

Comparative Methylation of ERVWE1/Syncytin-1 and Other Human Endogenous Retrovirus LTRs in Placenta Tissues

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Abstract

Human endogenous retroviruses (HERVs) are globally silent in somatic cells. However, some HERVs display high transcription in physiological conditions. In particular, ERVWE1, ERVFRDE1 and ERV3, three proviruses of distinct families, are highly transcribed in placenta and produce envelope proteins associated with placenta development. As silencing of repeated elements is thought to occur mainly by DNA methylation, we compared the methylation of ERVWE1 and related HERVs to appreciate whether HERV methylation relies upon the family, the integration site, the tissue, the long terminal repeat (LTR) function or the associated gene function. CpG methylation of HERV-W LTRs in placenta-associated tissues was heterogeneous but a joint epigenetic control was found for ERVWE1 5'/LTR and its juxtaposed enhancer, a mammalian apparent LTR retrotransposon. Additionally, ERVWE1, ERVFRDE1 and ERV3 5'/LTRs were all essentially hypomethylated in cytotrophoblasts during pregnancy, but showed distinct and stage-dependent methylation profiles. In non-cytotrophoblastic cells, they also exhibited different methylation profiles, compatible with their respective transcriptional activities. Comparative analyses of transcriptional activity and LTR methylation in cell lines further sustained a role for methylation in the control of functional LTRs. These results suggest that HERV methylation might not be family related but copy-specific, and related to the LTR function and the tissue. In particular, ERVWE1 and ERV3 could be developmentally epigenetically regulated HERVs.

Key words: HERV; LTR; methylation; syncytins; placenta

1. Introduction

Human endogenous retroviruses (HERVs) are constitutive elements of the human genome.¹ Now transmitted exclusively in a Mendelian way, they derived through infectious retrovirus integration in

the germ-line DNA and transmission to the offspring, millions of years ago. Different waves of retroviral endogenization and expansion by retrotranspositional events (copying mechanism) led to the formation of at least 31 phylogenetic multicopy families of HERVs, corresponding to over 400 000 HERV copies, dispersed throughout the genome.² Because of the accumulation of mutations, the contemporary HERV families consist of collections of heterogeneous HERV elements, ranging from full-length proviruses [gag, pol and env gene sequences flanked at each extremity

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by a long terminal repeat (LTR)] to isolated LTRs derived from inter-LTR recombination events.

Some families or family members of HERVs are naturally expressed in particular physiological contexts and display physiological functions. This is not only the case in several LTRs active as alternative promoters of cellular genes in placenta^{3,4} but also in proviruses driving the expression of their own retroviral protein, e.g. ERVWE1, ERVFRDE1 and ERV3, three phylogenically unrelated proviruses highly active in the placenta. ERVWE1 belongs to the HERV-W family and contains gag and pol pseudogenes but its envelope gene ORF has been preserved (for review see Gimenez and Mallet⁵). This domesticated envelope, dubbed Syncytin-1, was demonstrated to be involved in trophoblast fusion occurring during placental development⁶ and was also recently proposed to exert anti-apoptotic function⁷ and to induce proliferation.⁸ The ERVFRDE1 provirus from the HERV-FRD family⁹ and the ERV3/envR provirus from the ERV3/HERV-R family¹⁰ also code for functional envelope proteins that probably play a role in placental development, i.e. Syncytin-2 and ERV3 Env, respectively.^{9,11,12} Syncytin-2, which is fusogenic⁹ and immunosuppressive,¹³ appears strictly expressed in placenta.^{11,14} In contrast, ERV3 Env, which inhibits cell proliferation¹⁵ and exhibits immunosuppressive properties,¹³ is transcribed at various levels in almost all investigated tissues.^{11,16,17} Except for these few examples of localized physiological activities, HERV elements appear essentially silent in somatic cells.

HERV silencing is thought to be the result of the methylation of the LTRs (5' LTR, 3' LTR or solo LTR), which, as for infectious retroviruses, contain the regulatory elements and, in particular, the retroviral promoter in the LTR U3-subregion. This epigenetic process, which occurs in mammals on cytosines of 5'CpG dinucleotides, has the property to block the regulatory function of the targeted sequence.^{18,19} It is believed to have evolved primarily as a defense mechanism against the possible deleterious effects of retroelements and repeated sequences.²⁰ Indeed, whole genome methylation studies showed that repeated elements, including HERVs, are globally methylated in normal somatic cells.^{21–23} Methylation-mediated repression of HERV LTRs activity was illustrated *in vitro* by methylation assays on functional HERV-K 5'LTRs.²⁴ Conversely, demethylation-mediated derepression of copies from the HERV-K (HML-2) and HERV-E families was illustrated in cell culture by the use of the demethylating agent 5'-aza-deoxycytidine.^{25,26} *In vivo* HERV transcripts have been detected in numerous pathological situations and predominantly in autoimmune/inflammatory diseases^{26–30} and tumoral contexts.^{31–34} Methylation deregulations are strongly associated with tumors,^{22,23} and interestingly, tumoral transcriptional reactivations

of HERVs have been associated with LTRs hypomethylation for some contexts when compared with normal tissues. This is the case of one HERV-H copy in gastrointestinal cancers,³⁵ several HERV-K members in urothelial carcinomas³⁶ and human primary testicular tumors²⁵ and the HERV-W family in ovarian carcinomas.³⁷

Thus, the impact of DNA methylation in the control of HERV elements seems obvious. However, HERV methylation status in physiological conditions has been less considered. HERV-E LTRs acting as alternative promoters of cellular genes in placenta were shown to be unmethylated in this tissue, but hypermethylated in blood where they do not exert promoter function.³⁸ Likewise, the expression of the domesticated ERVWE1 locus has been correlated with hypomethylation of its 5'LTR in placenta in comparison with other tissues.³⁹ Thus, HERV activity in physiological context seems related to a lack of methylation. The HERV-W family contains ~80 full-length proviruses with flanking LTRs, 200 retrosequences with incomplete LTRs and 400 solitary LTRs.^{40,41} However, in contrast to ovarian carcinoma, expression of HERV-W family in placenta mainly results from the activity of the ERVWE1 locus.⁴² The high and specific placental expression of ERVWE1 has been attributed to the co-optation of its own retroviral promoter, localized in the 5' LTR, with an LTR retrotransposon of MaLR (mammalian apparent LTR retrotransposon) type, acting as a trophoblast-specific enhancer (TSE).^{43–46} Still neither the methylation status of this enhancer LTR nor the methylation status of family related LTRs is known. Similarly, the methylation status of other proviruses producing envelope proteins, like ERVFRDE1 and ERV3, are unknown.

Generally, the features determining HERV methylation status are unknown. In addition, it remains poorly understood whether methylation not only can repress but also regulate HERV activity. Here, we asked whether ERVWE1 5'LTR methylation is related to the HERV family, the LTR role, the LTR-surrounding genomic context, the tissue or the implication of its envelope gene in a physiological process. For this purpose, we investigated and compared the methylation profiles of ERVWE1 5'LTR with ERVWE1 MaLR LTR enhancer, different HERV-W LTRs and the 5'LTRs from HERVs probably involved in placenta development and maintenance. Whether transcriptional activity and methylation were correlated was further addressed using model cell lines.

2. Materials and methods

2.1. Biological samples

Placental tissues from the first trimester legally induced abortions (8–12 weeks of gestation) were

obtained from the Department of Obstetrics and Gynecology at the Broussais, Saint Vincent de Paul and Cochin Hospitals. Second trimester placental tissues were collected at the time of termination of pregnancy at 15 and 25 weeks of gestation (in weeks of amenorrhea) in trisomy 21-affected pregnancies. Fetal Down syndrome was diagnosed by karyotyping of amniotic fluid cells. The indication of amniocentesis was the maternal age. Term placentas were obtained after elective cesarean section from healthy mothers near term with uncomplicated pregnancies. With written informed consent of the pregnant woman, the following samples were collected at term: a fragment of the placenta, umbilical cord fetal blood and maternal blood. The placenta biopsy sample was collected at a depth of 1 cm and at a distance of 8 cm from the edge of the placenta, with the maternal side facing upward. The use of these biological samples was approved by local ethical committee. Villous cytotrophoblastic cells were isolated by sequential enzymatic digestion of chorionic villi from the first trimester, second trimester and term placenta and purified on a discontinuous percoll gradient, as described previously.⁴⁷⁻⁴⁹ These cells were positively stained for cytokeratin 7 at 95%, indicating the cytotrophoblastic origin of the cells. Placental fibroblasts were isolated by prolonged enzymatic digestion from first trimester chorionic villi as in Malassine et al.⁵⁰ Fibroblastic cells (1.25×10^5 cells/mL) were plated on 35-mm plastic dishes (TPP, Switzerland) and cultured for 48 h, and the culture medium was changed daily. These cultured fibroblasts were characterized using immunocytochemistry. It was found that 98% of the cells were vimentin positive and also positive for a monoclonal antibody against specific fibroblast antigen (clone ASO2, Dianova, Hamburg, Germany). Owing to the limited amount of cells, only DNA was extracted.

2.2. Cell lines

Human choriocarcinoma cells BeWo b30 were cultured in F-12K medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) (Invitrogen). Human ovarian carcinoma cells OVCAR-3 were cultured in the RPMI 1460 medium supplemented with 20% FCS (LGC Promochem, Molsheim, France). Human astrocytoma cells 85HG66 were cultured in the DMEM medium (Invitrogen) supplemented with 10% FCS (Invitrogen). Monocytoid cells U937 were cultured in the RPMI 1640 medium containing 10% FCS. All media were supplemented with antibiotics, i.e. 100 IU of penicillin/mL, 100 μ g of streptomycin/mL and 0.25 μ g/mL amphotericin B (Fungizone) (Invitrogen). Cells were maintained in culture until confluence before DNA and RNA extractions.

2.3. Sodium bisulfite modification of genomic DNA

Genomic DNA from confluent cell lines, blood samples and villous placenta section was isolated using the Nucleospin®Blood or Tissue kit following the manufacturer's instructions (Macherey-Nagel). Genomic DNA from cytotrophoblasts and placental fibroblasts was obtained following the overnight proteinase K lysis and phenol extraction procedure.⁵¹ To perform methylation studies, genomic DNA was submitted to sodium bisulfite treatment. This procedure converts all methyl-free cytosines into uracils, whereas methylated cytosines remain unchanged. For each sample, 1 μ g of genomic DNA was mixed with 3 μ g of carrier pUC18 DNA in a final volume of 100 μ L. The mixture was heated for 10 min at 65°C, and the DNA denatured in 20 μ L NaOH 3N for 30 min at 37°C. To convert unmethylated cytosines, denatured DNA was incubated in 50 μ L hydroquinone 0.1M and 350 μ L sodium bisulfite 3M for 16 h at 50°C. Modified DNA was then purified using Wizard DNA Clean-up System (Promega) and eluted in 50 μ L of sterile water. DNA modification was completed by incubation in 5 μ L NaOH 3N for 15 min at room temperature. DNA was washed by precipitation for 30 min at -20°C in 10 μ L of ammonium acetate 5M and 200 μ L of absolute ethanol, followed by centrifugation at 13 000 rpm at 4°C for 10 min. An additional wash was realized by centrifugation in 500 μ L ethanol 70% for 5 min. The DNA pellet was eluted in 30 μ L of sterile water.

2.4. DNA methylation analyses

Strand-specific primers were designed to specifically amplify the HERV LTR copy of interest on bisulfite modified DNA by nested PCR (Table 1). First-round PCR was accomplished in a 50 μ L mixture containing 3 μ L of modified DNA, 0.66 μ M of each primer, 1 mM of each dNTP, 1 U of DyNAzyme polymerase (Fynnzyme), 1 \times Mg-free DyNAzyme buffer (Fynnzyme) and 1.5 mM MgCl₂ (Fynnzyme). PCR cycling conditions were: 5 min at 94°C; 35 cycles as follows: 1 min at 94°C, 2 min at a temperature depending on the primer set (Table 1), 1 min at 72°C; and a final extension at 72°C for 5 min. Nested PCR was performed on 1 μ L of the first PCR products with internal primers in the same reaction mixture conditions. Nested PCR cycling conditions were 5 min at 94°C; 30 cycles as follows: 1 min at 94°C, 1 min at a temperature depending on a primer set (Table 1), 1 min at 72°C; and a final extension at 72°C for 5 min. PCR fragments were gel-extracted, cloned and sequenced using plasmid forward and reverse primers. To determine CpG methylation, and cytosine conversion rate, the sequence of each clone was aligned and compared

Table 1. Primer sets used for determination of HERV LTRs methylation

| Sequence amplified | Primer sequence for HERV LTRs methylation analyzes ^a | | | Length (bp) | Hyb. temp. (°C) |
|-----------------------------|---|--|---|-------------|-----------------|
| MaLR[LTR]–ERVWE1 [5'LTR] | PCR1 | U1 | 5'(-208)-AATTCATCAACATCCATTC | 1036 | 45 |
| | | L1 | 5'(+828)-GGT tt AATATT tt Att tt TTGGA | | |
| | Nested PCR | U2 | 5'(-177)-CTCT ta CCTTCCTACTCTCT taa A | 507 | 51 |
| L2 | | 5'(+330)-AGAGT gt AGTT gt AAGATTTAATAGAGT | | | |
| ERVWE1 [<i>env</i> -3'LTR] | PCR1 | U1 | 5'(-232)- a CT ta AAAACTACAAAT taa A CCCA | 984 | 50.9 |
| | | L1 | 5'(+752)-TG Att TTATGGTTGTGTT Att TT | | |
| | Nested PCR | U2 | 5'(-222)-TACAAAT taa A CCCA A ATa CA | 553 | 59.3 |
| L2 | | 5'(+331)-AGAGT gt AGTT gt AAGATTTAATAGAGT | | | |
| HW_4[5' LTR] | PCR1 | U1 | 5'(-128)-CCAACATCACTAACACAACC | 550 | 55 |
| | | L1 | 5'(+422)-GGGAGT tt AG ta AAGGGG tt TTG | | |
| | Nested PCR | U2 | 5'(-113)-CAACCT ta TT aa CAAAA ta AATT | 428 | 43.9 |
| L2 | | 5'(+315)-AGATTTAATAGAGTGAAA tt AGAG tt tt | | | |
| HW_12[solo LTR] | PCR1 | U1 | 5'(-13)- a CCCAATCCAAT taa A aa | 810 | 45 |
| | | L1 | 5'(+799)-GATGAATGTGTT tt AGGGG tt tt | | |
| | Nested PCR | U2 | 5'(+3)- Aa A aa CA aa ACT aa CT aa ATTCC | 313 | 46.2 |
| L2 | | 5'(+315)-AGATTTAATAGAGTGAAA tt AGAG tt tt | | | |
| ERVFRDE1 [5' LTR] | PCR1 | U1 | 5'(-36)-CA tt TCTATCA aa A aa CCATT | 677 | 54.7 |
| | | L1 | 5'(+641)-GGGGTAGGTTAGTAAG tt AGGA | | |
| | Nested PCR | U2 | 5'(-20)-CCATT aa TT aa CA tt CT ta TT aa | 568 | 50.9 |
| L2 | | 5'(+548)-GT tt TT tt Att tt TAGGGGAAGGTAGA | | | |
| ERV3[5' LTR] | PCR1 | U1 | 5'(-25)-GTTATA tt TTATAGG tt AGTGTATATG | 764 | 53.8 |
| | | L1 | 5'(+739)- aa TCAT taa A aa A aa CCAATCTCC | | |
| | Nested PCR | U2 | 5'(-19)- tt TT tt TA tt AGG tt AGTGTATATGAG tt | 644 | 57.8 |
| L2 | | 5'(+625)- Ta T aa TCTCCCTCT ta ACT | | | |

^aNucleotides in bold and lowercase letters represent changes in primers relative to the genomic sequence due to bisulfite treatment, as follows: **t** is C and **a** is G in the untreated sequence. The position relative to the LTR start is given in parentheses.

with the in silico modified reference sequence (with all cytosines converted into thymines independently of their context). CpG positions were located and the methylation status at each CpG site observed. To prevent false-positive interpretation, only sequences with more than 95% of converted bases (all cytosines out of CpG context) were taken in analyses. Independence of the sequences was determined based either on their within-sample unique methylation pattern and/or on unique non-conversion of cytosines out of CpG context. For some samples, conversion rate was too high to determine the molecular independence of sequences based on unconverted cytosines. In these cases, more than 10 sequences were systematically analyzed to ensure methylation pattern representativity, and results were confirmed on other samples.

2.5. Real-time qRT-PCR

HERV LTR-derived transcript-specific primer sequences were determined using Oligo 6 software (MBI, Cascade, USA) or taken from published work (Table 2). Systems specificity was checked by in silico PCR on the human genome (UCSC Genome Browser: <http://genome.ucsc.edu/cgi-bin/hgPcr>; reference assembly: March 2006).

Total RNA was isolated using the RNeasy Midi Kit including an on-column DNase treatment in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quantity was measured at OD₂₆₀ in a spectrophotometer, and RNA quality was assessed with a Bioanalyzer capillary electrophoresis device (Agilent, Palo Alto, USA). For each sample, 5 µg of total RNA was converted into cDNA in a final volume of 20 µL with random hexamer primers using ThermoScript RT-PCR System for first-strand cDNA synthesis (Invitrogen) following the manufacturer's recommendations. Reverse-transcriptase-free reactions were carried out simultaneously to verify the absence of contaminating genomic DNA.

Real-time PCRs were performed with the Stratagene Mx3005P quantitative PCR system (Stratagene, La Jolla, CA, USA) using the SYBR (with dissociation curve) option. Each quantitative PCR was performed on 10 µL of 100-fold diluted cDNA, in a PCR mix containing 0.2 µM of individual target primers, 10 µL of 2 × Brilliant SYBR Green QPCR Master Mix (Stratagene) and water to reach a volume of 25 µL. Housekeeping genes were analyzed in the same experiment as the target transcripts. The experiments were performed in duplicate and included RT-minus controls. Following a 10 min denaturation at 95°C, gene amplification was performed as follows:

Table 2. Primer sets used for analyses of HERV LTR-derived transcriptional activity

| Region amplified | | Primer sequence for real-time qRT-PCR (5'-3') | Length (bp) | Hyb. temp. (°C) |
|-----------------------------------|---|---|-------------|-----------------|
| ERVWE1- <i>env</i> ^a | f | CCCCATCGTATAGGAGTCTT | 207 | 55 |
| | r | CCCCATCAGACATACCAGTT | | |
| ERVWE1[3'LTR]-U5 | f | GTCCATGTTTCTTACGGCTTG | 402 | 55 |
| | r | GTGTTACTTGGGGGTCCTTG | | |
| HW_4[5'LTR]-U5 | f | GGTTCGTGCTAATTGAGCTG | 167 | 55 |
| | r | ATGGTGGCAAGCTTCTTGTT | | |
| HW_12[solo LTR]-U5 | f | CCATTGCTCCTGCACAGTTA | 99 | 55 |
| | r | TTAGAAGCAGTGGGTCATGGA | | |
| ERVFRDE1- <i>env</i> ^a | f | GCCTGCAAATAGTCTTCTTT | 114 | 55 |
| | r | ATAGGGGCTATCCCATTAG | | |
| ERV3- <i>env</i> ^a | f | CCATGGGAAGCAAGGGAAGT | 141 | 55 |
| | r | CTTTCCCCAGCGAGCAATAC | | |
| 18S | f | TTCGGAAGTGAAGCCATGAT | 101 | 55 |
| | r | TTTCGCTCTGGTCCGCTTG | | |
| HPRT | f | GTGATGATGAACCAGGTTATGACCTTG | 261 | 55 |
| | r | CTACAGTCATAGGAATGGATCTATCAC | | |

^aFrom De Parseval et al.¹¹

40 cycles at 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. Melting curve acquisition was performed within a 55–95°C range, and resulting amplicons were sequenced and analyzed to check for false-positive detection. 18S and HPRT were the most stable housekeeping genes among the samples (variation less than a factor of 4.05 and 4.25, respectively). The geometric mean of their expression average was calculated and used to normalize for differences in the amount of total RNA added to the reaction. The relative quantification of transcripts was then calculated with respect to an external standard calibration curve established using serial dilutions of purified genomic DNA amplification products. The copy number of LTR-derived transcripts was related to 1000 cells, corresponding to ~12.5 ng of total RNA.

2.6. In vitro methylation of ERVWE1 promoter constructs and luciferase assays

To investigate the influence of methylation on the different regulatory regions of the ERVWE1 promoter activity, we used the previously described reporter plasmid pGL3-basic firefly luciferase HW-67/310 (TSE-U3), HW-1/310 (U3 full-length) and HW-90/310 (minimal promoter) constructs.⁴³ Three micrograms of each pGL3-LTR vectors were methylated in a reaction mix containing 4 U of SssI CpG methylase, 1 × NEBuffer 2 and 160 μM S-adenosyl-methionine (SAM) in a final volume of 20 μL (New England Biolabs). The methylation reactions were realized at 37°C and stopped after 30, 60, 120 and 240 min by heat inactivation of SssI at 65°C for 20 min. The negative control (no methylation) underwent the same treatment lacking SAM. To determine the

methylation level of each reaction, 500 ng aliquots were digested with 0.5 U BstUI methylation-sensitive endonuclease (New England Biolabs) in 1 × NEBuffer for 1 h at 60°C, and restriction profiles were analyzed after electrophoresis on a 1% agarose gel. BeWo b30 choriocarcinoma cells at 60–70% confluence were seeded in 12-well plates. Twenty-four hours later, cells were co-transfected with 0.7 μg of pGL3-LTR firefly luciferase plasmid either methylated or not (-SAM) and 35 ng of pRL-TK Renilla luciferase plasmid for internal standardization (Kit Dual-Luciferase® Reporter Assay System, Promega) by use of Lipofectamine Plus (GibcoBRL-Life Technologies, Invitrogen) as recommended by the manufacturer. Cells were lysed 24 h after transfection with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured by using the Dual-Luciferase® Reporter Assay 1000 System (Promega) according to the manufacturer's instructions. Promoter activities were normalized to the internal Renilla luciferase control, i.e. (Firefly luciferase activity/Renilla luciferase activity) × 100. All reporter gene assays were performed in triplicate.

3. Results and discussion

3.1. Methylation of HERV-W LTRs in placenta and placenta-associated tissues

3.1.1. Description of the investigated HERV-W LTRs ERVWE1/Syncytin-1 (Ch7q21.2; AC007566) transcription is regulated by two distinct elements, i.e. the ERVWE1 5'LTR U3 promoter region and an adjacent upstream TSE. This enhancer is located within a MaLR solo LTR within which the

ERVWE1 provirus integrated ~25–40 millions years ago.^{43,46} We chose to analyze together the methylation status of these two co-opted but unrelated LTRs involved in Syncytin-1 regulation, further referred to as MaLR[LTR]–ERVWE1[5’LTR]. As a minimal model to test the family related methylation process hypothesis, three other HERV-W LTRs were studied: ERVWE1[3’LTR], HW_4[5’LTR] and HW_12[solo LTR]. ERVWE1[3’LTR] is the 3’LTR of ERVWE1 provirus and represents topographically and phylogenetically the closest HERV-W LTR relative to ERVWE1[5’LTR]. HW_4[5’LTR] is an another functional (data not shown) 5’LTR belonging to a HERV-W provirus located on chromosome 4 (Ch4p13; AC024 022). HW_12[solo LTR] is a full-length solitary HERV-W LTR localized on chromosome 12 (Chr12q24.32; AC005868), and a cDNA overlapping the 51 bp at the 3’ end of the U5 region was found to have 100% identity with this part of the U5 region (BC038735). These HERV-W LTRs exhibit common but also specific CpG sites. An alignment of the investigated LTR U3 regions with highlighted CpG location is presented in Fig. 1. About 200 bp of the upstream env region from the ERVWE1[3’LTR] and ~100 bp of the upstream region from the HW_4[5’LTR], which contains a MER110 type LTR, were co-amplified with

the respective HERV-W LTRs as controls of genomic environment methylation.

3.1.2. Methylation unequally affects ERVWE1 5’LTR and other HERV-W LTRs The methylation profiles of ERVWE1 and related HERV-W LTRs were investigated in villous placenta and in non-trophoblastic cells composing the placenta, i.e. placental fibroblasts, fetal blood cells and maternal blood cells (Fig. 2).

The phylogenetically related HERV-W LTRs schematized in Fig. 2A were found highly methylated in a majority of the investigated normal tissues (Fig. 2B–E). In blood particularly, the high global methylation of the HERV-W LTRs (from 85% to 98.2% of methylated CpGs) was similar to the methylation range observed for various LTRs of another HERV family, namely HERV-E.³⁸ In placental fibroblast, all LTRs were also highly methylated (76.9–93.9% of methylated CpGs) except for HW_12[solo LTR] whose methylation level was found at its lowest (38.5%).

The villous placenta appeared to be the tissue with the lower level of methylation for all the LTRs except HW_12[solo LTR]. In spite of it, both the methylation levels and the methyl-group distribution within the molecules were different between the LTRs. Thus, CpG

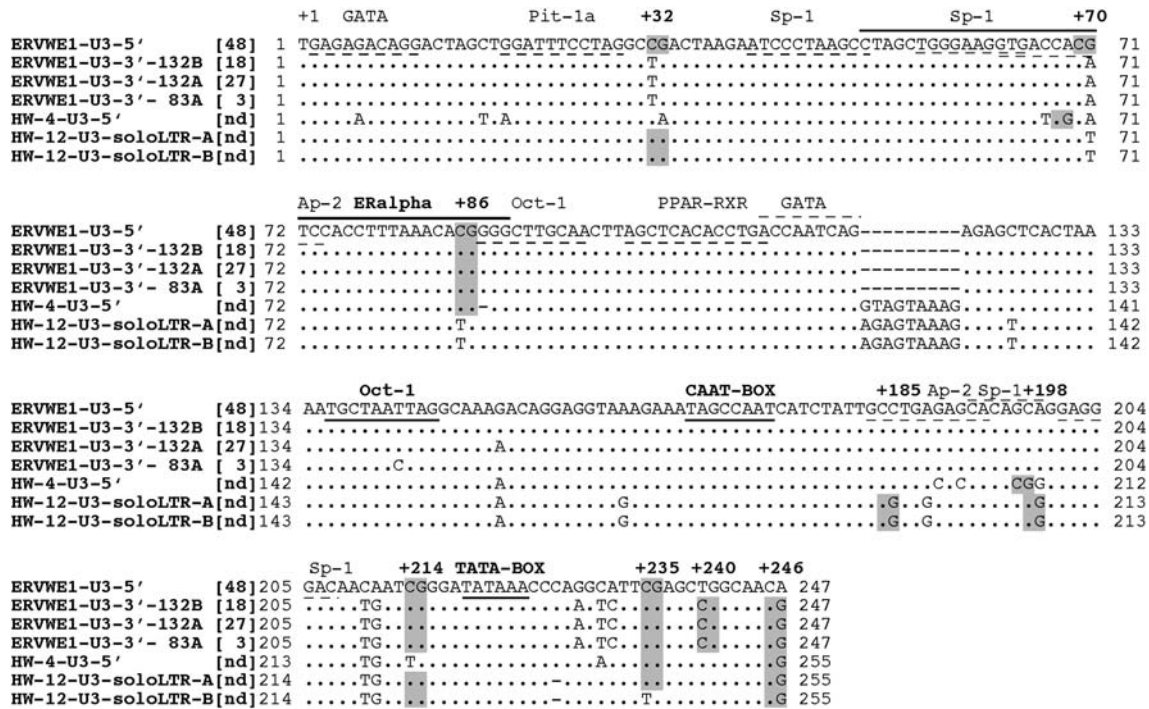


Figure 1. Alignment of HERV-W LTR U3 promoter. ERVWE1[5’LTR] and ERVWE1[3’LTR] U3 region allelic forms were previously determined on DNA amplified from 24 individuals (e.g. -132A, individual 132, allele A), i.e. 48 sequences.⁴⁵ HW_12[solo LTR] and HW_4[5’LTR] allelic forms derive from the annotated genome sequence (-A, reference allele; -B, alternative allele; SNP ID, rs6489086). Proportion of each allelic form is given in hooks (e.g. [3], 3 of the 48 sequences hold this allelic form; [nd], not determined). CAAT box, TATA box and transcription binding sites are indicated. Effective transcription factor binding sites are underlined; putative transcription factor binding sites are marked by interrupted lines. CpG sites are indicated by gray background, their position in the U3 region is given relative to ERVWE1 5’LTR sequence.

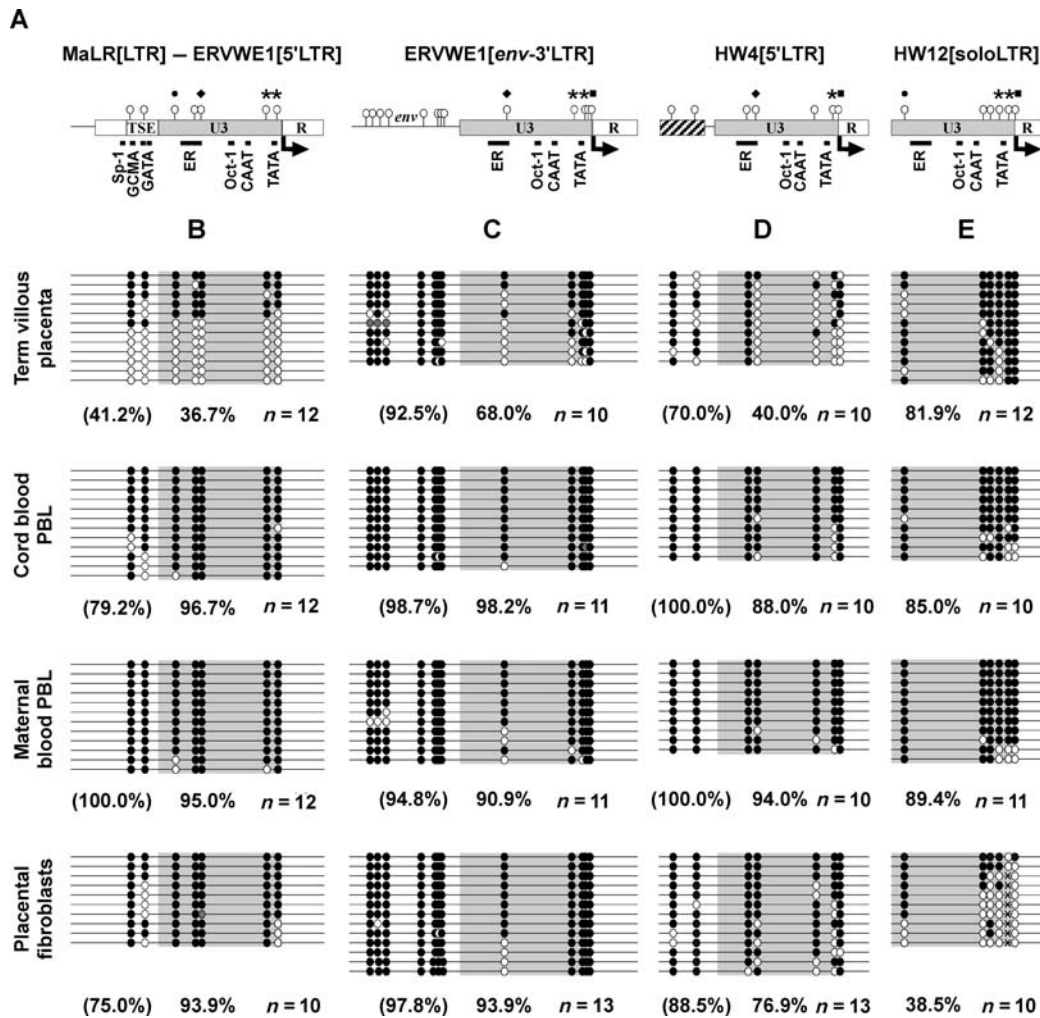


Figure 2. CpG methylation of HERV-W LTRs in placenta-associated tissues. **(A)** Schematic representation of MaLR[LTR]-ERVWE1[5'LTR], ERVWE1[env-3'LTR], HW_4[5'LTR] and HW_12[solo LTR] analyzed regions. LTR regions are represented by boxes and CpG dinucleotides by circles on vertical bars. The U3 region (light gray) constitutes the retroviral promoter, transcription starts at the U3/R boundary (arrow). For each LTR, CAAT and TATA boxes as well as putative and effective transcription factor binding sites proximal to or containing CpG are indicated. Symbols above CpGs point out conserved CpGs between the different LTRs, asterisk indicates CpGs conserved in all three LTRs. TSE, trophoblast-specific enhancer of ERVWE1 provirus, located in the 5' part of the MaLR LTR (white box at the 5' end); *env*, *env*-3'LTR region of ERVWE1 provirus. The MER114 LTR located upstream from HW_4[5'LTR] is also represented (hatched box). **(B-E)** CpG methylation of **(B)** MaLR[LTR]-ERVWE1[5'LTR], **(C)** ERVWE1[env-3'LTR], **(D)** HW_4[5'LTR] and **(E)** HW_12[solo LTR]. Methylation was determined by bisulfite sequencing PCR in villous trophoblast of term placenta, related fetal and maternal blood cells and in placental fibroblasts from chorionic villi of a first trimester placenta. Each sample result originates from the same conversion reaction. Each line represents an independent clone as determined by methylation and/or conversion differences. Methylated CpG are schematized by black circles, unmethylated CpGs by white circles, the mutated CpG (SNP) in HW_12 is represented by a cross and CpGs with undetermined methylation state by gray circles. Global methylation percentages in the U3 regions (highlighted in gray) as well as in the upstream regions (MaLR LTR, *env*, MER114 LTR) are given below the respective area for each sample (in parentheses for non-U3 regions).

methylation level was low for ERVWE1[5'LTR] and HW_4[5'LTR] (~40%), whereas it was relatively high for ERVWE1[3'LTR] (~70%) and even higher for HW_12[solo LTR] (~80%). Methyl-group distribution on ERVWE1[5'LTR] was bimodal, as molecules were either unmethylated (7 out of 12 clones) or densely methylated. This feature, first described by Matouskova et al.,³⁹ appeared to be specific to ERVWE1 promoter. The methylation defaults on the other HERV-W LTRs were more diffusely distributed. Thus, some molecules

were found repeatedly unmethylated on definite CpG sites, solely or together with contiguous CpGs (e.g. the first CpG of ERVWE1[3'LTR] and the second CpG of HW_4[5'LTR]), but no molecule was found fully unmethylated. On the contrary, some other CpG sites were found constitutively methylated (e.g. the first CpG of HW_4[5'LTR], the last CpG of ERVWE1[3'LTR] and the last two CpGs of HW_12[solo LTR]).

Interestingly in this tissue, the CpG site located at the end of the ERVWE1[5'LTR] ER binding site and

conserved in ERVWE1 [3′LTR] and HW_4[5′LTR] was not methylated in approximately the same proportion of clones in both LTRs (e.g. in 7 out of 10 clones and 9 out of 10 clones, respectively). Likewise, the CpG located before the TATA box, conserved in ERVWE1 [3′LTR] and HW_12[solo LTR], was not methylated in 4 out of 10 clones and 4 out of 12 clones. However, other conserved CpGs did not have the same pro-rata of methylation default (e.g. the CpG at the U3/R border was not methylated in 2 out of 10 clones of ERVWE1 [3′LTR] and 7 out of 10 of HW_4[5′LTR] and none of HW_12[solo LTR]), indicating that the ER binding site and TATA-box-associated CpGs might have particular methylation-targeting features.

These results demonstrate that LTRs from the same phylogenetic lineage can be unequally methylated within the same cellular type, suggesting that methylation establishment on HERV sequences might not be family dependent.

3.1.3. Methylation of ERVWE1 LTRs and associated MaLR LTR suggests a regional control By definition, ERVWE1[5′LTR] and ERVWE1[3′LTR] belong to the same proviral unit, but appeared differentially methylated in villous placenta. This unmethylation of the 5′LTR corresponds with its promoter function in placenta, as reported previously.³⁹ The 3′LTR methylation in return may prevent a local competition with ERVWE1 5′LTR for promoter activity as well as a 3′LTR-derived transcriptional interference with the nearby antisense-oriented ODAG/GATAD1 gene.⁴⁶ Such a differential methylation has been observed for HTLV-1⁵² and HIV-1 proviruses during viral latency.⁵³ For these two retroviruses, however, methylation favored repression of the 5′LTR promoter activity as the 5′LTR was methylated and the 3′LTR was not. This suggests different methylation features for exogenous/pathogenic and endogenous/domesticated proviruses but possibly a conserved strategy to prevent methylation spreading on both proviral LTRs, like the use of boundary elements as hypothesized for HTLV-1.⁵² The CTCF protein is a factor able to create boundaries between methylated and unmethylated genomic domains.⁵⁴ Interestingly, two domains containing potential binding sites for CTCF were identified within ERVWE1 provirus (www.essex.ac.uk/bs/molonc/spa.htm), respectively, downstream from the 5′ LTR and upstream from the 3′ LTR region that we found methylated. Further investigations are required to ascertain CTCF functional relevance in the context of proviruses. Nevertheless, it appears that methylation might be regionally controlled and linked either to the genomic environment or to the LTR function.

The upstream flanking sequence of three out of the four HERV-W LTRs was amplified along with the

respective LTRs. Except for one clone in the villous placenta tissue, the MaLR[LTR] directly upstream from ERVWE1 presented the same methylation profiles as the ERVWE1 [5′LTR], i.e. either unmethylated, partially methylated or highly methylated (for methylation values, see Fig. 2B). In contrast, the CpG sites contained within the ~200 bp of the env gene co-amplified with ERVWE1[3′LTR] appeared systematically highly methylated, including those in the villous placenta (with 92.5% of methylation level), despite a lower methylation (68%) of the 3′LTR in this tissue (Fig. 2C). Finally, methylation levels between HW_4[5′LTR] and the upstream MER114 [LTR] part were similar in PBL and placental fibroblasts, but in the villous placenta, HW_4[5′LTR] methylation level was much lower (40%) than the MER114 (70%) (Fig. 2D). Thus, except for ERVWE1[5′LTR] and the upstream MaLR[LTR], there was no clear correlation between the methylation of the HERV-W LTRs and their flanking sequence. Reiss et al.³⁸ also recently concluded that the hypomethylation of HERV-E LTRs acting as placenta promoters of cellular genes, when compared with random LTRs, cannot be explained by a difference in methylation of their flanking sequences, irrespective of its nature—repeated element or not.

The MaLR[LTR] and the ERVWE1[5′LTR] elements have been earlier demonstrated to be co-opted and to specifically control ERVWE1 placental expression.^{43,46} Thus, although belonging to distinct LTR types, their shared unmethylation status in placenta could be linked to their involvement in the regulation of Syncytin-1 transcription. Likewise, the heavy methylation of both LTRs in non-placenta cells supports a repression process outside placenta. Overall, these results are compatible with ERVWE1 env regulation as a *bona fide* gene.⁴⁵

3.2. 5′LTR methylation of HERVs coding for proteins associated with placenta morphogenesis

3.2.1. Description of the placentally expressed HERV included in the study ERVFRDE1 Env (Syncytin-2) and ERV3 Env together with ERVWE1 Env (Syncytin-1) are the three retroviral envelope proteins exhibiting a probable function in placenta development.⁵⁵ Syncytin-2 and ERV3 Env are, respectively, encoded by ERVFRDE1, a provirus belonging to the HERV-FRD family (HERV-FRD_6p24.1; AL136139) and the provirus ERV3 belonging to the ERV3/HERV-R family (HERV-R_7q11.2; AC073210). LTR borders and transcription start sites have already been published for ERVWE1 and ERV3.^{12,43} ERVFRDE1 LTR borders limits were determined using the RepeatMasker software (www.repeatmasker.org). ERVFRDE1[5′LTR] U3/R boundary, delineating the

transcription start site, was located at nt +492 or nt +504 from the [5'LTR] 5' end, using four human placental full-length or 5' end containing mRNAs displaying more than 99% identity with ERVFRDE1 genomic sequence (BC068585, AK123938, AL543429 and CR626159).

3.2.2. *The 5'LTR of placentally expressed HERVs display dissimilar methylation profiles in placenta tissues* We investigated and compared the 5'LTR methylation profiles of ERVWE1,

ERVFRDE1 and ERV3 proviruses (schematized in Fig. 3A), in the same placenta-associated tissues as done for HERV-W LTRs. It appeared that all three 5'LTRs shared a common lack of methylation in villous placenta but under different modalities. In addition, their methylation was widely variable in the non-trophoblastic cells associated with the placenta.

In villous placenta, ERVWE1[5'LTR] had a global methylation level of 36.7%, and molecules were bimodally methylated, as mentioned above. An

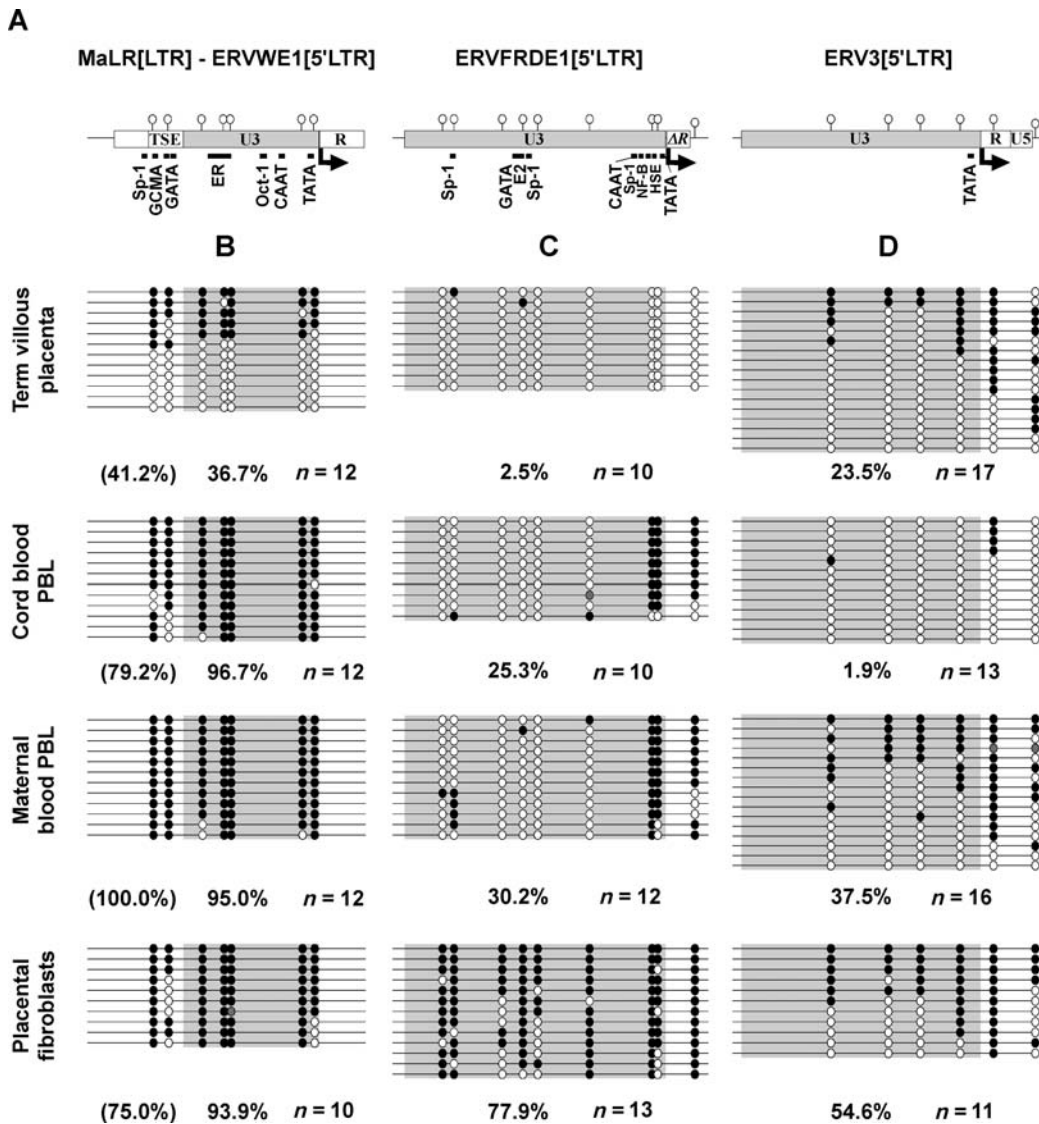


Figure 3. CpG methylation envelope-coding HERV 5'LTRs in placenta-associated tissues. **(A)** Schematic representation of MaLR[LTR]-ERVWE1[5'LTR] (as in Fig. 2A), ERVFRDE1[5'LTR] and ERV3[5'LTR] analyzed regions. LTR regions are represented by boxes and CpG dinucleotides by circles on vertical bars. The U3 region (light gray) constitutes the retroviral promoter, transcription starts at the U3/R boundary (arrow). Putative transcription factor binding sites proximal to or overlapping CpGs are indicated. Downstream horizontal bars are provirus internal sequence. **(B-E)** CpG methylation of **(B)** MaLR[LTR]-ERVWE1[5'LTR] (as in Fig. 1), **(C)** ERVFRDE1[5'LTR] and **(D)** ERV3[5'LTR]. Methylation was determined by bisulfite sequencing PCR in villous trophoblast of term placenta, related fetal and maternal blood cells and in placental fibroblasts from chorionic villi of a first trimester placenta. Each sample result originates from the same conversion reaction. Each line represents an independent clone as determined by methylation and/or conversion differences. Methylated CpG are schematized by black circles, unmethylated CpGs by white circles and CpGs with undetermined methylation state by gray circles. Global methylation percentage values in the U3 regions (highlighted in gray) as well as in the MaLR[LTR] (in parentheses) are given below the respective area for each sample.

important amount of unmethylated molecules (7 out of 12 clones) was observed (Fig. 3B). In contrast, methylation of ERVFRDE1 [5'LTR] was close to zero in the villous placenta, as only two CpGs were methylated in the whole sample (Fig. 3C). Finally, methylation of ERV3 [5'LTR] reached a level of 23.5% and was distributed within three principal patterns (Fig. 3D). Thus, completely unmethylated molecules were found in high proportion (10 clones out of 17), completely methylated molecules were found in very low proportion (2 clones) and the remaining molecules were methylated only at the U3 area borders (5 clones). Although the methylation distribution of ERVWE1, ERVFRDE1 and ERV3 [5'LTRs] was undoubtedly different, an important amount of totally unmethylated clones was found for all three HERV 5'LTRs in agreement with their promoter function and activity in trophoblast.¹¹

In placenta-associated non-trophoblastic cells, i.e. PBLs and placental fibroblasts, ERVWE1 [5'LTR] was found heavily methylated. ERVFRDE1 [5'LTR] was also essentially methylated in placental fibroblasts (77.9%), albeit CpGs 3, 5 and 8 were regularly unmethylated. In fetal cord blood cells on the contrary, the global methylation level of ERVFRDE1 [5'LTR] was only 25.3%. This was due to a quasi-systematic but exclusive methylation on the CpGs neighboring the TATA box. This accurate methylation pattern was conserved in maternal blood cells, although an occasional methylation of the second CpG site slightly increased the methylation level up to 30.2%. ERVWE1 high methylation level in non-trophoblastic cells and ERVFRDE1 high methylation in placental fibroblasts are in agreement with the lack of expression previously established for ERVWE1 and ERVFRDE1 *env* mRNAs in these tissues.^{11,14,50} In spite of this, there is no trivial link between ERVFRDE1 [5'LTR] low methylation level in PBLs and the described weak transcriptional activity of ERVFRDE1 *env* (200-fold less than in placenta).¹¹ One explanation could be that essential transcription factors needed for ERVFRDE1 transcriptional activity are missing in PBL. Nonetheless, another explanation could be related to the precise and accurate methylation at only the CpGs closed to the TATA box. Such a local methylation was observed for the RANKL gene promoter and impaired transcriptional induction by disturbing the attachment of the TATA binding protein.⁵⁶ Contrary to ERVWE1 and ERVFRDE1, methylation patterns of ERV3 [5'LTR] in maternal blood cells and placental fibroblasts were highly similar to those found in term trophoblast. However, the proportion of each pattern and consequently the global methylation level varied depending on the sample type. Thus, the global methylation of the ERV3 5'LTR U3 region reached 37.5% in maternal

blood (with 6 unmethylated clones out of 16) and 54.6% in placental fibroblasts (with 2 unmethylated clones out of 11). The variation in their relative proportion could explain the variable levels of ERV3 *env* transcription among human tissues in general and between placenta and PBL¹¹ in particular. An exception to this trend of ERV3 [5'LTR] generic methylation patterns was the finding of only one methylated CpG (equivalent to 1.9% of total methylation) in the fetal cord PBLs sample. This lack of methylation was all the more unexpected, since the LTRs of all the other HERVs analyzed in this study had a conserved methylation in fetal and adult PBLs. Whether this lack of methylation correlates with significant ERV3 *env* mRNA and protein levels in fetal PBLs deserves further investigation.

In conclusion, methylation status of these LTRs is globally in adequation with their respective promoter activity and could also be implicated in the modulation of their transcriptional level and consequently in the biological activity of their protein.

3.2.3. *Pregnancy-associated 5'LTR methylation changes in HERV coding for envelope proteins in cytotrophoblasts*

ERVWE1, ERVFRDE1 and ERV3 *env* expression is localized in cytotrophoblastic cells of the placenta and is temporally regulated during pregnancy, according to a dynamic specific to each envelope gene. We further investigate whether changes in promoter methylation of the 5'LTR of these HERVs may contribute to the observed transcriptional variations. Analyses were carried out in purified cytotrophoblasts from one normal first trimester placenta (placenta 1.1), trisomic 21 (T-21) second trimester placentas (placentas 2.1 and 2.2) and normal term placentas (placentas 3.1, 3.2 and 3.3) (for clarity, results from one sample for each time of gestation are shown in Fig. 4).

The bimodal methylation pattern previously observed for the ERVWE1 [5'LTR] domain³⁹ and herein shown to equally affect the associated MaLR [LTR] TSE in term villous placenta could have reflected an imprinted regulation of ERVWE1, as was suggested previously.⁵⁷ This process is regularly mediated by the methylation of one of the two alleles in a parent-of-origin manner and often related to genes involved in placenta morphogenesis.⁵⁸ However, the bimodal methylation was not clearly observed at all stages of pregnancy. Thus, in the first trimester placenta, the MaLR [LTR]–ERVWE1 [5'LTR] was not methylated at all (placenta 1.1 Fig. 4B). Among the two second trimester samples derived from T-21 placentas, placenta 2.1 also showed no methylation at all (not shown), whereas placenta 2.2 presented one fully methylated clone out of 12 (Fig. 4B). In contrast, in all the three

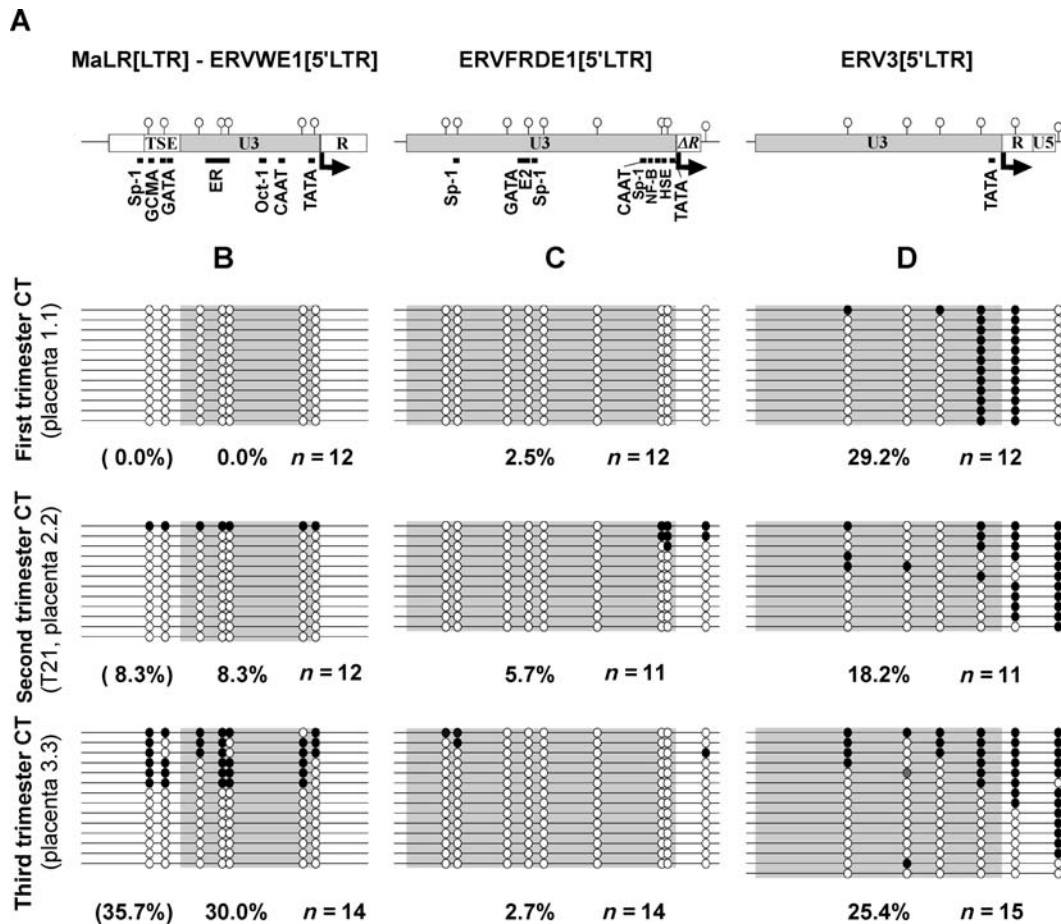


Figure 4. CpG methylation dynamics of envelope-coding HERV 5'/LTRs in cytotrophoblasts during pregnancy. (A) Schematic representation of the MaLR[LTR]-ERVWE1[5'/LTR], ERVFRDE1[5'/LTR] and ERV3[5'/LTR] analyzed regions. (B-D) CpG methylation of (B) MaLR[LTR]-ERVWE1[5'/LTR], (C) ERVFRDE1[5'/LTR] and (D) ERV3[5'/LTR]. Methylation was determined by bisulfite sequencing PCR in cytotrophoblasts (CT) at different times of gestation. One sample is represented here for each trimester i.e. CT of first trimester placenta from legally induced abortion (placenta 1.1), second trimester placenta from trisomy 21-affected pregnancy (T21, placenta 2.2) and term placenta from healthy mother (placenta 3.3). Each sample result originates from the same conversion reaction. Each line represents an independent molecule. Methylated CpGs are schematized by black circles and unmethylated CpGs by white circles.

CT samples derived from term placenta, the bimodal methylation pattern was clearly observed though with a significant sample-to-sample variation in the proportion of the two methylation patterns. More precisely, the number of methylated clones was 1 out of 12 in placenta 3.1 (not illustrated), 5 out of 12 in placenta 3.2 (not illustrated) and 6 out of 14 in placenta 3.3 (Fig. 4B), respectively. In short, the global amount of methylated CpGs in the MaLR[LTR]-ERVWE1[5'/LTR] region varied from 0% in the first trimester placenta to 33.3% at term (as observed in the term placental villi sample). The absence of stability in the bimodal repartition of molecules methylation throughout gestation argues against the proposed imprinting hypothesis. In contrast, the apparent increased frequency of hypermethylated sequences from first trimester to term is consistent with the decrease in ERVWE1 *env* expression observed between 37 and 40 weeks of

gestation.⁵⁹ In addition, the sample-to-sample variations are compatible with the inter-individual variation observed for the ERVWE1 expression level.⁵⁹ Consequently, ERVWE1 *env* mRNA down-regulation in placental dysfunction^{59,60} could be related to an early remethylation of ERVWE1 promoter. The discovery of a single atypical clone exhibiting a methylated MaLR[LTR] and an unmethylated ERVWE1[5'/LTR] in villous placenta (Fig. 3B) may reflect a 'targeted' remethylation process that would deserve further investigation.

Contrary to ERVWE1, ERVFRDE1[5'/LTR] was stably unmethylated in normal first and term cytotrophoblasts as well as in second trimester T-21 cytotrophoblasts, as overall methylation in the samples did not exceed 5.7% (0%, 5.7% and 2.7% in placenta 1.1, 2.2 and 3.3, respectively, see Fig. 4C; and 1.3%, 5.6% and 3.3% in placenta 2.1, 3.1 and 3.3, respectively, not illustrated). This stable unmethylation allows a

constitutive transcriptional activity but cannot account for transcriptional modulations. Thus, ERVFRDE1 *env* mRNA progressive decrease along gestation⁶¹ and down-regulation in T-21 affected placenta⁶² might preferentially be linked to a modulation of the transcription factor pool rather than to alteration of the LTR methylation.

ERV3 [5'LTR] methylation levels [investigated in one representative CT sample for each stage of pregnancy, i.e. placentas 1.1, 2.2 and 3.3 (Fig. 4D)] were somewhat same during all gestation. Hence, in the U3 region, 29.2% of the CpGs were methylated in the first trimester, 18.2% in the second trimester and 25.4% at term. Still, as in ERVWE1, ERV3[5'LTR] methylation pattern did change. However, these changes were not progressive but more drastically linked to the gestation stage, and differences were more particularly obvious between the first and the second trimester. Indeed and interestingly, ERV3[5'LTR] methylation profile in the first trimester was very similar to the one observed for ERVFRDE1[5'LTR] in blood, i.e. precisely and systematically associated with the TATA box. The fact that this precise promoter methylation has been found for the conventional RANKL gene,⁵⁶ and herein for two unrelated HERVs (ERV3 and ERVFRDE1, see Fig. 3C), might indicate an important regulation mechanism and would have to be studied further. ERV3 expression in placenta along gestation has been described sparsely. However, under the same hypothesis as proposed above for ERVFRDE1, this methylation pattern could correlate with the faint or absent ERV3 *env* expression in the first trimester highly proliferative CTs.^{63,64} In the second trimester CT sample, we could observe lower methylation of these CpGs leading to an unmethylated U3 region in 5 of the 11 sequences. In contrast, a new methylation occurred on the first CpG downstream from the LTR on all clones, which was never methylated in the first trimester. Finally, three methylation patterns were observed in term CTs, which were similar to those of the term villous placenta sample. Thus, over the 15 clones analyzed, 8 were completely unmethylated in the U3 area, whereas the others were methylated along the whole U3 region or only on the first and last CpGs. Correlating with ERV3 *env* highest expression,^{63,64} the maximal number of unmethylated ERV3[5'LTR] U3 sequences was found at this stage of pregnancy.

Altogether, these results support the hypothesis that the transcriptional activity of these domesticated proviruses is epigenetically controlled and that methylation might be further involved in the developmentally associated regulation of, at least, ERVWE1 *env* and ERV3 *env* transcriptional levels in placenta and possibly of ERV3 *env* in PBLs. Whether the epigenetic picture observed in the second trimester reflects

the 15–25 weeks of amenorrhea or the T-21 pathological context deserves further investigations.

3.3. Association between HERV LTRs methylation and transcriptional activity

To investigate whether a systematic correlation exists between LTRs methylation status and transcriptional activities, we conducted paralleled transcription and methylation analyses in various cell lines, namely BeWo (choriocarcinoma), U937 (monocytes), 85HG66 (astrocytoma) and OVCAR-3 (ovarian carcinoma). Although the situation appears complex (Fig. 5), we identified essentially four situations.

First, there is a strong correlation between transcription and methylation. Thus, ERVWE1 and ERVFRED1 *env* transcription in placental BeWo cells was correlated with their 5'LTR fully unmethylated status. Similarly, faint transcription (below 2 copies/10 cell) was mostly associated with a high and homogeneous methylation of LTRs (e.g. ERVWE1[5'LTR] in U397 and ERVFRDE1[5'LTR] in OVCAR-3).

Second, comparison of ERVWE1 LTRs and HW_12[solo LTR] suggests that the absence of expression is highly correlated with a huge methylation for functional LTRs (ERVWE1), in contrast to the HW_12[solo LTR] which displays a significant amount of unmethylated U3 regions in 85HG66 and OVCAR-3 cells. This LTR, which exhibits extremely heterogeneous methylation status, is probably not functional; even if we cannot exclude that the lack of related transcription could be attributed to transcription factors paucity.

Third, comparison of ERVFRDE1 and HW_4 in 85HG66 suggests different processes impairing the activity of these functional 5'LTRs. ERVFRDE1 lack of expression is surely due to dedicated transcriptional activators deficit rather than methylation as several molecules are unmethylated. In contrast, HW_4 lack of expression could be due to the systematic methylation of the CpGs at the 5'LTR U3/R boundary.

The fourth situation, concerning ERV3[5'LTR], is more complex. In U937 cells, the *env* region was very poorly expressed, although the 5'LTR had, so to say, no methylated CpGs. Yet, this permissive methylation is compatible with the induction of ERV3 *env* upon U937 stimulation with differentiating agents such as retinoic acid.⁶⁵ In contrast, in 85HG66, BeWo and to a lesser extent in OVCAR-3, we observed a high expression level of the *env* region, but meanwhile a strong methylation level of ERV3[5'LTR]. Analysis of ERV3 *env* containing mRNA using the USCS genome browser (<http://genome.ucsc.edu/cgi-bin/hgTracks>, chr7:64088622–64104466) suggests the existence of an alternative initiation site in addition to the published initiation site in the

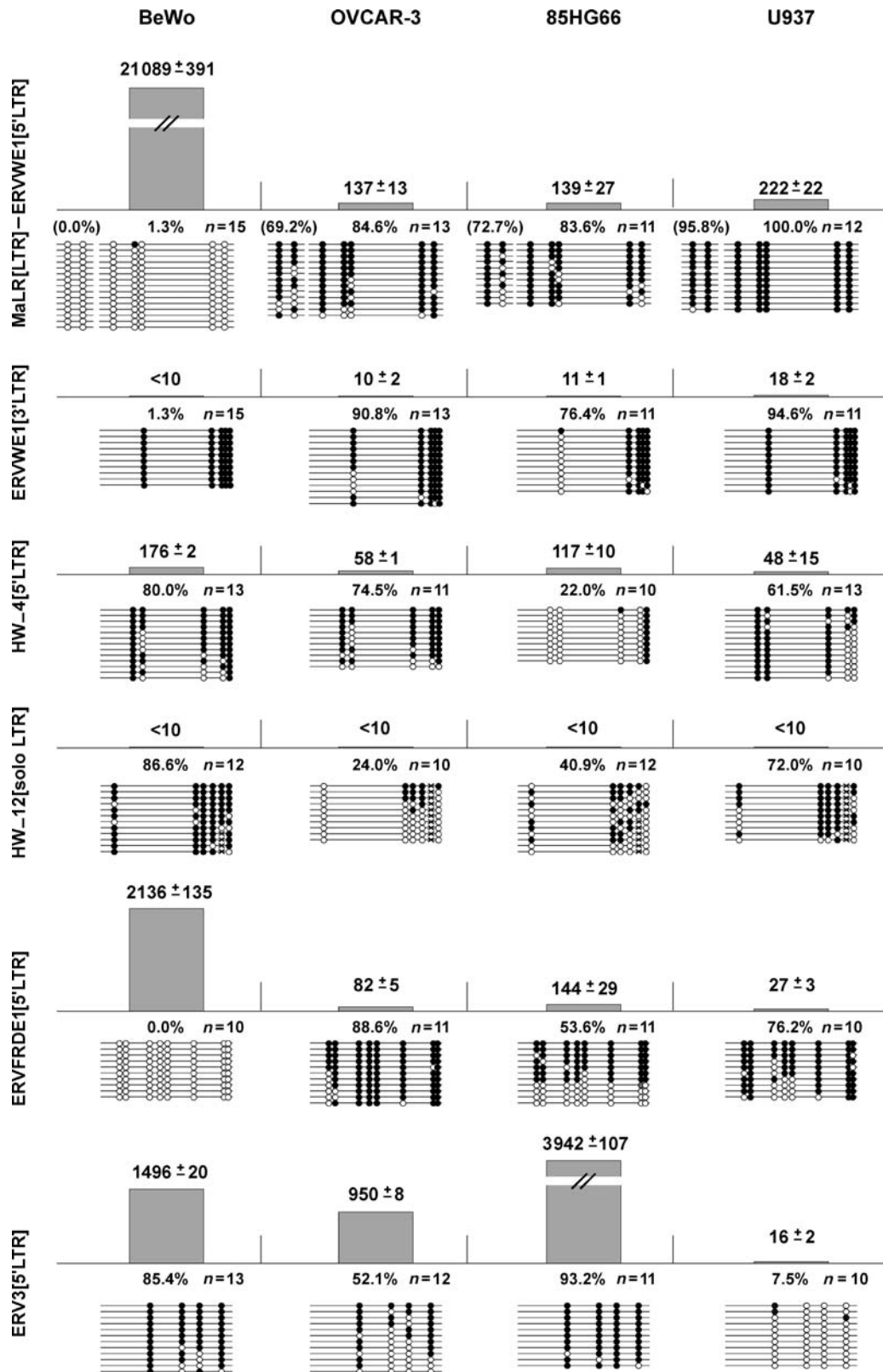


Figure 5. LTR promoter methylation and derived transcriptional activity. LTR-derived transcriptional activity in cell lines is represented by histograms, and the associated methylation profiles of the U3 promoter region of the LTRs are represented underneath. Real-time qPCR values were normalized by the geometric mean of HPRT and 18S housekeeping genes average and are expressed in copy number/1000 cells or 12.5 ng total RNA (numbers on the top of each bar). The associated methylation profiles were determined by bisulfite sequencing PCR. Each sample result originates from the same conversion reaction by bisulfite. Each line represents an independent molecule. Methylated CpGs are schematized by black circles and unmethylated CpGs by white circles. Percentage values express the global methylation level in the U3 promoter region. MaLR[LTR] methylation percentages are shown in parentheses.

ERV3[5'LTR].¹² The mRNA AK295189, which has 99.8% identity with the genomic sequence, notably supports this. It encompasses the full-length ERV3 env region, but not the retroviral sequence ahead, and starts about 7240 bp upstream from the ERV3[5'LTR] transcription start site. It is thus conceivable that in the cell lines 85HG66, BeWo and possibly in OVCAR-3, the observed transcription is related to such an alternate mRNA form. Alternatively although uncommon, we cannot exclude that the ERV3 promoter contains methylation-dependent transcription factor binding sites, as recently described for Epstein-Barr virus.⁶⁶

ERVWE1 is the only known HERV with a juxtaposed enhancer. This TSE is part of a MaLR LTR, and interestingly, all the MaLR CpG sites are located within the TSE. We were thus interested to know the extent to which methylation of the TSE could influence ERVWE1 transcriptional activity. Progressively 5'deleted MaLR[LTR]-ERVWE1[5'LTR] reporter constructs (simplified 'TSE-U3', 'U3 full-length' and 'U3 minimal' promoters, see Fig. 6A) were gradually methylated, transfected in BeWo cell and analyzed for luciferase activity (Fig. 6B). The TSE-U3 promoter activity was the maximal among the three promoter constructs in unmethylated state, but dropped dramatically when methylated. Thus, after 30 min of methylation (incomplete methylation), we observed a 5.4-fold decrease in comparison with only 3- and 1.8-fold for the U3 full-length and minimal promoters. Still, in this state, the TSE-U3 promoter activity remained the highest with 18.4% of the maximal activity, compared with only 10% for the TSE-deleted construct. Further increase in the methylation time to complete methylation reduced the activities to about the same level, i.e. 12.5% for TSE-U3 promoter, 8.5% for the full-length U3 promoter and 7.6% for the minimal U3 promoter. In line with the observed conjoint methylation status of the MaLR[LTR] and ERVWE1[5'LTR], these results underline the importance of the total absence of methylated CpGs on both LTRs to obtain maximal transcription and, conversely the need of a concomitant methylation of both LTRs for an efficient repression.

4. Conclusions

Our analyses provide evidence that methylation status of ERVWE1 and other HERV-W LTRs are not related to a family/phylogenetically related process, nor are they systematically correlated with the integration surrounding area. However, they support both tissue- and locus-specific methylation processes for functional LTRs. Still, methylation status seems to differ in relation to the promoter/enhancer role of

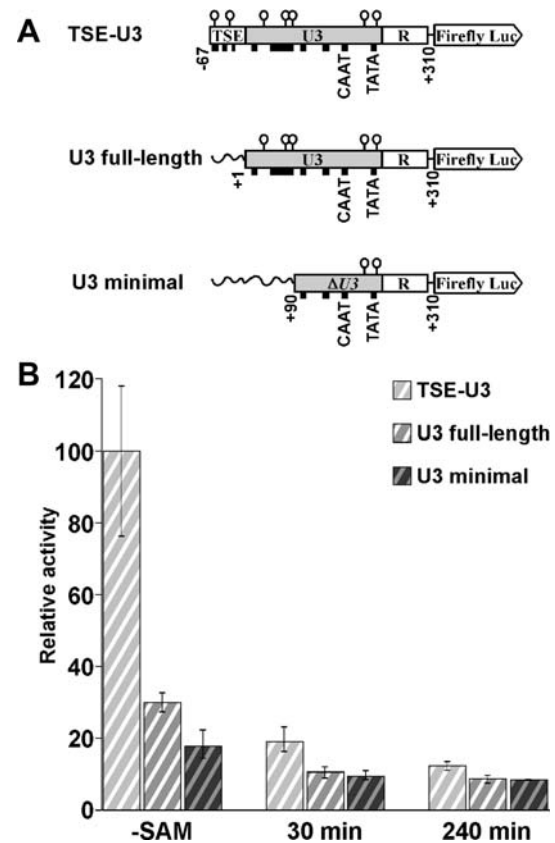


Figure 6. Effect of CpG methylation on MaLR[LTR]-ERVWE1 [5'LTR] promoter strength. **(A)** Schematic representation of TSE-U3, U3 full-length and U3 minimal promoter constructs. Numbering starts from the first position in ERVWE1 5'LTR. Effective and putative transcription factor binding sites associated with the MaLR[LTR] TSE region (Sp-1, c-myc, GCMA, GATA), with the ERVWE1 [5'LTR] U3 5'-subdomain (GATA, Pit-1a, ERalpha, Sp-1, Ap-2 and Oct-1), and CAAT and TATA boxes are indicated by black boxes. CpGs are depicted by circles on vertical bars. **(B)** Relative promoter activities in BeWo cells following *in vitro* methylation. pGL-LTR firefly luciferase plasmids were methylated *in vitro* during 30, 60 and 240 min, with and without -SAM. On the basis of restriction profiles with BstUI enzyme -SAM controls were, as expected, not methylated, and methylation level did not evolve anymore after 60 min indicating a similar methylation at 60 and 240 min. Plasmid methylated during 30 and 240 min and -SAM controls were transfected in BeWo b30 choriocarcinoma cells, which contain all the necessary factors for MaLR[LTR]-ERVWE1[5'LTR] promoter activity. Firefly luciferase activities were normalized to the activity of the co-transfected pRL-TK Renilla luciferase plasmids. The mean and standard deviation from at least three independent experiments are shown and expressed as a percentage of the maximal activity. Promoter activity of the vector backbone represents <0.3% of the larger TSE-U3 construct and <1.3% of the U3 minimal promoter construct (not shown).

the LTR or its use as polyadenylation signal. In addition, our results suggest that methylation of HERV LTRs could be involved in the modulation of their activity. Thus, this could be achieved in several ways, through an enhancer/promoter epigenetic co-regulation (e.g. MaLR[LTR] and ERVWE1 [5'LTR]) or through a variation

in cell proportion with unmethylated LTR (e.g. ERVWE1 [5'LTR] in CTs) or else, by targeting preferential CpG sites such as CpGs closed to the TATA box (e.g. ERVFRDE1 [5'LTR] in PBL, ERV3[5'LTR] in first trimester CTs). Thus, the selective and temporal unmethylation of ERVWE1 [5'LTR] in placenta during the first trimester may allow Syncytin-1-mediated cell differentiation/fusion.⁶ In contrast, increased methylation at term may limit Syncytin-1 production and consequent cell fusion or putative anti-apoptotic protection⁷ in accordance with CT limited fusion and higher apoptosis rate. Syncytin-1 transcriptional alterations observed in placental pathologies^{59,60} could similarly be associated with a temporally deregulated methylation of ERVWE1. Likewise, local methylation of the ERV3 promoter during the first trimester may limit ERV3 Env production and consequent inhibition of early CT proliferation.¹⁵ In contrast, hypomethylation of ERV3 promoter in the second trimester or term placenta and in fetal blood cells may allow ERV3 Env expression and consequently, contribute to immunosuppression.¹³ So, apparently convergent tropism and redundant functions, such as fusion for Syncytin-1 (ERVWE1) and Syncytin-2 (ERVFRDE1) or immunosuppression for Syncytin-2 and ERV3-Env, do not strictly match with similar methylation regulation. Sequence determinants and mechanisms involved in LTR methylation need to be further investigated to better understand HERV regulations and deregulations associated with pathologies.

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