ENDOGENOUS BETARETROVIRUSES IN THE OVINE UTERUS AND CONCEPTUS

A Dissertation

by

SARAH GRACE BLACK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Genetics

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Approved by:

Chair of Committee,	Thomas E. Spencer
Committee Members,	Fuller W. Bazer
	Gregory A. Johnson
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ABSTRACT

Endogenous Betaretroviruses in the Ovine Uterus and Conceptus. (August 2010)

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Chair of Advisory Committee: Dr. Thomas E. Spencer

Endogenous retroviruses (ERVs) comprise a significant portion of the genome of all mammals and have been implicated in placental development in multiple species. The ovine genome contains approximately 27 copies of endogenous betaretroviruses (enJSRVs) that are related to the exogenous Jaagsiekte sheep retrovirus (JSRV), an oncogenic retrovirus tropic to the lung. The enJSRV loci are abundantly expressed in the female reproductive tract and the conceptus, and they are essential to conceptus development.

Studies were conducted to determine: 1) the effect of exogenous progesterone administration on conceptus development after loss of enJSRV Env; 2) the specific enJSRV *env* expressed in the developing conceptus; and 3) if the uterus produces enJSRV viral particles that are capable of transducing the conceptus.

Study One determined the effects of exogenous progesterone on development of the conceptus in which enJSRV Env was ablated. Despite rescuing conceptus survival, the conceptuses were morphologically fragile and had reduced binucleate cell (BNC) numbers. These results suggest that mononuclear trophectoderm cell (MTC)

proliferation and differentiation is dependent on enJSRV Env, even in a uterine environment supported by exogenous progesterone.

Study Two assessed the enJSRV loci transcribed in the ovine conceptus during elongation before (day 13) and after (day 18) onset of BNC differentiation. The most represented loci in both day 13 and day 18 conceptuses encoded truncated Env proteins that did not contain membrane-spanning domains. Conceptuses from both time points contained evidence of the transcription of full-length, biologically active enJSRV Env, as well as completely intact proviral loci with the ability to produce viral particles *in vitro*.

Study Three utilized a transpecies embryo transfer experiment to determine if the intact enJSRVs loci could produce viral particles *in vivo*. The presence of enJSRV viral particles in the uterus was confirmed, as was their ability to transduce the conceptus.

Collectively, these studies provide evidence of truncated Env proteins, intact biologically active Env proteins, and enJSRVs viral particles within the ovine uterus and conceptus that are necessary to stimulate proliferation and differentiation of MTCs even in a uterine environment supported by exogenous progesterone.

DEDICATION

To my father,

Roy Walton Black

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Thomas E. Spencer, for his guidance and support throughout the course of my degree. I am very appreciative of the encouragement and guidance provided by my committee members, Drs. Fuller W. Bazer, Robert C. Burghardt, and Greg A. Johnson. In addition, the members of the Laboratory for Uterine Biology, IFRB trainees, and Genetics graduate students provided valuable assistance, patience, and friendship that made my graduate career possible, successful and entertaining.

This degree would not have been possible without the encouragement and support from my family. My mother, father, and sister have given me enormous amounts of love that has been a vital ingredient to my education. I also greatly appreciate Brad Christian for his positivity, advice and stimulating discussion throughout this experience.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Proper placental formation begins with formation of the trophectoderm and its continued growth is necessary for maternal recognition of pregnancy and continued production of progesterone from the corpus luteum (Spencer *et al.* 1996, Cameo *et al.* 2004). Progesterone induces production of growth factors and proteins from the uterus which stimulate trophectoderm proliferation, differentiation, and outgrowth (Spencer & Bazer 2002, Okulicz & Ace 2003, Spencer *et al.* 2004). In primates, rodents, and ruminants, the mononuclear trophectoderm cells differentiate into multinucleated syncytiotrophoblasts, which form the interface between maternal and fetal tissues (Cross 2000). These syncytiotrophoblasts are important in the exchange of nutrients and gases between the tissues, hormone production, and immune tolerance (Red-Horse *et al.* 2004). Failure of the trophectoderm to proliferate and differentiate into syncytiotrophoblasts is associated with pregnancy loss and complications (Redman & Sargent 2000, Dunlap *et al.* 2006b).

In humans and rodents, endogenous retroviruses (ERVs) have been implicated in the formation and function of the cytotrophoblasts and syncytiotrophoblasts via their envelope glycoproteins (Mi *et al.* 2000, Dupressoir *et al.* 2005). This phenomenon has also been seen in sheep, in which endogenous Jaagsiekte Sheep Retroviruses (enJSRVs) are highly expressed in the reproductive tract and placenta and are essential to survival of the periimplantation conceptus

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This dissertation follows the style of *Reproduction.*

(Palmarini *et al.* 2001a, Dunlap *et al.* 2005, Dunlap *et al.* 2006b). In ruminants, the mononuclear trophectoderm cells (MTC) rapidly proliferate and produce interferon tau (IFNT), the signal for maternal recognition of pregnancy (Spencer *et al.* 1996, Spencer & Bazer 2004). These cells differentiate into trophoblast giant binucleate cells (BNC) that will eventually fuse with cells of the luminal epithelium of the uterus as well as each other to form multinucleated syncytial plaques (Wooding 1984, Wooding 1992, Hoffman & Wooding 1993a). These plaques form the fetal contributions to the placental structures known as placentomes, which are sites of hematotrophic nutrition in the ruminant placenta (Igwebuike 2006).

Proliferation and differentiation of the MTCs is critical for fetal survival; however, this essential process is poorly understood in terms of it's cellular and molecular mechanisms. Evidence suggests that enJSRVs play an important role in these processes. The continued investigation into the role of enJSRVs in placental morphogenesis in sheep will contribute to fundamental knowledge of placental development in mammals.

CHAPTER II

LITERATURE REVIEW

Exogenous Retroviruses

Retroviruses are a large diverse family of viruses characterized by their replication strategy. Retroviral ribonucleic acid (RNA) is reverse transcribed into double-stranded deoxyribonucleic acid (DNA) which is then integrated into the genome of the host cell. This unique method of replication and their prevalence in mammals has led to intensive research into retroviruses; the study of retroviruses has impacted diverse areas of biology and medicine from carcinogenesis to biotechnology to evolutionary development.

Genomic organization. Exogenous retroviruses are divided into simple and complex classifications based upon their viral genomes (Vogt 1997b). Simple retroviruses consist of four major genes: *gag*, *pro*, *pol*, and *env*, and terminal noncoding sequences, which include two direct repeats (R), a U5 (5′unique), and a U3 (3′unique) sequence. The *gag* gene encodes matrix (MA), capsid (CA), and nucleocapsid (NC) proteins necessary for viral particle formation (Figure 2.1) (Vogt 1997b). The *pro* gene encodes a protease (PR) which is responsible for processing the viral proteins during viral particle assembly. The *pol* gene encodes both the reverse transcriptase (RT) and integrase (IN) enzymes. The *env* gene encodes the envelope glycoprotein (Env), consisting of both surface (SU) and transmembrane (TM) domains (Vogt 1997b). The Env is necessary for virus infectivity through receptor recognition and membrane fusion. Complex retroviruses encode additional regulatory proteins that perform various

functions. For instance, the Tax proteins in human T-lymphotropic virus (HTLV) promote transcription through interaction with long terminal repeat (LTR) sequences (Shimotohno *et al.* 1986). Retroviral genomes range in size between 7 and 11 kilobases (kB), and viral particles are 80 to 100 nanometers (nm) in diameter (Vogt 1997b).

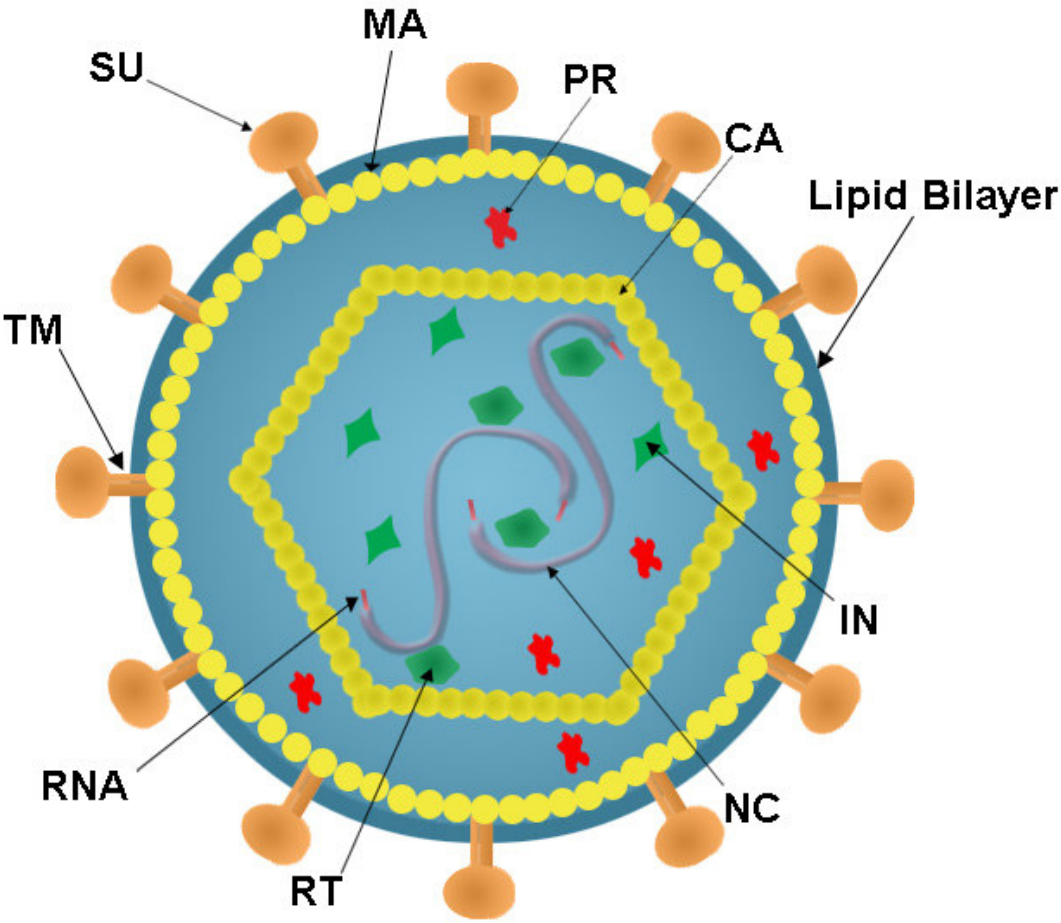

Figure 2.1. Schematic cross-section through a retroviral particle. The viral envelope (Env) consists of the transmembrane (TM) and the surface (SU) components linked by disulfide bonds and inserted in the lipid bilayer. The matrix (MA) protein, capsid (CA) protein, and nucleocapsid (NC) protein, all internal structural proteins, are encoded by the *gag* region of the viral genome. Major products of the *pol*-coding region are reverse transcriptase (RT) and integrase (IN). The protease (PR) is derived from the *pro* gene. Adapted from (Vogt 1997b).

Retroviruses can be further classified into seven groups defined by evolutionary relatedness (Table 2.1) (Weiss 2006). Five of the groups have oncogenic potential (alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses, and epsilonretroviruses), while the remaining two do not cause cancer but can still be pathogenic (lentiviruses and spumaviruses) (Vogt 1997b, Weiss 2006). Only those groups with simple genomes are known to become endogenized in host genomes, meaning they can be transmitted along with the chromosomes from parent to offspring (Weiss 2006).

Table 2.1. Seven genera of the retrovirus family. Adapted from (Vogt 1997b, Weiss 2006).

Genus	**Example**	**Genome Type**
Alpharetrovirus	Rous sarcoma virus (RSV)	Simple
	Mouse mammary tumor virus (MMTV)	Simple
Betaretrovirus	Jaagsiekte sheep retrovirus (JSRV)	
Gammaretrovirus	Feline leukemia virus (FLV)	Simple
	Bovine leukemia virus (BLV)	Complex
Deltaretrovirus	Human T-lymphotropic virus (HTLV)	
Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV)	Simple
Lentivirus	Human immunodeficiency virus (HIV)	Complex
Spumavirus	Simian foamy virus (SFW)	Simple

Life cycle. The life cycle of exogenous retroviruses begins when the viral particle enters the target cell through an interaction with the Env glycoprotein and specific receptor(s) on the plasma membrane (Figure 2.2). This interaction between Env and receptor is the main determinant of viral host range by defining the cell types (and species) for which the virus may enter (Vogt 1997b). For example, the human immunodeficiency virus (HIV) utilizes the CD4 antigen found primarily on T-helper cells and macrophages as its receptor, restricting the virus to infection in these cells (Dalgleish *et al.* 1984, Maddon *et al.* 1986). Once inside the cell, the RNA genome is transcribed into DNA by the viral reverse transcriptase. The resulting double-stranded DNA is then translocated into the nucleus and integrated into the chromosomal DNA of the host cell to form a provirus. This integration can occur throughout the genome, and the resulting provirus effectively becomes a gene within the host. The cellular RNA polymerase II can then transcribe the provirus under direction of the LTRs as promoters, leading to translation and assembly of proteins and progeny RNA into viral particles at the cell membrane. The particles bud from the plasma membrane, where the Env glycoproteins assemble on the surface. While many retroviruses are oncogenic, production of viral particles is not necessarily pathogenic, because viruses can be produced by most cells without negative effect on the organism (Rubin *et al.* 1961, Rubin *et al.* 1962).

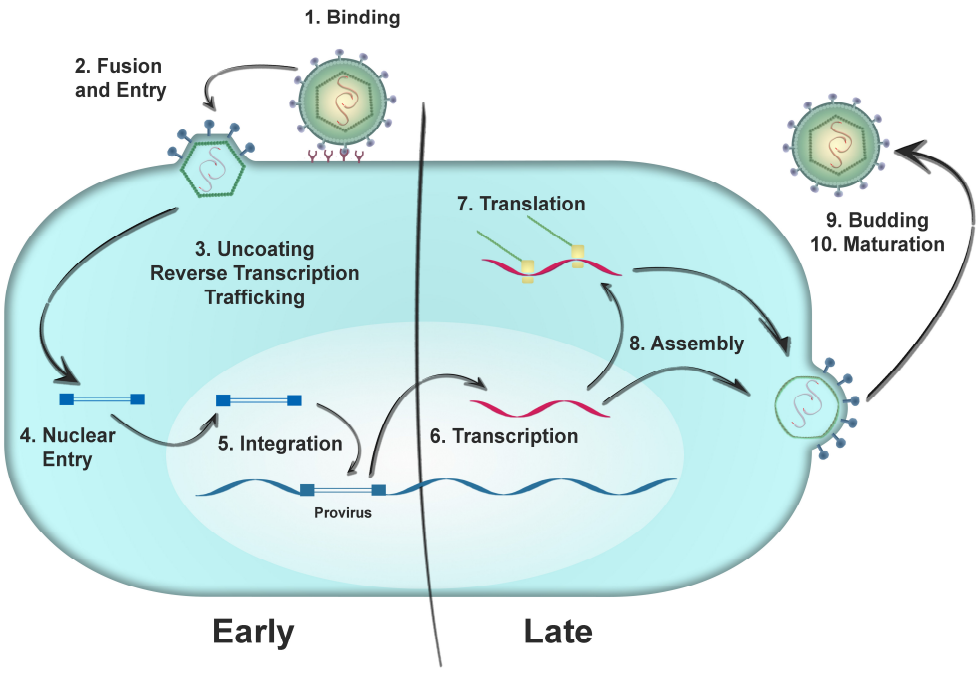

Figure 2.2. Schematic representation of the life cycle of retroviruses. The infecting virus attaches to a specific receptor on the cellular plasma membrane with the SU portion of the viral Env protein leading to fusion and entry. Reverse transcription then generates a double-stranded DNA copy of the RNA genome. The provirus is transported into the nucleus and integrated into chromosomal DNA. Transcription by the cellular machinery generates RNA copies that are then translated in the cytoplasm. Virion proteins and progeny RNA assemble at the cell boundary and the plasma membrane, and progeny virus is released into a mature viral particle.

Endogenous Retroviruses

The host cell machinery replicates the provirus along with the chromosomes, which allows for colonization of the germ line if the virus is integrated into the genome of a germ cell. It then has the potential to be vertically transmitted from parent to offspring in typical Mendelian fashion, which is termed “endogenization” and is termed

an endogenous retrovirus (ERV). ERVs have been found in all vertebrate species examined. Analysis of the human genome reveals that over 40% of the chromosomal DNA is derived from RNA via reverse transcription with 8-10% of retroviral origin (Bock & Stoye 2000, Lander *et al.* 2001). These ERVs are classified into ancient and modern according to when they integrated into the genome of the organism (Coffin 1990). Ancient retroviruses were inserted into the genome before speciation occurred as determined by the presence of the provirus in the same location of all individuals of the species; they can also be in the same location between related species (Boeke & Stoye 1997). Mutations are prevalent throughout the LTRs and coding sequences of ancient retroviruses. Modern ERVs are insertionally polymorphic between individuals of the species, and they have closely related exogenous counterparts (Boeke & Stoye 1997).

Genomic organization. A complete ERV provirus has the same general structure as an exogenous retrovirus: *gag*, *pro*, *pol*, and *env* genes flanked by two non-coding LTRs (Figure 2.3). The LTRs form during the process of reverse transcription, where the RT enzyme jumps from the U5 to the U3 end of the viral template. These sequences serve as promoters for the ERV genes, and can regulate transcription of nearby genes in a tissue-specific manner, as seen with the *amylase* and *pleiotropin* genes in humans (Ting *et al.* 1992, Schulte *et al.* 1996). While endogenous proviruses are often transcriptionally silent, some ERVs in several species, including human, mice, and sheep, have intact open reading frames for some or all proteins.

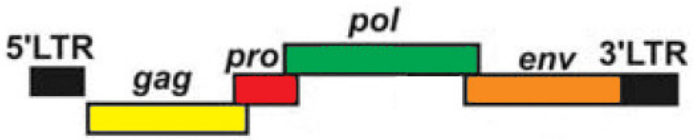

Figure 2.3. Basic genomic structure of ERVs. Various mutations, insertions, and deletions may ablate the ORFs of some or all of the genes in ancient ERVs, but genes with intact ORFs may survive. Adapted from (Arnaud *et al.* 2007a).

Effects of ERVs on host. ERVs are associated with detrimental effects to the host as well as beneficial roles in several species (Lower *et al.* 1996, Lower 1999, Bock & Stoye 2000). In mice and chickens, ERV proteins can recombine with other ERVs or exogenous retroviruses to form infectious virus that is oncogenic (Stoye *et al.* 1991, Benson *et al.* 1998). While examples of these recombination events have not been found in humans, some ERV products are detrimental and pathogenic. The human endogenous retrovirus type W (HERV-W) Env, ERVWE1, also known as syncytin 1, is involved in fusion in endometrial and breast cancer cell lines, as well as the redox-related cellular damage seen in multiple sclerosis (Antony *et al.* 2004, Bjerregaard *et al.* 2006, Strick *et al.* 2007). Some envelope glycoproteins in humans and mice are immunosuppressive due to a domain found in the TM section of the proteins (Cianciolo *et al.* 1985a). The Moloney Murine Leukemia Virus (MMLV) Env promotes tumor growth by suppressing the ability of the immune system to detect the transformed cells (Mangeney & Heidmann 1998). The immunosuppressive regions of human ERV Envs may play a beneficial role in protecting the developing placenta and/or fetus from the maternal immune response in pregnant females (Stoye & Coffin 2000, Mangeney *et al.* 2007).

Role of ERVs in cellular resistance to exogenous infection. Several ERV proteins offer an additional benefit to the host through cellular resistance to exogenous viral infection. In mice, the *Fv-4 env* gene confers resistance to the exogenous murine leukemia virus (MLV) by exerting a dominant negative effect on MLV Env (Takeda & Matano 2007). A HERV-W Env in humans confers resistance to the spleen necrosis virus, though the mechanism is unknown (Ponferrada *et al.* 2003). Infection by the exogenous Jaagsiekte Sheep Retrovirus (JSRV) can be blocked by endogenous JSRVs (enJSRVs) in sheep. The early mechanism of cellular resistance is conferred by enJSRV Env through receptor interference (Spencer *et al.* 2003). The second mechanism of resistance, known as JSRV late restriction (JLR), blocks JSRV replication with a transdominant Gag protein that prevents the exogenous virus from being released from the cell (Arnaud *et al.* 2007b, Murcia *et al.* 2007).

Role of ERVs in human placentation. In addition to offering the host cellular resistance to exogenous retroviral infection, available evidence supports the idea that ERV proteins are involved in placentation in humans, rodents, rabbits, and sheep. Expression of ERVs and presence of viral particles have been noted in placentae of many mammals including primates, cats, mice, guinea pigs, and Syrian hamsters since the early 1970s (Kalter *et al.* 1975, Smith & Moore 1988, Harris 1991, DeHaven *et al.* 1998). In the human, differentiation of the mononuclear villous cytotrophoblast is marked by the production of noninfectious HERV particles (Lyden *et al.* 1994). This differentiation includes fusion of the cytotrophoblasts to form the multinucleated villous syncytiotrophoblast, decreased proliferation, and increased hormone production (Lyden

et al. 1994). Due to their presence at the boundary layer between maternal and fetal tissues, the syncytiotrophoblasts are important in the maternal-fetal exchange of oxygen and nutrients, hormone production, and immune tolerance (Cross *et al.* 2003, Watson & Cross 2005). The significance of the expression of ERV proteins during placentation has been debated for years, and the hypothesis that ERVs have a fundamental role in placental morphogenesis was proposed nearly two decades ago (Harris 1991).

In humans, several ERV families [HERV-F, HERV-FRD, HERV-K (HML-2), HERV-R (ERV3), HERV-T, and HERV-W] are expressed in the placenta and have at least one loci that codes for an intact Env protein (de Parseval *et al.* 2003). The Env glycoproteins of most exogenous retroviruses contain a fusion domain and an immunosuppression domain involved in viral entry and escaping the host immune system; in ERVs, both of these domains could be adapted by the host to benefit placentation. Three HERV *env* loci that are expressed in the placenta have been studied in detail. ERV3 encodes an Env glycoprotein that includes the p15E immunosuppressive region found in exogenous Env proteins (Venables *et al.* 1995). *ERV3* is highly expressed in the syncytiotrophoblasts of the placenta, and is upregulated during differentiation of isolated cytotrophoblasts (Boyd *et al.* 1993, Lin *et al.* 1999). The upregulation of *ERV3* in human choriocarcinoma (BeWo) cells is accompanied by decreased cell growth, increased cell fusion, and induction of *beta chorionic gonadotropin* (*CGB*) mRNA (Lin *et al.* 1999). Although 1% of the Caucasian population contains a premature stop codon in ERV3, this may or may not affect the function of this protein in placental development (de Parseval & Heidmann 1998, Rote *et al.* 2004).

ERV3, while not directly involved in the fusion process, has been hypothesized to initiate the production of CGB and commit the trophoblast to cell cycle arrest (Rote *et al.* 2004). The precise function of ERV3 is still not known.

Syncytin 1, an HERV-W *env*, is preferentially expressed in the syncytiotrophoblasts and contains a fusion peptide charcteristic of exogenous viral glycoproteins (Blond *et al.* 2000). Syncytin 1 induces cell fusion in a variety of heterologous cell lines *in vitro*, and additional experiments in primary cytotrophoblast cells indicate a lack of fusion when syncytin 1 is inhibited (Blond *et al.* 2000, Mi *et al.* 2000, Frendo *et al.* 2003b). While it is difficult to determine cause and effect, placentae from patients with preeclampsia show decreased and/or aberrant expression of syncytin 1 (Lee *et al.* 2001, Knerr *et al.* 2002). In primary villous cytotrophoblasts, the loss of syncytin 1 decreased secretion of CGB as well as the number of multinucleated cells, indicating a role for syncytin 1 in both fusion and hormone regulation in the human placenta (Frendo *et al.* 2003b, Rote *et al.* 2004). The HERV-FRD Env, known as syncytin 2, also displays fusion activity *in vitro*, and is expressed in syncytiotrophoblasts (Blaise *et al.* 2003). A working hypothesis incorporating these three ERV Env proteins in the differentiation of villous cytotrophoblast was proposed by Rote and colleagues (Rote *et al.* 2004) in which the ERV Env proteins have overlapping roles in cell fusion, cell cycle control, and hormone production. While this theory is elegant, it is difficult to verify in humans given that the syncytiotrophoblast is one of the earliest cell types to differentiate in the conceptus.

Role of ERVs in mouse placentation. An *in silico* search identified genes in the murine genome that correspond to the human syncytins (Dupressoir *et al.* 2005). Two murine *env* genes were discovered that are phylogenetically unrelated or non-orthologous to human *syncytins 1* and *2*. Murine *syncytin A* (Gm52) and *syncytin B* (D930020E02Rik) are specifically expressed in the labyrinthine zone of the placenta, the region containing syncytiotrophoblasts. Additionally, the murine *env* genes have fusogenic capabilities *in vitro* (Dupressoir *et al.* 2005). The human and rodent ERVs appear to have been independently acquired with the *env* genes conserved in a functional state (Peng *et al.* 2007). Homozygous null mouse embryos (*SynA-/-*) die *in utero* between embryonic days (E) 11.5 and 13.5 (Dupressoir *et al.* 2009). Morphologically, the mutant mice showed growth retardation but no gross abnormalities, consistent with the presence of *syncytin A* expression in the placenta but not the embryo itself (Dupressoir *et al.* 2005, Dupressoir *et al.* 2009). Within the placenta of the *SynA-/-* mice, transport capacity was reduced, the labyrinth architecture was altered, and syncytiotrophoblast formation was reduced. Thus, syncytin A has a vital role in the placental morphogenesis of mice (Dupressoir *et al.* 2009). Syncytin B is both fusogenic and immunosuppressive, but the phenotype of syncytin B null mice has not been reported (Dupressoir *et al.* 2005, Mangeney *et al.* 2007). In addition to these *env* genes in rodents, an ERV *env* sequence has recently been identified in rabbits termed *syncytin-Ory1*. That gene encodes a fusogenic Env glycoprotein and is expressed in the placenta (Heidmann *et al.* 2009).

The retention of ERVs with ORFs is evidence of positive selection for a

beneficial role in development in multiple species. The intact *env* genes described above are expressed in the placentae of different species within which they elicit cell fusion or affect differentiation and have been preserved for millions of years. This phenomenon lends credence to the hypothesis that ERVs play an essential role in placental development in many species and have been positively selected for a beneficial and convergent role in the evolution of placental mammals (Villarreal 1997, Mi *et al.* 2000, Voisset *et al.* 2000, Dupressoir *et al.* 2009, Heidmann *et al.* 2009).

Jaagsiekte Sheep Retrovirus (JSRV)

Ovine pulmonary adenocarcinoma (OPA), initially known as Jaagsiekte, is a contagious form of lung cancer in sheep that is characterized by epithelial cell proliferation and excess production of lung fluids. OPA results from transformation of the type II alveolar epithelial cells and non-ciliated bronchiolar (Clara) cells of the lung, and results in death within a few weeks to months of tumor development (Demartini *et al.* 1988, Platt *et al.* 2002). Early reports found the infectious agent of OPA to be an RNA virus similar to other type B and type D retroviruses (Verwoerd *et al.* 1980, Sharp *et al.* 1983). Additional research revealed that the RNA virus Jaagsiekte Sheep Retrovirus (JSRV) is exclusively and consistently present in OPA tumor tissues and lung secretions from infected animals, and purified JSRV can transmit OPA to both sheep and goats (Tustin *et al.* 1988, Palmarini *et al.* 1996a). Recent evidence shows that colostrum, milk and lung secretions are major reservoirs of JSRV from infected animals and can transmit the infection to uninfected animals (Grego *et al.* 2008, Cousens *et al.* 2009). OPA resembles human bronchio-alveolar carcinoma, and is a model for human

retrovirus-associated lung cancers (Palmarini *et al.* 1997, Palmarini *et al.* 1999).

Genomic organization. JSRV is morphologically distinct and phylogenetic analysis classifies it as a betaretrovirus (Payne *et al.* 1983, Verwoerd *et al.* 1983). The viral particles range in size from 80 to 100 nm, and the genome of JSRV is 7.5 kB with simple organization, including U5 and U3 sequences, *gag*, *pro*, *pol*, and *env* genes (Hod *et al.* 1977). The JSRV provirus contains alternative splicing sites and premature polyadenylation sites that result in 6 virus-specific RNAs in addition to the normal full length RNA (7.5 kB) and the *env* spliced mRNA (2.4 kB) (Palmarini *et al.* 2002). JSRV also contains an ORF for a protein of unknown function, known as *orf-x*, that is similar to members of the G-protein coupled receptor family (York *et al.* 1992, Bai *et al.* 1999, Rosati *et al.* 2000, Palmarini & Fan 2003). Recent evidence suggests that the JSRV provirus also encodes a regulatory factor, Rej, that is necessary for the accurate translation of the Gag protein (Hofacre *et al.* 2009). The sequence encoding Rej also encodes the signal peptide of the envelope protein, which regulates viral gene expression post transcriptionally (Caporale *et al.* 2009, Hofacre *et al.* 2009). The JSRV provirus was identified in the DNA of lymphoid tissues before tumor formation, as well as in tumor tissues in infected animals, indicating probable dissemination of the virus through lymph tissues (Palmarini *et al.* 1996b, Holland *et al.* 1999). Endogenous sequences of JSRV (enJSRV) were also identified in all ovine tissues examined and could be distinguished from the exogenous provirus by sequences in the U3 region (York *et al.* 1992, Hecht *et al.* 1994, Bai *et al.* 1996).

The LTR sequences of the provirus are essential to the tissue specific expression

of JSRV. JSRV is preferentially active in type II pneumocytes and Clara cells (McGee-Estrada *et al.* 2005). The enhancer elements and proximal promoter of JSRV LTR contain potential binding sites for nuclear factor kappa B (NFKB1), which plays a key role in regulating immune response in many cells and is often dysregulated in cancer cells, and forkhead box M1 (FOXM1), which is a transcription factor specific to lung epithelia (Palmarini *et al.* 2000a). Subsequent research found that FOXM1 activated the JSRV LTR in type II pneumocytes, but not Clara cells *in vitro* (McGee-Estrada *et al.* 2002). The LTR also contains a binding site for the general transcription factor CCAAT/enhancer binding protein alpha (CEBPA) that is critical for its activity (McGee-Estrada & Fan 2006). Transgenic mice featuring the JSRV LTR driving a reporter gene preferentially express the reporter in type II pneumocytes (Dakessian & Fan 2008).

Transformation by JSRV. The mechanism of transformation in lung epithelia cells by JSRV was initially a mystery, due to the absence of a dedicated oncogene within the genome of JSRV (Hecht *et al.* 1996a). Surprisingly, further analysis found the Env is sufficient and necessary to develop lung tumors in sheep and mice (Palmarini *et al.* 1999, Caporale *et al.* 2006, Dakessian *et al.* 2007). The JSRV Env is a typical viral envelope glycoprotein of 69 kilodaltons (kD), with an SU domain and a TM domain separated by a furin cleavage site. The SU domain contains the signal or leader peptide that is cleaved after translation and the receptor binding site, while the TM domain contains the fusogenic domain, the membrane-spanning region, and a cytoplasmic tail (York *et al.* 1992). The cytoplasmic tail of JSRV Env has a phosphatidylinositol 3-kinase (PI3K) docking site (YXXM motif) which was initially found to be necessary for transformation

in NIH 3T3 cells (Palmarini *et al.* 2001b). Elevated levels of phosphorylated AKT1 are found in the transformed cells, indicating a potential role for the PI3K/AKT1 cell signaling pathway in JSRV transformation. The YXXM motif is not necessary for transformation in rodent cells, but transformation efficiency is markedly reduced when the motif is mutated (Liu *et al.* 2003b). Moreover, PI3K is dispensable to the transformation of mouse fibroblasts by JSRV (Maeda *et al.* 2003). AKT1-independent transformation by JSRV occurs in chicken cells, though efficiency of transformation is markedly increased in the presence of AKT1 phosphorylation (Allen *et al.* 2002, Zavala *et al.* 2003). AKT1 degradation may be responsible for the decreased proliferation of OPA tumor cells seen upon inhibition of HSP90, which blocks JSRV transformation (Varela *et al.* 2008). These *in vitro* studies in rodent and avian cell lines indicate that transformation by JSRV Env involves AKT1 phosphorylation through PI3K in addition to other signaling pathways. It is important to note that these studies investigating the signaling pathways activated by JSRV transformation were conducted using cells transfected and passaged for up to 6 weeks to allow growth of foci. These tumorogenic foci have markedly different expression profiles compared to their non-transformed counterparts. The YXXM motif in the cytoplasmic tail of JSRV Env is necessary for *in vivo* tumor formation in lambs (Cousens *et al.* 2007).

Inhibition of the Ras-MEK-Mitogen-Activated Protein Kinase (MAPK) p44/42 pathway strongly suppresses transformation by JSRV, while p38 MAPK inhibitors actually increase transformation of cells. Intriguingly, inhibition of the AKT1-mTOR pathway only slightly decreases transformation. These results indicate both the AKT1

and p44/42 pathways are involved, but the Ras-MEK-MAPK pathway plays a more prominent role in JSRV-induced cell transformation (Maeda *et al.* 2005). Mutational analysis of the cytoplasmic tail of JSRV Env confirms these results and indicates that one mutation consistently results in loss of signaling through the AKT1-MTOR pathway, with partial transformation occurring through Ras-Raf-MEK1/2. Some mutants show supertransforming abilities that are characterized by an increase in AKT1 signaling and a reduction in p38 MAPK cell signaling (Hull & Fan 2006). In support of this *in vitro* work, histological analysis of OPA tumors shows increased levels of activated RAF1 and MAPK1/2 in addition to the transcription factors RPS6KA1 (a kinase in the MAPK family), ELK1 (a serum responsive transcription factor activated by Ras-Raf-MAPK), and MYC (a transcription factor involved in cell cycle progression, apoptosis and cellular transformation) (Papavassiliou 1994, De Las Heras *et al.* 2006, Laurenti *et al.* 2009)

Current evidence indicates that transformation by JSRV is dependent upon the cytoplasmic tail of Env, through which signaling pathways are activated. Upon infection by JSRV Env, the AKT1-MTOR and Ras-MEK-MAPK pathways are activated to induce transformation, while the p38 MAPK pathway may work to decrease transformation. Activation of PI3K initiates a pathway shown to inhibit apoptosis and regulate transition through the cell cycle in some cells, with AKT1 as its central effector (Roche *et al.* 1994). AKT1 is an activator of ribosomal p70S6K (RPS6KB2) through MTOR. This activation of p70S6K regulates many cellular processes involved in mitogenic response (Chou & Blenis 1995). The Ras-MEK-MAPK pathway activated by JSRV is a classical signaling transduction pathway, in which activated Ras signals for growth and cell

division through MEK-MAPK. Oncogenic Ras may also trigger apoptosis via the p38 pathway (Maeda *et al.* 2005). Interestingly, both of these signaling pathways are involved in growth and differentiation of the trophoblasts in mice and humans (Pollheimer & Knofler 2005).

Receptor interactions. The cellular receptor for JSRV is a glycosylphosphatidylinositol (GPI) - anchored protein known as hyaluronidase 2 (HYAL2) that maps to human chromosome 3p21.3, a tumor suppressor region (Rai *et al.* 2000, Rai *et al.* 2001). JSRV is able to utilize HYAL2 as a receptor in sheep, human, monkey, bovine, dog, and rabbit cells, but cannot use mouse, rat, or hamster HYAL2 (Rai *et al.* 2000). Compared to other hyaluronidase family members, HYAL2 has very weak activity, but serves as an effective receptor for JSRV and enJSRV Env proteins (Rai *et al.* 2001, Spencer *et al.* 2003). The SU domain of the exogenous JSRV interacts directly with human HYAL2 to mediate viral entry into human and rat cells *in vitro* (Liu *et al.* 2003a). JSRV entry is pH-dependent, and, in cells overexpressing HYAL2, the SU domain of JSRV Env mediates fusion at low pH (Bertrand *et al.* 2008, Cote *et al.* 2008, Cote *et al.* 2009). JSRV utilizes a dynamin-dependent endocytosis pathway for entry into cells, possibly in a two-step entry process similar to avian leukosis and sarcoma virus (Bertrand *et al.* 2008).

Due to its location in a tumor suppressor region, HYAL2 was originally hypothesized to play some role in the oncogenic potential of JSRV; however, JSRV Env is able to induce transformation independent of its receptor (Chow *et al.* 2003). Initial studies indicated that HYAL2 was involved in the transformation of cells through JSRV

Env by interaction with RON tyrosine kinase, or macrophage stimulating 1 receptor (MST1R). Further analysis revealed that HYAL2 does not interact with MST1R, and JSRV Env is both necessary and sufficient to induce transformation of cells working independently of the receptor HYAL2 (Maeda *et al.* 2001, Chow *et al.* 2003, Miller *et al.* 2004). However, there is evidence that human HYAL2 can suppress transformation by Env in human and mouse cells through degradation of the Env protein (Liu *et al.* 2003a).

Endogenous JSRV (enJSRV)

The ovine genome contains at least 27 copies of enJSRVs that are highly related to two oncogenic exogenous retroviruses, JSRV and enzootic nasal tumor virus (ENTV) (Figure 2.4) (Palmarini *et al.* 2000b). Investigation of these 27 loci revealed potential beneficial roles for the host and a complex interaction between the endogenous and exogenous forms of the provirus. The evolutionary history of a particular ERV can be determined by examining its presence in different animals within the same species and within related species. The presence of the loci between two species indicates an integration event before the split of those species (Boeke & Stoye 1997). The age of a provirus can also be estimated through analysis of the sequence divergence between its LTRs (Johnson & Coffin 1999). Ancient ERVs will tend to have significant divergence between the 5' and 3' LTRs, while modern ERVs have sequences that are more similar. The enJSRVs have been integrating in the host genome for the past 5 to 7 million years, before the split between the Ova genus (domestic sheep and its wild relatives) and the Capra genus (domestic goat and its wild relatives) (Hernandez Fernandez & Vrba 2005, Arnaud *et al.* 2007a). Due to this phenomenon, the integration events of enJSRV can be

used to trace the domestication of sheep and its division into breeds (Chessa *et al.* 2009). Invasion of the sheep genome by JSRV is likely still happening today, with the most recent integration event occurring less than 200 years ago (Arnaud *et al.* 2007a).

Most contemporary families of ERVs are defective for viral replication due to mutations by substitutions, insertions, and deletions, and thus have lost their infectious abilities (Boeke & Stoye 1997). The presence of intact proviral genes within the sheep genome indicates that these loci are under positive selection for a biologically important role in sheep. Two enJSRV proviruses (enJS56A1 and enJSRV-20) encode Gag proteins that contain the transdominant mutation [arginine (R) to tryptophan (W)] capable of blocking replication of the exogenous JSRV via the JLR mechanism introduced earlier (Arnaud *et al.* 2007a). The dominant Gag protein (R21W) alters the localization of the non-dominant Gag proteins, inhibiting maturation and release of the viral particles (Murcia *et al.* 2007). The R21W Gag sequesters the non-mutated Gag proteins into microaggregates that prevent them from interacting with the recycling endosomes necessary for viral exit (Arnaud *et al.* 2007b). The two proviruses containing the transdominant Gag entered the genome independently and around the time of sheep domestication approximately 10,000 years ago (Arnaud *et al.* 2007a, Chessa *et al.* 2009). The enJSRV-26, which integrated into the sheep genome as recently as 200 years ago, is the only enJSRV known to escape JLR, highlighting the evolutionary antagonism between the exogenous and endogenous JSRV (Arnaud *et al.* 2007a, Arnaud *et al.* 2007b).

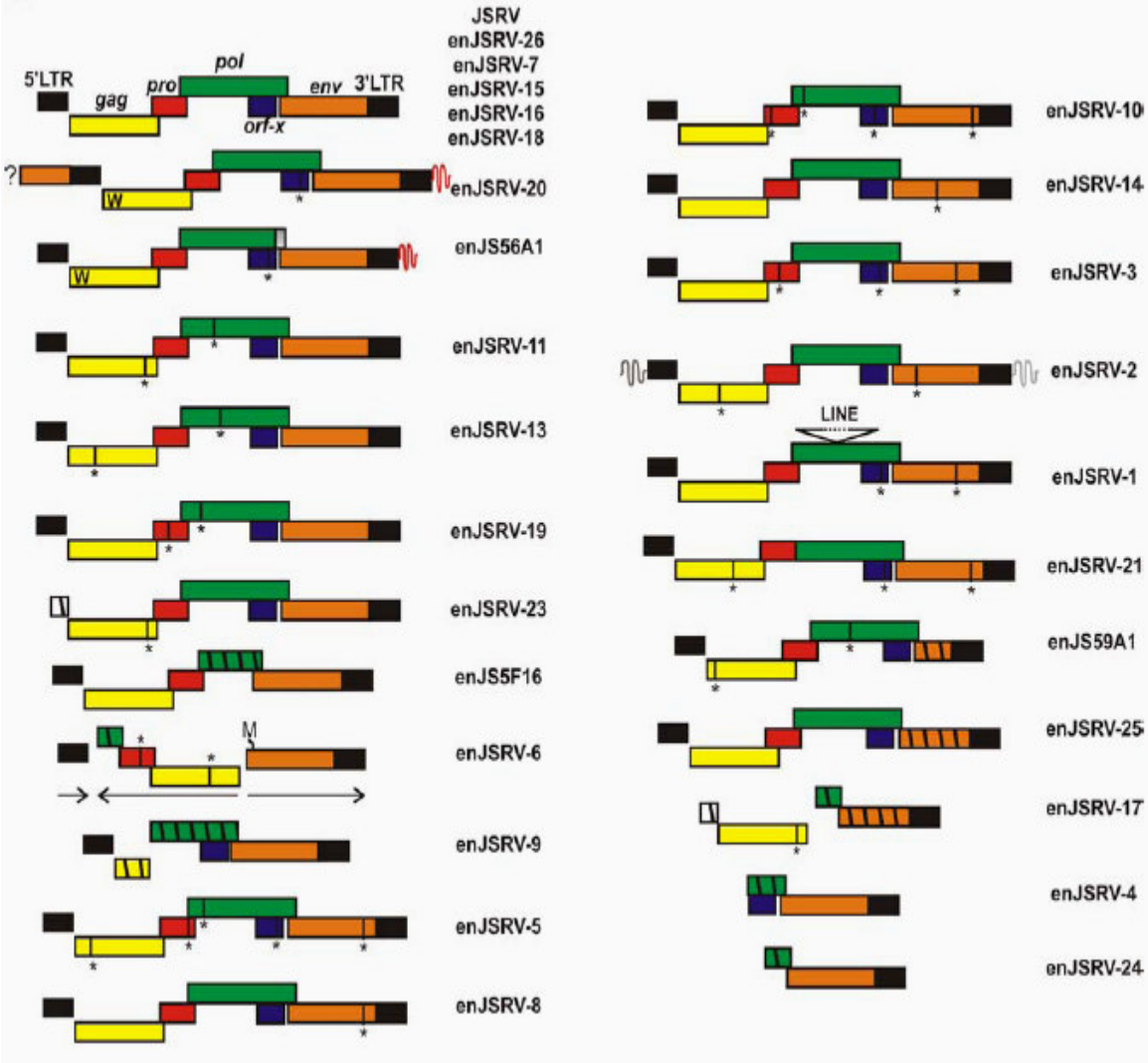

Figure 2.4. Genomic organization of the enJSRVs group. (From (Arnaud *et al.* 2007a)). Five proviruses contained the typical intact genomic organization of the replication competent exogenous JSRV (top). The ‘‘W’’ in the *gag* reading frame of enJS56A1 and enJSRV-20 indicates the R21W substitution present in these two transdominant proviruses. Before the proximal LTR, enJSRV-20 contains a portion of an *env* gene indicated by the red box and a question mark. Stop codons are indicated by vertical lines and an asterisk (*). Deletions in the proviruses are indicated by hatched boxes. 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. The enJSRV-1 has a LINE element within the *pol* reading frame.

Five of the enJSRV loci have intact ORFs for all the proviral genes and can release viral particles, unless blocked by the proviruses coding for the JLR Gag proteins (Arnaud *et al.* 2007a). Sixteen of the 27 proviruses contain ORFs for an intact Env protein. These intact and functional glycoproteins utilize the same receptor at similar efficiencies or better than the exogenous form and offer competition for receptor binding (Spencer *et al.* 2003). The enJSRV Env is similar to the exogenous JSRV Env, containing a signal peptide in SU domain that is cleaved and a furin cleavage site between the SU and TM domains. All of the enJSRV *env* loci contain a highly mutated region, termed variable region 3 (VR3), in the TM domain that corresponds to the cytoplasmic tail (Palmarini *et al.* 2000b, Arnaud *et al.* 2007a).

Expression profile of enJSRVs. The enJSRVs are predominantly expressed in the female reproductive tract of sheep and goats. Epithelia of oviduct, uterus, cervix, and vagina express enJSRVs, and the enJSRVs *env* is one of the most abundant RNAs in the ovine endometrium (Spencer *et al.* 1999b, Palmarini *et al.* 2000b, Palmarini *et al.* 2001a). At birth, the uterus does not express enJSRVs *env*, but expression begins at day 7 and continues into adulthood (Palmarini *et al.* 2001a). In cyclic ewes, expression is lowest at day 1, peaks on day 13, then decreases to day 15 (Palmarini *et al.* 2001a). In pregnant ewes, endometrial enJSRVs *env* mRNA levels are high on day 11, maximal on day 13, and then decline through day 19 (Palmarini *et al.* 2001a). The temporal pattern of expression of *env* indicates that it is potentially regulated by progesterone in the uterus. This is supported by *in vitro* evidence in ovine endometrium cells that enJSRV LTRs show increased transcriptional activity upon progesterone administration (Palmarini *et al.*

2001a). Additionally, exogenous progesterone administration stimulates enJSRVs *env* expression *in vivo* (Dunlap *et al.* 2005). The *enJSRVs* RNA has also been found in the thymus, lymph nodes, and lungs of the developing fetus (Sanna *et al.* 2002, Spencer *et al.* 2003). This expression is thought to tolerize the fetal immune system to infection by the exogenous JSRV, as most JSRV infections are characterized by a lack of immune response (Ortin *et al.* 1998, Spencer *et al.* 2003).

The presence of intact ORFs for ERVs within the domestic sheep genome is evidence for positive selection of these genes. Roles for enJSRV in protection from the exogenous JSRV have been described above. Endogenous JSRV is highly expressed in the reproductive tract, while the exogenous virus is tropic to the lungs (Palmarini *et al.* 1996b, Palmarini *et al.* 2000b). It is hypothesized that selective pressure from the JLR proviruses and intact Env proteins found in the reproductive tract drove the exogenous virus to an area with little *enJSRV* expression (i.e. the lung epithelia) (Palmarini *et al.* 2000b, Varela *et al.* 2009). The proposed role of enJSRV in placental development will be discussed later.

Biological Roles of ERVs in Placental Morphogenesis

The presence of retroviral particles in the placentas of mammals has intrigued investigators for many years. In humans and primates, these particles and retroviral proteins were determined to be C-type and of endogenous origins (Kalter *et al.* 1973a, Kalter *et al.* 1973b, Jerabek *et al.* 1984). ERV proteins and particles localize to the basal syncytiotrophoblast layer during the time of active cytotrophoblastic intercellular fusion (Johnson *et al.* 1990, Lyden *et al.* 1994). Further characterization of the proviruses

responsible for these proteins and particles in humans revealed multiple HERVs expressed in the placenta, with at least 5 coding for intact Env proteins (de Parseval *et al.* 2003).

ERV3. A single copy provirus on chromosome 7, named *ERV3*, produces three mRNAs of 3.5, 7.3, and 9 kB in length that are highly expressed in the placental chorion (O'Connell *et al.* 1984, Kato *et al.* 1987). All three ERV3 mRNAs lack the *gag* gene and most of the *pol* gene due to splicing. Interestingly, the two longer mRNAs also contain human cellular sequence from beyond the 3'LTR sequence (Kato *et al.* 1987). This non-viral portion of the mRNA encodes a zinc finger protein related to the transcription factor Krüppel (Kato *et al.* 1990). *ERV3* mRNAs are present in first trimester and full-term chorionic villi, specifically in the syncytiotrophoblasts but not the cytotrophoblasts (Cohen *et al.* 1988, Larsson *et al.* 1994). This restricted expression may explain the relatively low expression of *ERV3* in the choriocarcinoma cell lines, most notably BeWo cells, as these lines were developed from cytotrophoblasts (Boyd *et al.* 1993). The low expression of *ERV3* is proposed to be pathologically associated with susceptibility to choriocarcinoma formation (Cohen *et al.* 1988, Kato *et al.* 1988). High levels of *ERV3* are correlated with fusion in studies of expression in placentas, as well as in BeWo cells (Larsson *et al.* 1994, Lin *et al.* 1999). In BeWo cells, the addition of forskolin induces differentiation of single-celled cytotrophoblasts into multi-nucleated syncytia. This differentiation is characterized by an increase in production of CGB (Licht *et al.* 2001). Forskolin-mediated differentiation in BeWo cells also results in increased *ERV3* mRNA (Lin *et al.* 1999). Overexpression of *ERV3* alone also induces differentiation

characterized by decreased cell growth, increased nuclear size, a slight increase in cell fusion and induction of CGB production (Lin *et al.* 1999). ERV3 inhibits cell proliferation in BeWo cells through regulation of proteins cyclin B and p21, while inducing CGB secretion with PKA signaling (Lin *et al.* 2000).

Syncytin 1. *ERVWE1*, or *syncytin 1*, is an HERV-W *env* gene also located on chromosome 7 and expressed in the placenta with transcripts of 4 and 8 kB in length (Blond *et al.* 1999, Mi *et al.* 2000, Voisset *et al.* 2000). Expression is seen mainly in the syncytiotrophoblast, where the Env protein localizes to the basal cytoplasmic membrane in normal placentas (Mi *et al.* 2000, Lee *et al.* 2001). *ERVWE1* encodes a functional retroviral Env protein that is capable of mediating viral-cell fusion as determined by pseudotyping assays (An *et al.* 2001). As a fusogenic membrane glycoprotein, syncytin 1 is reported to be capable of fusion independent of a receptor, though there is conflicting evidence that it requires interaction with the type D mammalian retrovirus receptor to fuse cells (Blond *et al.* 2000, Mi *et al.* 2000). Syncytin 1 utilizes two sodium-dependent neutral amino-acid transporters (SLC1A4 and SLC1A5) as its receptor (Lavillette *et al.* 2002). *SLC1A5* is expressed in cytotrophoblasts of the human placenta adjacent to the *syncytin 1*-expressing syncytiotrophoblasts and, correspondingly, is downregulated in differentiating BeWo cells (Hayward *et al.* 2007).

The LTR of *ERVWE1* contains a CAAT box and an octamer module for basal transcriptional control in trophoblast cells and is responsive to cyclic adenosine monophosphate (cAMP) (Cheng *et al.* 2004, Prudhomme *et al.* 2004). This LTR interacts with a tissue specific enhancer that contains a binding site for GCM1, a placenta

specific transcription factor that transactivates *ERVWE1* (Yu *et al.* 2002, Prudhomme *et al.* 2004). Progesterone also positively regulates *syncytin 1* expression in first trimester trophoblast cells, though the exact mechanism for activation is unknown (Noorali *et al.* 2009).

To study the regulation of syncytin 1 and its potential function in the placenta, multiple studies were performed in BeWo cells and primary cytotrophoblasts, both of which naturally express *syncytin 1*. Increased *syncytin 1* expression accompanies syncytia formation and increased induction of CGB production in forskolin-mediated terminal differentiation of BeWo cells (Mi *et al.* 2000). Correspondingly, knockdown of syncytin 1 in BeWo cells and primary villous cytotrophoblast cells inhibits fusion and decreases CGB secretion (Mi *et al.* 2000, Frendo *et al.* 2003b). Overexpression of *GCM1* increases levels of syncytin 1 and fusion in BeWo cells (Yu *et al.* 2002). Other factors that affect *syncytin 1* expression *in vitro* include the gap junction protein GJA1 (connexin 43), which, when inhibited, decreases syncytin 1 and fusion (Frendo *et al.* 2003a). Overexpression of CD9, a membrane protein, results in a increased *GCM1* and *syncytin 1* mRNA, which is ablated by a PKA inhibiting compound (Muroi *et al.* 2009). Similarly, transfection of PKA into BeWo cells increases *GCM1* and *syncytin 1* expression (Knerr *et al.* 2005). Summarily, evidence reveals that GCM1 regulates *syncytin 1*, while it is regulated by other placental factors through the PKA pathway.

In addition to its role in fusion of cytotrophoblasts, syncytin 1 may also play a role in protecting differentiating cells from apoptosis. Forskolin-mediated terminal differentiation of BeWo cells increases expression of the ERVWE1 Env glycoprotein,

which is accompanied by decreased DNA synthesis (Mi *et al.* 2000). This decrease in DNA synthesis without apoptosis is also seen in cultured cells when transfected with syncytin 1, and reports indicate that syncytin 1 may be anti-apoptotic (Mi *et al.* 2000, Knerr *et al.* 2007, Knerr *et al.* 2008). ABCG2, a xenobiotic/lipid transporter also shows anti-apoptotic effects in BeWo cells, whereas inhibition of this membrane protein results in increased cell apoptosis, and decreased syncytin 1 and CGB expression without affecting fusion (Evseenko *et al.* 2007).

Preeclampsia is a condition in pregnant women that results in shallow placentation characterized by decreased syncytiotrophoblast formation (Lee *et al.* 2001). *Syncytin 1* mRNA is reduced and the protein is localized to the apical membrane of the syncytiotrophoblasts in preeclamptic placentae, as opposed to the normal basal membrane localization (Lee *et al.* 2001) (Chen *et al.* 2006); however, *SLC1A5* expression is not affected in preeclamptic placentas (Chen *et al.* 2006). Low oxygen conditions are used to mimic preeclampsia *in vitro*, and primary cytotrophoblast and BeWo cells induced with forskolin cultured under hypoxic conditions show retarded differentiation characterized by reduced fusion, CGB production, and levels of *syncytin 1* and *SLC1A5* mRNAs (Kudo *et al.* 2003, Wich *et al.* 2009). Hypoxia leads to decreased GCM1 by suppressing the PI3-AKT1 signaling pathway, leading to GSK3B activation, which phosphorylates GCM1 leading to ubiquitination and degradation (Chiang *et al.* 2009, Wich *et al.* 2009). This reduced GCM1 expression may explain the reduced syncytin 1 in cytotrophoblasts cultured under hypoxic conditions. In placentae affected by trisomy 21, there is a defect in syncytiotrophoblast formation and decreased production of CGB

(Frendo *et al.* 2000). *In vitro* evidence suggests that overexpression of *SOD1* (*superoxide dismutase 1* on chromosome 21) could be responsible for lack of differentiation including decreased *syncytin 1* expression (Frendo *et al.* 2001).

Syncytin 2. The HERV-FRD Env glycoprotein, syncytin 2, is fusogenic *in vitro* and expressed in the cytoplasm of cytotrophoblasts bordering the syncytiotrophoblasts (Blaise *et al.* 2003, Malassine *et al.* 2007). There are reports that *syncytin 2* expression decreases with syncytia formation *in vitro* and as pregnancy progresses *in vivo* (Kudaka *et al.* 2008, Malassine *et al.* 2008). Similar to syncytin 1, down-regulation of *syncytin 2* via siRNA in primary trophoblasts and in BeWo cells treated with forskolin results in decreased fusion (Vargas *et al.* 2009). Syncytin 2 utilizes the novel receptor MFSD2A, which is thought to be a carbohydrate transporter and is expressed in the syncytiotrophoblasts of the placenta and in BeWo cells (Blaise *et al.* 2003, Esnault *et al.* 2008). In preeclamptic placentas, expression of syncytin 2 is reduced, but can be stimulated upon addition of forskolin when cultured (Chen *et al.* 2008). Cytotrophoblasts from trisomy 21-affected placentas fuse poorly and show no decreased syncytin 2 when stimulated to differentiate (Malassine *et al.* 2008).

Evolutionary history of HERVs. Investigation into the conservation of the human ERVs involved in placentation reveals varying eras for integration events. *ERV3* integrated into the genome around 30 million years ago (MYA); *ERV3* sequences were amplified from DNA of great apes and Old World monkeys, but not New World monkeys or gorillas (Herve *et al.* 2004). While the ORF were conserved in all other species since integration, the *ERV3* locus was lost in gorillas (Herve *et al.* 2004). *ERV3*

does not code for a functional or fusogenic retroviral Env protein, having lost the fusion domain of its TM section; however, its function in placentae could be unrelated to fusion or immunosuppression (Rote *et al.* 2004). *ERVWE1* had an integration event around 25 MYA, and is present only in Old World monkeys and hominoids, not New World monkeys (Kim *et al.* 1999, Voisset *et al.* 1999). There is strong conservation of the LTRs, splice sites, and the ORF of the *ERVWE1* locus through human, chimpanzee, gorilla, and gibbon (Mallet *et al.* 2004). Evidence suggests that syncytin 1 in Old World monkeys is inactive, despite reports of conservation of the fusion domain (Mallet *et al.* 2004, Caceres & Thomas 2006). Syncytin 1 also contains a p15E immunosuppression domain, but it is not functional (Mangeney *et al.* 2007). *Syncytin 2* is the oldest, having integrated 40 MYA (Blaise *et al.* 2003, Renard *et al.* 2005). The ORF and fusogenic capabilities of Syncytin 2 are conserved in all simians, indicative of purifying selection (Blaise *et al.* 2003). Syncytin 2 also contains a functional p15E immunosuppression domain (Mangeney *et al.* 2007).

Trophoblast specific gene expression. In addition to the conserved *env* loci described above, there are several examples of ERV elements with active roles in placentae of both humans and mice. The human *pleiotropin* gene (*PTN*) encodes a placental specific transcript that is actually a fusion mRNA with HERV-C (*HERV-PTN*) (Schulte *et al.* 1996). The placenta-specific expression of this transcript is due to an Sp1 binding site in the LTR of HERV-C (Schulte *et al.* 2000). Ablation of this transcript in choriocarcinoma cells inhibits growth and invasiveness (Schulte *et al.* 1996). There are several other examples of genes in humans and some apes that are specifically expressed

in the placenta due to retroviral LTRs acting as promoters, including endothelin B receptor (EDNRB), insulin-like 4 (INSL4), midline 1 (MD1), leptin (LEP), and aromatase (CYP19A1) (Bi *et al.* 1997, Medstrand *et al.* 2001, Landry *et al.* 2002, Bieche *et al.* 2003, van de Lagemaat *et al.* 2003). The conservation of these promoter arrangements that include retroviral elements may have contributed to the evolution of the human placenta (Rawn & Cross 2008). Two ancient retroviral elements, PEG10 and RTL1, are also conserved in all eutherian mammals and are essential for placental morphogenesis (Ono *et al.* 2006, Sekita *et al.* 2008). Notably, both PEG10 and RTL1 are maternally imprinted (Ono *et al.* 2001, Lin *et al.* 2003). Thus, the acquisition of retroviral elements has likely shaped and driven the evolution of placentation in mammals.

Murine syncytins. An *in silico* search was performed to identify ERV *env* genes in the murine genome (Dupressoir *et al.* 2005). The placenta-specific *syncytin A* and *syncytin B* were both identified and found to code for functional retroviral envelopes capable of cell-cell fusion, though utilizing different receptors (Dupressoir *et al.* 2005). In the mouse placenta, there are two syncytiotrophoblast layers (SynTI and SynTII) in the labyrinth portion (Rawn & Cross 2008). SynTI is closest to the maternal blood sinusoids and surrounded by trophoblast giant cells. SynTII is adjacent to the fetal endothelial cells surrounding the fetal capillaries (Rawn & Cross 2008). Both of these layers have characteristics similar to the syncytiotrophoblasts of the human placenta, including nutrient exchange, hormone production, and immune regulation (Watson & Cross 2005). *Syncytin A* is expressed specifically in SynTI beginning around E8.5, while

syncytin B is expressed in SynTII during the same time points (Simmons *et al.* 2008). Similar to the human syncytin 1, there are GCM1 binding sites in both *syncytin A* and *B*; however, *syncytin A* is not responsive to GCM1 overexpression or ablation (Asp *et al.* 2007, Simmons *et al.* 2008). *GCM1* expression overlaps that of *syncytin B* in the SynTII layer and *syncytin B* is reduced in GCM1 mutants (Simmons *et al.* 2008). *In vitro* experiments using trophoblast stem cells induced to differentiate and fuse found that syncytin A levels increase with fusion and, correspondingly, inhibition of syncytin A in these cells results in decreased fusion (Gong *et al.* 2007). Indeed, embryonic death occurs between E11.5 and 13.5 due to placental defects in *syncytin A* null mice (*SynA-/-*) (Dupressoir *et al.* 2009). An in-depth analysis of the placenta of these mice revealed that the trophoblast stem cells in the labyrinth area failed to fuse into one of the syncytiotrophoblast layers (Dupressoir *et al.* 2009). The SynTI layer is ill-defined with a discontinuous and disrupted interface with SynTII, which appears to develop normally and express *syncytin B* and *GCM1* at normal levels (Dupressoir *et al.* 2009). Apoptosis and overexpansion of the trophoblast cells crowd the fetal capillaries resulting in decreased vascularization and nutrient transport, leading to fetal growth retardation (Dupressoir *et al.* 2009). Intriguingly, only syncytin B is capable of immunosuppression, although both proteins possess the domain. This reflects the situation in humans, with only syncytin 2 being immunosuppressive (Mangeney *et al.* 2007). The potential functional differences between syncytins in humans and mice lend confidence to the notion that pairs of *env* genes are conserved for convergent roles in placental morphogenesis in multiple species (Mangeney *et al.* 2007, Rawn & Cross 2008).

Rabbit syncytin. Recently, an *env* gene of retroviral origin was identified in the rabbit *Oryctolagus cuniculus*, termed *syncytin-Ory1*, that is phylogenetically distinct from the previously characterized syncytins (Heidmann *et al.* 2009). The placenta-expressed *env* gene encodes a protein that shows fusogenic activity in an *ex vivo* cell-cell fusion assay. In the rabbit, the junctional zone of the placenta contains the interface, or labyrinth, between the invading syncytial trophoblast and the maternal decidua. *Syncytin-Ory1* mRNA was specifically present at this level in the junctional zone of the rabbit placenta, decreasing with gestational age. Interestingly, the receptor for the rabbit syncytin-Ory1 was found to be the same as that for human syncytin 1, which is ASCT2. The identification of a novel *syncytin* gene within a third order of mammals which display syncytiotrophoblast formation during hemochorial placentation strongly supports the notion that retroviral infections have resulted in the independent capture of genes that were positively selected for a convergent physiological role in development of the placenta (Heidmann *et al.* 2009).

Proposed biological roles for ERVs in placental morphogenesis. The conservation of ERV ORF and retroviral elements that are expressed in the placenta support the idea that ERVs shaped the evolution of placentation in mammals. Intriguingly, animals within several different classifications (primates, rodents, rabbits, and sheep) have acquired ERVs with possible convergent biological roles in placentation through independent integration events (Mi *et al.* 2000, Dunlap *et al.* 2006b, Dupressoir *et al.* 2009, Heidmann *et al.* 2009). The proposed roles for these ERVs in the placenta include three main functions that are adapted by the host from sequences originally

evolved to help the exogenous retrovirus replicate.

(1) Trophoblast specific gene expression. The LTRs of proviruses exhibit tissue-specific gene expression originally evolved to regulate transcription of the viral mRNAs and production of the viral particles. Insertion of the provirus into the genome can lead to LTRs that drive expression of host genes in this tissue-specific manner. The placenta-specific transcripts of several ERV-driven genes (*PTN, EDNRB, INSL4, MD1, LEP,* and *CYP19A1*) are hypothesized to be involved in placentation and are under positive selection for these roles. In the case of *CYP19A1*, distinct placenta-specific promoters have evolved from LTRs in bovine, ovine, and human genomes (Vanselow *et al.* 1999, Kamat & Mendelson 2001, Rawn & Cross 2008). Other examples of ERV regulation of gene expression in placentae are conserved among humans and New World monkeys (Rawn & Cross 2008).

(2) Cell fusion. Formation of syncytiotrophoblasts is a key step in placental morphogenesis in several orders of mammals. These syncytiotrophoblast layers form the interface between maternal and fetal tissues and are involved in the transport of nutrients to the fetus, hormone production, and immune tolerance. Syncytiotrophoblasts form through fusion of the cytotrophoblasts, although the process is not fully characterized. The Env glycoprotein of exogenous retroviruses mediates cell entry and fusion of the viral particle with the cell. Adaptation of this function to facilitate formation of the syncytiotrophoblast in placentae has been proven or hypothesized in humans, mice, rabbits, and sheep (Mi *et al.* 2000, Dupressoir *et al.* 2005, Dunlap *et al.* 2006b, Heidmann *et al.* 2009). The ERV Env proteins are involved in the fusion process

through the TM unit, which contains a fusion peptide and leucine/isoleucine heptad repeats. Mutation of these domains ablates the fusion abilities of exogenous Env proteins (Salzwedel *et al.* 1993, Chen 1994, Wild *et al.* 1994). In humans and mice, at least one of the placenta-specific syncytins contains the fusion peptide and maintains the ability to elicit cell-cell fusion (Mi *et al.* 2000, Peng *et al.* 2007). Retention of these functional *env* genes within the genome supports the hypothesis that the Env proteins are involved in formation of the syncytiotrophoblasts in the placenta and have been under purifying selection for this role in both species.

(3) Immunosuppression. The placenta is an important organ within the pregnant female that protects the fetus from the maternal immune system. Because the fetus contains foreign (to the mother) genetic material, the maternal immune system must be modulated to avoid destroying the fetus and placenta. Placentally expressed ERVs have been proposed to provide one method of modulation of the maternal immune response through immunosuppressive domains. The *env* genes of most non-defective retroviruses encode a well-conserved immunosuppressive domain consisting of 17 hydrophobic amino acids, known as p15E (Boyd *et al.* 1993). Originally used by the exogenous retrovirus to evade the host immune response, the p15E domains of Envs within the placenta are hypothesized to repress the immune system through interaction with the maternal macrophages (Villarreal 1997). These domains have the ability to decrease proliferation of interlukin-2 dependent cells *in vitro*, as well as facilitating tumor growth in an *in vivo* mouse model (Cianciolo *et al.* 1985b, Mangeney *et al.* 2007). At least one of the syncytins in both humans (synyctin 2) and mice (syncytin B) contain an

immunosuppressive region that has been conserved throughout evolution, suggesting that it is involved in immune tolerance to the fetus (Rote *et al.* 2004). ERVs within the placenta may also offer cellular resistance to infection by exogenous retroviruses as noted in previous sections.

In summary, several pathological functions of ERVs have been conserved and adapted by hosts to facilitate placental morphogenesis. This process of purifying selection of ERVs is seen in at least four orders of mammals, including primates, rodents, rabbits, and ruminants in which open reading frames for the LTRs and/or proviral genes are conserved and maintain production of biologically active proteins.

Ovine Placental Development and enJSRVs

Placental development in the sheep is a highly coordinated process that involves the expansion of the compact morula into a blastocyst, shedding of the zona pellucida, and apposition, attachment, and adhesion of the trophoblast to the luminal epithelium (LE) of the endometrium (Guillomot *et al.* 1981, Guillomot 1995). In sheep, fertilization of the oocyte occurs in the oviduct, and the resulting zygote remains in the oviduct until reaching the morula stage at day 4 post-fertilization (Guillomot 1995, Spencer *et al.* 2004). At this stage, the embryo migrates into the uterus and begins to form a blastocyst by day 6 (205 μm). By day 8, the blastocyst hatches from the zona pellucida and begins interacting with the LE. By day 10, the conceptus (embryo and associated extraembryonic membranes) has become spherical or tubular in form (340 μm by 425 μm) and the MTCs produce IFNT, which is the signal for maternal recognition of pregnancy in ruminants (Spencer *et al.* 1996, Spencer *et al.* 2004). This protein

interrupts the luteolytic process, and production of progesterone to support pregnancy is maintained by the corpus luteum (CL) (Thatcher *et al.* 1995, Spencer *et al.* 1996). Progesterone and then IFNT act in concert on the LE and glandular epithelium (GE) to initiate production of proteins involved in conceptus elongation, migration, attachment, and adhesion to the LE (Spencer & Bazer 2002). Between days 12 to 16, the conceptus elongates from an its tubular shape to a filamentous form of 190 mm (Spencer *et al.* 2004). As this elongation occurs, the trophoblast apposes and attaches to the LE, and it undergoes extensive cell proliferation, growth, and remodeling (Wintenberger-Torres & Flechon 1974).

Progesterone, the hormone of pregnancy, plays a pivotal role in the establishment and maintenance of pregnancy in mammals. In sheep uteri, PR (progesterone receptor) is expressed in the endometrial epithelia and stroma during the early luteal phase, allowing direct regulation of a number of genes by progesterone via activation of the progesterone receptor (PR). However, continuous exposure of the endometrium to progesterone down-regulates PR expression in the endometrial epithelium (Spencer *et al.* 1995). Expression of PR protein is not detectable in endometrial LE and GE in sheep after days 11 and 13 of pregnancy, respectively (Spencer & Bazer 1995). Further, PR expression is only detected in stroma and myometrium throughout most of gestation in the ovine uterus. Thus, regulation of endometrial epithelial function during the peri-implantation period may be dependent on loss of epithelial cell PR and/or be directed by specific factors produced by PR-positive stromal cells (Spencer & Bazer 2002, Spencer *et al.* 2004). Loss of PR by the endometrial epithelium can be directly correlated with

reduced expression of certain genes, such as the antiadhesive protein MUC1. Furthermore, PR loss in the endometrial GE appears to be required for the onset of expression of other genes during pregnancy, such as galectin-15 and osteopontin (Spencer *et al.* 2004).

At the time of apposition and adhesion, the MTCs begin to differentiate into giant BNCs that comprise 15 to 20% of the conceptus trophectoderm by day 18 (Hoffman & Wooding 1993a). Throughout pregnancy, the BNC continue to form through a process known as mitotic polyploidy or endoreduplication in which cells undergo mitosis without cytokinesis (Wooding 1992). Some of these BNCs migrate and fuse with the LE to form trinucleate fetomaternal hybrid cells, and then fuse with each other to form multinucleated syncytial plaques containing as many as 25 nuclei by days 20 to 24 (Wooding 1984, Hoffman & Wooding 1993a). The syncytiotrophoblasts form over the aglandular caruncles of the uterus, and are the precursors to the fetal cotyledons. In most placental mammals, the term cotyledon refers to branched, tree-like folds of the trophoblast which act as a barrier and for transport of nutrients (Cross *et al.* 2003). In the sheep, the fetal cotyledons interdigitate with the maternal caruncles to form placentomes, which are the main source of hematotrophic nutrition to the fetus in ruminants (Igwebuike 2006). Without proper formation of the placentomes, the pregnancy will terminate due to reduced nutrients to the fetus (Mellor *et al.* 1977). The expansion of the syncytiotrophoblasts is presumably due to continued BNC differentiation and fusion. The BNCs and syncytiotrophoblasts have unique gene expression profiles, including *placental lactogen,* also known as *chorionic*

sommatomammotrophin one (CSH1) and *pregnancy associated glycoproteins (PAGs)* (Ushizawa *et al.* 2007). The BNCs in ruminants are comparable to the syncytiotrophoblasts in humans and rodent trophoblast giant cells with respect to polyploidy, migratory/invasive properties, lack of proliferation, and production of hormones (Hoffman & Wooding 1993a, Cross *et al.* 2003). In humans, rodents, and ruminants, the syncytiotrophoblasts are terminally differentiated cells responsible for nutrient and gas exchange between the mother and fetus, as well as hormone production (Hoffman & Wooding 1993a, Leiser & Kaufmann 1994). The cellular and molecular mechanisms that control the differentiation of ovine MTCs into BNCs and syncytiotrophoblasts are basically not known. The evidence of a role for ERVs in differentiation of the placental cells in humans and mice provides an intriguing hypothesis for differentiation of these cells in the sheep, where enJSRVs are highly expressed during pregnancy (Mi *et al.* 2000, Palmarini *et al.* 2001a, Dupressoir *et al.* 2009).

The enJSRVs *env* mRNA and protein are abundant in the uterine LE and GE of the uterus beginning on day 12 of pregnancy. Levels of enJSRVs *env* in the conceptus are first detected beginning on day 12 and *in situ* hybridization shows expression in the MTCs, but it is most abundant in the BNC and multinucleated syncytia (Dunlap *et al.* 2005). Also of note is the temporal and spatial pattern of HYAL2, which is first detected in the conceptus on day 16 and is observed only in the trophoblast giant BNC and multinucleated syncytia during pregnancy (Dunlap *et al.* 2005). This localization and abundance of enJSRVs *env* mRNA and its receptor HYAL2 suggests a possible role in

host reproduction. Given the co-expression of enJSRVs *env* and *HYAL2* in the BNCs, it is possible that HYAL2 binds to enJSRVs Env on the trophectoderm cells and promotes their fusion (Figure 2.5) (Varela *et al.* 2009)

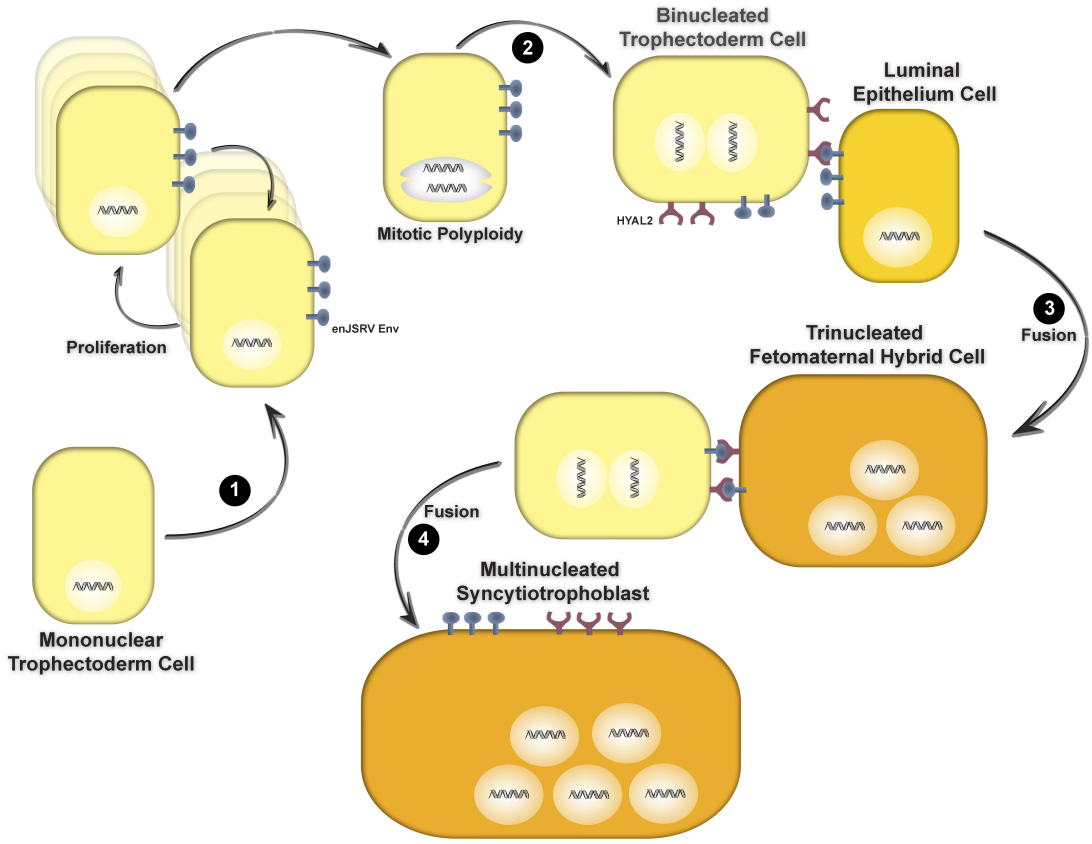

Figure 2.5. Hypothesis on the biological role of enJSRVs Env and HYAL2 in trophoblast differentiation in sheep. MTC begin to express enJSRVs *env* in the conceptus on day 12. BNC are derived from mitotic polyploidy. Next, the newly formed BNC that are co-expressing enJSRVs *env* and *HYAL2* fuse with enJSRVs *env*-expressing endometrial LE cells, forming a trinucleate cell. Other newly formed BNC fuse with trinucleate cells to form a multinucleated syncytial plaque.

ERVs were determined to be necessary for conceptus development in sheep using a morpholino (MAO-env) designed to inhibit splicing of all intact enJSRVs *env* mRNA

(Dunlap *et al.* 2006b). Control experiments established that the MAO-env abolished only production of the Env protein and did not inhibit production of enJSRV Gag. Morpholinos (MAO-env and two controls) were injected into the uterine lumen on day 8 of pregnancy, and the conceptuses were retrieved for analysis by flushing on day 16. While control conceptuses were all elongated and filamentous, the MAO-env-treated conceptuses showed altered MTC development, resulting in cytoplasmic vacuoles within most cells. MTC proliferation was also inhibited, as the MAO-env-treated conceptuses were elongated but not thickened as the controls were and produced less IFNT. The lack of BNC seen in the MAO-env treated conceptuses could be due to a lack of differentiation or due to a reduction in the number of MTCs. When the studies were carried out to day 20, the MAO-env treatment resulted in a reduced pregnancy rate. Evidence from the same study showed that MAO-env treatment also decreased proliferation of trophectoderm cells *in vitro*. These *in vivo* loss-of-function experiments in ewes determined that enJSRV Env is necessary for peri-implantation growth and elongation of the conceptus between days 12 and 16 (Dunlap *et al.* 2006b).

The physiological function of enJSRVs Env in the placenta is regulation of conceptus trophectoderm growth and differentiation, and conceptus-endometrial interactions during placentation (Spencer *et al.* 1999b, Palmarini *et al.* 2001a, Spencer & Bazer 2002, Dunlap *et al.* 2006b). However, the cellular and molecular mechanisms utilized by enJSRVs Env to mediate trophectoderm development are unknown. Both exogenous JSRV and enJSRV Envs contain a signal peptide at the amino terminus, a furin cleavage site between the SU and TM portions, a hydrophobic segment at the amino

terminus of the TM which maps to a fusion domain in other ERV Envs, and a cytoplasmic tail at the carboxy terminus of the TM (Varela *et al.* 2009). The proposed fusion domain of enJSRV Envs have not proven functional through testing at a neutral pH in a variety of cell lines (Varela *et al.* 2009). With some knowledge of the cell signaling pathways activated by the exogenous JSRV Env to induce transformation, it is tempting to speculate that enJSRV Env acts through some of the same pathways to affect placentation in the sheep. This may prove to be true through further investigation; however, the cytoplasmic tail of all enJSRV Env proteins is substantially different compared to the cytoplasmic tail of JSRV Env (Arnaud *et al.* 2007a). The cytoplasmic tail of the enJSRV Env lacks the characteristic YXXM transformation domains of JSRV Env due to an in-frame deletion. Trophoblast development has hallmarks similar to tumorigenesis and metastasis, including proliferation, migration and invasion, as well as evasion of the immune response. It is also intriguing to note that the pathways involved in JSRV Env transformation, Ras-MEK-MAPK and PI3K-AKT1-MTOR are both implicated in placental development in humans and mice (Pollheimer & Knofler 2005).

Conservation of the enJSRV ORFs within the sheep genome is indicative of a postive and purifying selection of these genes for a biologically important role in development. The transdominant Gag and intact Env proteins offer protection from the exogenous JSRV through restriction of viral exit and receptor competition (Spencer *et al.* 2003, Arnaud *et al.* 2007b). The Env proteins are involved in proliferation of the MTC, necessary to establish pregnancy in the sheep (Dunlap *et al.* 2006b). While the exact role and mechanisms for enJSRV involvement in proliferation and differentiation of the

MTCs and other placental cells remain to be elucidated, the presence of ancient ERVs and modern ERVs that are evolving within the sheep genome and interacting with exogenous counterparts provides an interesting model to study ERVs in placental morphogenesis.

CHAPTER III

EFFECTS OF PROGESTERONE ON DEVELOPMENT OF CONCEPTUSES WITH ENJSRV ENV LOSS-OF-FUNCTION

Introduction

In sheep, the conceptus elongates from an ovoid or tubular shape to a filamentous form in a process that involves proliferation and migration of MTCs. The MTCs produce IFNT, which is the signal for maternal recognition of pregnancy in ruminants (Spencer *et al.* 1996, Spencer *et al.* 2004). This protein interrupts the luteolytic process, and production of progesterone to support pregnancy is maintained by the CL (Thatcher *et al.* 1995, Spencer *et al.* 1996). Progesterone and IFNT act in concert on the LE and GE to initiate production of proteins involved in conceptus elongation, migration, attachment, and adhesion to the LE (Spencer & Bazer 2002). Between days 12 to 16, the conceptus elongates from an its tubular shape to a filamentous form (Spencer *et al.* 2004). As this elongation occurs, the trophoblast apposes and attaches to the LE, and it undergoes extensive cell proliferation, growth, and remodeling (Wintenberger-Torres & Flechon 1974). Beginning on day 14, some MTCs begin to differentiate into trophoblast giant BNCs that comprise 15-20% of the conceptus trophectoderm by day 18 (Hoffman & Wooding 1993a). The BNCs migrate and fuse initially with the LE as well as each other to form multinucleated syncytial plaques, which constitute the cotyledonary portions of the placenta. The cotyledons interdigitate into the endometrial caruncles of the uterus and form placentomes essential for supplying maternal nutrients to the

developing fetus (Hoffman & Wooding 1993a). The BNCs and syncytia have unique gene expression profiles, including *CSH1* and *PAGs* (Ushizawa *et al.* 2007).

An ERV of sheep, enJSRV, has at least 27 loci that are predominantly expressed in the epithelia of the female reproductive tract (Palmarini *et al.* 1995, Palmarini *et al.* 1999, Spencer *et al.* 1999b, Palmarini *et al.* 2000b, Palmarini *et al.* 2001a). At least 5 of the enJSRV loci contain intact ORFs for all proviral genes and are capable of forming viral particles in vitro (Arnaud *et al.* 2007a). Additionally, 16 of the 27 loci encode biologically active Env glycoproteins (Arnaud *et al.* 2007a). The *env* genes of enJSRVs are expressed highly in the LE and GE during the estrous cycle and pregnancy (Palmarini *et al.* 2001a, Dunlap *et al.* 2005). Maximal levels of *enJSRVs* RNA in the endometrial epithelia between days 11 and 16 of pregnancy coincides with the onset of conceptus elongation, when the MTCs are rapidly proliferating, migrating and producing IFNT, the pregnancy recognition signal that maintains the corpus luteum and thus progesterone production (Spencer *et al.* 1996). Progesterone acts on the uterus to induce genes that regulate conceptus elongation, migration, attachment, and adhesion to the LE (Spencer & Bazer 2002).

In the conceptus, enJSRVs *env* RNA is first detected beginning on day 12 as initial elongation occurs and is particularly abundant in the BNC and multinucleated syncytia throughout gestation (Dunlap *et al.* 2005). *In vivo* loss-of-function experiments in sheep found that enJSRVs Env is necessary for peri-implantation conceptus growth and perhaps formation of BNCs (Dunlap *et al.* 2006b). In that study, morpholino antisense oligonucleotides (MAO) were injected into the uteri of pregnant ewes on day

8. The MAO inhibited the splicing of the enJSRVs RNA, thereby inhibiting Env production. While control conceptuses were elongated and filamentous, the MAO-env-treated conceptuses on day 16 were growth retarded and with decreased MTC and lower production of IFNT. Further, the conceptuses lacked BNC, which could be contributed to the reduced number of MTCs or to a block in their differentiation. By day 20 of pregnancy, the MAO-env treatment resulted in loss of the conceptus, which was most likely caused by reduced IFNT production.

Establishment of pregnancy in the sheep is dependent on production of the IFNT by the conceptus to block destruction of the corpus luteum and maintain progesterone production (Spencer *et al.* 1996). Loss of enJSRVs Env results in decreased IFNT production, due to reduced MTC numbers. The reduced IFNT seen in MAO-env conceptuses was unable to block luteolysis, leading to loss of the conceptus. In the present study, we tested the hypothesis that exogenous progesterone administration would rescue development of conceptuses in which enJSRVs Env was inhibited by MAO.

Materials and Methods

Morpholino design. Morpholino oligonucleotides were designed and synthesized by Gene Tools, LLC (Philomath, Oregon, USA) as previously described (Dunlap *et al.* 2006b). The MAO-env had the sequence GCTTC GGCAT CCTGT GGAAA AACAC and targeted the enJSRVs *env* RNA overlapping the splice donor and acceptor region. The MO-5mis control morpholino had the sequence GGTTC GCCAT CCTCT GCAAA AAGAC (underlined differences with MAO-env). The MO-std had

the sequence CCTCT TACCT CAGTT ACAAT TTATA and targeted to a splice site mutant of *Homo sapiens* hemoglobin beta (*HBB*) gene (AY605051), which sheep do not posess. All morpholinos were synthesized with a 5' rhodamine modification for fluorescent detection.

Animal work. All animal experiments were approved by the Institutional Animal Care and Use Committee of Texas A&M University. Suffolk cross-bred ewes were mated at estrus (day 0) and on day 1 to rams of proven fertility. On day 7 post-mating, the ewes were subjected to a mid-ventral laparotomy. The base of the uterine horn ipsilateral to the corpus luteum was double ligated using non-absorbable umbilical tape to prevent migration and growth of the conceptus through the uterine body into the contralateral uterine horn. This surgical procedure does not affect conceptus implantation or fetal development in sheep (Bazer *et al.* 1979, Dunlap *et al.* 2006b). MO-std, MO-5mis, and MAO-env (100 nmol) were complexed with Gene Tools Endo-Porter delivery reagent (50 µl) and diluted to 1 ml final volume with OPTI-MEM (Invitrogen). The complex was then introduced into the lumen of the uterus (n=9 ewes/morpholino) via the uterotubal junction using a 1 ml syringe fitted with a 20-gauge catheter. After discharging the morpholinos into the uterine lumen, the catheter was withdrawn, and the uterine horn gently massaged to distribute the morpholinos throughout the uterine lumen. The outside of the uterus was rinsed with sterile 5% glycerol saline to prevent formation of adhesions and placed back in the body cavity. All ewes received daily intramuscular (i.m.) injections of progesterone (25 mg) from days 8 to 20.

All ewes were hysterectomized on day 20. The uterine horn injected with the morpholinos was opened along the mesometrial border to expose the conceptus. Portions of the conceptus (if present) and sections of the uterine horn containing the conceptus were frozen in OCT compound or fixed in 4% paraformaldehyde for histological analysis.

Fluorescence, histology, and immunohistochemistry. Effective delivery of the rhodamine-labeled morpholinos was analyzed by fluorescence microscopy (Dunlap *et al.* 2006b). Cryosections (8 μm) of the uteri and conceptuses were prepared, and a DAPI-containing mounting medium (ProLong Gold Antifade Reagent, Invitrogen, Carlsbad, CA) was used to visualize nuclei. Immunoreactive CSH1 and PAG proteins were assessed in the paraformaldehyde-fixed, paraffin embedded conceptus by using standard immunohistochemical procedures with antibodies to ovine CSH1 (rabbit polyclonal antibody was kindly provided by Russ V. Anthony, Colorado State University, Fort Collins, CO) and ovine PAG (rabbit anti-recombinant PAG3 polyclonal antibody was kindly provided by Jonathan A. Green, University of Missouri-Columbia, Columbia, MO) (Dunlap *et al.* 2006b). Rabbit serum was used in place of the relevant primary antibody as a negative control. The number of BNCs was quantified by determining the number of CSH1- and PAG-immunostained BNCs relative to MTCs in at least five nonsequential sections of each conceptus from each ewe. The nuclei were visualized after applying hematoxylin counterstain. Sections from all sheep and treatment groups were analyzed in duplicate in the same run, and images were captured by using standardized procedures.

Statistics. Pregnancy rate data were analyzed by χ^2 test. All quantitative data were subjected to least-squares ANOVA by using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Statistical models accounted for sources of variation, including the main effects of morpholino type and, where appropriate, histological section (BNC quantification). The data are presented as least-squares means with SEM.

Results

Pregnancy rates and morphology. Pregnancy rates and morphology of conceptuses recovered from morpholino-injected, progesterone-treated ewes are summarized in Table 3.1. Pregnancy rates were not different ($P > 0.10$) in control ewes receiving MO-std or MO-5mis control morpholinos, respectively. Though there was some variation in size, the control conceptuses were all morphologically normal. The MAO-env treated ewes had a 56% pregnancy rate that was not different ($P > 0.10$) when compared to the combined pregnancy rate for both control groups. Morphologically, the MAO-env conceptuses were elongated, but growth retarded and thinner.

Table 3.1 Effects of morpholinos on pregnancy and conceptus development in progesterone-treated ewes.

Morpholino	Pregnancy Rate % (ewes pregnant / total ewes per treatment)	Conceptus Morphology	BNC (#/100 cells) (LSM $\pm$ SE)
MO-std	100% (9/9)	Elongated, filamentous	12 ± 1.0^a
MO-5mis	66.7% (6/9)	Elongated, filamentous	14.4 ± 1.2^a
MAO-env	55.6% (5/9)	Growth retarded, filamentous	7.8 ± 1.1^b

Morpholino delivery. Fluorescence microscopy revealed that the morpholinos were delivered to the conceptus trophectoderm (Figure 3.1).

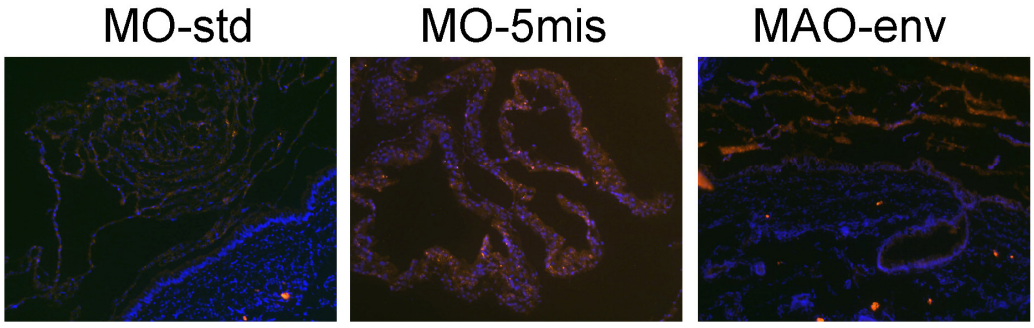

Figure 3.1 Fluorescence microscopy analysis of *in vivo* morpholino delivery. Portions of the conceptuses were frozen in OCT and sectioned. Sections were rinsed in phosphate-buffered saline and a coverslip affixed using DAPI-containing mounting medium. Fluorescence microscopy was used to detect the rhodamine-labeled morpholino (orange/red) and DAPI nuclei (blue).

Binucleate cell analysis. The conceptuses were evaluated for the presence of BNC using immunohistochemistry. Antibodies against the BNC-specific proteins CSH1 and PAGs were used as markers for these cells (Figure 3.2). Immunostaining for these proteins revealed the number of BNCs in both MO-std and MO-5mis conceptuses to be 12.0 and 14.4%, respectively, of the total trophoblast cell population, while the MAO-env conceptuses had 7.8% BNC ($P < 0.01$) (Table 3.1).

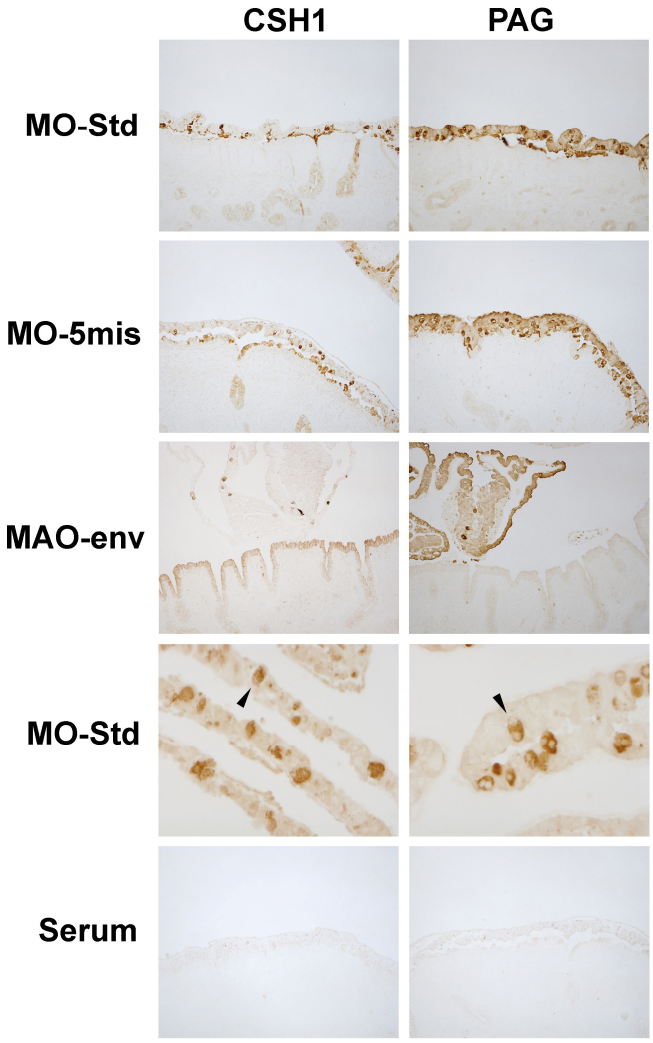

Figure 3.2 Immunohistochemical analyses of BNC. Immunoreactive CSH1 and PAG proteins were detected in paraformaldehyde-fixed, paraffin-embedded sections of conceptuses using a rabbit anti-ovine CSH1 antibody or anti-ovine PAG. Arrowheads indicate individual BNC. Width of field of view in rows 1,2,3 and 5 is 420 µm. Width of field of view of row 4 is 210 µm. Data are representative of conceptuses from all ewes.

Discussion

These results demonstrate that progesterone administration can partially rescue conceptus development in ewes injected with antisense morpholinos that inhibit

enJSRVs Env in the trophectoderm. A previous study found that MAO-env treatment in ewes that did not receive progesterone resulted in severe growth retardation of the conceptus on day 16 and ultimately pregnancy loss by day 20 (Dunlap *et al.* 2006b). The pregnancy loss on day 20 was likely due to insufficient production of IFNT by the growth-retarded conceptuses (Dunlap *et al.* 2006b). Without adequate IFNT, the CL regresses, progesterone production ceases, and ewes return to estrus (Spencer *et al.* 1996). Progesterone stimulates the LE and GE to produce histotroph, which is a complex mixture composed of growth factors, cytokines, enzymes, transport proteins, and other substances that stimulate conceptus development (Wimsatt 1950, Bazer 1975). In the present experiments, all ewes were given progesterone daily, and those sheep injected with MAO-env had a pregnancy rate of 56% on day 20, which was not significantly different compared to the control ewes but was substantially higher than observed in our prior study (Dunlap *et al.* 2006b). As mentioned above, the low pregnancy rate of the MAO-env treatment in the prior study was most likely due to reduced IFNT due to reduced proliferation of the MTC population, which progesterone administration was able to partially overcome in the current study.

Despite administration of progesterone and comparable pregnancy rates, the MAO-env conceptuses were morphologically fragile and had lower numbers of BNC, indicating a lack of trophoblast differentiation. Although exogenous progesterone administration created a permissive uterine environment in which conceptus development progressed, the effect of the loss of enJSRVs Env was evident in the growth retardation of the MAO-env conceptuses, just as in the previous loss-of-function

studies (Dunlap *et al.* 2006b). Conceptus development was delayed by the reduction of enJSRVs Env, indicating it is necessary for the proper proliferation and differentiation of the MTCs.

The cellular and molecular mechanisms utilized by enJSRVs Env to elicit biological effects, namely proliferation and differentiation, in the MTCs of the ovine conceptus are unknown. It is possible that enJSRV and JSRV Env proteins share common mechanisms by which they influence the cell cycle and cell proliferation, as the JSRV Env is a dominant oncoprotein and retroviral proteins have been shown to stimulate proliferation (Rosenberg & Jolicoeur 1997, Alberti *et al.* 2002b, Cousens *et al.* 2007). In order to understand the role of ERVs in placental morphogenesis in the sheep, it will be necessary to define the mechanisms whereby enJSRVs Env influences trophectoderm growth.

CHAPTER IV

ENDOGENOUS JSRVS ENV EXPRESSION IN THE DEVELOPING OVINE CONCEPTUS

Introduction

In sheep, conceptus elongation begins on day 11 with an ovoid or tubular conceptus which transforms into a filamentous form by day 14 in a process that involves proliferation and migration of MTCs. MTCs begin to differentiate into trophoblast BNC on day 14, and the BNC comprise 15-20% of the conceptus trophectoderm by day 18 (Hoffman & Wooding 1993a). The BNCs migrate and fuse initially with the LE as well as each other to form multinucleated syncytial plaques, which form the cotyledonary portions of the placenta. The cotyledons interdigitate into the endometrial caruncles of the uterus and form placentomes essential for supplying maternal nutrients to the developing fetus (Hoffman & Wooding 1993a).

The ovine genome contains at least 27 copies of enJSRV proviruses (Arnaud *et al.* 2007a). In the conceptus, enJSRVs *env* RNA is first detected beginning on day 12 as it begins to elongate and is particularly abundant in the BNC and multinucleated syncytia throughout gestation (Dunlap *et al.* 2005). *In vivo* loss-of-function experiments in sheep found that enJSRVs Env is necessary for peri-implantation growth and perhaps differentiation of BNCs (Dunlap *et al.* 2006b). Antisense morpholinos that decreased enJSRVs Env reduced MTC proliferation and resulted in decreased BNC numbers in day 16 conceptuses (Dunlap *et al.* 2006b). Additionally, by day 20 pregnancy loss was

observed in those ewes treated with the antisense morpholino, most likely due to reduced IFNT production by the MTCs (Dunlap *et al.* 2006b).

Sixteen of the 27 enJSRV loci within the sheep genome contain an *env* gene with an intact ORF and the ability to express a correctly spliced, full-length and biologically active Env protein that is capable of utilizing HYAL2 as a receptor for cell entry and, therefore, capable of interfering with JSRV receptor use (Arnaud *et al.* 2007a). Interestingly, 5 of the 27 enJSRV loci are completely intact with ORFs for all 5 genes (*gag, pro, pol, orf-x, env*) and produce viral particles *in vitro*. The objective was to determine the transcriptionally active enJSRV loci in the sheep conceptus during a critical period for trophoblast development and differentiation. Determining the sequence of enJSRVs *env* expressed by the conceptus and comparing them to known enJSRV loci will allow further analysis of how enJSRVs function *in vivo* to promote proliferation of MTC and formation of BNC and syncytia in the ovine conceptus.

Materials and Methods

Animals. Mature Suffolk-type ewes and Texel ewes (*Ovis aries*) were observed for estrus (designated as day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Study one. Suffolk cross-bred ewes and Texel ewes were mated at day 0 and on day 1 to rams of proven fertility. On day 13, the uteri (n=6 per breed) were surgically

flushed with 20 ml of 10 mM Tris-HCl. The recovered conceptuses were rinsed in the same medium and snap frozen at –80°C. RNA was extracted using the Rneasy Mini Kit (Qiagen, Valencia, CA), and cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The resulting cDNA was amplified with degenerate primers designed to amplify all of the enJSRV *env* loci within the sheep genome (Forward: 5'-GRYTTTCCRTGGGATAAGGTGAA-3', Reverse: 5'-ACAATCACYAGACCCTTACCATTG-3') with the following cycling conditions: 94°C x 1 min (94°C x 30s, 65°C x 1 min, 72°C x 45s) x 35, 72°C x 2 min. Standard PCR reactions using Ex Taq DNA polymerase (Takara Bio Inc, Otsu, Shiga, Japan) according to manufacturer's directions were performed on an MJ Research PTC-200 machine. The resulting PCR products were cloned into pCRII Dual Promoter vector using a T/A Cloning Kit (Invitrogen). For each individual ewe, a total of 8 individual plasmid clones containing *env* cDNAs were sequenced for each conceptus sample. Sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Forest City, CA).

Study two. On day 18 of pregnancy, Suffolk ewes (n=5) were hysterectomized. The uterine horns were opened along the mesometrial border to expose the conceptus. Portions of the conceptus were snap frozen at –80°C. RNA was extracted and RT-PCR was performed as in Study One.

Study three. The Eukaryotic Linear Motif Server (ELM) was used to identify short protein sequence motifs in the conceptus-expressed enJSRV Env and the exogenous oncogenic JSRV Env that are recognized by modular signaling domains, phosphorylated by protein Ser/Thr- or Tyr-kinases, or mediate specific interactions with protein or phospholipid ligands (Puntervoll *et al.* 2003, Gould *et al.* 2010).

Results

Study one. Comparisons of the *env* cDNA sequences from day 13 Suffolk conceptuses to the 27 endogenous proviral loci indicated that *env* from multiple enJSRV loci are transcribed in each conceptus (Figure 4.1B). The most represented *env* locus in day 13 Suffolk conceptuses was enJSRV-8 with 40% of sequences matching this provirus. The *env* cDNA sequences from day 13 Texel conceptuses resulted from multiple enJSRV loci (Figure 4.1A). The best represented *env* locus in day 13 Texel conceptuses was also enJSRV-8 with 26% of sequences matching this provirus.

Day 13 conceptuses from both breeds expressed enJSRV loci that code for intact, functional Env proteins, as well as expressing *env* genes from proviruses that can produce complete viral particles. Intriguingly, conceptuses from both breeds on day 13 expressed a high proportion (18 – 21%) of enJSRV-11, an enJSRV locus that produces an intact, functional Env protein.

Study two. Comparisons of the *env* cDNA sequences from the day 18 Suffolk conceptuses to the 27 endogenous proviral loci indicated that *env* from multiple enJSRV loci are transcribed in each conceptus (Figure 4.1C). Although the most represented (70% of sequences) locus was enJSRV-1, which contains a stop codon in its TM

domain, these analyses also provided evidence for expression of intact enJSRV loci that are capable of producing viral particles, and for those loci that encode intact, functional Env proteins. Additionally, both enJSRV-7 and enJSRV-14 were present only in day 18 conceptuses.

There were differences in the enJSRV loci present between the Texel and Suffolk breeds as well. Several loci (enJSRV-26, -17, and –15) were represented in the Texels but not in the Suffolk, while enJSRV-1 is present in both day 13 and day 18 Suffolk conceptuses, but not in the Texel conceptuses.

Study three. Analysis of the amino acid sequences of the highly represented enJSRV loci was performed using the Eukaryotic Linear Motif (ELM) Server (Gould *et al.* 2010). The TM portions of JSRV Env, enJSRV-26, enJSRV-16, enJSRV-11, enJSRV-1, and enJSRV-8 were aligned using Clustal-W (http://www.ebi.ac.uk/Tools/clustalw2/index.html (Figure 4.2). The enJSRV-26 is the most recently integrated enJSRV provirus, and was found only in the Texel conceptuses (Arnaud *et al.* 2007a). The enJSRV-16 locus, which is identical to enJSRV-18, contains intact ORFs for all genes in the provirus and can produce viral particles *in vitro* (Arnaud *et al.* 2007a). A functional Env protein is coded for by enJSRV-11, which has been shown to utilize the same receptor as the exogenous JSRV. Sequences matching enJSRV-11 were present only in day 13 conceptuses (regardless of breed). As mentioned above, the loci that were best represented in both day 13 and day 18 conceptuses (enJSRV-8 and enJSRV-1) both contain stop codons in their TM domain, which truncates their putative membrane-spanning domain.

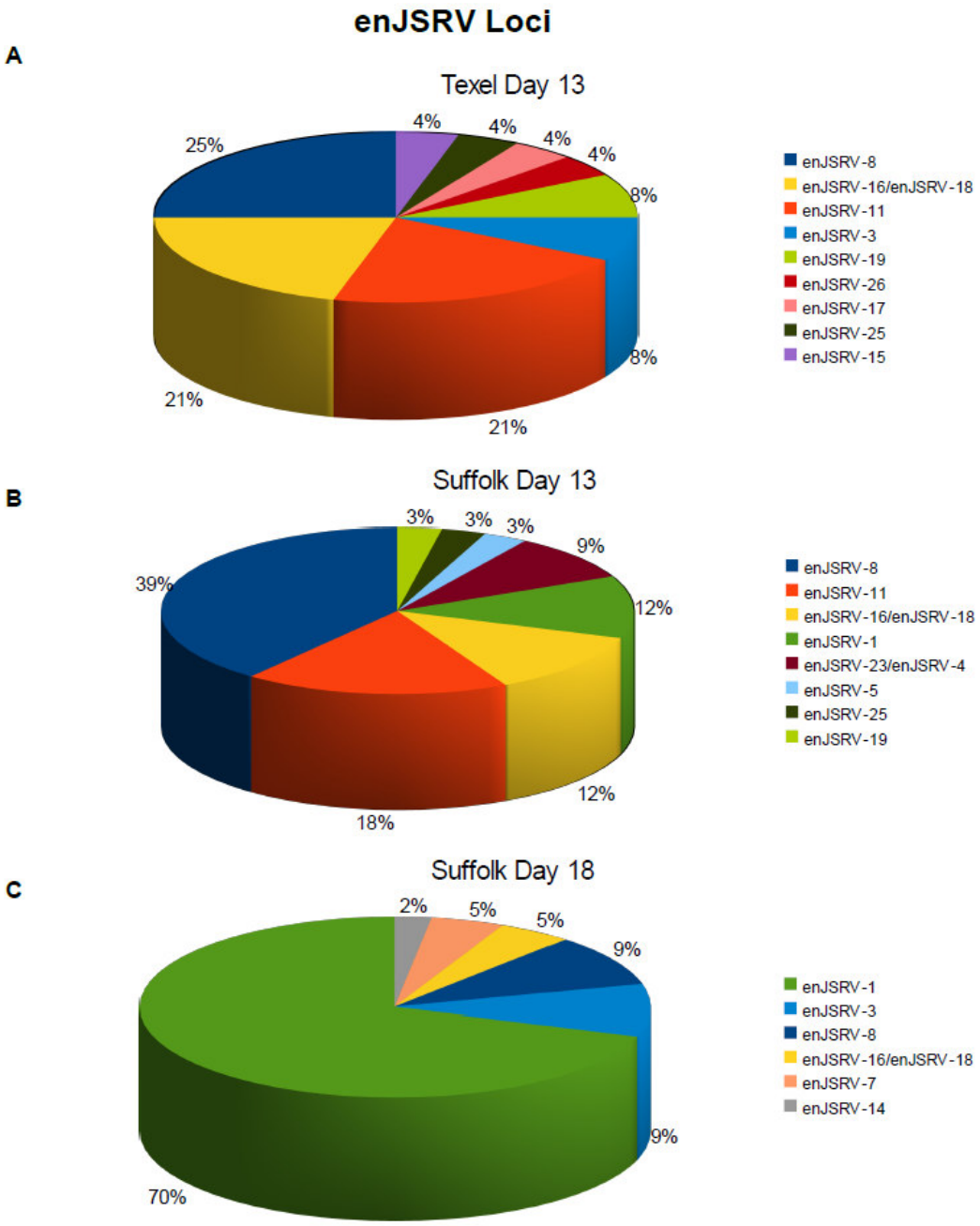

Figure 4.1 Representative chart of enJSRV *env* transcript abundance in conceptuses. A) The enJSRV *env* transcript abundance in Texel day 13 conceptuses shows enJSRV-8 is the best represented locus, with enJSRV-11 as the second most common. B) The enJSRV *env* transcript abundance in Suffolk day 13 conceptuses shows enJSRV-8 is also the most represented locus in the Texel breed. C) The enJSRV *env* transcript abundance in Suffolk day 18 conceptuses shows enJSRV-1 is the best represented with 70% of transcripts matching that locus.

The JSRV Env and three full-length enJSRVs Env (enJSRV-26, enJSRV-16, and enJSRV-11) all contain two hydrophobic domains in the TM portions. The first hydrophobic domain occurs just beyond the furin cleavage site, which separates the SU and TM domains, and corresponds to fusion peptides in other retroviral Env proteins (Hunter 1997, Nakayama 1997). The hydrophobic domain at the C-terminus of the Env protein corresponds to a membrane-spanning domain, leaving a cytoplasmic tail of about 40 amino acids through which signaling may occur. ELM analysis confirmed the location of the YXXM PI3K-binding domain in the cytoplasmic tail of the JSRV Env, which is essential for the oncogenic potential of this glycoprotein. As expected, the YXXM domain was not found in the enJSRVs Env cytoplasmic tails, and further analysis found no common signaling domains in these regions (Figure 4.2).

Analysis of the TM portions of enJSRV-1 and enJSRV-8 revealed the hydrophobic domain adjacent to the furin cleavage site, but no second hydrophobic domain due to truncation by a stop codon. These truncated TMs may have altered confirmation, which could result in soluble forms of the enJSRVs Env TM or use of the prototypical fusion peptide as a membrane-spanning domain, creating a cytoplasmic tail of 33 amino acids for enJSRV-8 and 97 residues for enJSRV-1 (Figure 4.2).

```
JSRV21          RPKRGLSLII LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV
enJSRV-26       RPKRGLSLII LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV
enJSRV-16       RPKRGLSLII LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV
enJSRV-11       RPKRGLSLII LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV
enJSRV-1        RTKRSLSLIV LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV
enJSRV-8        RPKRGLSLII LGIVSLITLI ATAVTASVSL AQSIQAAHTV DSLSYNVTKV

JSRV21          MGTQEDIDKK IEDRLSALYD VVRVLGEQVQ SINFRMKIQC HANYKWICVT
enJSRV-26       MGTQEDIDKK IEDRLSALYD VVRVLGEQVQ SINFRMKIQC HANYKWICVT
enJSRV-16       MGTQEDIDKK IEDRLSALYD VVRVLGEQVQ SINFRMKIQC HANYKWICVT
enJSRV-11       MGTQEDIDKK IEDRLSALYD VVRVLGEQVQ SINFRMKIQC HANYKWICVT
enJSRV-1        MGTQEDIDKK MEDRLSALYD VVRVLGEQVQ STSFRMKIQC HANYKWICVT
enJSRV-8        MGTQEDIDKK NRR..... .......... .......... ..........

JSRV21          KKPYNTSDFP WDKVKKHLQG IWFNTNLSLD LLQLHNEILD IENSPKATLN
enJSRV-26       KKPYNTSDFP WDKVKKHLQG IWFNTNVSLD LLQLHNEILD IENSPKATLN
enJSRV-16       KKPYNTSDFP WDKVKKHLQG IWFNTNVSLD LLQLHNEILD IENSPKATLN
enJSRV-11       KKPYNTSDFP WDKVKKHLQG IWFNTNVSLD LLQLHNEILD IENSPKATLN
enJSRV-1        KKAYNASDFP WDKVKNIYKE FGLILFL... .......... ..........
enJSRV-8        .......... .......... .......... .......... ..........

JSRV21          IADTVDNFLQ NLFSNFPSLH SLWKTLIGVG ILVFIIIVVI LIFPCLVRGM
enJSRV-26       IADTVDNFLQ NLFSNFPSLH SLWRSIIAMG AVLTVVLIII CLAPCLIRSI
enJSRV-16       IADTVDNFLQ NLFSNFPSLH SLWRSIIAMG AVLTVVLIII CLAPCLIRSI
enJSRV-11       IADTVDNFLQ NLFSNFPSLH SLWRSIIAMG AVLTVVLIII CLAPCLIRSI
enJSRV-1        .......... .......... .......... .......... ..........
enJSRV-8        .......... .......... .......... .......... ..........

JSRV21          VRDFLKMRVE MLHMKYRNML QHQHLMELLK NKERGDAGDD P
enJSRV-26       VKEFLHMRVL IH....KNML QHQHLMELLK NKERGAAGDD P
enJSRV-16       VKEFLHMRVL IH....KNML QHQHLMELLK NKERGAAGDD P
enJSRV-11       VKEFLHMRVL IH....KNML QHQHLMELLK NKERGAAGDD P
enJSRV-1        .......... .......... .......... .......... .
enJSRV-8        .......... .......... .......... .......... .
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Figure 4.2 Alignment of the TM portions of the JSRV Env and the amino acid sequences from the most represented enJSRV loci. The gray highlighted region corresponds to the furin cleavage site, while the yellow highlighted regions represent hydrophobic residues within the sequences. JSRV Env contains a YXXM PI3K docking site in the region noted in blue that is not present in the enJSRVs.

Discussion

The expressed enJSRVs *env* in day 13 and day 18 ovine conceptuses represent many of the enJSRVs loci present in the ovine genome. Our studies revealed that the *env* gene of intact loci were expressed in day 13 and day 18 Suffolk and Texel conceptuses, and these intact sequences encode functional Env proteins and/or be loci that form enJSRV viral particles *in vitro* (Arnaud *et al.* 2007a). Interestingly, enJSRV-11, which codes for an intact functional Env glycoprotein, represented about 20% of sequences in both day 13 Suffolk and day 13 Texel conceptuses, but was not seen in day 18 Suffolk conceptuses. This could indicate a specific role for enJSRV-11 in the proliferation of MTCs at this point in conceptus development. In the cases of enJSRV-7 and enJSRV-14, the loci were represented in day 18, but not day 13 conceptuses, possibly indicating a role for these two loci in the differentiation of the BNC population. It is also interesting to note that while all conceptus samples contained *env* from full-length enJSRV proviruses, none of these enJSRVs contain the transdominant Gag mutation capable of JLR interference with viral particle release. This indicates that the enJSRV loci that form viral particles *in vitro* could form viral particles *in vivo*, since several of these intact loci are expressed and there is not an intereference with their release.

The best represented enJSRV loci in the conceptuses for both days were enJSRV-1 or enJSRV-8, which both contain stop codons in the TM portion of the Env, rendering them without a membrane-spanning domain. These proteins are either soluble or transmembrane due to the hydrophobic region adjacent to the furin cleavage site.

Despite the absence of the membrane-spanning domains, enJSRV-1 and enJSRV-8 encode Env glycoproteins which could function in novel ways to stimulate proliferation and differentiation of the MTCs. In humans, ERV3 encodes a truncated Env protein that is implicated in differentiation and hormone production of the syncytiotrophoblast despite not having a transmembrane domain or a fusion domain (Larsson *et al.* 1994, Rote *et al.* 2004).

Due to the ongoing endogenization of enJSRVs within the sheep genome, different breeds of sheep contain different enJSRV loci due to their varying genetic backgrounds. These modern loci entered the ovine genome after speciation and can be used to trace the lineages of different breeds of sheep across the world (Chessa *et al.* 2009). Therefore, it is not surprising to note that there are differences in the loci represented between the Texel and Suffolk conceptuses. Previously, enJSRV-26 was identified in only one animal, which happened to be a Texel ram used to create the CHORI-BAC library from which the initial enJSRV loci were sequenced (Arnaud *et al.* 2007a). It appears that enJSRV-26 integrated into the ovine genome less than 200 years ago, and is present only in a subset of sheep within the Texel breed (Arnaud *et al.* 2007a). The Suffolk conceptuses from both days also contained evidence of enJSRV-1, a truncated Env which was not present in the Texel conceptuses. The continued endogenization of enJRSV sequences and the expression of ERV genes from intact ORFs suggest an evolutionarily important role for enJSRV in sheep reproduction.

The JSRV Env glycoprotein is oncogenic, and an extensive analysis of the mechanisms involved in the transformation action revealed the involvement of PI3K

through the YXXM motif (Liu *et al.* 2003b, Hull & Fan 2006, Cousens *et al.* 2007). Both the Ras-MEK-MAPK and PI3K-AKT pathways have been implicated in JSRV-induced cell transformation, but it remains to be determined how the cytoplasmic tail engages the cell-signaling network to activate these pathways (Palmarini *et al.* 2001b, Maeda *et al.* 2005, De Las Heras *et al.* 2006, Varela *et al.* 2006). As mentioned earlier, the enJSRV Env proteins do not contain the YXXM domain due to an in-frame deletion of these residues (Palmarini *et al.* 2001b). Perhaps the other residues involved in enhancing transformation by JSRV also function in the enJSRV Env proteins to promote proliferation and differentiation in MTCs in the developing ovine conceptus.

Multiple hallmarks of oncogenesis are mirrored in the events of early pregnancy and establishment of the placenta in sheep (Soundararajan & Rao 2004, Ferretti *et al.* 2007). These processes include proliferation, migration, and differentiation, but the signaling mechanisms governing these systems in placental morphogenesis are not well defined in sheep. *In vivo* loss-of-function studies indicate that enJSRV Env is involved in regulation of peri-implantation conceptus growth and differentiation. However, the cellular signaling pathways activated by enJSRV Env are not known. The current study defines which enJSRV loci are represented in the developing conceptuses and defines the TM portions of the most represented loci within the conceptuses. A further functional analysis utilizing a mutational approach could elucidate the signaling domains within the enJSRVs Env necessary for conceptus growth and differentiation. Indeed, the expression of *env* from loci that code for intact, functional Env proteins and/or produce

viral particles *in vitro* is evidence of a purifying and positive selection of enJSRVs for a role in placental morphogenesis.

CHAPTER V

VIRAL PARTICLES OF ENDOGENOUS JSRVS ARE RELEASED IN THE SHEEP UTERUS AND INFECT THE CONCEPTUS TROPHECTODERM IN A TRANS-SPECIES EMBRYO TRANSFER MODEL

Introduction

Retroviruses have the unique ability to integrate their proviral DNA into the genome of most eukaryotes (Boeke & Stoye 1997). As a result of their integration step, retroviruses can be found in nature as either "exogenous" or "endogenous" viruses. Exogenous retroviruses are transmitted, like any other virus, horizontally from infected to uninfected host. Occasionally, exogenous retroviruses gain access to the germline by infecting the germ cells of the host, resulting in the stable integration of the viral genome (termed "provirus") into the host genome. In these cases, ERVs are transmitted from generation to generation in a typical Mendelian fashion. ERVs are present in the genome of all animal species, and a complete ERV provirus has the same general structure as an exogenous retrovirus: *gag*, *pro*, *pol*, and *env* genes flanked by two non-coding LTRs. The *gag* gene encodes matrix, the major capsid protein, and nucleocapsid proteins necessary for viral particle formation. The *pro* gene encodes the viral protease, while the *pol* gene encodes the enzymes reverse transcriptase, integrase and RNAseH. The *env* gene encodes the envelope glycoprotein (Env), consisting of both surface and transmembrane domains, that is necessary to interact with the cellular receptor for virus entry (Vogt 1997a). Most ERVs are defective for viral replication due to either mutations, substitutions, insertions, and/or deletions that alter the provirus genome,

thereby preventing these elements from producing infectious viral particles and being transmitted horizontally (Boeke & Stoye 1997). However, ERVs with an intact genomic structure are present in various animal species and, in general, represent proviruses that have integrated into their host relatively recently from an evolutionary point of view (Boeke & Stoye 1997, Eiden 2008, Jern & Coffin 2008).

Sheep offer an interesting model to study the biological impact of ERVs and their interactions with exogenous retroviruses and their host (Arnaud *et al.* 2007a). The ovine genome contains multiple copies of enJSRVs highly related to two oncogenic exogenous retroviruses, JSRV and the enzootic nasal tumor virus (ENTV) (Cousens *et al.* 1996, Cousens *et al.* 1999, Palmarini *et al.* 1999, Palmarini *et al.* 2000b, Caporale *et al.* 2006, Arnaud *et al.* 2007a). The infectious and pathogenic JSRV and ENTV are tropic for the respiratory tract of sheep, while enJSRVs are predominantly expressed in the female reproductive tract (Palmarini *et al.* 1995, Palmarini *et al.* 1999, Spencer *et al.* 1999b, Palmarini *et al.* 2000b, Palmarini *et al.* 2001a). There are at least 27 enJSRV proviruses that have integrated in the host genome throughout the last 5-6 million years during the evolution of Caprinae (including sheep and goats and their wild relatives) (Arnaud *et al.* 2007a). Interestingly, endogenization of enJSRVs is still occurring, and there are several proviruses (known as "insertionally polymorphic") that have integrated in the last few thousand years and are present only in some domestic sheep (Arnaud *et al.* 2007a, Chessa *et al.* 2009). The enJSRVs possess several biological features that helped shape host evolution (Spencer *et al.* 2003, Spencer *et al.* 2007, Arnaud *et al.* 2008, Spencer *et al.* 2008, Varela *et al.* 2009). For example, some enJSRV loci are able to block viral exit

of related exogenous and endogenous retroviruses at late stages of the replication cycle (Mura *et al.* 2004, Arnaud *et al.* 2007a, Murcia *et al.* 2007). The enJSRVs can also block entry of related exogenous retroviruses by receptor competition, as both group of viruses use HYAL2 as a cellular receptor (Rai *et al.* 2001, Alberti *et al.* 2002a, Spencer *et al.* 2003).

Importantly, enJSRVs play essential roles in sheep reproduction (Dunlap *et al.* 2006a). In the female reproductive tract, the epithelia of oviduct, uterus, cervix, and vagina express enJSRVs (Spencer *et al.* 1999b, Palmarini *et al.* 2000b, Palmarini *et al.* 2001a). Of particular note, the enJSRVs *env* RNA is very abundant in the endometrial luminal and glandular epithelia during the estrous cycle and pregnancy (Palmarini *et al.* 2001a). Maximal levels of *enJSRVs* RNA in the endometrial epithelia coincide with the onset of conceptus elongation, when the mononuclear trophectoderm cells are rapidly proliferating and producing interferon tau (IFNT), the pregnancy recognition signal that maintains ovarian progesterone production (Spencer *et al.*). In sheep, the conceptus elongates from an ovoid or tubular shape on day 11 to a filamentous form by day 14 in a process that involves proliferation and migration of mononuclear trophectoderm cells. Beginning on day 14, some mononuclear trophectoderm cells begin to differentiate into trophoblast giant binucleate cells that comprise 15-20% of the conceptus trophectoderm by day 18 (Hoffman & Wooding 1993a). The binucleate cells migrate and fuse initially with the luminal epithelium as well as each other to form multinucleated syncytial plaques, which comprise the cotyledonary portions of the placenta that interdigitate into the endometrial caruncles of the uterus and form placentomes essential for supplying

maternal nutrients to the developing fetus (Hoffman & Wooding 1993a).

In the conceptus, enJSRVs *env* RNA is first detected on day 12 as it begins to elongate and is most abundant in the binucleate cell and multinucleated syncytia throughout gestation (Dunlap *et al.* 2005). Indeed, *in vivo* loss-of-function experiments in sheep found that conceptus elongation and binucleate cell formation was compromised when the production of enJSRVs Env was inhibited, which supported the idea that enJSRVs Env play a role in conceptus development (Dunlap *et al.* 2006b). Sixteen of the 27 enJSRV loci contained an *env* gene with an intact open reading frame, and 5 of the 27 enJSRV loci isolated so far have an intact genomic structure and can produce viral particles *in vitro*, as well as utilize ovine HYAL2 as a receptor for cell entry (Arnaud *et al.* 2007a). The intact enJSRV proviruses integrated in the host genome after sheep domestication and are insertionally polymorphic in the host (Arnaud *et al.* 2007a).

In this study we found that enJSRVs produce viral particles that are released into the uterine lumen of the ewe. Using *in vitro* produced bovine embryos transferred into the ovine uterus, we found that enJSRVs can potentially transduce the conceptus trophectoderm. Interestingly, only the "evolutionary young" enJSRVs proviruses, which integrated around or after sheep domestication, were found to be consistently expressed in the uterine endometrium and form the genomes packaged into the released viral particles present in the uterine lumen, which could influence development and differentiation of the conceptus trophectoderm.

Materials and Methods

Animals. Ewes of Suffolk and Texel breed (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16-18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Blastocyst production and embryo transfer. Bovine ova were obtained from a local abattoir and matured *in vitro* using methods described previously (Golding *et al.* 2006). The ova were fertilized *in vitro* using standard procedures and then cultured *in vitro* using G_1/G_2 embryo culture medium at 38.5°C in an atmosphere of 5% CO_2/5% O_2/90% N_2. Recipient ewes were synchronized to estrus via a 12-day treatment with progesterone using an intravaginal insert (controlled internal drug release, CIDR) (Pfizer, New York, NY). On day 12, the CIDR was removed and each ewe was administered 20 mg Lutalyse (Pfizer) intramuscularly. Estrus was detected using vasectomized rams fitted with a marking harness. Two *in vitro* produced bovine blastocysts were transferred into each synchronized ovine recipient (n=30) on day 7 post-estrus. All ewes received a CIDR at transfer. On day 20 of pregnancy (13 days post-transfer), bovine conceptuses were recovered by gently flushing the ovine uterus with 20 ml of 10 mM Tris (pH 7.0). Recovered bovine conceptuses were frozen in Tissue-Tek Optimal Cutting Temperature Compound (OCT; PELCO International, Redding, CA) and stored at -80°C. The ovine uterus was then obtained, and the

endometrium removed by physical dissection and stored at -80°C. Uterine flushes were clarified by low-speed centrifugation (3000 x *g*) immediately following collection, aliquoted, and stored at -80°C.

Laser capture microdissection. All instruments and reagents used for laser capture microdissection and nucleic acid extraction were obtained from MDS Analytical Technologies (Sunnyvale, CA) unless otherwise noted. The conceptuses frozen in OCT were sectioned at 8 μM using a cryostat. Sections (at least 6 per conceptus) were placed on PEN (polyethylene naphthalate) membrane slides and stored at -80°C until use. Slides were stained using a HistoGene LCM Frozen Section Staining Kit. Immediately following the staining, LCM was performed using a Veritas Microdissection System (Molecular Devices, Sunnyvale, CA) to isolate conceptus trophectoderm cells. Trophectoderm cells were transferred to CapSure Macro LCM Caps for RNA and DNA extraction using a PicoPure Extraction Kit. The PicoPure RNA or DNA Extraction kit was used according to manufacturer's recommendations, including DNase treatment using the RNase-free DNase Set from Qiagen (Valencia, CA).

Polymerase chain reaction. Total RNA was analyzed by RT-PCR using primers and annealing temperatures listed in Table 5.1. The cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Input RNA could not be quantified accurately due to the nature of the extraction process from the LCM cells. The cDNA was

Table 5.1. Primer Sequences and Cycling Conditions for RT-PCR and PCR analyses.

Gene Symbol	Species	NCBI Accession Number	Forward sequence (5' – 3')	Reverse sequences (5' – 3')	Cycling conditions
LGALS15	*Ovis aries*	EU009326	GTTTCTGGTACTGCGATGG	ACGATATCTCCCCTCACAACC	95°C x 2 min (95°C x 1 min, 68°C x 1 min, 72°C x 1 min) x 35, 72°C x 2 min
CSH1	*Bos taurus*	M33268	GGCAGTCACAGAGTTGTTGC	ACTCGAATCCCTGTGTAGGC	95°C x 5 min (95°C x 1 min, 55°C x 1 min, 72°C x 1 min) x 35, 72°C x 7 min
IFNT	*Bos taurus*	NM_001015511	CTCTCCTCATCCCTGTCTGC	TCTTTCTCTCCCATCACTGG	95°C x 5 min (95°C x 1 min, 62°C x 1 min, 72°C x 1 min) x 35, 72°C x 7 min
MT-CO1	*Ovis aries*	AF039171	TTAAAGACTGAGAGCATGATA	ATGAAAGAGGCAAATAGATTTTCG	94°C x 2 min (94°C x 1 min, 58°C x 1 min, 72°C x 1 min) x 30, 72°C x 5 min
MT-CO1	*Bos taurus*	J01394	GCCATATACTCTCCTTGGTGACA	GTAGGCTTGGGAATAGTACGA	94°C x 2 min (94°C x 1 min, 58 x 1 min, 72°C x 1 min) x 30, 72°C x 5 min
HYAL2	*Ovis aries*	NM_001009754	CTGAAGCCCACAGCACCACCCATC	GACTGACCTCCCCACCGAGTTGAAGTG	(94°C x 30s, 72°C x 3 min) x 5, (94°C x 30s, 70°C x 3 min) x 5, (94°C x 30s, 68°C x 30s, 72°C x 3 min) x 35
enJSRVs env	*Ovis aries*	EF680301	GRYTTTCCRTGGGATAAGGTGAA	ACAATCACYAGACCCTTACCATTG	94°C x 1 min (94°C x 30s, 65°C x 1 min, 72°C x 45s) x 35, 72°C x 2 min

resuspended in 20 µl of ddH_2O, and 2 µl was used in the subsequent PCR reactions. The resulting cDNA was amplified using the primers in standard PCR reactions using Ex Taq DNA polymerase (Takara Bio Inc, Otsu, Shiga, Japan) according to manufacturer's directions on an MJ Research PTC-200 machine. Each reaction contained 2 µl cDNA template, 2 µl Ex Taq buffer, 1.6 µl Ex Taq dNTPs, 0.4 µl of each 10µM primer, 0.25 µl Ex Taq DNA polymerase, and water to 20 µl.

Genomic DNA was analyzed by PCR using Ex Taq DNA Polymerase and an MJ Research PTC-200 machine. Each genomic DNA PCR reaction contained 5 µl DNA template, 5 µl Ex Taq buffer, 4 µl Ex Taq dNTPs, 1 µl of each 10 µM primer, 0.5 µl Ex Taq DNA polymerase, and water to 50 µl. RT- PCR and PCR products (10 µl aliquots) were visualized using a 1% agarose gel in TAE buffer containing ethidium bromide.

Western blot analyses. Uterine flush samples (2 ml per animal) were diluted approximately 4-fold in Dulbecco's PBS (Sigma-Aldrich) and centrifuged over a 1.5 ml 29% sucrose-PBS (weight/volume) cushion at 100,000 x *g* at 4°C for 1 h. The resulting pellets were resuspended in 20 µl 2X SDS-PAGE loading buffer (Ausubel 1988) for subsequent Western blot analyses or 30 µl Dulbecco's PBS for RNA extraction and transcriptional profiling. For Western blot analyses, concentrated viral particles were denatured, separated using SDS-PAGE, and transferred to nitrocellulose. Western blot analyses were performed using methods described previously (Spencer *et al.* 1999a). Enhanced chemiluminescent detection (Immun-Star WesternC Kit, BIO-RAD, Hercules, CA) on X-OMAT AR X-ray film (Kodak, Rochester, NY) was performed according to manufacturer's recommendations. The presence of Gag proteins was analyzed with

rabbit polyclonal sera against the JSRV major capsid (CA) protein (Caporale *et al.* 2009) diluted at 1:10,000 in PBS containing 1% bovine serum albumin (BSA).

enJSRVs transcriptional and phylogenetic analysis. Total RNA was isolated from the ultracentrifuge pellets of uterine flushes and resuspended in PBS using the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions. Total RNA was isolated from the LCM-isolated bovine conceptus trophectoderm samples using a PicoPure RNA Extraction kit, according to manufacturer's recommendations, including DNase treatment using the RNase-free DNase Set from Qiagen (Valencia, CA). Total RNA was also extracted from samples of Texel endometrial using Trizol (Invitrogen). Texel ewes were used for this phylogenetic analysis in order to be consistent with previous analyses (Arnaud *et al.* 2007a). RT-PCR was conducted using total RNA from individual samples of viral particles purified from each of the uterine flushes, the LCM-isolated bovine conceptus trophectoderm cells, and the ovine endometrium. Primers designed to amplify enJSRVs *env* and the U3 region of the LTRs (see Table 1 for sequences and cycling conditions) were utilized for RT-PCR analysis. PCR products were cloned into pCRII Dual Promoter vector using a T/A Cloning Kit (Invitrogen). For each individual ewe, a total of 100 individual plasmid clones containing *env* cDNAs were sequenced for each endometrial sample, and 50 individual plasmid *env* cDNAs were sequenced for samples of purified uterine flush viral particles and LCM-isolated bovine conceptus trophectoderm samples. Sequencing was carried out using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Forest City, CA).

For each ewe, the 200 partial *env* sequences from endometrium, uterine flush and conceptus, as well as homologous sequences from the 27 known enJSRV loci were aligned using the Clustal W algorithm (Thompson *et al.* 1994) as implemented in program Geneious. From these alignments (566 bp), Bayesian phylogenies were estimated in MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) under a general time reversible model with among site variation (GTR+G). Initial analyses indicated that sampled trees had unrealistically high branch lengths, a known problem with some MrBayes analyses (Brown & Lemmon 2007). Final runs were therefore not started from random trees but rather from NJ trees calculated under a GTR+G model in Paup* (Swofford 2002). Branch length priors were rescaled using the formula supplied by Brown et al. (Brown & Lemmon 2007) based on average branch length from the NJ tree. Two independent chains were run for 5 million generations each of which 1 million were later removed as burn-in. Parameters and trees were sampled every 2,000 steps and convergence among runs was assessed visually using Tracer 1.5 (Rambaut & Drummond 2007). Majority rule consensus trees and posterior support for individual branches were then found based on the final sample of 4,000 trees.

Results

Mature enJSRV particles are released in the uterine lumen. Western blot analysis of uterine flush samples, clarified and ultracentrifuged over a sucrose cushion, detected the enJSRVs major capsid protein (CA) from all ewes in this study (n=16) (Figure 5.1). As expected, the immunoreactive protein identified was of ~25 kDa in size, which is the predicted size of the mature enJSRVs CA derived from the cleavage of

Gag by the viral protease. These data strongly support the hypothesis that intact enJSRV particles are assembled in the uterine endometrial epithelia and released into the uterine lumen.

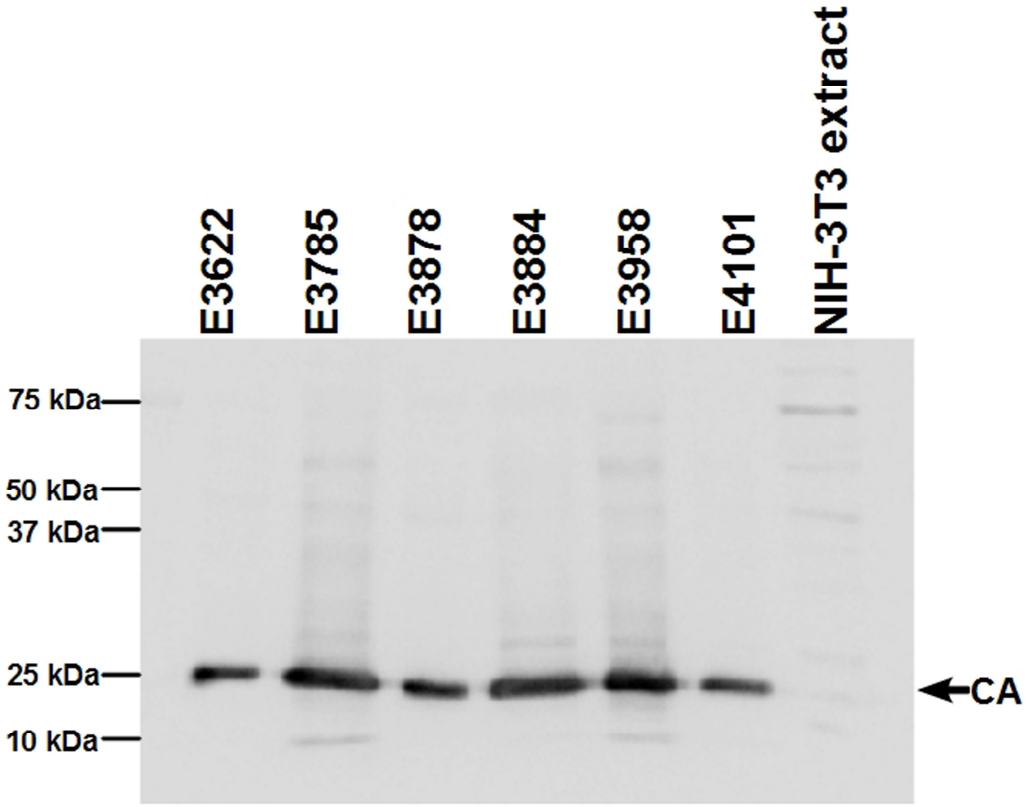


Figure 5.1 Western blot analysis of enJSRV viral particles isolated from the lumen of the ovine uterus. Viral particles were purified by ultracentrifugation of uterine flush samples over a sucrose cushion. Proteins were separated by 12% SDS-PAGE, and Western blot analysis was conducted using a rabbit antiserum towards the major capdis protein of JSRV. An immunoreactive protein of ~25 kDa in size, which is the correct size of the mature enJSRVs capsid, was detected in uterine flushings from all ewes (n=16). Representative results from 6 ewes are presented. Cell lysates from NIH 3T3 cells were used as a negative control.

enJSRVs *env* mRNA is present in implanted bovine conceptus trophectoderm. In order to test whether the enJSRVs particles released in the uterus could potentially infect the developing conceptus, we conducted a trans-species embryo transfer experiment in which *in vitro* produced bovine embryos (Rexroad & Powell 1999, Talbot *et al.* 2000) were transferred into the ovine uterus, gestated for 13 days, and then recovered and analyzed for the presence of enJSRVs (Figure 5.2). The use of bovine embryos was necessary because these species do not harbor enJSRV loci in their genome. The "background" of enJSRVs in sheep embryos would make it exceedingly difficult to detect uterine-derived enJSRVs.

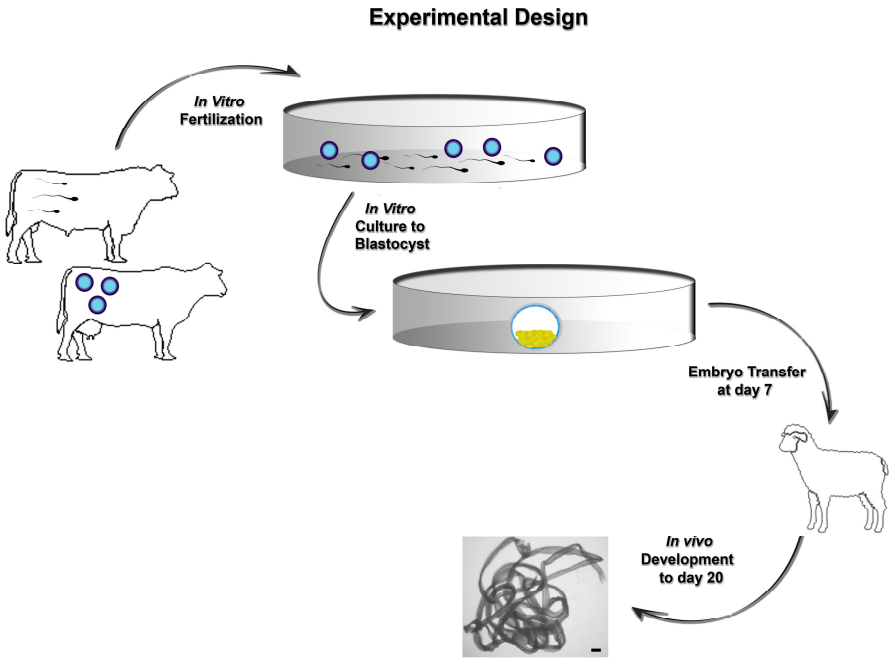


Figure 5.2 Experimental design for trans-species embryo transfer. Schematic illustrating experimental design of trans-species embryo transfer conducted to test the working hypothesis that enJSRV particles are released from the uterine endometrial epithelia and infect the conceptus trophectoderm.

The spherical blastocyst changes morphology during early pregnancy that involves proliferation and outgrowth of the trophoblast, a process termed conceptus elongation (Spencer *et al.* 2004). Sixteen fully elongated and filamentous conceptuses (8-10 cm in length) were recovered at 13 days post-implantation in 16 of the 30 recipient ewes that received two *in vitro* produced bovine blastocysts. Trophectoderm cells from the bovine conceptuses were isolated by laser capture microdissection and RNA analyzed by RT-PCR for *IFNT*, *LGALS15* and *CSH1* mRNAs. *IFNT* is expressed exclusively in the mononuclear trophectoderm cells of the conceptus (Farin *et al.* 1989). As expected, all of the bovine trophectoderm samples contained *IFNT* mRNA (Figure 5.3 and Table 5.2). *LGALS15* is a gene expressed specifically in the uterine endometrial luminal epithelium in the sheep and goat but not in the conceptus (Gray *et al.* 2004, Lewis *et al.* 2007). Although the *LGALS15* gene is present in cattle, *LGALS15* mRNA is not detectable in the bovine uterus or conceptus or in other bovine tissues (Lewis *et al.* 2007). *CSH1* is expressed specifically by the trophoblast giant binucleate cells in both cattle and sheep, but is not expressed in the uterus (Hoffman & Wooding 1993b). Thus, the presence of *LGALS15* mRNA from the microdissected bovine trophectoderm cell samples would indicate contamination by cells of ovine endometrial luminal epithelium, whereas the presence of *CSH1* mRNA would indicate the presence of differentiated trophoblast giant binucleate cells in the conceptus trophectoderm. In total, we detected *LGALS15* and/or *CSH1* mRNA in 6 of the LCM-isolated bovine trophectoderm samples (Figure 5.3 and Table 5.2); these samples were excluded from subsequent analysis.

HYAL2 mRNA was detected in all of the bovine trophectoderm samples. Importantly, *enJSRVs* RNA was present in all bovine trophectoderm RNA samples (n=16/16), including the 10 samples with no evidence for contamination by ovine uterine endometrial luminal epithelium.

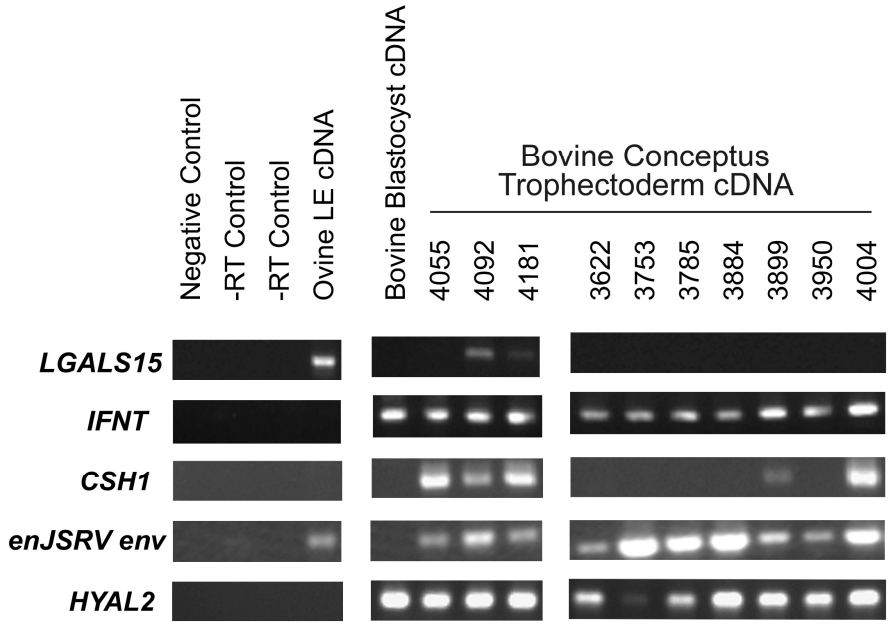


Figure 5.3 RT-PCR analysis of bovine conceptus trophectoderm. RNA was extracted from microdissected bovine conceptus trophectoderm cells and analyzed by RT-PCR for *LGALS15*, *IFNT*, *CSH1*, and *enJSRVs* mRNAs. Representative results are shown from 10 different bovine conceptuses. Negative controls included water as template and no reverse transcriptase (-RT) to assess genomic DNA contamination.

Table 5.2. Summary of RNA and DNA analysis of microdissected bovine conceptus trophectoderm samples.

Conceptus	***IFNT* mRNA**	***LGALS15* mRNA**	***CSH1* mRNA**	***enJSRVs env* mRNA**	***HYAL2* mRNA**	**Bovine *MT-CO1* DNA**	**Ovine *MT-CO1* DNA**	***enJSRVs env* DNA**
3622	+	-	-	+	+	+	-	-
3753	+	-	-	+	+	+	-	+
3785	+	-	-	+	+	+	-	-
3884	+	-	-	+	+	+	-	-
3899	+	-	+	+	+	+	-	-
3950	+	-	-	+	+	+	-	-
3958	+	-	-	+	+	+	-	+
4004	+	-	+	+	+	+	-	+
4055	+	-	+	+	+	+	+	+
4092	+	+	+	+	+	+	+	+
4181	+	+	+	+	+	+	+	+
7900	+	-	-	+	+	+	-	+
7912	+	-	-	+	+	+	-	-
7919	+	-	+	+	+	+	+	+
7935	+	-	-	+	+	+	-	-
7937	+	-	-	+	+	+	-	-
Total	16	2	6	16	16	16	4	8

[1]Presence (+) or absence (-) of mRNA or DNA determined by PCR analyses of microdissected bovine conceptus trophectoderm samples (see Table 1 for primer and conditions and Methods for protocol).

Analysis of DNA from bovine conceptus trophectoderm. Collectively, these results indicated that: (i) enJSRVs release viral particles from the endometrial epithelium into the lumen of the uterus; and (ii) these particles then enter the trophectoderm of the transplanted bovine conceptuses. The trophectoderm nourishes the developing conceptus in the early stages of pregnancy by phagocytosis of secreted products from the uterine epithelia that are present in the uterine lumen (Guillomot *et al.* 1981). Thus, the presence of *enJSRVs* RNA in the conceptus trophectoderm could arise from either non-specific trophoblast phagocytosis or true receptor-mediated entry. In order to differentiate these processes, we tested whether we could detect *enJSRVs* DNA in bovine trophectoderm, indicative of reverse transcription. We purified DNA from the microdissected bovine trophectoderm and ruled out the presence of ovine DNA contamination by PCR using primers that amplify the ovine mitochondrial gene *MT-CO1* (Lahiff *et al.* 2001). As illustrated in Figure 5.4 and summarized in Table 5.2, all of the bovine conceptus trophectoderm samples were positive for bovine *MT-CO1* (Lahiff *et al.* 2001). On the other hand, ovine *MT-CO1* was amplified only from 4 samples [4055, 4092, 4181, and 7919 (not shown in the figure)]. Thus, 10 conceptuses had no indication of ovine cell contamination as tested by RT-PCR of the *CSH1* and *LGALS15* mRNAs and PCR of ovine *MT-CO1*. enJSRVs DNA was amplified in total DNA isolated from 3 of the 10 bovine conceptus trophectoderm samples (3753, 3958, and 7900) that were not contaminated with ovine DNA or RNAs. These results support the idea that enJSRV viral particles from the ovine uterus entered into the bovine

conceptus trophectoderm and, in some cases, their genome was reverse transcribed indicative of receptor-mediated cell entry.

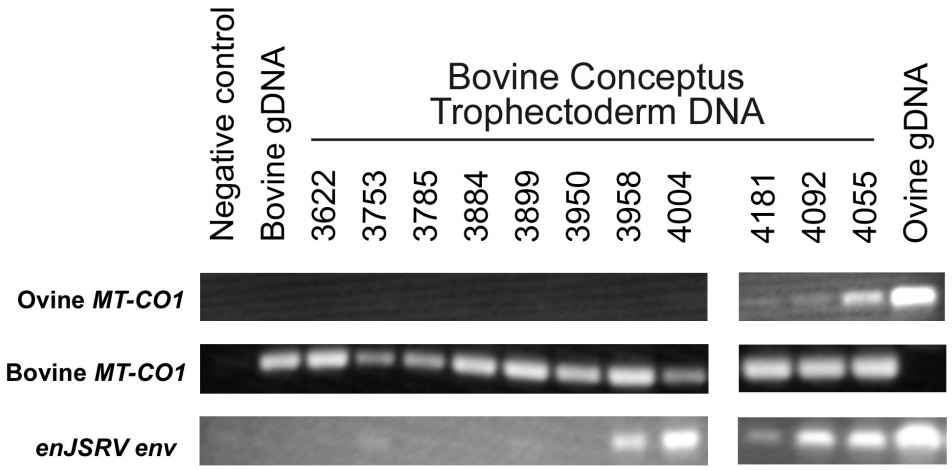


Figure 5.4 PCR analysis of bovine conceptus trophectoderm. DNA was extracted from microdissected bovine conceptus trophectoderm cells and analyzed by PCR for ovine and bovine *MT-CO1* genes as well as *enJSRVs env.* Representative results are shown from 11 bovine conceptuses. The negative control used water as template, and the positive controls were bovine or ovine genomic DNA (gDNA).

Transcriptional profile of enJSRV loci in the ovine endometrium. We next analyzed the transcription profile of the enJSRV loci that were: (i) expressed in the ovine endometrium; (ii) packaged in viral particles released in the lumen of the endometrium and; (iii) present in the bovine conceptuses. We performed RT-PCR analysis of RNA extracted from the endometrium, viral particles purified from flushes of the uterine lumen, and microdissected trophectoderm of the bovine conceptuses from four ewes (7900, 7912, 7935, 7937). PCR products were then cloned and from each animal with 100 colonies derived from ovine endometrium, 50 from viral particles purified from

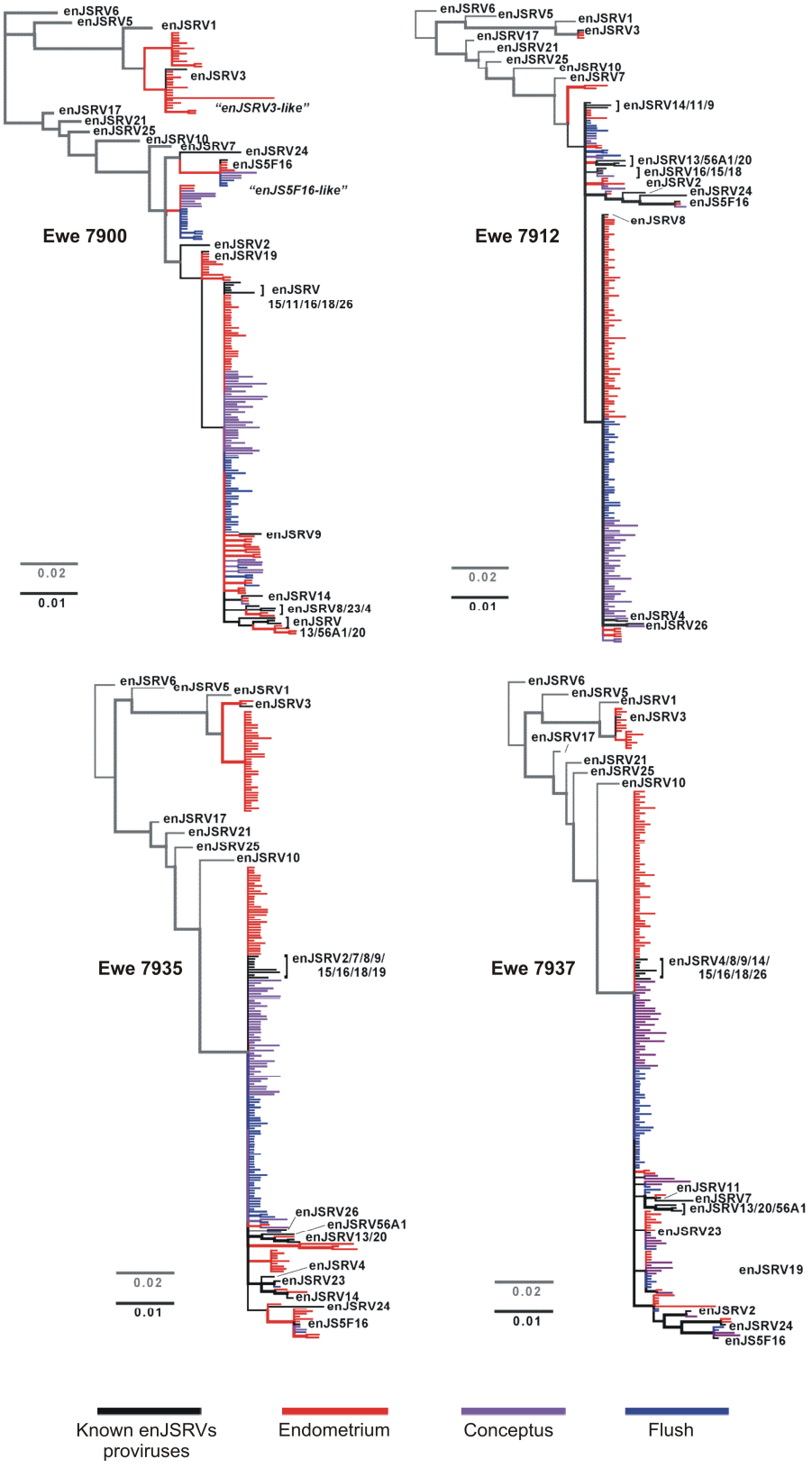
uterine flushes, and 50 from microdissected bovine conceptus trophectoderm were sequenced and Bayesan phylogenies estimated as described in the Materials and Methods. The enJSRV loci have a very high degree of sequence similarity between each other, which makes it difficult to identify each provirus with certainty (Arnaud *et al.* 2007a). In addition, some of these loci are insertionally polymorphic and entered the host genome only in the last few thousand years. Consequently, different individuals may possess different enJSRV proviruses with similar/identical sequences while the current nomenclature is based to enJSRV loci that have been cloned from a single BAC library isolated from a single ram (of Texel breed). However, sequence differences in the amplified region (consisting of the transmembrane domain of Env and the U3 region of the LTR) are sufficient at least to distinguish proviruses with similarity to either evolutionary "younger" (after domestication) or "older" (before domestication) enJSRV loci.

As illustrated in Figure 5.5, all four ewes analyzed showed a consistent transcriptional profiling. The great majority of the enJSRV sequences amplified from the endometrium of the four ewes clustered with sequences amplified from the viral particles present in the uterine flushes and from the transferred bovine embryos. In all cases, the majority of sequences clustered with the youngest enJSRV loci that are present only in domestic sheep (and not to the wild relatives within the *Ovis* genus). From 3 of the 4 ewes, the majority of the enJSRVs sequences expressed clustered with the insertionally polymorphic proviruses (such as enJSRV-16, enJSRV-18). In one of the ewes (7912), the majority of the expressed sequences appear to cluster more

specifically with enJSRV-8. enJSRV-8 was detected in our previous study (Arnaud *et al.* 2007a) in domestic sheep, but not in wild relatives. However, the enJSRV-8 provirus contained a stop codon in *env* that was not present in the enJSRV sequences recovered from ewe 7912. Sequences similar to some of the oldest proviruses, such as enJSRV-3, were detected at a variable proportion in all the endometrial samples. Interestingly these sequences were not recovered in the viral particles isolated from uterine flushes.

The enJS56A1 locus, which has a transdominant mutation in *gag*, is able to block viral exit of all the intact enJSRVs proviruses with the exception of enJSRV-26 that escapes this viral restriction (Arnaud *et al.* 2007a). The enJS56A1-like sequences were rarely expressed in the endometrium of Texel sheep analyzed in this study, suggesting weak expression in these animals of this transdominant proviral locus compared to the other enJSRVs. Sequences similar to enJSRV-26 were detected in the conceptuses of two animals (7912/7935). The enJSRV-26 is the only provirus that is able to escape restriction induced by enJS56A1. Overall, these results suggest that recently integrated enJSRV proviruses are abundantly expressed in the sheep endometrium and are able to release viral particles. The low abundance of enJS56A1 and the presence of enJSRV-26-like escape mutants might allow those enJSRVs to efficiently release infectious viruses that subsequently enter/infect the bovine conceptus trophectoderm.

Figure 5.5 Phylogenetic analysis of enJSRVs expressed in the uterus. Bayesian majority rule consensus trees of 200 partial *env* sequences from endometrium (red), uterine flush (blue) and conceptus (purple) of four ewes receiving bovine blastocytes. Sequences from 26 known endogenous betaretroviruses (enJSRVs) were also included (in black); enJSRV-6 was used to root the tree. Branch width represents the level of posterior support with most internal branches receiving 100% support. For easier visual representation, deeper parts of the phylogeny (grey branches) are shown at a different scale.


enJSRV6
enJSRV5
enJSRV1
enJSRV3
"enJSRV3-like"
enJSRV17
enJSRV21
enJSRV25
enJSRV10
enJSRV7
enJSRV24
enJS5F16
"enJS5F16-like"
Ewe 7900
enJSRV2
enJSRV19
] enJSRV 15/11/16/18/26
enJSRV9
0.02
0.01
enJSRV14
] enJSRV8/23/4
] enJSRV 13/56A1/20
enJSRV6
enJSRV5
enJSRV1
enJSRV3
enJSRV17
enJSRV21
enJSRV25
enJSRV10
enJSRV7
] enJSRV14/11/9
] enJSRV13/56A1/20
] enJSRV16/15/18
enJSRV2
enJSRV24
enJS5F16
Ewe 7912
enJSRV8
0.02
0.01
enJSRV4
enJSRV26
enJSRV6
enJSRV5
enJSRV1
enJSRV3
enJSRV17
enJSRV21
enJSRV25
enJSRV10
Ewe 7935
] enJSRV2/7/8/9/ 15/16/18/19
enJSRV26
enJSRV56A1
enJSRV13/20
enJSRV4
enJSRV23
enJSRV14
enJSRV24
enJS5F16
0.02
0.01
enJSRV6
enJSRV5
enJSRV1
enJSRV3
enJSRV17
enJSRV21
enJSRV25
enJSRV10
Ewe 7937
] enJSRV4/8/9/14/ 15/16/18/26
enJSRV11
enJSRV7
] enJSRV13/20/56A1
enJSRV23
enJSRV19
enJSRV2
enJSRV24
enJS5F16
0.02
0.01
Known enJSRVs proviruses
Endometrium
Conceptus
Flush

Discussion

Collectively, results presented here provide strong evidence that enJSRVs are expressed in the sheep endometrium and release viral particles that have the potential to infect the trophoblast of the developing conceptus. In fact, the presence of viral particles in the mammalian placentae has been documented in many species, including humans, baboons, and mice (Kalter *et al.* 1973a, Kalter *et al.* 1973b, Smith *et al.* 1975, Lyden *et al.* 1994). Although it has been assumed that these particles derive from ERVs expressed in the placenta, it is also possible, as shown in this study, that these particles are released from the uterine endometrium and then enter into the mononuclear trophectoderm cells of the gestating conceptus.

The present study utilized a trans-species embryo transfer model in which *in vitro* derived bovine blastocysts were transferred to recipient ovine uteri. Utilization of bovine blastocysts provided a unique opportunity to assess the presence of the enJSRV RNA/DNA into the bovine conceptus, as these enJSRVs are not present in the bovine genome (Hecht *et al.* 1996b, Arnaud *et al.* 2007a). The bovine conceptuses gestated in the ovine uterus developed appropriately, as they were elongated, filamentous in nature, and contained *IFNT* mRNA. While the bovine genome does not contain enJSRVs sequences, the presence of *enJSRVs* RNA and, in some cases, DNA in the bovine conceptuses is evidence that enJSRV viral particles, released by the ovine uterine endometrial epithelia, are able to enter the mononuclear trophectoderm cells of the bovine conceptus.

The mechanisms by which the enJSRV particles enter the conceptus trophectoderm are not clear. One possibility is that they use HYAL2, the cellular receptor for JSRV and enJSRVs. Although *HYAL2* mRNA was detected in all of the bovine conceptus trophectoderm samples, bovine HYAL2 is much less efficient at mediating entry of the enJSRV particles than ovine HYAL2 (Arnaud *et al.* 2007a). Alternatively, enJSRV particles could enter the mononuclear trophectoderm cells via a non-receptor mediated process such as phagocytosis. Indeed, the trophectoderm is highly phagocytic in nature due to reliance on substances secreted by the endometrium and present in the uterine lumen for conceptus development (Wintenberger-Torres & Flechon 1974, Gray *et al.* 2001). Although *enJSRVs* RNA was detected in 10/10 conceptuses, *enJSRVs* DNA was detected in only in 3/10 trophectoderm samples of bovine conceptuses. These data suggest that receptor-mediated entry and subsequent reverse transcription did not occur in all of the bovine conceptuses and that enJSRVs in the trophectoderm could be derived from both receptor-mediated entry and phagocytosis/pinocytosis.

We recently found that oBST2/tetherin is upregulated in the ovine endometrium during early pregnancy by conceptus IFNT (Arnaud *et al.* 2010). The oBST2/tetherin is a restriction factor that can inhibit enJSRV exit *in vitro*; however, it is upregulated only in the stromal cells of the endometria of pregnant ewes and is not present in the endometrial luminal epithelium (Arnaud *et al.* 2010). Thus, the host appears to have evolved mechanisms that favor (or at least do not hamper) enJSRVs expression and release of viral particles from the uterine luminal epithelium. Available evidence suggests that the enJSRV ERV particles are beneficial to the developing conceptus,

possibly by stimulating trophectoderm cell proliferation and differentiation. Previous research from our laboratory supports this hypothesis as *in utero* enJSRV Env knockdown resulted in growth-retarded conceptuses that exhibited reduced MTC proliferation and an absence of BNC formation (Dunlap *et al.* 2006b). In that study, morpholinos were used to inhibit the splicing and translation of enJSRVs *env* RNA in the conceptus trophectoderm, but the *in utero* injections also reduced the amount of enJSRVs Env in the uterine LE. Thus, it is possible that the morpholino knockdown of enJSRVs Env in the uterine LE reduced the amount of enJSRV viral particles shed by the LE, thereby impacting MTC proliferation and BNC differentiation. Therefore, enJSRVs expressed in the developing conceptus after day 12 may be derived from proviruses expressed in the trophoblast itself and/or from those expressed in the maternal uterus that are subsequently transmitted to the trophoblast.

It is feasible to hypothesize that at least some enJSRV loci present in the ovine genome are the result of integration of new proviruses derived from enJSRVs produced by the uterine epithelia that infect the inner cell mass (ICM) of the developing embryo that gives rise to the embryo/fetus proper. However, one would expect that transmission of enJSRVs from the trophectoderm to the ICM is not readily achieved by enJSRVs; otherwise there would be an acquisition of too many proviruses in each generation. From our results, the enJSRVs abundantly expressed in the uterus and producing functional Env proteins are those recently integrated in the *Ovis aries* genome. Therefore, the ongoing endogenization and *de novo* integrations in the genome of intact

enJSRVs might be vital for the host in order to maintain the expression of functional envelope proteins in the ovine reproductive tract generation after generation.

Collectively, findings from the current study provide evidence that fully intact proviral loci are present within the sheep genome and capable of producing viral particles that are shed from the uterine epithelia expressing enJSRV loci. Further, the resultant viral particles enter the cells of the conceptus and integrate into the genome of the bovine trophectoderm cells in a trans-species embryo transfer model.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The role of ERVs in mammalian placental morphogenesis has been debated and hypothesized for many years. Within the last decade, reports in multiple species of placental specific retroviral gene expression and induced syncytia formation *in vitro* have supported the hypothesis that the Env glycoproteins of ERVs are involved in formation of the placenta (Mi *et al.* 2000, Dupressoir *et al.* 2005, Heidmann *et al.* 2009). In the sheep, a loss-of-function approach clearly demonstrated the requirement for enJSRVs Env, specifically in the outgrowth and differentiation of the trophoblast in the peri-implantation period (Dunlap *et al.* 2006b). Recently, a knockout mouse model found that an ERV Env, syncytin A, is necessary for proper placental development and successful pregnancy in rodents, because the null phenotype was embryonic lethal (Dupressoir *et al.* 2009). Thus, ERVs are essential to placentation in at least two species and have been conserved over thousands of years in primates, rodents, rabbits, and sheep. The studies presented here contribute to the body of knowledge regarding the role of ERVs in placental morphogenesis in the sheep.

Summary. The experiments detailed in Chapter III investigated the effect of progesterone on growth and development of conceptuses containing reduced enJSRVs Env. An *in vivo* loss-of-function approach was described in which morpholino antisense oligonucleotides inhibited translation of enJSRVs Env in the uterus and conceptus. The results of this loss-of-function were decreased MTC growth and differentiation on day 16, followed by conceptus loss by day 20 (Dunlap *et al.* 2006b). In ewes treated with

progesterone, pregnancy rates between morpholino treatments were comparable; however, the BNC numbers were lower in the MAO-env treated conceptuses. Exogenous progesterone administration induces production of histotroph, which includes nutrients, extracellular matrix proteins, growth factors and other proteins from the LE and GE of the uterus that enhance proliferation and outgrowth of the conceptus. The permissive uterine environment maintained by the progesterone administration allowed the MAO-env conceptuses to develop to day 20; however; the conceptuses were still developmentally delayed compared to controls. This study demonstrates that when a progestenized environment is maintained through day 20, conceptus development is still negatively affected by enJSRVs Env loss-of-function, reinforcing evidence for a requirement of enJSRVs Env for proper trophoblast proliferation and differentiation.

In Chapter IV, the specific enJSRV loci expressed by the conceptus during critical developmental time points were determined by matching to sequenced *env* mRNA. The corresponding protein sequences were aligned with the JSRV Env amino acid sequence to determine possible structural and signaling domains. Conceptuses were collected on day 13, at the beginning of elongation and rapid MTC proliferation, and day 18, after the onset of BNC differentiation (Spencer *et al.* 2004). Both Suffolk and Texel breeds were used for the day 13 sequence analysis, while only Suffolk conceptuses were obtained for the day 18 study. Intriguingly, both enJSRV-1 and enJSRV-8, which were the best represented among all conceptuses, encode truncated Env proteins, and upon sequence analysis, these proteins were found to have no membrane-spanning domain. In all three conceptus groups, sequencing analysis revealed that enJSRV loci were

expressed that code for intact, functional Env proteins, as well as coding for all of the proteins necessary for viral particle formation *in vitro*.

The experiments in Chapter V utilized a transpecies embryo transfer experiment in which *in vitro* derived bovine conceptuses were transferred to recipient ovine uteri. This study provides the first evidence for production of endogenous betaretroviral particles by the endometrium of the uterus, presence of endogenous viral particles in the uterine lumen, and entry of those viral particles into MTCs of the developing conceptus. Evaluation of bovine conceptuses gestated in the ovine uterus revealed that they were morphologically similar to sheep conceptuses at a similar developmental stage, as they were elongated, filamentous and expressed *IFNT* mRNA. While the bovine genome does not contain enJSRV sequences, the presence of enJSRVs *env* RNA and, in three cases, the enJSRVs *env* gene in DNA from the bovine conceptus is evidence that enJSRV viral particles shed by the ovine uterine epithelia are able to enter the MTCs of the bovine conceptus.

The transcriptional profiling in Chapter V revealed the majority of sequences from the sheep endometrium clustered with the youngest enJSRV loci that are present only in the domestic sheep. These include enJSRV-16, which is a completely intact proviral loci and enJSRV-8, which contains a stop codon in *env* that is not present in the enJSRV sequences recovered from the endometrium. These loci also matched sequences in the sheep conceptuses studied as described in Chapter IV. Sequences similar to some of the oldest proviruses, such as enJSRV-3, were detected at a variable proportion in all the endometrial samples from Chapter V, just as in the conceptus

samples from Chapter IV. Interestingly, these "older" sequences were not recovered in the viral particles isolated from uterine flushes.

Conclusions. The results from experiments presented here contribute to the body of knowledge regarding expression and function of ERVs in the reproductive tracts and placentae of mammals. The presence of enJSRVs *env* RNA and protein within the ovine uterus and conceptus is well established and is crucial for MTC proliferation and differentiation.

In Chapter III, progesterone administration did rescue pregnancy rates in conceptuses with reduced enJSRV Env. Despite administration of progesterone and comparable pregnancy rates, the MAO-env conceptuses were developmentally delayed, with lower numbers of BNC which is indicative of a lack of differentiation. These results support the hypothesis that enJSRVs are necessary for proper trophoblast development and differentiation in the sheep, even in uterine environment that is supportive for pregnancy. As enJSRVs RNA and protein are present in both the conceptus and the endometrium, it is not clear from where the required enJSRVs are originating.

Investigation into the enJSRV loci transcribed by the sheep conceptus revealed that enJSRV Env proteins in the conceptus come from multiple loci. Two of the most represented loci encode truncated Env proteins, similar to the truncated ERV3 in humans. *ERV3* in humans is highly expressed in the syncytiotrophoblast layer and associated with hormone production by these cells, despite not containing the traditional domains of an Env glycoprotein (Larsson *et al.* 1994, Rote *et al.* 2004). Without the membrane-

spanning domain, the Env proteins of enJSRV-1 and enJSRV-8 are likely soluble, and could function as signaling molecules. The conservation of the signal peptide in the two proteins indicates that these truncated Envs may be trafficked as extracellular proteins just as for normal SU peptides. Intriguingly, the transcriptional profiling in Chapter V revealed that enJSRV-8 is highly represented in the endometrium of at least one Texel ewe on day 20, although the sequences do not contain the stop codon as is the case for sequences from day 13 and day 18 conceptuses. This could indicate the presence of an additional enJSRV locus within the sheep genome that has not been fully characterized. Currently, enJSRV-26 is thought to be the "newest" enJSRV provirus, having been identified only in the Texel breed and being capable of escaping the JLR late interference mechanism. This information is supported by evidence from Chapter IV, in which enJSRV-26, as well as enJSRV-17 and enJSRV-15, were present only in the Texel conceptuses on day 13. Additionally, enJSRV-1 was identified only the Suffolk conceptuses. These insertionally polymorphic proviral loci are evidence of ongoing endogenization of the enJRSV loci within the sheep genome, and can be used to determine the lineages of breeds within different species of sheep, as well as lineages before speciation in the order of Caprinae (Arnaud *et al.* 2007a, Chessa *et al.* 2009). The presence of the newly integrated enJSRVs in the ovine genome and their expression in the uterus and conceptus support the hypothesis that enJSRVs play an evolutionarily important role in sheep.

Both day 13 and day 18 conceptuses contained evidence of full-length Env proteins that are capable of interaction with HYAL2, the receptor for both enJSRVs and

JSRV Env. These enJSRVs Env are structurally very similar to the exogenous JSRV Env, except for the variable region in the cytoplasmic tail. This region contains mutations which render the enJSRVs Env non-oncogenic. Despite the lack of a transformative domain, the enJSRVs Env may share other signaling domains with JSRV, which is capable of activating several cell signaling pathways through domains that have not been identified. It is probable that enJSRVs activate similar pathways as JSRV which enhance proliferation and differentiation of MTC population. Indeed, pathways activated by JSRV, including PI3K/AKT1 and MAPK, are implicated in placental morphogenesis in mice (Pollheimer & Knofler 2005). Similarly, the cellular changes seen in cancer and tumorogenesis mirror changes seen in early pregnancy, such as outgrowth and rapid proliferation, migration, and differentiation (Ferretti *et al.* 2007). The full-length enJSRV Env proteins in the developing conceptus likely activate pathways important for these functions. Additionally, enJSRV-11, which encodes a fully functional Env protein, is transcribed in day 13 conceptuses, but was not present in the day 18 conceptuses. This specific temporal pattern of expression could be indicative of an important role for enJSRV-11 in proliferation of the day 13 MTCs before differentiation progresses.

The presence of viral particles in the mammalian placentae has been documented in many species, including humans, baboons, and mice. In fact, the differentiation of the syncytiotrophoblast cells of the placenta in humans is marked by production of noninfectious HERV particles (Kalter *et al.* 1973a, Kalter *et al.* 1973b, Smith *et al.* 1975, Lyden *et al.* 1994). Interestingly, expression of ERV genes has been documented in uteri of both humans and monkeys, but the endometria and uterine lumen were not analyzed for

the presence of endogenous viral particles (Okulicz & Ace 2003, Kim *et al.* 2006, Yi & Kim 2007). As detailed in Chapter V, the expression of completely intact proviral loci in the ovine uterus led to the discovery that viral particles are produced by the uterus and can transduce the conceptus (Figure 6.1).

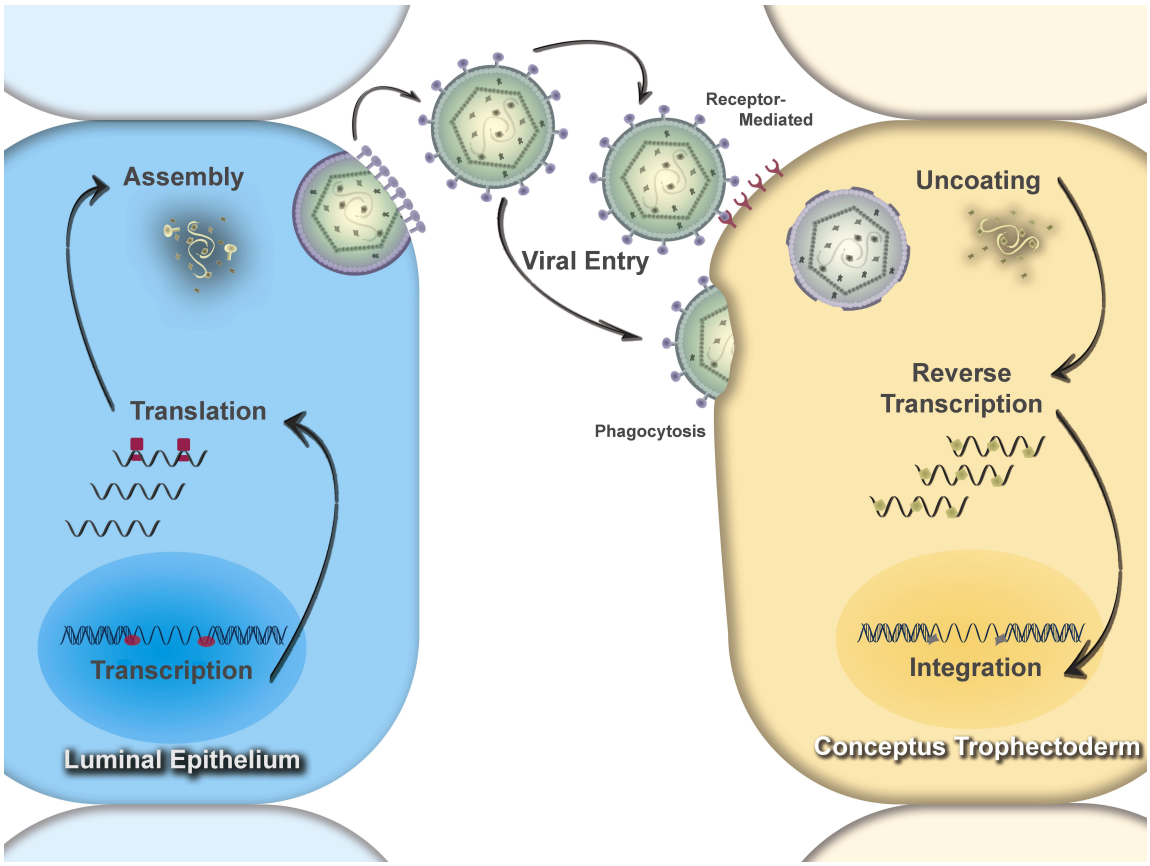


Figure 6.1 Schematic illustrating production and shedding of enJSRV viral particles from the endometrial epithelia into the uterine lumen and their entry into the conceptus trophectoderm. Once reverse transcribed and integrated into trophectoderm DNA, the enJSRVs Env influences trophoblast growth and differentiation.

These endogenous viral particles are potentially responsible for the enJSRV-stimulated proliferation and differentiation of the MTC population in the conceptus. While the term "infection" typically implies a pathological effect associated with exogenous retroviral infection, the ERV particles may offer a potential benefit to the developing conceptus, specifically by stimulating cell proliferation and differentiation. Previous research from our laboratory and research related in Chapter III support this hypothesis as *in utero* enJSRV Env knockdown resulted in growth-retarded conceptuses that exhibited reduced MTC proliferation and an absence of BNC formation (Dunlap *et al.* 2006b). In that study, morpholinos were used to inhibit the splicing and translation of enJSRVs *env* RNA in the conceptus trophectoderm, but the *in utero* injections also reduced the amount of enJSRVs Env in the uterine LE. Thus, it is possible that the morpholino knockdown of enJSRVs Env in the uterine LE reduced the amount of enJSRV viral particles shed by the uterine LE, thereby impacting the MTC proliferation and subsequent BNC differentiation. This raises the question of the origins of enJSRVs that affect trophoblast development. Either acquired enJSRVs from the uterus, innate enJSRVs from the conceptus or both could be activating signaling in the MTCs of the conceptus.

Additionally, the enJSRVs abundantly expressed in the uterus and producing functional envelope proteins are those recently integrated in the ovine genome. Therefore, the ongoing endogenization and new integrations in the genome of intact enJSRVs (such as enJSRV-26) might be vital for their host in order to maintain the expression of functional envelope proteins in the ovine reproductive tract generation after

generation. The preservation of proviral genes capable of forming functional proteins and the maintenance of their ability to produce viral particles suggest that enJSRVs do have an evolutionarily important role in sheep.

Placental morphogenesis in sheep is dependent on enJSRVs Env to stimulate a specific population of MTC to proliferate and differentiate into BNC, which will form the major source of hematotrophic nutrition to the fetus (Dunlap *et al.* 2006b, Igwebuike 2006). The presence of the whole variety of enJSRV loci originating from either the conceptus, the uterus, or both, reinforce the idea that multiple loci may be involved in trophoblast differentiation, including those encoding a truncated Env protein, an intact functional Env protein that can possibly stimulate trophoblast development through its cytoplasmic tail, and an endogenous viral particle which may stimulate proliferation upon entry into the cell similar to other viral particles (Rosenberg & Jolicoeur 1997) (Figure 6.2).

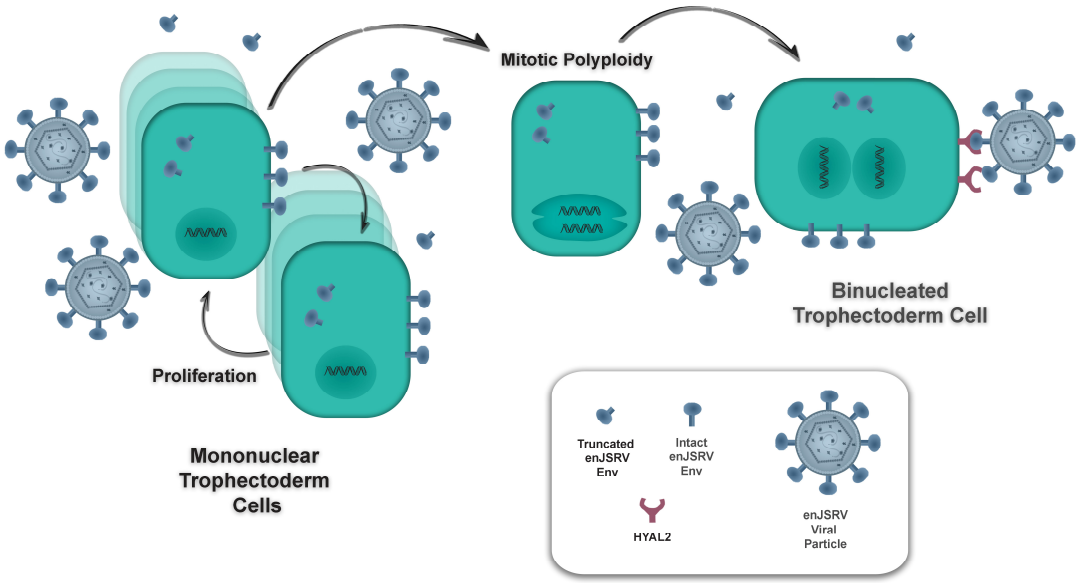


Figure 6.2 Hypothesis on the role of enJSRVs in trophoblast differentiation. Both fully intact enJSRVs Env and truncated Env proteins are present and stimulate proliferation and differentiation of the MTC population. Endogenous JSRV viral particles are produced from the uterine lumen and can interact with this stem cell population as well as the BNC to stimulate proliferation and differentiation as well.

With the contribution of the current experiments, *in vivo* loss-of-function studies in the sheep and mouse, and multiple *in vitro* experiments in the human, the role of ERVs in placental morphogenesis is well supported. Collectively, these studies in multiple species have generated fundamental knowledge concerning the cellular and molecular mechanisms regulating trophoblast proliferation and differentiation. The following additional research into enJSRVs will continue to elucidate these mechanisms and further contribute to the body of knowledge regarding ERVs and their role in placental morphogenesis: (1) determining the origin of enJSRVs in the conceptus – innate from the conceptus genome or acquired from the uterus via enJSRV viral particles; (2) determining the signaling mechanisms utilized by enJSRV Env; (3) investigating the role of the

enJSRV receptor HYAL2 in placental morphogenesis; and (4) studying the potential fusogenic abilities of enJSRV Env.

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