

**BIOLOGY OF THE ENVELOPE GLYCOPROTEINS
OF SHEEP BETARETROVIRUSES**

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Abstract

Retroviruses possess several biological features that differentiate them from all other infectious agents. The obligatory integration step of the retrovirus genome into the host genome has allowed these viruses to associate, modulate and alter the biology of the cell with a variety of unique mechanisms. Integration of retroviruses into the germ line of the host results in the formation of vertically transmitted “endogenous” retroviruses (ERVs). It is now becoming apparent that ERVs have often been selected as they provided evolutionary advantages to the host. Sheep Betaretroviruses provide a unique biological system to study the complex interaction between retroviruses and their hosts. Jaagsiekte sheep retrovirus (JSRV) is the causative agent of ovine pulmonary adenocarcinoma (OPA), a naturally occurring lung cancer of sheep. The JSRV Env glycoprotein is a dominant oncoprotein and its expression is sufficient to induce cell transformation *in vitro* and *in vivo*. Thus, OPA is a unique large animal model for the study of lung carcinogenesis. The sheep genome harbours at least 27 copies of ERVs highly related to JSRV (enJSRVs). Studies on enJSRVs have provided evidence supporting the idea that ERVs, exogenous retroviruses and the host have coevolved through a dynamic process throughout evolution. enJSRVs play a critical role in conceptus development and placental morphogenesis, and can block JSRV replication *in vitro* at both early and late stages of the replication cycle. The work presented here focuses on the study of the exogenous and endogenous JSRV Envs and their role in cell transformation and trophoblast differentiation respectively. We were able to show that: I) the JSRV Env transforms epithelial cells *in vitro* independently from its cellular receptor; II) both the exogenous and endogenous JSRV Envs interact with the receptor tyrosine kinase RON and that the cytoplasmic tail of the Env is the major determinant modulating the biological effects of the Env-RON interaction; III) the molecular chaperone Hsp90 regulates JSRV Env induced cell transformation, in part by downregulating Akt; and IV) OPA is a useful large animal model for the evaluation of new anti-cancer therapeutic agents. Moreover, we characterized the transforming properties, receptor usage and fusogenic activity of enJSRVs Envs to gain insight into their role in placental morphogenesis. The studies described in this thesis contributed to the understanding of JSRV induced cell transformation and the biology of enJSRVs.

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List of Accompanying Material

Copies of the following articles are included in this thesis:

Varela, M., Chow, Y.H., Sturkie, C., Murcia, P., Palmarini, M. (2006). Association of RON tyrosine kinase with the Jaagsiekte sheep retrovirus envelope glycoprotein. *Virology* 350(2): 347-57.

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Author's Declaration

I hereby declare that the work presented in this thesis is original and was conducted by the author under supervision except where stated.

I certify that no part of this thesis has been submitted previously for the award of a degree to any University but has been reproduced in parts in the following scientific papers:

Varela, M., Chow, Y.H., Sturkie, C., Murcia, P., Palmarini, M. (2006). Association of RON tyrosine kinase with the Jaagsiekte sheep retrovirus envelope glycoprotein. *Virology* 350(2): 347-57.

Dunlap, K.A., Palmarini, M., Varela, M., Burghardt, R.C., Hayashi, K., Farmer, J.L., Spencer, T.E. (2006). Endogenous retroviruses regulate periimplantation placental growth and differentiation. *Proceedings of the National Academy of Sciences USA* 103(39): 14390-5.

Varela, M., Golder, M., Archer, F., de las Heras, M., Leroux, C., Palmarini, M. (2007). A large animal model to evaluate the effects of Hsp90 inhibitors for the treatment of lung adenocarcinoma. *Virology*. Epub ahead of print.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
ALV	Avian leukosis virus
APOBEC	Apolipoprotein B mRNA editing enzyme
BaEV	Baboon endogenous retrovirus
BASCs	Bronchioalveolar stem cells
BLV	Bovine leukaemia virus
BNCs	Trophoblast giant binucleate cells
bp	Base pairs
C/EBP	CCAAT-Box/enhancer binding protein
CA	Capsid
CMV	Cytomegalovirus immediate early promoter
CoIP	Co-immunoprecipitation
CT	Cytoplasmic tail
CTE	Constitutive transport element
DLS	Dimer linkage structure
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EIAV	Equine infectious anaemia virus
ENA	Enzootic nasal adenocarcinoma
enJSRV	Endogenous Jaagsiekte sheep retrovirus
ENTV-1	Enzootic nasal tumour virus-1
env	Envelope
ER	Endoplasmic reticulum
ERV	Endogenous retrovirus
FBS	Foetal bovine serum
FeLV	Feline leukaemia virus
FISH	Fluorescence in situ hybridization

FIV	Feline immunodeficiency virus
gag	Group-specific antigen
GE	Glandular epithelium
GPI	Glycosylphosphatidylinositol
Grb-2	Growth factor receptor binding protein-2
GTP	Guanosine triphosphate
HA	Hemagglutinin
HERV-L	Human endogenous retrovirus L
HIV	Human immunodeficiency virus
HTLV	Human T-lymphotropic virus
Hyal-2	Hyaluronidase 2
IF	Immunofluorescence
IHC	Immunohistochemistry
IN	Integrase
INSL4	Insulin growth factor 4
JLR	JSRV late restriction
JSRV	Jaagsiekte sheep retrovirus
JSRV-SA	JSRV South African
Kb	Kilobases
L domain	Late domain
LTR	Long terminal repeat
MA	Matrix
MAO	Morpholino antisense oligonucleotides
MAPKs	Mitogen-activated protein kinases
MCF	Mink cell focus forming viruses
MDCK	Madin-Darby canine kidney cells
MHR	Major homology region
MMTV	Mouse mammary tumour virus
MoMLV	Moloney murine leukaemia virus

MSP	Macrophage stimulating factor
MTC	Mononuclear trophoblast cells
MVV	Maedi-Visna virus
NC	Nucleocapsid
oLE	Ovine endometrial luminal epithelium
OPA	Ovine pulmonary adenocarcinoma
ORF	Open reading frame
oST	Uterine stroma cells
oTr	Ovine trophoblast cells
PAGs	Pregnancy associated glycoproteins
PBS	Primer binding site
PI3K	Phosphatidyl inositol-3 kinase
PIC	Preintegration complex
pol	Polymerase
PPT	Polypurine tract
RBD	Receptor binding domain
RSV	Rous sarcoma virus
RT	Reverse transcriptase
RTK	Receptor tyrosine kinases
SA	Splice acceptor
Sag	Superantigen
SD	Splice donor
SFFV	Friend spleen focus-forming virus
sf-Stk	Short form of Stk
SIV	Simian immunodeficiency virus
SP-A	Surfactant protein A
SP-C	Surfactant protein C
SU	Surface domain
TBS-T	Tris buffer saline-Tween

TE	Transposable elements
TM	Transmembrane domain
U3	Unique 3
U5	Unique 5
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VR1-2-3	Variable regions 1-2-3
WB	Western blot
WDSV	Walleye dermal sarcoma virus

Chapter 1

Introduction

Historic perspectives

Retroviruses were originally discovered at the beginning of the 20th century by Vilhelm Ellermann and Oluf Bang (1908) and Peyton Rous (1911) as “ultrafiltrable” agents associated with neoplastic diseases of veterinary interest (chicken leukosis and sarcomas) and much of our current knowledge of cancer development arose from their study as well as the study of DNA tumour viruses (Goff 2001). Although the earliest descriptions of cancer date back to approximately 1600 B.C. in the Edwin Smith papyrus (Smith 2007), it wasn’t until the discovery of oncogenes and tumour suppressor genes, through retroviruses and DNA viruses respectively that key events in cancer causation began to be elucidated. However, it is surprising that what the Egyptian writing says about the disease, “there is no treatment”, is still valid for many forms of cancer.

Several milestones in the history of retrovirology have been made using Rous sarcoma virus (RSV) one of most informative viruses of this family. RSV was one of the first retroviruses to be discovered (Vogt 1997) and studies on its replication led to the “provirus” theory and the anti-dogmatic hypothesis that the virus used RNA as a template for the synthesis of DNA. In addition, work with the Bryan strain led to the hypothesis of the presence of an “endogenous” envelope that allowed the release of infectious virus in the absence of a helper virus (Weiss 2006), introducing the concept of endogenous retroviruses. Moreover, the use of molecular probes derived from a RSV gene that was responsible for transformation (later known as *src*) allowed the identification of cellular oncogenes (Stehelin, Guntaka et al. 1976) that dramatically changed the understanding of carcinogenesis.

Howard Temin was probably one of the investigators that made the most significant contributions to modern retrovirology: I) in 1958, together with Harry Rubin, he developed cell culture focus-forming assay for RSV (Temin and Rubin 1958) allowing for the first time measurement of virus infectivity and transformation, and setting up the experimental platform for seminal studies on the retroviral cycle and viral

oncogenes that began in the 1960s; II) in 1970, simultaneously with David Baltimore, he isolated an RNA-dependent DNA polymerase, the reverse transcriptase (RT), that not only unravelled the conundrums that had puzzled retrovirologists for decades, but also provided an invaluable biotechnological tool. Temin and Baltimore were awarded a Nobel Prize in Physiology or Medicine in 1975.

HIV/AIDS has dominated the last thirty years of retrovirology. The discovery of virus-induced tumours in higher mammals sparked the search for human retroviruses. Over the years, many false “first human retroviruses” were to be discovered and subsequently identified as cell culture contaminants from animal sources. However, the epidemiological patterns of adult T-cell leukaemias in Japan raised suspicion on the presence of a transmissible agent. In 1980, thanks to the development of a long term cell culture system for T lymphocytes, Human T-cell leukaemia virus 1 (HTLV-1) was identified as the first human oncogenic retrovirus (Poiesz, Ruscetti et al. 1980). Coincidentally, the AIDS epidemic arose around the time HTLV-1 was discovered, and the research on this virus provided the foundations for the discovery of HIV and its identification as the causative agent of AIDS (Barre-Sinoussi, Chermann et al. 1983; Gallo 2005). Robert Gallo commented in 2006: “...if AIDS had to come, we were lucky (scientifically speaking) it came at a very good time” (Gallo 2005). He refers to the accumulated knowledge on the retroviral cycle and the modern tools in molecular biology that were developed in the seventies. Although the HIV/AIDS epidemics came at a “good time” HIV has proven to be unique in its transmission, pathogenesis and replication, hampering the way to the discovery of a vaccine and an effective therapy.

Taxonomy

Retroviruses comprise a large family of positive stranded RNA enveloped viruses found in a variety of vertebrates. Their replication strategy presents two unique events that indubitably differentiate them from other virus families: I) upon entry into the target cell the RNA genome is reverse-transcribed (by the virion associated reverse transcriptase, RT) into DNA that is II) then stably integrated into the host cell genome. This peculiar life cycle allows retroviruses to establish persistent infections and possibly vertical transmission when they infect the germ line of the host giving rise to endogenous retroviruses (ERV).

Retroviruses were originally divided into four groups according to the morphology and intracellular position of the nucleocapsid core visible by electron microscopy (Vogt 1997). A-Type viruses were characterized as intracellular structures with a thick shell and a hollow, electron-lucent centre representing an immature capsid. This term is now used to refer to the intracytoplasmic particles formed by some retrotransposons (intracisternal A-type particles, IAPs). B-type viruses assemble in the cytoplasm and possess a round and eccentrically positioned core. C-type viruses on the other hand assemble at the plasma membrane and contain a central, spherical inner core (Bouillant and Becker 1984). D-type viruses display a bar-shaped core upon budding and they assemble in the cytoplasm. Retroviruses are commonly divided into simple or complex according to the array of genes encoded by their genomes. Simple viruses encode only the Gag, Pro, Pol and Env gene products, while complex viruses encode the same genes plus a number of regulatory proteins such as Tat, Rev, Vif etc in HIV-1. The family *Retroviridae* has recently been re-classified by the International Committee on Taxonomy of Viruses into two subfamilies (*orthoretrovirinae* and *spumaretrovirinae*) and seven genera that are summarized in Table 1.

Subfamily	Genera	Genome	Morphology	Examples
Orthoretrovirinae	Alpharetrovirus	Simple	C-type	Avian leukosis virus Rous sarcoma virus
	Betaretrovirus	Simple	B/D-type	Jaagsiekte sheep retrovirus
	Gammaretrovirus	Simple	Type-C	Murine leukaemia viruses
	Deltaretrovirus	Complex	Type-C	Bovine leukaemia virus
	Epsilonretrovirus	Complex	Type-C	Walleye dermal sarcoma virus
	Lentivirus	Complex	Type-C	Human immunodeficiency virus type 1
Spumaretrovirinae	Spumavirus	Complex	N/A	Human foamy virus

Table 1. Retrovirus taxonomy.

Alpharetroviruses have a simple genomic organization, with a C-type morphology and comprise a number of avian exogenous and endogenous viruses including Avian leukosis virus (ALV) and Rous sarcoma virus (RSV). Betaretrovirus have only been isolated from mammals, assemble in the cytoplasm with either a B or a D-type morphology and comprise both endogenous and exogenous viruses. This genus includes Mouse mammary tumour virus (MMTV), Mason-Pfizer monkey virus (M-PMV) and Jaagsiekte sheep retrovirus (JSRV). Gammaretrovirus have so far the largest number of members, infecting more than one vertebrate class, like murine leukaemia viruses (MLV), feline leukaemia viruses (FeLV) and avian reticuloendotheliosis viruses among others. They are simple viruses (although the recent discovery of Rev-like protein in MMTV may challenge this notion (Mertz, Simper et al. 2005)) and endogenous retroviruses are also found in this genus. Deltaretroviruses and lentiviruses share some similarities although they are phylogenetically different. Both are complex viruses, restricted to mammals and they do not comprise oncogene-containing members. Deltaretrovirus include bovine leukaemia virus (BLV) and HTLV. Lentiviruses include both human and animal pathogens such as human immunodeficiency virus type 1 and 2 (HIV-1 or 2), equine infectious anaemia virus (EIAV) and feline immunodeficiency virus (FIV) among others. Epsilonretroviruses include fish and reptile complex viruses with a C-type morphology like Walleye dermal sarcoma virus (WDSV). Finally, spumaviruses include a number of viruses with complex genomes widespread in mammals that have not been associated so far with disease (Goff 2001; Gifford and Tristem 2003).

Genomic organization of retroviruses

The retroviral genome consists of two identical positive stranded RNA molecules held together as a homodimer, through a self complementary region, the dimer linkage structure (DLS) present at the 5' end. Thus, the virions are operationally diploid. Each RNA molecule is between 7 to 13 kb in length and being generated by the cellular machinery they possess features of a cellular mRNA molecule including a "cap" at the 5' end by a methylated GDP attached to the first encoded nucleotide, and a polyadenosine tail at the 3' end (Vogt 1997).

The retroviral genome is organized into the following regions as illustrated in Figure 1.

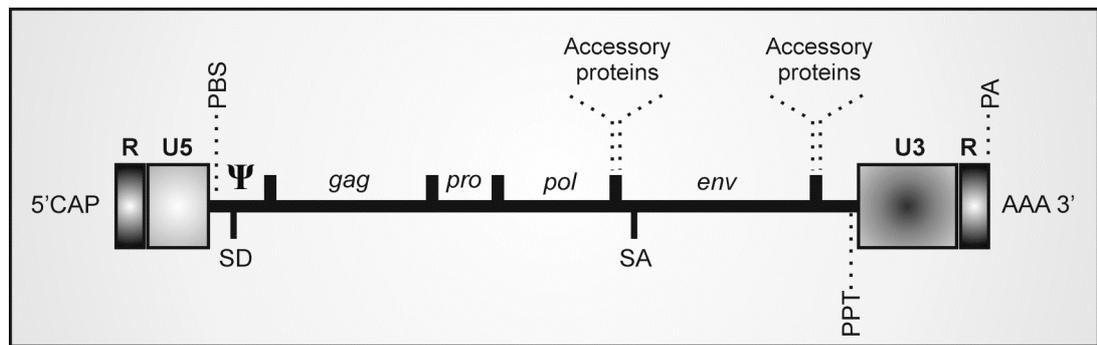


Figure 1. Schematic organization of the retroviral genome.

Adapted from *Retroviruses*, 1997, Cold Spring Harbour Laboratory press.

The repeated region (R) is a small sequence present at both ends of the genome, immediately downstream of the cap at the 5' end and just upstream of the poly(A) tail. It is required for the generation of the two complementary DNA molecules during reverse transcription (see below). Downstream of the 5' R there is the 5' unique region or U5 that contains one of the *att* sites required for integration. U5 is followed by an 18 nucleotide sequence, the primer binding site (pbs) that accommodates a host tRNA required for the initiation of reverse transcription. The packaging (encapsidation) signal (Ψ) is found downstream of the pbs and allows packaging of the viral RNA into the viral particle. This region usually contains the splice donor sequences necessary for the generation of subgenomic mRNA. The genes encoding for the viral proteins occupy the sequences downstream of this region and the majority of the genome. All replication competent retrovirus carry at least four genes: *gag* (for group-specific antigen), *pro* (for protease), *pol* (for polymerase), and *env* (for envelope). The gene *gag* encodes for the major structural protein of retroviruses. Gag is initially synthesized as a polyprotein that is cleaved upon budding by the viral protease (PR) (encoded by *pro*) during a process referred to as maturation. In mature viral particles Gag is proteolytically processed into at least three proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). In addition, in some retroviruses spacer peptides are present between MA and CA and between CA and NC. The gene *pol* encodes for two enzymes: I) the reverse transcriptase (RT) that mediates the conversion of the viral RNA into a double stranded copy of DNA; and II) the integrase (IN) that joins this double stranded DNA copy with the DNA of the host cell. The *env* gene encodes for the envelope glycoprotein (Env) that is inserted in the lipid bilayer of the cell membrane to form the viral envelope. Env mediates adsorption and penetration of the virus into susceptible cells. The envelope protein is cleaved by cellular proteases into the surface (SU) domain, responsible for the interaction with the

cellular receptor, and the transmembrane (TM) domain that fixes the SU to the lipid bilayer (Vogt 1997; Goff 2001).

Simple retroviruses contain only the four genes mentioned above. On the other hand, complex retroviruses express an array of small regulatory proteins that are translated from subgenomic mRNA and regulate viral gene expression and infectivity at different levels. HIV for example expresses a small protein called Rev that mediates the nuclear export of unspliced RNA.

Some oncogenic retroviruses display a particular genomic organization as they have acquired (“transduced”) cellular genes (oncogenes) that give them transforming properties. Consequently these viruses are replication defective and require the presence of a “helper” virus in order to complete their life cycle. An exception to this rule is presented by some strains of RSV where the captured *src* oncogene is located downstream of *env* and consequentially these viruses retain their ability to replicate (Vogt 1997; Goff 2001).

The polypurine tract (ppt) is located at the 3' end of the region encoding the viral genes and it is used as a primer for the start of the synthesis of the plus strand of DNA since it survives the RNase H activity of the RT during reverse transcription. The last two regions of the genomic RNA comprise the unique region 3 (U3) and the 3' copy of R. U3 contains numerous cis-acting elements that are required for viral gene expression as well as the other *att* site needed for integration. R is followed by the poly(A) tail.

Virion structure and proteins

When retroviral virions are released from the infected cells they display an “immature” morphology given by the unprocessed nature of the Gag and Gag-Pol precursors. These immature virions are spherical and characterized by an electron-lucent centre. After proteolytic cleavage, mediated by PR, the CA protein “collapses” inducing the condensation of the core. The mature viral particles are approximately 100 nm in diameter, although the size varies within a viral preparation (Goff 2001).

Gag is formed in all retroviruses by at least three domains placed invariably in the same order: MA (matrix), CA (major capsid protein) and NC (nucleocapid). The core of the viral particle is formed by the CA protein that surrounds the viral RNA which is kept in

a highly condensed state through interactions with the NC protein. A peculiar characteristic of retroviruses is that members of different genera display a different shape of the core, from spherical to cylindrical or conical. The MA protein forms a shell around the core, and the whole structure is enclosed by a lipid bilayer, derived from the host cell, modified by the insertion of the Env glycoprotein. Virions not only contain the structural proteins that give shape to the viral particle but also viral enzymes and some of the regulatory proteins, like Vpr in the case of HIV-1. It is interesting to note that cellular proteins are also packaged into the virions. One of the cellular proteins packaged by HIV-1 is APOBEC3G, a cytidine deaminase that catalyzes the conversion of cytosine to uracil. APOBEC3G acts as a restriction factor at early stages of the retroviral cycle (Goff 2004) and it is a paradox that a virus carries factors impeding the next round of replication! Figure 2 shows a schematic representation of a retroviral particle.

MA harbours the so-called membrane domain (M domain), composed in most retroviruses by a myristyl group and a patch of basic amino acids that target Gag to the cell membrane. MA is also able to interact with Env in a non-specific manner, allowing the incorporation of heterologous Envs, a phenomenon known as pseudotyping (Vogt 1997; Goff 2001).

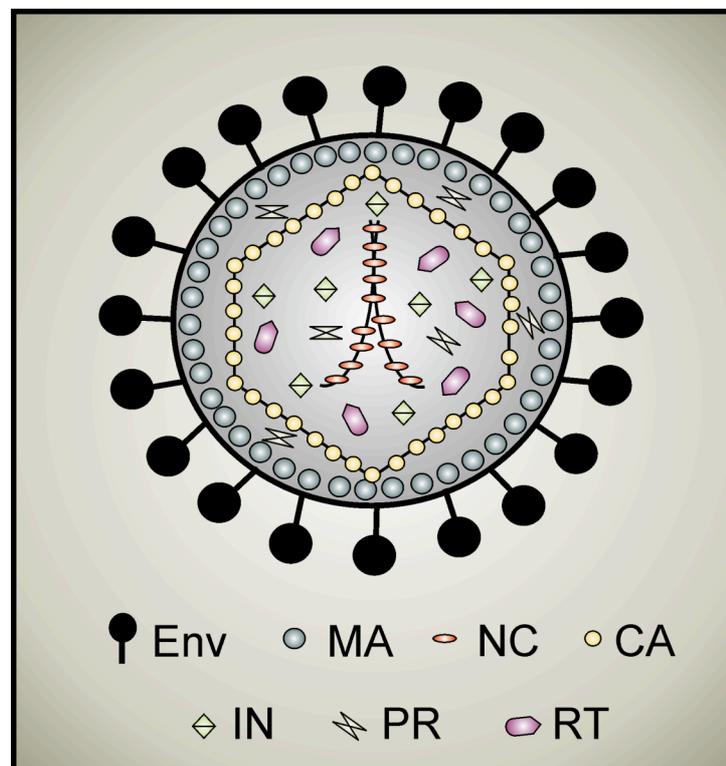


Figure 2. Retroviral particle.

The CA protein is relatively conserved among different genera and contains a highly conserved motif among retroviruses the major homology region (MHR) (spumaretroviruses do not have a recognisable MHR). The function of MHR is not completely understood, however its mutation impairs assembly of some viruses, (e.g. M-PMV) (Strambio-de-Castillia and Hunter 1992).

NC is a small basic protein, cleaved from the carboxy terminus of Gag, which is found in association with the viral RNA to form the core of the virion. The affinity of NC for RNA can be attributed to the presence of basic residues and one or two Cys-His motifs with the sequence CX₂CX₄HX₄C. NC recognizes the packaging signal (Ψ) in the viral RNA thus differentiating them from the cellular mRNAs (Berkowitz, Ohagen et al. 1995). This intrinsic affinity for the viral RNA also promotes the dimerization of the two copies of the viral genome as well as the formation of the duplex between the tRNA and the sequences of the primer binding site. NC also bears the interaction domain (I) that is involved in Gag-Gag associations. Mutations on this domain reduce or block assembly and reduce the incorporation of Gag precursors into the viral particle (Goff 2001).

As previously mentioned, in most retroviruses Gag has also spacer peptides between MA and CA or between CA and NC. Their function is not clear, however it is worth mentioning that the L (late) assembly domains often lie on these peptides. L domains are involved in the late stages of the retroviral cycle since L-domain mutants induce the accumulation of viral particles under the cell membrane. Some viruses contain more than one L domain. They can be located at different positions in Gag and in some cases they are interchangeable (Goff 2001).

The Env glycoprotein is originally translated as a precursor from a sub-genomic singly-spliced mRNA. The Env precursor is subsequently cleaved into SU and TM proteins by cellular proteases while crossing the Golgi complex. The SU protein is the external, hydrophilic portion of the Env complex and it is attached to the TM protein through non-covalent interactions and in some viruses also by disulfide bonds. TM anchors the Env to the host cell membrane that decorates the viral lipid bilayer. The Env protein mediates receptor recognition and viral entry, determining cell tropism (Vogt 1997).

The JSRV Env exhibits unique features among retroviruses as it is oncogenic both *in vitro* (Maeda, Palmarini et al. 2001; Allen, Sherrill et al. 2002) and *in vivo* (Wootton,

Halbert et al. 2005; Caporale, Cousens et al. 2006) while the Env of endogenous retroviruses highly related to JSRV (enJSRVs) are involved in conceptus development and placenta morphogenesis (Dunlap, Palmarini et al. 2006).

Retroviral replication cycle

As illustrated in Figure 3, retroviruses replicate through a complicated cycle that involves the following steps:

- I) Receptor recognition
- II) Entry
- III) Uncoating
- IV) Reverse transcription
- V) Nuclear entry and integration
- VI) Transcription and splicing of viral RNA
- VII) Nuclear export of spliced and unspliced viral RNA
- VIII) Translation of viral proteins
- IX) Assembly of virions
- X) Budding of the newly formed virions
- XI) Virion particle maturation

The first five steps, starting from the attachment of the viral particle to the cell surface to the integration of the viral cDNA, are generally referred to as the early phases of the replication cycle. The late phase begins with the expression of the viral genes and culminates with the release and maturation of the newly formed viral particles.

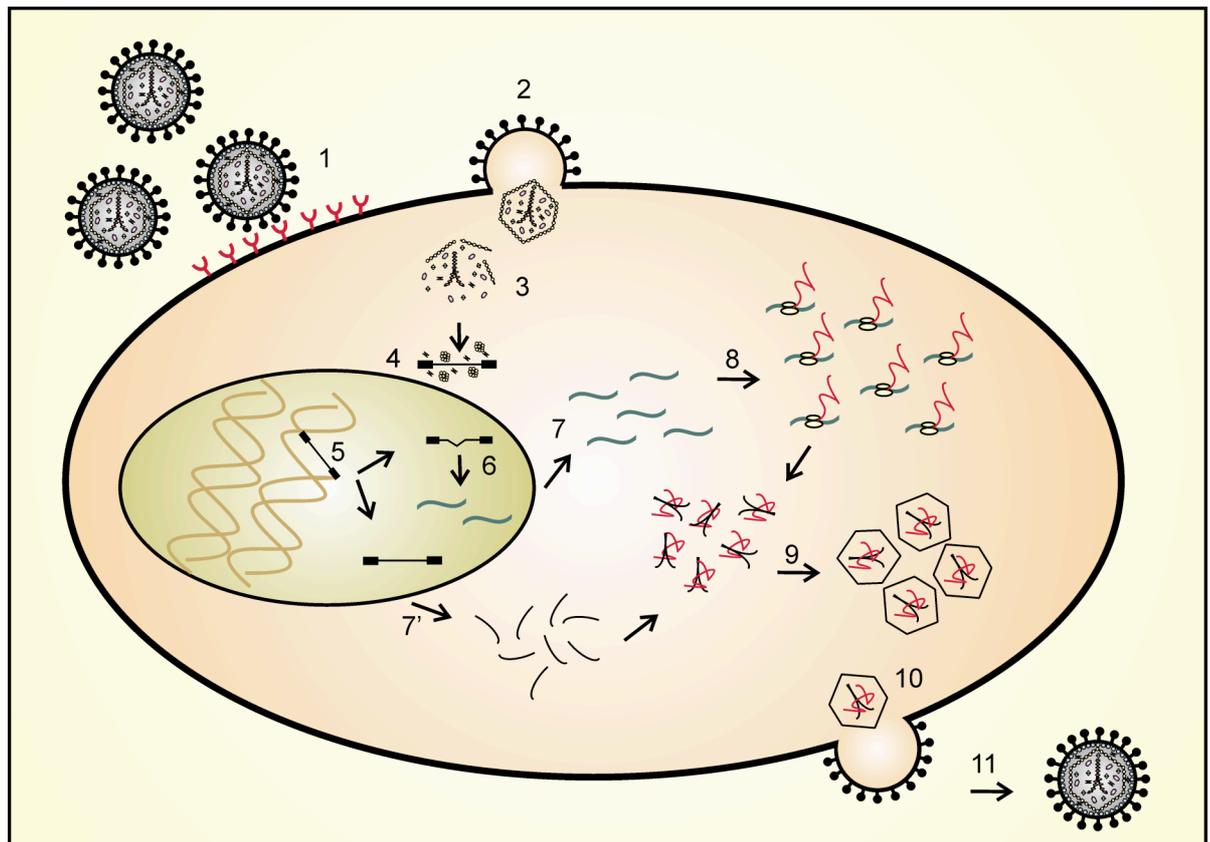


Figure 3. Schematic representation of the retroviral cycle.

1) Recognition of the cellular receptor. 2) Viral entry. 3) Uncoating. 4) Reverse transcription. 5) Integration. 6) Transcription and splicing of viral RNAs. 7) Nuclear export of spliced RNAs. 7') Nuclear export of unspliced RNAs. 8) Translation of viral RNAs. 9) Assembly. 10) Budding. 11) Maturation.

Viral entry

The first event to take place during the retroviral replication cycle is the adsorption of the viral particles to the cell membrane of the target cell. This process is believed to be independent from the cellular receptor but dependent on surface molecules, like heparin sulphate proteoglycan in the case of HIV (Nisole and Saib 2004). HIV-1, HIV-2 and Simian immunodeficiency virus (SIV) also bind to the surface of dendritic cells via the C-type mannose lectins DC-SIGN and DC-SIGNR, which are believed to allow the transport of the virions from the site of infection to the peripheral lymph nodes where they can encounter cells expressing the appropriate receptors (Pohlmann, Soilleux et al. 2001).

Once the virions are attached to the cell membrane, the interaction of the Env glycoproteins with specific receptors allows the penetration of the virus into the cell. This specific Env-receptor interaction determines in part the cell tropism of each virus.

A particular feature of retroviruses is the diverse set of molecules that they use as receptors as shown in Table 2.

Avian leukosis/sarcoma viruses, for example, are divided into ten different viral subgroups (A-J) based in part on their receptor usage. From the genus gammaretrovirus, MLVs are divided into four groups according to the distribution of their receptor in different species and to the viral interference patterns: I) ecotropic viruses can only infect mouse or rat cells through the use of a cationic, basic amino acid transporter (mCAT-1); II) xenotropic viruses are endogenous viruses that only infect non-murine cells; III) amphotropic viruses can infect cells derived from a variety of species using a sodium-dependent phosphate symporter (PiT-2); and IV) polytropic viruses are also endogenous viruses with a wide host range including murine cells. Lentiviruses such as HIV-1, HIV-2 and SIV require not only the presence of CD4, which was the first retroviral receptor to be identified, but also the presence of a second molecule, the co-receptor (often CCR5 or CXCR4) for efficient infection (Maddon, Dalgleish et al. 1986; Hoxie, Haggarty et al. 1988; Sattentau, Clapham et al. 1988; Alkhatib, Combadiere et al. 1996; Dragic, Litwin et al. 1996; Feng, Broder et al. 1996; Hunter 1997; Goff 2001).

The series of events that ultimately allow the entry of the viral particles into the cell are very complex and have been extensively studied in the HIV system. In HIV, the SU and TM proteins (gp120 and gp41 respectively) are arranged as trimers which recognise the cellular receptor (CD4). This interaction leads to a conformational change in both gp120 (SU) and CD4 allowing the recruitment of the co-receptor (CXCR4 or CCR5). A new conformational change takes place inducing the insertion of the hydrophobic signal peptide present in gp41(TM) into the target cell membrane. This is followed by the dissociation of gp120 from gp41 and the formation of a six-helix bundle that ultimately promotes complete fusion and the release of the viral core into the cytoplasm. It appears that the efficiency of this process depends on the amount of Env, CD4 and co-receptor molecules that interact in one particular event (Gallo, Finnegan et al. 2003).

Virus	Receptor	Function
MLV ecotropic	CAT-1	Basic amino acid transporter
MLV amphotropic	Ram-1/GLVR2/PiT-2	Phosphate transporter
MLV 10A1; FeLV; GaLV	GLVR1/PiT-1	Phosphate transporter
MLV xenotropic, polytropic	Rmc1/XPR1	G-coupled receptor
M813 ecotropic	SMIT-1	Na/inositol transporter
FeLV-C	Flvcr	Organic anion transporter
MMTV	TfR1	Transferrin receptor
ASLV-A	tv-a	LDLR-like
ALV-B,D,E	tv-b, -e	Fas receptor like
ALV-C	tv-c	Butyrophilin-like
Perv-A	HuPAR-1, -2	G-coupled receptor
RD114, BaEV, MPMV, HER-W	RDR, RDR2/ASCT1,2	Neutral amino acid transporter
BLV	Blvr	AP-3 delta subunit-like
JSRV	HYAL2	Hyaluronidase receptor
HTLV-1	GLUT-1	Glucose transporter
HIV-1, HIV-2, SIV	CD4 and CCR5, CXR4	T-cell differentiation marker

Table 2. Retrovirus receptors¹.

Reverse transcription

Soon after fusion, uncoating of the viral core and reverse transcription take place. The mechanism and signals leading to the disassembly of the viral core and initiation of reverse transcription are poorly understood, however these two events seem to be coupled. It is believed that exposure of the core to the high deoxyribonucleotide concentration of the cytoplasm and the presence of viral and cellular proteins in the viral particle trigger uncoating and reverse transcription. In HIV-1 the lack of the viral proteins Nef and Vif and the cellular protein Cyclophilin A is associated with reduced

¹ Adapted from Fields of Virology, 2001, Fifth edition.

infectivity, attributed to defects in the early phases of the retroviral cycle (von Schwedler, Song et al. 1993; Aiken and Trono 1995; Braaten, Franke et al. 1996).

The hallmark of the retroviral life cycle is the process of reverse transcription that generates a DNA duplex containing duplicated ends, known as long terminal repeats (LTR), which are not present in the viral RNA template (Figure 4). This complex reaction is mediated by the RT, thanks to its dual enzymatic activity: DNA polymerase and RNase H. The DNA polymerase activity lies at the amino terminal domain of *pol* and allows the incorporation of nucleotides to a growing 3'OH end. RT requires the presence of a DNA or RNA primer as well as a DNA or RNA template which can be used with the same efficiency. The enzyme has low fidelity and generally no proofreading activity, allowing the virus a high mutagenic rate and the chance to evade the immune system and develop drug resistance. The endonuclease activity of the RNase H of the RT is present in the carboxyterminal domain and permits the release of 3'OH and 5'PO₄ only when the RNA is in a duplex form, mainly RNA:DNA. This activity allows the generation of primers that are used in the initiation of the DNA synthesis (Goff 2001).

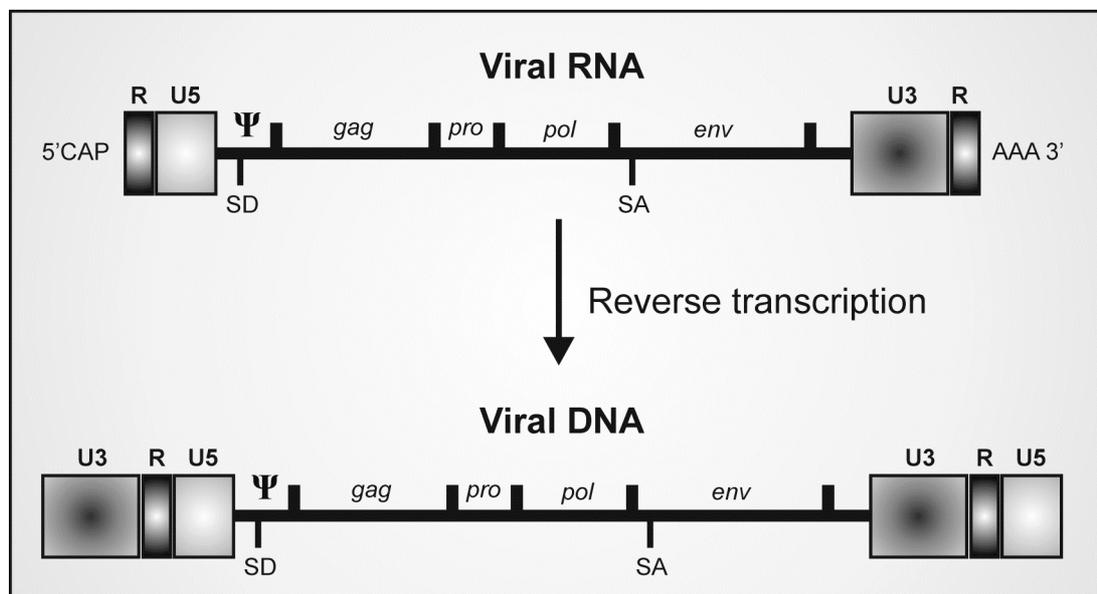


Figure 4. Comparison of viral RNA and DNA after reverse transcription and the duplication of the U5 and U3 regions.

Adapted from *Retroviruses*, 1997, Cold Spring Harbour Laboratory press.

Reverse transcription starts with the synthesis of the minus-strand strong-stop DNA (-sssDNA) which is initiated near the 5' end of the viral RNA using as a primer a tRNA annealed in the PBS (Figure 5). The -sssDNA is relatively short and contains U5 and R sequences. The RNA that forms part of the newly formed RNA:DNA hybrid is degraded by the RNase H activity of the RT and the first "jump" occurs where the -sssDNA anneals in the R region of the 3' end of the RNA molecule, a process that seems to be facilitated by NC. Once in the 3' end, the synthesis of the minus strand continues until the PBS, while the RNA template is degraded by an incomplete RNase H digestion that leaves behind the PPT which serves as a primer for the synthesis of the plus strand DNA, using the minus strand DNA as a template. Polymerization extends until it reaches the tRNA generating the plus-strand strong-stop DNA (+sssDNA). At this point the tRNA is removed by the RNase H activity of the RT exposing complementary sequences in the 3' end of +sssDNA and allowing the second "jump" where these sequences anneal in the PBS present in the 3' end of the minus strand DNA. This process creates a circular intermediate that permits the complete elongation of both the minus and the plus strands (Telesnitsky and Goff 1997; Goff 2001).

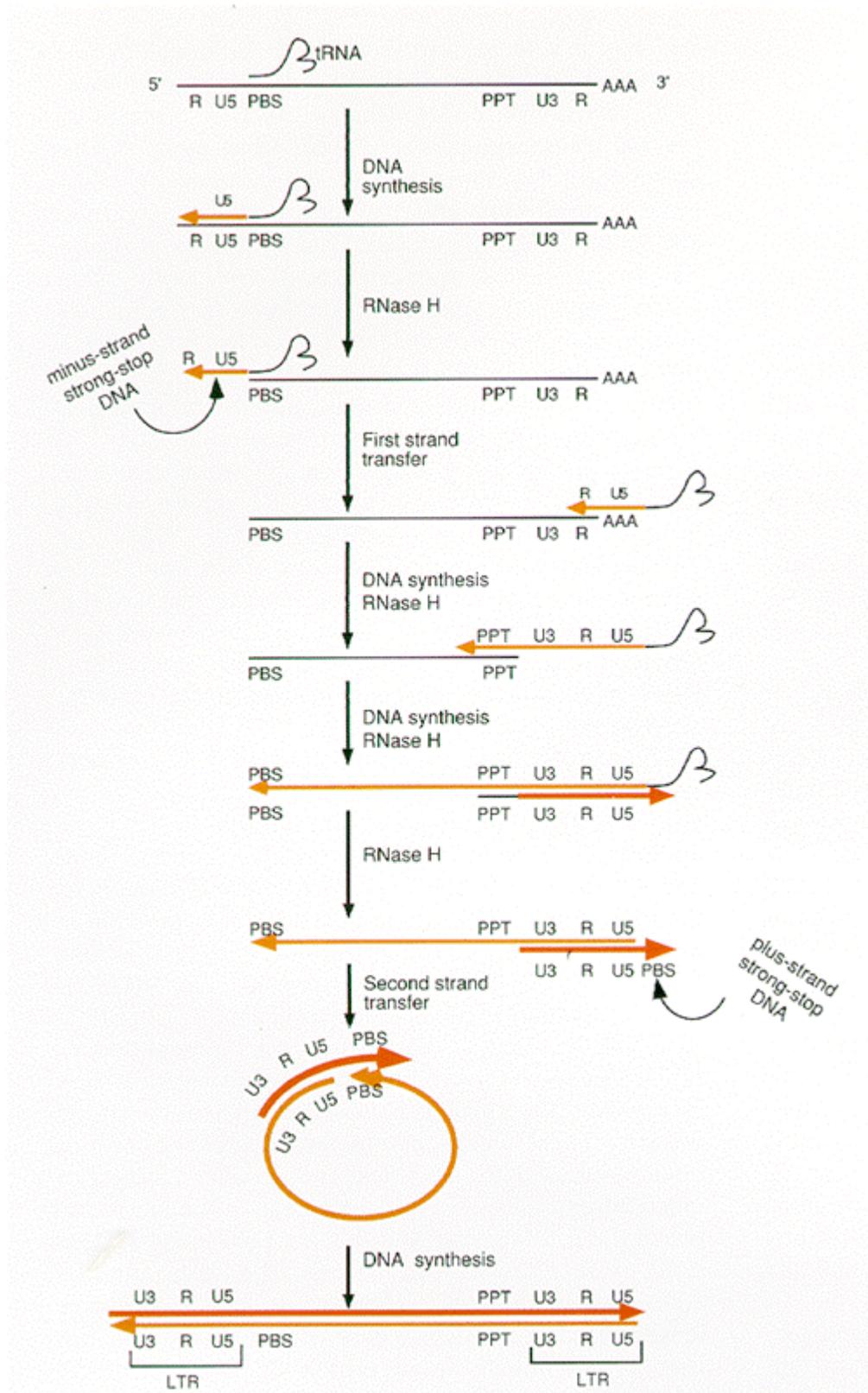


Figure 5. Reverse transcription

The black line represents the viral RNA; the red light line the minus-strand DNA and the bold red line, the plus-strand DNA. From *Retroviruses* (Cold Spring Harbour Laboratory Press, with permission).

Integration

The newly synthesized retroviral DNA genome has to gain access to the nucleus where it will become permanently integrated into the host genome. This is a crucial step that allows retroviruses to persist in the infected cell and be transmitted to daughter cells in a classical Mendelian fashion and perpetuating indefinitely viral production. The process of integration also gives retroviruses the chance to enter the germ cells and become a permanent element in the host genome (see endogenous retroviruses below). Although integration into the host genome provides retroviruses with certain advantages, the road to chromatin is complex and hazardous, that is why the retroviral DNA does not travel alone but as part of a big complex: the preintegration complex (PIC). The components of the PIC are not completely known, however in simple retroviruses it seems to contain CA, RT, IN and possibly other viral proteins while the PIC of complex retroviruses contains MA, NC, Vpr, RT and IN. These variations of the PIC composition within different retroviruses might reflect differences in the mechanisms used to gain access to the nucleus. Most retroviruses can infect only dividing cells during mitosis. In contrast, lentiviruses can infect non-dividing cells thanks to their ability to cross the nuclear envelope, through the nuclear pores. Although the mechanisms facilitating infection of non dividing cells are not yet well characterized there is evidence supporting the role of HIV-1 CA and some tRNAs species (Goff 2001; Zaitseva, Myers et al. 2006; Yamashita, Perez et al. 2007).

Once in the nucleus, the PIC has to find a suitable site to integrate into the host DNA and, although it usually occurs in a non sequence-specific manner, it is believed that the structure of the surrounding chromatin and interactions with host proteins influences the choice of the target site. The integration reaction is mediated by the viral IN into two steps (Figure 6). Firstly, the two terminal nucleotides at both 3' ends of the viral DNA are removed just downstream of a highly conserved CA sequence, leaving two protruding 5' ends. Secondly, the 3'OH ends of the viral DNA attack the phosphodiester bonds of the target host DNA and produce a new bond between the extremes of the viral and host DNA. This reaction results in the formation of short gaps in the host DNA and unpaired bases in the viral DNA that are repaired (filled in) presumably by host enzymes, leading to the duplication of the target sequence that now flanks the integrated provirus (Goff 2001).

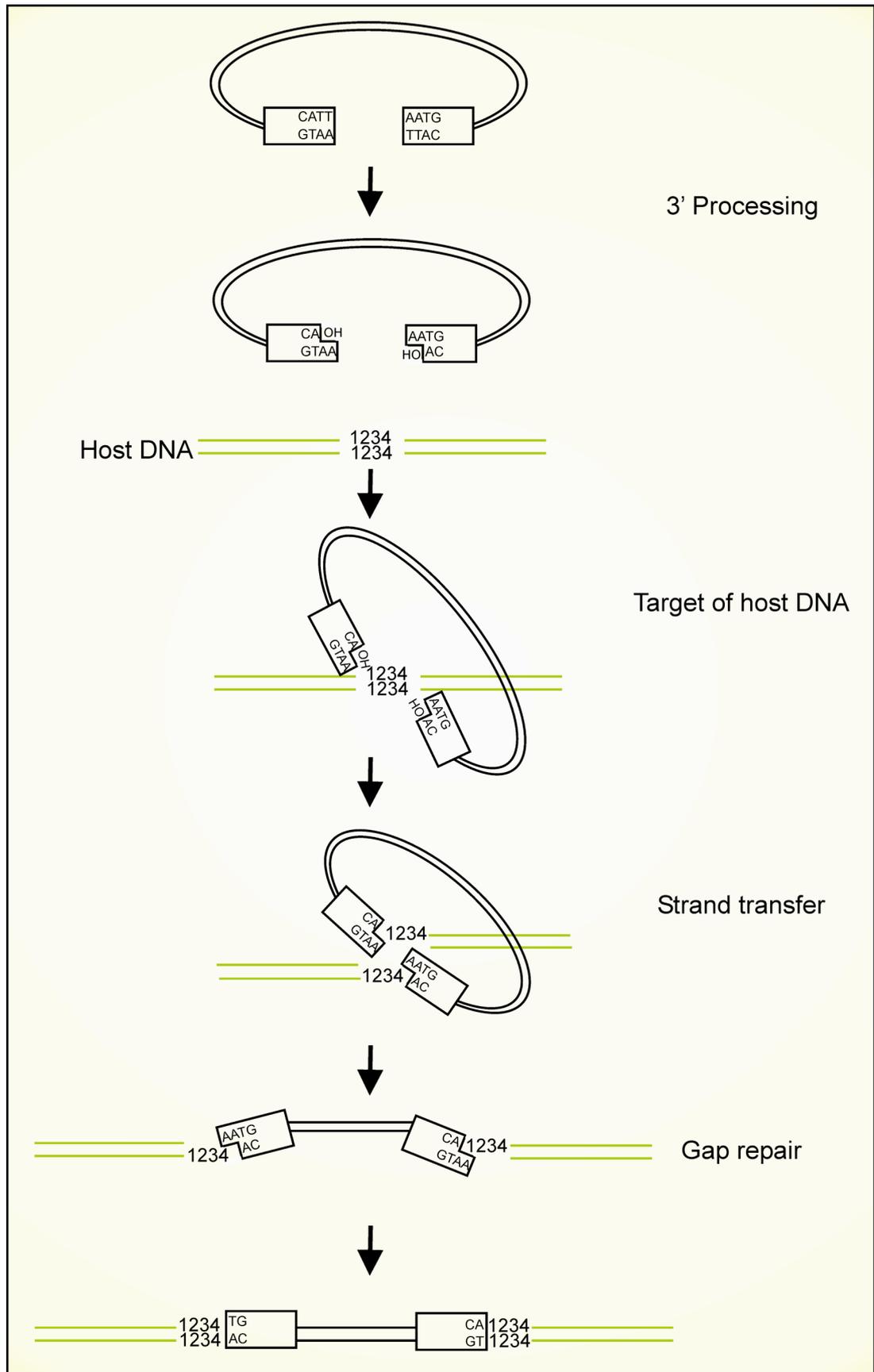


Figure 6. Integration

Adapted from Fields of Virology, (Goff 2001).

Expression of viral RNA

The post-integration phase of the retroviral replication cycle begins with the expression of the viral RNA. This and other late phases of the viral cycle utilise mostly cellular proteins that are part of the normal transcription and translation machinery, as opposed to the early phase, where the most relevant reactions are mediated by viral proteins. The objective of the virus is to produce all the elements required for the formation of newly infectious viral particles, including proteins and viral genomes. The proviral LTRs contain the promoter that is recognized by the cellular RNA polymerase II complexes to initiate transcription in the U3-R border. The promoter in the 5' LTR is much more efficiently used than the one in the 3' LTR. The U3 contains enhancer binding motifs that regulate viral expression and is one of the main determinants of viral tropism. Ultimately the cell type, its physiological state and the integration site determine the levels of activity of the retroviral promoter (Rabson and Graves 1997; Goff 2001).

The retroviral primary transcript displays features of a cellular mRNA, capped in the 5' end and polyadenylated in the 3' end, and follows one of two pathways: I) it is spliced to yield subgenomic mRNA for the synthesis of Env, in simple retroviruses, or Env and accessory proteins in the case of complex retroviruses; or II) it is exported to the cytoplasm, unspliced, where it is used for the translation of Gag or Gag-Pol polyproteins or as genomic RNA for encapsidation. Some retroviruses like MLV use two distinct populations of genomic RNAs for protein translation and for encapsidation while complex retroviruses use only one, interchangeably (Balvay, Lopez Lastra et al. 2007). Simple and complex retroviruses use different mechanisms to export unspliced RNA into the cytoplasm. While the RNAs of simple retroviruses are exported via the action of cis-acting elements, the constitutive transport element (CTE); complex retroviruses export their RNAs through interactions between accessory proteins, responsive elements and cellular factors (Bray, Prasad et al. 1994; Ernst, Bray et al. 1997; Goff 2001).

Gag, Pro and Pol are expressed as polyproteins from the unspliced RNA in a complicated fashion that varies between different viruses but that ultimately ensures the presence of the correct amount of each protein in the viral particle. Gag is the first protein to be synthesized by the ribosomes until they reach a stop codon. From here each virus will use a different strategy to synthesize Gag-Pro-Pol polyproteins considering that sometimes their open reading frames (ORF) are separated from *gag* by

a stop codon or sometimes they are present as a separate ORF. In gammaretroviruses and epsilonretroviruses, Gag and Pro-Pol ORF are all in the same reading frame but separated by a stop codon and they are translated by a mechanism referred to as translational readthrough. When ribosomes reach the stop codon they do not terminate translation but introduce a normal amino acid (usually glutamine) in its position and translation continues through the reading frame of *pro-pol* (Yoshinaka, Katoh et al. 1985). Another mechanism known as translational frameshifting is used when the ORF of *pro-pol* or *pol* are in different reading frames. In this case when ribosomes encounter the stop codon they slip back one nucleotide and proceed with translation with the new ORF. This last mechanism is dependent on the presence of a heptanucleotide slippery sequence upstream of an RNA pseudoknot (Balvay, Lopez Lastra et al. 2007). In betaretroviruses and deltaretroviruses two frameshifting events take place since both *pro* and *pol* are in different reading frames. Spumaviruses are unique in that they synthesize Pro-Pol through a subgenomic mRNA (Goff 2001).

The *env* gene is expressed through a spliced mRNA where the majority of *gag*, *pro* and *pol* are removed. Translation starts in the cytoplasm and soon after is relocated to the rough endoplasmic reticulum (ER) through signals provided by the first hydrophobic amino acids of the nascent Env, the signal peptide. In the ER the signal peptide is removed and the protein is glycosylated. The cotranslational insertion of the Env inside the ER reaches a stop by signals provided by a hydrophobic segment near the end of the protein which anchors it to the membrane. Before accessing to the Golgi apparatus, Env is folded and oligomerized. In the Golgi the sugar residues get further modified and the Env is cleaved by furin proteases into the SU and TM subunits. From here it travels to the cell membrane likely by using cellular vesicular transport systems (Goff 2001).

Viral assembly and budding

Once all the components required for the formation of the viral particle are synthesized they have to recognise each other at particular cellular locations, assemble and bud from the cell membrane. These processes are mainly orchestrated by uncleaved Gag precursors. For retroviruses with a type C morphology assembly takes place at the cell membrane, where Gag precursors are targeted via the M domains and aggregate by side to side contacts through the I domains (Jouvenet, Neil et al. 2006). As the aggregate grows bigger, the cell membrane bends until the structure takes the form of a sphere attached to the cell membrane only by a slim stalk. Finally the virion pinches off and the

cell membrane is sealed. As previously mentioned the L domains play a critical role in the late phases of assembly and it is believed that they mediate virus-cell separation, since mutant Gag proteins form spherical structures that remain tethered to the cell surface by a membrane stalk (Freed 2002). For some betaretroviruses, assembly occurs in the cytoplasm. In particular for M-PMV assembly is believed to occur in the pericentriolar region, and the immature capsids then travel to the cell membrane by uncertain mechanisms, probably assisted by the Env protein (Sfakianos, LaCasse et al. 2003). Interestingly, one amino acid change in M-PMV Gag (MA) changes its site of assembly from the cytoplasm to the cell membrane, indicating that the two mechanisms are not extremely different (Rhee and Hunter 1990; Goff 2001).

Maturation

The final step of the retroviral cycle is the processing (cleavage) of the viral proteins by the PR. The viral protease is an aspartyl protease that functions only as a dimer. Viral maturation is a controlled process that takes place during and after budding but only when the protein precursors are assembled. The structural changes that lead to the change in morphology associated with maturation are due to the cleavage of Gag and are required for infectivity. In murine leukaemia viruses a short peptide (the R peptide) is removed from the carboxyterminal of TM upon maturation, which is required to activate the fusogenic activity of the Env (Goff 2001; Bukrinskaya 2004).

Retroviral oncogenesis

As previously mentioned, much of the current knowledge on the molecular mechanisms leading to cancer development arose from studies of oncogenic retroviruses. The discovery that retroviruses carry genes with transforming properties (v-oncogenes) derived from cellular genes (c-oncogenes or proto-oncogenes) lead to the conclusion that cancer was due to genetic alterations. This concept was supported by the observation that ALV induced tumours in a high percentage of chickens although it lacked an oncogene. It was then found that these tumours were caused by the integration of the virus adjacent to the *c-myc* gene inducing its over expression (Hayward, Neel et al. 1981). From here on the study of oncogenes and their role in cancer development exploded and it became apparent that cancer is a genetic disease resulting from the successive accumulation of genomic alterations. Thus cancer development is a multistep process that could be explained by central precepts of Darwinian medicine as follows:

cancer is the result of somatic mutations selected during tumour evolution and that susceptibility to cancer is the result of design limitations and flaws of our evolutionary legacies, that persist as long as they are not detrimental for reproductive fitness (Greaves 2007).

Hallmarks of cancer

We cannot approach the subject of retroviral oncogenesis without understanding first the basic mechanisms leading to cell transformation. It is now accepted that cancer is the manifestation of six fundamental alterations in cell physiology that lead to malignant transformation (Hanahan and Weinberg 2000).

Growth independence

Insensitivity to anti-growth signals

Evasion of apoptosis

Immortalization

Sustained angiogenesis

Tissue invasion and metastasis

Genome instability provides the enabling context for these alterations to develop.

Growth independence

To proliferate, normal cells require mitogenic growth signals provided by the surrounding microenvironment. This dependence on other cells and the extracellular matrix creates a homeostatic balance where the behaviour of each cell type within a tissue is highly controlled (Lodish, Berk et al. 2003). Tumour cells have a reduced dependence on the surrounding environment thanks to three different strategies. Many cancer cells create positive feedback loops by autocrine stimulation. In other words, cancer cells produce their own growth factors and activate themselves. Another strategy is to modify cell surface receptors that transduce signals from growth factors to the interior of the cell by switching the type of extracellular matrix receptor displayed or by receptor overexpression. Massive receptor overexpression can lead to ligand independent activation, which can also be achieved by expression of a modified receptor. Growth independence can also be acquired by altering the intracellular

pathways that transduce the signals provided by growth factors (Hanahan and Weinberg 2000).

Insensitivity to anti-growth signals

Cell proliferation is ultimately the result of the balance between mitogenic and anti-growth signals, thus cancer cells not only have to take advantage of signals that promote growth but also escape antiproliferative mechanisms. Antigrowth signals can function in two ways: I) they can promote the entry of cells into a reversible quiescent state (G_0); or II) they can induce cells to enter into a postmitotic state, frequently associated with terminal differentiation, with the loss of their replication capability. In the majority of cases, inhibitory signals are conducted by the retinoblastoma protein and its relatives, p107 and p130. If hypophosphorylated retinoblastoma protein sequesters transcription factors controlling the expression of genes which promote the transit from G1 to S phase of the cell cycle if the conditions dictate so. In cancer cells this circuit can be disturbed in several different ways (Hanahan and Weinberg 2000).

Evasion of apoptosis

Programmed cell death or apoptosis and cell proliferation are the major mechanisms used by tissues to regulate the number of cells of which they are composed. In cancer cells the balance is shifted towards proliferation, and resistance to apoptosis is a major contributor of this imbalance. The apoptotic machinery is triggered by the action of “sensors”, that detect abnormalities in the extracellular and intracellular environment, and “effectors” that ultimately commit the cell to death. The release of cytochrome C by the mitochondria activates a group of intracellular proteases, the caspases, which destroy organelles, cellular structures and the genome. The pro-apoptotic members of the Bcl-2 family of proteins (Bax, Bak, Bid, Bim) can activate the release of cytochrome C and many cancer cells evade apoptosis by the loss of the *p53* tumour suppressor gene, which upregulates the expression of Bax under circumstances of DNA damage (Hanahan and Weinberg 2000).

Immortalization

Cancer cells not only have to overcome the barriers mentioned above but also the fact that normal cells have an intrinsic program that restricts their multiplication. Cells in culture can divide a certain number of times after which they stop and undergo

senescence. It is now known that this process is controlled by the size of the ends of the chromosomes, the telomeres. The inability of the DNA polymerase to completely replicate the 3' ends of the chromosomes after each replication cycle generates a shortening of the telomeres. When this erosion can no longer protect the ends of the chromosomes, the ends fuse causing genomic abnormalities that lead to cell death. So far there are two known mechanisms evolved by cancer cells to escape death by telomere shortening: I) overexpression of the telomerase enzyme, that adds hexanucleotide repeats to the telomeric DNA; or II) regeneration of telomeres through recombination-based interchromosomal exchange of sequence information, a process also known as "alternative lengthening of telomeres" (Hanahan and Weinberg 2000).

Sustained angiogenesis

The ability of a tissue to develop depends on the oxygen and nutrients provided by the vasculature. Therefore for a tumour to grow, angiogenesis has to be turned on (Carmeliet 2003). The formation of new blood vessels, as any other cellular mechanism, is regulated by the action of positive and negative signals. It seems that tumour cells induce angiogenesis in mid-stage lesions, via an "angiogenic switch", before the onset of macroscopic lesions. The vascular endothelial growth factor (VEGF) plays a critical role in the angiogenic switch and it has been shown to be overexpressed in many tumours. Other tumours choose to downregulate the expression of inhibitors, but it is now evident that different types of tumour cells choose different mechanisms to turn on the angiogenic switch (Hanahan and Weinberg 2000).

Tissue invasion and metastasis

Ninety percent of human cancer deaths are due to metastasis, which is the process by which tumour cells invade the surrounding tissues, access the body's circulation system and establish secondary areas of growth (Lodish, Berk et al. 2003). Apart from the previously mentioned changes required for uncontrolled cell growth, additional modifications are necessary for the acquisition of an invasive and metastatic phenotype. These modifications include changes in: I) molecules that mediate cell-cell adhesion, like members of the immunoglobulin and calcium dependent cadherin families which are required for the transmission of antigrowth signals; II) molecules that connect cells to the extracellular matrix, like integrins, to be able to face the changes of the new microenvironments; III) as well as changes in extracellular matrix degrading proteases (Hanahan and Weinberg 2000).

Transforming retroviruses

Oncogenic retroviruses have been classically divided in two groups according to the rapidity with which they induce disease: acute and slow transforming viruses (Rosenberg and Jolicoeur 1997). Acute transforming retroviruses induce tumours very rapidly after infection and in a high percentage of the infected animals and can transform cells *in vitro*. They are also referred to as “transducing” retroviruses since they mediate transformation through the expression of viral oncogenes which derive from cellular genes. This group of viruses is replication defective since they lack part of their coding sequences in exchange for the cellular oncogene. However, since they retain all their cis-acting elements, they can replicate with the assistance of a replication competent (helper) virus. An exception is RSV that encodes the *v-src* oncogene but retains the fully coding regions for Gag, Pol and Env and thus is replication competent (Muriaux and Rein 2003). Some examples of acute transforming retroviruses are: Abelson murine leukaemia virus, encoding the non-receptor tyrosine kinase *abl* as a gag-Abl fusion protein; the MC29, CMII, OK10 and MH2 avian retroviruses encoding *myc* and the murine sarcoma virus 3611 which encodes the *raf* gene (Fung, Fadly et al. 1981; Rapp, Reynolds et al. 1983; Rosenberg and Jolicoeur 1997).

Transducing retroviruses are produced by a complex mechanism that involves recombination events at the DNA and RNA levels, thus their occurrence is a very rare event even in animals with high levels of viremia. Although the genomes of transducing retroviruses are very distinct they retain the cis-acting elements required for replication: LTRs, PBS, ppt and packaging signals. Many of the regions encoding for the structural genes are lost and are occupied by the host sequence to be transduced, that can be expressed as a separate unit or fused to Gag, Pol or Env. These rearrangements can lead to drastic changes in the expression patterns of the viral oncogene compared to the cellular counterpart. In addition, often the fusion protein between a retroviral gene and an oncogene can acquire subsequent mutations and display different localization, stability and activity that render it a fully transforming protein (Goff 2001). The currently accepted model to explain the formation of a transducing virus comprises the following steps (Muriaux and Rein 2003):

- 1) Firstly, a replication competent virus integrates upstream of a cellular gene to be incorporated.

- 2) Transcription of the viral RNA is initiated from the 5'LTR and generates a long transcript containing the viral sequences fused to the downstream cellular gene by "readthrough" transcription ignoring the normal termination signals in the 3'LTR. This process is favoured by lesions or deletions in the 3'LTR.
- 3) The chimeric RNA is then spliced and packaged into a viral particle along with a full molecule of genomic RNA.
- 4) The newly formed viral particles infect other cells and during reverse transcription non homologous recombination takes place between the chimeric RNA and the RNA of the helper virus through a template switch performed by the RT. This process generates a provirus containing host sequences flanked by viral termini.

This whole mechanism is supported by the ability of retroviruses to package chimeric and long RNAs as well as cellular mRNAs and allows recombination events between them. At the moment the possibility that transducing retroviruses arise through recombination at the DNA level, albeit at a lower rate cannot be excluded (Muriaux and Rein 2003).

Slow transforming retroviruses are replication competent, since they harbour the fully coding sequences required for replication; they produce tumours with longer incubation periods and do not cause cell transformation *in vitro*. They mediate transformation by proviral insertional mutagenesis that leads to the activation of proto-oncogenes (Nervis 2001). Examples of slow transforming retroviruses are the Rous-associated viruses types 1 and 2, that integrate within the *myc* gene and MMTV whose integration promotes the rearrangement of the Notch family of proteins and the p48 component of the eukaryotic translation initiation factor-3 (Callahan and Smith 2000).

Each retroviral integration event could be considered a somatic mutation, most of the time being harmless, not causing a significant disruption of gene expression or affecting only one allele creating a recessive mutation. However, occasionally a dominant mutation arises that leads to a disruption of the normal mechanisms controlling cell proliferation and induces the clonal expansion of the cell that ultimately will form a tumour. Several mechanisms have been identified that induce proto-oncogene activation by insertional mutagenesis as illustrated in Figure 7 (Goff 2001):

- 1) **Promoter insertion:** in this case the provirus integrates upstream or within the gene in the same transcriptional orientation and the promoter and enhancer elements in one of the LTRs can induce an increased level of gene expression. Transcription can be initiated in either LTR but it is usually favoured for the 3'LTR that reads into the gene. This results in the creation of a transcript with R-U5 sequences in the 5' end that can be of the same size, longer or truncated with regard to the normal mRNA and expressed at abnormal levels. This is the mechanism by which *c-myc* is activated in 80 % of ALV-induced tumours (Fung, Fadly et al. 1981; Uren, Kool et al. 2005).
- 2) **Enhancer insertion:** the provirus inserts upstream of the cellular gene in the opposite transcriptional orientation or downstream of it in the same or opposite orientation. This allows the positioning of the enhancers present in U3 in a suitable location to activate the promoters of cellular genes leading to the altered expression of normal transcripts. *c-myc* is also activated by this mechanism in MLV-induced tumours (Rosenberg and Jolicoeur 1997) (Uren, Kool et al. 2005).
- 3) **Read-through transcription:** gene expression can be driven by the 5' or 3' LTR of a provirus if it is integrated within a gene in the sense orientation. Provirus transcription initiates in the 5' LTR and continues into the gene usually due to defects in the 3' LTR allowing read through transcription resulting in the formation of a chimeric transcript that is then spliced in a complex fashion. In ALV-induced erythroblastosis a provirus is integrated within the *erbB* gene leading to the expression of a truncated form of the gene (Nilsen, Maroney et al. 1985).
- 4) **Posttranscriptional stimulation of expression:** proviral insertions downstream of the coding regions can remove regulatory or destabilizing signals or provide a polyadenylation signal that increases the formation of stable transcripts. This mechanism is used by MLV to remove a portion of the 3' untranslated region (UTR) of the *Pim-1* gene that reduces mRNA stability (Selten, Cuypers et al. 1985; Uren, Kool et al. 2005).

Insertional mutagenesis can also lead to gene inactivation by inducing premature transcript termination or aberrant splicing events which can result in the production of a truncated, unstable or inactive protein. These mutations are usually silent since the other

allele is still able to produce a functional protein. A loss of function arises when the second allele is mutated leading to loss of tumour suppressor gene activity and consequent tumour induction (Goff 2001).

Although the classification of acute and slow transforming retroviruses is widely used it is incomplete since some oncogenic retroviruses do not fit in either of these two categories. Deltaretroviruses, such as Human T-cell leukaemia virus type 1 (HTLV-1), Simian T-cell leukaemia virus type 1 (STLV-1) and Bovine leukaemia virus (BLV), do not harbour cellular derived oncogenes, instead transformation is initiated by the viral accessory protein, Tax, which functions as a transcriptional activator. HTLV-1 Tax protein induces transformation by the activation of cyclin-dependent kinases, NfκB and the Akt pathways, silencing of p53 and spindle assembly checkpoints as well as the creation of chromosomal instability, centrosome amplification and abrogation of DNA repair systems. Tax is not required to maintain transformation, which seems to depend on the function of HBZ (HTLV-1 basic leucine zipper factor) encoded by the minus strand of the provirus (Matsuoka and Jeang 2007). The interplay between Tax, HBZ, cellular proteins and possibly other viral proteins that ultimately leads to leukaemia development is poorly understood at the moment.

JSRV is a replication competent retrovirus lacking cellular derived oncogenes (Fan, Palmarini et al. 2003). In natural conditions JSRV induces disease with a long incubation period, usually appearing in animals aged 1 to 4 years (De las Heras, Gonzalez et al. 2003). These are characteristics of a slow transforming retrovirus, however when newborn lambs are experimentally inoculated with lung fluid from infected animals clinical signs appear within weeks (De las Heras, Gonzalez et al. 2003), consistent with an acute transforming virus. It is now known that JSRV mediates transformation through its Env glycoprotein (Fan, Palmarini et al. 2003), a unique feature among retroviruses, which will be described in detail in the following sections.

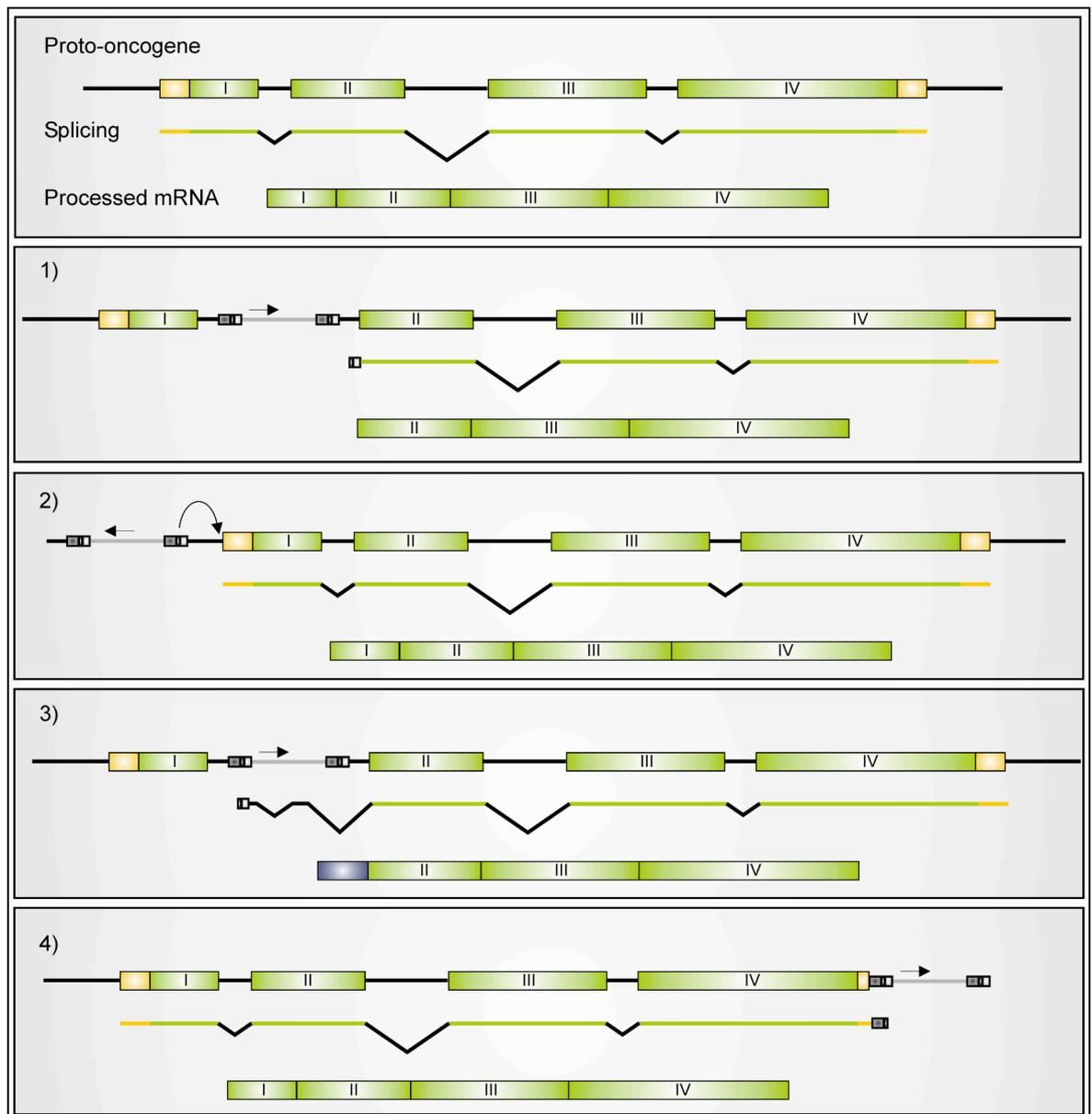


Figure 7. Mechanisms of proto-oncogene activation by proviral insertion.

Top panel shows a hypothetical proto-oncogene formed by four exons. Green boxes exons and protein products after splicing. Black lines introns. Yellow boxes untranslated regions. Blue box sequences in the protein encoded by the virus. Linear arrows indicate transcriptional orientation of the provirus. 1) Promoter insertion. 2) Activation by enhancer sequences. 3) Read-through transcription. 4) Posttranscriptional stimulation of expression. Adapted from *Retroviruses*, 1997, Cold Spring Harbour Laboratory press.

Endogenous retroviruses

During the retroviral cycle the viral genetic information (provirus) is integrated into the chromosomal DNA of the infected cell, which is then passed on to the descendants of that cell after division as any other cellular gene. Occasionally, some retroviruses infect the germ cells of the host, resulting in the transmission of the provirus to the somatic cells of the descendants of the individual in whom infection originally took place. If the

provirus (in this case known as “endogenous” retrovirus, ERV) does not exert any pathogenic effect it is then transmitted from generation to generation and becomes fixed in the host species (Coffin 2004). Since changes in the genomic DNA of a species take place very slowly, the presence of ERVs gives us insights into the ecology and evolution of ancient viruses (Coffin 2004). ERVs have heavily colonized the genome of all animal species and account for approximately 8% of the human genome (Lander, Linton et al. 2001).

The frequency of an ERV in a population may increase over time as a result of genetic drift (the statistical effect that chance has on the survival of alleles), hitchhiking (the process by which an evolutionary neutral allele or mutation is spread through the gene pool by means of being linked to a beneficial mutation) or positive selection if the provirus was to provide a beneficial effect to the host (Arnaud, Caporale et al. 2007). Retrotransposition or reinfection of the germ line can generate further insertions augmenting the number of a particular lineage in the genome (Gifford and Tristem 2003). ERVs are destined to extinction if their expression brings deleterious consequences for the host. Their persistence in the host genome is the result of a fine balance reached throughout evolution which usually renders them replication defective due to the accumulation of mutations, deletions, rearrangements and methylation (Boeke and Stoye 1997). However, replication defective ERVs can be rescued if complemented in *trans* or recombined with another ERV within the same cell or with an exogenous retrovirus, should those retain the essential regulatory sequences, as is the case for the endogenous gammaretrovirus BaEV (baboon endogenous retrovirus) that bears a betaretrovirus *env* gene (Gifford and Tristem 2003).

ERVs fall into the category of transposable elements (TE). TE are stretches of DNA that cut themselves out of the genome and splice themselves into another region, contributing to genetic diversity in a variety of organisms (Biemont and Vieira 2006). Forty five percent of the human genome is composed of transposable elements (TE) (Lander, Linton et al. 2001). As shown in Figure 8 they can be divided into DNA transposons (Class II) which act via a DNA intermediate and retrotransposons (Class I) that use a RNA intermediate. DNA transposons comprise 2.8% of the TEs of the human genome, while the remaining 42.2% are retrotransposons. Retrotransposons can be further divided into non LTR elements (33.9%), comprising the long and short interspersed elements (LINEs and SINEs respectively), and LTR elements (8.3%), comprising ERV.

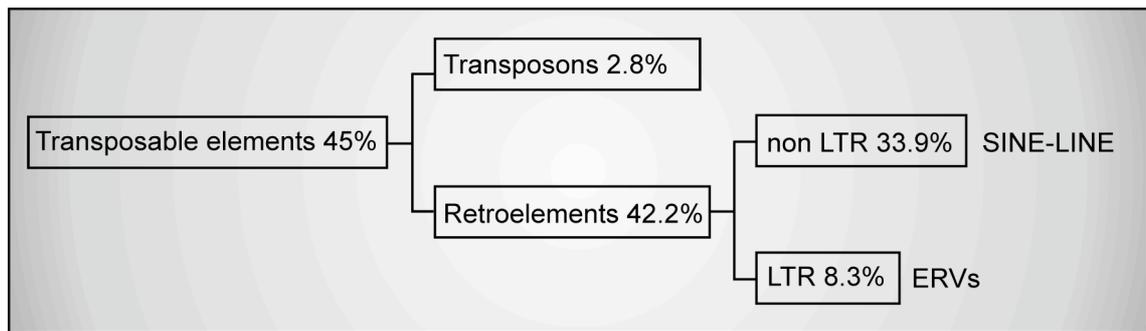


Figure 8. Classification of transposable elements.

ERVs are restricted to vertebrates, indeed they have been found in every vertebrate class analyzed with the exception of the most basal vertebrates (Herniou, Martin et al. 1998). LTR retrotransposons are also found in plants, fungi and insects. ERVs belong to most of the retroviral genera, although there is no consensus on how they should be incorporated into the current retroviral classification. Some ERVs are highly related to exogenous retroviruses like JSRV, MMTV, FeLV, ALV, and thus can be included in a specific genera, albeit they constitute a minority of the cases (Boeke and Stoye 1997). They can also be classified into recent and ancient. Recent ERVs integrated into the host genome after speciation and in some cases are closely related to exogenous viruses that are still infectious. Some recent ERVs are still able to produce infectious virus. These ERVs haven't accumulated inactivating mutations and are often insertionally polymorphic since they are not completely fixed in the population and are still undergoing endogenization. This is the case for Koalas and sheep which are presently being invaded by the Koala retrovirus (KoRV) (Tarlinton, Meers et al. 2006) and enJSRVs (Arnaud, Caporale et al. 2007) respectively. Ancient ERVs invaded the genomes before speciation and thus are present in all vertebrates and in every individual at the same genomic location. They are replication defective due to the accumulation of mutations and genetic damage (Coffin 2004).

ERVs in general can be used as genetic markers to perform phylogenetic analysis of the host species. The presence of specific ERV in the same genomic location in two different species indicate that invasion took place in a common ancestor since provirus integration is a random event and it is highly unlikely that the same provirus integrated exactly in the same genomic location in two different hosts (Coffin 2004).

Interactions between endogenous and exogenous retrovirus

Several studies have indicated that ERVs are able to modulate the outcome of infection of exogenous retroviruses both in a beneficial or a detrimental manner to the host. The expression of some ERVs can confer resistance to superinfection by receptor blockage, where the expression of endogenous *env* genes impedes interaction of exogenous viruses with their receptors. An example of this interference mechanism is provided by mice expressing the *Fv4* locus which encodes a mutated Env protein whose receptor binding domain resembles the one of ecotropic MLV and blocks infection by exogenous MLVs (Boeke and Stoye 1997). Another mechanism by which ERVs modulate exogenous retroviral infections is provided by the expression of the *Fv1* locus in particular strains of mice. *Fv1* determines susceptibility to MLVs infection, by as yet not completely characterized mechanisms. *Fv1* is homologous to the Gag protein of human endogenous retrovirus L (HERV-L) and presumably interference takes place by interactions with CA altering the binding of the latter to the PIC (Nethe, Berkhout et al. 2005).

Some ERVs can shape the immune response towards retroviral infections or other microorganisms, like bacteria (Bhadra, Lozano et al. 2006). MMTV is transmitted to newborn pups through the milk of infected females. The virus enters the small intestine and infects B lymphocytes and dendritic cells of the underlying lymphoid tissue which then express a viral encoded protein referred to as superantigen or Sag. Expression of *sag* induces a T cell response that results in the proliferation of cells susceptible to MMTV. These cells act as a reservoir of infection and transmit the virus to the dividing mammary gland during puberty. Laboratory mice harbour between 2 to 8 replication defective endogenous MMTVs which express *sag* genes early in life leading to a clonal deletion of responsive T cells and thus preventing infection by exogenous MMTV. However, it has recently been shown that laboratory mice lacking endogenous MMTVs are not only resistant to mammary tumours but also to *Vibrio cholerae* and this phenomenon is reverted by the restoration of any of the endogenous MMTVs present in that particular mice strain (Bhadra, Lozano et al. 2006). Another example is provided by ALVs-related ERVs which reduce immunoresponses to exogenous ALVs augmenting the risk of infection, although their expression prevents the development of wasting syndrome (Gifford and Tristem 2003).

ERVs RNAs can be copackaged with the genomes of exogenous viruses which can result in recombination and the appearance of novel pathogenic variants, as is the case for avian, murine and feline leukaemia viruses (Boeke and Stoye 1997). This

phenomenon also generates concern in the preparation of vectors for gene therapy since ERVs genomes present in packaging cell lines can contaminate therapeutic vectors.

Physiological functions

The biological role of ERVs has been debated for the last twenty years. Initially ERVs were mostly deemed as “junk DNA”. However, recent studies have shown that in some cases ERVs have been selected as they conferred beneficial effects to the host besides protecting it against exogenous virus infections. One example is given by ERV-induced expression of the α -amylase gene in humans. The α -amylase gene family in humans comprise five active genes clustered in chromosome 1, including two pancreatic and three salivary genes, which are all associated with insertions of two TEs : a γ -actin pseudogene and an endogenous retrovirus. Using transgenic mice it was shown that the endogenous provirus contains specific enhancer sequences which promote expression in the salivary gland, although other mechanisms regulating gene expression might be present since Old World monkeys show high levels of salivary amylase and lack proviral insertion (Ting, Rosenberg et al. 1992; Samuelson, Phillips et al. 1996).

Another example for the selection of ERVs with a physiological function is provided by the expression of the *env* gene of enJSRVs in trophoblast cells of the sheep placenta. enJSRV Env expression has been found to be critical for conceptus development and placenta morphogenesis of sheep as will be described later (Dunlap, Palmarini et al. 2006). A similar role has been attributed to the *env* gene of HERV-W, which is highly expressed in the human placenta although for obvious reasons this has not been confirmed by *in vivo* experimentation (Mi, Lee et al. 2000).

Endogenous retroviruses and disease

It is implicit that ERVs cannot have deleterious effects otherwise they would be counter selected during evolution. However, ERVs may persist if the deleterious effects they induce are intermittent or if they are counterbalanced by beneficial consequences. Most of the associations between disease and the expression of ERVs remain speculative, in particular the association with autoimmune diseases. The strongest evidence supporting a role for ERVs in pathogenic processes is provided by some strains of mice selected for high incidence of tumours. AKR mice develop spontaneous lymphoma in the absence of exogenous virus, probably from retroviral insertional activation of oncogenes. The

oncogenic agents are a group of viruses referred to as mink cell focus forming viruses (MCF) which arise by recombination events from three different endogenous viruses (Stoye, Moroni et al. 1991).

Another example is provided by the proviral loci *Mtv1* and *Mtv2* which induce mammary tumours in mice not exposed to MMTV. Expression of these loci releases infectious virus in the lactating mammary gland, with reinfection of the same tissues followed by transformation (Boeke and Stoye 1997).

Jaagsiekte sheep retrovirus

Historical introduction

Jaagsiekte sheep retrovirus (JSRV) is the causative agent of ovine pulmonary adenocarcinoma (OPA) a naturally occurring lung cancer of sheep (Palmarini, Sharp et al. 1999), occasionally diagnosed also in goats and wild moufflons, although more rarely (De las Heras, Gonzalez et al. 2003). “Jaagsiekte” was the name given to the disease in South Africa which derives from the Afrikaner word *Jaag*, for chase, and *siekte*, for sickness, as sheep affected by the disease showed signs of respiratory distress and consequently looked as if they had been chased (Tustin 1969). The earliest documentations and descriptions of the disease date from the nineteenth century in South Africa, although it was later recognised throughout the world (York and Querat 2003). In 1891 a description performed by a South African veterinarian suggested that the disease was contagious (Hutcheon 1891), but it wasn't until 1929 that transmissibility of the disease was shown by de Kock (De Kock 1929). In 1974 viral particles were observed in lung lesions of affected animals (Perk, Michalides et al. 1974), and soon after the disease was reproduced experimentally in lambs using viral particles containing reverse transcriptase (RT) activity that were derived from tumours and lung fluids of OPA-affected sheep (Martin, Scott et al. 1976; Verwoerd, Williamson et al. 1980). Soon after Verwoerd et al. also showed that the incubation period of experimentally reproduced OPA in lambs was inversely proportional to the amount of RT present in the inoculum (Verwoered and Williamson 1981). Experimental transmission of OPA in goats was demonstrated a few years later although in this animal species tumours were not induced as readily as in sheep (Sharp, Angus et al. 1986; Tustin, Williamson et al. 1988).

Molecular studies on OPA were hampered initially by the difficulty of isolating a virus associated with the disease. The difficulty in associating JSRV to OPA was due to the combination of several factors including: I) the impossibility to grow the virus in cell culture; II) the presence of contaminants in the “purified” samples obtained from lung secretions; III) the simultaneous infection with Maedi-Visna virus (MVV) and IV) the presence of endogenous retrovirus related to JSRV. It was by the use of antisera against M-PMV and MVV that JSRV and MVV could be distinguished (Sharp and Herring 1983). In 1991 York and colleagues deduced the nucleotide sequence of JSRV by constructing a cDNA library from viral particles purified from lung fluids of OPA-affected sheep (York, Vigne et al. 1991) and provided evidence of the presence of endogenous retroviruses highly related to JSRV in sheep and goats (York, Vigne et al. 1992).

The development of reagents and techniques to differentiate enJSRVs from the exogenous JSRV (Bai, Zhu et al. 1996; Palmarini, Cousens et al. 1996) and the finding that JSRV was detected only in tumour tissues (Palmarini, Holland et al. 1996), ruled out the possibility that oncogenesis was the result of reactivation of enJSRVs. Finally, the construction of a molecular clone, referred to as JSRV₂₁, by Palmarini et al. allowed the production of virus *in vitro* and firmly established that JSRV was sufficient to induce OPA (Palmarini, Sharp et al. 1999). JSRV₂₁ proved to be an invaluable tool for several molecular studies that followed its isolation including the identification of the *env* gene as the major determinant of cell transformation (Maeda, Palmarini et al. 2001), a unique feature among oncogenic retroviruses. In addition, the isolation of enJSRV molecular clones led to the discovery of a novel mechanism of retroviral interference acting at late stages of the replication cycle (Mura, Murcia et al. 2004).

OPA

OPA has been recognized in Europe, Africa, Asia and America (Sharp and DeMartini 2003). It was eradicated in Iceland after its introduction in 1933 from Russian Karakul rams imported from Germany, which introduced not only OPA but also MVV and Paratuberculosis and resulted in the death of more than 60 percent of the stocks of the island (York and Querat 2003). It does not affect the bulk sheep population of Australia and New Zealand and it is endemic in Peru, Europe and South Africa. A recent longitudinal study estimated that the prevalence of infection in a commercial flock can reach 35 percent, although the majority of animals do not show clinical signs during

their commercial life span (Caporale, Centorame et al. 2005). More studies are required to reveal the true prevalence of OPA in natural settings in different regions now that JSRV can be detected by high sensitive PCR techniques from peripheral blood samples.

Under natural conditions, most OPA cases appear in animals aged 1-4 years, although all ages are susceptible under experimental settings. The incubation period ranges between 6 to 8 months in natural occurring cases and 5 to 12 months in experimentally infected adult sheep and lambs of several months of age. Very rapid onset of clinical signs occurs (ranging from 4 to 6 days to 3-6 weeks) when newborn lambs are experimentally infected. It appears that the natural mode of transmission for JSRV is the respiratory route, albeit maternal-foetal intra-uterine or perinatal transmission through colostrum and milk cannot be excluded (De las Heras, Gonzalez et al. 2003) (Caporale, Centorame et al. 2005).

Clinical manifestations appear once the lesions are big enough to interfere with lung function. Firstly, sheep appear less active, they tend to be left behind by the rest of the flock when walking and look exhausted. They are afebrile and loose weight. This is followed by tachypnoea, intense movements of the abdominal wall and moist rales due to the accumulation of fluids in the respiratory airways. This sero-mucous fluid is discharged through the nostrils and can cause spasmodic coughing. The animals will ultimately die after variable lengths of time depending on the presence of secondary pulmonary infections (De las Heras, Gonzalez et al. 2003).

OPA can occur in two pathological forms: classical and atypical. In classical OPA the tumours appear mainly in the cranioventral parts of all lung lobes as diffused or nodular lesions with a moist surface. They are usually multiple and tend to coalesce giving a diffuse glandular appearance. Metastases are rare but occasionally can occur in the regional lymph nodes and extrathoracic organs. In the atypical form they tend to be more nodular and mainly in the diaphragmatic lobe. They are very hard in consistency and well demarcated from the surrounding tissue with a dry surface. Both forms may be present in a flock and in individual sheep with no differences in JSRV at the molecular level (De las Heras, Gonzalez et al. 2003).

The tumour cells derive from type II pneumocytes or Clara cells, given their ultrastructure and the expression of markers such as surfactant protein A (SP-A), surfactant protein C (SP-C) for type II pneumocytes and Clara cell 10 kDa protein (CC-

10) for Clara cells (Sharp and DeMartini 2003). Both cell types have secretory properties and this is the reason for the large amount of secretions that accumulate in the lungs of most affected sheep. Histologically, the lesions are characterized by the neoplastic proliferation of epithelial cells of the alveolar and bronchiolar walls, forming foci that compress the surrounding tissues. The structure of the alveolar wall is preserved although the flat type I pneumocytes are replaced by columnar cells. The stroma supporting the lesions is composed of variable amounts of lymphoid cells, depending on the presence of secondary infection, and connective tissue fibres, which tend to be more abundant in advanced lesions leading to the fibrosis of the centre. The neoplastic focus is surrounded by macrophages which delineate the lesion. The terminal bronchioles are occupied by polypoid ingrowths composed of prismatic epithelial cells that can occlude their lumen. Myxomatous tissue composed of fusiform cells contained in a homogeneous matrix can be found mainly in association with alveolar neoplastic proliferations, although it not clear whether these are transformed cells as well. Atypical OPA lesions are mainly characterized by the same features, however their arrangement is more acinar than papillary and they display a higher inflammatory infiltrate of cells and connective fibres. An example of a classical and an atypical lesion are shown in Figure 9.

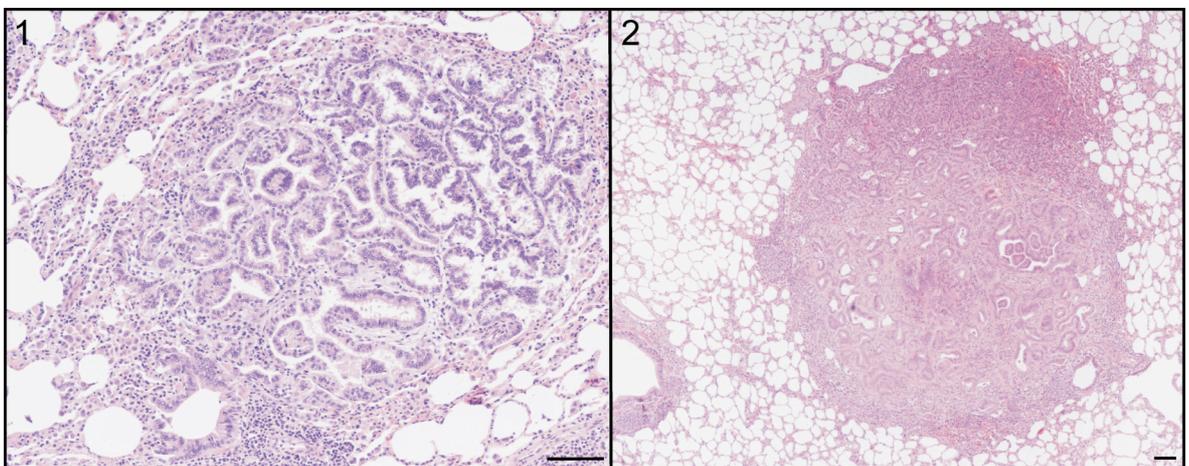


Figure 9. Histological sections of classical and atypical OPA.

Histological section stained with hematoxylin and eosin of a classical (1) and an atypical (2) OPA lesion. Bars represent 100 μm . Images kindly provided by Dr. Marcelo de las Heras.

Genetic organization of JSRV

JSRV is a Betaretrovirus phylogenetically related to M-PMV and MMTV. It is considered a type-B/D retrovirus since the *gag*, *pro* and *pol* genes are more closely

related to M-PMV while the *env* gene is more related to MMTV (York and Querat 2003). There are three completely sequenced JSRV strains: the South African JSRV strain (JSRV-SA) (York, Vigne et al. 1991; York, Vigne et al. 1992) and the UK JSRV₂₁ (Palmarini, Sharp et al. 1999) and JSRV_{JS7} (DeMartini, Bishop et al. 2001) strains. All the strains share a high degree of similarity, although JSRV₂₁ is the most thoroughly studied (Palmarini and Fan 2003). The genome of JSRV is approximately 7.5 Kb in length and has the typical retroviral genes *gag*, *pro*, *pol* and *env* (Figure 10). It also contains an additional ORF referred to as *orf-x* overlapping *pol* of unknown function (Palmarini and Fan 2003).

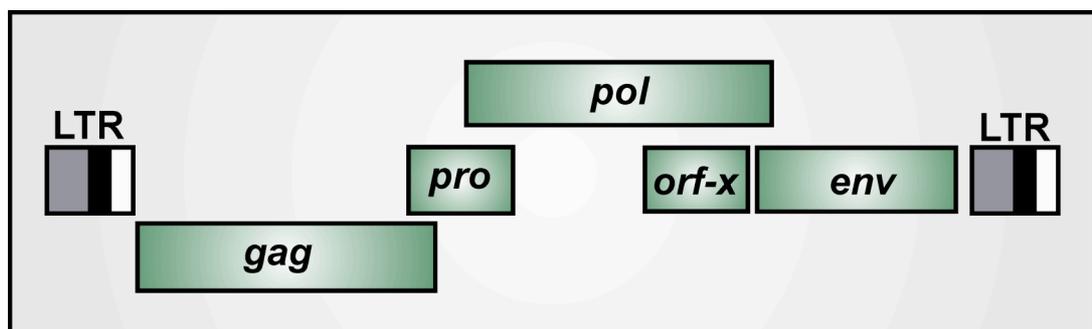


Figure 10. JSRV genetic organization.

The U3 region of JSRV₂₁ is 266 base pairs (bp) long and contains enhancer binding motifs, a TATA box and a polyA signal. The R region is 13 bp and U5 115 bp. The JSRV LTR, along with the JSRV Env, determine tissue tropism. *In vivo*, high levels of JSRV proteins are found only in the tumour cells of OPA-affected sheep although DNA and RNA are found in lymphoid tissues (Palmarini, Dewar et al. 1995; Palmarini, Holland et al. 1996; Palmarini, Sharp et al. 1999). This restricted tissue expression is not due to the confinement of the JSRV receptor to these cells but to the preferential activity of the JSRV LTR in type II pneumocytes and Clara cells of the lung. The JSRV LTR contains multiple putative factor binding sites, however it seems that its activity is driven by the presence of HNF-3, a transcription factor of the hepatocyte nuclear factor -3/forkhead homology protein family (McGee-Estrada and Fan 2006). HNF-3 is expressed in liver and lungs, in particular in type II pneumocytes and Clara cells, where it influences the expression of SP-B and CC-10. The activity of the JSRV LTR also seems to be under the influence of the CCAAT-Box/enhancer binding protein (C/EBP), since mutations or deletions of its putative site considerably reduce transcription in MLE-15 and mtCC1-2 cells (McGee-Estrada and Fan 2006). C/EBP has been implicated in the regulation of expression of SP-A and D as well as CC-10 (Cassel and Nord 2003).

JSRV proteins

The JSRV Gag polyprotein is cleaved into at least five products: MA-p15-CA-NC-p4 in this order (Murcia, Arnaud et al. 2007). MA contains in its N-terminus a consensus myristilation signal which forms part of a M domain that abrogates viral particle release *in vitro* when mutated (Mura, Murcia et al. 2004). P15 harbours two putative L domains (Murcia, Arnaud et al. 2007). Pro and Pol are encoded in different ORFs, as in all betaretroviruses, probably encoding for a deoxyuridine triphosphatase (dUTPase) and protease in the case of *pro*, and a reverse transcriptase and integrase in the case of *pol* (Palmarini and Fan 2003). The Env glycoprotein is synthesized from a single spliced transcript of 2.4 Kb in length (Palmarini, Murgia et al. 2002). The SU domain contains a hydrophobic signal peptide that is cleaved at residue 81 (Murcia, Arnaud et al. 2007). The cleavage between the SU and TM probably takes place between residues 385 and 386 (RPKR-GLS) (Palmarini and Fan 2003). The TM contains a short cytoplasmic tail of approximately 44 amino acids.

The JSRV receptor

The JSRV receptor was identified by the phenotypic screening of human/hamster radiation hybrid cell lines (Rai, Duh et al. 2001) as the glycosylphosphatidylinositol (GPI)-anchor protein, hyaluronidase 2 (Hyal-2). Hyal-2 is located in the p21.3 region of chromosome 3 (Rai, DeMartini et al. 2000), that is commonly deleted in human cancers (Lerman and Minna 2000; Petursdottir, Thorsteinsdottir et al. 2004), thus genes present in this zone are suspected to be tumour suppressors. However, as it will be described later, the role of Hyal-2 in JSRV induced cell transformation is controversial (Chow, Alberti et al. 2003; Danilkovitch-Miagkova, Duh et al. 2003).

Hyal-2 belongs to the hyaluronidase gene family (Csoka, Frost et al. 2001) which degrade hyaluronan, a component of the extracellular matrix. The biological role of normal Hyal-2 is not known at the moment. Its hyaluronidase activity is low compared to other members of the family (Vigdorovich, Miller et al. 2007) and is not required for its ability to function as the JSRV receptor.

Endogenous JSRVs

After the viral genome of JSRV was sequenced it was clear that the sheep and goat genomes harboured proviral sequences related to JSRV (York, Vigne et al. 1992). By the use of CA and SU probes, these proviruses were later found to be widely distributed in the *Ovis* and *Capra* genera (Hecht, Stedman et al. 1996). A recent study (see attached paper) which screened a sheep genomic BAC library revealed that sheep possess at least 27 individual enJSRV proviruses, including the previously cloned enJS5F16 and enJS56A1 and predicts the existence of another 10 proviruses (Arnaud, Caporale et al. 2007).

The genomic organization of the known enJSRV proviruses is shown in Figure 11. Five of the 27 enJSRV proviruses, named enJSRV-7-15-16-18 and -26 contain an intact genomic organization with uninterrupted ORF for all the retroviral genes. These proviruses are presumed to be recent integrations since four of them have identical 5' and 3' LTRs and enJSRV-16 and -18 are identical to each other at the nucleotide level. In addition, these enJSRV loci are insertionally polymorphic and present only in a portion of the sheep populations. enJS56A1 maintains intact ORFs for all the viral genes except for *orf-x* due to the presence of a premature stop codon. It also contains a -2 bp deletion at the end of *pol* which causes a frameshift that would render a protein 14 residues shorter and with 33 amino acids different at the carboxy terminus. There is a 85-89% identity at the nucleotide level between the various enJSRVs and the JSRV₂₁ molecular clone. The major differences lie in U3 and in three regions along Gag and Env referred to as variable regions 1, 2 and 3 (VR1-2-3) (Palmarini, Hallwirth et al. 2000). VR1 and VR2 are 50 residues apart and reside in MA while VR3 comprises the last 67 amino acids of TM.

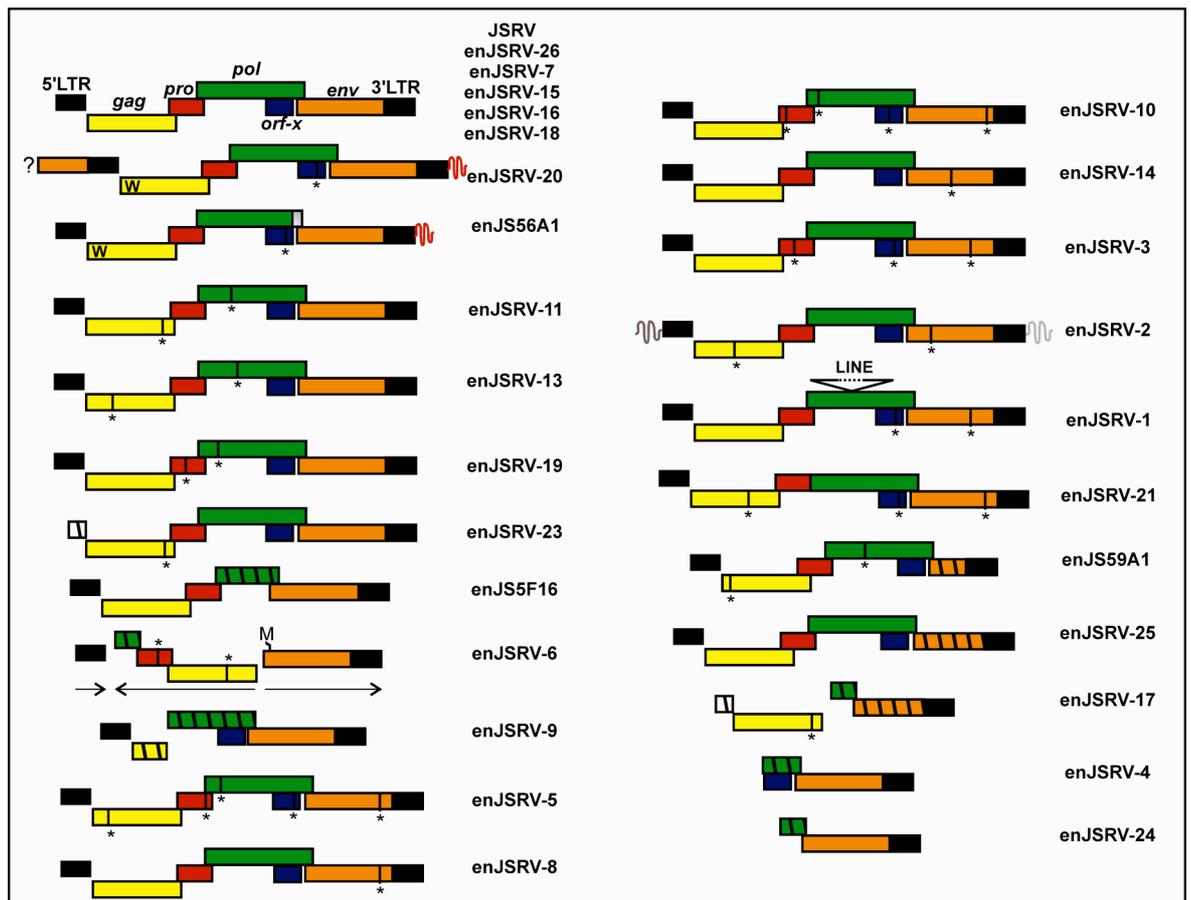


Figure 11. Genomic organization of enJSRV proviruses.

Note that five enJSRV loci maintain an intact genomic organization. The “W” in the *gag* reading frame indicates the R21W substitution present in enJS56A1 and enJSRV-20. enJSRV-20 contains a portion of an *env* gene before the proximal LTR which is indicated by the orange box and a question mark. Stop codons are indicated by vertical lines and an asterisk (*). Hatched boxes indicate large deletions in the proviruses. The letter M in enJSRV-6 indicates the position of the first methionine (M) in *env* after the usual start codon present in the other enJSRV loci and the exogenous JSRV. EnJSRV-6 contains a recombined structure with internal sequences present in the opposite direction compared to the 5'/3' LTRs and the *env* gene (indicated by horizontal arrows). EnJSRV-1 presents a LINE element within the *pol* reading frame. The 3' flanking region of enJS56A1 and enJSRV-20 are identical. Adapted from Arnaud et al. (Arnaud, Caporale et al. 2007).

The characterization of the evolutionary history of these proviruses together with the current knowledge of ruminant evolution suggest that the integration of enJSRVs began before the split between the genus *Ovis* and the genus *Capra*, approximately 5 to 7 million years ago, and continued after sheep domestication (9000 years ago). Some proviruses are found in none or a few sheep tested indicating their recent integration. enJSRV-26 in particular was found only in the Texel ram whose DNA was used to construct the BAC library used by Arnaud et al. (Arnaud, Caporale et al. 2007). None of the 330 sheep DNA samples (including 150 obtained from Texel sheep) tested harboured enJSRV-26. Given the history of the selection of the Texel breed it appears

that enJSRV-26 integrated within the last 200 years and may be a unique integration event in a single animal (Arnaud, Caporale et al. 2007).

enJSRV-induced restriction

enJSRVs have been found to interfere with JSRV replication at early and late stages of the retroviral cycle. Expression of enJSRVs blocks viral entry probably by receptor interference since JSRV is not able to enter a cell line derived from the ovine endometrial luminal epithelium (oLE) which expresses high levels of enJSRVs. JSRV entry is not affected when the assay is performed using a cell line derived from the uterine stroma (oST) which does not express enJSRVs (Spencer, Mura et al. 2003). The notion that enJSRVs block JSRV entry by receptor blockage is supported by the fact that enJSRVs and JSRV use the same cellular receptor for entry. This has been measured by the ability of MLV-based retroviral vectors pseudotyped with the Env proteins of enJSRVs and JSRV to transduce cell lines expressing the JSRV receptor (Spencer, Mura et al. 2003) (see chapter 5 and attached paper).

enJSRVs can also block JSRV replication at the late stages of the retroviral cycle. enJS56A1 displays a novel mechanism of retroviral interference known as JSRV late restriction (JLR). Cells transfected with an expression plasmid for enJS56A1 do not release viral particles in the supernatant despite the fact that Gag can be detected in the cell lysates (Palmarini, Hallwirth et al. 2000). This defect is transdominant over JSRV and has been mapped to residue 21 of enJS56A1 Gag (Mura, Murcia et al. 2004). JSRV displays an arginine (R) in position 21 which is highly conserved among members of the genus betaretrovirus, while enJS56A1 harbours a tryptophan (W). A single JSRV mutant where R21 is replaced with a tryptophan residue recapitulates the phenotype of enJS56A1 (Mura, Murcia et al. 2004). The molecular mechanisms of JLR are not completely understood. It has been recently shown that JSRV Gag is targeted to the microtubule organization centre (MTOC) and then colocalizes with pericentriolar recycling endosomes which are assumed to facilitate Gag trafficking to the cell membrane. JLR occurs before Gag reaches the MTOC. In particular, enJS56A1 induces the accumulation of Gag in microaggregates that develop into aggresomes when the proteasome machinery is inhibited (Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007). These studies suggest that mutations in MA can induce conformational alterations resulting in the production of defective particles that are unable to interact with the cellular trafficking machinery and accumulate in the cytoplasm where they are

degraded by the proteasome (Arnaud, Murcia et al. 2007). Interestingly, another provirus (enJSRV-20) bearing a W in position 21 has been recently identified and displays a defective transdominant phenotype like enJS56A1 (Arnaud, Caporale et al. 2007). Interestingly, the same study shows that enJS56A1 and enJSRV-20 originally possessed the R21 residue in Gag when first entered the host genome. W21 was acquired later during evolution and was positively selected for its ability to interfere with replication competent retroviruses. Interestingly, enJSRV-26 is able to produce viral particles *in vitro* and escapes the JLR. These results highlight the idea that some enJSRVs act as restriction factors and were selected around sheep domestication supporting the hypothesis that ERVs could help the host to fight retroviral infections (Arnaud, Caporale et al. 2007).

enJSRVs and sheep reproductive biology

enJSRVs play a critical role in conceptus development and placental morphogenesis of sheep. The earliest hints that enJSRVs could participate in some aspect of sheep reproductive biology came from the observation that high levels of enJSRV expression were limited to the reproductive tract by *in situ* hybridization, although enJSRV RNA could be detected by PCR-based assays in a variety of organs including lungs, kidneys, thymus, bone marrow, spleen, mediastinal lymph nodes and leukocytes. Highest levels of enJSRVs expression are observed in the endometrial luminal and glandular epithelium of the uterus and the epithelium of the oviducts and cervix (Spencer, Stagg et al. 1999; Palmarini, Hallwirth et al. 2000; Palmarini, Gray et al. 2001). Lower levels of enJSRVs RNA are also detected in the posterior and anterior regions of the vagina. The lamina propria of the gut also shows strong signal by *in situ* hybridization (Palmarini, Gray et al. 2001). enJSRVs expression is also observed in the mononuclear trophoctoderm cells of the developing placenta, indeed the highest levels of expression are observed in the trophoblast giant binucleate cells (BNCs) and multinucleated syncytial plaques of the placentomes (Dunlap, Palmarini et al. 2005). Expression of enJSRVs Env in trophoblast cells starts at day 12 after mating which is coincident with conceptus elongation. As it will be described in detail later, inhibition of enJSRV Env expression by morpholino antisense oligonucleotides *in utero* retarded blastocyst elongation and inhibited BNC differentiation, with consequent loss of pregnancy (Dunlap, Palmarini et al. 2006). The results of this study demonstrate how some ERVs have been positively selected to provide an essential physiological role for the host.

Moreover, the level of enJSRV expression in the endometrium is influenced by the oestrous cycle. There is an increase in the levels of enJSRV RNA between days 1 to 13, followed by a decrease by day 15 which correlates with an increase in the levels of progesterone and progesterone receptor. Constant exposure to progesterone decreases the levels of progesterone receptor in the uterine epithelium and this may be linked to the reduction in enJSRV expression that follows (Spencer and Bazer 1995; Palmarini, Gray et al. 2001). Moreover, the LTR of enJS59A1 can be activated by progesterone in transient transfection assays (Palmarini, Gray et al. 2001).

enJSRV expression has also been investigated in sheep fetuses by *in situ* hybridization with an enJSRV *env* probe (Spencer, Mura et al. 2003). These experiments revealed positive signal in the lymphoid cells of the lamina propria of the gut, in bronchial epithelial cells of the lungs and in the cortico-medullary junction of the thymus where it is thought that the final selection of T lymphocytes takes place. Expression of enJSRVs in these regions might tolerize sheep towards related exogenous betaretroviruses and this could explain the lack of circulating antibodies against JSRV in affected sheep.

All the studies conducted so far highlight that the relationship between JSRV, enJSRVs and the host is a dynamic process and that the invasion of the sheep genome is still taking place. It is speculated that the expression of enJSRV in the genital tract protected sheep/goats from related exogenous betaretroviruses present at that time. This generated a selective pressure for viruses with a different tropism from the genital tract (i.e. respiratory tropism). The different tropism might have given JSRV/ENTV the chance to replicate in a host with high levels of expression of enJSRVs and establish successful infection. The fixation of enJSRVs in the sheep genome then contributed to conceptus implantation and the development of the ruminant placenta (Palmarini, Mura et al. 2004).

JSRV induced cell transformation

The study of the molecular mechanisms leading to cell transformation by JSRV started with the development of the molecular clone JSRV₂₁ (Palmarini, Sharp et al. 1999). Originally, JSRV was thought to be an acute transforming retrovirus bearing an oncogene since it could induce multi-focal tumours very rapidly in experimentally inoculated lambs. However, the JSRV sequence did not reveal the presence of apparent cell derived oncogenes. JSRV was then shown to transform NIH 3T3 cells which

indicated that JSRV contains a gene capable of transforming cells *in vitro* and probably being an acute transforming retrovirus. (Maeda, Palmarini et al. 2001). Using deletion mutants it was possible to identify *env* as the gene with the oncogenic potential (Maeda, Palmarini et al. 2001). Since then, a number of studies have shown that the JSRV Env is able to transform a variety of cell lines *in vitro* including: mouse, rat and chicken fibroblasts as well as human bronchial, canine and rat epithelial cells (Figure 12) (Rai, Duh et al. 2001; Allen, Sherrill et al. 2002; Danilkovitch-Miagkova, Duh et al. 2003; Zavala, Pretto et al. 2003; Liu and Miller 2005; Varela, Chow et al. 2006). Moreover, the sole expression of the JSRV Env is able to induce lung tumours in immunocompromised mice with similar characteristics to OPA when expressed by replication-incompetent adeno-associated virus vectors (Wootton, Halbert et al. 2005). More importantly, the JSRV Env is able to induce lung adenocarcinomas in immunocompetent sheep when expressed by a JSRV based vector under the control of the JSRV LTR (Caporale, Cousens et al. 2006). This study not only demonstrates that JSRV Env is a dominant oncoprotein but also that JSRV can induce OPA without viral spread, a unique feature among oncogenic retroviruses.

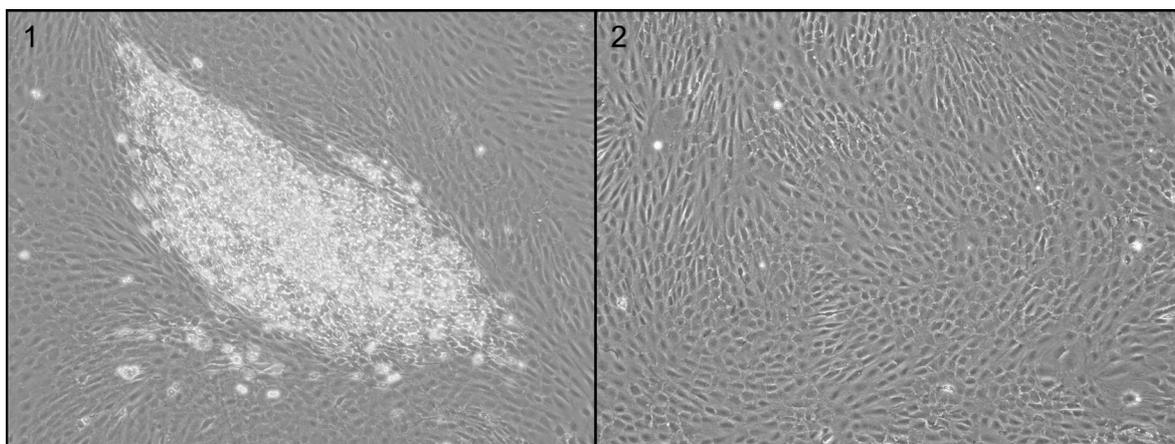


Figure 12. Focus of transformed cells induced by the JSRV Env in 208F cells.

208F cells transfected with 1) an expression plasmid of the JSRV Env and 2) empty vector, photographed two weeks after transfection.

The cytoplasmic tail of the TM domain of the JSRV Env glycoprotein bears a YXXM motif (Y for tyrosine, X for any amino acid and M for methionine), which has been deemed critical for transformation (Palmarini, Maeda et al. 2001). This is a distinctive feature from other retroviruses which normally harbour YXX Φ motifs (Φ for any amino acid with a bulky hydrophobic chain) which are implicated in trafficking, endocytosis and pathogenesis (Ye, Bu et al. 2004). Mutations in the YXXM motif of the JSRV Env indicate that the Y in position 590 is crucial for transformation although its importance

depends on the experimental conditions and the cell type used to perform the assays (Palmarini, Maeda et al. 2001; Allen, Sherrill et al. 2002; Liu, Lerman et al. 2003; Liu and Miller 2005). Y590 forms part of a potential binding site for p85, the regulatory subunit of phosphatidylinositol-3 kinase (PI3K). In addition, JSRV-derived transformed cell lines show activation of Akt which is an important PI3K downstream effector (Palmarini, Maeda et al. 2001; Liu, Lerman et al. 2003; Zavala, Pretto et al. 2003; Varela, Chow et al. 2006). Transformation by the JSRV Env is considerably reduced when assays are performed under the presence of a PI3K inhibitor (LY294002) (Alberti, Murgia et al. 2002; Liu, Lerman et al. 2003). However, the effects of PI3K inhibitors vary according to the cell type used to perform the assays (Maeda, Inoshima et al. 2003) and indeed transformation of chicken fibroblasts is not critically dependent on Y590 (Allen, Sherrill et al. 2002; Zavala, Pretto et al. 2003). Furthermore, a crucial step in the activation of the PI3K-Akt pathway is the interaction between p85 and the phosphorylated tyrosine present in the YXXM motif. No interaction between p85 and the JSRV Env in GST pull-down assays and co-immunoprecipitation (CoIP) assays and no Y590 phosphorylation were ever observed (Liu, Lerman et al. 2003). It is now clear that the mechanisms of cell transformation induced by the JSRV Env are more complicated than originally thought and depend on the cell type used to study them. However it is agreed that the tyrosine present in the YXXM motif is crucial for transformation since Y590 mutant viruses are unable to induce OPA *in vivo* (Cousens, Maeda et al. 2007) and that the activation of Akt is both PI3K dependent and independent. The role of Akt in JSRV Env transformation *in vivo* remains elusive since no phosphorylation is detected by immunohistochemistry (IHC) of OPA lung sections (Zavala, Pretto et al. 2003), although its activation has been shown in 10 out of 27 samples of OPA lung tumours analysed by western blotting (Suau, Cottin et al. 2006).

The role played by the SU domain in JSRV Env transformation has been controversial and forms part of the work presented in this thesis. In rodent fibroblasts, replacement of the receptor binding domain (RBD) and proline rich region of the SU of the JSRV Env with that of Moloney murine leukaemia virus (MoMLV) does not considerably affect transformation (Chow, Alberti et al. 2003). This chimeric Env does not use Hyal-2 as a receptor to mediate entry when pseudotyped with MoMLV vectors. Other studies have also shown that overexpression of mouse Hyal-2 does not modify the outcome of transformation assays (Liu, Duh et al. 2003). Taken together these results indicate that the SU domain of the JSRV Env is not important in the induction of cell transformation and that the JSRV receptor is not involved in this process, at least in rodent fibroblasts.

However, others have shown that large deletions or small insertions in SU abolished transformation of rodent fibroblasts and that transformation may require both SU and TM (Hofacre and Fan 2004).

In contrast to the scenario in rodent fibroblasts, the SU domain and Hyal-2 seem to be important in Env transformation of BEAS-2B human bronchial epithelial cells. In normal BEAS-2B cells Hyal-2 is apparently bound to the receptor tyrosine kinase RON, inhibiting its activation. It is proposed that the JSRV Env triggers transformation by interacting with HYAL-2 and inducing its degradation thus liberating RON which is able to dimerize, autophosphorylate and initiate signals that ultimately will lead to cell transformation (Danilkovitch-Miagkova, Duh et al. 2003). Since the RBD is responsible for the interaction with Hyal-2, it is assumed that the SU is required to transform these cells.

Beside the PI3K/Akt pathways, the Raf-MEK-MAPK signalling cascade has also been implicated in JSRV induced cell transformation. The mitogen-activated protein kinases (MAPKs) are expressed generally in all cell types although they regulate different responses according to the cell type (Dhanasekaran and Johnson 2007). The key to their specific biological functions lies in their spatial and temporal regulation within cells. As shown in Figure 13, MAPKs, which include ERK1/2, are phosphorylated and activated by MAPK kinases (MAPKKs). MAPKK kinases (MAPKKKs) phosphorylate and activate MAPKKs. MAPKKKs possess different regulatory domains that selectively govern the localization and activation status of the associated MAPKKs and MAPKs (Dhanasekaran and Johnson 2007). The ERK pathway is deregulated in one third of all human cancers and is the best studied of the MAPKs pathways (Dhillon, Hagan et al. 2007). ERK1/2 are phosphorylated and thus activated by MEK1/2, which are themselves activated by phosphorylation by Raf (A-Raf, B-Raf and C-Raf) (Figure 13). The activation of the ERK pathways can be triggered by several extracellular signals. Usually ligand activation of receptor tyrosine kinases (RTK) causes the loading of Ras with guanosine triphosphate (GTP), which then recruits Raf to the cell membrane for activation (Dhillon, Hagan et al. 2007). Once activated, ERKs are able to phosphorylate a variety of cytoplasmic and nuclear factors that in turn will lead to diverse cellular responses like proliferation, differentiation, survival, migration, angiogenesis and chromatin remodelling. It is now clear that the signals from RTK to ERKs are highly dynamic and form part of the complex regulated processes. The role of MAPKs pathways in JSRV induced transformation is partially understood. Activated MEK1/2

and Erk1/2 have been found by IHC in lung sections of naturally occurring and experimentally induced OPA (Caporale, Cousens et al. 2006; De Las Heras, Ortin et al. 2006). Additionally, transformation assays performed under the presence of the MEK1 and Ras inhibitors reduce transformation in a dose dependent manner (Maeda, Fu et al. 2005). However, no Erk1/2 phosphorylation is detected in JSRV Env transformed rodent fibroblasts. This could be explained by the fact that Erk activation is transient or unstable or it takes place in specific cell compartments and is “lost” when analyzed in whole cell extracts. The picture is complicated even more by the fact that inhibition of p38 (a MAPK protein) increases transformation by the JSRV Env and enhances the phosphorylation status of Mek1/2 and Erk1/2 in JSRV-derived transformed cells, indicating cross-talk between p38 and Erk pathways (Maeda, Fu et al. 2005). These results show that a fine tuning regulates the signal pathways involved in JSRV induced cell transformation.

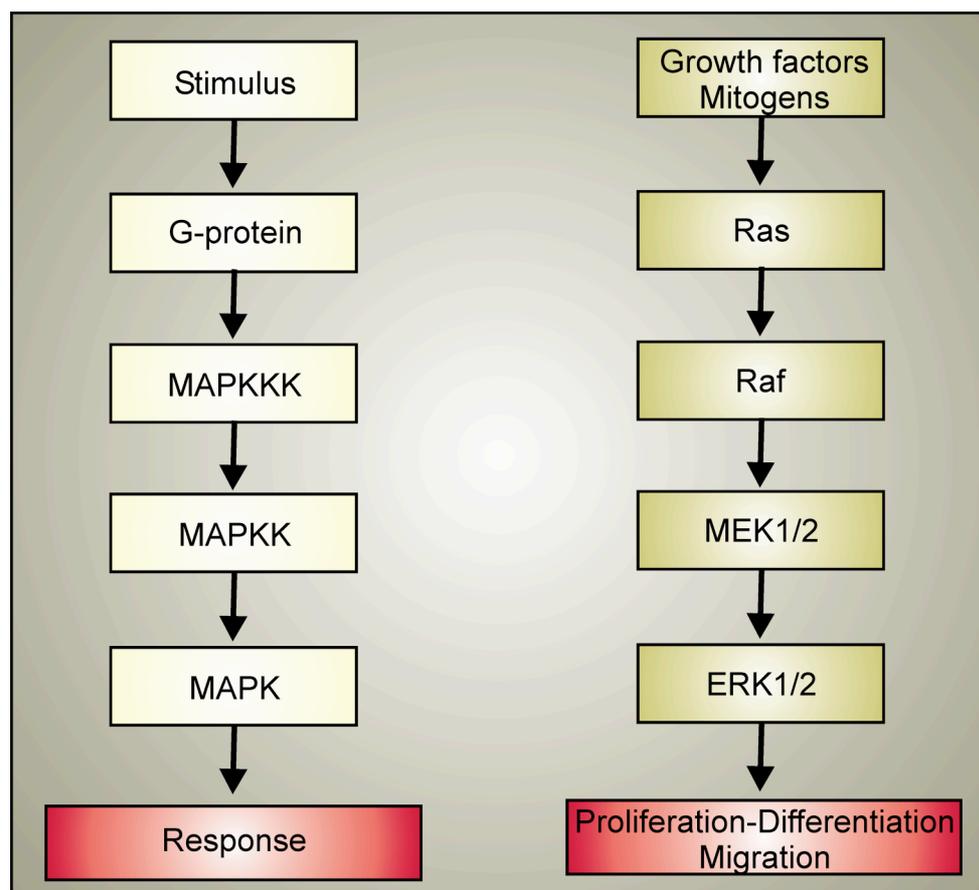


Figure 13. Overview of MAPK pathway.

Adapted from Dhillon et al., 2007.

In conclusion, all the evidence collected so far supports the fact that JSRV Env induces transformation at least in part by the activation of Akt and the Raf-Mek-Erk pathways.

The relevance of each pathway might vary depending on the cell type analyzed and it is likely that transformation is ultimately the result of the combinatory effects of these two pathways among others not yet identified. This notion is supported by the fact that in natural settings the development of OPA takes months to years, emphasizing the multistep essence of JSRV-induced cancer progression and the probable requirement of several genetic alterations for its full expression. The early steps in this process, including the mechanism used by the JSRV Env to engage the cell signalling network, remain the least understood area in the study of JSRV oncogenesis.

Enzootic nasal tumour virus

Enzootic nasal adenocarcinoma (ENA) is a contagious tumour of the gland cells of the nasal mucosa of sheep and goats. Enzootic nasal tumour virus-1 (ENTV-1) and ENTV-2 have been associated with the disease in sheep and goats respectively (Cousens, Minguijon et al. 1999; Ortin, Cousens et al. 2003). ENTV-1 and -2 are highly related to JSRV; however they have enough differences to be considered distinct betaretroviruses. Affected animals are usually 2 to 4 years of age and clinical signs are characterized by the presence of variable amounts of seromucous fluid coming from the nostrils. The tumour affects the nasal chambers and expands in all directions penetrating the frontal and nasal sinuses (De las Heras, Ortin et al. 2003). The neoplasm is characterized by the presence of epithelial cells that proliferate in an acinar, tubular, papillary or cystic pattern. As for JSRV, the affected animals do not mount an immune response towards the virus.

ENTV uses Hyal2 as a cellular receptor, thus the differences in tissue specificity may lie in the transcription factors required for LTR activation (Dirks, Duh et al. 2002). This is supported by sequence analysis of the ENTRV LTR which reveals differences in transcriptional regulators (Cousens, Minguijon et al. 1999). The major determinant of transformation is the *env* gene as is demonstrated by its ability to transform cells *in vitro* (Dirks, Duh et al. 2002) and both the PI3K/Akt and the Raf-Mek-Erk pathways have been implicated in the mechanism leading to cell transformation (Zavala, Pretto et al. 2003; De Las Heras, Ortin et al. 2006).

Conclusions

Betaretrovirus of sheep have proven to be a fascinating system to study several aspects of the biology of retroviruses. JSRV is unique among oncogenic retroviruses since one of its structural proteins behaves as a dominant oncogene both *in vitro* and *in vivo*, allowing the study of basic mechanisms leading to cell transformation. In addition, OPA serves as an animal model for the study of lung carcinogenesis where novel diagnostic and therapeutic interventions can be investigated. enJSRVs are able to block JSRV replication at early and late stages of the retroviral cycle. This together with the characterization of the evolutionary history and molecular virology of these enJSRVs have provided evidence suggesting that selection of ERVs acting as restriction factors could be a mechanism used by the host to fight retroviral infections. Moreover, the Envs of enJSRVs are essential for conceptus development and placental morphogenesis and their study has provided the first *in vivo* evidence of a role for retroviral Envs in reproductive biology. The profound role that the Envs of both exogenous and endogenous JSRV play in OPA pathogenesis and sheep reproductive biology merits further investigation. The aim of the study presented here is to provide a better understanding of the biology of JSRV and enJSRVs Envs.

Chapter 2

Materials and Methods

Plasmids

pCMV3JS21ΔGP, the expression plasmid for the JSRV₂₁ envelope, has been previously described (Maeda, Palmarini et al. 2001). pJSE-V5 expresses wild type JSRV Env fused with the V5 epitope in the carboxyterminal of the TM domain. pMoMLVprrr-JSE, which contains the MoMLV receptor binding domain (RBD) and proline rich region of Moloney murine leukaemia virus (MoMLV) and the remaining portion of the envelope from the JSRV Env, has been previously described (Chow, Alberti et al. 2003). MoMLVprrr-JSE-F, derives from MoMLVprrr-JSE and contains a FLAG epitope in the carboxyterminal of the TM domain (Chow, Alberti et al. 2003). pEnvEn2, the expression plasmid for the Env of enJS5F16, has been described before (Palmarini, Maeda et al. 2001). JSEY590D-F and JSE-En2-Flag express respectively the JSRV Env with a Y590D mutation (Palmarini, Maeda et al. 2001) in the CT and the enJS5F16 Env (Palmarini, Hallwirth et al. 2000). In these two plasmids both Envs are fused in their carboxyterminal with the FLAG epitope. See Figure 14 for a schematic representation of the various *env* expression plasmids. pCEE-F, expressing the MMuLV Env with a C-terminal FLAG epitope was derived from pCEE+ (MacKrell, Soong et al. 1996) and was kindly provided by Paula Cannon.

pCMV3JS21ΔGP-neo, pMoMLVprrr-JSE-neo, pCMV3JS21ΔGPY590D-neo, pCMV3JS21ΔGPY590F-neo and pEnvEn2-neo express the respective envelopes and the neomycin resistance gene. They were constructed by PCR amplification of the neomycin resistance cassette from pcDNA3.1+ (Invitrogen) and introduction into the *env* expression plasmids between HindIII and KpnI restriction sites.

pCMV₁-F-hHyal2 is an expression plasmid for the human HYAL-2 gene containing a preprotrypsin leader sequence and the FLAG epitope at the N-terminal of the human HYAL-2 gene and was constructed by introducing the human HYAL-2 cDNA sequence (Gene Bank accession number: BC000692.2; cDNA clone: MGC-1922) into pFLAG-CMV-1 (Sigma). pC1-neo-hRON, an expression plasmid for the human RON tyrosine kinase, was kindly provided by Pam Correll. pRK5-HERc, the expression plasmid for

Epidermal growth factor receptor was provided by Silvia Gartner. pCDNA 3.1-STK-HA, pCDNA 3.1-Ron-Ha and MSV-Neo-MYC-SFSTK expressing the mouse (Stk) and human Ron proteins and a short form of Stk that lacks most of the extracellular domain but retains the transmembrane and tyrosine kinase sequences (Persons, Paulson et al. 1999) respectively were kindly provided by Pam Correll.

The envelopes of enJSRV1, 4, 6, 7, 9, 11, 13, 15, 16/18, 19, 20 and 26 were PCR amplified from the original BAC clones and cloned into pCMV3JS21ΔGP by replacing the exogenous JSRV Env. The same envelopes were also cloned into the retroviral vector pLNCX2JS21ΔGP, which expresses the JSRV Env. F-JSE expresses the JSRV Env and was kindly provided by Claudio Murgia. F-enJSRV6,13 and 26 are homologous to F-JSE expressing the respective enJSRV Envs. F-JSEY590F was obtained by site directed mutagenesis (SDM) from F-JSE.

pLNCX2ovHyal2 was cloned by removing by restriction enzyme digestion the ovine Hyal2 gene present in pCR3.1ovineHyal2 (using HindIII and NotI sites) and introducing it into the same sites into the retroviral vector pLNCX2 (Clontech). pLNCX2goatHyal2 was constructed by introducing the cDNA of the goat Hyal2 gene derived from RNA extracted from TIGEF cells into pLNCX2 using XhoI and Sall restriction sites. pLNCX2BovHyal2 was constructed by PCR amplification of the *Bos taurus* Hyal2 from cDNA (Clone BC102042) obtained from Geneservice (I.M.A.G.E. number: 7928340) and introduced into pLNCX2 between XhoI and Sall .

For cloning purposes, PCR were performed using the *Pfu* polymerase (Stratagene). Ligation reactions were done using the Rapid Ligation Kit (Roche). Plasmid DNA was produced in DH5α and Top 10 strains of *E. coli* (Invitrogen), using the DNA Maxiprep kit from Invitrogen. SDM was performed using the Quickchange site directed mutagenesis kit from Stratagene following the manufacturers' instructions. Primers for site-directed mutagenesis were designed with PrimerX, a web-based software that automatically designs mutagenic PCR primers (<http://bioinformatics.org/primerx/>).

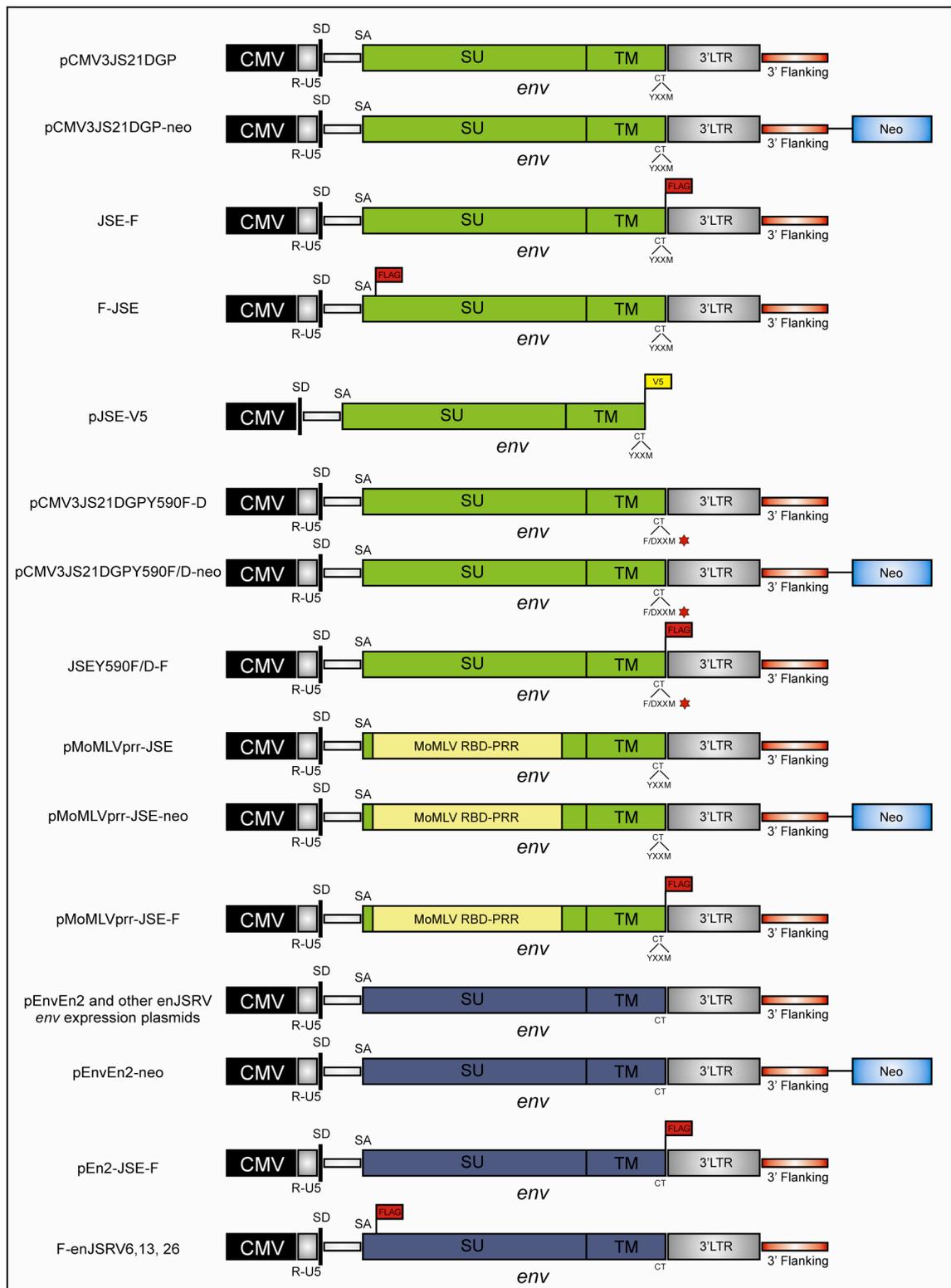


Figure 14. Schematic representation of *env* expression plasmids used in this study.

Cell culture

All the cells used in this study were cultured at 37°C in a 5% CO₂ atmosphere and 95% humidity. 208F, human 293, 293T, COS, TIGEF (Da Silva Teixeira, Lambert et al. 1997) and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM)

with high glucose (4.5 g/l) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. NIH 3T3 cells were grown in the presence of new born calf serum instead of FBS. Rat epithelial IEC-18 cells (ATCC CRL-1589) were grown in DMEM supplemented with 5% foetal bovine serum, 0.1 U/ml of bovine insulin. JS8, a cell line derived from an OPA tumour, and oST (Johnson, Burghardt et al. 1999) cells were grown in DMEM nutrient mixture F-12 Ham (Sigma) media. oTr cells were grown in DMEM/F-12 Ham media supplemented with 10% FBS, 1% of penicillin-streptomycin, 1% non essential amino acids, 1% sodium piruvate, 1% glutamine and 8 µg/ml of human recombinant insulin (Invitrogen).

Cell line	Origen
293	Immortalized human Embryonic kidney cells
HeLa	Immortalized human cervical cancer cells
208F	Immortalized rat fibroblasts
NIH 3T3	Immortalized mouse fibroblasts
IEC-18	Immortalized normal ileum epithelium
COS	Immortalized African green monkey kidney cells
TIGEF	Immortalized goat embryo fibroblasts
oTr	Primary ovine trophoblast cells
oLE	Primary ovine luminal epithelium cells
JS8	Immortalized ovine lung transformed cells

Table 3. Cell lines used in this study.

Transfections

Cells were transfected using either the Calphos Mammalian transfection kit (Clontech) or lipofectamine (Invitrogen) following manufacture's instructions. In some cases cells were transfected using Polyethylenimine (PEI) 25kD linear form (Polysciences via Park Scientific Labs). When PEI was used, cells were plated in a 10 cm dish and allowed to reach between 80 and 90% confluence. Ten µg of plasmid DNA were then diluted in 500 µl of DMEM and 400 µl of PEI (1 mg/ml in distilled water, pH 7) and incubated for 10 minutes at RT. The transfection mix was added drop by drop to the cells and

incubated for 2 hours in a 37°C in a 5% CO₂ atmosphere and 95% humidity incubator. Before the addition of the transfection mix, 5 ml of fresh media were added to the cells. Dishes were then washed twice with PBS and cells were grown in normal culture media until transgene expression was analysed (Boussif, Lezoualc'h et al. 1995).

Stable cell lines

NIH-3T3 expressing sheep (sheep-Hyal2), goat (goat-Hyal2), or bovine (bovine-Hyal2) Hyal-2 and 208F cells expressing various enJSRV Envs were produced as follows. Ecophoenix cells (kindly provided by Linda Hanlon) were transfected with retroviral vectors expressing the genes in question and supernatants were collected 24 and 48 hours after transfection. Supernatants were clarified by centrifugation at 900 rpm for 5 minutes and polybrene was then added to a final concentration of 8 µg/ml. Supernatants were then used to infect NIH 3T3 or 208F cells twice with a 24 hour interval. Eight hours after the last infection cells were split at different ratios and stable clones were selected by the addition of a G418 (500 µg/ml) to the culture media.

Transformation assays

2.5×10^5 IEC-18 rat epithelial cells were seeded in a 6 cm diameter plate. Each plasmid (10.4 µg) was transfected in 4 X 6 cm plate using the Calphos Mammalian transfection (Clontech). Approximately 16 hours after the transfection, cells were washed three times with phosphate-buffered saline (PBS) and split into 4 X 10 cm plates. Media was replaced every other day for four weeks when foci of transformed cells were counted. The same procedure was used when transformation assays were performed with the Env constructs expressing the neomycin resistance gene, except that cells were cultured in the presence of G418 (500 µg/ml).

Transformation assays performed to test the effects of inhibitors of various signal transduction pathways on JSRV Env transformation were performed by transfecting 5×10^5 208F cells (per 10 cm diameter plate) with pCMV3JS21ΔGP (an expression plasmid of the JSRV Env) (Palmarini, Sharp et al. 1999; Rai, Duh et al. 2001; Maeda, Inoshima et al. 2003) or an empty vector as a negative control using Calphos mammalian transfection kit (Clontech). Cells were washed 12-16 hours after transfection with PBS and split into 4 x 6 cm plates. Cell culture medium was replaced every other day for one week with the addition of 1 µM of dexamethasone. Thereafter,

two cell culture dishes were treated with inhibitor and the remaining two with DMSO as negative control. Foci of transformed cells were counted 14 days post transfection. 208F cells were co-transfected with 1 μ g of pCMV3JS21 Δ GP and increasing amounts of a dominant negative form of Src (SrcMF) (kindly provided by Valerie Brunton) (Timpson, Jones et al. 2001) in order to test the ability of the latter to block JSRV Env transformation. Foci of transformed cells were counted 14 days post transfection. Analogous experiments were performed using the enJSRVs Env as possible inhibitors of JSRV Env-induced cell transformation.

Entry assays

The ability of the enJSRV Env proteins to mediate cell entry was assessed by standard entry assays using murine leukaemia virus (MLV)-based vectors. 293-GP-AP is a packaging cell line that expresses MLV Gag and Pol and a MLV-based retroviral vector expressing the alkaline phosphatase gene (Chow, Alberti et al. 2003). 293-GP-AP cells were transfected with the expression plasmids for the various enJSRV Env or with plasmids expressing the exogenous JSRV Env as a control. Supernatants were collected 48 and 72 hours after transfection and stored at -70°C . Subsequently, naïve NIH-3T3, NIH-3T3 expressing either ovine (sheep-Hyal2), goat (goat-Hyal2), or bovine Hyal2 (bovine-Hyal2) or oST cells were exposed to 10-fold serial dilutions of vector supernatants supplemented with 8 μ g/ml of polybrene. Two days post-infection cells were fixed with 0.5 % of glutaraldehyde for 5 minutes, washed twice with PBS and incubated at 55°C for an hour to inactivate endogenous alkaline phosphatase activity. Cells were then washed once with staining solution (1X NBT, 1X XPHOS in buffer 3; Buffer 3: 100 mM Tris pH 8.5, 100 mM NaCl, 50 mM MgCl₂; 100X NBT: 50 mg/ml of nitro blue tetrazolium in 70% dimethylformamide and 30% water; 100X XPHOS 10 mg/ml of 5bromo-4chloro-3indolyl phosphate in water). Alkaline phosphatase-positive foci were revealed after staining ON. Viral titer is expressed as alkaline phosphatase foci per ml (APF/ml). Experiments were performed at least twice with two replicates tested for each dilution.

Cell proliferation assays

The effect of Hsp90 inhibitors on the proliferation of JS8 cells was measured using the WST assay from Roche following manufacture's instructions. 10^3 cells were plated in a 96 well plates and cultured in normal growth media with the addition of 17-DMAG or

radicol at concentrations ranging from 0.025 to 0.1 μM . Cell media was replaced every day for 3 days. Experiments were repeated three times with at least three replicates each time.

The effect of enJSRV Env expression on the growth of 208F cells was measured using the WST assay from Roche. Briefly naïve 208F and 208F stabling expressing various enJSRVs Envs were plated in 96 well plates (10^3 cells/ well) and cell proliferation was measured every day for 5 days. Two independent cells lines were prepared for each Env construct and each cell line was analysed twice with at least three replicates each time.

The effect of enJSRV Env expression on the growth of oTr cells was measured using the WST assay (Roche) as above. oTr cells were transfected with the respective enJSRV Env expression plasmids using PEI as described above. Two hours after transfection cells were seeded at a ratio of 10^3 per well into a 96 well plate and cell proliferation was measured after 72 hours of culture in normal growth media without the addition of insulin.

Inverted cell invasion assay

A 100 μl of a 1:1 dilution of complete matrigel (BD Biosciences) in PBS was pipetted into a 6.5 mm diameter polyester transwell chambers with a pore size of 8 μm (Corning) and was left to sit for at least 30 minutes at 37°C . 1.45×10^4 cells were pipetted into the underside of the membrane (by inverting the chambers) and then covered with the bottom part of a 24 well plate so that the base of the plate contacted the droplet of cell suspension. The plates were incubated between 2 to 4 hours to allow cell attachment. Thereafter the plates were turned upside down and each well was sequentially dipped three times into 1 ml of serum free media as a washing step. The transwells were left into the third washing media and 100 μl of serum free media was pipetted on top of the transwell. The plates were then incubated for 4 days at 37°C in a 5% CO_2 atmosphere and 95% humidity incubator. Cells were then stained with a $4\mu\text{M}$ solution of calcein AM (Molecular Probes) in serum free media for 1 hour at 37°C in a 5% CO_2 atmosphere and 95% humidity incubator. Cells invading the matrigel were then imaged using a Leica GMIR2 confocal microscope as follow. At least three optical sections (Z-sections) per transwell were scanned using a 20X objective at 15 μm intervals (Z-steps), moving up from the underside of the membrane, into the matrigel producing a series of images. Images were quantified using the Image J software, which gives each on-screen

pixel a value from 0 to 255 (Abramoff, Magelhaes et al. 2004). The background pixel value is operator-defined as the pixel value on the 0 to 255 scale at which only cells from that individual section are visible with no bleed-through from bordering sections (for this experiments it was set to 75). Only pixels with an intensity value greater than background were then quantified. Each image was quantified and the sum of the values for each optical section was used. Since three optical sections were analyzed per transwell and each experiment was done in duplicate, the average of six sections was used to create the charts shown as results.

Cell migration assay

Migration assays were performed in 6.5 mm diameter polyester transwell chambers with a pore size of 8 μm (Corning). A morpholino antisense oligonucleotide (MAO) was designed to specifically inhibit the expression of enJSRV *env* mRNAs (MAO-*env*) while a five-mismatch (MAO-5mis) was designed as a negative control. oTr (10^4) cells were plated inside the chamber in their normal growth media with the exception of FBS and insulin. The bottom chamber contained complete growth media and Endo-Porter, the delivery reagent (8 $\mu\text{l/ml}$), MAO-5mis (80 μM) or MAO-*env* (80 μM) as required for each experiment. After three days in culture cells were stained with calcein AM as described above. Cells on the inside of the chamber were carefully removed with a cotton soap and cells that migrated to the other side of the membrane were imaged using a Leica GMIR2 confocal microscope with a 20X objective and quantified basically as described above using the Image J software.

Antibodies

RON C-20 polyclonal antibody against the β chain of RON, CD4 MT310 and Hsp90 α/β (H-114) antibodies were purchased from Santa Cruz Biotechnology. Monoclonal Anti Flag M2 and Anti Flag polyclonal antibodies were purchased from Sigma. Anti-HA monoclonal and polyclonal antibodies were purchased from Covance and Abcam respectively. Monoclonal anti phosphotyrosine antibody clone G410 and Anti-EGF Receptor clone LA22 were purchased from Upstate Biotechnology. Anti MYC, Akt and phosphorylated AKT antibodies were purchased from Cell Signaling. Anti-V5 antibody was purchased from Invitrogen. Secondary anti-rabbit IgG peroxidase linked F(ab') fragment from donkey was purchased from Amersham Biosciences. Peroxidase conjugated goat anti mouse antibody was purchased from Jackson Research. Goat anti-

rabbit IgG labeled with Alexa-488 and donkey anti-mouse IgG labeled with Alexa-594 were purchased from Molecular Probes.

Cell lysates and immunoprecipitations

For the evaluation of AKT phosphorylation, transformed and parental IEC-18 cells were grown up to approximately 80% confluency, serum starved overnight and lysed with Triton lysis buffer (Cell Signaling) with the addition of a protease inhibitors cocktail (Complete- Roche) and 1 mM PMSF. To test RON phosphorylation, human 293 cells were transfected with the appropriate plasmids (see Results section) using the Calphos Mammalian transfection kit (Clontech); 48 hours after transfection cells were serum-starved for 12-16 hours and lysed as described above.

Co-immunoprecipitation assays shown in Chapter 3 and 4 were performed in 293T cells transfected with the appropriate plasmids using the Calphos Mammalian transfection kit. 48 hours after transfection cells were lysed with SDS-NP40 lysis buffer [0.5% Sodium Deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.5% NP-40, 50 mM TRIS, 150 mM NaCl] with the addition of a protease inhibitors cocktail (Complete-Roche) and 1 mM PMSF. Lysates were sonicated and then centrifuged for 20 minutes at 10,000 rpm to remove insoluble material. Protein concentration was determined using the Lowry method provided by Biorad. For the evaluation of protein-protein interactions 200 µg of whole cell extracts were rocked with 20 µl of protein A agarose beads (Santa Cruz Biotechnology) and primary antibody at 4°C for 3 hours. After three washes with lysis buffer, beads were resuspended in SDS loading buffer, boiled for 5 minutes, subjected to SDS-PAGE and western blotting using standard protocols (Ausbel, Brent et al. 2000). Detection was achieved by using the appropriate secondary antibodies labelled with horseradish peroxidase followed by enhanced chemiluminescence (ECL) detection using SuperSignal West Pico chemiluminescent reagent (Pierce). When necessary membranes were stripped with restoring buffer (Pierce) and used again with another antibody. Each experiment has been repeated at least twice independently.

Co-immunoprecipitation assays shown in Chapter 5 were performed in 293T cells transfected with the appropriate plasmids using the Calphos Mammalian transfection kit. 48 hours after transfection cells were lysed with a lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 25 mM of NaF, 1 mM of EDTA with the addition of a protease inhibitors cocktail (Complete- Roche) and 1 mM

PMSF. Lysates were rocked for 30 minutes at 4°C and then centrifuged for 20 minutes at 10,000 rpm to remove insoluble material. 500 µg of cell lysates were pre-cleared with 10 µl of protein A agarose beads (Santa Cruz Biotechnology) for 30 minutes at 4°C. Lysates were then centrifuged for 5 minutes at 12,000 rpm to remove beads and were immunoprecipitated for 12-16 hours using 20 µl of protein A agarose beads and 1 µl of Flag M2 antibody (Sigma). Immunoprecipitates were then washed and analyzed as described above.

Western blot analysis

50 µg of protein extracts obtained from cell lysates (or whole pellets resulting from immunoprecipitations) were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Hybond, Amersham), and blocked for 1 hour at RT with blocking buffer (5% skimmed milk in TBS/T [0.1% of Tween 20]). After blocking, membranes were rinsed with TBS/T three times for five minutes and further incubated with the selected primary antibody. This step was performed either for 1 hour at RT or overnight at 4°C. Primary antibodies were diluted as follows:

Anti-FLAG: 1:5000 in 5% BSA in TBS/T.

Anti-HA: 1:10000 in 5% BSA in TBS/T.

Anti-phosphotyrosine: 1:1000 in 5% BSA in TBS/T.

Anti-RON: 1:5000 in 5% BSA in TBS/T.

Anti-Akt and phospho-Akt: 1:1000 in 5% BSA in TBS/T.

Anti-EGFR: 1:1000 in 5% BSA in TBS/T.

Anti-CD4: 1:2000 in 5% BSA in TBS/T.

Anti-MYC: 1:1000 in 5% BSA in TBS/T.

Anti-HSP90: 1:1000 in 5% BSA in TBS/T.

Anti-γ tubulin: 1:000 in 5% BSA in TBS/T.

Anti-JSRV Env: 1:500 in blocking buffer

After incubation with primary antibody, membranes were rinsed three times in TBS/T for five minutes and further incubated with the appropriate peroxidase-conjugated secondary antibodies for 1 hour at RT. Secondary antibodies used were donkey anti-rabbit (F[ab']₂ fragment) from Amersham (GE), used at a working dilution of 1:40000

in blocking buffer; and goat anti-mouse (IgG [H+L]) from Jackson Research, diluted 1:10000 also in blocking buffer. Chemiluminescence was developed using ECL (Amersham, GE). If membranes were to be reblotted with a different primary antibody, they were first stripped with Restore (Pierce) for an hour at 37°C, and washed three times with TBS/T for 5 minutes.

Confocal microscopy

HeLa or COS (6.5 x 10⁴ cells/well) cells were plated onto two well-chambered glass slides (Lab-Tek, Nalge Nunc International) and cultured as described above. Cells were transfected with Lipofectamine (Invitrogen) supplemented with Plus reagent (Invitrogen) according to the manufacturer's instructions. Cells were kept with the transfection mixture (without serum or antibiotics) between 3 and 5 hours. After this time the transfection mixture was replaced by complete medium. Forty-eight hours after transfection cells were fixed with 3% paraformaldehyde (PFA) for 15 minutes at RT or with methanol for 5 minutes at -20°C. After fixation cells were quenched with 10 mM ammonium chloride (not when cells were fixed with methanol) and further permeabilized with PBS containing 0.1% Triton X-100 (10 minutes at RT). After permeabilization, slides were blocked twice for five minutes, firstly with PBS containing 0.4% fish skin gelatine and 0.2% Tween 20, and secondly with PBS containing 2.5% normal goat serum and 0.2% Tween 20. The primary antibody was diluted in PBS containing 2.5% normal goat serum and 0.2% Tween 20 and incubated for 45 minutes at 37°C. Slides were further washed with PBS containing 0.2% Tween 20 and blocked a second time as described above. Fluorescently-labelled secondary antibodies (Alexa 488 and 594 [Molecular Probes, Invitrogen]) were diluted in PBS containing 2.5% normal goat serum and 0.2% Tween 20 and incubated for 30 minutes at 37°C. Slides were washed with PBS containing 0.2% Tween 20 and mounted with Vectashield (Vector Labs) mounting medium with DAPI. Slides were analyzed with a Leica TCS SP2 confocal microscope.

To detect HA and FLAG epitopes, mouse monoclonal anti-HA (Covance) and anti-FLAG polyclonal (Sigma) were diluted 1:500 and 1:200, respectively. V5 epitopes were detected using anti-V5 antibodies (Invitrogen) at 1:200 dilution. To detect JSRV/enJSRV Envs an antibody raised against the JSRV TM domain was used at a 1:100 dilution.

The Image J parameters used to analysed colocalization were for minimum ratio between channels: 0.5; red channel lower threshold: 100; green channel lower threshold: 100 (Abramoff, Magelhaes et al. 2004).

RT PCR

Total RNA from cultured cells was extracted using the Qiagen RNeasy mini kit following manufacture's instructions. Contaminating genomic DNA was removed by DNase treatment as follows. 10 µg of RNA were diluted in 25 to 100 µl volume using DEPC water and 2U/µl of DNase (DNase I RNase-free, Ambion) were added after the addition of 10X reaction buffer. Treatment was performed for one hour at 37°C. Thereafter, EDTA was added at a final concentration of 5 mM and the DNase activity was stopped by heat inactivation at 75°C for 5 minutes. cDNA was prepared using Omniscript reverse transcriptase from Qiagen. RNase inhibitors and oligod(T) used were obtained from Ambion and used at respectively 10U/µl and 10 µM.

The RT reaction was prepared as follows and performed for 1 hour at 37°C:

RNA	2 µg
10X buffer	2 µl
dNTPs (5mM)	2 µl
oligod(T) (10 µM)	2 µl
RNase inh. (10U/ µl)	1 µl
RT (4U/ µl)	1 µl
Water	up to 20 µl

5 µl of the cDNA was later used in the PCR reaction with specific primers.

Cell staining

Cells were stained with basic fuchsin/methylene blue for the visualization of cell-cell fusion. Cells were first washed three times with PBS and then covered for 20 min with a 1% methylene blue and 0.25% basic fuchsin in methanol solution. Thereafter the dishes were washed with water and examined.

oTr cells were stained with May-Grunwald and Giemsa to examine the appearance of BNCs. Briefly cells were washed three times with PBS and fixed with methanol for 15 minutes at RT. Cells were then stained with a 50% solution of May-Grunwald dye in buffered water (pH 6.8) for 5 minutes. After 3 washes with buffered water cells were stained with an 8% solution of Giemsa in buffered water for 8 minutes. The stain was removed after another three washes with buffered water.

Hydrophobic profile

The hydrophobic profile of enJSRV Envs and wild type JSRV Env was calculated by the Kyte and Doolittle method implemented by the Proscale program provided by Expasy.

Chapter 3

Summary

This chapter will describe studies aimed to understand: I) the role of the SU domain of the JSRV Env glycoprotein in the process of transformation of epithelial cells *in vitro*, and II) the interplay between the receptor tyrosine kinase RON and the Env protein of both exogenous and endogenous JSRVs.

Introduction

JSRV is a replication competent oncogenic retrovirus that induces transformation using different mechanisms from other oncoretroviruses. Its Env glycoprotein is a dominant oncoprotein that alone is able to transform a variety of cell lines *in vitro* (Maeda, Palmarini et al. 2001; Rai, Duh et al. 2001; Allen, Sherrill et al. 2002; Danilkovitch-Miagkova, Duh et al. 2003; Zavala, Pretto et al. 2003; Liu and Miller 2005). Moreover, the sole expression of the JSRV Env is capable of inducing lung adenocarcinomas in immunocompromised mice when expressed by adeno-associated virus vectors (Wootton, Halbert et al. 2005). More importantly, when the JSRV Env is expressed in immunocompetent sheep by a JSRV based vector it causes OPA in a high percentage of animals (Caporale, Cousens et al. 2006). Thus the JSRV Env can function as a powerful oncoprotein both *in vitro* and *in vivo*.

The mechanisms used by the JSRV Env to induce cell transformation are not completely understood and both receptor dependent and independent mechanisms have been proposed. In the receptor independent mechanisms the cytoplasmic tail (CT) of the TM of the JSRV Env is the main determinant of transformation (Palmarini, Maeda et al. 2001). In particular, a tyrosine in position 590, that is part of a SH-2 binding domain, has been shown to be critical for transformation although its influence in JSRV Env-induced cell transformation varies and depends on the amino acid substitution, the cell line used to perform the assays and experimental conditions (Liu, Wang et al. 2001; Palmarini, Maeda et al. 2001; Allen, Sherrill et al. 2002; Zavala, Pretto et al. 2003; Hofacre and Fan 2004; Liu and Miller 2005; Cousens, Maeda et al. 2007). The Ras-MEK-MAPK and the PI3K/Akt pathways have been implicated in JSRV-induced cell transformation but how JSRV Env engages the cell signalling network is not clear at the

moment (Maeda, Palmarini et al. 2001; Palmarini, Maeda et al. 2001; Liu and Miller 2005; Maeda, Fu et al. 2005; Caporale, Cousens et al. 2006; De Las Heras, Ortin et al. 2006). Akt activation has been found in several cell lines transformed by the JSRV Env and it seems to be dependent on Y590. Both PI3K dependent and independent mechanisms have been implicated in Akt activation (Liu, Lerman et al. 2003; Maeda, Inoshima et al. 2003), however its role *in vivo* has proven harder to demonstrate (De Las Heras, Ortin et al. 2006; Suau, Cottin et al. 2006).

JSRV Env receptor-dependent mechanisms have been proposed in the transformation of human BEAS-2B cells (Danilkovitch-Miagkova, Duh et al. 2003). HYAL-2 is a glycosylphosphatidylinositol (GPI)-anchor protein that functions as a cellular receptor for exogenous and endogenous JSRVs (Rai, Duh et al. 2001; Spencer, Mura et al. 2003). In humans, HYAL-2 is located in the p21.3 region of chromosome 3 (Rai, DeMartini et al. 2000) which is commonly deleted in lung and other types of human cancers (Lerman and Minna 2000; Petursdottir, Thorsteinsdottir et al. 2004), indicating the possible presence of tumour suppressor genes in this location. BEAS-2B cells are human bronchial epithelial cells immortalized with an adenovirus-12 SV40 hybrid virus (Reddel, Ke et al. 1988). It was shown in this cell line that HYAL-2 binds to the receptor tyrosine kinase RON leading to an inactive complex. However, when the JSRV Env is overexpressed, HYAL-2 binds to it, inducing their degradation and allowing the release of RON which then is able to dimerize, autophosphorylate and activate downstream signal pathways, including PI3K/Akt and MEK/MAPK pathways, that ultimately induce cell transformation (Danilkovitch-Miagkova, Duh et al. 2003). The fact that a dominant negative kinase inactive form of RON blocked transformation by the JSRV Env in these cells demonstrated that RON was required for transformation. The proposed model is illustrated in Figure 14. This model was also proposed as the mechanism for transformation of epithelial cells in general however, subsequent studies performed by the same group in Madin-Darby canine kidney cells (MDCK) did not find RON or canine Hyal-2 involved in the mechanism of transformation by the JSRV Env (Liu and Miller 2005). In addition, HYAL-2 was unable to modulate the basal or macrophage stimulating factor (MSP)-induced RON activity in 208F cells (Miller, Van Hoeven et al. 2004) arguing against a direct regulation of RON by HYAL-2. In addition both our group and other groups have been unable to transform BEAS-2B cells with the JSRV Env (Palmarini, M., personal communication).

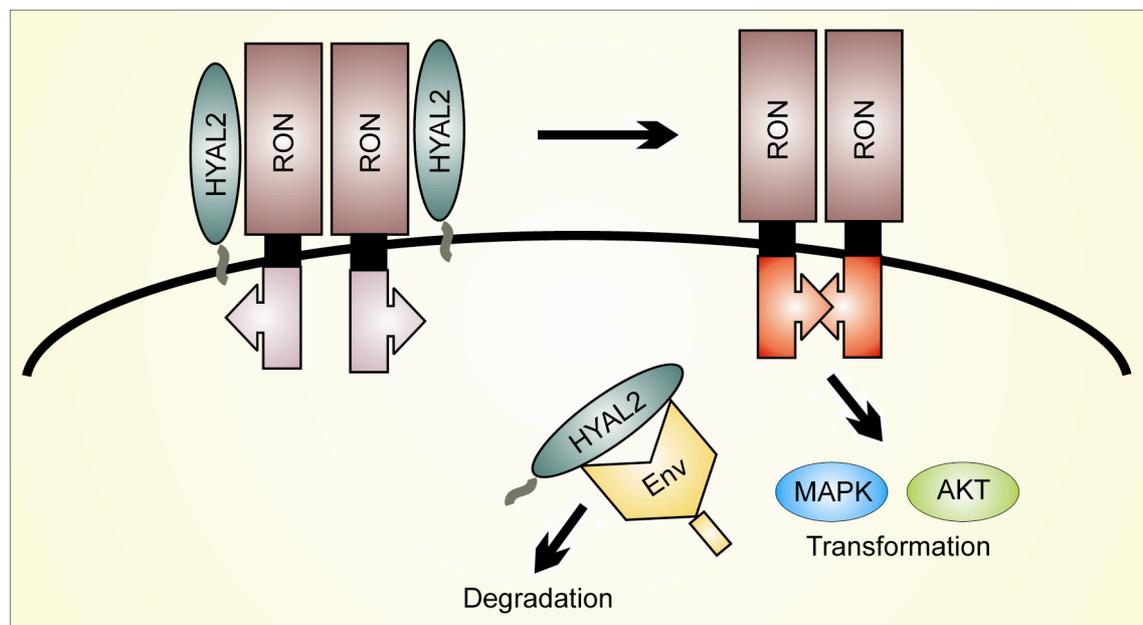


Figure 15. Model of JSRV Env mediated transformation of BEAS-2B cells.

RON is expressed at the cell membrane as a dimer and maintained in an inactive state thanks to its association with HYAL-2. Expression of the JSRV Env sequesters HYAL-2 and induces its degradation in a proteasomal-dependent manner. This allows the liberation of RON which undergoes conformational changes that trigger its catalytic activity and the activation of the Akt and MAPK pathways that lead to cell transformation. Adapted from Danilkovitch et al. 2003.

The receptor d'origine nantais, RON, is a receptor tyrosine kinase that structurally belongs to the MET proto-oncogene family, which contains only two members, MET and RON (Manning, Whyte et al. 2002). The RON protein is synthesized as a single chain precursor that matures at the cell membrane leading to a 180 kDA heterodimeric protein composed of a 40 kDA α -chain and a 150 kDA transmembrane β -chain with intrinsic kinase activity (Gaudino, Follenzi et al. 1994; Wang, Ronsin et al. 1994). It is mainly expressed in cells of epithelial origin and proteins highly homologous to it have been identified in mouse, chicken, xenopus and puffer fish (Wang, Yao et al. 2006). The only known ligand identified so far of RON is a serum-derived protein referred to as MSP (Gaudino, Follenzi et al. 1994; Wang, Ronsin et al. 1994). RON is required for normal embryogenesis since RON gene knockouts lead to the death of mouse embryos at early stages (Muraoka, Sun et al. 1999). RON overexpression has been identified in breast, colon, lung, bladder and ovarian human cancers and is usually correlated with increased activation and the presence of splice variants (Wang, Yao et al. 2006). At the moment there are six known spliced variants of RON and two have shown tumour-producing activity (Chen, Zhou et al. 2000; Wang, Kurtz et al. 2000; Zhou, He et al. 2003). The tumorigenic potential of RON has been demonstrated by mutational analysis of the kinase domain that confers RON the ability to cause cell transformation,

tumour growth and metastasis in nude mice (Santoro, Penengo et al. 1998; Williams, Longati et al. 1999; Peace, Hughes et al. 2001). Transgenic mice overexpressing RON in the distal lung epithelial cells develop multiple adenomas and adenocarcinomas, demonstrating its role in tumour formation *in vivo* (Chen, Zhou et al. 2002). RON has also been implicated in the induction of cell spreading, dissociation, migration, matrix invasion and tubular formation in a variety of cancerous cells as well as increasing the metastatic potential of tumours (Santoro, Penengo et al. 1998; Peace, Hughes et al. 2001) further demonstrating its role in the acquisition of malignant phenotypes.

The oncogenic potential of RON depends on the efficiency of the kinase activity and at the moment there are three mechanisms which can abnormally upregulate it: overexpression, mutation and truncation. Upon activation RON can transduce a variety of signal transduction pathways including SOS, Grb2, Ras, PI3K, MAPK/Erk1/2, JNK, β -catenin, FAK, integrins, Smad 2/3 and the NF- κ B complex via the formation of a multifunctional docking site through the phosphorylation of tyrosine residues in the carboxy terminal tail (Wang, Yao et al. 2006).

It is interesting to note that beside JSRV two replication defective retroviruses exert their transforming activity through the expression of part of their Env glycoproteins, via RON activation. However, both retroviruses possess, unlike JSRV, Envs that are not functional. The S13 avian erythroblastosis retrovirus is a replication defective oncogenic retrovirus that induces small fibrosarcomas and expansion of the erythroid compartment resulting in severe and fatal anaemia in chickens (Beug, Hayman et al. 1985). Interestingly, the oncogenic factor of this virus is a fusion protein composed by an extracellular and juxtamembrane domain encoded by the viral *env* gene and a cytosolic region encoded by c-SEA sequences, the chicken orthologue of RON (Hayman, Kitchener et al. 1985). The *env* encoded sequences promote dimerization and autophosphorylation and thus constitutive activation of the fusion protein leading to cell transformation (Smith, Vogt et al. 1989; Morimoto and Hayman 1994). Stk, the mouse counterpart of RON, is also involved in the pathogenicity of another retrovirus, the Friend spleen focus-forming virus (SFFV). SFFV is a replication incompetent retrovirus that induces erythroleukaemia in susceptible strains of mice. The major determinant of transformation of SFFV is the gp55 Env protein that has unique characteristics and associates with the erythropoietin receptor at the cell membrane allowing the proliferation of erythroid cells in the absence of erythropoietin (Ruscetti 1999). Susceptibility to SFFV is strain specific and depends on different host genes (Coffin,

Hughes et al. 1997). One of these genes is Fv-2 which encodes the Stk tyrosine kinase. Susceptible mice encode a short form of Stk (sf-Stk) from an internal promoter that lacks most of the extracellular domain (Persons, Paulson et al. 1999). It has been demonstrated that sf-Stk interacts with SFFV gp55 in haematopoietic cells expressing the erythropoietin receptor and that this interaction induces the activation of sf-Stk (Nishigaki, Thompson et al. 2001). Thus, it appears that SFFV induces its biological effects through the activation of sf-STK.

The role of the interactions between RON, HYAL-2 and the JSRV Env in JSRV induced cell transformation are not clear and warrant further investigation. In this section I will describe several experiments that demonstrate that the JSRV Env can transform epithelial cells independently from its cellular receptor. In addition, I will show that the JSRV Env associates with RON and that the cytoplasmic tail of the TM domain is the major determinant of the biological response of the RON-Env interaction.

Results

The JSRV Env transforms IEC-18 cells independently from the receptor binding domain

HYAL-2 has been found to negatively regulate RON in BEAS-2B cells. Thus, the interaction between the JSRV Env and HYAL-2 and their consequent intracytoplasmic degradation, has been deemed critical for RON activation and transformation (Danilkovitch-Miagkova, Duh et al. 2003). If this were the case, the Env of enJSRVs would also induce cell transformation because they use as well HYAL-2 as a cellular receptor (Spencer, Mura et al. 2003). It has been shown in previous studies that the Env of enJS5F16 does not induce cell transformation due to differences between exogenous and endogenous Env proteins that mapped in the CT (Palmarini, Gray et al. 2001; Palmarini, Maeda et al. 2001).

Initial experiments were performed using the BEAS-2B cells as this was the cell line used to demonstrate the involvement of RON in JSRV Env-induced transformation in the first place (Danilkovitch-Miagkova, Duh et al. 2003). However, we could not reliably induce transformation of BEAS-2B cells with the JSRV Env. Other groups, including the authors of the original paper also confirmed this difficulty in subsequent publications (Miller, Van Hoeven et al. 2004). Thus, we performed standard

transformation assays in another epithelial cell line, rat epithelial IEC-18 (Quaroni and Isselbacher 1981). Typical results obtained in transformation assays are shown in Figure 15 and Table 3 summarizes the data obtained. Two different types of transformation assays were performed. In the first set of experiments IEC-18 cells were transfected with expression plasmids of the wild type (pCMV3JS21ΔGP-neo), endogenous (pEnv-En2-neo), mutant (pCMV3JSΔGPY590D or F-neo) and chimeric (pMoMULVpr-r-JSE-neo) JSRV Envs that also encoded for the neomycin resistant gene. G418 resistant clones were selected and foci of transformed cells were counted four weeks after transfection. For the second set of experiments no G418 selection was used.

The transformation efficiency of the JSRV Env in IEC-18 was relatively low compared to other cell lines such as 208F cells or NIH/3T3. However, foci of elongated refractile cells that lost the epithelial phenotype of the parental cells were clearly visible four weeks after transfection (Figure 15 panel 1). The JSRV Env mutants Y590D and Y590F and the Env of enJS5F16 did not induce cell transformation (Figure 15 panels 3 to 6). On the other hand, a chimeric Env formed by the receptor binding domain (RBD) and the proline rich region of MoMLV with the remaining portions of SU and TM domain from JSRV (pMoMMLVpr-r-JSE) (Chow, Alberti et al. 2003) induced transformation with variable efficiency between experiments. In general however, foci of transformed cells induced by this chimeric Env were smaller in size with respect to those induced by the JSRV Env. We were unable to quantify the transfection efficiency of each individual *env* expression plasmid, thus comparisons regarding the ability of different construct to induce cell transformation should be raised with caution.

Foci from IEC-18 cells transformed by JSRV and the chimeric pMoMMLVpr-r-JSE Env were picked and expanded. The resulting lines consisted of refractile cells with a fibroblast-like phenotype that could be easily differentiated from the parental IEC-18 cells. Both cell lines were able to form colonies in soft agar (not shown) and in both of them increased levels of phosphorylated Akt with respect to the parental IEC-18 cell line were found (not shown). We could not detect phosphorylated RON in both JSRV and the chimeric pMoMMLVpr-r-JSE Env-transformed derived cell lines although RON expression in these cells (and in the parental IEC-18) was at the limits of detection with the available reagents (not shown).

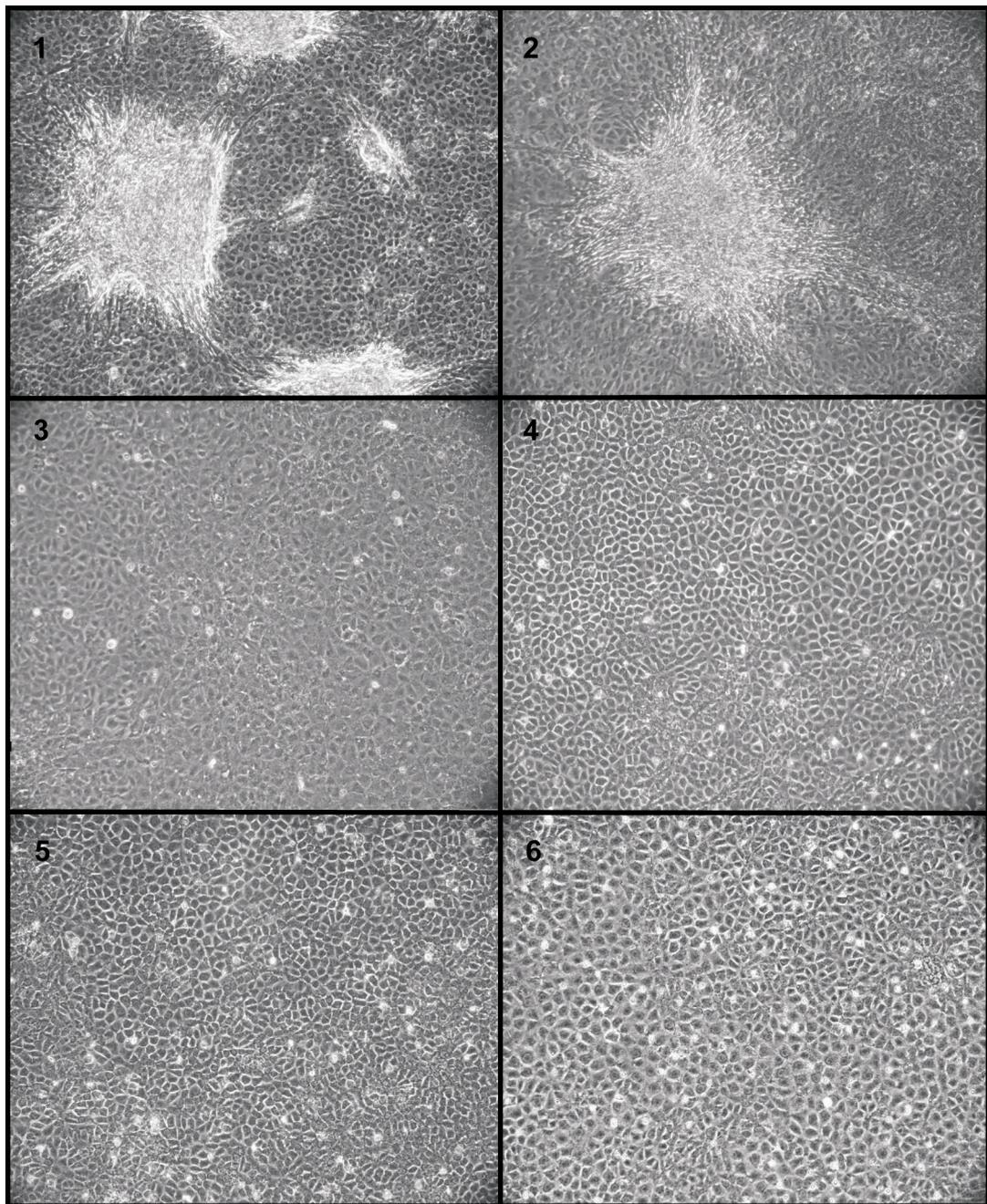


Figure 16. Transformation assays in rat epithelial cells (IEC-18).

IEC-18 cells were transfected, G418 selected and foci of transformed cells were counted four weeks after transfection. Wild type JSRV Env and MoMLVppr-JSE (JSRV-MoMLV chimeric Env) induced foci of transformed cells 1) and 2) respectively. Panels 3 to 6 show typical results obtained by transfecting IEC-18 cells with pCMV3JSΔGPY590D-neo, pCMV3JSΔGPY590F-neo, mock and pEnv-En2-neo (enJS5F16 Env expression plasmid) respectively.

Plasmid	Number of foci			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Mock	0	0	0	0
pCMV3ΔGP	n/a ²	n/a	18	21
pMoMLVprrr-JSE	n/a	n/a	4	10
pCMV3ΔGP-neo	32	5	n/a	n/a
pMoMLVprrr-JSE-neo	63	13	n/a	n/a
pCMV3ΔGPY590D-neo	0	0	n/a	n/a
pCMV3ΔGPY590F-neo	0	0	n/a	n/a
pEnv-En2-neo	0	0	n/a	n/a

Table 4. Transformation assays in IEC-18 cells.

JSRV, enJS56A1, chimeric MoMLV-JSRV and MoMLV Envs coimmunoprecipitate with RON

Next, we further investigated the nature of the JSRV Env-RON association considering that the model of Env induced transformation of BEAS-2B cells does not fit with the mechanisms of Env transformation of other epithelial cell lines (Liu and Miller 2005; Maeda, Fu et al. 2005).

We performed co-immunoprecipitation studies in human 293T cells by transfecting them with an expression plasmid of the human RON gene and expression plasmids of JSRV Env (JSE-F), JSRV Env mutant Y590D (JSEY590D-F), enJS5F16 Env (JSE-En2-F), chimeric MoMLV-JSRV Env (MoMLVprrrJSE-F), MoMLV Env (pCEE-F) and human HYAL-2 (F-hHYAL2). All the Env plasmids were tagged with the FLAG epitope at the carboxy terminal of the CT. HYAL-2, also tagged with a FLAG epitope but at its amino terminal portion, was used as a positive control and normal rabbit serum was used as a further immunoprecipitation control. All the Env proteins used in this study were able to associate with RON and co-immunoprecipitation was shown both using an anti-RON or an anti-FLAG serum (Figure 16) and different transfection reagents (not shown). Interestingly, only the full length Env proteins were pulled down by anti-RON antibodies suggesting that RON-Env interaction might occur co-translationally in the Golgi and this association interferes with Env cleavage. We noticed that while JSRV, enJSRV and MoMLV Env proteins used in this study are normally processed (into the SU and TM domains), we were not able to detect a processed TM for the MoMLV-JSRV Env chimera (bottom panel Figure 16). This is

² Not analysed.

not entirely surprising given the fact that this is a chimeric envelope protein formed by the receptor binding domain and proline rich region of MoMLV and the remaining portion of the SU and TM from JSRV (Chow, Alberti et al. 2003). We speculate that the folding of the SU and TM in this chimeric molecule hampers the access of the cleavage site to cellular proteases considering that the SU-TM boundary in this particular chimera is identical to the JSRV Env. The MoMLV-JSRV chimera has indeed a reduced ability to transform cells (with respect to JSRV) and grossly reduced infectivity when used to pseudotype retroviral vectors (Chow, Alberti et al. 2003). On the other hand, it has been shown that failure to cleave the MLV Env does not necessarily preclude its ability to reach the cell surface, incorporation into virion particles and mediate infection (Zavorotinskaya and Albritton 1999).

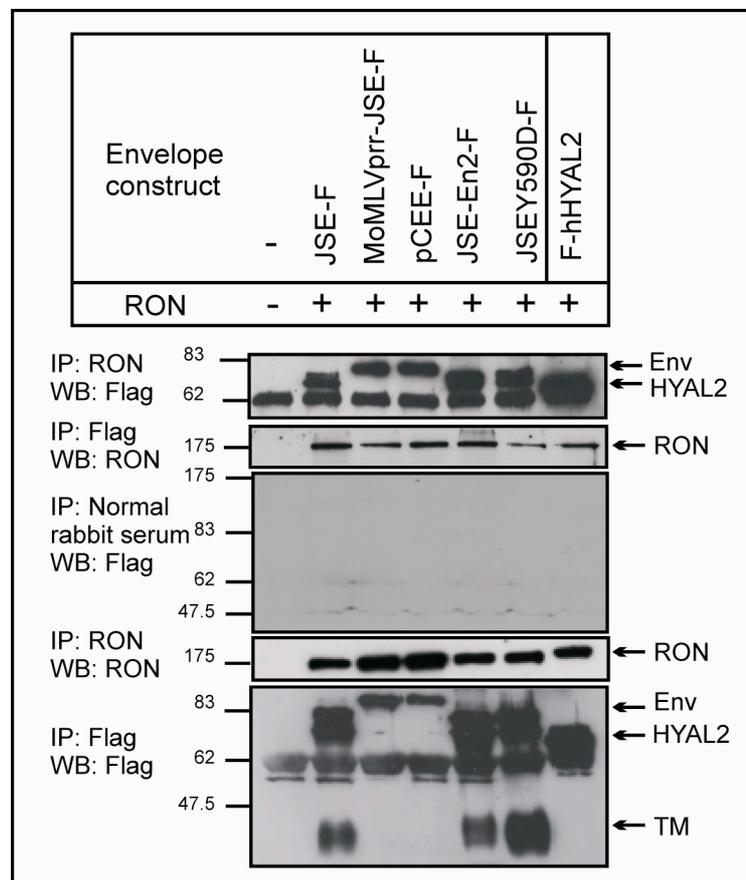


Figure 17. Wild type, endogenous, chimeric and mutant of the JSRV Env co-immunoprecipitate with RON.

293T cells were co-transfected with an expression plasmid for human RON and one of the following Env expression plasmids: wild type JSRV Env (JSE-F), chimeric JSRV-MoMLV Env (MoMLVpr-r-JSE-F), MoMLV Env (pCEE-F), enJS5F16 Env (JSE-En2-F), Y590D mutant (JSEY590D-F) and HYAL-2 (F-hHYAL2). Forty eight hours after transfection cells were lysed, and lysates were immunoprecipitated (IP) and analyzed by WB. Antisera used for IPs and WB are indicated beside each panel. Please note that the MoMLV Env (pCEE-F) is processed correctly but the TM domain (p15) is not visible in the figure because the gels shown are usually run for long periods of time in order to resolve HYAL-2 from the IgG heavy chain and better visualize proteins of high molecular weight such as RON.

It is also noticeable that two bands of similar molecular weight are present for the full length JSRV, enJS5F16 and JSRV Y590D Env proteins. This phenomenon is probably due to the immunoprecipitation of partially (newly synthesized) and fully glycosylated forms of the Env proteins.

All the Env proteins described above were still able to co-immunoprecipitate with RON if Triton-X-100 rather than SDS was used in the lysis buffer (Figure 17). Moreover, we observed co-immunoprecipitation when variable amounts of plasmid DNA were used in transfections as well as different amounts of proteins in the immunoprecipitates (not shown).

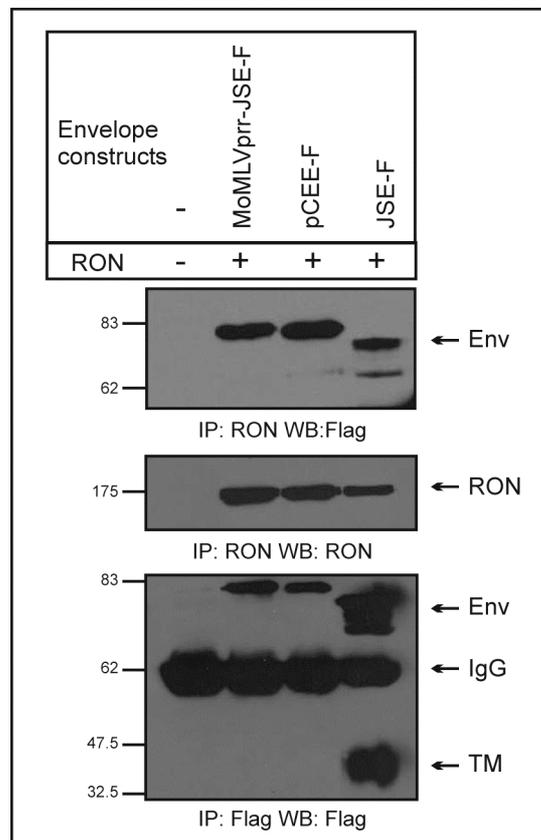


Figure 18. Various Envs bind RON when lysed with Triton-X-100 lysis buffer.

293T cells were co-transfected with an expression plasmid for human RON and one of the following Env expression plasmids: chimeric JSRV-MoMLV Env (MoMLVpr-JSE-F), MoMLV Env (pCEE-F) and wild type JSRV Env (JSE-F). Forty eight hours after transfection cells were lysed, and lysates were immunoprecipitated using a Triton-X100-based lysis buffer and analyzed by WB.

The interaction between RON and the JSRV is a bona fide association

We performed a series of experiments aimed to determine whether the co-immunoprecipitation of RON with the various Env proteins was due to a real association or to artefacts of the experimental conditions. First, we checked if the Env-RON interaction was preserved when membrane lipids rafts were disrupted by cholesterol depletion prior to cell lysis. This experiment was performed in order to rule out the possibility that the association found by co-immunoprecipitation was merely due to the presence of both proteins in the same membrane microdomain. As shown in Figure 18, all the Env used in this study associated with RON when lipid rafts were disrupted by treatment with methyl- β -cyclodextrin (Niu, Mitchell et al. 2002).

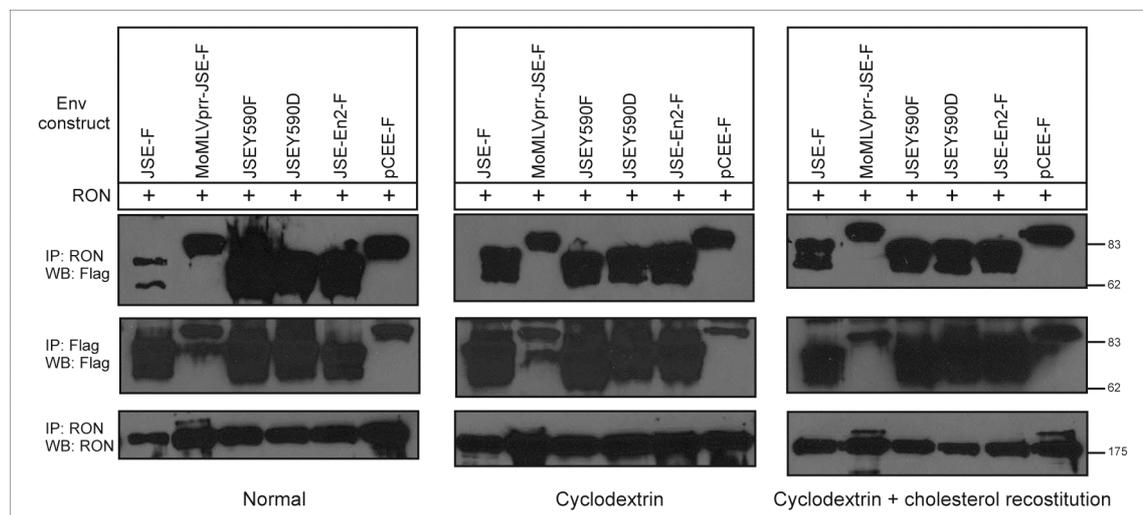


Figure 19. Lipid raft disruption does not affect the binding between RON and various JSRV Envs.

293T cells were transfected with RON and various Env expression plasmids in triplicate. One of the dishes was left untreated while the other two were incubated with 8 mM of Methyl- β -cyclodextrin for one hour at 37°C. One of the two treated dishes was cholesterol reconstituted by a further incubation with cholesterol as a control. Thereafter all the dishes were lysed, immunoprecipitated and analysed by WB as indicated for each panel.

Secondly, we increased the stringency of the co-immunoprecipitation of RON with the JSRV Env and other Env proteins by disrupting cells with SDS lysis buffer for 25 minutes at 37° C (Figure 19A) and by incubating the immunoprecipitates with increasing amounts of sodium chloride in the last washing step for 30 minutes at room temperature (Figure 19B). Under both experimental conditions all the tested proteins were still able to coimmunoprecipitate with RON.

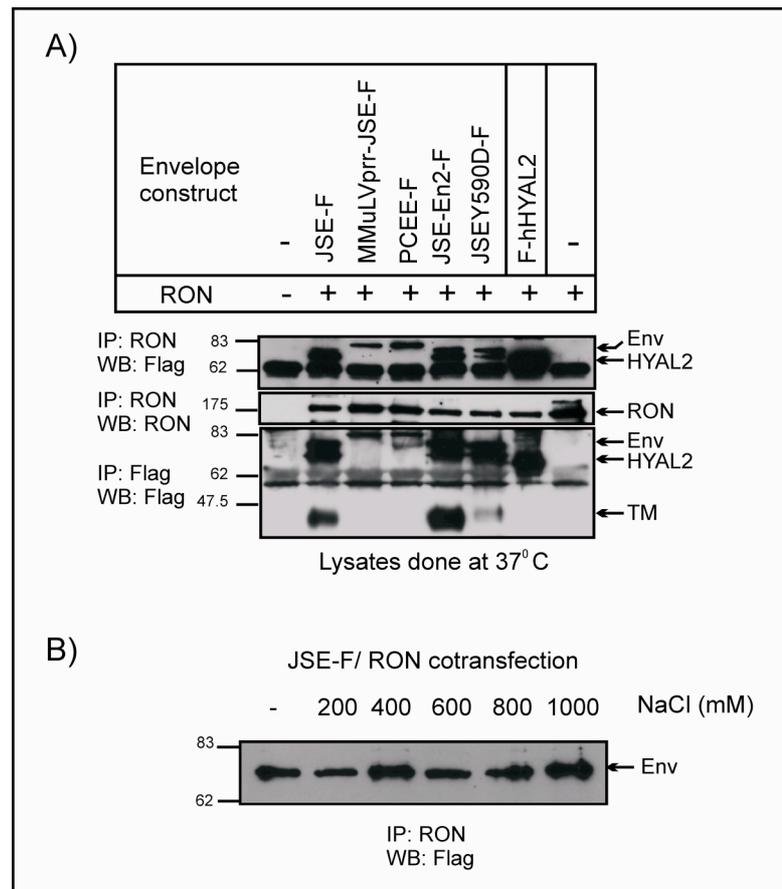


Figure 20. Stringent conditions do not modify the association between RON and Env proteins.

A) 293T cells were cotransfected as described in Materials and Methods. Forty eight hours after transfection cells were lysed at 37°C for 25 minutes. Cell lysates were then immunoprecipitated (IP) with RON or FLAG antibodies and analyzed by WB as indicated beside each panel. B) Immunoprecipitates from cells co-transfected with RON and JSE-F were washed with increasing amounts of sodium chloride for 30 minutes at room temperature and analyzed by WB as indicated.

Thirdly, we repeated the co-immunoprecipitation experiments described above replacing RON with another receptor tyrosine kinase, the epidermal growth factor receptor (EGFR). As shown in Figure 20A EGFR did not co-immunoprecipitate with any of the Env proteins employed above or with HYAL-2. An additional membrane protein, CD4, did not co-immunoprecipitate with RON (Figure 20B).

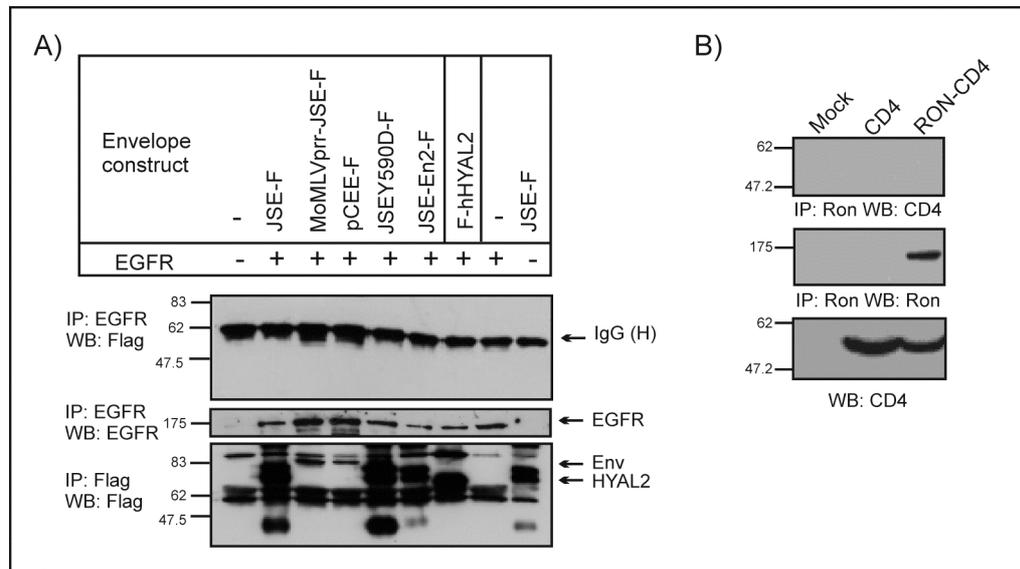


Figure 21. The JSRV Env does not co-immunoprecipitate with EGFR and RON does not co-immunoprecipitate with CD4.

293T cells were lysed, immunoprecipitated and analyzed as described for Figure 16.

We then investigated whether the RON-Env association could be specifically competed by the use of a JSRV Env plasmid without the FLAG epitope at the carboxy terminus (pCMV3JSΔGP, named JSE in the figures due to space restrictions). Co-immunoprecipitations were performed from lysates of cells co-transfected with fixed amounts of RON and JSE-F and increasing amounts of pCMV3JS21ΔGP (JSE). As shown in Figure 21A, untagged JSRV Env competes with tagged JSRV Env for its association with RON since the interaction between RON and JSE-F diminishes when high amounts of JSE are present in the lysates. We found also MoMLV Env to compete with the JSRV Env for RON association (data not shown). A similar experiment was performed from lysates of cells co-transfected with fixed amounts of RON tagged with the HA epitope and JSE-F and increasing amounts of non tagged RON. Figure 21B shows that the RON-HA/JSE-F association can be competed by untagged RON.

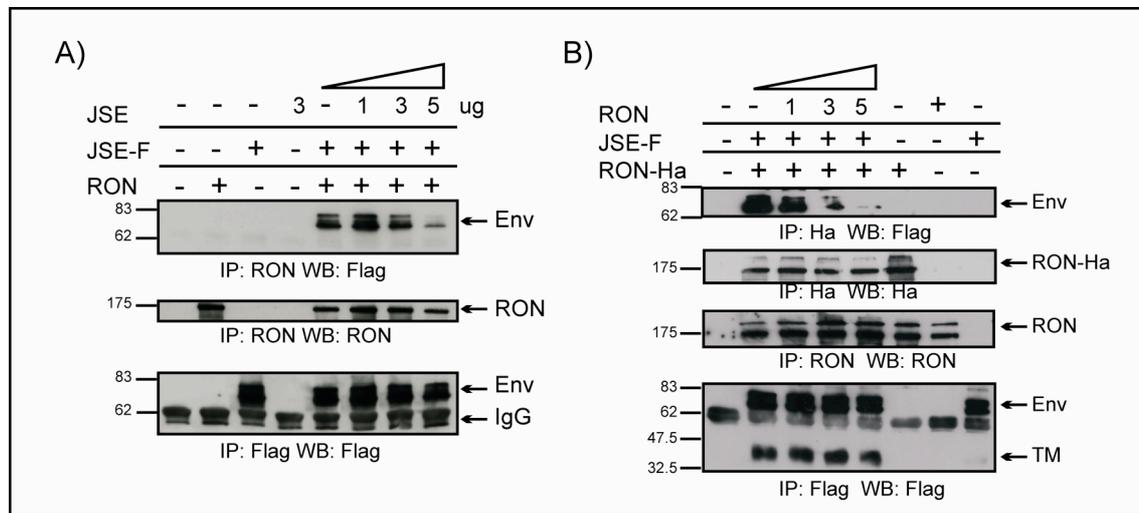


Figure 22. The binding between RON and the JSRV Env can be specifically competed.

293T cells were cotransfected with 3 μ g of RON (A) or 3 μ g of RON-HA (B) expression plasmids, 1 μ g of the expression plasmid for the JSRV Env tagged with the FLAG epitope (JSE-F) and increasing amounts of the non tagged JSRV Env (JSE) (panel A) or non tagged RON (panel B). Cells were lysed 48 hours after transfection. RON and RON-HA were immunoprecipitated using RON or HA antibodies, while the tagged JSRV Env was immunoprecipitated with FLAG antibodies and analyzed by WB as indicated below each panel. Note that RON-Ha cross-reacts also with anti-RON antibodies.

Many studies on the transforming properties of the JSRV Env have been performed in cell lines of mouse and rat origin. We then tested whether Stk, the mouse counterpart of RON, was also able to co-immunoprecipitate with the JSRV Env (and the other Envs employed in this study) when co-expressed in the same cells. As shown in Figure 22A, all the Env proteins employed in this study (and HYAL-2) co-immunoprecipitated with Stk. In agreement with the results with RON, the membrane protein CD4 did not co-immunoprecipitate with Stk (not shown). It was also tested whether the short form of Stk (sf-Stk) that lacks most of the extracellular domain but retains the transmembrane and tyrosine kinase sequences (Persons, Paulson et al. 1999) was able to associate with the different Envs. sf-Stk was unable to co-immunoprecipitate with the JSRV Env and other Env proteins (Figure 22B) suggesting that the RON/Stk extracellular domain is necessary to associate with the JSRV Env.

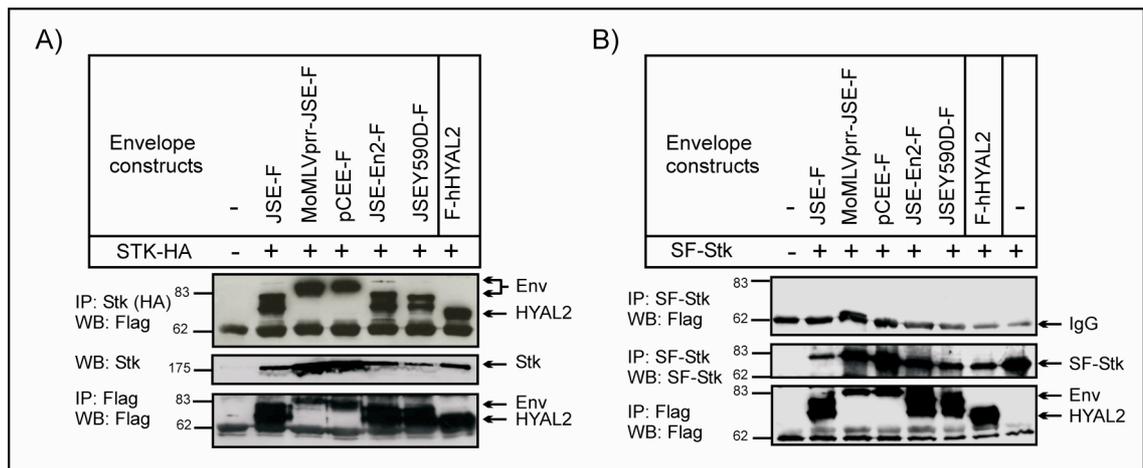


Figure 23. Stk but not sf-Stk co-immunoprecipitate with the various JSRV Envs tested.

293T cells were cotransfected as in Figure 16 with the exception that expression plasmids for mouse Stk (panel A) or SF-Stk (panel B) tagged with the HA epitope were used instead of RON. Cells were lysed, immunoprecipitated (IP) and analyzed by WB with the antibodies indicated beside each panel.

The surface domain of the JSRV Env is not required to associate with RON

By the use of a truncated form of the JSRV Env (Δ GPSU Δ 103-352 HA) that has a large deletion in the SU domain (Hofacre and Fan 2004) we tested whether the SU is required for the interaction with RON. Co-immunoprecipitations were performed with lysates of 293T cells transfected with RON and Δ GPSU Δ 103-352 HA and analysed by WB. As shown in Figure 23 the SU truncated form of the JSRV Env co-immunoprecipitated with RON.

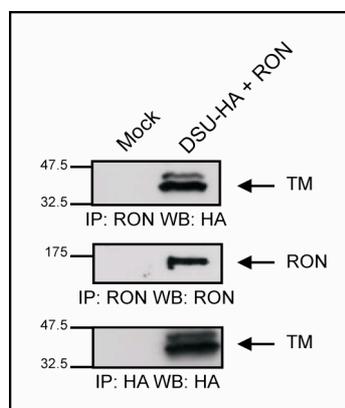


Figure 24. The SU of the JSRV Env is not required for the interaction with RON.

The sole expression of RON is not sufficient to allow entry of JSRV into NIH 3T3 cells

NIH 3T3 cells stably expressing the human RON protein were prepared and used in standard entry assays using MLV retrovirus vectors pseudotyped with the JSRV Env. As shown in Table 4, the expression of RON did not render these cells permissive for JSRV entry. Figure 24 shows RON expression in cells used to perform the entry assay.

	Exp. 1	Exp. 2	Exp.3	Average
NIH 3T3	10^2	6.5	0.75	$3.6 \cdot 10^1$
NIH 3T3- RON	$4.5 \cdot 10^2$	$2.9 \cdot 10^2$	5	$2.2 \cdot 10^2$
NIH 3T3-HYAL2	$1.7 \cdot 10^6$	$4.9 \cdot 10^5$	$4.3 \cdot 10^5$	$8.7 \cdot 10^5$

Table 5. Entry assays in NIH 3T3 RON-expressing cells.

NIH 3T3 cells expressing human RON or HYAL-2 were transduced with retroviral vectors expressing alkaline phosphatase and pseudotyped with the JSRV Env. Results are expressed as alkaline phosphatase foci per ml.



Figure 25. RON expression in NIH 3T3-RON cells.

NIH 3T3, NIH 3T3-RON and NIH 3T3-HYAL2 cells were lysed, immunoprecipitated and analysed by WB using anti-RON antibodies.

All the experiments described above point to the fact that RON is a promiscuous protein and, besides binding to HYAL-2, also associates with the Envs of JSRV, enJSRVs, JSRV mutant Y590D, MoMLV and MoMLV/JSRV chimeric Env protein. However, JSRV cannot use RON as cellular receptor or enhance transformation in 208F cells (data not shown) similarly to what has been shown by other authors in dog MDCK cells (Liu and Miller 2005).

JSRV and enJSRV Envs co-localize with RON

Since RON and the various JSRV Envs were found to interact in co-immunoprecipitation studies, we performed confocal microscopy to determine whether they co-localize when co-expressed in the same cells. HeLa cells were transfected with an expression plasmid of the human RON protein and wild type JSRV Env tagged with the V5 epitope at the carboxy terminus (JSE-V5) or the Env of enJS5F16 tagged with the Flag epitope (also at the carboxy terminus) and subsequently analysed by immunofluorescence (IF) and confocal microscopy. JSRV and enJS5F16 JSRV Env proteins showed membrane and broad cytoplasmic distribution, consistent with a protein synthesized in the endoplasmic reticulum and transported to the cell membrane. Co-localization of RON with both, JSE-V5 and JSE-En2-F was observed at the cell membrane and in the cytoplasm as shown in Figure 25.

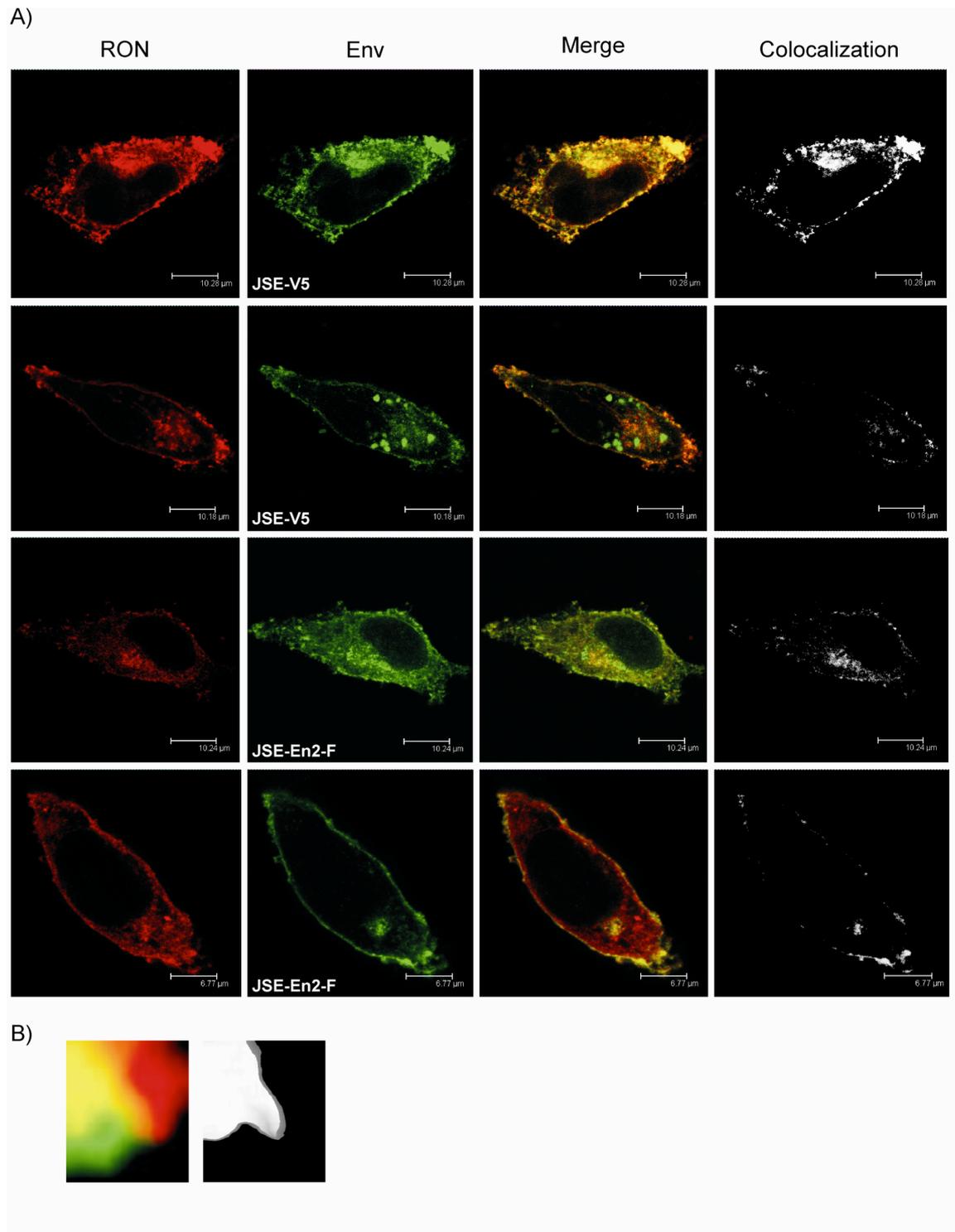


Figure 26. The JSRV Env co-localizes with RON.

A) Photomicrographs showing representative examples of the major phenotypes observed in cells expressing JSE-V5 or JSE-En2-F and RON. Specific staining for all proteins was mainly observed at the cell surface or diffused within the cytoplasm. Co-localization is especially observed at the cell membrane. Right panels show areas of co-localization in white determined by RGB Image J analysis. B) Co-localization parameters as determined by RGB Image J analysis. First panel shows all red and green colours. Second panel shows co-localization in black and white.

Endogenous but not exogenous JSRV Env inhibits RON autophosphorylation

Next we investigated whether direct Env-RON association could lead to RON activation. We performed co-transfection experiments using different amounts of RON expression plasmid that would either lead to its autophosphorylation (3 μ g) or that would keep RON expression detectable but with no or limited signs of activation (100ng – 1 μ g) under our experimental conditions.

We were unable to detect any increase in RON phosphorylation when 293 cells were co-transfected with 1 μ g of RON and increasing amounts of the various Env expression plasmids (not shown). Thus the JSRV Env (or any other Env used in this study) was not able by itself to induce RON activation under our experimental conditions. However, by co-transfecting 293 cells with 3 μ g of RON expression plasmid, an amount of DNA sufficient to induce its autophosphorylation, and increasing amounts of Env or HYAL-2 expression plasmids we discovered a different outcome resulting from the interaction of RON with the various Env proteins (Figure 26). Wild type JSRV Env and MoMLV Env did not affect RON autophosphorylation (Figure 26A-B). HYAL-2 blocked RON activation as shown before by other authors (Figure 26C) (Danilkovitch-Miagkova, Duh et al. 2003). Surprisingly, enJS5F16-Env blocked RON autophosphorylation too. The degree of the block of RON phosphorylation by enJS5F16 was variable (as shown in Figure 26D-E) but we could not identify any technical reason behind this variability. JSEY590D displayed an intermediate phenotype between the JSRV and enJS5F16 Env (Figure 26F). As shown in Figure 27 the same results were obtained when Stk was used instead of RON.

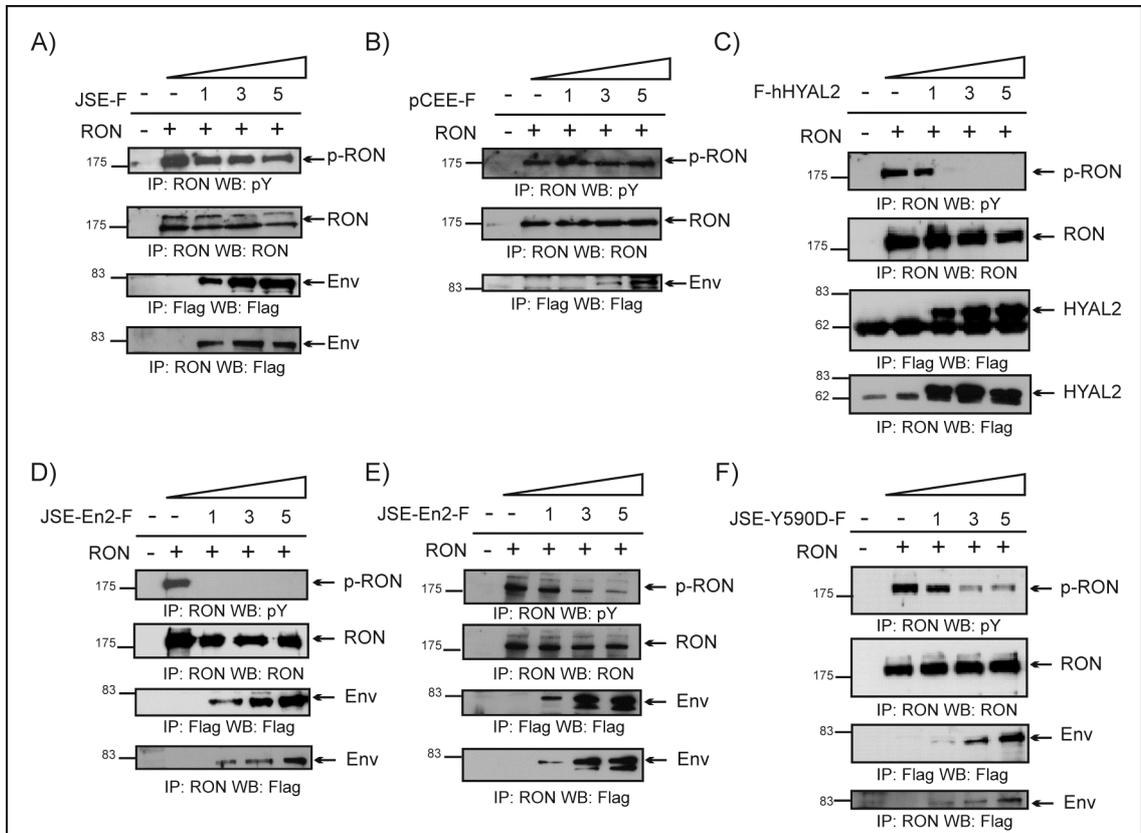


Figure 27. The cytoplasmic tail of various Envs used in this study modulates the biological effects of the RON-Env interaction.

293 cells were cotransfected with 3 μ g of the RON expression plasmid and increasing amounts of expression plasmids for the various Envs tagged with a FLAG epitope at the carboxy terminus and HYAL-2 as a control. Forty eight hours after transfection, cells were serum starved overnight and lysed. Lysates were immunoprecipitated and analyzed by WB as indicated below each panel. D) and E) show two independent experiments where the enJS5F16 Env blocks RON phosphorylation at variable degrees.

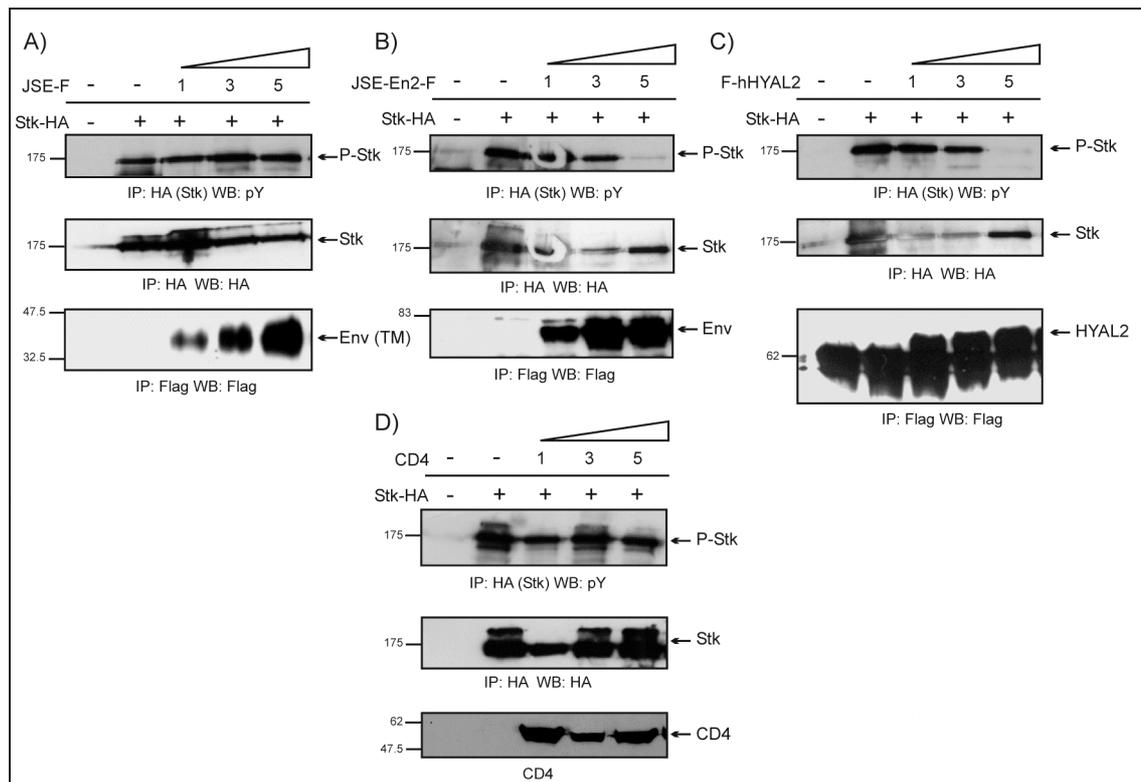


Figure 28. The cytoplasmic tail of various JSRV Envs modulates the biological effects of the Stk-Env interaction.

293 cells were cotransfected with 3 μ g of the Stk expression plasmid tagged with the HA epitope and increasing amounts of expression plasmids for the various Envs tagged with a FLAG epitope at the carboxy terminus. HYAL-2 and CD4 were used as controls considering that the former but not the latter blocks Stk phosphorylation. Forty eight hours after transfection, cells were serum starved overnight and lysed. Lysates were immunoprecipitated and analyzed by WB as indicated below each panel.

These experiments suggest that the CT of the TM domain of the JSRV/enJS5F16 Env (and Y590 in particular) influences the effects of the interaction with RON despite the fact that both, JSRV and enJS5F16, utilize HYAL-2 as cellular receptor (Rai, Duh et al. 2001; Spencer, Mura et al. 2003). Furthermore, these experiments reinforce the concept that RON can associate with the JSRV Env. To further support the hypothesis that the JSRV Env interacts with RON and that the effects on RON phosphorylation are HYAL-2 independent, RON activation was measured in the presence of the JSRV or enJS5F16 Env or HYAL-2. In agreement with previous studies, when increasing amounts of the JSRV Env are co-transfected with fixed amounts of RON and HYAL-2 expression plasmids, RON autophosphorylation increases accordingly (Figure 28A) (Danilkovitch-Miagkova, Duh et al. 2003). The authors state that this phenomenon takes place because the binding of the JSRV Env to HYAL-2 liberates RON from the inhibitory effects of the latter (Danilkovitch-Miagkova, Duh et al. 2003). When the same experiment was performed with the enJS5F16 Env, a decrease in RON phosphorylation was detected

(Figure 28B). These observations support the idea that: I) the JSRV Env can associate with RON directly; II) that the effects in RON phosphorylation mediated by the CT of the JSRV Env are HYAL-2 independent, since they take place regardless of its presence; and III) that transformation of BEAS-2B cells mediated by the JSRV Env could involve the activation of RON but occurs by another mechanism than the one proposed by Danilkovitch-Miagkova et. al. As mentioned before, unfortunately our group and others (Miller, Van Hoeven et al. 2004; Maeda, Fu et al. 2005) were unable to reproduce the results of transformation of BEAS-2B cells with the JSRV Env due to the high level of background that did not allow us to distinguish transformed from non-transformed cells. Thus, the role of RON and HYAL-2 in JSRV Env-induced transformation of BEAS-2B cells remains elusive.

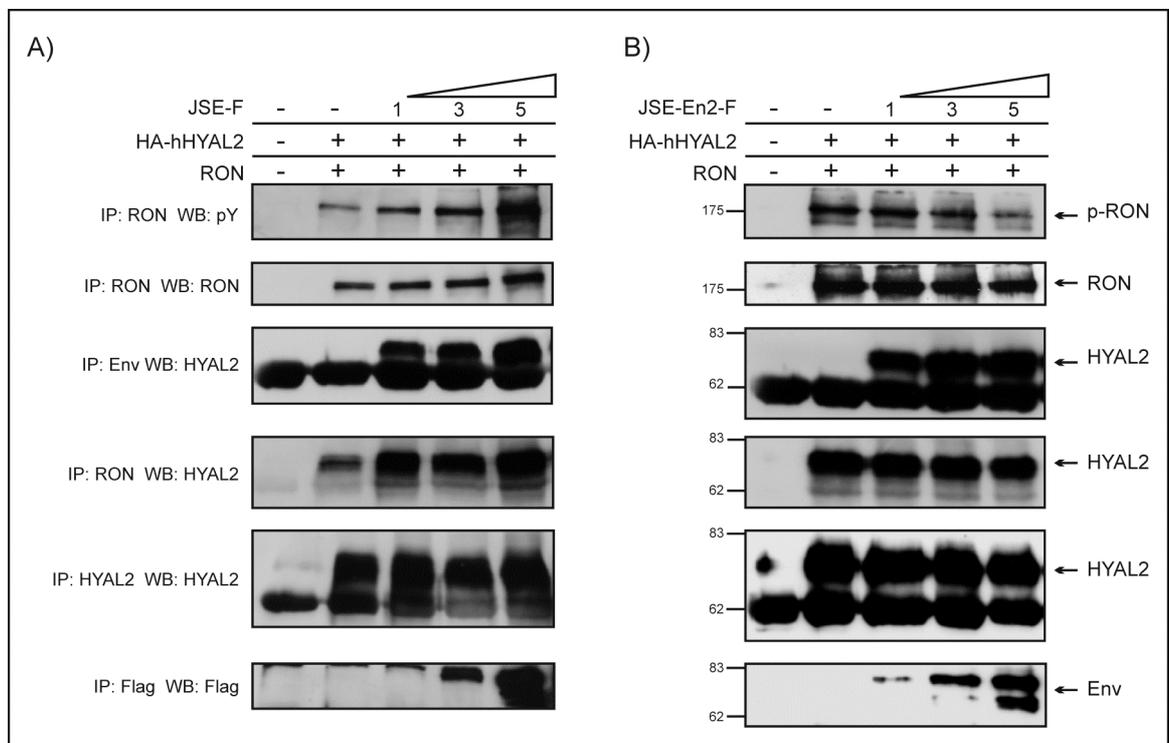


Figure 29. The Env of enJS5F16 blocks RON activation even in the presence of HYAL2.

293 cells were transfected with 5 μ g of RON, 5 μ g of HYAL2 (tagged with the HA epitope at the amino terminus) expression plasmids and increasing amounts of the JSRV or enJS5F16 Envs (tagged with the Flag epitope at the carboxy terminus). Forty eight hours after transfection, cells were serum starved and treated with a proteasome inhibitor (ALLN) overnight to preserve the Env-HYAL2 interaction and lysed. Cell lysates were immunoprecipitated and analyzed by WB as indicated beside each panel.

Discussion

In this section we showed that the interaction between the JSRV Env, HYAL-2 and RON tyrosine kinase is more complex than previously thought. We demonstrated that the JSRV Env can associate with RON and the CT of the JSRV/enJS5F16 Env influences the biological effects of Env-RON association. Furthermore, data obtained in IEC-18 cells suggests that transformation of epithelial cells by the JSRV Env is likely to occur independently of an interaction with HYAL-2 and through a different mechanism than the one proposed by Danilkovitch-Miagkova, 2003. A model of JSRV Env-induced transformation of epithelial cells has been suggested based on the results obtained in the human bronchial epithelial cell line BEAS-2B by these authors. The model suggests that the JSRV Env induces transformation of epithelial cells by sequestering HYAL-2 and inducing its degradation, allowing the release of RON from the inhibitory association with HYAL-2 and its subsequent activation (Danilkovitch-Miagkova, Duh et al. 2003). Thus, transformation of epithelial cells by the JSRV Env was thought to occur through a receptor-dependent mechanism distinct from the essentially receptor-independent mechanism seen in virus-induced transformation of fibroblasts. However, JSRV induced transformation also of the canine epithelial cell line MDCK (Liu and Miller 2005) and rat epithelial RK3E cells (Maeda, Fu et al. 2005) with mechanisms similar to those observed in fibroblasts and largely dependent upon the CT of the Env. In addition, adeno-associated vectors overexpressing the JSRV Env induced lung adenocarcinomas in immunocompromized mice (Wootton, Halbert et al. 2005) despite the fact that mouse HYAL-2 does not bind the JSRV Env (Rai, Duh et al. 2001; Liu, Duh et al. 2003).

The rat epithelial cell line, IEC-18, was used in this study and we determined that the interaction of the JSRV Env with HYAL-2 is not critical to induce cell transformation of epithelial cell lines because: I) chimeric MoMLVpr-r-JSE bearing the receptor binding domain of MoMLV (and consequently not using HYAL-2 as cellular receptor) induced cell transformation (Chow, Alberti et al. 2003); while II) the Env of the JSRV-related endogenous retrovirus enJS5F16 (that uses HYAL2 as cellular receptor) (Spencer, Mura et al. 2003) did not induce cell transformation. Thus, as a whole, it appears from the results of this and previous studies that transformation of epithelial cells is mainly HYAL-2 independent (Liu and Miller 2005; Maeda, Fu et al. 2005; Wootton, Halbert et al. 2005).

Given the apparent differences between transformation of BEAS-2B cells and other epithelial cell lines including the one used in this study we decided to investigate further the interaction of the JSRV Env with RON tyrosine kinase. Initially, we confirmed the results of previous studies showing that RON binds HYAL-2 and that this interaction inhibits RON autophosphorylation. However, we also found that the JSRV Env co-immunoprecipitates with RON. We took great care in controlling for artefacts of the immunoprecipitation assays since the envelope of MoMLV, another retrovirus highly divergent from JSRV, also co-immunoprecipitated with RON. A major concern was the possibility that both RON and Env are targeted to membrane lipid rafts and the results of the co-immunoprecipitations do not reflect a real association but merely the fact that the proteins tested are present in the same membrane micro domain that is not disrupted by our lysis procedures. Thus lysates and co-immunoprecipitations were performed with lysis buffers containing different detergents and under different conditions (Figures 16 and 17 and not shown). It was also demonstrated that lipid raft disruption by cholesterol depletion did not alter the binding between the various Envs and RON. In addition, we showed that the Env-RON association is maintained if co-immunoprecipitations are performed from cells lysed at 37°C in SDS-based lysis buffer and if the immunoprecipitates are washed with increasing concentrations of salts. Furthermore, the JSRV/enJS56A1 Env co-localize with RON at the cell membrane and in the cytoplasm as determined by confocal microscopy. Applying the same experimental conditions used in the Env/RON co-immunoprecipitations, we also determined that the JSRV Env (and other Env proteins described in this study) do not bind EGFR (Downward, Yarden et al. 1984), another receptor tyrosine kinase, and that RON does not bind CD4, a membrane protein.

Most importantly, JSRV and enJSRV Env proteins had a different biological effect on RON phosphorylation. The non-oncogenic enJS5F16 Env blocked RON autophosphorylation while the JSRV Env did not affect it at all. The main differences between the JSRV and enJS5F16 Env proteins are present in the CT of the TM domain (Palmarini, Hallwirth et al. 2000). Indeed, JSRV Env mutant JSEY590D that contains a single amino acid mutation in the CT (Palmarini, Maeda et al. 2001), also blocked RON autophosphorylation although to a lesser degree than the endogenous enJS5F16 Env. These results strongly suggest that the CT of the JSRV/enJSRVs Env modulates the biological effect of RON-Env interaction. It is interesting to note that RON generates downstream signals mainly through phosphorylation of two sites in its CT (Ponzetto,

Bardelli et al. 1994); thus a possible interaction between the CTs of RON and Env could be envisaged.

This study, however, has not been able to demonstrate that the JSRV Env interacts with RON in a biologically relevant system, such as in transformed cells. We failed, with the available reagents, to show RON activation in IEC-18 cells, similar to other studies in MDCK and 208F cells (Miller, Van Hoeven et al. 2004; Liu and Miller 2005). The same reagents were also not able to detect Ron expression, and thus Ron activation, in lysates of lung tumour samples from OPA affected animals by WB, which is likely due to the inability of the available antibodies to recognise sheep Ron (not shown). Overexpression of proteins in a transient transfection system can lead to artefacts that have to be interpreted with caution. RON has been shown to associate with a variety of proteins including MET, EGFR, integrins and adhesion proteins (Danilkovitch, Miller et al. 1999; Follenzi, Bakovic et al. 2000; Peace, Hill et al. 2003). Unfortunately RON activation is quite difficult to detect *in vivo* and in most studies RON/Stk activation is shown in cells that overexpress it (either transiently or stably)(Wang, Iwama et al. 1995; Follenzi, Bakovic et al. 2000; Rai, Duh et al. 2001; Peace, Hill et al. 2003; Angeloni, Danilkovitch-Miagkova et al. 2004; Miller, Van Hoeven et al. 2004; van den Akker, van Dijk et al. 2004; Yokoyama, Ischenko et al. 2005). Thus, it is unclear if the association between JSRV Env and RON has a biological significance. However, the experimental conditions employed in this study have also confirmed the previously described data on RON-HYAL-2 association and the downregulation of RON by HYAL-2 (Danilkovitch-Miagkova, Duh et al. 2003). However, in side-by-side experiments, we have also showed that the envelope of a JSRV-related endogenous retrovirus can inhibit RON autophosphorylation. Thus, it appears that as a whole, the biological relevance of RON-HYAL-2-Env association in JSRV-induced cell transformation needs still to be clarified.

The block of RON autophosphorylation by enJSRVs Env is striking and appears to be determined by differences between the CTs of JSRV and enJS5F16 Env. Possibly, the CT of enJS5F16 masks (directly or via other interacting proteins) RON phosphorylation sites. Further studies will be necessary to investigate the mechanisms of enJS5F16 Env induced-block of RON phosphorylation.

The interaction between RON and the Env of enJSRVs could be relevant in conceptus development and implantation. enJSRVs are highly expressed in a spatial and temporal

regulated fashion in the genital tract of the ewe, in particular in the binucleate cells of the conceptus trophoblast (Dunlap, Palmarini et al. 2005). When enJSRV Env expression is blocked *in utero* using morpholino antisense oligonucleotides, trophoectoderm growth and differentiation is severely impaired (Dunlap, Palmarini et al. 2006). On the other hand, Ron^{-/-} mice embryos fail to survive after periimplantation (Muraoka, Sun et al. 1999) and Ron transcripts have been found within the trophoblast cell mass of E3.5 embryos and in the giant trophoblast cells of the placenta (Hess, Waltz et al. 2003), suggesting a possible role in implantation. At the moment it is not clear which particular enJSRV loci are transcriptionally active in the sheep placenta. It is also unknown whether all of the enJSRV loci express Env proteins that elicit the same block in RON phosphorylation as shown in this study for the Env of enJS5F16. However, the alternative expression of different enJSRV loci with the ability to inactivate RON at variable degrees in a spatial and temporal fashion could contribute to the fine tuning that lies behind the modulation of its activation. Moreover, it could be speculated that enJSRV Envs could also regulate the activation of other receptor tyrosine kinases and signalling molecules that are important in conceptus development, implantation and placenta morphogenesis.

The association of the JSRV Env with RON might have some effect on the overall pathogenesis of JSRV infection in sheep. It has been recently demonstrated that clinical disease is not the most common outcome of JSRV infection during the commercial lifespan of sheep (Caporale, Centorame et al. 2005). Thus, the association of the JSRV Env with RON might not be critical for transformation but might have some influence on JSRV pathogenesis. For example, JSRV has been found to infect adhering cells/macrophages (Holland, Palmarini et al. 1999) whose activation is also known to be mediated by RON (Correll, Morrison et al. 2004). Further studies will be necessary to investigate this point.

Chapter 4

Summary

This chapter will describe the experimental work performed with the objectives of: I) identifying signalling pathways involved in JSRV Env-induced cell transformation; and II) to establish the basis for the use of OPA as a large animal model for lung cancer.

Introduction

The understanding of the molecular mechanisms governing pulmonary oncogenesis has increased tremendously throughout the last decade (Minna, Roth et al. 2002). However, lung cancer is still the most common cause of death of cancer patients worldwide and its survival rate after 5 years is extremely poor, highlighting the urgent need for the development of better therapies and early detection strategies (Parkin, Bray et al. 2005). To this end, appropriate animal models can be of great help in understanding the molecular basis of lung cancer, designing candidate therapeutic interventions, new surgical procedures and testing novel imaging technologies for early diagnosis.

A variety of mouse models are available for lung cancer (Dutt and Wong 2006). Transgenic and especially “conditional” mouse models, had a dramatic effect in understanding the contribution of oncogenes in the onset and maintenance of cancer (Varmus, Pao et al. 2005). In the pre-clinical settings, treatment of xenograft mouse models is routinely the first step used to test new anticancer drugs. However, most anticancer drugs fail in phase I and II clinical trials (Rothenberg, Carbone et al. 2003).

Neoplasms of domestic animals are not extensively used as cancer models. The large body of knowledge in mouse genetics, the possibility to manipulate their genome and the availability of biological reagents make rodents the natural choice as disease model organisms. Large and domestic animals are more difficult and generally more expensive to manage compared to mice or rats. However, the completion of the sequencing of the genome of several domestic animal species and the development of new cloning and transgenic techniques open the possibility to explore other animal species as cancer models (Khanna, Lindblad-Toh et al. 2006).

Among retroviruses, JSRV follows unique mechanisms to induce cell transformation, since its Env glycoprotein functions as a dominant oncoprotein both *in vitro* (Palmarini and Fan 2003) (Palmarini, Sharp et al. 1999; Maeda, Palmarini et al. 2001; Rai, Duh et al. 2001; Allen, Sherrill et al. 2002) and *in vivo* (Wootton, Halbert et al. 2005; Caporale, Cousens et al. 2006). The molecular mechanisms underlying JSRV Env-induced transformation have not been fully characterized but several pieces of evidence point to the involvement of the Ras-MEK-MAPK and PI3K-AKT pathways (Palmarini, Maeda et al. 2001; Maeda, Fu et al. 2005; De Las Heras, Ortin et al. 2006).

OPA shares some similarities with some forms of human lung adenocarcinomas (Palmarini and Fan 2001; Mornex, Thivolet et al. 2003). In addition, OPA has several features suggesting that it can be developed into a useful animal model for lung cancer: I) sheep and humans have a comparable lung size and tumour to body mass ratio; II) tumours in OPA can grow for a long time in the presence of a functional immune system; III) the disease is experimentally reproducible (Sharp, Angus et al. 1983; Palmarini and Fan 2003) and the location/extent of the induced lesions can be modulated by the use of replication defective viruses delivered to specific sites with an intrabronchial delivery (Caporale, Cousens et al. 2006).

In this section I will describe an array of experiments aimed at identifying signalling pathways involved in JSRV mediated transformation and to establish the basis for the use of OPA as a model to study the effects of small molecule inhibitors in cancer development. Data is provided showing that several Hsp90 inhibitors efficiently block transformation of rodent fibroblasts by the JSRV Env and revert the phenotype of cells already transformed by this oncoprotein. This phenomenon was due at least in part to Akt degradation, which is normally activated in JSRV-mediated transformation (Palmarini, Maeda et al. 2001; Caporale, Cousens et al. 2006). Importantly, Hsp90 was found expressed in tumour cells of sheep with naturally occurring OPA and Hsp90 inhibitors reduced proliferation of primary and immortalized cell lines derived from OPA tumours. Targeting of the Hsp90 molecular chaperone has great potential for cancer therapy (Workman 2004). Thus, OPA could be used as a large animal model for comprehensive studies investigating the effects of Hsp90 inhibitors.

Results

Effects of signal transduction inhibitors in JSRV-induced cell transformation of rodent fibroblasts

The first goal of these studies was to identify inhibitors of signal transduction pathways that efficiently blocked JSRV Env-induced cell transformation. We assessed a total of 22 inhibitors in two different experimental settings. In the first series of experiments, we used a cell line transformed by the JSRV Env (208F-tr) and determined whether the addition of various inhibitors reverted the phenotype of the transformed cells to the parental cell line. In the second set of experiments, we performed standard transformation assays in 208F cells by transfecting an expression plasmid for the JSRV Env (pCMV3JS21ΔGP) and cultured them in the presence or absence of each inhibitor for 15 days when the number of foci of transformed cells was counted. In the first set of experiments, each inhibitor was used at a concentration corresponding to respectively 10X and 1X its reported IC₅₀. The second set of experiments was performed using the highest concentration of each inhibitor that was found not to induce toxicity. Each experiment was repeated at least twice. Results obtained are summarized in Table 5.

Inhibitors against the Janus protein kinase (JAKs), vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) did not affect transformation by the JSRV Env since no or minimal reduction in the number of foci was observed in cultures treated with inhibitors compared to the control ones treated with DMSO. Inhibitors against platelet-derived growth factor receptor (PDGF) reduced the number of transformed foci induced by the JSRV Env from 30 to 60% as compared with cells treated with DMSO alone. However, the PDGF inhibitors used had a noticeable toxic effect in 208F cells and consequently the reduction in the number of transformed foci could be due simply to this phenomenon. Neither the PDGF inhibitors nor the inhibitors mentioned above were able to revert the phenotype of 208-tr. These data indicate that signalling through the JAKs, VEGF receptor, PDGF receptor and EGFR do not play a major role in JSRV induced cell transformation of rodent fibroblasts.

Pathway	Inhibitor name	Inhibitor concentration (uM)	Inhibition of transformation ³ (%)	Reversion of transformed phenotype	
Janus protein tyrosine kinase	JAK inhibitor I	0.001	N/A ⁴	No	
		0.005	N/A	No	
		0.01	N/A	No	
		0.015	N/A	No	
		0.05	N/A	No	
		0.15	0	No	
VEGFR	VEGF receptor 2 kinase inhibitor III	1	0	No	
		20	N/A	Toxic	
SRC (family)	PP2	5	41	No	
		10	73.4	No	
	PP1	0.0015		No	
		0.015	0	No	
	Genistein	25	11.9	No	
	Inhibitor I	0.044	N/A	No	
		0.088	N/A	No	
		0.44	N/A	No	
		0.88	0	No	
	Inhibitor II	1.2	0	No	
		12	Toxic	Toxic	
	Emodin	0.018	N/A	No	
		18.5	0	No	
		185	N/A	Toxic	
	SU 6656	0.28	N/A	No	
1		22	N/A		
Lavendustin C	2.8	65.9	Yes		
	2	4	No		
Damnacanthal	0.017	N/A	No		
	0.17	0	No		
Platelet derived growth factor receptor (PDGF)	Inhibitor I	0.2	N/A	No	
		2	N/A	No	
	Inhibitor II	1.1	N/A	No	
		11	57.5	No	
	Inhibitor III	0.05	N/A	No	
		0.08	20 ⁵	No	
		0.5	N/A	No	
		0.8	0	No	
	AG 1296	1	N/A	No	
		10	N/A	No	
AG 2043	1	N/A	No		
	10	29.2	No		
EGFR	PD 153035	0.000025	N/A	No	
		0.00025	0	No	
	AG 1478	0.003	N/A	No	
		0.03	0	No	
Herbimycin A	0.2	96	N/A		
	0.4	100	Yes		
HSP 90	Geldanamycin	0.0017	0	N/A	
		0.0075	74	N/A	
		0.0187	90	N/A	
		0.0375	97.9	Yes	
		0.075	Toxic	Yes	
		0.75	Toxic	Yes	
		Radicicol	0.27	N/A	Yes
			2.7	55.7	Yes
17-DMAG	0.5	88.8	Yes		

Table 6. Effect of inhibitors in JSRV-induced cell transformation of 208F cells.

³ The percentage of inhibition of transformation represents the average of at least two experiments and was calculated comparing the dishes treated with inhibitor versus DMSO.

⁴ not analysed.

⁵ Toxicity

SRC contributes to JSRV-induced cell transformation

As shown in Table 5, seven of nine inhibitors against the Src family of non receptor tyrosine kinases neither reverted the phenotype of 208F-tr cells nor reduced the number of foci of transformed cells in standard JSRV Env transformation assays. However, SU6656 reverted the transformed phenotype of 208F-tr cells to a flatter and less translucent morphology and slightly reduced transformation. In addition, when transformation assays were performed in the presence of PP2 the number of foci of transformed cells induced by the JSRV Env was drastically reduced (~70%).

The differences on the effects seen among the various Src inhibitors are not surprising since the specificity and potency towards each Src family member varies (Blake, Broome et al. 2000; Karni, Mizrachi et al. 2003). In addition, PP2 was shown previously to have an effect on JSRV Env-induced cell transformation (Hull and Fan 2006). To further understand the role of Src in JSRV Env mediated transformation we co-transfected 208F cells with the expression plasmid for the JSRV Env (pCMV3JS21ΔGP) and increasing amounts of a dominant negative form of Src (SrcMF) (Timpson, Jones et al. 2001). As shown in Figure 29, we found a dose dependent inhibition of JSRV Env-induced transformation by SrcMF. As a whole the data described above suggest that Src may be involved in the mechanisms of JSRV Env-induced cell transformation.

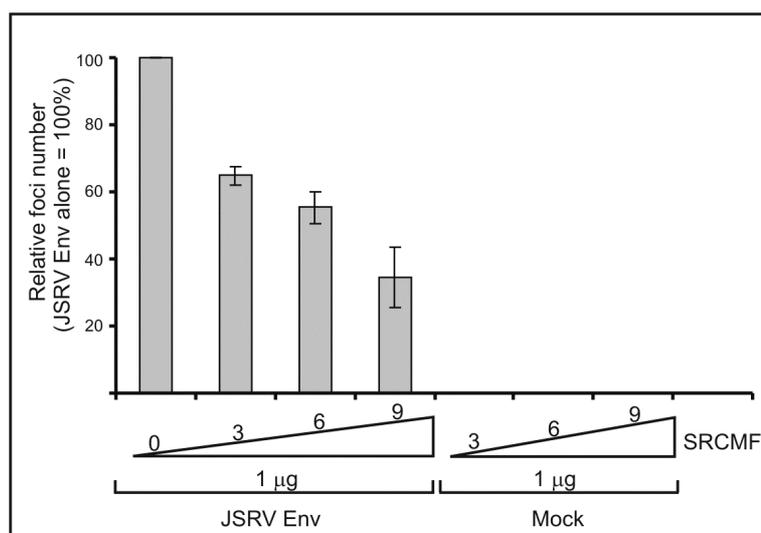


Figure 30. Src contributes to JSRV Env induced cell transformation.

208F cells were co-transfected with 1 μg of an expression plasmid for the JSRV Env and increasing amounts of a plasmid expressing a dominant negative form of Src (SrcMF). Results show the average of two independent experiments and are expressed as a percentage of transformation where the number of foci resulting from transfection of 1 μg of JSRV Env alone is considered 100%. Bars represent standard deviation.

Hsp90 inhibitors block transformation by the JSRV Env.

We next examined several Hsp90 inhibitors including herbimycin A (HA), geldanamycin (GA), radicicol and 17-DMAG. All the above mentioned inhibitors suppressed transformation in a dose-dependent manner (Table 5) and reverted the transformed phenotype of 208F-tr cells to a flatter and less translucent morphology compared to control 208-tr cells (Figure 30). Once the drugs were removed from the culturing media, cells returned to display their original transformed phenotype demonstrating that the drugs had no effect on integration and expression of the JSRV Env plasmid (Figure 30 bottom panel). These results indicate that Hsp90 is involved in the initiation and progression of the transformation process mediated by the JSRV Env as well as in the maintenance of the transformed phenotype *in vitro*.

Hsp90 is a molecular chaperone that participates in the folding, assembly, maturation and stabilization of “client” proteins including a variety of signalling molecules and transcription factors that are crucial for oncogenesis such as AKT, HER2, c-SRC, NFκB, IGFR1, p53 and RAF among others (Zhang and Burrows 2004). Hsp90 inhibition leads to proteasome-dependent degradation of its client proteins and the consequent disruption of critical pathways involved in tumour progression and survival. Hsp90 suppression could conduce to the simultaneous disruption of multiple signal pathways, ergo Hsp90 inhibitors are promising therapeutic reagents (Blagg and Kerr 2006). In particular, 17-AAG has completed Phase I (Goetz, Toft et al. 2005) and Phase II trials (Ronnen, Kondagunta et al. 2006).

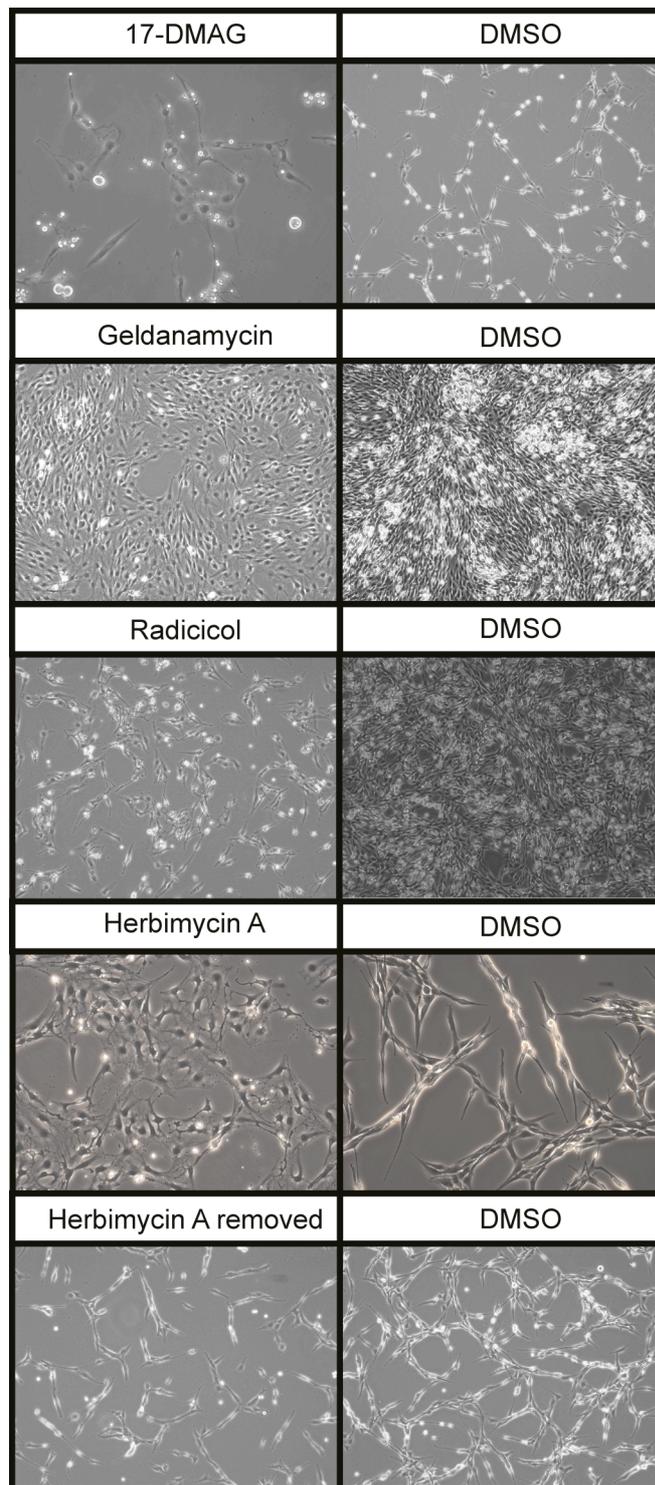


Figure 31. Reversion of the transformed phenotype of 208F-tr cells.

208-tr cells derive from a focus of cells transformed by the JSRV Env. These cells were cultured in the presence of Hsp90 inhibitors (or DMSO as negative control) and their morphology was monitored for 5 days. Illustrative examples of 208-tr cells cultured at higher or lower density in the presence of Hsp90 inhibitors are shown.

To further understand the mechanisms underlying the effects of Hsp90 inhibitors in JSRV-transformed cells, we examined whether the JSRV Env was an Hsp90 client protein. If this was the case, the block in transformation and the reversion of the

transformed phenotype seen with the various Hsp90 inhibitors would be due to the association of Hsp90 with the JSRV Env followed by proteosomal degradation. To this end, we assessed the expression of the JSRV Env by WB in total cell lysates extracted from transformed 208F-tr cells or from 208F-tr cells that reverted to a flatter morphology in the presence of Hsp90 inhibitors (Figure 31A). We could not detect down-regulation of the JSRV Env in 208F-tr cells when the phenotype was reverted to a more flat morphology in the presence of geldanamycin or herbimycin A. Moreover, we did not find association between the JSRV Env and Hsp90 by co-immunoprecipitation assays performed under different stringency conditions (Figure 31B) strongly suggesting that the JSRV Env is not an Hsp90 client protein.

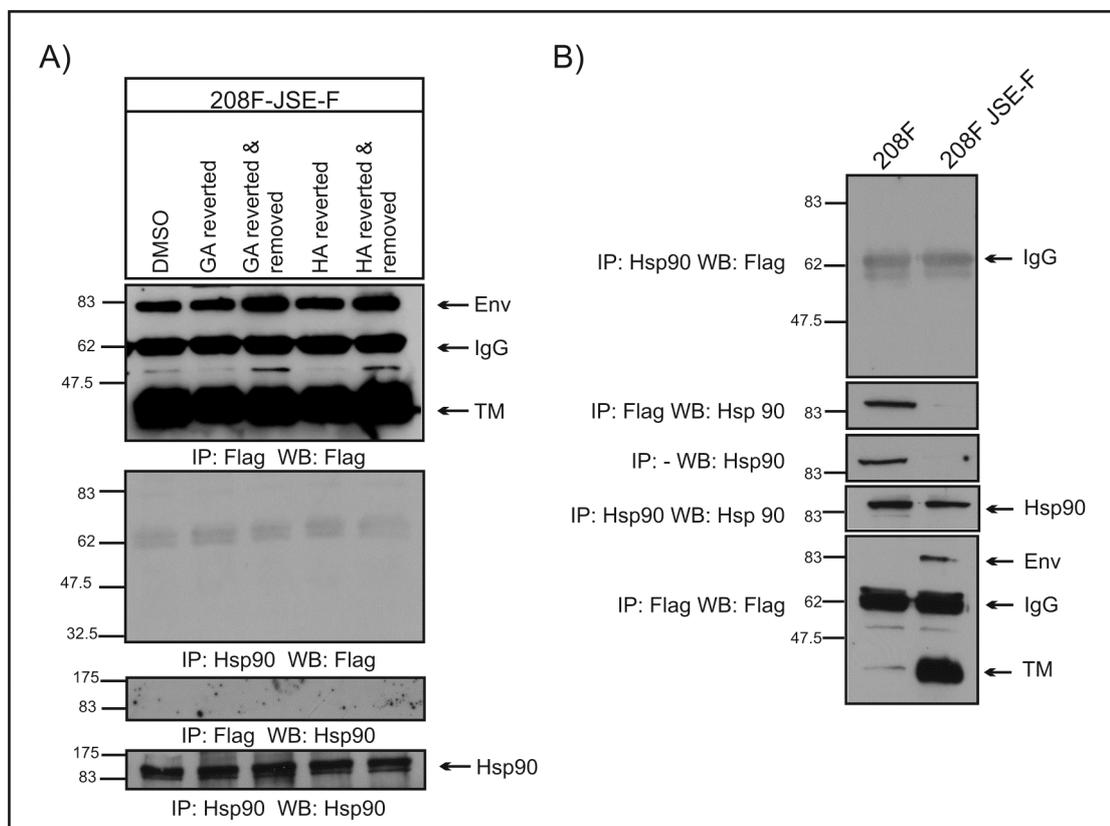


Figure 32. The JSRV Env does not associate with Hsp90 and it is not degraded by Hsp90 inhibitors.

A) Total cell extracts were obtained from 208F-tr cells in the presence of DMSO (negative control) or from 208F-tr that had reverted their transformed phenotype in the presence of geldanamycin (GA) or herbimycin A (HA). In addition, cell extracts were also obtained from 208F-tr that reverted to the transformed phenotype once GA or HA were removed from the culturing media (GA reverted & removed). 200 μ g of cell extracts were immunoprecipitated and analysed by WB as indicated below each panel. Note that the JSRV Env expressed in 208F-tr is tagged with a FLAG epitope at its carboxy terminus. TM indicates the transmembrane domain of the Env glycoprotein. B) Lysates of normal and 208F-tr cells (expressing the JSRV Env tagged with the flag epitope) were lysed and immunoprecipitated with milder detergents to preserve weak protein-protein interactions and analysed by WB as indicated beside each panel. A protein of approximately 90 kDa binds non specifically to the agarose beads since it was detected in samples where no antibody was added (third panel).

Hsp90 inhibitors induce Akt degradation.

Akt is an Hsp90 client protein and the association between Hsp90 and Akt modulates the kinase activity of the latter (Sato, Fujita et al. 2000). Akt activation plays an important role in JSRV Env-mediated transformation of 208F cells (Palmarini, Maeda et al. 2001; Chow, Alberti et al. 2003; Liu, Lerman et al. 2003). Considering that the JSRV Env itself is not an Hsp90 client protein we tested whether Hsp90 inhibitors caused changes in the expression (or phosphorylation status) of Akt in Env-transformed cells. To address this point, we cultured 208F-tr cells in serum free media with the addition of 17-DMAG (0.5 μ M) (or DMSO as control) for a period of 3, 6, 12 and 24 hours. Thereafter, total cell lysates were analysed by WB. We observed time dependent Akt degradation and dephosphorylation at serine 473 when cells were cultured with 17-DMAG while no changes were seen in the expression of the JSRV Env or γ -tubulin that was used as loading control (Figure 32). No changes in the phosphorylation status or expression of Akt or the JSRV Env were observed and no changes in the transformed morphology of these cells were noticeable when cells were cultured with DMSO as a control. Akt degradation was observed when the same experiment was performed in the presence of radicicol, while no changes were noticeable in the level of expression of the JSRV Env or γ -tubulin (not shown). These data indicate that the reversion of the transformed phenotype seen with the Hsp90 inhibitors could be due at least in part to the degradation of Akt.

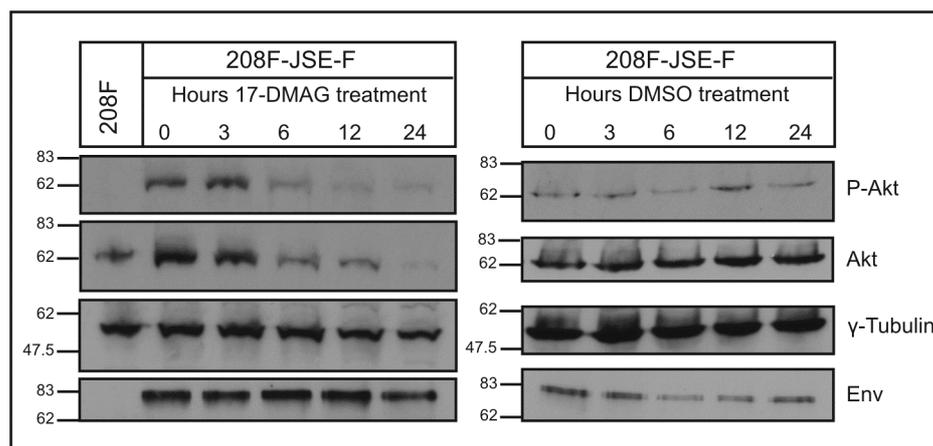


Figure 33. Hsp90 inhibitors induce AKT degradation and dephosphorylation in 208F transformed by the JSRV Env.

208F-tr cells were cultured in serum free media with the addition of 17-DMAG or DMSO as a negative control for 3, 6, 12 and 24 hours and cells were lysed and analysed by WB for the presence of Akt and pAkt. Detection of γ -tubulin was used as loading control.

Hsp90 inhibitors reduce proliferation of OPA-derived immortalized and primary cell lines.

In order to better assess the effects of Hsp90 inhibitors on JSRV-induced transformation we analyzed their effects on the growth of tumour cells derived from OPA lesions. JS8 is an immortalized cell line derived from a lung tumour of a sheep affected by OPA (Jassim 1988). Cells were grown for 72 hours in the presence of increasing amounts of radicicol and 17-DMAG and their proliferation was measured as described in materials and methods. We found statistically significant inhibition ($p=0.0002$) in cell proliferation when cells were grown in the presence of 17-DMAG and radicicol at all the concentrations tested (Figure 33). Although the reduction in proliferation was modest it should be taken into consideration that this cell line has been passaged extensively and does not express JSRV viral particles in the supernatants (data not shown).

Moreover, a significant reduction ($p=0.04$) in the growth of tumoural alveolar type II cells from naturally occurring OPA cases as compared to the normal type II pneumocytes was observed in the presence of increasing amounts of radicicol while the effects of 17-DMAG were more variable (not shown). The normal type II pneumocytes used in these proliferation assays were found to express markers such as SP-A, SP-C and presented lamellar bodies by electron microscopy (Archer, Jacquier et al. 2007). Tumour cells were confirmed to express JSRV by the detection of reverse transcriptase activity in the culture supernatants and the detection of the viral major capsid protein by WB (Archer, Jacquier et al. 2007).

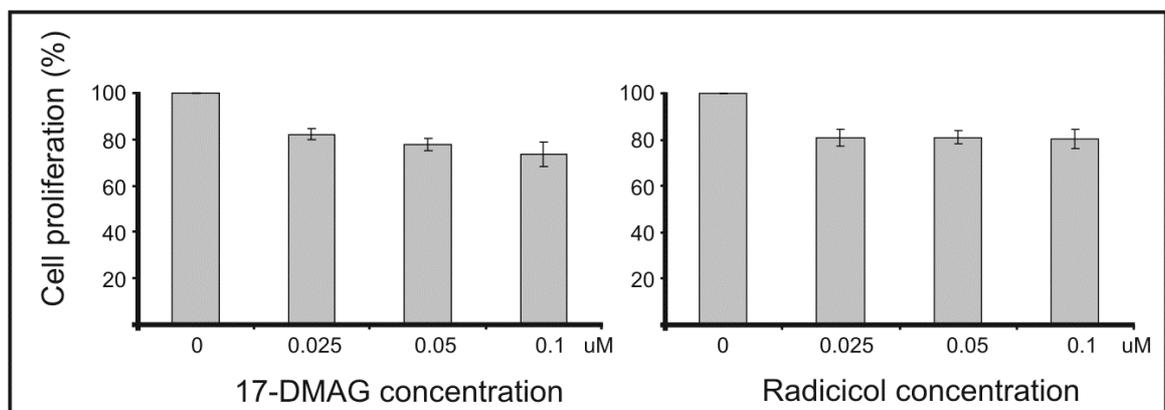


Figure 34. Hsp90 inhibitors reduce proliferation of OPA derived cells.

17-DMAG and radicicol significantly reduce ($p 0.0002$) proliferation of JS8 cells. Bars in both panels represent standard deviation.

Discussion

The aim of this study was to identify signalling pathways involved in JSRV induced cell transformation by the use of drugs that could efficiently block transformation by the JSRV Env *in vitro* and to establish the functional basis for the development of OPA as a large animal model for lung cancer. JSRV is unique among oncogenic retroviruses because its envelope glycoprotein functions as a dominant oncoprotein (Palmarini, Sharp et al. 1999; Maeda, Palmarini et al. 2001; Rai, Duh et al. 2001; Allen, Sherrill et al. 2002; Palmarini and Fan 2003). Transfection of a variety of cell lines with expression plasmids for the JSRV Env readily results in the induction of foci of transformed cells. In addition, adeno-associated viral vectors expressing the JSRV Env induce lung cancer in immunosuppressed mice (Wootton, Halbert et al. 2005). Furthermore, replication defective JSRV vectors expressing only the viral Env induce lung cancer in sheep, the natural host of JSRV infection (Caporale, Cousens et al. 2006). Thus, the JSRV/OPA model is an excellent system where the significance of findings obtained *in vitro* can be immediately translated *in vivo*.

We found that the Src pathway and the molecular chaperon Hsp90 are involved in the mechanisms of cell transformation induced by the JSRV Env and that OPA could be used as an alternative large animal model for the development of Hsp90 inhibitors and the study of the molecular mechanisms underlying their effects in cancer development. These conclusions are based on the fact that various Hsp90 inhibitors efficiently blocked transformation by the JSRV Env and reverted the morphology of cells already transformed by it and we could in part understand the mechanism behind these effects. We found that the JSRV Env is not an Hsp90 client protein considering that I) Hsp90 and the JSRV Env do not co-immunoprecipitate and II) Hsp90 inhibitors do not affect the levels of expression of the JSRV Env in 208-tr cells with a phenotype reverted to a flatter untransformed morphology. We also demonstrated that Hsp90 inhibitors reduced the levels of Akt expression in 208F cells transformed by the JSRV Env. Activation of the PI3K/ Akt pathway is one of the features displayed by cells transformed by the JSRV Env and the inhibitory effects of the Hsp90 inhibitors in this system could be due, at least in part, to Akt degradation. Akt is activated in a variety of cell lines transformed by the JSRV Env (Palmarini, Maeda et al. 2001; Liu, Lerman et al. 2003; Zavala, Pretto et al. 2003; Varela, Chow et al. 2006) and in cell lines derived from lung tissues of sheep affected by OPA (Suau, Cottin et al. 2006) thus, the results obtained here are in agreement with the important role played by Akt in JSRV induced cell transformation.

The Akt kinases (Akt1, Akt2 and Akt3) are critical intermediate molecules in signalling pathways that are involved in the regulation of cell proliferation and survival by inactivating proapoptotic factors like BAD, procaspase-9 and Forkhead (Hennessy, Smith et al. 2005). Several human cancers including breast, gastric and renal cancer, among others, display Akt activation that has been correlated with poor prognosis and/or advance disease (Altomare and Testa 2005). Importantly, several studies demonstrate the involvement of Akt in human lung carcinogenesis, in particular 67 % of samples of non-small cell lung cancer (NSLC) showed immuno-reactivity for phosphorylated AKT (Lee, Kim et al. 2002). Moreover, cell lines derived from NSLC showed activated AKT that promoted cell survival and resistance to chemotherapy (Brognard, Clark et al. 2001). Thus, therapies targeting Akt could be effective in the treatment of lung cancer. However, since Akt is involved in the regulation of many physiological processes its inhibition could lead to serious side effects and a consequent low therapeutic index. OPA could provide a useful system to study the efficacy as well as the short and long term effects of these therapies.

Finally, we demonstrated proliferation of OPA-derived tumour cells *in vitro* is inhibited by radicicol and 17-DMAG and that Hsp90 is expressed in OPA tumour cells by IHC [experiments performed by Fabienne Archer and Marcelo de las Heras, (Varela, Golder et al. 2007)].

Lung cancer is a multi-step process that involves the accumulation of genetic and epigenetic alterations that cause the activation of several signal pathways simultaneously (Girard, Zochbauer-Muller et al. 2000; Digel and Lubbert 2005). Ideally, therapeutic interventions for cancer should be able to interfere with a variety of signal transduction pathways that are involved in cell transformation. Heat shock proteins have been found to be overexpressed in several haematological and solid human cancers, including lung cancer (Yufu, Nishimura et al. 1992; Chant, Rose et al. 1995; Santarosa, Favaro et al. 1997; Senju, Sueoka et al. 2006). For reasons that yet remain to be fully clarified, Hsp90 extracted from tumour cells has a higher binding affinity for 17-AAG than Hsp90 extracted from normal tissue, allowing the accumulation of the drug in tumours (Kamal, Thao et al. 2003). Moreover, Hsp90 inhibitors have been shown to reduce proliferation of several human lung cancer cell lines and induce further growth inhibition when combined with irradiation (Senju, Sueoka et al. 2006). The ability of Hsp90 inhibitors to disrupt a variety of signalling

pathways that are involved in the development of cancer makes them ideal therapeutic agents for the treatment of lung cancer.

The mechanisms of cell transformation by the JSRV Env are not completely clarified but involve the PI3K-Akt, the Ras-MEK-MAPK pathways and possibly, as shown in this study, also Src considering that two Src inhibitors and a dominant negative Src (SrcMF) reduced JSRV Env transformation. All these pathways have been implicated in the development of human lung cancer (Brognard, Clark et al. 2001; Kolch 2002; Vivanco and Sawyers 2002; Zhang, Kalyankrishna et al. 2007). Thus, JSRV-mediated transformation can be a useful model to study the molecular mechanisms underpinning the effects of Hsp90 inhibitors on particular cell signalling molecules in tumours where several pathways are activated simultaneously, both *in vitro* and *in vivo*.

There is an increasing need of animal models for studying the safety and efficacy of the numerous anticancer drugs that are under development (Collis 2006). OPA can be experimentally reproduced with a short incubation period (~4-12 weeks) when lambs are inoculated intratracheally with concentrated virus preparations. Under these circumstances, the primary target cells of infection produce new infectious virus that is able to infect and hence transform new cells leading to the appearance of lesions of different sizes that tend to coalesce (Figure 34). It could be argued that the use of this model could be “overpowering” even for effective drugs, given that new infectious virus expressing a dominant oncoprotein is continuously produced. However, a JSRV-replication-defective virus (JS-RD) has been recently developed that proved to be oncogenic in a high percentage of inoculated lambs (Caporale, Cousens et al. 2006). In addition, JS-RD can be inoculated by bronchoscopy in well defined anatomical regions of the lungs, increasing the opportunity to develop *intravital* imaging techniques where lesion development is continuously monitored.

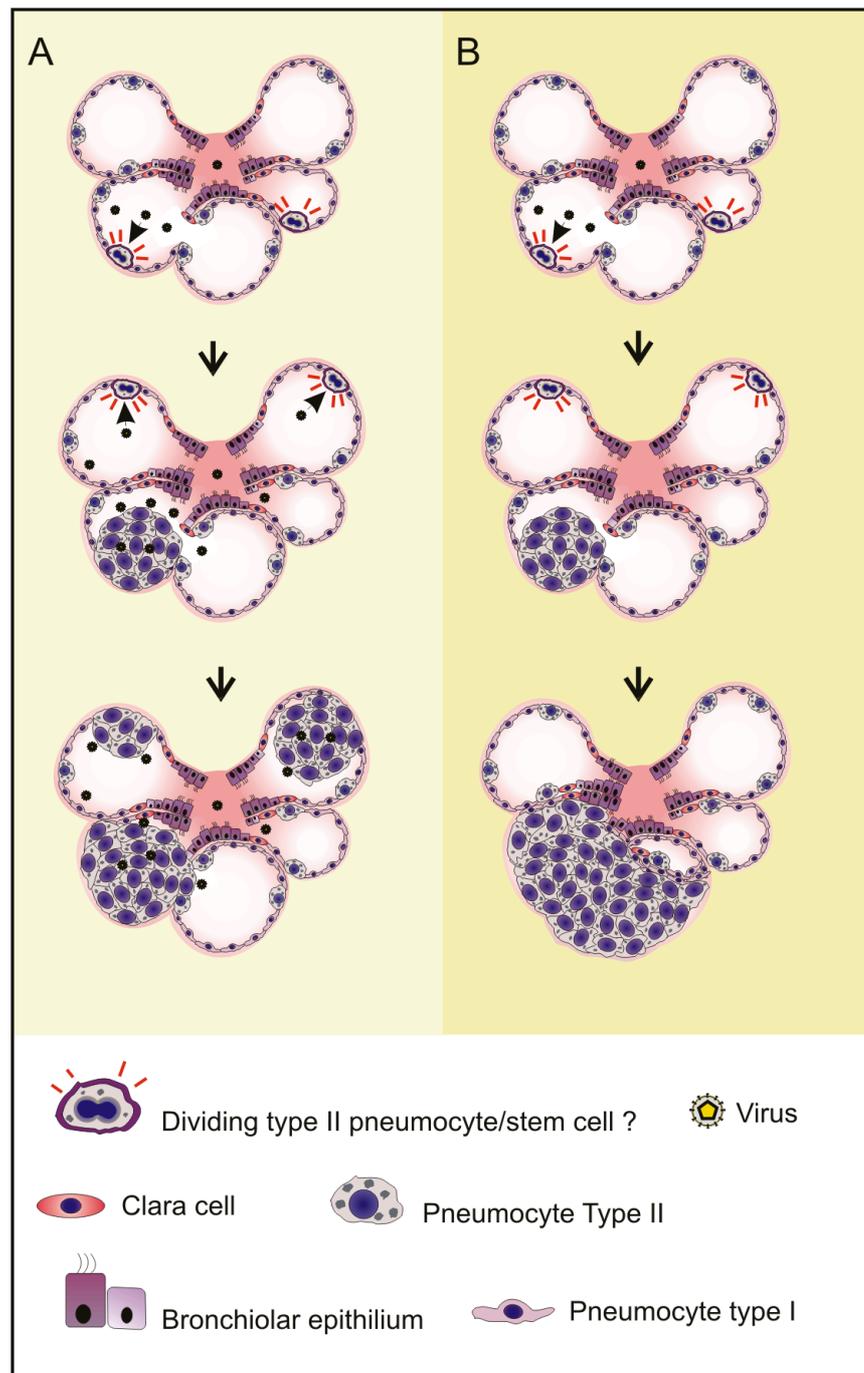


Figure 35. Extension of the OPA lesions can be modulated by the use of replication competent or incompetent viruses.

Cartoon representing two experimental models of OPA. (A) Young lambs have many available target cells (pneumocytes type II or possibly pulmonary stem cells) that can be infected and transformed by JSRV. Tumour cells produce infectious virus that can then infect and transform other target cells resulting in many satellite and coalescing lesions. On the other hand, experimental inoculation of young lambs with replication incompetent JS-RD (B) will result in infection and transformation of target cells that do not produce infectious virus, resulting in tumour nodules derived from a single transformed cell. The cartoon is only a schematic representation of the histopathological lesions in OPA. Tumour cells in OPA grow usually in a well organized manner along the alveolar walls. Image kindly provided by Marco Caporale.

The finding that the effects of inhibitors of Hsp90 in cell transformation can be studied in this system demonstrates that OPA could be used as tool for the development and improvement of other Hsp90 inhibitors. Although animals affected by OPA have not been used to test the therapeutic potential of any drugs so far, inhibitors of Hsp90 offer an interesting opportunity to challenge OPA in this regard considering the promising *in vitro* findings shown in this study. In conclusion, OPA offers a system that allows: a) the study of the molecular mechanisms leading to the development of lung cancer both *in vivo* and *in vitro*; b) the study and improvement of early detection techniques like imaging tools and serum biomarkers that so far have been unsuccessful in the prevention of human lung cancer (Shaw, Kirsch et al. 2005) and III) the evaluation of new therapeutic agents before they reach the clinic as well as the experimentation and development of radiotherapy and surgery. In this respect, OPA (and in general large animal cancer models) can be a valid alternative to rodent models.

Chapter 5

Summary

This chapter describes a series of experiments performed to characterize the enJSRV Envs present in the sheep genome and their role in placental morphogenesis of sheep. A set of results described in this chapter formed part of a larger study published in 2006 (Dunlap, Palmarini et al. 2006) and provided the framework for the design of the majority of the experiments reported here.

Introduction

ERVs are present in the genome of all vertebrates as stable inherited Mendelian genes (Boeke and Stoye 1997). They are thought to arise from ancient germ line infections by exogenous retroviruses and their copy number increase via retrotransposition or germ line reinfection (Boeke and Stoye 1997). As a result of the accumulation of different types of mutations (i.e. insertions, deletions, substitutions) the great majority of ERVs are non infectious and non pathogenic (Gifford and Tristem 2003). The biological relevance of the presence of ERVs in the mammalian genome is controversial. They have been characterized as junk DNA as well as critical for mammalian development (Bock and Stoye 2000). However, the fact that ERVs comprise approximately 8% of the human genome (Lander, Linton et al. 2001) and that various animal species possess transcriptionally active proviruses with intact open reading frames millions years after integration support the idea that they might provide benefits to their hosts. A possible beneficial role for the expression of ERVs is protection from exogenous retrovirus infection. As demonstrated in mice, chickens and cats ERVs can confer resistance to superinfection by exogenous retrovirus by receptor blockage (Boeke and Stoye 1997). Another example is provided by *FvI* locus that encodes an endogenous Gag-like protein and blocks infection by the Friend strain of murine leukaemia virus (Best, Le Tissier et al. 1996; Boeke and Stoye 1997). Physiological functions have been also attributed to ERV, for example the tissue specific expression of salivary amylase. Amylase-associated proviruses are thought to contain transcriptional control elements that are specifically active in the parathyroid gland, thus suggesting that specific salivary expression is due to proviral insertion (Ting, Rosenberg et al. 1992; Samuelson, Phillips et al. 1996). The high expression of ERVs in human and mouse placenta, in particular

the presence of intact *env* genes in the syncytiotrophoblasts which have been preserved over thousands of years together with the observation that they elicit fusion of cells *in vitro*, has led to the speculation that they are important for placental morphogenesis and evolution and that they have been positively selected (Mi, Lee et al. 2000; Voisset, Bouton et al. 2000; Dupressoir, Marceau et al. 2005).

HERV-W, HERV-FRD and ERV-3 are three human ERVs (HERV) whose *env* coding genes display a high level of expression in the human placenta (Venables, Brookes et al. 1995; Blond, Lavillette et al. 2000; de Parseval, Lazar et al. 2003). HERV-W is not present in the human genome as a complete provirus, however its Env protein, also referred to as syncytin-1, is preferentially expressed in the syncytiotrophoblast which is a multinuclear tissue forming the outer surface of the foetal part of the placenta providing a barrier with the maternal blood. The syncytiotrophoblast is produced by intercellular fusion of trophoblast cells and is responsible for the transport of oxygen, nutrients and waste products, hormone production and immune tolerance (Benirschke and Kaufmann 2000). Syncytin is a 80 kDa glycosylated protein and possesses characteristic features of other retroviral Env proteins such as the presence of a leader peptide, a potential proteolytic site, a fusion peptide-like sequence and a putative immunosuppressive region. It also contains a hydrophobic membrane-spanning domain that suggests its insertion into the plasma membrane (de Parseval, Lazar et al. 2003). There is considerable information suggesting that syncytin is involved in the fusion of the cytotrophoblasts to form the syncytiotrophoblast. Transfection of a variety of cell lines with HERV-W *env* results in increased cellular fusion and this phenomenon is reduced when the cell cultures are treated with an antibody against the HERV-W Env protein (Blond, Lavillette et al. 2000; Mi, Lee et al. 2000). In addition, induction of fusion of BeWo cells, (a human trophoblastic choriocarcinoma cell line) (Pattillo, Gey et al. 1968), by forskolin increased expression of *syncytin* (Mi, Lee et al. 2000). Moreover, anti-sense inhibition of syncytin expression in primary trophoblast cells reduced the number and size of syncytia formed during culture (Frendo, Olivier et al. 2003).

The Env glycoprotein of HERV-FRD, referred to as syncytin-2, is structurally similar to syncytin-1, however it entered the primate genome before the split of the New World and the Old World Monkeys, more than 40 million years ago while syncytin-1 entered the primate genome approximately 25 millions years ago and is not present in Old

World Monkeys (de Parseval and Heidmann 2005). It also elicits cell fusion when transiently transfected in several cell lines (Blaise, de Parseval et al. 2003).

The Env protein of ERV-3 is also expressed in the syncytiotrophoblast and was the first endogenous retroviral Env to which a physiological function was attributed (Boyd, Bax et al. 1993). It has a long ORF although is prematurely terminated by the presence of a stop codon in the TM region which truncates the hydrophobic domain that is required for anchoring to the cell membrane (Cohen, Powers et al. 1985). It also lacks a leader and a fusion peptide and although it harbours a region with homology to an immunosuppressive domain its function is likely diminished by the lack of membrane anchorage (Rote, Chakrabarti et al. 2004). ERV-3 Env does not elicit cell fusion, however its mRNA levels increase in BeWo cells treated with forskolin. When ERV3 Env is stably expressed in undifferentiated BeWo cells it induces changes characteristic of trophoblast differentiation such as increased levels of β -hCG, growth inhibition and altered morphology (Lin, Xu et al. 1999). Considering that ERV-3 Env is expressed in a variety of normal tissues and in particular hormone producing organs, including adrenal and sebaceous glands and testis, it could be speculated to play a general role in hormone production (Rote, Chakrabarti et al. 2004). However, 1% of 150 healthy Caucasian individuals were found to be homozygous for a premature stop codon that potentially could result in a severely truncated non-functional protein (de Parseval and Heidmann 1998). Thus, it is debatable whether ERV-3 Env could really play any critical biological function.

Interestingly, two murine ERV *env* genes have been identified which are specifically expressed in the placenta at the level of the syncytiotrophoblast-containing labyrinthine zona. They are highly fusogenic in *ex vivo* transfection assays and are present in all Muridae tested which suggests positive selection (Dupressoir, Marceau et al. 2005).

Sheep possess at least 27 copies of betaretroviruses in their genomes highly related to the exogenous and pathogenic JSRV (York, Vigne et al. 1992; Arnaud, Caporale et al. 2007). Both endogenous and exogenous JSRVs use hyaluronidase 2 (Hyal2) as a cellular receptor and enJSRVs can block JSRV replication at early stages of the retroviral cycle probably by receptor interference (Spencer, Mura et al. 2003). Moreover, two enJSRV loci can block JSRV replication at a late stage in the retroviral replication cycle, a block referred to as JSRV late restriction (JLR) (Mura, Murcia et al. 2004). JLR probably occurs by the production of defective viral particles that are unable

to interact with the trafficking cellular machinery and accumulate in the cytoplasm where they are degraded by the proteasome (Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007). These observations support the hypothesis that enJSRV protect the host against pathogenic retroviruses.

enJSRVs are highly expressed in the female reproductive tract (Palmarini, Hallwirth et al. 2000; Dunlap, Palmarini et al. 2005). In particular, in the uterus enJSRVs RNA and protein are observed in the endometrial luminal epithelium (LE) and in the glandular epithelium (GE) (Spencer, Stagg et al. 1999; Palmarini, Hallwirth et al. 2000; Palmarini, Gray et al. 2001). In addition, enJSRV RNA is detected in the trophoectoderm in a temporal fashion that is coincident with key events in the development of the sheep conceptus (Dunlap, Palmarini et al. 2005). In particular enJSRV expression is most abundant in the trophoblast giant binucleate cells (BNC) and multinucleated syncytial plaques of the placentomes.

The ruminant placenta is classified as cotyledonary on the basis of its anatomical features. It is characterized by discrete areas of attachment, the placentomes, which are formed by the interaction of areas of the chorioallantois and the endometrium. The foetal part of the placentome is the cotyledon and the maternal parts are the caruncles (Igwebuike 2006). In sheep, morula-stage embryos enter the uterus by day 4-5 after mating and by day 6 the blastocysts contain a blastocoele surrounded by a monolayer of trophoectoderm (Guillomot 1995; Spencer, Johnson et al. 2004). By day 11 they have hatched from the zona pellucida and develop into a tubular form and by day 12 they begin to elongate, reaching 25 cm or more by day 17. The elongation of the blastocysts is critical for the production of interferon (IFN- τ), which is the pregnancy recognition signal, and for proper implantation (Spencer, Johnson et al. 2007). Apposition of the trophoectoderm and the LE takes place between days 14 and 19 and is then followed by the attachment and interdigitation of cytoplasmic projections of the trophoectoderm and the microvilli of LE. Trophoblast giant BNC start to appear on day 14 (Wooding 1984) and it is believed that they are the result of consecutive nuclear divisions without cytokinesis of trophoblast mononuclear cells (MTC), a process referred to as mitotic polyploid (Wooding 1992). The BNCs then migrate to the microvilliar junction and fuse with individual LE cells to form trinucleate fetomaternal hybrid cells (Wooding 1984). BNCs continue to migrate and fuse with trinucleate cells while cells of the LE get displaced to ultimately form the syncytial plaques by day 24 (limited to 20-25 nuclei in sheep) which will cover the surface of the endometrial caruncles to aid the development

of the placentomes. The placentomes are formed by interdigitation of foetal placental cotyledons and endometrial caruncles and are the functional units that will provide hemotrophic nutrition to the conceptus. The functions of BNCs are mainly: I) the formation of the hybrid fetomaternal syncytia for successful implantation and the cotyledonary growth of the placentome; and II) the production and synthesis of proteins and hormones like placental lactogen, pregnancy associated glycoproteins (PAGs) and progesterone (Wooding 1992). Trophoblast BNCs of the sheep placenta are analogous to the giant cells of the syncytiotrophoblast of the human placenta (Hoffman and Wooding 1993).

The RNA of enJSRVs is first detected by RT-PCR in the conceptus on day 12 which is coincident with their elongation and the production of IFN- τ . *Hyal2* mRNA is detected in the conceptus, exclusively in the BNC and the syncytial plaques of the cotyledons, starting from day 16 when trophoblast giant BNC differentiation takes place (Dunlap, Palmarini et al. 2005). These results led to the hypothesis that expression of enJSRVs and *Hyal2* are important for peri-implantation trophoectoderm differentiation in sheep (Spencer, Johnson et al. 2007). The authors propose that the expression of enJSRVs in trophoblast cells starting on day 12 increase their proliferation by undefined mechanisms. It is possible that BNCs derive from division of MTCs without cytokinesis or by fusion of MTCs. In the latter scenario some MTCs would begin to express *Hyal2* and fusion would take place by the interaction of *Hyal2* with the Env of enJSRVs expressed in another MTC. By day 16 the newly formed BNCs start to migrate and fuse with the LE to form trinucleate cells. During this period both BNCs and the LE express enJSRV Env while only BNCs express *Hyal2*, therefore the formation of trinucleate cells could also be the result of cell fusion elicited by the interaction between enJSRV Env and *Hyal2*. Fusion would continue throughout most of gestation and the co-expression of enJSRVs and *Hyal2* in the same cells supports the idea that *Hyal2* binds the enJSRV Env in the surface of BNCs inducing fusion and the formation of multinucleated syncytia (Spencer, Johnson et al. 2007).

All of the evidence reported so far for primates, rodents and sheep points to the suggestion that ERVs have influenced mammalian evolution and have been positively selected for a physiological role in placenta morphogenesis. In this section I will describe an array of experiments aimed to help understand the role of enJSRV Envs in the morphogenesis of the sheep placenta *in vitro* as well as the characterization of enJSRV Envs present in the sheep genome.

Results

The expression of enJSRV Envs can be blocked with morpholino antisense oligonucleotides

A morpholino antisense oligonucleotide (MAO) was designed to specifically inhibit the expression of enJSRV *env* mRNAs (MAO-*env*). The ultimate goal of the experiment was to use these MAOs to block enJSRV Env expression *in utero* to test the hypothesis that enJSRVs are essential in periimplantation ovine conceptus development and placenta morphogenesis. MAOs are short chains of morpholino subunits comprised of a nucleic acid, a morpholino ring and a non-ionic phosphorodiamidate intersubunit linkage. Morpholinos act via a steric block mechanism that is RNase-H independent, inhibiting splicing and/or translation (Summerton 1999). They are effective when designed to complement the nucleotide region around the start codon and/or possibly splicing sites of a given gene mRNA. Since the nucleotide sequence around the splice acceptor and start codon of the exogenous JSRV and the enJSRVs loci known at the time are highly conserved, one common MAO should be able to inhibit splicing and translation of most enJSRV proviral loci expressing intact *env* genes (Palmarini, Hallwirth et al. 2000). A series of *in vitro* experiments were conducted to test the morpholino effectiveness in human 293T cells transiently transfected with an expression plasmid of the *env* of enJS5F16 (pSV-En2-EnvFlag) driven by the simian virus 40 promoter and tagged with the FLAG epitope at the carboxy terminus (Figure 35). MAO-*env* very effectively blocked the expression of enJS5F16 Env (Figure 35B) in a dose dependent manner (Figure 35C), while a five-mismatch (MAO-5mis) and a standard control MAO (MAO-std) had no effect (Figure 35B-C). MAO-*env* and controls had no effect in the expression of enJSRV Gag (Figure 35D) since Gag is synthesized from a full length genomic mRNA, whereas Env is produced only from correctly spliced mRNA (Figure 35A) (Vogt 1997).

These results prompted the use of MAO-*env* *in utero* to test the effects of loss of function (Dunlap, Palmarini et al. 2006). Dunlap and colleagues at Texas A&M injected MAO-*env* into the lumen of the ovine uterus on day 8 after mating and the effects were determined on day 16 or 20. Two control groups were used where ewes were injected with MAO-std and MAO-5mis. The conceptuses removed from ewes injected with the control MAO at day 16 were normal in appearance in contrast to the ones removed from

MAO-env treated ewes that were fragile and smaller. Histological examination revealed the presence of fewer trophoblast cells in the MAO-env conceptuses with an abnormal vacuolated cytoplasm, which correlated with a reduction in the amount of IFN- τ collected from uterine flushes. Most importantly, the amount of trophoblast BNCs was severely reduced to almost complete absence in MAO-env treated conceptuses. When the same experiment was repeated and the results evaluated at day 20 most of the MAO-env treated ewes exhibited oestrous at day 17-18 after mating which is indicative of early pregnancy loss due to inadequate production of IFN- τ . When normal day 15 trophoblast cells were isolated and cultured *in vitro* they were shown to express enJSRV *env* and *gag* and their proliferation was reduced by 33% in the presence of MAO-env. The results of this study are reported in Dunlap et al. 2006. The data strongly supports the hypothesis that enJSRVs play a critical role in mononuclear cell outgrowth and differentiation of trophoblast giant BNCs during periimplantation (Dunlap, Palmarini et al. 2006).

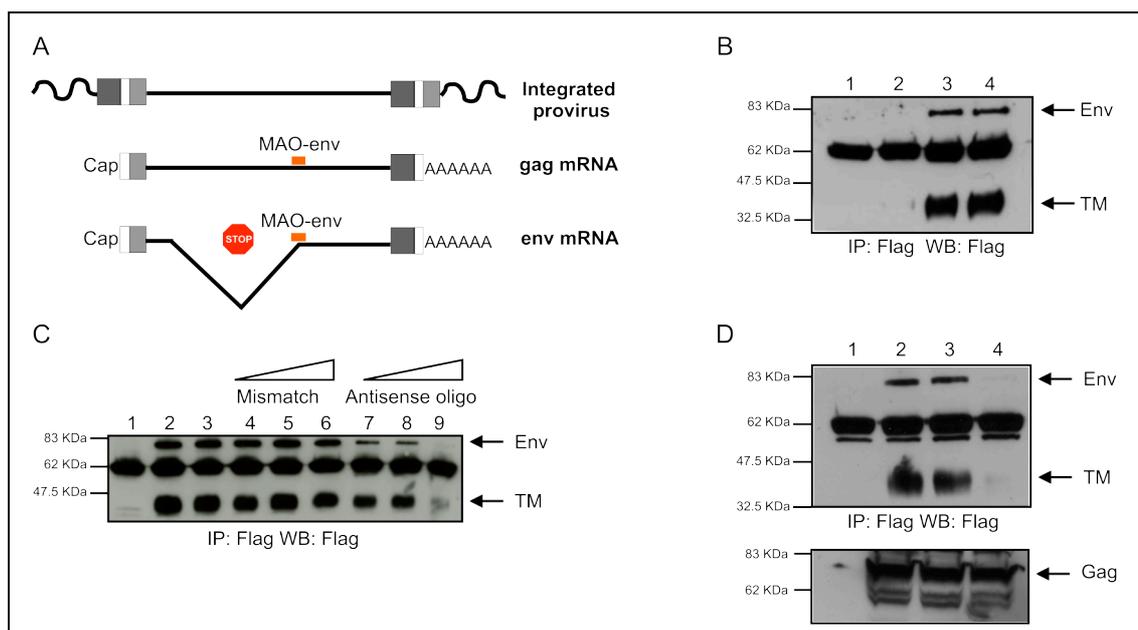


Figure 36. Design and effect of morpholinos on enJSRV Env expression in vitro.

A) MAO-env were designed to inhibit splicing and translation of enJSRV *env* mRNA but not expression of full length genomic RNA. B) 293T cells were mock transfected (lane1) or transfected with an expression plasmid of the enJS5F16 Env tagged with the FLAG epitope at the carboxy terminus (lanes 2-4). Cells were then treated with MAO-env (lane 2), MAO-5mis (lane 3) or MAO-std (lane 4) as controls and lysed after 48 hours, immunoprecipitated and analyzed by WB. C) Cells were mock transfected (lane1) or transfected with an expression plasmid for the enJS5F16 Env (lanes 2-9) and then treated with Endo-Porter alone (the delivery reagent) (lane 2), MAO-std as a control (lane 3), increasing amounts of MAO-5mis (lanes 4-6; 20, 40 and 80 μ M respectively) and MAO-env (lanes 7-9; 20, 40, 80 μ M respectively). After 48 hours cells were lysed, immunoprecipitated and analyzed by WB. D) 293T cells were mock transfected (lane 1) or cotransfected with an expression plasmid of the *env* of enJS5F16 and pCMV2enJS5F16 expressing the full length enJS5F16 clone (lanes 2-4). Cells were then treated with Endo-Porter alone (lanes 1 and 2), MAO-5mis (lane 3) or MAO-env (lane 4) and analysed as described for the other panels B and C.

enJSRV Env characterization

First we wanted to determine if the various Env glycoproteins expressed by different enJSRV loci possessed the same biological characteristics. Frederic Arnaud in our laboratory screened a sheep genomic BAC library (CHORI-243) and isolated 26 individual enJSRV proviruses, including the previously cloned enJS56A1 and enJS5F16 (Arnaud, Caporale et al. 2007).

Sixteen enJSRV loci were identified that contained an intact *env* ORF. The newly-cloned enJSRV *envs* maintain the characteristic nucleotide sequence and domains of the previously identified enJS5F16 and enJS56A1 *env* glycoproteins. Sequence alignment revealed 92.2 to 99.7% identity among the enJSRV Envs and 87.7 and 92.4 between exogenous and enJSRVs Envs at the amino acid level. The predicted hydrophobic profiles of the JSRV Env and the Env of enJS5F1613, as prototype of an enJSRV Env, are shown in Figure 36. The sequence alignment shown in Figure 37 allows the identification of characteristic Env domains: I) a signal peptide is present at the amino terminus portion of Env [SU starts at amino acid 81(Murcia, Arnaud et al. 2007)]; II) a furin consensus cleavage site (R-X-[R/K]-R) (Nakayama 1997) is present between the SU and TM domains; III) a hydrophobic segment is present at the amino terminus of TM which in other retroviral Envs maps to the location of the fusion peptide (Hunter 1997); IV) a hydrophobic transmembrane anchor domain; and V) a cytoplasmic tail at the carboxyterminal of TM lacking the characteristic transformation domains present in the JSRV Env.

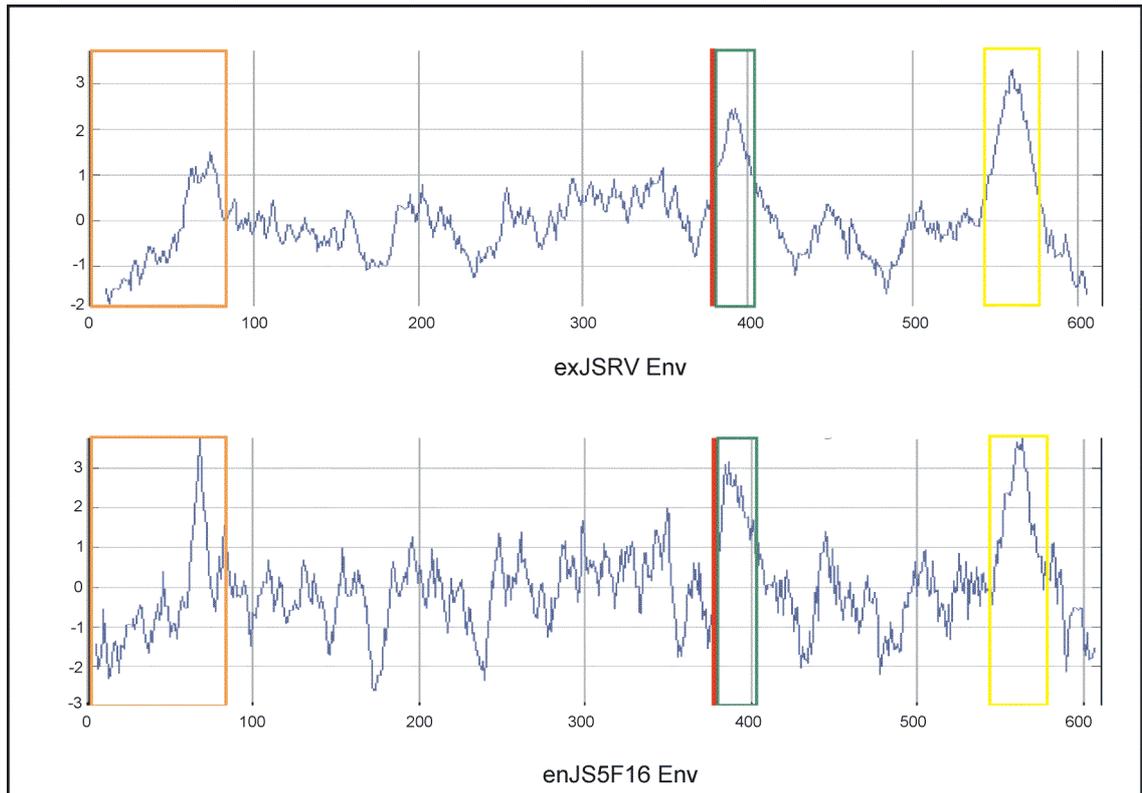


Figure 37. Hydrophobic profiles and characteristics of betaretroviral Envs of sheep.

The hydrophobic profile of enJSRV Envs and JSRV Env was calculated by the Kyte and Doolittle method. Only the hydrophobic profile of enJS5F16 is shown as an example. The orange box indicates the signal peptide; the red line indicates the consensus proteolytic cleavage site separating SU from TM; the green and yellow boxes correspond to hydrophobic regions associated with the fusion peptide and the membrane spanning domain respectively.

We next sub-cloned twelve of the sixteen *envs*, into expression plasmids and assessed the cellular distribution of the Env proteins by immunofluorescence and confocal microscopy. Some of the enJSRVs *env* were not further used. For example the *env* of enJS5F16 was already cloned (Palmarini, Maeda et al. 2001). *envs* of enJSRV16 and enJSRV18 are identical therefore only the *env* of enJSRV16 was cloned and enJSRV4, enJSRV23 and enJSRV24 lack the 5'LTRs. The use of an antibody raised against the TM of the exogenous JSRV Env revealed that enJSRV Envs have a broad cytoplasmic distribution, as expected for proteins synthesized in the ER, and possibly membrane expression as shown in Figure 38. We could not detect Env expression in cells transfected with expression plasmids for enJSRV-7, enJSRV-11, enJSRV-15 and enJSRV19 Env glycoproteins. The possible explanations for this observation are that: I) the *env* carried by these loci are somehow defective and readily degraded; or II) that these constructs do not express sufficient levels of Env.

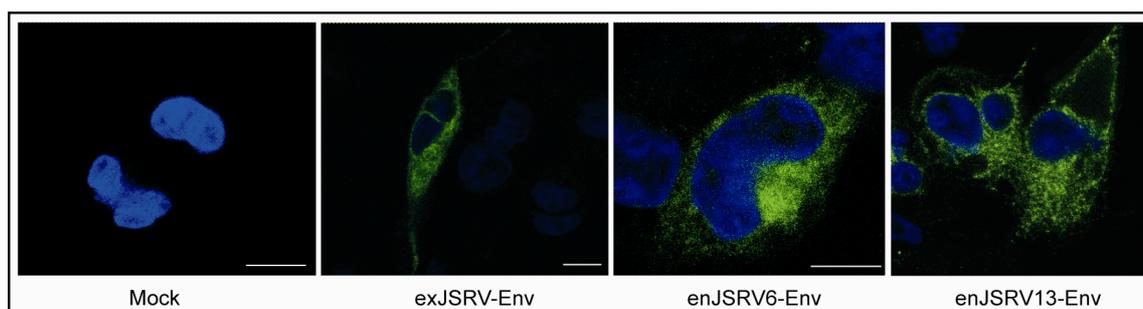


Figure 39. Cellular distribution of enJSRV Envs as determined by immunofluorescence and confocal microscopy.

Photomicrographs showing representative examples of the major phenotypes observed in COS cells transiently expressing enJSRV Envs. Specific staining was mainly observed diffused within the cytoplasm (second and third panels) and possibly at the cell surface (fourth panel). Bars represent 10 μ m.

We then used standard retroviral vectors pseudotyped by various enJSRV Envs to determine their ability to utilize Hyal2 of different species as a cellular receptor. We engineered NIH 3T3 stably expressing the sheep, goat or bovine *Hyal2* and performed standard viral entry assays. As shown in Figure 39 the Envs of enJSRV-4, enJSRV-16/18 and enJSRV-26 showed titers similar to the vectors pseudotyped with the exogenous JSRV Env in both NIH 3T3 cells stably expressing the sheep and the goat *Hyal2* genes. The Envs of enJSRV-6, enJSRV-7, enJSRV-11, enJSRV-15, enJSRV-16, enJSRV-19 and enJSRV-20 mediated entry less efficiently, while the Envs of enJSRV-9, enJSRV-13 and enJS56A1 did not mediate entry at all in the same cells lines. The inability of the last three Envs to mediate cell entry could not be explained by lack of

expression of the constructs since, as shown above, they were found expressed when analyzed by immunofluorescence and confocal microscopy, although it is possible that they are not functional. Interestingly, these Envs possess two to eight amino acid differences within the receptor binding domain that could explain their inability to use Hyal2 as a cellular receptor. As described above, we were not able to detect expression of the Envs of enJSRV-7, enJSRV-11, enJSRV-15 and enJSRV-19 by confocal microscopy. However, their ability to mediate cell entry, albeit at a lower efficiency, indicates that these constructs are being expressed at some level. Entry assays were also performed in the ovine uterine stromal oST cell line, with virus pseudotyped with those enJSRV Envs that did not mediate entry efficiently, to rule out the possibility that these Envs do not interact with Hyal2 but with other receptors. As shown in Figure 40 none of the enJSRV Envs tested were able to mediate entry in this cell line in contrast to the exogenous JSRV that was used as a positive control.

None of the enJSRV Envs utilized bovine Hyal2 as a cellular receptor as efficiently as sheep or goat Hyal2 as previously reported by the exogenous JSRV Env (Dirks, Duh et al. 2002). This could explain the lack of enJSRV colonization of *Bos taurus* (Arnaud, Caporale et al. 2007). It should be taken into consideration that the reduced ability of the Envs of enJSRV-7, enJSRV-9, enJSRV-11, enJSRV-13 enJSRV-15, enJSRV-20, enJS56A1 and enJSRV-19 to mediate viral entry could be due to the inability of these Envs to be efficiently packaged.

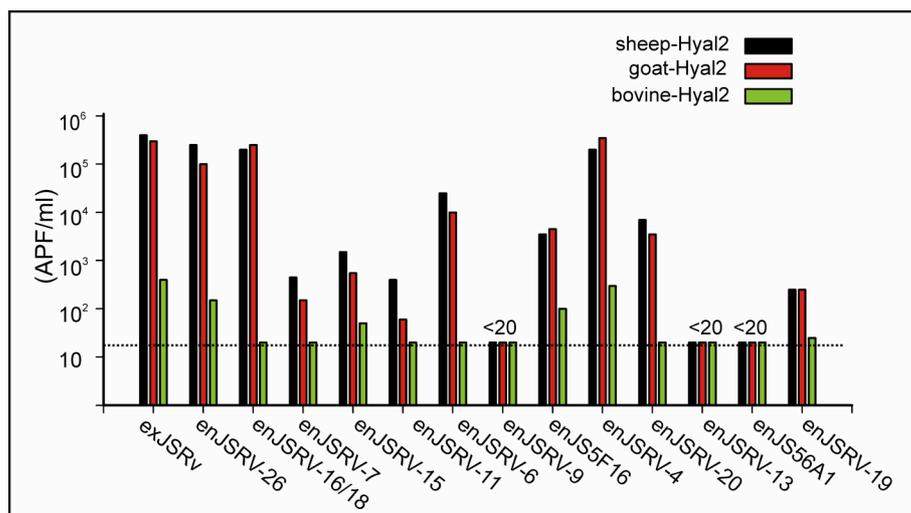


Figure 40. Receptor usage of exogenous and endogenous Betaretroviruses of sheep.

Viral entry assays were performed in NIH 3T3 cells stably expressing the ovine, goat or bovine Hyal2 gene. Cells were transduced with MLV-based retroviral vectors expressing the alkaline phosphatase gene and pseudotyped with various enJSRV Envs. Results are expressed as alkaline phosphatase foci per ml (APF/ml) and are indicated as <20 when the titer was less than 20 APF/ml.

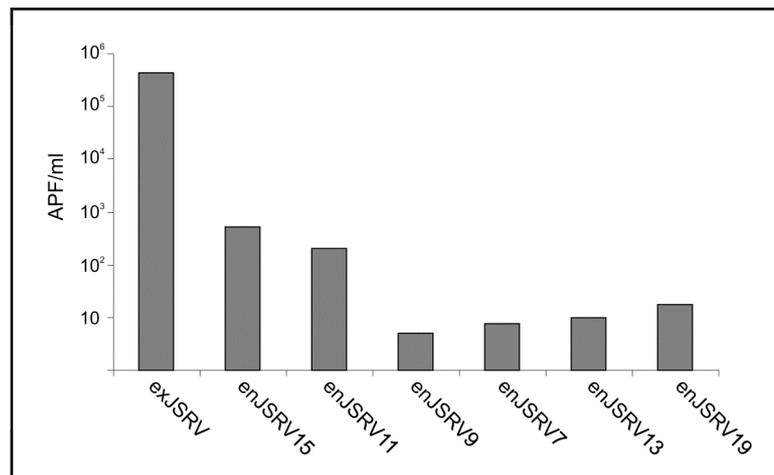


Figure 41. Entry assays in oST cells.

Ost cells were transduced as described for the previous figure. Results are expressed as alkaline phosphatase foci per ml (APF/ml).

As shown in Figure 37, none of the identified enJSRV *envs* encoded for a VR3 region similar to the exogenous JSRV *env*, in particular all of them lacked a YXXM motif in the cytoplasmic tail of the TM domain which is critical for transformation by the exogenous JSRV Env (Liu, Wang et al. 2001; Maeda, Palmarini et al. 2001; Palmarini, Maeda et al. 2001; Zavala, Pretto et al. 2003; Liu and Miller 2005; Cousens, Maeda et al. 2007). Standard transformation assays were performed in 208F cells to test the transforming potential of these enJSRV Envs. As expected, none of the enJSRV Envs induced transformation two weeks after transfection (Table 6).

enJSRV Env construct	Number of foci ⁶
JSRV-Env	289.5
enJS56A1-Env	0
enJS5F16-Env	0
enJSRV4-Env	0
enJSRV6-Env	0
enJSRV7-Env	0
enJSRV9-Env	0
enJSRV11-Env	0
enJSRV13-Env	0
enJSRV15-Env	0
enJSRV16/18-Env	0
enJSRV10-Env	0
enJSRV20-Env	0
enJSRV26-Env	0

Table 7. enJSRV Env transformation assays.

⁶ The numbers of foci represent the average of at least two experiments.

enJSRV Envs do not interfere with JSRV-Env induced cell transformation

The mechanisms used by the JSRV Env to induce cell transformation are not completely understood, however several pieces of evidence point to the involvement of the PI3K-AKT and the Ras-MEK-MAPK pathways (Palmarini, Maeda et al. 2001; Maeda, Fu et al. 2005; De Las Heras, Ortin et al. 2006). Recent work performed in our laboratory using mass spectrometry analysis identified the association of the JSRV Env with the signal transducer B-Raf (Murgia and Palmarini, unpublished results). The mass-spec data was validated by co-immunoprecipitations assays performed in transformed 208F cells as well as transiently transfected 293T cells. Interestingly, B-Raf is directly and indirectly involved in the modulation of the Ras-Raf-Mek-Erk and the PI3K/Akt pathways (Stork 2003; O'Neill and Kolch 2004; Kolch 2005; Stork and Dillon 2005) which are activated in JSRV transformed cells. We were interested in testing whether the non transforming enJSRV Envs and the Y590 mutant were also able to interact with B-RAF to gain insight into the domains involved in this association. As shown in Figure 41 we found interaction between enJSRV Envs and B-RAF by immunoprecipitation studies performed in transiently transfected 293T cells as well as with the Y590 mutant.

Next, we tested whether the enJSRV Envs could function as dominant negative proteins and prevent transformation by the exogenous JSRV Env. Indeed, enJSRVs Envs may interact with some of the same proteins that are possibly used by the JSRV Env to mediate transformation, To this end standard transformation assays were performed in 208F cells with pCMV3JS21ΔGP, an expression plasmid of the JSRV Env, and increasing amounts of various enJSRV Env expression plasmids. None of the enJSRV Envs tested were able to reduce significantly the number of foci induced by the JSRV Env (Figure 42).

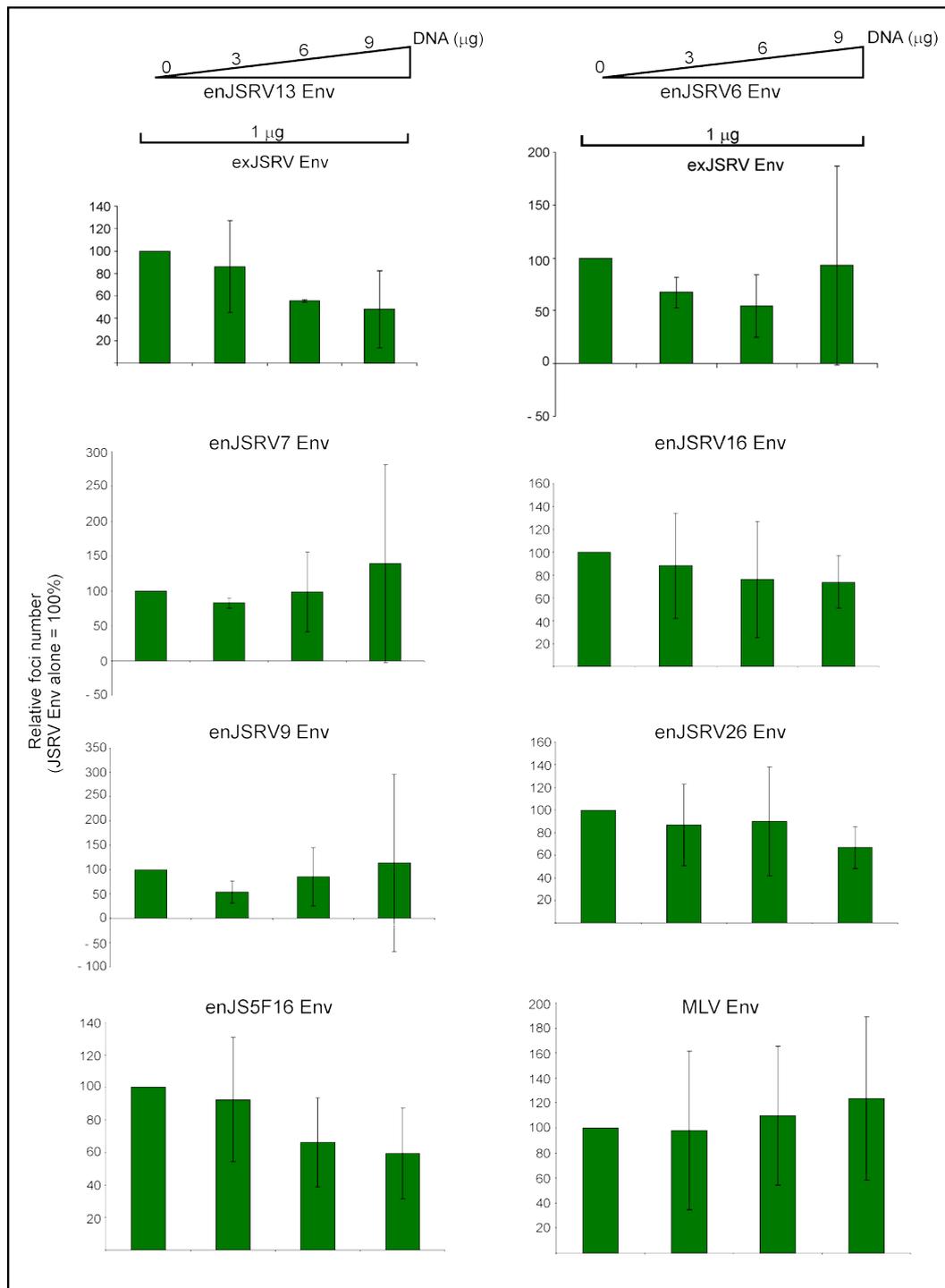


Figure 43. enJSRV Env do not prevent transformation by the JSRV Env.

Transformation assays in 208F cells were performed by transfecting 1 μg of the expression plasmid of the JSRV Env (exJSRV) and 3, 6 and 9 μg of expression plasmids of various enJSRV Envs and the Env of MLV as a control, as indicated in the top of each chart. Results represent the average of four experiments and are expressed as a percentage of transformation where the number of foci obtained with the JSRV Env alone is considered a hundred percent. Bars represent standard deviation.

To rule out the possibility that the efficiency of the cotransfection was low (a significant percentage of cells taking up and expressing the JSRV Env plasmid did not take up the enJSRV Env plasmid), cell lines stably expressing enJSRV Envs were produced by infecting 208F cells with retrovirus vectors encoding the various enJSRV Envs and the neomycin resistance gene. Colonies of neomycin resistant clones were pooled and used in standard transformation assays by transfecting the JSRV Env plasmid. Stable cell lines were shown to express enJSRV Envs by RT-PCR (see below, Figure 47). As shown in Figure 43 no changes in the number of foci were detected between the assays performed in cell lines expressing an empty vector and cells lines expressing enJSRV Envs. Altogether, these results indicate that enJSRV Env cannot prevent transformation by the exogenous JSRV Env *in vitro*.

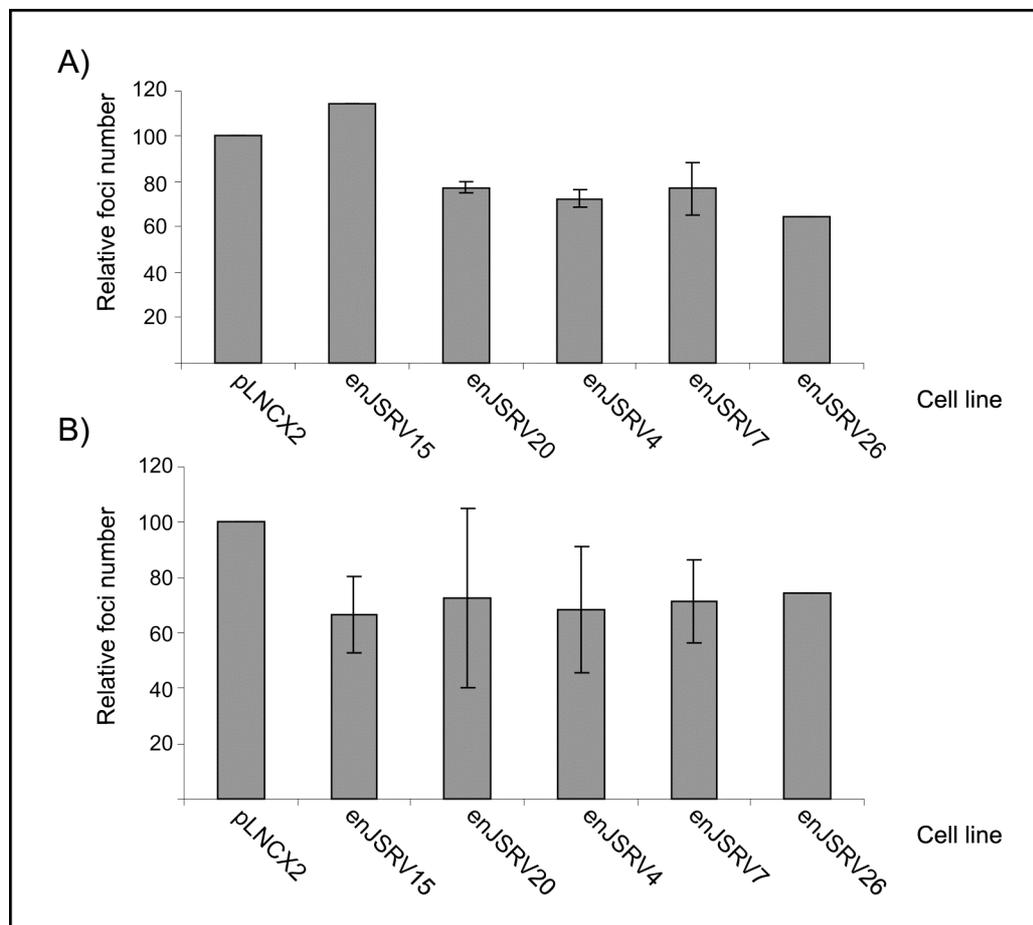


Figure 44. Transformation assays in cell lines stably expressing enJSRV Envs.

208F cells stably expressing an empty vector (pLNCX2) or various enJSRV Envs were prepared as described in Materials and Methods and used in standard transformation assays transfecting two different amounts of DNA of the JSRV Env (0.1 μ g and 1 μ g of DNA, panel A and B respectively). Foci were counted two weeks after transfection. Results represent the average of two experiments and are expressed as a percentage of transformation where the number of foci obtained in the cell line expressing the empty vector is considered a hundred percent. Bars represent standard deviation.

enJSRV Envs are not fusogenic in vitro

The cellular mechanisms underlying mononuclear trophoectoderm proliferation and differentiation into trophoblast giant BNCs in ruminants are poorly understood. The temporal and spatial expression of enJSRV Envs and Hyal2 in the sheep placenta together with retarded blastocyst elongation and failure in the differentiation of BNCs after enJSRV Env inhibition *in utero* suggest their involvement in trophoblast proliferation and differentiation (Dunlap, Palmarini et al. 2005; Dunlap, Palmarini et al. 2006). We hypothesized that the formation of giant BNCs, trinucleate fetomaternal hybrid cells and ultimately the establishment of the multinucleated syncytia could be the result of cell fusion elicited by the expression of enJSRV Envs and Hyal2. This idea is supported by the fact that Env proteins of ERVs present in the primate and mouse genomes, and highly expressed in the placenta, induce cell fusion *in vitro* (Mi, Lee et al. 2000; Blaise, de Parseval et al. 2003; Dupressoir, Marceau et al. 2005). Therefore we investigated the fusogenic potential of enJSRV Envs *in vitro*. To this end 293, COS, HeLa and NIH-3T3 cells stably expressing the sheep *hyal2* gene were transiently transfected with expression plasmids of the various enJSRV Envs and cells were monitored for 48 hours. As shown in Figure 44 none of the enJSRV Envs elicited cell fusion in the analyzed cell lines in contrast to the Envs of HERV-W or HTLV that were used as positive controls.

It is possible that the lack of fusogenic activity of enJSRV Envs observed in the previous experiments was due to the absence of other molecules required for fusion besides from Hyal2 in the tested cell lines. To test this possibility, the same assays were performed in ovine trophoblast cells (oTr) from day 15 conceptuses (kindly provided by Dr. Thomas Spencer) (Dunlap, Palmarini et al. 2006). This cell line was indirectly shown to express Hyal2 since it allowed entry of retroviral vectors pseudotyped with the JSRV Env (not shown). As shown in Figure 45 we could not detect cell fusion of oTr cells expressing enJSRV Envs.

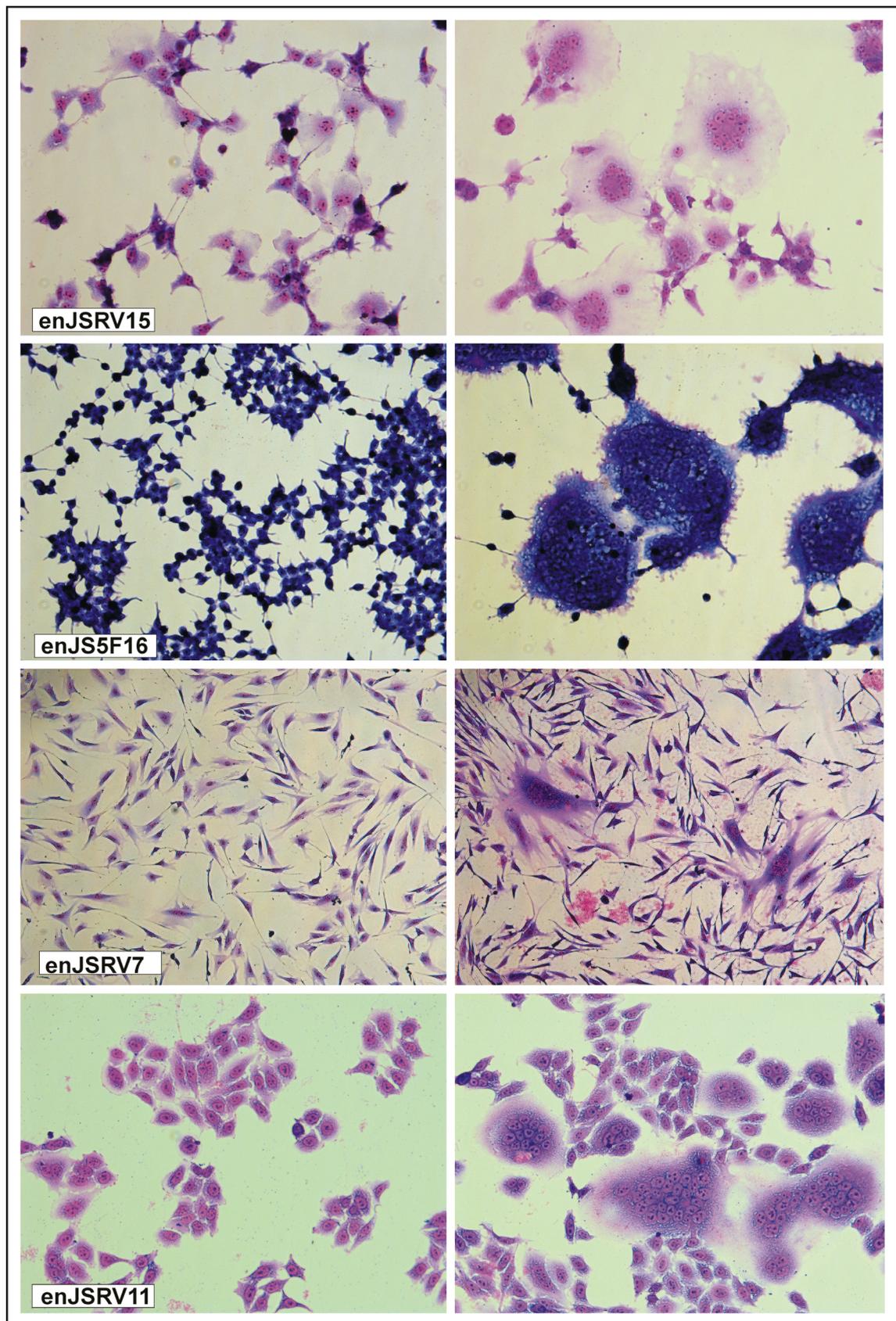


Figure 45. Fusion assays.

From top to bottom fusion assays performed in COS, 293, NIH 3T3-ovine Hyal2 and HeLa cells. Left panels show representative micrographs of the results obtained when cells express enJSRV Envs. Right panels show fusion induced by the Env of HERV-W that was used as a positive control.

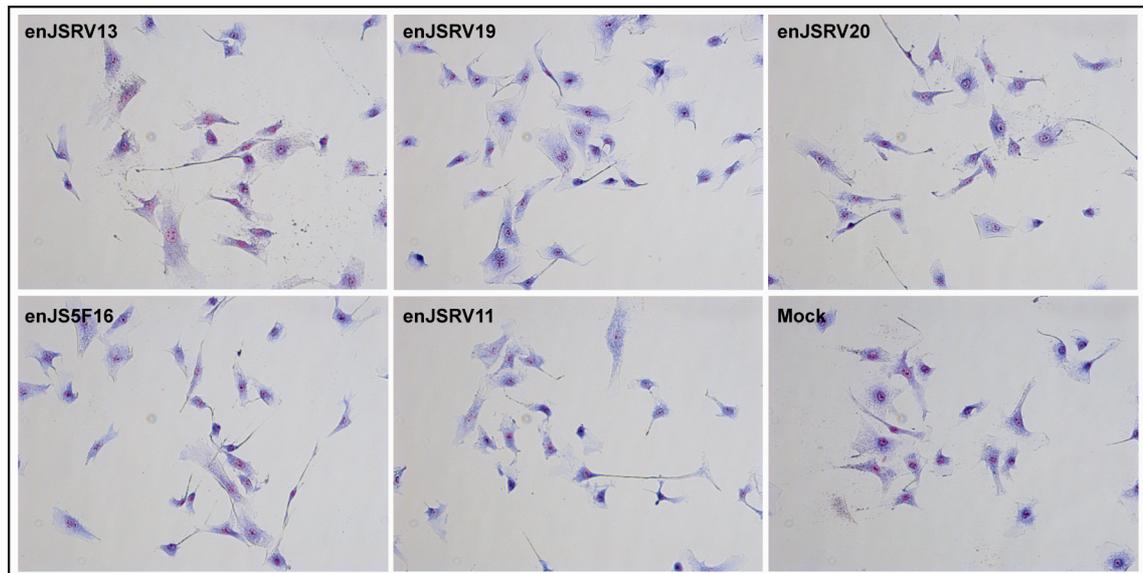


Figure 46. EnJSRV Envs do not induce fusion of oTr cells.

OTr cells were transiently transfected with various enJSRV Env expression plasmids and monitored for seven days for the presence of cell fusion. Cells were stained with basic fuchsin two and seven days after transfection and photographed.

We also tested whether oTr expressing enJSRV Envs could fuse with cells of the ovine luminal epithelium (oLE) (Johnson, Burghardt et al. 1999) to mimic the formation of fetomaternal hybrid cells. As shown in Figure 46 we could not detect cell fusion between oTr cells expressing enJSRV Envs and oLE cells expressing ovine *Hyal2*.

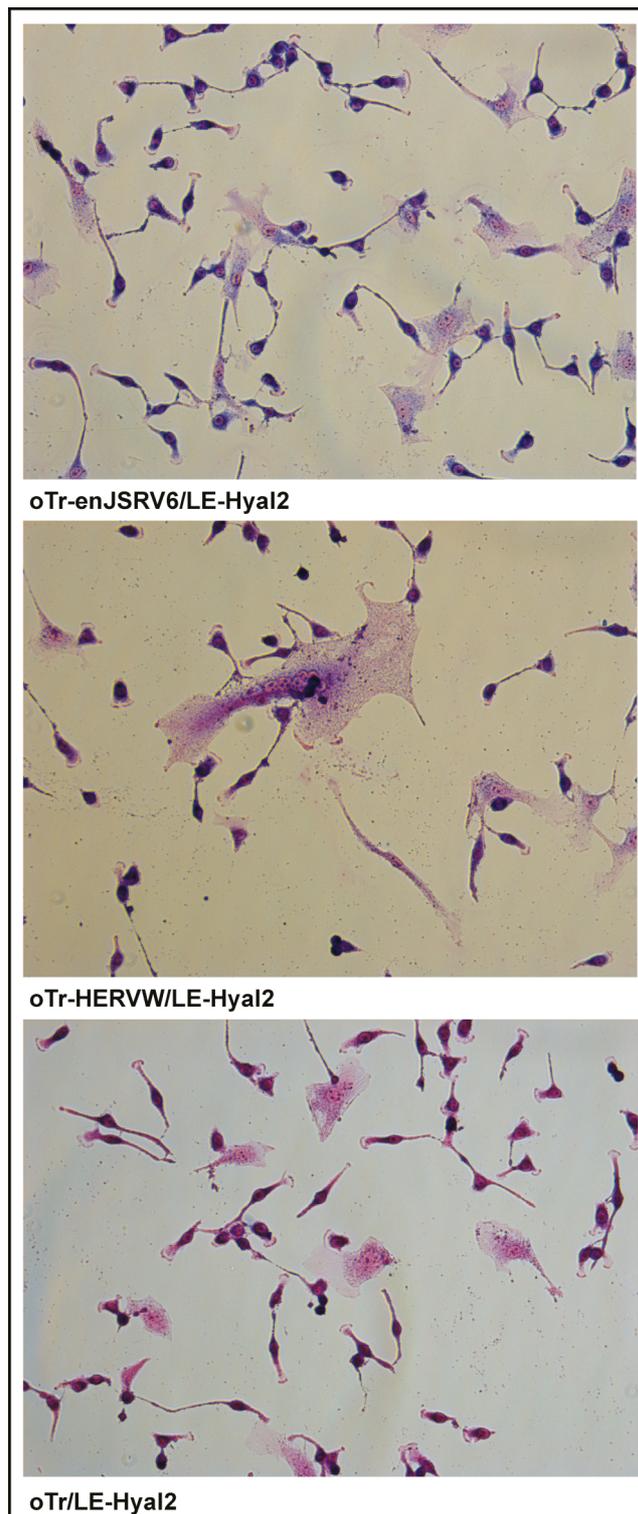


Figure 47. EnJSRV Env do not induce fusion of oTr with LE cells.

oTr cells were transiently transfected with expression plasmids of enJSRV Envs and plated in 6 well plates. Two hours later they were overlaid with oLE cells transiently expressing the ovine *hyal2* gene and the presence of cell fusion was monitored for 72 hours when cells were stained with basic fuchsin and photographed. Expression of HERV-W Env induced fusion of oTr cells.

enJSRV Envs do not enhance proliferation of 208F cells

Since oTr cells were not available to us at the time, we used rat 208F cells (that have been extensively utilized in the study of the transforming properties of the JSRV Env) as a first approach into the elucidation of the molecular mechanisms inducing cell proliferation and differentiation of trophoblast cells by enJSRV Envs (Chow, Alberti et al. 2003; Liu, Lerman et al. 2003).

Since the proliferation of oTr cells isolated from day 15 conceptuses is reduced by 33% when enJSRV Env expression is blocked by MAO-env (Dunlap, Palmarini et al. 2006) we wanted to test whether 208F cells expressing enJSRV Envs displayed an increased proliferation rate compared to cells expressing empty vector. If so, we would investigate further the molecular mechanisms underpinning this effect. To this end 208F cells stably expressing various enJSRV Envs were prepared as described in Materials and Methods and cell proliferation was measured using the WST assay. enJSRV Env expression was determined by RT-PCR as shown in Figure 47. Two different cells lines were prepared and each cell line was assessed in triplicate in two independent experiments. Experiments were also performed by culturing cells in the presence of 2 or 5% FBS in order to be able to detect subtle changes in cell proliferation that otherwise would not be recognized if high concentrations of serum were used. As shown in Figure 48 no changes in cell proliferation were detected under any of the experimental conditions tested when 208F cells express enJSRV Envs. The same results were obtained when individual cell clones were analyzed (not shown).

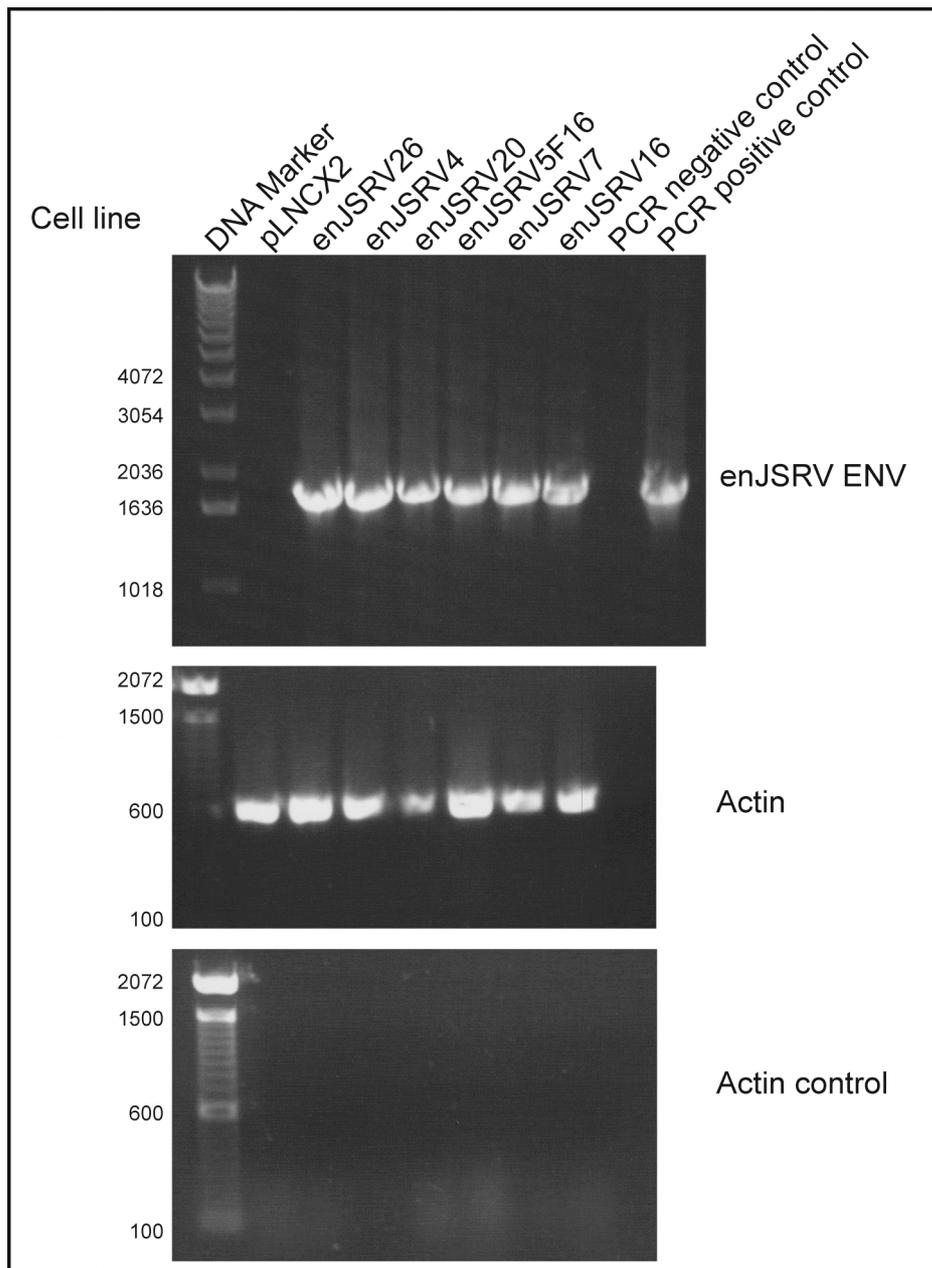


Figure 48. enJSRV Env expression in 208F stable cell lines as demonstrated by RT-PCR.

EnJSRV Env expression was determined by RT-PCR from RNA extracted from the indicated stable cell lines. Primers were designed in conserved regions so that one pair would amplify all the envelopes and the full length product. Amplification of rat β -actin was used as a positive control (middle panel) and no PCR product was obtained when the cDNA was prepared without the addition of reverse transcriptase, indicating the absence of DNA contamination (bottom panel). Numbers on the left indicate base pairs.

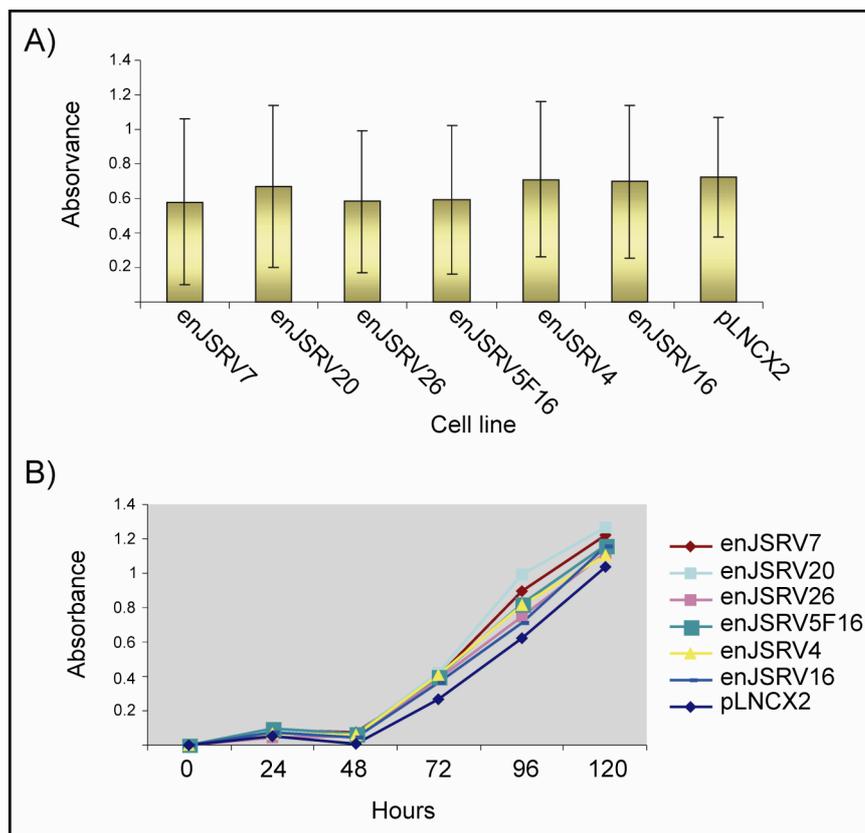


Figure 49. EnJSRV Envs do not enhance proliferation of 208F cells.

A) Cell proliferation of 208F cells stably expressing the indicated enJSRV Envs or empty vector (pLNCX2) after 120 hours of culture in media supplemented with 5% FBS. Each column represents the combined data of four experiments done in triplicate each time. Bars indicate standard deviation. B) Growth curve of one representative experiment of 208F cells stably expressing the indicated enJSRV Envs or empty vector and cultured in media supplemented with 5% FBS to demonstrate that no differences in the cell proliferation rate were observed at any of the time points analyzed.

enJSRV Envs do not promote invasion of 208F cells

Trophoblast giant BNCs have differentiated from the MTC by day 16 and migrate through the apical tight junctions of the chorion to fuse apically with the cells of the endometrial LE of the uterus forming trinucleate hybrid fetomaternal cells throughout the whole gestational period (Wooding 1992). Giant and BNC typically exhibit the capacity of migration and invasion, albeit the degree of migratory activity varies within species. The synepitheliochorial placentation of sheep could be characterized by a “restricted” invasion since trophoblast BNCs migrate towards the endometrial LE but do not go beyond it in contrast to the haemochorial human placenta where these cells cross several layers of the uterine wall (Wooding 1992; Spencer, Johnson et al. 2004). We wanted to test whether enJSRV Env expression could promote cell invasion. To this end a tridimensional inverse invasion assay with a matrigel reconstituted basement

membrane matrix was performed using 208F cells stably expressing enJSRV Envs. As shown in Figure 49 we could not detect differences in the invasive phenotype in cells expressing enJSRV Envs compared to the control cells. Of great concern was the observation that control cells, expressing the empty retroviral vector, displayed a highly invasive phenotype and that we obtained variable results within experiments. To rule out the possibility that the selection of cells with neomycin was playing a role in this phenomenon, we prepared new set of cell lines that were not subject to neomycin selection. Under the same experimental conditions we still observed a highly invasive phenotype in control cells and big variations within experiments (not shown).

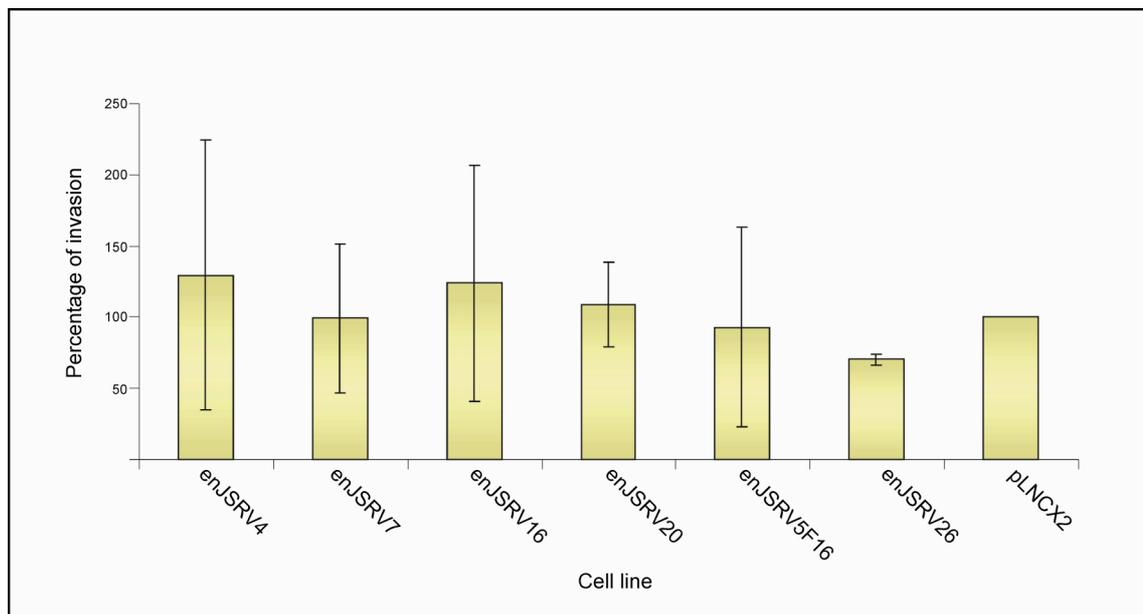


Figure 50. Invasion assays of 208F cells expressing enJSRV Envs.

Invasion assays were performed in duplicates and three independent fields per sample were analyzed and quantified. Invasion was visualized by staining cells directly with calcein followed by confocal microscopy analysis and quantification using the Image J software. Only cells in the 75 μ m section or above were considered invasive for quantification purposes. A composition of these sections was quantified and results are expressed as a percentage of invasion where the 100% value is taken by cells expressing the empty vector alone. The chart shows the average of three experiments and bars represent standard deviation.

enJSRV Env expression does not increase proliferation, the appearance of binucleate cells or migration of oTr cells

We next analyzed the effect of enJSRV Env expression in oTr cells. We wanted to address whether enJSRV Env over-expression increases oTr cell proliferation considering that a 33% reduction in their growth was observed when cultured in the presence of MAO-env (Dunlap, Palmarini et al. 2006). To this end oTr cells were

transiently transfected with expression plasmids of various enJSRV Envs and cell proliferation was measured using the WST assay after 72 hours of culture. As shown in Figure 50, no difference in the proliferation rate was detected in oTr cells overexpressing enJSRV Envs.

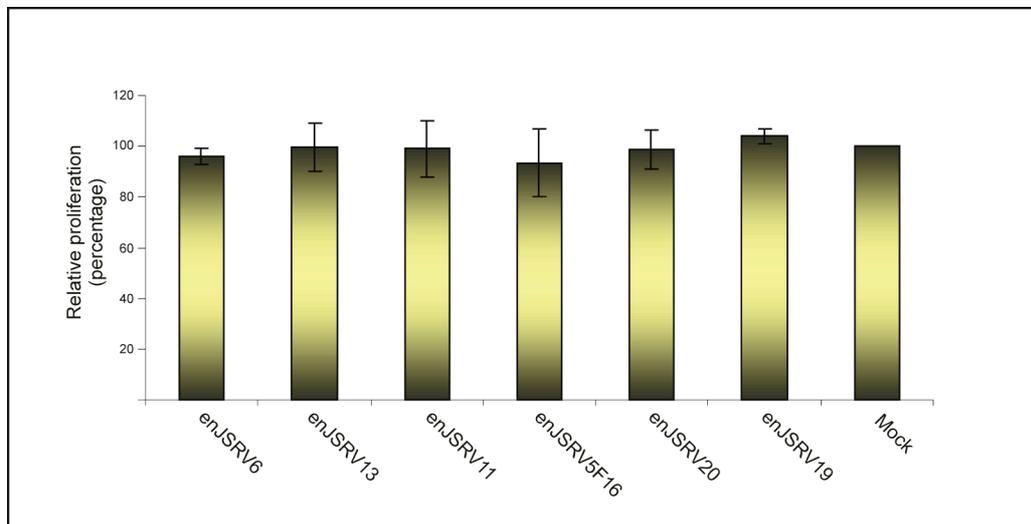


Figure 51. EnJSRV Env overexpression does not increase proliferation of oTr cells.

Data are shown as a % of proliferation where growth of mock transfected cells is considered as 100%. Each column represents the average of four independent experiments with eight replicates for each experiment. Bars represent standard deviation.

Based on the fact that enJSRV Env inhibition of expression *in vivo* was associated with an undeveloped conceptus and an almost complete lack of trophoblast giant BNCs (Dunlap, Palmarini et al. 2006), we were interested in testing if enJSRV Env expression induced the appearance of BNCs. OTr cells transfected with the respective enJSRV Env plasmids were plated in 6 well dishes 2 hours after transfection. After 72 hours cells were fixed, stained with May Grunwald Giemsa and the number of BNCs counted. As shown in Figure 51 no changes in the number of BNCs were observed after enJSRV Env over-expression.

We next investigated if enJSRV Env over-expression would induce cell migration of oTR cells. Standard migration assays were performed in transwell chambers with oTr cells transiently expressing enJSRV Envs. As shown in Figure 52 we could not detect differences in the migratory properties of any of the cells analyzed.

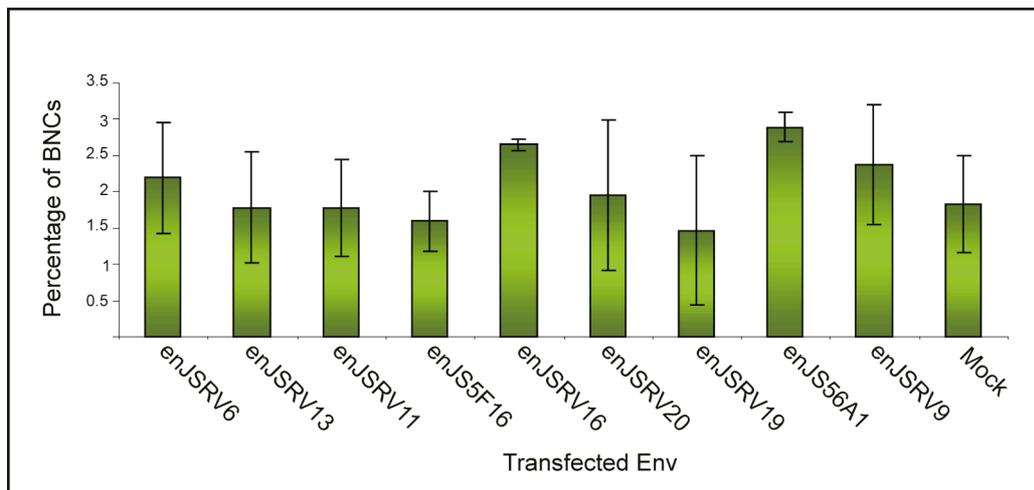


Figure 52. Percentage of BNCs after enJSRV Env expression.

Between 400 and 700 cells were counted for each well and scored as mononuclear or binuclear and then the percentage was calculated. The experiment was repeated six independent times. Bars represent standard deviation.

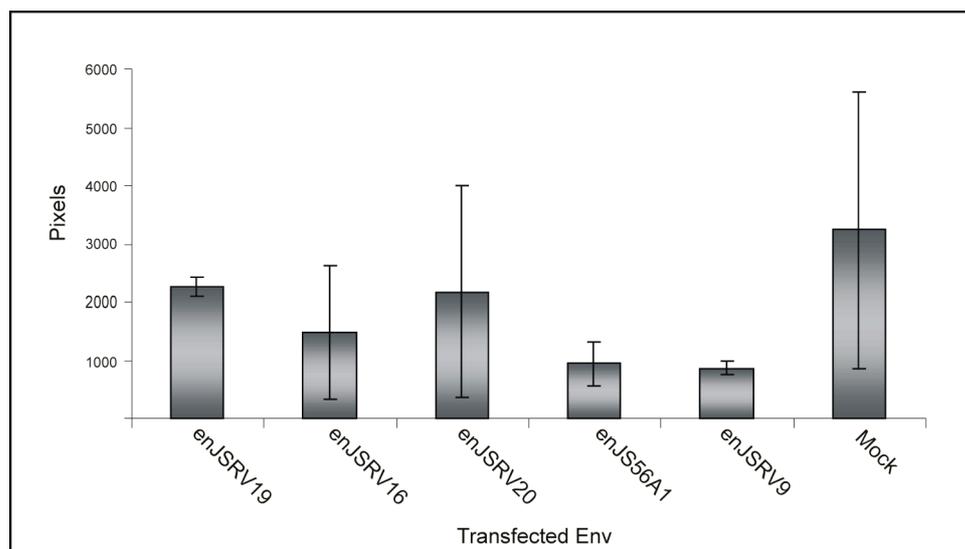


Figure 53. OTr cells migration when over-expressing enJSRV Envs.

Migration assays were performed in transwell chambers as described in Materials and Methods. Cell migration was quantified after 1 day (not shown) or 3 days of culture. Cells were stained with calcein and five random fields were photographed using a confocal microscope and quantified using the Image J software. Each column represents the average of two experiments. Bars represent standard deviation.

Finally, we performed migration assays with oTr cells that were treated with only Endo-Porter (the delivery agent), MAO-5mis or MAO-env. Figure 53 shows that no statistically significant differences were observed in the migration of oTr cells in which enJSRV Env expression is inhibited.

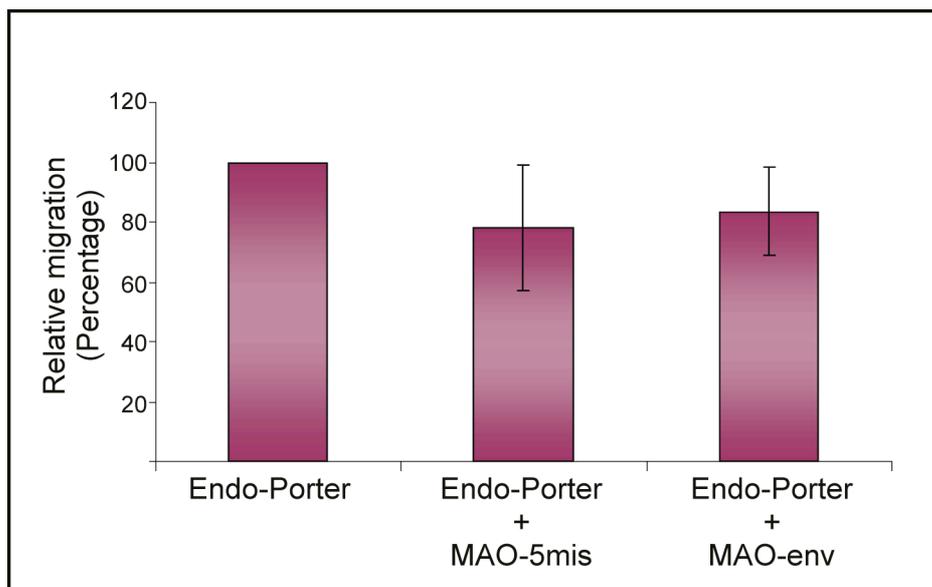


Figure 54. Migration assay in oTr treated with MAO.

OTr cells were plated in transwell chambers with media containing Endo-Porter alone or Endo-Porter plus MAO-5mis or MAO-env and incubated for 48 hours. Cells were then stained and ten pictures per wells were taken and quantified as described above. Experiments were repeated twice independently. Bars represent standard deviation.

Discussion

In this section, a series of experiments were performed with the intention to elucidate the mechanisms behind the regulation of ovine trophoblast growth and differentiation by enJSRV Envs. We hypothesized that enJSRV Envs and Hyal2 regulate placenta morphogenesis in sheep by modulating cell-cell fusion and/or intracellular signalling pathways. Our hypothesis was based on the observation that the block of enJSRV Env expression *in utero* resulted in retarded conceptus growth and BNC differentiation in sheep (Dunlap, Palmarini et al. 2006). Moreover, HERV-W Env (syncytin) has been found to regulate trophoblast growth and differentiation *in vitro* (Lin, Xu et al. 1999) and human and mouse syncytins elicit cell fusion *in vitro* (Mi, Lee et al. 2000; Dupressoir, Marceau et al. 2005).

To experimentally address our hypothesis, we first characterized the currently known enJSRV Envs that possess an intact ORF (Arnaud, Caporale et al. 2007). By standard viral entry assays we demonstrated that most of the Envs analyzed were able to mediate viral entry using goat and ovine Hyal2. None of the enJSRV Envs utilise the bovine

Hyal2 protein probably justifying the reason why enJSRVs have not been found in *Bos taurus* (Arnaud, Caporale et al. 2007).

We showed that these enJSRV Envs do not induce cell transformation *in vitro* and cannot interfere with transformation induced by the exogenous JSRV although they are able to interact with B-Raf, one of the cellular molecules known to mediate transformation by the JSRV Env (Murgia and Palmarini, unpublished results).

It is interesting to note that enJSRV Envs have a high degree of similarity with the oncogenic JSRV Env. It is tempting to speculate that both endogenous and exogenous JSRV Envs share similar mechanisms to induce trophoblast proliferation/differentiation and cell transformation respectively, since placenta morphogenesis has been regarded as a “pseudo-tumour” or a “physiological metastasis” (Soundararajan and Rao 2004; Ferretti, Bruni et al. 2007). Although many of these parallels come from comparisons made with the human placenta, trophoblast cells in general are characterized by a high proliferative rate, migratory and invasive properties and the capacity to evade the immune system, which are also characteristics of cancer cells. Interestingly, human cytotrophoblast cells express functional tumour-associated genes and are capable of engaging in autocrine stimulatory loops, rendering them less dependent on survival and growth factors from the surrounding tissue (Ferretti, Bruni et al. 2007). Moreover, the growth stimulatory effects can be amplified by signals provided by the neighbouring cells through paracrine loops (Ferretti, Bruni et al. 2007). However, the ultimate fate of trophoblast cells is terminal differentiation, which regulates their tumour-like attributes and the progression to senescence and apoptosis. The difference between malignant cell transformation and normal trophoblast development is that in the latter, the cellular and molecular events leading to cell proliferation/migration/invasion are spatially and temporally regulated, following a highly controlled plan. Thus, trophoblast cells are an ideal model for the study of the regulation of cell growth, differentiation, migration/invasion and carcinogenesis. enJSRV and JSRV Envs could mediate their effects through the activation of similar pathways considering that both are able to interact with B-Raf that is implicated in JSRV mediated transformation and in the growth and migration of human trophoblast cells (Pollheimer and Knofler 2005).

We tested the possibility that enJSRV Envs are important for BNC and syncytiotrophoblast formation by eliciting cell fusion as proposed for syncytins in humans and mice (Mi, Lee et al. 2000; Dupressoir, Marceau et al. 2005). We could not

detect cell-cell fusion in several cell lines transiently transfected with enJSRV Envs or in oTr cells. The lack of fusogenic activity of enJSRV Envs could be due to several reasons. Firstly, the limitations of the *in vitro* systems used should be considered. It is possible that other molecules beside enJSRV Envs and Hyal2 are required for the induction of cell fusion and these are absent in the cell lines used. We have used oTR cells to safeguard us from this problem; however there is no guarantee that oTR cells *in vitro* faithfully represent trophoblast cells *in vivo*.

The *in vivo* loss of function experiments using MAO-env indicated that enJSRV Env influences mononuclear trophoectoderm cell growth and differentiation during conceptus elongation. This process precedes the formation of multinucleated syncytia (Dunlap, Palmarini et al. 2006). Thus, we initially tested whether enJSRV Env over-expression could increase the proliferation rate and the migratory/invasive properties of 208F cells (as a first approach) and oTr cells. We detected no changes in proliferation and migration/invasion in either cell type. enJSRV Env over-expression did not induce the appearance of BNCs either. Failure to detect a different phenotype, measured by changes in cell proliferation/migration/invasion, after enJSRV Env expression in 208F is not surprising since these are immortalized rodent fibroblasts whose characteristics are far from similar to the attributes of conceptus trophoblast cells. The inability to induce changes in oTr cells after enJSRV Env over-expression could be attributed to the fact that the day 15 oTr cells used in these experiments already express enJSRV Envs and therefore their over-expression by transient transfection does not induce profound effects. However, we did not induce a reduction in cell migration after blocking enJSRV expression with MAO-env.

It is also possible that multiple enJSRV Envs are necessary to induce cell proliferation/migration/invasion since that several Envs appear to be co-expressed in the placenta (Rote, Chakrabarti et al. 2004; Seifarth, Frank et al. 2005). In conclusion, we were unable to experimentally reproduce *in vitro* the effects of enJSRV Env *in vivo*. However, as mentioned before it should be highlighted that oTr cells used in these studies were primary cultures that had been frozen twice and passaged at least eight times and thus any conclusions arising from these studies should be considered with caution.

An obligatory step in the understanding of the role of enJSRVs in placenta morphogenesis and the cellular and molecular mechanisms underpinning their effects is

the identification of the enJSRV loci expressed in the placenta. In particular it would be interesting to know the specific loci that are expressed during the periimplantation period. The knowledge of which enJSRV loci are expressed in the placenta could have facilitated the design of the *in vitro* studies presented here and the evaluation of Envs that are unable to encode full length products because of the presence of stop codons, as is the case of enJSRV1. As previously mentioned, the Env protein of ERV3 contains a premature stop codon that prevents the expression of the membrane spanning domain, it also lacks signal and fusion peptides (Rote, Chakrabarti et al. 2004). Despite these unusual characteristics, it seems to be involved in trophoblast differentiation and thus the role of prematurely terminated enJSRV Envs in BNC differentiation, which were not included in this study, should be taken into consideration. Moreover, it would be interesting to learn if enJSRV insertions could regulate neighbouring genes in a tissue and temporal specific fashion by the creation of alternative promoters, enhancers or polyadenylation signals as it is the case for the HERV-K element, which is inserted in proximity of the insulin-like growth factor gene and seems to mediate its expression during human syncytiotrophoblast formation (Bieche, Laurent et al. 2003).

In conclusion, all of data reported to date in sheep, primates and mice suggests that ERVs have been positively selected for a physiological role in placenta morphogenesis and possibly, the diversity of placenta structures found among species could reflect the differential use of ERV Env glycoproteins among mammals (Villarreal 1997).

Chapter 6

Discussion

Retroviruses have the ability to integrate their genetic information into the host genome, the capacity to transduce cellular genes and the opportunity to colonize the germ line of the host. ERVs are present in the genomes of all vertebrates (Gifford and Tristem 2003) and can be used as DNA fossils to unravel virus-host coevolution over millions of years (Coffin 2004). ERVs have protected the host against exogenous retroviruses (Gardner, Rasheed et al. 1980; O'Brien, Berman et al. 1983; Arnaud, Caporale et al. 2007) maintained genomic plasticity (Hughes and Coffin 2001) and play a critical role in placental morphogenesis (Mi, Lee et al. 2000; Dunlap, Palmarini et al. 2006).

Sheep betaretroviruses constitute a perfect model to study retrovirus biology. JSRV is the causative agent of OPA, a naturally occurring lung cancer of sheep. The expression of one of its structural proteins (Env) is sufficient to induce cell transformation both *in vitro* and *in vivo*, which is a unique feature among oncogenic retroviruses (Maeda, Palmarini et al. 2001; Rai, Duh et al. 2001; Allen, Sherrill et al. 2002; Danilkovitch-Miagkova, Duh et al. 2003; Zavala, Pretto et al. 2003; Liu and Miller 2005; Caporale, Cousens et al. 2006). OPA, besides being a disease that has important economic consequences for the farming industry represents a large animal model to study pulmonary carcinogenesis and to develop novel therapeutic and diagnostic interventions (Palmarini and Fan 2001) (this thesis). The sheep genome harbours at least 27 copies of ERVs highly related to the exogenous and pathogenic JSRV (enJSRVs). Two of the enJSRV loci are transdominant towards related exogenous viruses using a unique mechanism of viral interference acting at the late stages of the retroviral cycle [JSRV late restriction (JLR)] (Mura, Murcia et al. 2004; Arnaud, Caporale et al. 2007; Arnaud, Murcia et al. 2007; Murcia, Arnaud et al. 2007). A recent study from our laboratory strongly supports the hypothesis that selection of transdominant enJSRV loci has protected sheep against infection with related exogenous retroviruses. Thus, endogenization and selection of ERVs that may act as restriction factors is another mechanism used by the host against retroviral infections (Arnaud, Caporale et al. 2007). enJSRVs have also evolved to play a critical role in placental morphogenesis by regulating conceptus development (Dunlap, Palmarini et al. 2006). Thus the interaction

between JSRV, enJSRVs and their host provides a unique model to study many aspects of retrovirus biology

The aim of the first part of this thesis was to gain insights into the molecular mechanisms underpinning JSRV Env induced cell transformation. In addition, experiments were performed to determine whether the knowledge gained into the JSRV/OPA system could be used for the development of OPA as a large animal model for lung carcinogenesis. The objective of the second part of this study was to unravel how enJSRV Envs regulate trophoblast growth and differentiation. The two major points studied in this thesis might seem unrelated at first. However, some of the major biological consequences of JSRV and enJSRVs interaction with the host, such as oncogenesis and placental development, are both mediated by the viral envelope glycoprotein. In addition, placental morphogenesis has been regarded as “pseudo-tumorigenesis” and similar molecular pathways seem to be shared by cancer and trophoblast cells to turn on their highly proliferative and invasive phenotypes (Soundararajan and Rao 2004). Thus, we used our knowledge of the mechanisms of cell transformation induced by the JSRV Env to understand the role of enJSRV Envs in placental morphogenesis.

We first investigated the role of the receptor tyrosine kinase RON and Hyal2 in JSRV Env induced transformation. We showed that: I) RON interacts with both the exogenous and endogenous JSRV Envs and the cytoplasmic tail of the Env appears to be the major determinant of the biological effects of the RON-Env interaction; and II) Hyal2 is not involved in the transformation of epithelial cells. We then investigated the signal pathways involved in JSRV mediated transformation by using a variety of signal transduction inhibitors. As a result of these studies we found that the molecular chaperone Hsp90 regulates JSRV induced cell transformation at least in part by downregulating Akt expression. Thus, we could use in the future the JSRV/OPA model as a tool for the evaluation of the mechanisms of action and efficacy of new therapeutic agents.

We wanted then to elucidate the mechanisms by which enJSRV Envs regulate trophoblast growth and differentiation. Our original goal was to set-up an *in vitro* system where we could study the role of enJSRV Envs in cell proliferation, migration/invasion. Unfortunately we were unable to set-up this *in vitro* approach.

Thus, it still remains to be determined how enJSRV Envs contribute to conceptus development and placenta morphogenesis.

What is the relevance of studying the mechanisms of transformation of an oncogenic retrovirus of sheep? OPA, aside from being one of the most important viral diseases of sheep, also has striking similarities with some forms of human lung adenocarcinomas (Palmarini and Fan 2001). Thus the understanding of the molecular mechanisms governing JSRV induced cell transformation could provide insights into the development of human lung cancer (Palmarini and Fan 2003). Lung cancer is the most common cause of death among human cancer patients worldwide and the survival rate is very poor, which stresses the lack of effective therapies and early detection techniques (Parkin, Bray et al. 2005). Thus, the use of animal models other than the mouse could provide new insights especially for the development of new therapeutic and diagnostic strategies. It is clear that better animal models are required to facilitate drug development considering that most of the currently available models are based on conditional transgene expression or conditional gene knockouts that usually do not reflect the tumour spectrum observed in humans. In this scenario, OPA offers an alternative animal model where cancer development can be studied in a natural setting. As previously mentioned the JSRV Env is a dominant oncoprotein both *in vitro* and *in vivo*. The JSRV Env has been shown to activate the PI3K/Akt and the Ras-MEK-MAPK pathways (Palmarini, Maeda et al. 2001; Maeda, Fu et al. 2005; De Las Heras, Ortin et al. 2006). These pathways are involved in cellular responses to a variety of stimuli that activate membrane, cytoplasmic and nuclear signalling networks (Dhanasekaran and Johnson 2007). It is now apparent that they can transduce a diverse array of signals into specific cellular functions through their spatial and temporal regulation within the cell. Although at the moment it is not clear how the JSRV Env engages the cell signalling network to activate these pathways, the elucidation of the multi-protein complexes that might be assembled upon JSRV Env expression could provide insight into the understanding of the regulation of the cell signalling network. OPA can be experimentally reproduced in lambs with a short incubation period and in the presence of a functional immune system. Thus, the JSRV/OPA system allows us to immediately address *in vivo* in the sheep, observations gained from experiments in tissue culture.

In the work presented here we demonstrated that several Hsp90 inhibitors suppressed JSRV Env-induced transformation *in vitro*. The JSRV-OPA model could be used for the

development of new Hsp90 inhibitors for the treatment of lung adenocarcinoma. Hsp90 inhibitors are believed to be good candidates for cancer therapy considering that: I) lung cancer is the result of genetic and epigenetic alterations that lead the activation of several signal transduction pathways simultaneously (Girard, Zochbauer-Muller et al. 2000; Digel and Lubbert 2005) and II) Hsp90 inhibitors are able to disrupt various signal transduction pathways simultaneously (Blagg and Kerr 2006).

Our laboratory is interested in investigating whether bronchioalveolar stem cells (BASCs) are the actual target of JSRV transformation, rather than type II pneumocytes and Clara cells. JSRV expression is found by IHC in tumour cells but not in the normal type II pneumocytes and Clara cells surrounding the lesions (Palmarini and Fan 2003; Caporale, Centorame et al. 2005). It is currently believed that some types of cancers develop and are maintained by a minority of cells, cancer stem cells, that have the capacity of self renewal and give rise to more progenitor-like and differentiated cells in a disorganized fashion (Burkert, Wright et al. 2006). The fact that BASCs have been proposed to maintain type II pneumocytes and Clara cells and give rise to adenocarcinomas in mice (Kim, Jackson et al. 2005) supports the idea that JSRV targets BASCs.

ERVs have been described as either junk DNA or as essential for mammalian evolution (Bock and Stoye 2000). They have also been linked with both detrimental and beneficial roles for the host. One of the most fascinating aspects of the biology of ERVs is their possible role/s in placental morphogenesis. ERVs have been speculated to play a physiological role in placenta morphogenesis for almost three decades considering that retroviral particles have been detected frequently in the reproductive tract of several animal species (Kalter, Helmke et al. 1973; Vernon, McMahon et al. 1974; Kalter, Heberling et al. 1975; Smith and Moore 1988; Harris 1991; DeHaven, Schwartz et al. 1998). A few years ago, the identification of HERVs expressing intact *env* ORFs in the human placenta and the ability of these Envs to elicit cell fusion *in vitro* provided some evidence for the involvement of ERVs in placental development (Mi, Lee et al. 2000). Moreover, a systematic *in silico* analysis identified two fusogenic retroviral murine *env* genes with similar characteristics to the human genes supporting the idea that independently acquired ERVs were positively selected and contributed to the development of the placenta in different species (Dupressoir, Marceau et al. 2005). The study of enJSRVs has provided the first piece of evidence of a physiological role played by ERVs in conceptus and placental development *in vivo* (Dunlap, Palmarini et al.

2006). By blocking enJSRV Env expression *in utero* by MAO we demonstrated that they are essential for sheep trophoblast outgrowth and BNC differentiation. Early pregnancy loss was observed in MAO-env-treated ewes probably due to the reduced production of INF- τ by the growth retarded conceptus. The understanding of the molecular mechanisms governing sheep trophoblast differentiation and the role of ERVs in this process would provide invaluable information for comparative physiology and pathology, considering that for ethical reasons similar experiments can not be performed in humans.

One of the objectives of this thesis was to study how enJSRVs regulate trophoblast cell growth and differentiation. Despite the fact that we were unable to identify phenotypic changes in cells upon enJSRV Env expression, it is interesting to mention how the knowledge of the mechanisms of cell transformation induced by the JSRV Env could be translated in the cellular machinery engaged by enJSRV Envs to promote trophoblast differentiation. Interestingly, Ron^{-/-} mouse embryos fail to survive after the periimplantation period (Muraoka, Sun et al. 1999). This, together with the fact that Ron transcripts have been detected within the trophoectoderm surrounding the inner cell mass of E3.5 embryos, in particular in the giant trophoblast cells of later stage embryos, and that Ron overexpression promotes an invasive phenotype, suggests that Ron might be required for embryo implantation and trophoblast viability (Muraoka, Sun et al. 1999; Hess, Waltz et al. 2003). MSP (the Ron ligand) deficient mice are phenotypically normal and do not display reproductive problems (Bezerra, Carrick et al. 1998), thus it may be possible that in sheep Ron could be activated by other means including enJSRV Envs. In this study we have demonstrated that the Env of enJS5F16 inhibits Ron activation. Since constitutive Ron activation has been found to promote cell spreading, dissociation, migration and invasion (Wang, Yao et al. 2006), which are some of the attributes of trophoblast BNCs, it is feasible that enJSRV Envs, by inhibiting Ron activation, contribute to the less invasive phenotype of the ruminant placenta. We have no information at this time of which enJSRV loci are expressed in the placenta and if they all display the same level of inhibition towards Ron. However, it is possible that the spatial and temporal expression of different enJSRV loci with diverse activities towards Ron and with different affinity for Hyal2 (that can also inhibit Ron activation (Danilkovitch-Miagkova, Duh et al. 2003)), mediate the highly regulated series of events that culminate with the appearance of BNCs and their migration towards the LE. Ron splice variants have been found in association with altered Ron expression in cancer cells (Wang, Yao et al. 2006). enJSRV Envs could also have different biological

effects on these splice variants that might be expressed and/or required in a “tumour like” tissue like the placenta. Considering that Ron forms a multichaperone complex with Hsp90 (Germano, Barberis et al. 2006), the latter could be involved in the stabilization of these splice variants or activated Ron itself. Even though all these scenarios could possibly be taking place in trophoblast cells, the signals that ultimately dictate the activation of particular enJSRV loci during the periimplantation period remain to be determined. The development of an appropriate *in vitro* system will be required to dissect these mechanisms.

It could be speculated that enJSRVs could contribute to placental morphogenesis not only by the expression of Env proteins but also by transcriptional regulatory control of their LTRs by the creation of alternative promoters, enhancers and polyadenylation signals. The insertion of HERV-E in the 5' UTR of the growth factor pleiotropin provides an example of a trophoblast specific promoter created upon retroviral insertion which seems to contribute to the invasive phenotype of the trophoblast (Schulte, Lai et al. 1996). Another example is provided by the expression of the insulin growth factor 4 (INSL4) in the placenta which seems to be driven by a HERV-K insertion (Bieche, Laurent et al. 2003).

Another aspect to discuss is the possible role of ERV Envs in maternal immune tolerance to the foetus. Many retroviral Envs seem to mediate immunosuppression by means of the expression of a stretch of conserved amino acids present in the TM domain (Cianciolo, Copeland et al. 1985). Syncytin 1 and 2 harbour this immunosuppressive region and it has been speculated that they contribute to the creation of an immune tolerant environment for the foetus although the mechanism by which this region is immunosuppressive remains unclear (Villarreal 1997; Mi, Lee et al. 2000). On the other hand it is unlikely that full immune suppression is due to the expression of a single retroviral gene. None of the currently known enJSRV Envs harbour a known immunosuppression domain, although this does not exclude the possibility that they do play a role in maternal tolerance to the foetus.

In conclusion, although a role of ERVs in the reproductive biology of several animal species has been speculated for quite some time, the study of enJSRVs has provided the first *in vivo* evidence for their involvement in placental morphogenesis (Dunlap, Palmarini et al. 2006). However, the lack of knowledge of the cellular and molecular mechanisms governing trophoblast outgrowth and differentiation during early stages of

pregnancy in ruminants (Cammass, Reinaud et al. 2005) has made it difficult to understand how enJSRV Envs exert their biological effects. Our early speculation, that enJSRV Env are essential for BNCs and syncytia formation by eliciting cell to cell fusion through a mechanism similar to viral-cell fusion, as proposed for syncytins in humans and mice, now seems unlikely given that none of the enJSRV Envs tested induced fusion when overexpressed in a variety of cell lines (although technical issues could account for this observation). Given the complexity and the temporal and spatial regulation of the signalling pathways that command pregnancy recognition and conceptus implantation in sheep (Spencer, Johnson et al. 2007) it is feasible that enJSRV Envs are just one of the components that contribute to the chain of events that ultimately result in a successful pregnancy. It still remains to be determined which enJSRV loci are expressed in the ovine placenta. It seems likely that cooperation between several *env* genes would be required since both humans and mice present two related *syncytins* genes with possibly redundant properties. On the other hand, there is evidence to support the hypothesis that ERV have been positively selected for a beneficial role in placental morphogenesis and possibly have contributed to the evolution of placental mammals (Harris 1991).

Overall, ERVs provide an excellent model to study the evolutionary interplay between exogenous retroviruses, endogenous retroviruses and their host. Despite all the discoveries made in these last years on this fascinating group of viruses, there are still many critical aspects on the pathogenesis of OPA, on the mechanisms leading to JLR and the role of enJSRVs in reproductive biology that remain to be elucidated that would provide the background required for the development of preventive/therapeutic strategies. Unravelling of these unknown aspects of JSRV and OPA biology provides an exciting challenge for the next generation of PhD students!

Accompanying material

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