in multiple sclerosis (Christensen 2010).

2.3.3 The Placenta, Where Everything Converges

In the 1970s, electron microscopy has described the presence of virus related particles in placental chorionic villous tissues of humans and primates (Kalter et al. 1975). Further studies then revealed some retroviral characteristics of these particles such as ultrastructural features and RT activity (Lyden et al. 1994). Apart from particles, mRNA expression from different HERV families have been reported early in the placenta (Lower et al. 1996) and was followed by the detection of retroviral envelopes using immunohistochemical techniques in human (Venables et al. 1995) and in baboon (Langat et al. 1999). Together with osteoclasts, skeletal muscles and sperm-oocyte fusion, the placenta is a tissue where cells fuse in physiological conditions what may strongly suggest the involvement of retroviral fusogens (Pérot et al. 2011). More, the ability of HERV and HERV-related sequences to modulate the immune system (Mangeney et al. 2001; Rolland et al. 2006) seemed very adapted to provide new insights on feto-maternal tolerance mechanisms.

Placenta and LTRs

The study and the description of transcriptional mechanisms that involve non-retroviral cellular genes and their neighboring retroviral LTRs were fruitful in the placenta. Indeed, the integration of retroviral elements near some genes provided them various cell expression tropisms, depending on the activation of native non-retroviral or additionally-acquired retroviral promoters. PTN, CYP19A1, NOS3, INSL4 and IL2RB are some significant examples discussed here of genes whose expression in the placenta is solely due to the presence of a promoter LTRs and thus can be regarded as exaptation events (see also Cohen et al. 2009). Expression of pleiotrophin (PTN) in the central nervous system during the perinatal period is controlled by a non-retroviral promoter, whereas expression in the normal trophoblast is controlled by a promoter HERV-E LTR inserted upstream of the first exon

(Schulte et al. 1996). CYP19A1, encoding the P450 aromatase, uses a MER21A LTR as placenta-specific promoter in addition to several non-LTR promoters in other tissues (Conley and Hinshelwood 2001; Kamat et al. 1998; Sun et al. 1998; Toda et al. 1996; van de Lagemaat et al. 2003). The nitric oxide synthase 3, NOS3, which mediates VEGF-induced angiogenesis, uses one LTR of the HERV-I family as a predominant promoter in the placenta (Cohen et al. 2009). The 3'LTR of a HERV-K element inserted near the INSL4 (insulin-like growth factor) gene has been exapted as primary promoter and regulates the placental specific expression of this gene during the formation of the syncytiotrophoblast (Bieche et al. 2003; Rawn and Cross 2008). Finally, the cytokine receptor subunit β , IL2RB, involved in the activation of T and NK cells, has been described more recently to rely on the alternative promoter function of a THE1D LTR in the placenta specifically (Cohen et al. 2011). Additional somewhat less major contributions of promoter LTRs to placental gene expression can also be mentioned, like LTRs of the HERV-E family playing a role in the expression of the endothelin receptor B EDNRB (Medstrand et al. 2001) and the Optiz syndrome-associated midline 1 MID1 genes (Landry et al. 2002), given that transcripts from native promoters in these cases are also detected in numerous tissues. Overall, the ability of the LTRs to act as enhancer elements should not be neglected, as it was reported in the case of leptins. The leptin gene, LPT, is expressed in mice adipocytes but the insertion of a MER11 LTR in the natural promoter of LPT confers an activating effect in the human placenta (Bi et al. 1997). Although it remains difficult to identify enhancer LTRs due to the genomic distances that can theoretically disconnect them from their target gene, it is likely that a large number of retroviral enhancers exist throughout the human genome.

Placenta and Syncytins

The keen interest for endogenous retroviral proteins expression in placentas is fed by *in vivo* or *ex vivo* demonstrations that directly link retroviral envelopes with fusion events during the development of many eutherian species. The molecular characterization of the HERV-W family relied on the isolation of placental cDNA clones, including one complete *RU5-env-U3R-polyA* sequence containing an *env* full length viral ORF (Blond et al. 1999). In 2000, protein truncation tests confirmed that this *env* ORF was unique among the genome and has the coding ability for a putative envelope gene, in association with a functional U3 promoter (Voisset et al. 2000). One year later, Blond and Mi concomitantly associated a HERV-W envelope protein with fusion events in TE671 and BeWo cells, and the name Syncytin was proposed by Mi in reference to the resulting syncytia (Blond et al. 2000; Mi et al. 2000). The amino-acid transporters hASCT2 (Blond et al. 2000) and hASCT1 (Lavillette et al. 2002) were identified as the receptors/fusion partners of Syncytin-1. Note that the connexin 43 was also demonstrated to play an important role in the fusion by interacting with hASCT2 in the syncytiotrophoblast basal membrane (Dunk et al. 2012). Heidmann and colleagues conducted a genome wide screening that identified a second envelope protein, belonging to the HERV-FRD family, and

Fig. 2 Structure, phylogeny and fusion capacities of Syncytins involved in placenta development (a) Envelopes structures of Syncytins and schematic representation of their cognate receptors. FP: fusion peptide; tm: transmembrane domain; cyt: cytoplasmic tail. Black dots indicate the predicted N-glycosylation sites. SDGGGX2DX2R, consensus motif conserved in type D retroviral interference group, is indicated in human Syncytin-1 and rabbit Syncytin-Ory1. (b) Phylogenetic tree depicting the conservation among species of the six envelope-open reading frames harbouring retroviral canonical motifs (branches of the tree are only illustrative). NWM: new world monkeys; OWN: new world monkeys. (c) **1st column:** Assays reporting the biological effect of Syncytins.

expressed exclusively in the human placenta. They named Syncytin-2 this second fusogenic *env* protein (Blaise et al. 2003) whose receptor is the carbohydrate transporter MFSD2 (Esnault et al. 2008). A similar *in silico* approach was done then in the murine genome, identifying the two coding envelopes genes present as unique copies and with a placenta specific expression Syncytin-A and Syncytin-B (Dupressoir et al. 2005) but without receptor identification to date, and later in the rabbit genome, identifying the Syncytin-Ory1 gene that unexpectedly shares ASCT2 as a receptor (Heidmann et al. 2009). If the situation is much more different in the ovine genome, where approximately 27 copies of endogenous betaretrovirus (enJSRVs) were detected, RT-PCR and *in situ* hybridization clearly indicate a conceptus (embryo/fetus and extra embryonic membranes) localization of enJSRVs *env* transcripts during gestation (Dunlap et al. 2006). JSRV uses the GPI-anchored cell surface HYAL2 protein to enter the cells (Arnaud et al. 2008) (Fig. 2a, b). Retroviral envelope sequences have also been detected in the placenta of cat, dog, guinea pig, as well as in bovine binucleate cells (Baba et al. 2011; Koshi et al. 2011; Vernochet et al. 2011), although the demonstration of a function remains to be established to date.

Although the role of Syncytins in human placentation awaits a definitive demonstration (e.g. infertility associated mutation), knock-out gene experiments in mice clearly achieved this goal in rodent model and demonstrated for the first time the critical role of Syncytin-A in placenta morphogenesis. Using a homologous recombination strategy, Syncytin-A null mouse embryos exhibited growth retardation with an altered placenta labyrinth architecture and died in utero (Heidmann et al. 2009), while Syncytin-B null placenta displays impaired formation of syncytiotrophoblast layer II (Dupressoir et al. 2011). This is consistent with previous *in vitro* works that used specific antibodies and antisense oligonucleotides to show a decrease in syncytia cell formation after Syncytin-A inhibition (Gong et al. 2007). In addition, the endogenous retroviruses of sheep, enJSRVs, play a fundamental role in sheep conceptus growth and trophoctoderm differentiation *via* their envelope glycoproteins. Indeed, *in vivo* experiments using an enJSRV envelope-specific morpholino injection trigger the loss of pregnancy by day 20 after injection (Dunlap et al. 2006). These kind of *in vivo* experiments obviously cannot be performed in human. Yet, primary cultures of human villous cytotrophoblasts cells give a unique opportunity to study placenta cells as closely related as possible to tissue environment.

2nd column: *Ex vivo* or *in vivo* specific inhibition of Syncytins expressions. From top to bottom: Syncytin-1-induced human primary trophoblasts fusion and differentiation results in syncytia formation *ex vivo* (1st column). Inhibition by specific antisense oligonucleotide largely reduces syncytia formation (2nd column). Electron micrograph of Syncytin-A wild type mouse placenta shows tight apposition of the syncytiotrophoblast I and II layers (ST-I; ST-II); stgc: sinusoidal trophoblast giant cells (1st column). Syncytin-A knock out mouse embryo interhemal domains shows unfused trophoblast I cells (T-I) (2nd column). Micrograph of the normal development of a sheep conceptus (1st column). Retarded growth of a sheep conceptus recovered after an envelope enJSRV morpholino antisense oligonucleotide injection (2nd column)

Thus, by using specific antisense oligonucleotides and siRNA strategies, expression of Syncytin-1 mRNA and protein as well as the syncytium formation by cell fusion events were dramatically reduced (Frendo et al. 2003; Vargas et al. 2009). In addition to that, Vargas and colleagues compared these results using the same targeting strategy against Syncytin-2, and interestingly showed that Syncytin-2 inhibition in primary cells culture also leads to a decrease in fusion index that is more important than for Syncytin-1 (Vargas et al. 2009). They concluded that Syncytin-2 could also be a major determinant of trophoblast cell fusion, and in a coherent vision this underlines there should be more than one HERV envelopes proteins acting upon trophoblast cell fusion in human. Those parallel procedures demonstrating the involvement of human, mouse and sheep Syncytins in placenta development are illustrated in Fig. 2c.

3 Domestication Inside, the Case of ERVWE1 and Genemates

We now discuss the domestication processes through the better exemplified case of retroviral envelope gene, the ERVWE1/Syncytin-1 (Fig. 3a), at different regulation levels: insertion, transcription, maturation and function.

3.1 *Sequence Features*

3.1.1 LTR and MaLR Provide Together a Bipartite Control Element

Like any conventional retroviruses, endogenous retroviruses may display all the signals required for the transcription initiation and regulation within their LTRs. The U3 region of the ERVWE1 5'LTR possesses the promoter activity. The core promoter domain within the U3 region contains the CAAT box and the TATA box located upstream of the CAP site, marking the beginning of the R region (Prudhomme et al. 2004). Mutant analyses have confirmed the functional role of these boxes. Moreover, the 5' end of the U3 region harbors multiple binding sites contributing to overall promoter efficiency including GATA, Sp-1, AP-2, Oct-1, and PPAR- γ /RXR. Although Sp-1 and Ap-2 binding sites remain putative, they have been found to be essential for LTR activity (Prudhomme et al. 2004). It is noteworthy that Syncytin-1 regulation elements not only include the 5'LTR but also a so-called Upstream Regulatory Element (URE), a cellular 436 bp sequence located immediately upstream the Syncytin-1 proviral integration site, that define together with the 5'LTR a bipartite control element (Prudhomme et al. 2004) (Fig. 3b, c). This URE is composed of two main domains : (i) a distal regulatory region, including the previously mentioned putative binding sites found in the promoter core, as well as binding sites

Fig. 3 Schematic representation of the retroviral-enriched PEX1-ODAG intergenic region and functional analysis of the bipartite element (MaLR and ERVWE1 LTRs) which controls Syncytin-1 placental expression (a) Flanking black boxes correspond to the 24th exon and the 5th exon of the PEX1 and ODAG genes, respectively. Host nonretroviral DNA is represented by a line. LTR elements are depicted as red boxes (MaLR LTR), green boxes (ERV-P LTR), purple tri-partite boxes (HERV-H provirus) and grey tri-partite boxes (ERVWE1 provirus). The U3, R, and U5 regions of HERV-H and ERVWE1 proviruses are labelled. The Env ORF is indicated by an orange arrow. (b) ERVWE1/Syncytin-1 transcriptional regulatory element: ERVWE1/Syncytin-1 expression is regulated by a bipartite element consisting of a cyclic AMP-inducible LTR retroviral promoter (ERVWE1 5'LTR U3 region) adjacent to an upstream regulatory element (URE) of composite origin. This URE consists of a 208 bp non-retroviral, non-repeated/transposable cellular sequence (non-TE region) and a 228bp MaLR LTR containing a trophoblast specific enhancer (TSE). True (top black boxes) and putative (bottom grey boxes) transcription factor binding sites along ERVWE1 5'LTR and URE are indicated. The positive (+) or negative (–) involvement of regulatory domains in placental tissue is annotated below the schematic representation. The CAP transcription initiation site (arrow) is located at the 5' end of the R region. (c) Trophoblast specific enhancer role of the MaLR element. The ERVWE1 5' LTR (5' LTR, white bars) and the MaLR – ERVWE1 5' LTR bipartite element (MaLR 5'LTR) (red bars) were used to transfect 8 human cell types (BeWo, Jeg-3, N-Tera-2, HBL-100, HBL-CeB6, HeLa, U373, and LCS) corresponding to seven organs. Luciferase relative activities from at least three independent experiments are shown, illustrating that MaLR element defined placenta tropism. Note that MaLR cloned upstream from a heterologous SV40 promoter or in a reverse orientation far downstream from the ERVWE1 5' LTR increased both promoters efficiencies in BeWo cells what confirmed enhancer function (not illustrated)

for the NF- κ B and AP-1 important for the stimulation by TNF α , IFN γ , IL-1 β , IL-6, and the inhibition by IFN β (Mameli et al. 2007) (ii) a MaLR ancestor retrotransposon with binding sites for glucocorticoid and progesterone receptors, that features a trophoblast specific enhancer with putative sequences for ubiquitous Ap-2 and Sp-1, but also placenta specific GCMA binding sites (Prudhomme et al. 2004).

Sequencing of a human panel showed that the 780 bp ERVWE1 5'LTR exhibits an unusually low polymorphism of one variable site every 18.0 kb as compared to a variability of one in 0.47 kb and one in 0.31 kb described for noncoding sequences and repeated sequences, respectively (Nickerson et al. 1998). This highlights a strong selection pressure in this region (Mallet et al. 2004). Moreover, comparative genomic analysis of the human 7q21.2 syntenic regions in eutherians showed the conservation of the MaLR-LTR tandem from human to gibbon, and the juxtaposition of the MaLR of Hominidae with their related LTRs induces a drastic increase of the transcriptional activity in human trophoblastic cells (Prudhomme et al. 2004).

3.1.2 Cross-Species Transcription Regulation Exemplified with GCM

Glial cell missing is a transcription factor family that has gradually attracted the attention of placenta researches. Originally isolated from a *Drosophila melanogaster* mutant line, two GCM homologues (GCMA and GCMb) have then been reported in mice, rats and humans (Keryer et al. 1998). GCMA is characterized by a zinc-coordinating DNA binding domain of β -sheets that recognizes an octomeric GCM binding motif 5'-ATGCGGGT-3' (Cohen et al. 2003). GCMA is primarily expressed in the placenta in humans and highly expressed in the labyrinthine trophoblast cells in mice (Basyuk et al. 1999). Two binding sites by which GCMA can specifically transactivate Syncytin-1 have been described (Yu et al. 2002) and functional GCMA-binding sites were also identified in Syncytin-2 and MFSD2 promoters (Liang et al. 2010). Moreover, GCMA regulation has been linked to AMPc, protein kinase A signaling pathways (Chang et al. 2005, 2011; Knerr et al. 2005) and hypoxia levels (Klase et al. 2009). In agreement with these observations, the Syncytin-1 5'LTR core promoter is cAMP-inducible (Prudhomme et al. 2004). Interestingly, a microarray approach that aimed to identify GCMA target genes reported Syncytin-A to be downregulated in murine GCMA-deficient placenta (Schubert et al. 2008). siRNA GCMA inhibition in BeWo cells led to a decrease in syncytialization upon fusion events (Baczyk et al. 2009), and a reduced placental GCMA expression has been reported as a causative factor in defective syncytiotrophoblast differentiation in human preeclampsia (Bainbridge et al. 2012). More, GCMA have been identified as an upstream regulator of the connexin 43, a partner-protein engaged alongside with hASCT2 in cell-cell fusion (Dunk et al. 2012). Altogether, these data argue that GCM acts as a major regulator in the humans and mice Syncytins expression as well as in placenta maintenance and development.

3.1.3 Splicing Strategies

ERVWE1 is a 10.2 kb-long locus located on chromosome 7q21.2 that can produce different spliced transcripts depending of the context (Fig. 4a). Historically, three single-spliced transcripts were detected in the placenta (Blond et al. 1999; Gimenez et al. 2010; Mi et al. 2000; Smallwood et al. 2003). The first mono-spliced transcript, 7.4 kb-long, contains the *gag*, the *pro/pol* pseudogenes and the *env* gene. The second one, 3.1 kb-long, strictly includes the open reading frame for the envelope protein Syncytin-1. Additionally, early northern blot experiments also detected a largely-spliced 1.3 kb transcript in the placenta (Blond et al. 1999). Alongside with the placenta, the testis exhibits different ERVWE1 mRNAs. The 7.4 kb form was seen early by northern blot in normal testis samples (Mi et al. 2000) and later by RT-PCR (Gimenez et al. 2010), and the 3.4 kb mRNA, which embarks the envelope-coding capacity, have been detected by RT-PCR in seminoma samples (Gimenez et al. 2010; Trejbalova et al. 2011). Note that the genomic full-length transcript of ERVWE1, from R (5'LTR) to R (3'LTR) has never been observed by northern blot although it was recently detected by RT-PCR in seminoma (Trejbalova et al. 2011). All these observations are in line with complex retroviruses transcription patterns such as MMTV or HTLV, for which several genomic and subgenomic transcripts derive from a single locus by alternative splice variations. Although the biological significance of non-coding splice forms of ERVWE1 in cancers is subject to discussions, the fact that the 3.1 kb *env*-coding RNA, in normal tissues, is constricted to the organ in which a physiological function exists but can re-appear in particular cancers argues that splicing variations may represent one additional level of control to the expression of domesticated retroviral sequences, balanced by other epigenetic mechanisms (Trejbalova et al. 2011).

3.1.4 Above the Battlefield: Epigenetics

Methylation of the LTRs

Methylation pattern studies of the ERVWE1 5'LTR revealed an inverse correlation between CpG methylation and locus expression indicating that demethylation of the 5'promoter is a prerequisite for the Syncytin-1 expression in trophoblasts cells (Matouskova et al. 2006). In an attempt to gain epigenetic characterization, Gimenez and colleagues (Gimenez et al. 2009) compared the methylation profiles of different HERV-W LTRs, including ERVWE1 5'LTR and 3'LTR, in villous placenta and in various non-trophoblastic cells (Fig. 4b). They showed that ERVWE1 5'LTR has the lowest methylation rate in villous placenta compared to others HERV-W LTRs, whereas all these LTRs including ERVWE1 5'LTR were broadly methylated in non-trophoblastic cells, a result reinforced by others (Macaulay et al. 2011). More, ERVWE1 5'LTR and 3'LTR, that both belong to the same locus, shared different methylation pattern since the 3'LTR remained highly methylated in villous placenta. Differential methylation between 5'LTR and 3'LTR is known for HTLV-1

(Koiwa et al. 2002) and HIV-1 (Ishida et al. 2006) during stages of viral latency but in a mirror scenario in which the 5'LTR is methylated as opposed to the 3'LTR demethylation. This suggests different methylation features whether we consider exogenous (pathogenic) or endogenous (domesticated) proviruses albeit with a common strategy that likely prevents the spread of methylation from one LTR to the other like the use of boundary elements as hypothesized for HTLV-1 (Koiwa et al. 2002). In the case of the ERVWE1 3'LTR, this could be crucial to keep active the Syncytin-1 promoter as well as to safeguard the use of the 3'LTR as a competitive or disrupting alternative promoter. Conversely, ERVWE1 5'LTR and MaLR behave quite similarly, e.g. in term villous placenta where all but one clone present a similar methylation profile whether we consider the MaLR or the 5'LTR. Thus, although belonging to distinct LTR types, these two elements could be linked and be involved jointly in the regulation of ERVWE1/Syncytin-1, what seems consistent with previous co-optation demonstrations (Bonnaud et al. 2005; Prudhomme et al. 2004) and the perspective of a *bona fide* gene (Mallet et al. 2004).

Changes of methylation patterns within ERVWE1 during pregnancy were also studied by a comparison involving first and third trimester samples (Gimenez et al. 2009). Methylation of the ERVWE1 5'LTR reaches 40% at term while completely

← Fig. 4 Transcriptional and epigenetic control of Syncytin-1

(a) ERVWE1 splicing strategy in placenta and normal and tumoral testis. Left panel: the CAP transcription initiation site (*right arrow*) is located at the 5' end of the R region of the 5'LTR. The polyadenylation signal (*left arrow*) is located toward the 3' end of the R region belonging to the 3'LTR. ERVWE1 appears to produce four single-spliced transcripts, a genomic 9.6 kb, the subgenomic 7.4-kb and 3.1-kb mRNAs and the fully-spliced 1.3-kb mRNA. Only the 3.1-kb variant is responsible for Syncytin-1 translation. Splice donor (SD) and acceptor (SA) sites are indicated by blue right and yellow left arrows, respectively. SD and SA were identified by screening a placental cDNA library. Right panel: these four transcripts have been evidenced either by Northern blot (NB), RT-PCR or as almost complete cDNA clones in the tissues mentioned at the top of the table. References: 1. Blond et al. (1999), 2. Mi et al. (2000), 3. Smallwood et al. (2003), 4. Gimenez et al. (2010) and 5. Trejbalova et al. (2011). (b) Comparative epigenetic control of 5' and 3' ERVWE1 LTRs in placenta (1. Tropism) and convergent modulation of bipartite element MaLR-ERVWE1 during gestation (2. Development) versus potentially sequential in tumoral context (3. Escape). Promoter regions are indicated as boxes and CpG schematized by circles. MaLR, LTR containing trophoblast specific enhancer, U3, ERVWE1 LTR promoter, R, transcription initiation site. CpG methylation is determined by bisulfite sequencing PCR in the indicated tissues. Each line represents an independent molecule. Methylated CpGs are schematized by black circles and unmethylated CpGs by white circles. (1. **Tropism**) Schematic representation of MaLR[LTR]–ERVWE1[5'LTR] and ERVWE1[env-3'LTR] analyzed regions. Methylation analysis was performed in villous trophoblast of term placenta and in placental fibroblasts from chorionic villi of a first trimester placenta. Each line represents an independent clone. Methylated CpG are schematized by black circles, unmethylated CpGs by white circles. (2. **Development**) Schematic CpG methylation dynamics of envelope-coding HERV 5'LTRs in cytotrophoblasts during pregnancy. Methylation MaLR[LTR]–ERVWE1[5'LTR], was in cytotrophoblasts (CT) at different times of gestation, i.e. CT of first trimester placenta from legally induced abortion and term placenta from healthy mother. Partial apparent remethylation may suggest an imprinting scenario (3. **Escape**) MaLR LTR and ERVWE1 5'LTR global methylation comparison in normal, peritumoral and tumoral testis. Half of the molecules are hypomethylated in the MaLR domain for the peritumoral tissue, suggesting a preferential route for hypomethylation

absent at the beginning of the pregnancy. Thus, the selective and temporal unmethylation of the ERVWE1 locus in placenta during the first trimester may allow Syncytin-1-mediated cell differentiation and fusion, while, in contrast, increased methylation at term may limit Syncytin-1 production and consequent cell fusion or putative anti-apoptotic protection (Knerr et al. 2007) in accordance with cytotrophoblast limited fusion and higher apoptosis rate (Chen et al. 2011). Interestingly, ERVFRDE1/Syncytin-2 and ERV3 proviruses which are involved in fusion and immunomodulation, or proliferation, respectively (Andersson et al. 2005; Blaise et al. 2003; Kato et al. 1987; Mangeney et al. 2007) exhibit different and independent methylation patterns than the ERVWE1 locus in the placenta, what may reflect complementary and ordered physiological functions for these three provirus sequences (Gimenez et al. 2009).

A CpG hypomethylation status of the domesticated ERVWE1 5'LTR was reported in seminoma samples although at different extent, what may result from the small number of tumour samples or various degrees of differentiation (Gimenez et al. 2010; Trejbalova et al. 2011). The work on normal testis in addition to tumoral and peritumoral samples tends to show a switch from methylated to unmethylated DNA induced by a permissive escape of the MaLR (Fig. 4b//3.Escape). This illustrates the need for the host to develop strong epigenetic defences in order to turn HERV sequences into domestic partners that can play physiological roles.

Changes in the Histone Code

Together with methylation levels, histones marks begun few years ago to change the appreciation of how chromatin is organized in normal development, cellular reprogramming and cancers (for an overview, see Baylin and Jones 2011). Recent works have investigated epigenetics hallmarks of the ERVWE1/Syncytin-1 and ERVFRDE1/Syncytin-2 loci surrounding their 5'LTR in BeWo and HeLa cells, and showed that the level of H3K9 trimethylation correlates perfectly with the CpG methylation of both proviruses (Trejbalova et al. 2011). The authors also associated the high density of H3K36 trimethylation along the intron-exon boundary of the Syncytin-1 envelope with high expression and efficient splicing form of the envelope gene. If these findings suggest at least partial redundancy within levels of control, histones modifications can also be seen as additive and multi-layers adaptations of the cell to guard against unfavorable effects of HERV elements. In line with this idea, we showed for instance that tissue specificity of the URE does not completely prevent weak and basal expression of ERVWE1 5'LTR in non-placental cell lines (Prudhomme et al. 2004).

Despite little is known about the general mechanisms of H3K9me3-dependant repression of ERVs sequences, different partner 'readers' proteins have been described to bind methylated lysines and to establish silent chromatin state in mouse, like isoforms HP1 α , HP1 β and HP1 γ , but also the chromodomain proteins CDYL, CDYL2, CBX2, CBX4, CBX7 and the M-phase phosphoprotein 8 protein (MPP8). In an attempt to clarify the role of these readers, Maksakova and coworkers recently

performed experiments in mice embryonic stem cells and demonstrated that neither the depletion of HP1s nor the knockdown of the remaining known H3K9me3 readers lead to significant proviral reactivation (Maksakova et al. 2011). This suggests H3K9me3 might directly maintain ERVs in silent state in mice embryonic stem cells, and consequently this invites to take an interest in what would happen in early stages of development.

3.2 Protein Properties

3.2.1 Physiological Cell-Cell Fusion Requires Crucial Sequence Adaptations

The fusogenic form of viral envelope glycoproteins is the outcome of a succession of maturation events. More precisely, class I fusion proteins are synthesized as glycosylated precursors in the lumen of the endoplasmic reticulum and are first modified by the cotranslational addition of N-glycans to the polypeptidic chain as well as by disulfide bond formation. After that a trimerization step involving a leucine zipper-like motif $LX_6LX_6NX_6LX_6L$ occurs in the ER before a proteolytic cleavage involving the cellular furin-like endoprotease gives rise to the two subunits SU and TM in the Golgi apparatus. Then a disulfide bond is established between SU and TM using the $C\Phi\Phi C$ and the $CX6CC$ motives, respectively. The final maturation step for γ -retroviruses envelopes, during viral budding, involves a 16-amino-acid carboxy-terminal peptide of TM, named R peptide, which is proteolytically cleaved by the viral protease what enables envelopes to ultimately trigger membrane fusion, as described by mutagenesis experiments (Yang and Compans 1996). Note that the cytoplasmic tails of most retrovirus envelope glycoproteins contain a $YXX\Phi$ (Φ is an amino acid with a bulky hydrophobic side chain [Leu, Ile, Phe, Val, or Met]) tyrosine-based sorting signal which plays a key role in subcellular distribution and adaptin-mediated endocytosis of plasma membrane-bound glycoproteins. Interestingly, the $YXX\Phi$ motif is located in the R peptide for MLVs and Mason-Pfizer monkey virus Env but is missing in Syncytin-1. These conserved motives are depicted in Fig. 5a. Altogether, these maturation steps are essential for the acquisition of the envelope protein's fusogenic activity and therefore virion infectivity as illustrated in Fig. 5b.

We illustrate how Syncytins used various strategies that diverge from envelopes of infectious retroviruses to adapt to their physiological functions in Fig. 5c. Surprisingly, sequences comparison of the Syncytin-1 locus with all other HERV-W envelope elements revealed a 12-bp (corresponding to four LQMV amino acids) deletion in its cytoplasmic tail (Bonnaud et al. 2004) just downstream from the R-like ERVWE1 counterpart region. Moreover, insertion of these four amino acids into Syncytin-1 tail completely abolished the fusogenic potential (Bonnaud et al. 2004). This result argues that Syncytin-1 is constitutively fusion competent, as opposed to exogenous retroviruses envelopes, and is coherent with a domestication point of view since no viral protease open reading frames exist anymore in the human genome (Voisset et al. 2000). Furthermore, the role of the cytoplasmic

domain of Syncytin-1 has been systematically investigated by producing a series of C-terminal truncated variants, leading to the conclusion that residues adjacent to the membrane domain are required for optimal fusion probably by forming a helical structure, while final C-terminal residues more likely act as a fusion inhibitor domain (Cheynet et al. 2005; Drewlo et al. 2006). Remarkably, a truncation mutant which shortens the cytoplasmic tail precisely at the site of the LQMV-deletion motif exhibits higher fusogenic properties than the wild-type protein (Cheynet et al. 2005). Even if no work on Syncytin-2 has been done in an exhaustive way to assess the fusogenic properties modulation of its cytoplasmic tail, we identified a stop codon in the cyt of Syncytin-2, as opposed to the RepBase prototype, resulting in a shortening of the tail. More, the protease cleavage site appears absent as regard to the FRD family consensus genome. Studies on the cytoplasmic tail of JSRV envelope protein first focused on the VR3 region that was described as the least conserved region between exogenous and endogenous forms. The VR3 region includes the putative membrane-spanning domain as well as the cytoplasmic tail, and series of envelope chimeras revealed that mutations in a YXXM motif of the cytoplasmic tail of JSRV *env* were sufficient to inhibit or modulate its transforming abilities (Hull and Fan 2006; Palmarini et al. 2001). Further mutational amino acid substitutions have proven the tyrosine residue to be essential for transformation of exogenous JSRV.

Fig. 5 Structure and maturation of retroviral envelope leading to virus-host cell membrane fusion and comparative evolution of Syncytins cytoplasmic tails (a) Schematic portrait of an envelope prototype. SP, signal peptide (grey). SU, surface unit contains RBD, receptor binding domain (orange) and C, C-terminal domain (light) with CΦΦC motif (Φ=L,I,V,F,M or W), (K/R) X(K/R)R, furin cleavage site (red box). TM, transmembrane unit contains FP, fusion peptide (red); leucine zipper motif (LX₆LX₆NX₆LX₆L) with HR1 (blue) and HR2 (green) heptad repeats followed by the CX6CC motif; tm, trans-membrane anchorage domain (red, hatched); The ectodomain part of the TM contains a so-called immunosuppressive domain labelled isd, (QNRX2LDXLX5GXC); cyt, cytoplasmic tail with C-terminal R peptide (blue) containing YXXΦ motif. (b) Schematic representation of the maturation and conformational changes leading to virus-cell membrane fusion, beginning with the fusion competency acquisition of the envelope glycoprotein (1) based on R peptide release by viral protease and ending with the gathering of viral and cellular membranes (4) induced by the anchorage of the fusion peptide into the cell membrane. Red arrow symbolizes the R peptide cleavage. (c) The first five amino acids correspond to the transmembrane domain. Experimentally determined (GaLV, MLV, exoJSRV) and putative (W Rep. and FRD Rep.) protease cleavage site (black line) and YXXΦ signaling motif are indicated in lowercase. Comparison of the Syncytin-1 protein (Syn-1) with the HERV-W family consensus sequence obtained from Repbase (W Rep.) shows a four amino acids deletion (LQMV) in the domesticated fusogenic protein, overlapping the ancestral viral protease cleavage site. The underlined leucine indicates a C-terminal truncation mutant exhibiting hyperfusogenic activity and significant pseudotyping capacity. Comparison of the Syncytin-2 protein (Syn-2) with the Repbase FRD consensus sequence (FRD Rep.) shows a stop codon that shortens the Syncytin-2 cytoplasmic tail and no evidence of viral protease cleavage site. Alignment of enJSRV and exoJSRV shows the placenta-expressed enJSRV has accumulated mutations surrounding the protease cleavage site and lacks downstream tyrosine (Y) residue. Genebank accession numbers: MLV: M14702; GaLV: AF055060, Syncytin-1: GQ919057, Syncytin-2: HEU27240, enJSRV: enJS56A1 and exoJSRV: AF105220

We observed that the VR3 region of all exogenous stains of JSRV sequences exhibit this tyrosine residue whereas all the enJSRVs envelopes described so far lacked this motif critical for JSRV transformation (Fig. 5c).

3.2.2 Immunomodulation, That Makes the Switch

Given that the placenta is an extra-embryonic tissue, half paternal and half maternal genetically inherited, the past decades have gathered reproductive immunologists researches to solve the fetal semi-allograft problem. Regulatory T cells are responsible for the establishment of tolerance by modulating the immune response, and uterine natural killer cells direct placentation by controlling trophoblast invasion (Munoz-Suano et al. 2011). As an example the contact zone between mother uterus and fetus extravillous cells of spiral arterioles appears to be one of these predictive immunological conflict zones, where Syncytin-1 has also been shown to be expressed (Malassine et al. 2005). So, beside extravillous cytotrophoblast-expressed HLA-G immunoregulatory proteins, immunomodulation properties of retroviruses sequences (Mullins and Linnebacher 2012; Rolland et al. 2006; Wang-Johanning et al. 2008) may contribute to answer the tolerance during pregnancy. Thus, a first mechanism of Syncytins-mediated immunosuppressive activity may be due to the presence of a putative immunosuppressive region conserved among murine, feline, and human retroviruses (Cianciolo et al. 1985), depicted hereafter for MPMV, SRV, SNV and BAEV so-called immunosuppressive retroviruses: LQNRRLDLLTAE**QGGICLA**. The analysis of this domain for the human and mouse Syncytins in a mouse model of transplant rejection has revealed an immunosuppressive activity for Syncytins-2 and -B but not for the Syncytins-1 and -A (Mangeney et al. 2007). More precisely, two amino acids have been described as commutator points that can be alternatively turned ‘on’ or ‘off’ in substitution experiments and trigger a switch from immunosuppressive to non-immunosuppressive activity (see bold letters in sequence above). This suggests a possible co-operation in tandem of the Syncytins pairs in Primates and Muridae, with complementary fusion and immunosuppression functions adapted to cellular and physiological contexts. Additional recent findings also suggest that envelopes coming from the HERV-K family may contribute to placentogenesis or provide immune protection to the fetus (Kammerer et al. 2011), what reinforces the idea of complementary functions within HERV envelope partners in the placenta. A second potential mechanism links immune response and amino acid balance. During pregnancy, maternal tryptophan is required for the T lymphocytes activation and ‘immunosuppression by starvation’ is the consequence of tryptophan depletion experiments (Mellor and Munn 1999). Besides, a tryptophan-catabolizing enzyme, the indoleamine 2,3-dioxygenase, is particularly expressed in the syncytiotrophoblast. Thus, the lymphocyte regulation appears to be strongly mediated by the ability of the apical membrane to incorporate the tryptophan into the syncytiotrophoblast (Kudo et al. 2001). In other words, the tolerance towards the allograft is locally conditioned by the CD98/LAT1 tryptophan transporter and the resulting amino acid balance changes. If we consider that the Syncytin-1 interacts with

amino-acid transporters from one side (Blond et al. 2000; Lavillette et al. 2002) and with the TLR4 and the pathogen-recognition receptor DC-SIGN *in vitro* from the other side (Rolland et al. 2006; Cheynet et al. 2005), the modulation of the immune system *via* amino-acid balances and TLR4 stimulations would become an axis of understanding the tolerance, articulated around the Syncytins. Even if the Syncytin-1 and Ory-1 ASCT2 receptor only mediates the transport of small amino acids, and consequently probably not tryptophan, considerations about balance changes that could impact the immune system response are maybe not so far. On one hand, infection of cells with Syncytin-1 phylogenetically-related RD114/simian immunosuppressive type D retroviruses results in impaired amino acid transport, a mechanism proposed to mediate virus immunosuppression (Rasko et al. 1999). On the other hand the glutamine, a small amino-acid accepted by both ASCT1 and ASCT2 transporters, was shown to influence the balance within the T lymphocytes sub-populations, potentially influencing the host response (Chang et al. 1999). Interestingly, recent findings suggest that Syncytin-1 is shed from the placenta into the maternal circulation in association with microvesicles, and modulates immune cell activation (Holder et al. 2012). Surprisingly, similar effect was demonstrated with a recombinant protein encompassing amino acids 116–225, i.e. excluding the TM immunosuppressive domain but conserving part of the SU subunit which includes most of the SDGGGX₂DX₂R-conserved motif previously seen to be directly involved in Syncytin-1/hASCT2 receptor recognition (Cheynet et al. 2006).

3.3 *A Price to Pay: ‘That said, the wolf ran off, and he is running still’*

As illustrated above, the multiple levels of control exemplified with the Syncytin-1 may suggest that Syncytins expression is tightly regulated to be constrained to the placenta, where physiological functions take place. However, diseases of the placenta have been linked with Syncytins deregulations, and various pathological contexts have reported Syncytin-1 expression. We give here an overview of the price to pay to the domestication of such retroviral elements.

3.3.1 Syncytins and Diseases of the Placenta

Pre-eclampsia and HELLP syndrome are disorders associated with abnormal placentation, including defects in syncytiotrophoblast formation. Numerous studies have associated PE and HELLP with Syncytin-1 and Syncytin-2 significant reduction (Chen et al. 2006, 2008; Knerr et al. 2002; Lee et al. 2001; Strick et al. 2007). Syncytin-2 expression was more importantly impaired than Syncytin-1 in severe pre-eclampsia (Vargas et al. 2011). Interestingly, a redistribution of Syncytin-1 within the syncytiotrophoblast polarized cell layer was observed for patients with PE (Lee et al. 2001). Though, cultured cytotrophoblast cells from PE and HELLP showed higher apoptotic

rates (Strick et al. 2007). A reduced Syncytin-1 expression has also been reported in placenta from intra uterine growth restriction and was associated with an overall disorganized syncytiotrophoblast layer with fewer nuclei (Ruebner et al. 2010).

3.3.2 Expression of Syncytin-1 in Autoimmune Diseases and Cancers

Syncytin-1 is expressed in astrocytes, glial cells and activated macrophages in brain regions affected by multiple sclerosis. Syncytin-1 expression in astrocytes mediates neuroimmune activation and death of oligodendrocytes by inducing the release of cytotoxic redox reactants (Antony et al. 2004). In astrocytes, Syncytin-1 induces the expression of OASIS, an endoplasmic reticulum stress sensor, which in turn increases the expression of inducible NO synthetase and concurrent suppression of cognate hASCT1 receptor, resulting in a diminished myelin protein production (Antony et al. 2007). What mechanisms reactivate Syncytin-1 in the brain in MS is still not clear. This could be the result of viral infection of the brain, such as herpes simplex virus, which has previously been shown to transactivate Syncytin-1 expression (Nellaker et al. 2006), or cytokine deregulation (Perron et al. 2001). Indeed it has been shown in astrocyte cultures that MS detrimental cytokines, IFN- γ and TNF- α are able to induce Syncytin-1 expression through NF- κ B activation, while MS protective IFN- β inhibits its expression (Mameli et al. 2007). In addition, Syncytin-1 induction by exogenous TNF- α into the corpus callosum, a region of the brain frequently exhibiting demyelination in MS, leads to neuroinflammation, reduction of myelin proteins level and neurobehavioural deficits in Syncytin-1-transgenic mice, as observed in MS (Antony et al. 2007). Interestingly as a parallel between MS and cancers, NO production in tumor vessels correlates with an increase of the over-all survival as well as the decrease of metastatic potency in experimental systems (Mortensen et al. 2004). On line with this, the level of Syncytin-1 expression represented a positive prognostic indicator for recurrence-free survival of breast cancer patients (Larsson et al. 2007). Conversely, increased Syncytin-1 expression was associated with decreased overall survival in rectal but not in colonic cancer patients (Larsen et al. 2009). The situation appears unclear in endometrial carcinoma where the increase of Syncytin-1 expression in normal endometrium of patients may possibly influence the development of endometriosis (Oppelt et al. 2009). Thus, the prognostic impact of Syncytin-1 expression appears to vary with the tumor type potentially, due to different functions associated with different pathways of reactivation. In a more general way, Syncytin-1 expression was observed for about one-third of breast cancer patients, and additionally, neighbouring endothelial cells were shown to express hASCT2 receptor (Bjerregaard et al. 2006). *In vitro* studies confirmed the involvement of Syncytin-1 in the fusion process between breast cancer cell lines and endothelial cells (Bjerregaard et al. 2006). Syncytin-1 was also found to be expressed in leukemia and lymphoma cells while no expression was identified in blood samples of normal individuals (Sun et al. 2010). Syncytin-1 associated cell-cell fusion was identified in EnCa tumors *in vivo*, but interestingly, *in vitro* studies showed the implication of Syncytin-1 in both the fusion and the proliferation of EnCa cells (Strick et al. 2007). Syncytin-1 up-regulation *via* the

cAMP pathway leads to cell-cell fusion while induction by steroid hormones (estradiol) leads to proliferation. This molecular switch is apparently controlled by TGF- β 1 and TGF- β 3 which are induced by steroid hormones and may override Syncytin-1 mediated cell-cell fusions (Strick et al. 2007).

4 Conclusion

Our life starts with the sperm-egg fusion, yet this princeps phenomenon remains partly to be elucidated (Kawano et al. 2011). As the embryo develops, skeletal muscle differentiation depends on the fusion of mononucleated myoblasts to form multinucleated muscle fibers. Recent in vitro findings showed that Syncytin-1 and its receptors hASCT1 and hASCT2 are expressed in human myoblasts and involved in myoblast fusion (Bjerregaard et al. 2011). In the adult body, macrophages can fuse to form either multinucleated osteoclasts that control the maintenance of the bones or multinucleated giant cells that are important for the immune response. We begin to know that Syncytin-1 interacts with hASCT2 in differentiating osteoclasts and is expressed in human iliac crest biopsies (Søe et al. 2011). All these findings continue to feed both mechanistic and biological knowledge on gene domestication. More generally, genetic exchanges and their impacts on living structures remain today the crucial evolutionary force it was in ancient time, and in our point of view, retrovirology may significantly support comparative genomics. Definitely, endogenous retroviruses and more broadly retrotransposons represent an impressive mass of insufficiently solicited witnesses of such forces in action.

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Dedicate On behalf of past and present members of the Mallet’s group, we would like to dedicate this chapter to the memory of our colleague and friend Olivier Bouton who substantially contributed to the human and scientific adventure that was the MSRV/HERV-W/ERVWE1 discovery.

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