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Review

Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation

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The endogenous betaretroviruses of small ruminants offer an excellent model to investigate the biological relevance of endogenous retroviruses (ERVs). Approximately twenty copies of endogenous betaretroviruses (enJSRVs) are present in the genome of sheep and goats. enJSRVs are highly related to Jaagsiekte sheep retrovirus (JSRV) and the Enzootic nasal tumour virus (ENTV), the causative agents of naturally occurring carcinomas of the respiratory tract of sheep. enJSRVs interact/interfere at different levels both with the host and with their exogenous and pathogenic counterparts. enJSRVs blocks the exogenous JSRV replication by a novel two-step interference mechanism acting both early and late during the virus replication cycle. enJSRVs are highly active, they are abundantly and specifically expressed in the epithelium of most of the ovine female reproductive tract. The specific spatial and temporal expression of enJSRVs supports a role in trophoblast development and differentiation as well as conceptus implantation. In addition, enJSRVs are expressed during fetal ontogeny leading to the apparent tolerance of sheep towards the pathogenic JSRV. Thus, the sheep/enJSRVs system is a model that can be utilized to study many different aspects of ERVs and retrovirus biology. The impressive technologies developed to study the sheep reproductive biology, in conjunction with the knowledge gained on the molecular biology of enJSRVs, makes the ovine system an ideal model to design experiments that can functionally address the role of ERVs in mammalian physiology.

Introduction

Retroviruses possess the unique ability to integrate their genome in the DNA of the host cell. The required integration step for the retrovirus replication cycle has provided an opportunity for these viruses to colonize (during evolution) the germline of virtually all eukaryotes. Consequently, retroviruses are transmitted horizontally, as 'exogenous' viruses (e.g. as any other virus), as well as vertically, as 'endogenous' viruses (ERVs) inherited in a classical Mendelian fashion (Vogt, 1997). ERVs account for a substantial portion of the genetic pool of every single animal but the biological significance of these elements has represented a biological puzzle for several years (Boeke & Stoye, 1997).

ERV–host interactions

Eukaryotic transposons can spread rapidly in sexual species in the absence of positive selection at the cellular or organism level (Hickey, 1982). Thus, ERVs could have 'survived' in the genome of eukaryotes without furnishing any obvious beneficial effect. However, some positive (and more rarely negative) roles for ERVs have been described and many more hypothesized. In general, ERVs are proposed to contribute in shaping the genome of the host and influencing gene expression by leading chromosomal rearrangements through homologous recombination between distant loci (Hughes & Coffin, 2001) and by directly influencing gene expression (Ting *et al.*, 1992).

Interesting scenarios are envisaged for two human ERVs, ERV-3 and HERV-W (*Human endogenous retrovirus W*). ERV-3 (Venables *et al.*, 1995) is conserved throughout primate evolution and is highly expressed in the trophoblast of the placenta (Boyd *et al.*, 1993). The similarity between a portion of the transmembrane (TM) glycoprotein of ERV-3 and a putative immunosuppressive region (termed p15E) (Haraguchi *et al.*, 1995) of gammaretroviruses led to the speculation that ERV-3 may protect the foetus from immune attack by the mother (Venables *et al.*, 1995). An even more intriguing example on how an ERV could be beneficial to its host is represented by HERV-W. HERV-W is specifically expressed in the syncytiotrophoblast of

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the human placenta. The HERV-W envelope protein, like many retroviral envelope proteins, induces formation of syncytia when expressed *in vitro*, thereby favouring the hypothesis that HERV-W is involved in human placental morphogenesis (Blond *et al.*, 2000; Mi *et al.*, 2000; Frendo *et al.*, 2003).

ERVs may also protect the host against infection by related exogenous retroviruses. For example, ERV (*ev*) loci of chickens (subgroup E) express envelope proteins that confer resistance to *Rous sarcoma virus* subgroup E infection, presumably by receptor interference (Payne & Pani, 1971). A similar situation has been observed in some feral mice, wherein the *fv-4*^{r} locus blocks ecotropic receptors for *Murine leukaemia virus* (MLV) through an endogenous ecotropic gp70 synthesis (Kozak *et al.*, 1984).

An interference mechanism at the levels of post-entry and pre-integration is present in some strains of mice possessing the MLV-resistant locus *fv-1* (Lilly, 1970). This locus was cloned, sequenced, and found to be related to HERV-L *gag*, an ERV only weakly related to MLV (Best *et al.*, 1996; Stoye, 1998; Towers *et al.*, 2000). The precise mechanism of action of Fv-1 remains to be elucidated.

Endogenous interference through the immune system is well established in the *Mouse mammary tumour virus* model (MMTV) through the expression of a superantigen (Golovkina *et al.*, 1992; Held *et al.*, 1993).

ERVs can also have detrimental effects and have been associated with some human diseases but for space limitations this topic will not be covered here.

The exogenous and pathogenic Jaagsiekte sheep retrovirus

Jaagsiekte sheep retrovirus (JSRV) is an exogenous and pathogenic retrovirus (Palmarini & Fan, 2001). JSRV is the cause of ovine pulmonary adenocarcinoma (OPA), a major infectious disease of sheep (Sharp, 1987; Sharp & Angus, 1990; DeMartini & York, 1997; Palmarini *et al.*, 1997).

The JSRV genome has a simple genetic organization, characteristic of the replication-competent betaretroviruses (Hunter *et al.*, 2000), containing the canonical structural retroviral genes *gag*, *pro*, *pol* and *env* (York *et al.*, 1991, 1992; Palmarini *et al.*, 1999a) (Fig. 1). The *gag* gene encodes the structural proteins of the viral core; *pro* and *pol* encode virion-bound enzymes (PR, RT and IN); and the *env* gene encodes the proteins in the envelope (surface and transmembrane).

JSRV is the only virus inducing a naturally occurring lung cancer. JSRV induces transformation of type II pneumocytes and Clara cells. The JSRV long terminal repeats (LTRs; containing the viral promoter and enhancers) are preferentially active in differentiated epithelial cells of the lungs and interact with lung-specific transcription factors (Palmarini *et al.*, 2000a; McGee-Estrada *et al.*, 2002). Of particular note, expression of JSRV Env alone is sufficient to induce cell transformation *in vitro* (Maeda *et al.*, 2001; Palmarini *et al.*, 2001b; Chow *et al.*, 2003; Danilkovitch-Miagkova *et al.*, 2003).

Another exogenous betaretrovirus related to JSRV and enJSRVs is ENTV (*Enzootic nasal tumour virus*) (Cousens *et al.*, 1996, 1999). The biology of ENTV is very similar to the

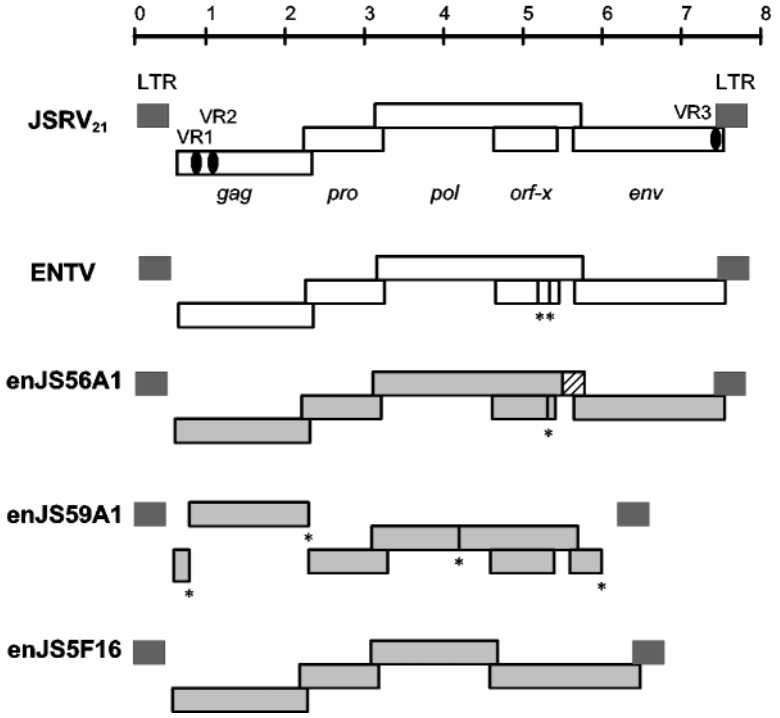

Fig. 1. Genomic structure of endogenous and exogenous sheep betaretroviruses. The numbered bar at the top indicate distances in kb. The exogenous JSRV and ENTV show the canonical retroviral *gag*, *pro*, *pol* and *env* with *pro* in a different open reading frame from *pol*, the same for all betaretroviruses. An additional open reading frame (*orf-x*) overlapping *pol* is present in JSRV but is interrupted by two stop codons in ENTV. Premature stop codons are indicated by a vertical bar underlined by an asterisk. enJS56A1 is the only one of the three complete endogenous proviruses cloned to maintain full (or nearly full) open reading frames in all the structural genes. enJS59A1 has premature stop codons in *gag* and *pol* and a major deletion in *env*. enJS5F16 has a deletion in *pol*. Different peptide sequences at the 3′ end of the *pol* gene in enJS56A1 due to a frame shift are indicated by cross-hatching. The regions of main divergence among the sheep betaretroviruses (VR1, VR2 and VR3) are indicated by a black oval in the JSRV genome.

highly related JSRV, including the capacity of ENTV Env to transform rodent fibroblasts *in vitro* (Alberti *et al.*, 2002; Dirks *et al.*, 2002).

Endogenous betaretroviruses of sheep related to JSRV

Sheep harbour in their genome about 20 copies of endogenous betaretroviruses (York *et al.*, 1992; Hecht *et al.*, 1994, 1996; DeMartini *et al.*, 2003) highly related to the exogenous and pathogenic JSRV (hence the name enJSRVs) (York *et al.*, 1992; De las Heras *et al.*, 1993; DeMartini & York, 1997; Palmarini *et al.*, 1997, 1999a). The genome of the enJSRVs loci is highly related to JSRV with 90–98 % identity at the amino acid level in most parts of the genome (Bai *et al.*, 1996, 1999; Palmarini *et al.*, 1996a, 2000b; Rosati *et al.*, 2000).

We isolated, sequenced and functionally characterized three complete enJSRV proviruses (enJS56A1, enJS5F16 and enJS59A1) (Palmarini *et al.*, 2000b) derived from a sheep genomic DNA λ phage library. All three proviruses contained open reading frames encoding at least one or more structural genes. enJS56A1 is a virtually full-length provirus with open reading frames for *gag*, as well as most of *pol* and *env* (Fig. 1). In transiently transfected cells, enJS56A1 is unable to release viral particles, even when expressed under control of the CMV immediate early promoter (pCMV2enJS56A1) (Palmarini *et al.*, 2000b). The use of JSRV/enJS56A1 chimeras determined that the main defect for particle formation resided in the first two-thirds of *gag*. Two short regions (VR1 and VR2) were identified in the enJS56A1 *gag* that contained major differences between ovine endogenous and exogenous betaretroviruses. In particular, VR1 contains a proline-rich region with SH2 and SH3 domains that are present in both JSRV and ENTV, but are absent in the homologous regions of the enJSRV proviruses. VR1 and VR2 belong to JSRV p23 (M. Mura & M. Palmarini, unpublished), a previously identified virion protein (Palmarini *et al.*, 1999b). A chimeric exogenous JSRV construct, wherein a region including the VR1 (Gag amino acid residues 89–142) was replaced with the homologous region from the endogenous enJS56A1, is unable to produce viral particles in the supernatant (M. Mura & M. Palmarini, unpublished results). Thus, the VR1 region (or amino acid residues immediately adjacent the VR1) is a determinant for the release of JSRV viral particles. Understanding the nature of this defect is particularly important, as enJS56A1 also blocks the release of viral particles from the exogenous JSRV, underlining a novel mechanisms of retroviral interference acting late in the replication cycle (see below).

Besides VR1 and VR2, a third region (VR3) located in the carboxy-terminal portion of the transmembrane (TM) protein of the viral envelope, is divergent between the exogenous JSRV and enJSRV sequences (Palmarini *et al.*, 2000b). Our studies indicate that this region is a main determinant of JSRV oncogenesis (see below) (Palmarini *et al.*, 2001b; Alberti *et al.*, 2002; Chow *et al.*, 2003; Zavala *et al.*, 2003).

enJSRVs interfere with exogenous JSRV entry by receptor interference

One of the possible reasons explaining the widespread fixation of ERVs in the mammalian germline is to protect the host from infection by related exogenous and pathogenic retroviruses. We hypothesized that enJSRVs interfered with the exogenous JSRV by receptor competition. The cellular receptor for JSRV was recently identified as the product of the *hyaluronidase-2* (*hyal-2*) gene (Rai *et al.*, 2000, 2001). enJSRVs can also utilize Hyal-2 as a cellular receptor, based on assays using retroviral vectors pseudotyped by the enJS5F16 envelope (Spencer *et al.*, 2003). To assess whether enJSRVs could interfere with JSRV at entry, the enJS5F16 Env was stably expressed in an ovine endometrial stromal cell line (oST-enEnv). The oST cell line was established from sheep uterine endometrial stroma cells that do not express enJSRVs. The oST-enEnv cell line was approximately 300-fold less infectable than the parental oST cell line by exogenous JSRV Env pseudotyped retroviral vectors. Collectively, these results support the hypothesis that enJSRVs can interfere with the exogenous and pathogenic JSRV at the level of virus entry (Spencer *et al.*, 2003).

enJSRVs interfere with JSRV late in the replication cycle: a novel mechanism of retroviral interference

As explained previously, ERVs have been found to interfere with their exogenous counterpart at the entry (e.g. the *fv-4 locus*) (Kozak *et al.*, 1984) or post-entry (but pre-integration) levels as in the case of *fv-1* (Lilly, 1970; Best *et al.*, 1996).

enJSRVs provide another example of retroviral interference, as one of the enJSRVs loci (enJS56A1) blocks exogenous JSRV viral particle formation late in the replication cycle at a post-integration step. As described above, enJS56A1 is unable to release viral particles in transfected cells but expresses abundant quantities of intracellular Gag (Palmarini *et al.*, 2000b). Intriguingly, the defect in viral particle release possessed by enJS56A1 is trans-dominant over the capacity of JSRV to make viral particles (M. Mura & M. Palmarini, unpublished data). In particular, release of viral particles in the supernatant of 293T cells transfected with JSRV plasmid was inhibited if the cells were co-transfected with enJS56A1. The dominant negative activity shown by enJS56A1 is specific for ovine betaretroviruses. enJS56A1 inhibits JSRV particle release, but does not interfere with the exit of *Moloney murine leukaemia virus* (MMuLV) or *Mason–Pfizer monkey virus* (MPMV) (M. Mura & M. Palmarini, unpublished data). The biological significance of these data is enhanced by *in vivo* observations of enJSRVs Gag protein expression in the epithelium of the ovine uterus (see below; Palmarini *et al.*, 2001a).

An obvious possibility is that some enJSRV loci do not encode the so-called late (L) domains within their Gag protein whose disruption result in normal virus assembly, with the exception of particle release (Wills *et al.*, 1994;

Xiang *et al.*, 1996; Puffer *et al.*, 1998; Yasuda & Hunter, 1998; Yuan *et al.*, 1999). L domains function by recruiting cellular factors such as Tsg101, Nedd4 and ESCRT-I and exploit the cellular endocytic trafficking machinery to release viral particles (Freed, 2002). Classical L domains are present in the JSRV21 VR2, but these motifs are also conserved in the VR2 of enJS56A1. Analysis and functional characterization of enJSRVs might reveal novel retroviral late domains or even help to understand the mechanisms of exit from the cells of retroviral particles.

enJSRVs thus appear to block JSRV at two levels (Fig. 2). The first block acts at the level of virus entry by receptor interference while the second step blocks most likely viral particle transport or exit. This is a powerful example that supports the hypothesis that ERVs have protected their host against infection of related pathogenic retroviruses.

enJSRVs Env lack the determinants of cell transformation present in the JSRV envelope

The JSRV envelope (Env) has the unique ability to transform cells *in vitro* (Maeda *et al.*, 2001; Rai *et al.*, 2001; Allen *et al.*, 2002; Zavala *et al.*, 2003). The enJSRVs Env of two loci tested so far are not able to induce cell transformation (Palmarini *et al.*, 2001b). Chimeras formed by the VR3 region of enJSRVs in the context of the exogenous JSRV Env are unable to transform either rodent or chicken fibroblasts (Palmarini *et al.*, 2001b; Zavala *et al.*, 2003). Several studies in the last few years have addressed the molecular mechanisms governing JSRV-induced cell transformation (Palmarini *et al.*, 2001b; Alberti *et al.*, 2002; Chow *et al.*, 2003; Zavala *et al.*, 2003). In the JSRV Env, the cytoplasmic tail and the TM domain contain major determinants of cell transformation (Chow *et al.*, 2003; Liu *et al.*, 2003; Palmarini

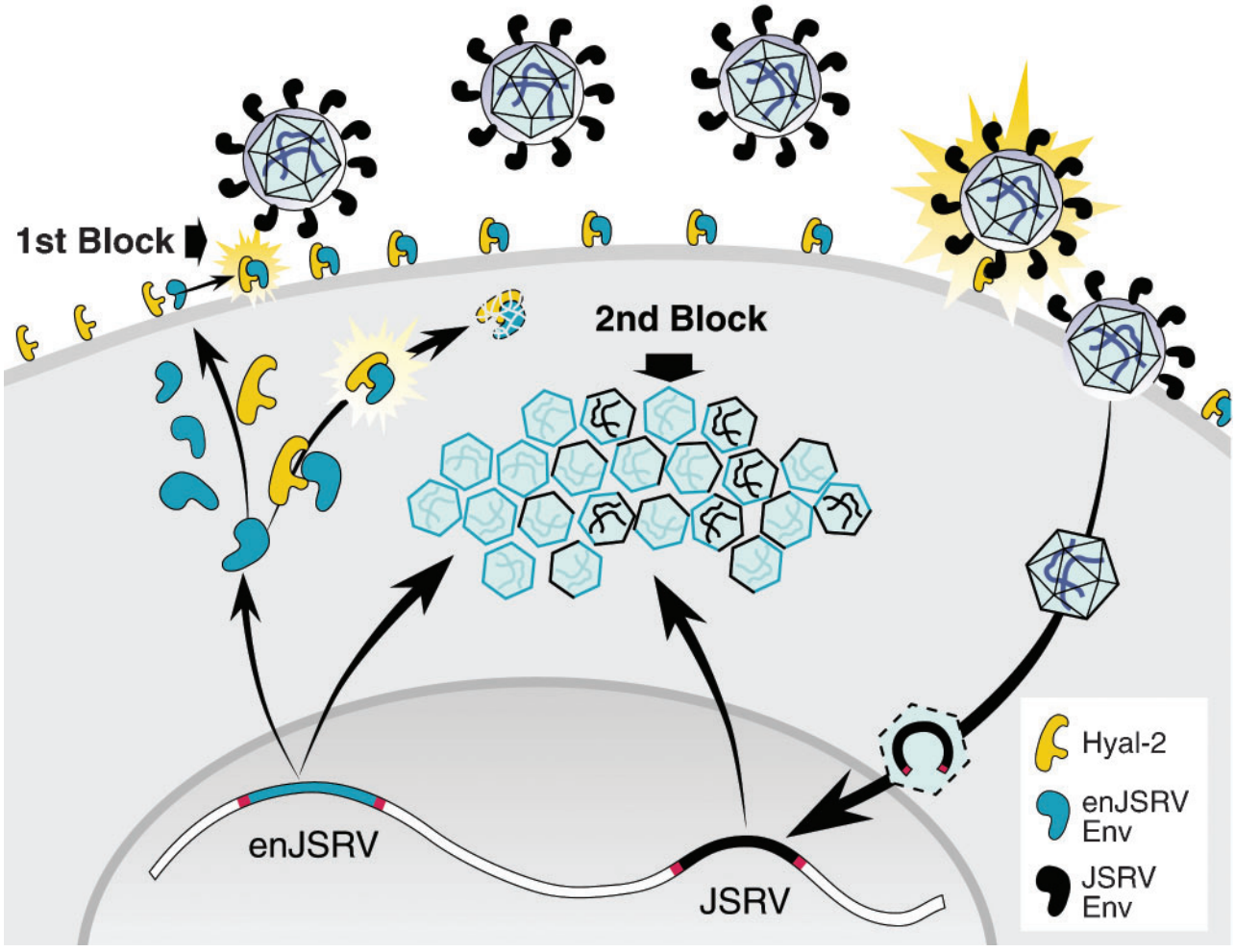

Fig. 2. enJSRVs-induced blocks of JSRV replication. Cells expressing enJSRVs are protected by exogenous JSRV infection at two different levels. The first step is provided by expression of the enJSRVs Env. The Env–Hyal-2 interaction (either at the membrane or in the cytoplasm) decreases the JSRV receptor availability at the cell surface, inhibiting in this way virus entry. The second block is provided by Gag expression of some enJSRVs loci (e.g. enJS56A1). Viral particles formed by enJS56A1 cannot exit the cell and inhibit JSRV exit when both proviruses are expressed in the same cell.

& Fan, 2003; Zavala *et al.*, 2003). Interestingly, the enJSRV Envs analysed to date lack these domains, suggesting that the transforming properties of the betaretrovirus Envs may have evolved relatively recently. Alternatively, ancestral ovine betaretroviruses may have possessed transforming-inducing Env, but those were counterselected during evolution, as they would have not allowed normal development of the host. A role for the surface domain of the JSRV Env in virus-induced cell transformation has recently been hypothesized for the transformation of epithelial cells (Danilkovitch-Miagkova *et al.*, 2003). The recently described model hypothesizes that JSRV-induced cell transformation derives from the activation of the Ron tyrosine kinase. Hyal-2 (the cellular receptor for JSRV) constitutively binds Ron at the cell surface. JSRV Env expression sequesters Hyal-2 and allows Ron dimerization and activation leading to cell transformation. It is interesting to note that enJSRVs, as mentioned above, use Hyal-2 as cellular receptor too and we have found high expression of these elements in the genital tract of the ewe (see below). Thus, it is difficult to reconcile the model hypothesized with the physiological expression of enJSRVs unless additional mechanisms, besides Ron activation, are necessary to cause JSRV-induced cell transformation.

Possible roles of enJSRVs in sheep reproductive biology

enJSRVs are highly expressed in the genital tract of the ewe. The localization, level and timing of expression of enJSRVs lend support to the hypothesis that these loci are important in female reproductive tract and placental biology. Indeed, expression of ERVs in the genital tract and placenta of various animal species has been described for at least three decades (Kalter *et al.*, 1973, 1975; Vernon *et al.*, 1974; Smith & Moore, 1988; Harris, 1991; DeHaven *et al.*, 1998).

Embryo development in sheep

In sheep, the ovulated oocyte is fertilized and develops into a morula embryo in the oviduct and then is transported from the oviduct into the uterus (Guillomot, 1995). Between Days 12 and 16, the conceptus rapidly elongates to a filamentous form that achieves contact with most of the luminal epithelial cells lining the uterine endometrium (Guillomot *et al.*, 1981). The morphological development of the sheep blastocyst from spherical, to tubular, to filamentous conceptus during the peri-implantation period coincides with the production of large amounts of interferon-tau (IFN-τ) from the mononuclear trophectoderm (Spencer *et al.*, 1996; Bazer *et al.*, 1997; Spencer & Bazer, 2002). These events ensure survival of the corpus luteum which produces progesterone, the hormone of pregnancy (Spencer *et al.*, 1996).

Implantation is initiated by the conceptus on Days 14 to 16 of pregnancy. As the blastocyst develops into an elongated conceptus, the outer trophectoderm transiently contacts uterine endometrial luminal epithelial cells in preparation for implantation (Guillomot *et al.*, 1981; Guillomot, 1995). Apposition of conceptus trophectoderm and endometrial luminal epithelium is initiated on Day 14, followed quickly by attachment on Day 15, and firm adhesion on Days 16 to 18 (Guillomot *et al.*, 1981). Between Days 15 and 16, the binucleate syncytiotrophoblast cells of the placenta differentiate from the mononuclear trophectoderm cells.

enJSRVs expression

enJSRVs are abundantly expressed in the epithelia of female reproductive tract tissues (Fig. 3) (Spencer *et al.*, 1999; Palmarini *et al.*, 2000b, 2001a). This finding may reflect tropism for the female reproductive tract by an ancestral exogenous retrovirus that was the predecessor of enJSRVs. Using sensitive PCR analyses, enJSRVs RNA can be detected in a variety of tissues, including lungs, kidneys, thymus, bone marrow, spleen, mediastinal lymph nodes and leukocytes (Palmarini *et al.*, 1996b). However, the highest levels of enJSRVs RNA expression are observed in the female reproductive tract. In the uterus, abundant *en*JSRVs expression was observed solely in the endometrial luminal epithelium and glandular epithelium of the uterus (Spencer *et al.*, 1999; Palmarini *et al.*, 2000b, 2001a).

Temporal and spatial expression of enJSRVs coincides with blastocyst maturation and peri-implantation period

Expression of enJSRVs RNA in the endometrial epithelia increases 12-fold between Days 1 and 13 of the oestrous cycle and pregnancy (Palmarini *et al.*, 2000b). In pregnant ewes, endometrial enJSRV RNA expression is high on Day 11, increases at Day 13, and then decreases at Day 19. Expression of enJSRVs is not limited to RNA, because enJSRV capsid and envelope proteins were also observed on the apical surface of the endometrial epithelia by immunofluorescence using antisera to the highly related exogenous JSRV capsid or envelope proteins (Palmarini *et al.*, 2001a).

The increase in epithelial enJSRVs expression occurs during a period when the blastocyst hatches from the zona pellucida on Day 9, transitions from a spherical to tubular conceptus by Day 11, and then undergoes rapid elongation beginning on Day 12 to a filamentous conceptus that occupies the entire uterine horn by Day 16 (Guillomot *et al.*, 1981; Guillomot, 1995; Palmarini *et al.*, 2001a). These developmental changes in the conceptus involve rearrangement and proliferation of the mononuclear trophectoderm cells which produce IFN-τ, a novel Type I IFN that is the pregnancy recognition signal (Spencer & Bazer, 2002). The timing of enJSRVs expression is coincidental with the period of conceptus implantation. We hypothesize that an interaction between the enJSRVs Env (expressed in the uterine epithelium) and Hyal-2 (in the trophoblast) facilitates the process of conceptus implantation in the uterus.

enJSRVs are highly expressed in the placental binucleate cells

In the uteri of pregnant ewes, expression of enJSRVs is also observed in the developing placenta. This phenomenon is

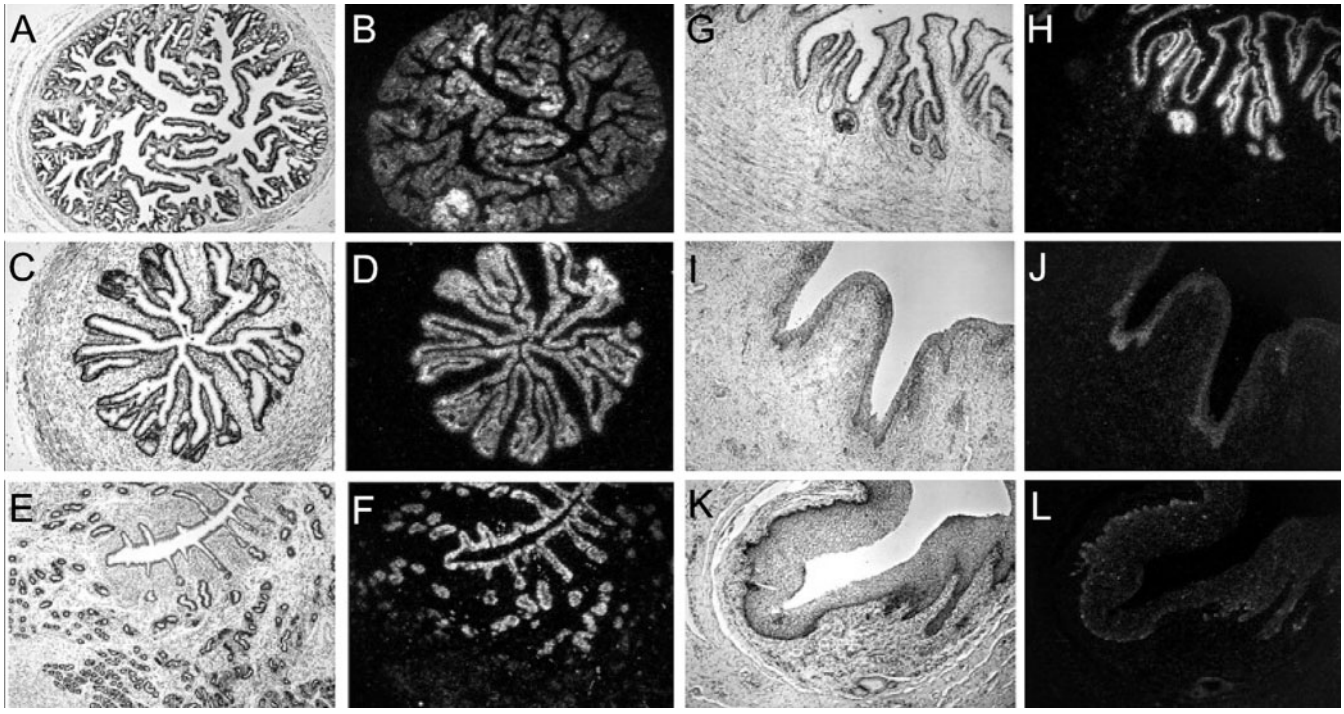

Fig. 3. *In situ* hybridization analysis of enJSRV expression in the adult sheep female reproductive tract. Cross-sections of different regions of the female reproductive tract from Day 9 pregnant ewes were hybridized with ^{35}S-labelled antisense or sense ovine enJSRV *env* cRNA probes. Protected transcripts were visualized by liquid-emulsion autoradiography for 1 week and imaged under bright-field (A, C, E, G, I, K) or dark-field illumination (B, D, F, H, J, L). A and B, ampulla; C and D, isthmus; E and F, uterus; G and H, cervix; I and J, anterior vagina; K and L, posterior vagina. Magnification, 260 ×. Figure modified from Palmarini *et al.* (2001a) and printed with permission from the American Society for Microbiology.

remarkably similar to the expression of HERV-W in the human placenta (Blond *et al.*, 2000). The syncytiotrophoblast is the outer layer of the placenta that evolves from the mononuclear cytotrophoblast. In the ruminant placenta, the mononuclear cells of the trophoblast are the source of binucleate cells that arise from their cell duplication without subsequent division (Wooding, 1982). Interestingly, we detected both enJSRVs RNA and immunoreactive proteins in the sheep placental binucleate cells (Fig. 4) (Palmarini *et al.*, 2001a). The binucleate cells first develop in the placenta on Day 16 and continue to develop until Days 60–80 when placentation and placentome formation is complete. The binucleate cells form the syncytiotrophoblast by fusing with the endometrial luminal epithelium in both caruncular and intercaruncular areas. The binucleate cells display invasive properties and they are abundantly present in the placentome. The placentomes are formed mainly by binucleate syncytiotrophoblast cells fused with the uterine endometrial luminal epithelium. The binucleate cells solely synthesize and secrete placental lactogen, a key hormone in pregnancy that stimulates endometrial gland morphogenesis and differentiated function for fetal nutrition (Spencer & Bazer, 2002).

The invasive properties of the binucleate cells are reminiscent of some attributes possessed by transformed cells. As we explained above the JSRV Env induces cell transformation and functions essentially as an oncoprotein. Although the enJSRVs cloned so far do not possess the ability to transform cells, we cannot rule out that some of these loci are able to induce some degree of cell transformation. Thus it is tempting to speculate that enJSRVs expression in the binucleate (and not the mononuclear) cells of the trophectoderm is directly correlated with the invasive properties and the formation of syncytia exhibited by these cells.

The sheep model is thus uniquely suited to test the biological relevance of ERVs in placental morphogenesis given the ample similarities between enJSRVs and HERV-W and the impressive techniques available in sheep reproductive biology.

*en*JSRVs expression is regulated by progesterone *in vitro* and *in vivo*

In the ovine endometrium, the expression of *en*JSRVs RNA is correlated with circulating levels of progesterone and epithelial progesterone receptor (PR) expression, suggesting that the enJSRV LTR (or the LTR of some enJSRV loci), containing the viral promoter and enhancers, is influenced by progesterone. In transient transfection assays, the LTR of the enJS59A1 locus was transactivated almost 10-fold by progesterone, but the effects of progesterone on the LTRs of exogenous JSRV was minimal (Palmarini *et al.*, 2000b). The

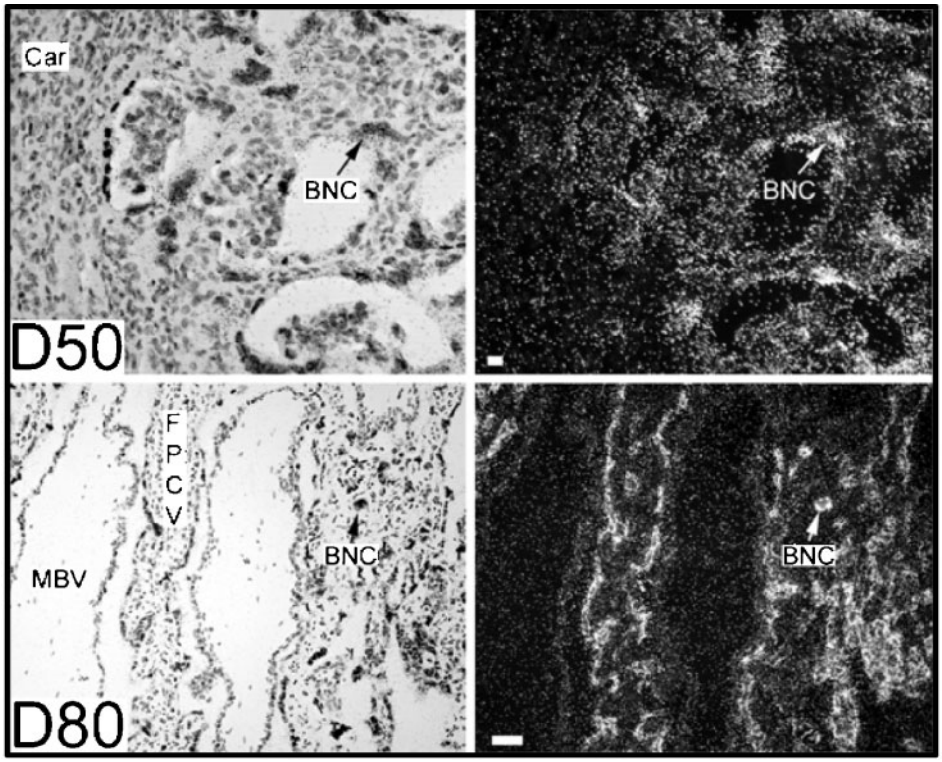

Fig. 4. *In situ* localization of enJSRV *env* RNA in the ovine placentomes. *In situ* hybridization of the sheep placenta collected at Day 50 (D50) and 80 (D80) of pregnancy. Sections were hybridized with ^{35}S-labelled antisense or sense ovine enJSRV *env* cRNA probes. Protected transcripts were visualized by liquid-emulsion autoradiography for 1 week and imaged under bright-field (left panels) or dark-field illumination (right panels). enJSRVs expression was localized in the binucleate cells (BNC) of the fetal placental cotyledonary villus (FPCV). CAR, maternal caruncle; MBV, maternal blood vessel; Bar, 10 μm.

LTR of the exogenous JSRV is activated by lung-specific transcription factors, such as HNF-3β (Palmarini *et al.*, 2000a; McGee-Estrada *et al.*, 2002), while the tested enJSRV LTRs are not affected by HNF-3*β*. These results support the hypothesis that the exogenous JSRV and ENTV developed their pulmonary tropism relatively recently and quite possibly after the integration of the enJSRV loci in the sheep germline.

Recent data indicate that enJSRVs expression is directly regulated by progesterone and progesterone receptor in the ovine endometrial epithelium also *in vivo* (K. E. D. Dunlap & T. E. Spencer, unpublished data). *In situ* hybridization analyses of uteri collected from sheep treated with progesterone and progesterone receptor antagonists showed a substantial reduction in enJSRVs expression. On the other hand, IFN-τ did not affect enJSRVs expression. These *in vivo* observations confirm those from *in vitro* experiments indicating that progesterone, acting through PR, increases expression of enJSRVs in the endometrial lumenal and glandular epithelia in a temporal manner coincident with the beginning of conceptus elongation and implantation.

Distribution of enJSRVs in Artiodactyla

Sheep have approximately 20 enJSRVs loci as determined by Southern blotting hybridization (Hecht *et al.*, 1994, 1996). Closely related viruses are found in goats (*Capra hircus*) in similar copy numbers as sheep; the goat hybridization pattern is different from sheep, but one that is generally conserved among goats and wild goats. However, the differences in restriction enzyme profiles between sheep and goat lineages suggest that much of the amplification from founding viruses within the respective genomes occurred after the divergence of goats and sheep approximately 4–10 million years ago (Irwin *et al.*, 1991; Miyamoto *et al.*, 1993; Honeycutt *et al.*, 1995; Reza Shariflou & Moran, 2000).

Recent data indicate that two of the twenty enJSRVs loci have a conserved chromosomal location in sheep and goats mapping on chromosome 1 (1q45) and 2 (2q41) (Carlson *et al.*, 2003). This observation strongly suggests that these two loci were fixed in the germline of a host that existed before the divergence of the genus *Ovis* from the genus *Capra*. Interestingly, one to three bands hybridizing at high stringency with JSRV probes were found in cattle and in some members of the Cervidae. The domestic cattle and deer diverged from the other ruminants between 18 to 19 million years ago. Artiodactyls that diverged much earlier, such as the domestic pig (55 millions years ago), do not show enJSRVs sequences by Southern blotting, although endogenous betaretrovirus sequences have been detected

(Ericsson *et al.*, 2001). Strikingly, the more recently diverged species, such as sheep, goats and domestic cattle, have evolved an increased number of placentomes (Fig. 5). The domestic pig has no placentomes, no binucleate cells and no syncytiotrophoblast. Therefore, it is plausible that integration of enJSRVs into the ruminant germline may have assisted the selective pressure towards the formation of placentomes and syncytiotrophoblast. However, by Southern blotting no JSRV-related bands were found in the DNA of Mountain goat (*Oreamnos americanus*) and this piece of data would be against the presence of some enJSRVs loci common to all ruminants (Hecht *et al.*, 1996). More hybridization studies are necessary to further investigate the distribution of enJSRVs in ruminants and artyodactyla by using probes derived from the more ancient enJSRV loci.

Could ERVs play a role in mammalian placentation?

The placenta is a complex organ that provides nutrients for the fetus, manages waste products, regulates gas exchanges and suppresses immunological rejection by the mother. The evolution of complex organs (like the eye for example) is one of the oldest puzzles in evolutionary biology: how did adaptation allow for the evolution of so many complicated functions in a single organ?

The placenta has evolved repeatedly in different groups of organisms, including fish, amphibians, reptiles and mammals (Blackburn, 1999). A model of placental evolution can be derived from fish of the genus *Poeciliopsis*. These fish display variation in live-bearing embryos that range from species that maintain eggs after fertilization with no maternal provision to those that have various degrees of maternal provisioning after fertilization. The latter are associated with maternal and fetal membranes that are functionally similar to a mammalian placenta. Recent data indicate that placentas (or pseudo-placentas) in *Poeciliopsis* evolved independently multiple times in 750 000 years or less (Reznick *et al.*, 2002). This is the same time-scale suggested by theoretical calculations for the evolution of complex eyes.

Many viviparous species exhibit placentas or similar membranes to nourish embryo development. The placenta evolved concomitantly with viviparity, because thinning of the eggshell allowed for apposition of the extraembryonic membranes to the uterine lining. The evolutionary implications are remarkable. Given that viviparity has evolved on over 100 separate occasions among squamates, placental organs must have also originated as frequently (Blackburn, 1999). No other organ is known to have originated on so many occasions, and no other organ shows such wide structural variability. In Eutheria (e.g. all mammals with the exceptions of marsupial and monotremes), the wide differences among major taxa suggest a polyphyletic origin. Thus, ERVs might have played one or more major roles in placental morphogenesis but not necessarily in all the species. Given the structural diversity of placentas and their likely polyphyletic origin, a 'common' ERV with a biological role in the reproductive biology of all Eutheria most likely does not exist.

enJSRVs expression in the ovine foetus

enJSRVs are highly expressed in the sheep fetus (Fig. 6) (Spencer *et al.*, 2003). Specific expression of enJSRVs RNA is observed in the lamina propria of the gut. Expression of *en*JSRV genes during fetal development may explain some aspects of the pathogenesis of the disease induced by the related exogenous JSRV after birth. Sheep affected by OPA or ENT do not develop circulating antibodies towards JSRV or ENTV (Sharp & Herring, 1983; Ortin *et al.*, 1998). Indeed, expression of enJSRVs RNA is detected in Peyer's

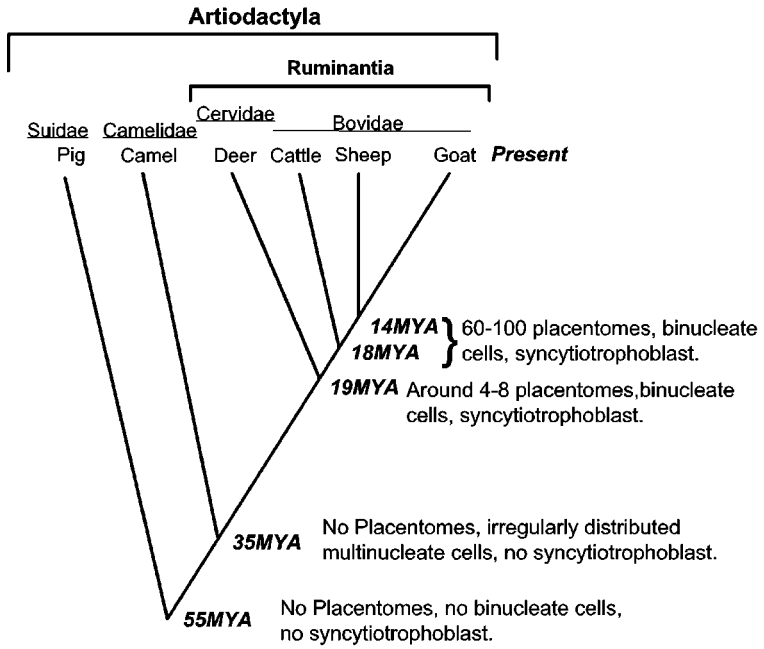

Fig. 5. Artiodactyla phylogeny. The approximate time of divergence of various artiodactyla is shown (MYA = million years ago) along the characteristics of the placenta of the respective species. Data were taken from Miyamoto *et al.* (1993) and Honeycutt *et al.* (1995). Figure reproduced (with modifications from Reza Shariflou & Moran, 2000) with permission from the Society for Molecular Biology and Evolution.

patches and thymus of fetal sheep (Spencer *et al.*, 2003). In particular, expression of enJSRVs in the thymus is detected predominantly in the cortico-medullary junction. The final selection of T cells occurs in this region of the thymus (Griebel, 1998). These results support the hypothesis that sheep are tolerized towards the exogenous viruses by expression of enJSRVs in the fetus during development of the immune system. The observation that antibodies can be detected in sheep immunized with recombinant JSRV capsid or surface proteins in adjuvant (Sharp & DeMartini, 2003) does not contrast with a possible enJSRVs-induced tolerance. All processes involving tolerance, both central and pheripheral, are recurring events and may be broken. Several reports in the litterature show that tolerance can be broken when self-antigens (especially in large amounts) are detected in the presence of pro-inflammatory signals (e.g. adjuvants) that promote the maturation of antigen-presenting cells (Burt *et al.*, 2002; Ohashi & DeFranco, 2002).

How the induction of tolerance to an exogenous retrovirus could be beneficial for its host is not readily apparent. Ancient enJSRV-related viruses might have been the cause of immuno-mediated disorders, so that induction of tolerance by related viruses integrated in the germline of the host provided an evolutionary advantage. Another hypothesis is that a uterine viral infection (induced by an ancestral exogenous ovine betaretrovirus) could have been the cause of an inflammatory process that would compromise development of the conceptus. The induction of immunotolerance in this case would have been beneficial for host evolution.

The expression of enJSRVs in the fetus also has implications for the design of strategies to control JSRV infection. OPA is one of the major infectious diseases of sheep. Given the extensive homology between JSRV and enJSRVs it is difficult to hypothesize a vaccine that can elicit a strong immune response in the sheep, as most viral epitopes would be recognized as self-antigens. Moreover, enJSRVs proteins are highly expressed in the sheep genital tract, and consequently even if a hypothetical effective JSRV vaccine was to be found this could have adverse effects on normal host cells. A critical evaluation of reproductive performances of sheep immunized with JSRV-based vaccines will have to be introduced in future safety and efficacy trials.

Conclusions

Many theories on the biological relevance of ERVs have been advanced during the last 20 years but few model systems have been investigated to substantiate experimentally these hypotheses. We speculate that enJSRVs were originally selected as they protected their host. enJSRV expression in the genital tract might have conferred an evolutionary advantage for sheep/goats through resistance to infection from related exogenous betaretroviruses circulating at that time. This could have provided a selection pressure for betaretroviruses with tropism towards the respiratory tract (e.g. JSRV and ENTV) rather than the genital tract (Fig. 7). Sheep betaretroviruses with tropism for the respiratory tract might have had a higher chance to establish a successful infection in a host with high-level expression of enJSRVs in the genital tract. Once fixed in the germline of the host, we speculate that enJSRVs expression favoured the process of conceptus implantation and influenced the placental morphogenesis of its host by contributing to the formation of the syncytiotrophoblast and of the placentomes of the ruminant placenta.

The enJSRVs/sheep model is perfectly suited to address many biological questions on ERVs and retroviral biology with practical applications of great relevance (Fig. 8). The enormous progress made in recent years in reproductive biology techniques has created a window of opportunity to experimentally address the question of whether ERVs participate as regulators of mammalian reproductive physiology. These studies would not be possible in humans for

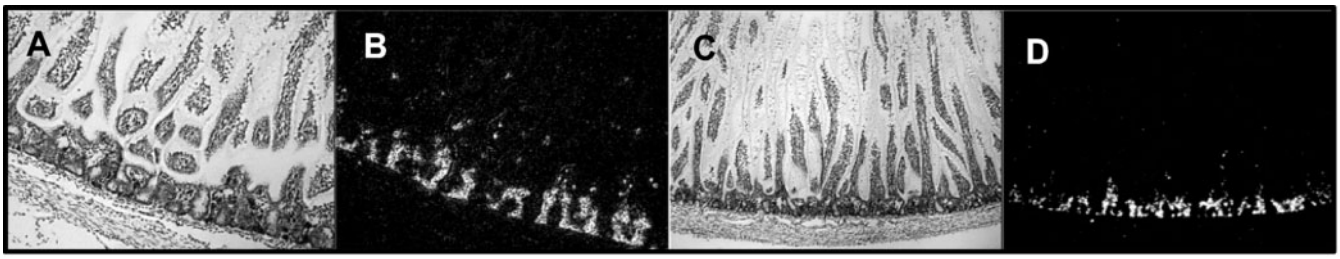

Fig. 6. enJSRVs expression in the ovine fetus. *In situ* hybridization analysis of enJSRV mRNA expression in Peyer's patch tissue collected from the small intestine of fetal lambs (Day 120 gestation). Cross-sections of different regions of the small intestine from sheep foetuses were hybridized with ^{35}S-labelled antisense ovine enJSRV *env* cRNA probes. Protected transcripts were visualized by liquid-emulsion autoradiography for 1 week and imaged under bright-field (A, C) or dark-field illumination (B, D). (A) Bright field of jejunal Peyer's patch tissue stained with haematoxylin. (B) *In situ* hybridization reveals a high degree of enJSRVs expression that localizes to cells within the lymphoid aggregates of the jejunals Peyer's patch. (C) Bright field of ileal Peyer's patch tissue stained with haematoxylin. (D) *In situ* hybridization reveals enJSRV RNA expression that is localized to cells within the lymphoid aggregates of the Peyer's patch. Figure modified from Spencer *et al.* (2003) and reproduced with permission from the American Society for Microbiology.

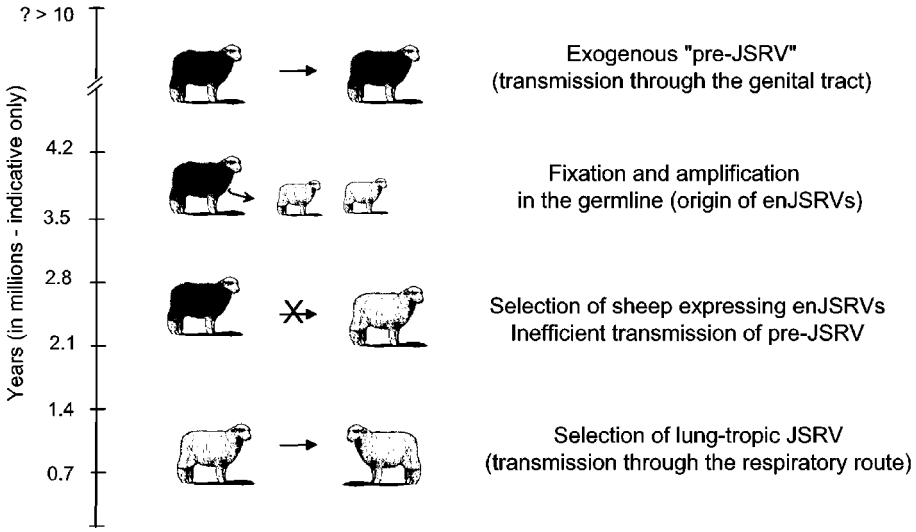

Fig. 7. Proposed model for sheep betaretrovirus evolution. The high level expression of *en*JSRVs in the sheep genital tract suggests that at least some of the ancestral exogenous forms of ovine betaretroviruses ('pre-JSRV') may have been transmitted from sheep to sheep through coitus. The selection of respiratory-tropic exogenous betaretroviruses might have been favoured by the endogenization of enJSRVs. Sheep shown in black represent sheep before the fixation of enJSRVs in their germline. The time of endogenization and subsequent events shown in the figure is purely indicative. Figure from Spencer *et al.* (2003) and reproduced with permission from the American Society for Microbiology.

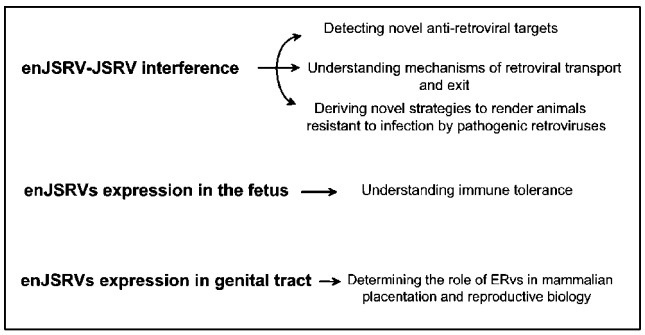

Fig. 8. Areas of research that can be explored with the sheep/enJSRVs model.

obvious ethical reasons. The presence of exogenous and pathogenic enJSRV-related viruses infecting sheep allows studying retroviral interference in an outbred animal species. Understanding the mechanisms of ERV interference towards exogenous viruses will be useful to further understand the late steps in the retrovirus replication cycle and identify novel anti-retroviral targets. In addition, novel methodologies offer the possibility of generating transgenic sheep resistant to JSRV infection by redirecting enJSRVs expression in type II pneumocytes and Clara cells, although these strategies would have to be carefully considered.

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