

## Research Article

# Tropism, Cytotoxicity, and Inflammatory Properties of Two Envelope Genes of Murine Leukemia Virus Type-Endogenous Retroviruses of C57BL/6J Mice

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Received 16 January 2011; Accepted 9 March 2011

Academic Editor: Y. Mandi

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Envelope (*env*) proteins of certain endogenous retroviruses (ERVs) participate in various pathophysiological processes. In this study, we characterized pathophysiological properties of two murine leukemia virus-type ERV (MuLV-ERV) *env* genes cloned from the ovary of C57BL/6J mice. The two *env* genes (named ENV<sub>OV1</sub> and ENV<sub>OV2</sub>), with 1,926 bp coding region, originated from two MuLV-ERV loci on chromosomes 8 and 18, respectively. ENV<sub>OV1</sub> and ENV<sub>OV2</sub> were ~75 kDa and predominantly expressed on the cell membrane. They were capable of producing pseudotype murine leukemia virus virions. Tropism trait and infectivity of ENV<sub>OV2</sub> were similar to the polytropic *env*; however, ENV<sub>OV1</sub> had very low level of infectivity. Overexpression of ENV<sub>OV2</sub>, but not ENV<sub>OV1</sub>, exerted cytotoxic effects and induced expression of COX-2, IL-1 $\beta$ , IL-6, and iNOS. These findings suggest that the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> are capable of serving as an *env* protein for virion assembly, and they exert differential cytotoxicity and modulation of inflammatory mediators.

## 1. Introduction

Ancient infection of germline cells with exogenous retroviruses established a genome-wide random embedment of proviruses, called endogenous retroviruses (ERVs), and Mendelian genetics governs their inheritance to the offsprings [1]. ERVs are reported to exist in the genome of all vertebrates and constitute approximately 8% of the human genome and 10% of the mouse genome [2–4]. The majority of ERVs identified so far are reported to be defective primarily based on their inability to encode intact polypeptides for *gag* (group specific antigen), *pol* (reverse transcriptase), and *env* (envelope) genes, which are essential for the retroviral life cycle [5]. However, recent studies identified a number of ERVs, which retain intact coding potentials for *gag*, *pol*, and/or *env* genes, and some of them are reported to be associated with a range of normal physiology (e.g., placental morphogenesis) as well as pathogenic processes (e.g., multiple sclerosis, schizophrenia,

injury, and chronic fatigue syndrome) [6–10]. On the other hand, biology of porcine ERVs (PERVs) has been studied extensively because of the potential transmission of PERVs to humans as an adverse side effect of xenotransplantation [11].

The *env* glycoproteins of certain human ERVs (HERVs) have been implicated in diverse disease processes [12–16]. For instance, the *env* glycoproteins of HERV-K, HERV-E, and ERV-3 were characterized as tumor-associated antigens in different types of cancer [15–18]. The HERV-W *env* glycoprotein, called syncytin-1, is highly expressed in glial cells within central nervous system of multiple sclerosis, an autoimmune disease, patients [13]. It is proposed that potent proinflammatory properties of syncytin-1 contribute to neuronal inflammation and resultant damage to oligodendrocytes during the progression of multiple sclerosis [12]. On the other hand, syncytin-1 and HERV-FRD *env* glycoprotein, called syncytin-2, are reported to play an essential role during embryonic development by controlling formation of placental syncytiotrophoblasts primarily through their highly

fusogenic properties [19–22]. Additional *env* glycoproteins have been identified and characterized from murine ERVs (syncytin-A and syncytin-B) and endogenous Jaagsiekte sheep retrovirus (enJSRV), and their roles in placenta morphogenesis are similar to syncytin-1 and syncytin-2 [7, 23, 24]. The findings from recent studies provide evidence suggesting that *env* glycoproteins of certain ERVs play a critical role in biological processes of normal physiology as well as diseases.

During a survey of expression profile of MuLV-ERV subgenomic *env* transcripts in various normal tissues of C57BL/6J mice, two putative full-length *env* transcripts were identified in the ovary. In this study, the biological characteristics of these two MuLV-ERV *env* genes, named ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, were investigated by examining a selective set of pathophysiologic parameters.

## 2. Materials and Methods

**2.1. Animals.** Female C57BL/6J mice (approximately 12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, Me) and housed according to the guidelines of the National Institutes of Health. The Animal Use and Care Administrative Advisory Committee of the University of California, Davis, approved the experimental protocol. Three mice were sacrificed by cervical dislocation for tissue collection without any pretreatment, and tissue samples were snap-frozen.

**2.2. RT-PCR Analyses.** RNA isolation and cDNA synthesis were performed primarily according to the relevant protocols provided by the kit manufacturer. Briefly, total RNAs were extracted using an RNeasy kit (Qiagen, Valencia, Calif) and cDNAs were synthesized using 100 ng of total RNA from each sample (tissue or cell) and the Sensiscript reverse transcriptase (Qiagen). The primers capable of amplifying the full length as well as subgenomic MuLV-ERV transcripts were designed based on the MAIDS (murine acquired immunodeficiency virus) virus-related provirus (GenBank No. S80082) [25]: forward, 5'-CAT TTG GAG GTC CCA CCG AGA-3' (MV1K) and reverse, 5'-CTC AGT CTG TCG GAG GAC TG-3' (MV2D). The following are the primer sets used for inflammatory mediators: COX-2 (forward, 5'-ACA CAG TGC ACT ACA TCC TGA C-3' and reverse, 5'-ATC ATC TCT ACC TGA GTG TC-3'), ICAM-1 (forward, 5'-AGC TGT TTG AGC TGA GCG AGA-3' and reverse, 5'-CTG TCG AAC TCC TCA GTC A-3'), IL-1 $\beta$  (forward, 5'-GAC AGT GAT GAG AAT GAC CTG-3' and reverse, 5'-GAA CTC TGC AGA CTC AAA CTC CA-3'), IL-6 (forward, 5'-GCC TTC CCT ACT TCA CAA GTC CG-3' and reverse, 5'-CAC TAG GTT TGC CGA GTA GAT CTC-3') [26], iNOS (forward, 5'-ACA AGC TGC ATG TGA CAT CGA-3' and reverse, 5'-CAG AGC CTG AAG TCA TGT TTG C-3'), and TNF- $\alpha$  (forward, 5'-GCA TGA TCC GCG ACG TGG AA-3' and reverse, 5'-AGA TCC ATG CCG TTG GCC AG-3') [27]. In addition,  $\beta$ -actin (forward, 5'-CCA ACT GGG ACG ACA TGG AG-3' and reverse, 5'-GTA GAT GGG CAC AGT GTG GG-3') was used as an internal expression control [28]. The density of amplified products (applied only for inflammatory

mediators) was measured using KODAK Molecular Imaging Software ver. 4.5 (Carestream Health, Rochester, NY), and it was normalized to  $\beta$ -actin control.

**2.3. Cloning and Sequencing of *env* Transcripts.** The RT-PCR products of the MuLV-ERV subgenomic transcripts (~2.9Kb) were cloned into the pGEM-T Easy vector (Promega, Madison, Wis) followed by plasmid DNA preparation using a kit from Qiagen, and sequencing analysis at Davis Sequencing Inc (Davis, Calif) or Molecular Cloning Laboratory (South San Francisco, Calif). DNA sequences were analyzed using Vector NTI-ver. 10 (Invitrogen, Carlsbad, Calif) or Editseq and MegAlign program within DNASTAR ver. 8.0.2 (DNASTAR, Madison, Wis).

**2.4. Construction of ENV<sub>OV1</sub> and ENV<sub>OV2</sub> Expression Vectors.** The coding regions of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> were amplified by PCR from their respective original cDNA clones using a set of primers embedded with restriction enzyme sites for cloning into the pcDNA4/HisMax (Invitrogen): forward with *NotI*, 5'-CGC GGC GGC CGC ATG GAA GGT CCA GCG TTC TC-3', ENV<sub>OV1</sub>-reverse with *XhoI*, 5'-GGC TCG AGT TAT TCA CGT GAT TCC ACT TTT TCT GG-3', and ENV<sub>OV2</sub>-reverse with *XhoI*, 5'-GGC TCG AGT TAT TCA CGT GAT TCC ACT TCT TCT GG-3'. The amplified coding sequences after 10 PCR cycles were cloned into the pGEMT-Easy vector (Promega) followed by digestion with *NotI* and *XhoI* and subsequently cloned into pcDNA4/HisMax (Invitrogen).

**2.5. Cell Lines.** The GP2-293 packaging cells (purchased from Clontech, Mountain View, Calif), tsA201 cells (a derivative of HEK293 cells), COS-7 cells, and COS-1 cells were maintained in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, streptomycin, and penicillin G. Five other cell lines (HeLa, Neuro-2a, MDCK, HCT 116, and NIH3T3) were cultured according to the protocols recommended by the American Type Culture Collection (Manassas, Va).

**2.6. Assays for Production, Tropism, and Infectivity of Pseudotype LacZ-MuLV Virions.** The GP2-293 cells, which were seeded onto a 6-well plate at a concentration of  $5 \times 10^5$  cells per well, were cotransfected with pQCLIN (Clontech, Mountain View, Calif) and pcDNA4/HisMax-ENV<sub>OV1</sub> or pcDNA4/HisMax-ENV<sub>OV2</sub> plasmid using Lipofectamine 2000 (Invitrogen). The following *env* proteins were used for tropism and infectivity controls: ecotropic (pEco), 4070A amphotropic (pAmpho), 10A1 amphotropic (p10A1) with a broader host range than 4070A, and G glycoprotein of the vesicular stomatitis virus (VSV-G) (Clontech). Culture supernatants containing pseudotype viral particles were passed through a 0.45  $\mu$ M filter (Fisher Scientific, Pittsburgh, Pa). Transfection efficiency was estimated by counting the stained cells under the microscope after X-gal staining.

For each cell line (a total of 8 cell lines) employed for tropism and infection analysis,  $5 \times 10^4$  cells/well were seeded onto a 24-well plate and incubated overnight in preparation of viral transduction. Subsequently, the medium

was replaced with 0.5 mL of serial dilutions of culture supernatants containing pseudotype LacZ-MuLV virions, in which Polybrene (Sigma, Milwaukee, Wis) was added (8  $\mu$ g/mL), followed by washing after 4 hours and incubation with 0.5 mL of fresh media for 2 days. The infected cells were treated with fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) and stained with X-gal solution. Cells stained blue were counted under the microscope as an infection unit.

**2.7. Western Blot Analyses.** To confirm expression of the pcDNA4/HisMax-ENV<sub>OV1</sub> or pcDNA4/HisMax-ENV<sub>OV2</sub> construct, Western blot analysis was performed following transfection into tsA201 cells using Fugene 6 reagent (Roche, Mannheim, Germany). At 2 days after transfection, the cells were harvested, and Western blot analysis was performed. Briefly, the membrane, blocked in 5% nonfat dry milk (NFDm), was incubated with a goat antibody specific for gp69/71 of Rauscher MuLV (1:2000 dilution with 5% NFDm in TBST [Tris-buffered saline with Tween 20]) obtained from ViroMed Biosafety Laboratories (Camden, NJ) followed by an anti-goat-HRP antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, Calif). The protein signal was visualized using ECL reagents (GE healthcare, Pittsburgh, Pa). A similar protocol was used to detect *env* glycoprotein from supernatants of the GP2-293 cells producing pseudotype LacZ-MuLV virions.

**2.8. Immunocytochemistry.** HeLa cells, which were transfected with the pcDNA4/HisMax-ENV<sub>OV1</sub> or pcDNA4/HisMax-ENV<sub>OV2</sub> construct, were harvested and transferred into 0.1% poly-L-Lysine coated coverslips and incubated for 1 day. Cells were then immunostained with a goat antibody specific for gp69/71 (1:200 diluted in culture medium, ViroMed Biosafety Laboratories) and fixed with both 4% paraformaldehyde. Fixed cells were incubated with a Texas-Red-conjugated antigoat IgG secondary antibody (1:200 diluted in PBS, Vector Laboratories, Burlingame, Calif) and stained cells were visualized by a Zeiss microscope using AxioVison software version 4.5 (Carl Zeiss, Jena, Germany).

**2.9. Cytotoxicity and Cell Proliferation Assays.** HeLa cells, which were transfected with the pcDNA4/HisMax-ENV<sub>OV1</sub> or pcDNA4/HisMax-ENV<sub>OV2</sub> construct, were subjected to cytotoxicity assay using a Cytotoxicity Detection Kit (Roche, South San Francisco, Calif) according to the protocol recommended by the manufacturer. Absorbance was measured at 490 nm with a reference at 600 nm using a reader from Molecular Devices (Sunnyvale, Calif). Cell proliferation rate was measured from these cells using the colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, Milwaukee, Wis) assay as described previously [29]. Absorbance was read at 560 nm with a reference at 600 nm using a reader (Molecular Devices). All experiments were performed at least in triplicate, and 4 independent experiments were repeated.

**2.10. Analysis of Inflammatory Mediators.** RAW264.7 cells, which were transfected with the pcDNA4/HisMax-ENV<sub>OV1</sub>

or pcDNA4/HisMax-ENV<sub>OV2</sub> construct, were harvested at 1 day after transfection, and they were examined for expression of a set of inflammatory mediators at mRNA levels by RT-PCR, and the relevant protocols and reagents are described in Section 2.2 above.

**2.11. Statistical Analysis.** Statistical analysis was performed using two-tailed Student's *t*-test and statistical significance was determined as \**P* < .05 and \*\**P* < .01.

### 3. Results

**3.1. Identification and Initial Characterization of Two MuLV-ERV *env* Subgenomic Transcripts Expressed in the Ovary of C57BL/6J Mice.** The expression profiles of MuLV-ERV *env* genes in various normal tissues (liver, lung, salivary gland, adrenal gland, brain, skin, ovary, and uterus) of C57BL/6J mice were investigated. A number of putative subgenomic transcripts with varying sizes, ranging from ~1 Kb to ~5 Kb, which may be generated by splicing and/or deletion, were differentially expressed in each tissue. Among them were ~2.9 Kb bands presumed to be amplified from full-length MuLV-ERV *env* transcripts, and their expression was evident in the ovary and uterus as well as other tissues (Figure 1(a)). Sequencing analysis revealed that the two 2,892 bp transcripts were *env* mRNAs, which were generated by a single splicing using the well-characterized donor and acceptor signals [30]. A subsequent open reading frame analysis revealed that the two full-length MuLV-ERV *env* genes, named ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, retain intact coding potential for *env* glycoproteins of 641 amino acids. While the nucleotide and polypeptide sequences of the ENV<sub>OV2</sub> was identical to an *env* gene of an polytropic murine leukemia virus (MuLV)-related retroviral sequence from NFS/N mice, the ENV<sub>OV1</sub> has not been reported yet [31].

Prior to the functional characterization of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, they were aligned with four different reference *env* polypeptides displaying different host tropisms: ecotropic, xenotropic, polytropic, and modified polytropic (Figure 1(b)). It turned out that both ENV<sub>OV1</sub> and ENV<sub>OV2</sub> had a higher level of sequence similarity to the polytropic/modified polytropic *env* polypeptides compared to the others. Both the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> share the identical sequence in the variable region A and proline rich region, while one amino acid residue was different in the variable region B and R peptide, respectively. To identify the putative MuLV-ERVs encoding the ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, respectively, the C57BL/6J genome sequence (Build 37.1) from the National Center for Biotechnology Information (NCBI) was surveyed with the respective *env* nucleotide sequences using the BLAST program [32]. The putative proviruses presumed to encode the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> were mapped to ideogram data of chromosome 8 and ideogram data of chromosome 18, respectively. Both MuLV-ERVs retained the coding potential for *env* polypeptide, and ENV<sub>OV2</sub> also had the coding potential for *pol* polypeptide (Figure 1(c)).

To examine whether the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> are able to produce full-length *env* polypeptides, they were overexpressed in a human cell line followed by Western blot

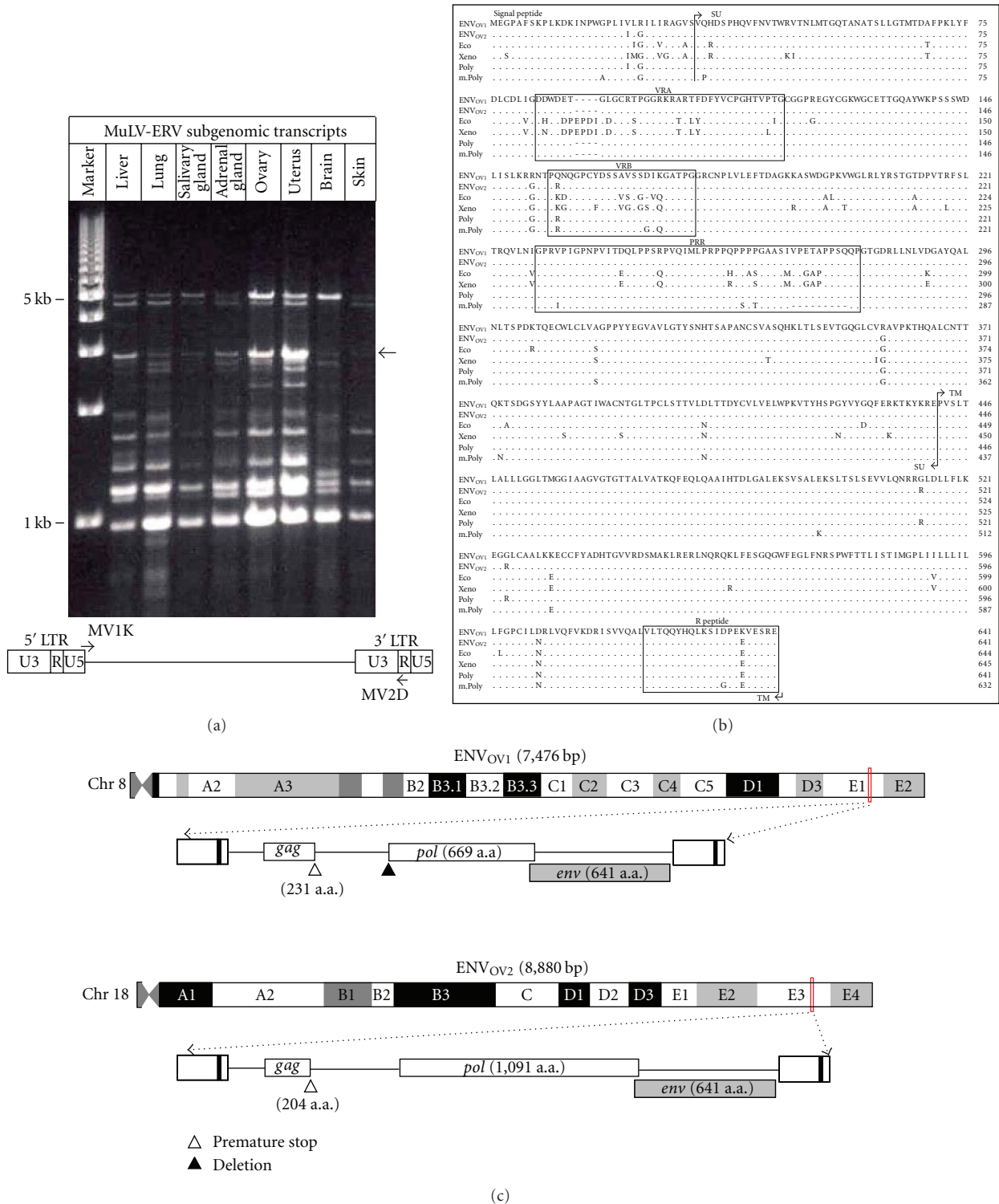


FIGURE 1: Identification of full-length *env* transcripts from the ovary of C57BL/6J mice. (a) A number of MuLV-ERV subgenomic transcripts were expressed in normal tissues (liver, lung, salivary gland, adrenal gland, brain, and skin) of C57BL/6J mice. A schematic diagram indicates the locations of primers used for amplification of the subgenomic transcripts. (b) The amino acid sequences of two intact *env* genes, named ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, which were isolated from the ovary (indicated with an arrow in panel (a)), were compared to reference *env* polypeptides with known tropism traits (GeneBank accession number: AAG39911 (Eco), ACY30460 (Xeno), AAO37283 (Poly), and AAA88318 (m.Poly)). (c) The putative MuLV-ERV proviruses harboring the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> genes were mapped to chromosomes 8 and 18 of C57BL/6J genome, respectively. SU (surface domain), TM (transmembrane domain), VRA (variable region A), VRB (variable region B), PRR (proline rich region), LTR (long terminal repeat), R (repeat), and U (unique region).



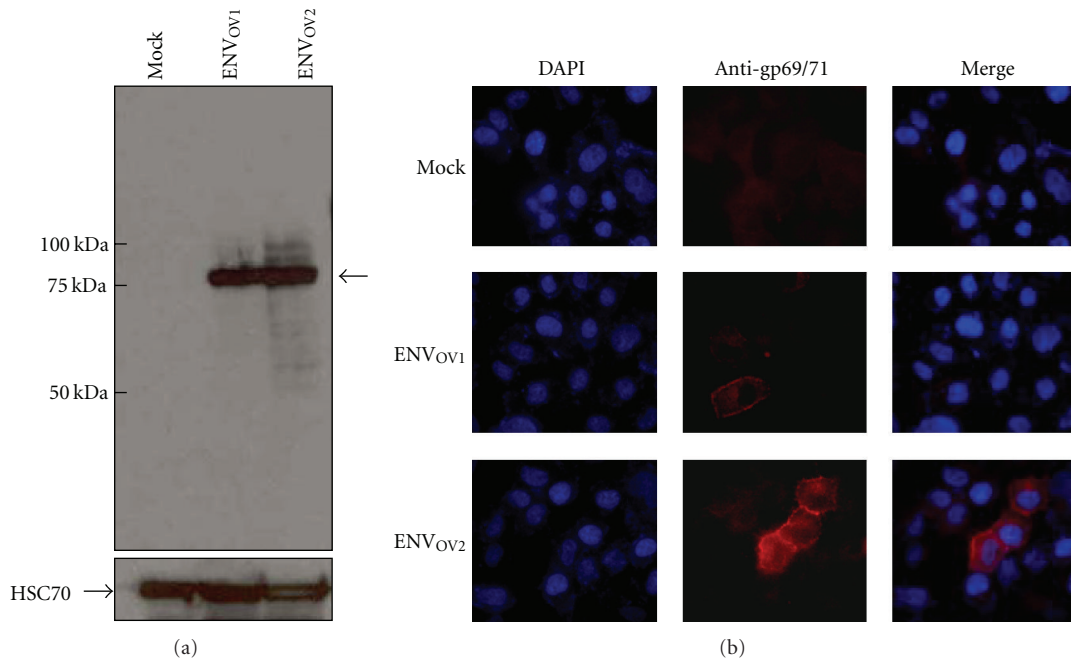


FIGURE 2: Coding potential and membrane localization of ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides. (a) The coding potential of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides was confirmed by overexpression followed by Western blot analysis using antibody against Rauscher MuLV gp69/71 *env* polypeptide. (b) The cellular distribution of the overexpressed ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides was examined by immunocytochemistry using antibody against Rauscher MuLV gp69/71 polypeptide, and their membrane staining pattern was evident. The cells transfected with a blank plasmid serves as a negative control (mock). DAPI (4',6-diamidino-2-phenylindole).

detection using an anti-gp69/71 (*env*) antibody. A protein band of ~75 kDa, which was about the size of MuLV-ERV *env* polypeptides, was detected (Figure 2(a)). Moreover, the subcellular distribution of these *env* polypeptides was examined by transient transfection followed by immunocytochemistry using the same antibody used for the Western blot analysis. Both the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> proteins were evidently expressed on the cell membrane as was expected from the retroviral *env* polypeptides (Figure 2(b)).

**3.2. Infectivity and Tropism Traits of ENV<sub>OV1</sub> and ENV<sub>OV2</sub> Polypeptides.** Two relevant characteristics, tropism and infectivity, of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides were determined using a retroviral packaging system and compared to reference *env* proteins with known host tropisms: ecotropic, amphotropic, and pantropic. Prior to the analyses of infectivity and tropism traits, the packaging potential of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides and release of pseudotype LacZ-MuLV virions were confirmed by detection of ~75 kDa bands in the culture supernatants collected after transfection (Figure 3). Interestingly, a markedly higher level of *env* protein was detected in the supernatants presumed to contain ENV<sub>OV2</sub>-packaged pseudotype virions compared to the ENV<sub>OV1</sub> samples. This finding may suggest that the ENV<sub>OV2</sub> polypeptide is more efficiently produced and/or packaged during the course of virion assembly compared to the ENV<sub>OV1</sub>. The infectivity and tropism traits of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides were then examined by infection of various cell types derived from human, nonhuman primate, mouse, and dog. It revealed that

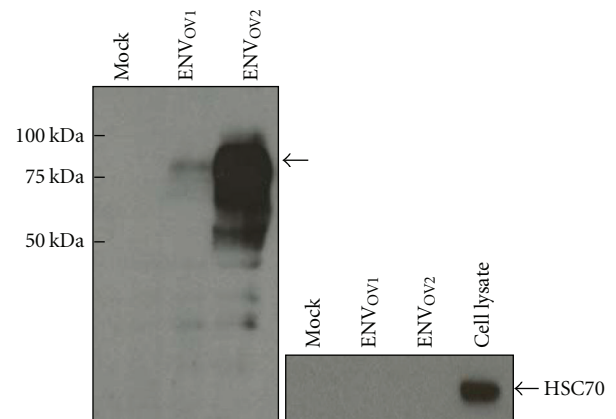


FIGURE 3: Production of pseudotype LacZ-MuLV virions. Presence of the pseudotype LacZ-MuLV virus particles in culture supernatants of the GP2-293 packaging cells was confirmed by detection of the *env* polypeptides using antibody against Rauscher MuLV gp69/71. Arrow indicates the *env* polypeptides. Supernatants collected from cells transfected with a blank plasmid serves as a negative control (mock).

the pseudotype LacZ-MuLV virions packaged with either ENV<sub>OV1</sub> or ENV<sub>OV2</sub> were capable of infecting both mouse as well as nonmouse cells suggesting their polytropic tropism trait, which is consistent with the alignment data presented in Figure 1 (Table 1). While the pseudotype ENV<sub>OV2</sub>-LacZ-MuLV virions demonstrated infectivity that is very similar to

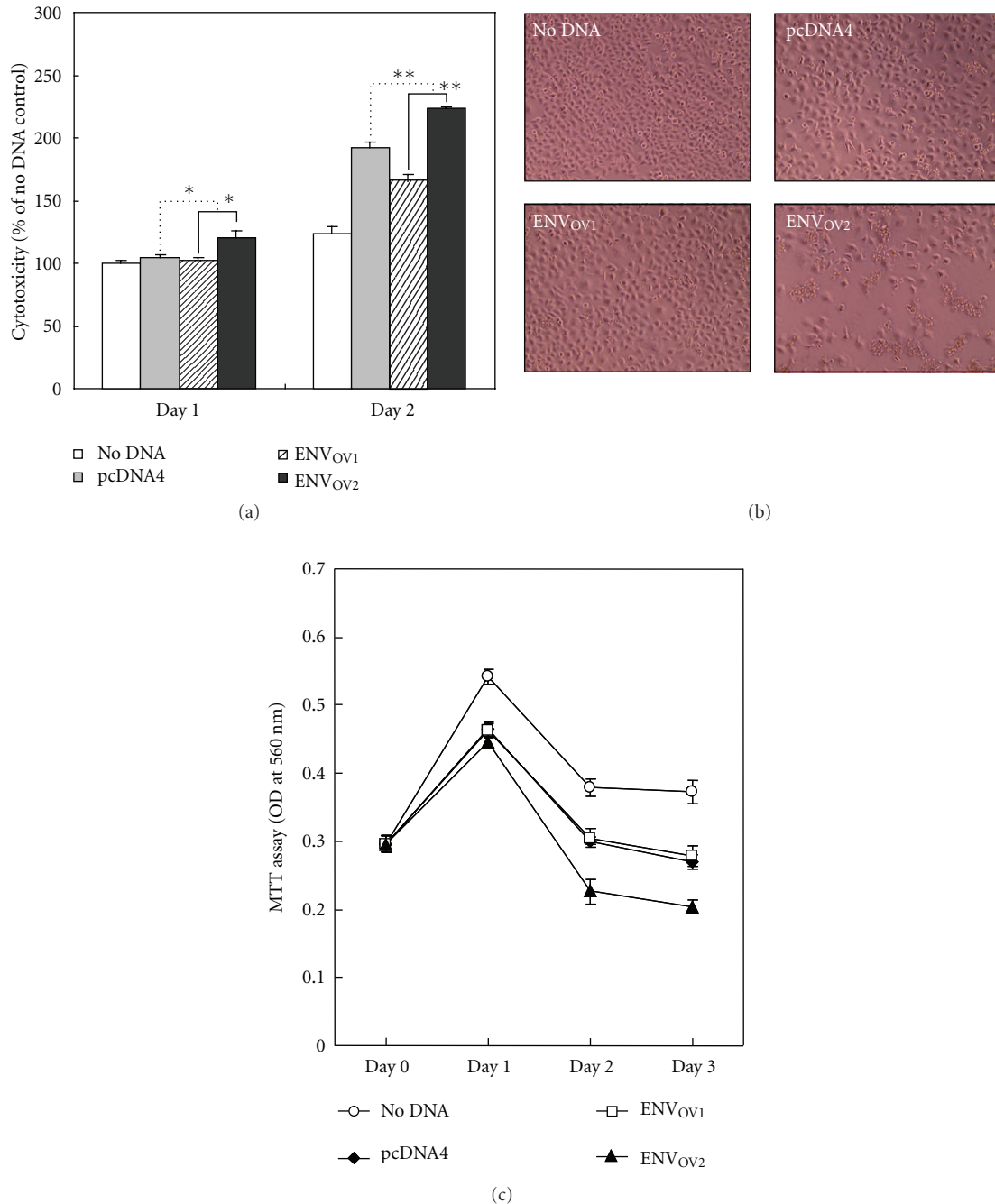


FIGURE 4: Cytopathic effects of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides. (a) and (b) Cytotoxic property of the ENV<sub>OV2</sub> polypeptide, but not ENV<sub>OV1</sub> polypeptide, was observed during cytotoxicity assay by measurement of lactate dehydrogenase release and morphologic examination of cells (200x magnification). The degree of cytotoxicity was normalized to the no DNA (Panel (a)). (c) ENV<sub>OV2</sub> polypeptide's inhibitory effect on cell proliferation was demonstrated by MTT assay, which measures cell viability, following overexpression. All experiments were performed in triplicate. \* and \*\* indicate statistical significance (\* $P < .05$ ; \*\* $P < .01$ ).

the amphotropic and pantropic controls, the ENV<sub>OV1</sub>-LacZ-MuLV virions had substantially low infection titers compared to the controls, probably due to low expression level and/or inefficient packaging potential during virion assembly.

### 3.3. Cytopathic Characteristics of ENV<sub>OV1</sub> and ENV<sub>OV2</sub> Polypeptides.

In this experiment, the cytopathic effects of

the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides were examined by overexpression followed by measurement of cytotoxicity and inhibition of cell proliferation. Cytotoxic property of the ENV<sub>OV2</sub> polypeptide was clearly demonstrated by both colorimetric quantitative assay and microscopic examination of morphological characteristics, including adherence to culture plate (Figures 4(a) and 4(b)). In contrast,

TABLE 1: Tropism trait and infectivity of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides. Infection titer unit (U/mL): number of LacZ positive cells per mL of supernatant containing virus particles. ND: not detectable.

Cell lines	ENV <sub>OV1</sub>	ENV <sub>OV2</sub>	Infection titer (U/mL)			
			pEco	pAmpho	p10A1	pVSVG
Human						
HeLa	$1.6 \times 10^1$	$1.5 \times 10^4$	ND	$2.2 \times 10^4$	$2.2 \times 10^4$	$4.7 \times 10^4$
tsA201	$3.1 \times 10^1$	$2.0 \times 10^6$	ND	$2.8 \times 10^6$	$1.4 \times 10^6$	$8 \times 10^6$
HCT116	$2.0 \times 10^1$	$3.2 \times 10^4$	ND	$2.7 \times 10^4$	$4.0 \times 10^4$	$1.7 \times 10^4$
Nonhuman primate						
COS-1	$4.0 \times 10^1$	$2.0 \times 10^5$	ND	$2.0 \times 10^5$	$3.8 \times 10^5$	$1.2 \times 10^5$
COS-7	$6.9 \times 10^1$	$8.4 \times 10^5$	ND	$1.3 \times 10^6$	$3.2 \times 10^6$	$5.2 \times 10^6$
Mouse						
NIH3T3	$2.0 \times 10^1$	$9.5 \times 10^4$	$4.5 \times 10^5$	$1.7 \times 10^4$	$3.3 \times 10^4$	$1.4 \times 10^4$
Neuro2a	ND	$2.1 \times 10^4$	$4.4 \times 10^3$	$2.7 \times 10^3$	$2.1 \times 10^3$	$4.8 \times 10^3$
Dog						
MDCK	ND	$3.2 \times 10^1$	ND	$3.2 \times 10^2$	$2.6 \times 10^2$	$1.2 \times 10^2$

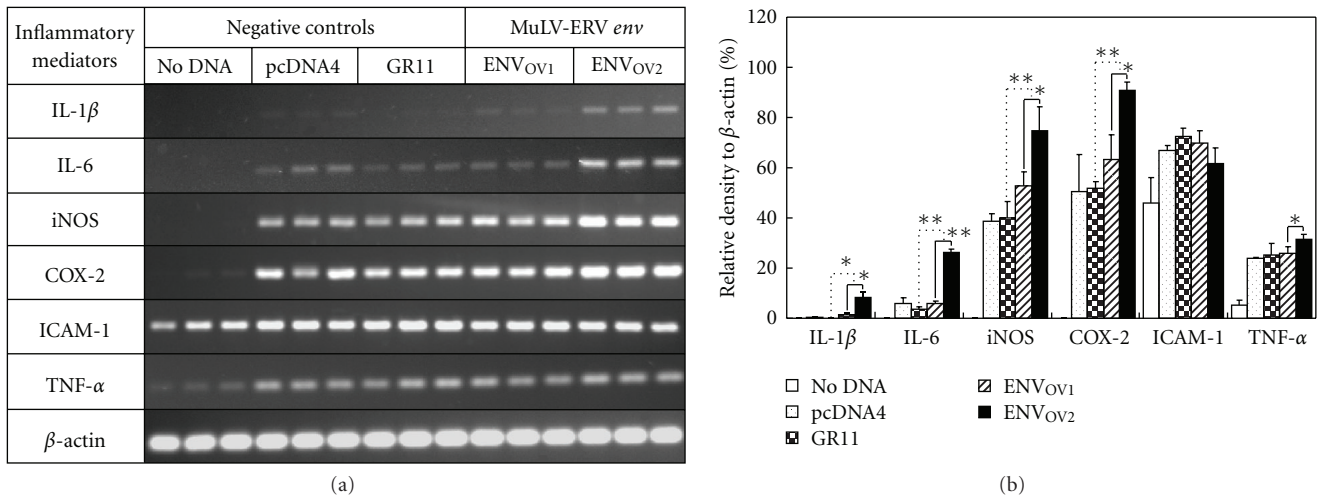


FIGURE 5: Effects of ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides on expression of inflammatory mediators. (a) and (b) The effects of the ENV<sub>OV1</sub> or ENV<sub>OV2</sub> polypeptide in RAW264.7 on the expression of various inflammatory mediators are presented. Differential modulation potentials for inflammatory mediators were observed between ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides. The densitometric value of each inflammatory mediator was normalized to  $\beta$ -actin, and a graph was formulated. Three different forms of negative control were included in this experiment: no DNA, pcDNA4 (blank pcDNA4/HisMax plasmid), and GR11 (similar insert size as ENV<sub>OV1</sub> and ENV<sub>OV2</sub>: mouse glucocorticoid receptor in pcDNA4/HisMax). The assay was performed in triplicate. \* and \*\* indicate statistical significance (\* $P < .05$ ; \*\* $P < .01$ ).

no significant cytotoxic effects were observed in the cells overexpressed with the ENV<sub>OV1</sub> polypeptide compared to negative controls. On the other hand, the overexpression of the ENV<sub>OV2</sub> polypeptide, but not ENV<sub>OV1</sub> polypeptide, evidently inhibited cell proliferation, which was measured by colorimetric quantitation of cell growth (Figure 4(c)). It is likely that inhibition of cell proliferation by the ENV<sub>OV2</sub> polypeptide is linked to its cytotoxic effect, and it is unclear how its high infection titer correlates with the cytopathic characteristics.

**3.4. Modulation of Inflammatory Mediators by ENV<sub>OV1</sub> and ENV<sub>OV2</sub> Polypeptides.** To investigate whether the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> play a role in inflammation, changes in mRNA expression of a set of inflammatory mediators

were surveyed following their overexpression in RAW264.7 alveolar macrophage cells. The set include proinflammatory mediators of COX-2 (cyclooxygenase-2), ICAM-1 (intercellular adhesion molecule 1), iNOS (inducible nitric oxide synthase), IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . The expression of four proinflammatory mediators, COX-2, iNOS, IL-1 $\beta$ , and IL-6, was significantly increased by overexpression of ENV<sub>OV2</sub> polypeptide but not ENV<sub>OV1</sub> (Figures 5(a) and 5(b)). In contrast, no significant changes in ICAM-1 and TNF- $\alpha$  levels were detected following the overexpression of either the ENV<sub>OV1</sub> or the ENV<sub>OV2</sub> polypeptide. The findings from this study suggest that the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides differentially participate in certain signaling events controlling the production of inflammatory mediators.

#### 4. Discussion

Two MuLV-ERV *env* genes with intact coding potential, named ENV<sub>OV1</sub> and ENV<sub>OV2</sub>, were isolated from the ovary of normal C57BL/6J mice and their biological properties were characterized. Although the sequence of one (ENV<sub>OV2</sub>) of the two *env* genes has been reported previously, its biological functions have not been characterized [31]. The findings from this study suggest that both the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides, which were determined to confer polytropic tropism, participate in a range of biological processes, such as retroviral packaging, cell death, proliferation, and inflammation.

The results from this study suggest that putative MuLV-ERVs, or unidentified exogenous retroviruses, which are packaged with either ENV<sub>OV1</sub> or ENV<sub>OV2</sub> polypeptide, are capable of infecting cells of mice as well as other species, such as humans, nonhuman primates, and dogs. *De novo* as well as stress-related activation of the MuLV-ERVs, which are packaged with these *env* polypeptides, may be followed by infection of specific cells of local as well as distant. In addition to the potential cytopathic effects examined in this study, the genomic random integration of the proviral DNAs may be directly linked to various pathogenic outcomes following infection. Further *in vivo* studies are needed to determine infectivity of the MuLV-ERVs packaged with these *env* polypeptides in mice as well as other species.

ERVs have been associated with a range of diseases, such as sepsis, multiple sclerosis, and injury whose central pathology includes inflammatory conditions [12, 33–37]. Some reports provided evidence that certain ERV *env* gene products, but not the relevant virus particles, play a role in the inflammatory processes associated with various pathologic phenotypes [38, 39]. The HERV-W syncytin-1 exerted its inflammatory effects by induction of proinflammatory mediators, such as IL-1 $\beta$ , IL-6, IL-12, iNOS, and TNF- $\alpha$ , leading to neuron inflammation in multiple sclerosis patients [12, 40]. The findings from this study that the ENV<sub>OV2</sub> polypeptide is capable of modulating inflammatory mediators suggest its potential roles in immunologic homeostasis as well as in various diseases involving inflammatory conditions, such as sepsis [41, 42].

A markedly higher level of packaging and subsequent release of pseudotype LacZ-MuLV virions was predicted with the ENV<sub>OV2</sub> compared to the ENV<sub>OV1</sub>, based on the detection of abundant *env* polypeptide in the culture supernatants of ENV<sub>OV2</sub> samples. It is consistent with the finding that the ENV<sub>OV2</sub>-packaged virions (pseudotype ENV<sub>OV2</sub>-LacZ-MuLVs) had higher infection titers compared to the ENV<sub>OV1</sub>-packaged virions (pseudotype ENV<sub>OV1</sub>-LacZ-MuLVs). It is possible that the putative high packaging rate with the ENV<sub>OV2</sub> polypeptide is directly linked to its efficient transcription and/or translation as well as stability. On the other hand, abundant presence of the ENV<sub>OV2</sub> polypeptide in the cytoplasm may explain, at least in part, the characteristics of cytotoxicity and inhibition of proliferation compared to the ENV<sub>OV1</sub>. Throughout the entire coding sequences, nine amino acid residues (V22I, R24G, R158G, Q161R, R362G, G518R, G528R, D608, and K640E) were

different between the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides. Further investigation may be needed to learn the roles of these polymorphic residues in stability as well as pathogenic characteristics, including infectivity, of the ENV<sub>OV1</sub> and ENV<sub>OV2</sub> polypeptides.

#### 5. Conclusions

The findings from this study indicate that certain MuLV-ERV *env* polypeptides, such as ENV<sub>OV2</sub>, may participate in a range of pathophysiologic processes as an envelope of MuLV-ERV virions and/or independently.

#### Acknowledgments

This study was supported by grants from Shriners of North America (no. 86800 to K. Cho and no. 84308 to Y.-K. Lee [postdoctoral fellowship]) and the National Institutes of Health (R01 GM071360 to K. Cho).

#### References

- [1] J. D. Boeke and J. P. Stoye, "Retrotransposons, endogenous retroviruses, and the evolution of retroelements," in *Retroviruses*, J. M. Coffin, S. H. Hughes, and H. E. Varmus, Eds., pp. 343–435, Cold Spring Harbor Press, Cold Spring Harbor, NY, USA, 1997.
- [2] D. J. Griffiths, "Endogenous retroviruses in the human genome sequence," *Genome Biology*, vol. 2, no. 6, article 1017, 2001.
- [3] E. Herniou, J. Martin, K. Miller, J. Cook, M. Wilkinson, and M. Tristem, "Retroviral diversity and distribution in vertebrates," *Journal of Virology*, vol. 72, no. 7, pp. 5955–5966, 1998.
- [4] R. H. Waterston, K. Lindblad-Toh, E. Birney et al., "Initial sequencing and comparative analysis of the mouse genome," *Nature*, vol. 420, no. 6915, pp. 520–562, 2002.
- [5] R. Gifford and M. Tristem, "The evolution, distribution and diversity of endogenous retroviruses," *Virus Genes*, vol. 26, no. 3, pp. 291–316, 2003.
- [6] J. Clausen, "Endogenous retroviruses and MS: using ERVs as disease markers," *International MS Journal*, vol. 10, no. 1, pp. 22–28, 2003.
- [7] K. A. Dunlap, M. Palmarini, M. Varela et al., "Endogenous retroviruses regulate periimplantation placental growth and differentiation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 39, pp. 14390–14395, 2006.
- [8] H. Karlsson, S. Bachmann, J. Schröder, J. McArthur, E. F. Torrey, and R. H. Yolken, "Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4634–4639, 2001.
- [9] V. C. Lombardi, F. W. Ruscetti, J. D. Gupta et al., "Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome," *Science*, vol. 326, no. 5952, pp. 585–589, 2009.
- [10] R. H. Yolken, H. Karlsson, F. Yee, N. L. Johnston-Wilson, and E. F. Torrey, "Endogenous retroviruses and schizophrenia," *Brain Research Reviews*, vol. 31, no. 2-3, pp. 193–199, 2000.



- [11] C. Patience, Y. Takeuchi, and R. A. Weiss, "Infection of human cells by an endogenous retrovirus of pigs," *Nature Medicine*, vol. 3, no. 3, pp. 282–286, 1997.
- [12] J. M. Antony, K. K. Ellestad, R. Hammond et al., "The human endogenous retrovirus envelope glycoprotein, syncytin-1, regulates neuroinflammation and its receptor expression in multiple sclerosis: a role for endoplasmic reticulum chaperones in astrocytes," *Journal of Immunology*, vol. 179, no. 2, pp. 1210–1224, 2007.
- [13] J. M. Antony, G. van Marle, W. Opii et al., "Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination," *Nature Neuroscience*, vol. 7, no. 10, pp. 1088–1095, 2004.
- [14] N. de Parseval, G. Forrest, P. J. W. Venables, and T. Heidmann, "ERV-3 envelope expression and congenital heart block: what does a physiological knockout teach us," *Autoimmunity*, vol. 30, no. 2, pp. 81–83, 1999.
- [15] F. Wang-Johanning, A. R. Frost, B. Jian et al., "Detecting the expression of human endogenous retrovirus E envelope transcripts in human prostate adenocarcinoma," *Cancer*, vol. 98, no. 1, pp. 187–197, 2003.
- [16] F. Wang-Johanning, A. R. Frost, B. Jian, L. Epp, D. W. Lu, and G. L. Johanning, "Quantitation of HERV-K env gene expression and splicing in human breast cancer," *Oncogene*, vol. 22, no. 10, pp. 1528–1535, 2003.
- [17] E. Larsson, A. C. Andersson, and B. O. Nilsson, "Expression of an endogenous retrovirus (ERV3 HERV-R) in human reproductive and embryonic tissues—evidence for a function for envelope gene products," *Uppsala Journal of Medical Sciences*, vol. 99, no. 2, pp. 113–120, 1994.
- [18] F. Wang-Johanning, J. Liu, K. Rycaj et al., "Expression of multiple human endogenous retrovirus surface envelope proteins in ovarian cancer," *International Journal of Cancer*, vol. 120, no. 1, pp. 81–90, 2007.
- [19] J. L. Frendo, D. Olivier, V. Cheynet et al., "Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation," *Molecular and Cellular Biology*, vol. 23, no. 10, pp. 3566–3574, 2003.
- [20] A. Malassiné, S. Blaise, K. Handschuh et al., "Expression of the fusogenic HERV-FRD Env glycoprotein (syncytin 2) in human placenta is restricted to villous cytotrophoblastic cells," *Placenta*, vol. 28, no. 2-3, pp. 185–191, 2007.
- [21] M. Sha, X. Lee, X. P. Li et al., "Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis," *Nature*, vol. 403, no. 6771, pp. 785–789, 2000.
- [22] J. L. Blond, D. Lavillette, V. Cheynet et al., "An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor," *Journal of Virology*, vol. 74, no. 7, pp. 3321–3329, 2000.
- [23] A. Dupressoir, G. Marceau, C. Vernochet et al., "Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 3, pp. 725–730, 2005.
- [24] R. Gong, L. Huang, J. Shi et al., "Syncytin-A mediates the formation of syncytiotrophoblast involved in mouse placental development," *Cellular Physiology and Biochemistry*, vol. 20, no. 5, pp. 517–526, 2007.
- [25] B. C. Cho, J. D. Shaughnessy, D. A. Largaespada et al., "Frequent disruption of the Nfl gene by a novel murine AIDS virus-related provirus in BXH-2 murine myeloid lymphomas," *Journal of Virology*, vol. 69, no. 11, pp. 7138–7146, 1995.
- [26] E. Meylan, J. Curran, K. Hofmann et al., "Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus," *Nature*, vol. 437, no. 7062, pp. 1167–1172, 2005.
- [27] K. Cho, L. K. Adamson, and D. G. Greenhalgh, "Parallel self-induction of TNF- $\alpha$  and apoptosis in the thymus of mice after burn injury," *Journal of Surgical Research*, vol. 98, no. 1, pp. 9–15, 2001.
- [28] Y. K. Lee, A. Chew, H. Phan, D. G. Greenhalgh, and K. Cho, "Genome-wide expression profiles of endogenous retroviruses in lymphoid tissues and their biological properties," *Virology*, vol. 373, no. 2, pp. 263–273, 2008.
- [29] M. Horiuchi, A. Itoh, D. Pleasure, and T. Itoh, "MEK-ERK signaling is involved in interferon- $\gamma$ -induced death of oligodendroglial progenitor cells," *Journal of Biological Chemistry*, vol. 281, no. 29, pp. 20095–20106, 2006.
- [30] S. M. Mount, "A catalogue of splice junction sequences," *Nucleic Acids Research*, vol. 10, no. 2, pp. 459–472, 1982.
- [31] L. H. Evans, M. Lavignon, M. Taylor, and A. S. M. Alamgir, "Antigenic subclasses of polytropic murine leukemia virus (MLV) isolates reflect three distinct groups of endogenous polytropic MLV-related sequences in NFS/N mice," *Journal of Virology*, vol. 77, no. 19, pp. 10327–10338, 2003.
- [32] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, "Basic local alignment search tool," *Journal of Molecular Biology*, vol. 215, no. 3, pp. 403–410, 1990.
- [33] K. Cho, S. Chiu, Y. K. Lee, D. Greenhalgh, and J. Nemzek, "Experimental polymicrobial peritonitis-associated transcriptional regulation of murine endogenous retroviruses," *Shock*, vol. 32, no. 2, pp. 147–158, 2009.
- [34] K. Cho and D. Greenhalgh, "Injury-associated induction of two novel and replication-defective murine retroviral RNAs in the liver of mice," *Virus Research*, vol. 93, no. 2, pp. 189–198, 2003.
- [35] K. Cho, T. Pham, L. Adamson, and D. Greenhalgh, "Regulation of murine endogenous retroviruses in the thymus after injury," *Journal of Surgical Research*, vol. 115, no. 2, pp. 318–324, 2003.
- [36] Y.-K. Lee, A. Chew, L. Fitzsimon, R. Thomas, D. Greenhalgh, and K. Cho, "Genome-wide changes in expression profile of murine endogenous retroviruses (MuERVs) in distant organs after burn injury," *BMC Genomics*, vol. 8, article 440, 2007.
- [37] H. B. Rasmussen, C. Geny, L. Deforges et al., "Expression of endogenous retroviruses in blood mononuclear cells and brain tissue from multiple sclerosis patients," *Multiple Sclerosis*, vol. 1, no. 2, pp. 82–87, 1995.
- [38] A. Rolland, E. Jouvin-Marche, M. Saresella et al., "Correlation between disease severity and in vitro cytokine production mediated by MSR/V (multiple sclerosis associated retroviral element) envelope protein in patients with multiple sclerosis," *Journal of Neuroimmunology*, vol. 160, no. 1-2, pp. 195–203, 2005.
- [39] M. Saresella, A. Rolland, I. Marventano et al., "Multiple sclerosis-associated retroviral agent (MSRV)-stimulated cytokine production in patients with relapsing-remitting multiple sclerosis," *Multiple Sclerosis*, vol. 15, no. 4, pp. 443–447, 2009.
- [40] A. Rolland, E. Jouvin-Marche, C. Viret, M. Faure, H. Perron, and P. N. Marche, "The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses," *Journal of Immunology*, vol. 176, no. 12, pp. 7636–7644, 2006.

- [41] R. C. Bone, "Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation," *Critical Care Medicine*, vol. 24, no. 1, pp. 163–172, 1996.
- [42] Z. Ozbalkan, A. K. Aslar, Y. Yildiz, and S. Aksaray, "Investigation of the course proinflammatory and anti-inflammatory cytokines after burn sepsis," *International Journal of Clinical Practice*, vol. 58, no. 2, pp. 125–129, 2004.