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Yingguang Liu

Liberty University College of Osteopathic Medicine

Tam D. Nguyen

Liberty University College of Osteopathic Medicine

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INTEGRATED REGULATION OF CLASS II HUMAN ENDOGENOUS RETROVIRUSES IN A BREAST CANCER CELL LINE

Yingguang Liu, Liberty University College of Osteopathic Medicine, 306 Liberty View Lane, Lynchburg, VA 24502
yliu@liberty.edu

Tam D Nguyen, Liberty University College of Osteopathic Medicine, 306 Liberty View Lane, Lynchburg, VA 24502
tdnguyen5@liberty.edu

ABSTRACT

Endogenous retroviruses (ERVs) are still regarded as foreign invaders by most biologists. Because of structural and positional homology of ERVs in human and ape genomes, they have been considered molecular evidences of common ancestry. Using a breast cancer cell line, we analyzed the regulatory features of a group of human endogenous retroviruses (HERV-K), and found that they contain multiple sequence motifs subjecting them to regulation by sex hormones, a stem cell-specific transcription factor (OCT4), and DNA methylation. Mutation of the OCT4 motif abrogates their response to sex hormones, while methylation of a progesterone-response element enhances receptor-binding. We also found that solo LTRs of HERVK enable hormonal regulation of downstream cellular genes. The findings support the hypothesis that ERVs are integral parts of eukaryotic genomes and are designed to regulate interspersed genes, especially in reproduction and development.

KEY WORDS

gene regulation, cancer, progesterone, DNA methylation, long terminal repeat

INTRODUCTION

Creationists generally believe viruses were created for purposes beneficial to their hosts, the ecosystem, or mankind, and some became pathogenic only as a result of corruption (Bergman, 1999). Endogenous retroviruses (ERVs) are repetitive DNA elements in eukaryotic genomes that resemble DNA forms of retroviruses. Because humans and apes share similar ERVs at similar genomic locations, and ERV insertions are thought to be largely random, ERVs have been used by evolutionists as strong evidences of common ancestry (Weiss 2006). However, during the past decade, biologists are increasingly convinced that ERVs play important functions for their host organisms, including reproduction and early embryonic development (Grow et al. 2015; Mitchell 2015). Functionality and cellular regulation of ERVs will support the creationist model that ERVs were created in the cell, and homologies between human and ape ERVs are due to functional necessity (Fabich 2015).

In the Seventh ICC, we reported *in vivo* correlation between ERV expression and plasma levels of estradiol and progesterone in reproductive-age women (Mackey et al. 2013). Since then, we have studied the expression of various ERV elements in a human breast cancer cell line, T47D. Because T47D cells have receptors for female sex hormones, they provide an *in vitro* model to analyze the effects of these hormones on cellular and molecular levels. As early as in 1987, it was discovered that transcription of HERVK10 in T47D breast cancer cells is enhanced by a sequential treatment with estradiol and progesterone (Ono et al. 1987). More recent research found that female sex hormones also induce the release of retroviral particles from T47D cells (Patience et al. 1996). The goal of this project was to investigate the mechanisms of hormonal control of HERVs, which should shed light on the functional integration between the HERV elements and the rest of the genome.

MATERIALS AND METHODS

1. Cell lines and culture

T47D breast cancer cell line was a gift from Dr. Joseph Brewer. Cells were maintained in 10% FBS (ATLANTA Biologicals),

2 mM L-glutamine (HyClone), 1X antibiotic antimycotic solution (CORNING) containing penicillin, streptomycin, and amphotericin B, 10 mM HEPES free acid (HyClone), and basal medium RPMI 1640 (HyClone). Primary mammary epithelial cells were obtained from ATCC and were maintained in Mammary Epithelial Cell Basal Medium (ATCC) supplemented with the Mammary Epithelial Cell growth kit (ATCC) containing rH-insulin, L-glutamine, epinephrine, apo-transferrin, rH-TGF α , Extract P, and hydrocortisone hemisuccinate.

2. Extraction of RNA and reverse transcription

Total cell RNA was extracted from cultured cells using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA was prepared using the Quantitect Reverse Transcription Kit (Qiagen). All RNA samples were examined for integrity using electrophoresis. Total cell RNA was incubated with the gDNA Wipeout Buffer in the reverse transcription kit for 10 minutes to eliminate residual genomic DNA.

3. Quantitative PCR

The reference gene, YWHAZ, was selected using the GeNorm and NormFinder programs by comparing the stability of expression of several candidate genes in peripheral leukocytes of women during the menstrual cycle (Mackey et al. 2013). Primers for the reference gene and some HERV genes were also described in the same paper. Primers for Syncytin-2 were designed and validated by Toufaily, et al. (2015). Primers for the human pleiotrophin gene were 5'-GAGCTGAGTGCAAGCAAACC-3' and 5'-TTCAGGGCTGTGTTTCAGGTC-3', whose product and amplification efficiency were verified by electrophoresis and quantitative PCR. Primers for SLC4A8 and IFT172 genes were designed and verified by Gogvadze et al. (2009). We used their "LTR for" as the shared forward primer, and "SLC4A8 for1" and

“IFT172 for6” as the reverse primers. Primers for OCT4 (POU5F1) were purchased from RealTimePrimers.com (Item# VHPS-7107).

Realtime PCR was performed using the LightCycler 96 (Roche) and SsoAdvanced Universal SYBR Green Supermix (Bio-rad). Primer concentrations and cycling conditions were optimized for each pair of primers, but most HERV genes and the reference gene were amplified at 95°C 15”, 57°C 15”, 72°C 45”, 78°C 5” for 40 cycles. Fluorescence were read at 78°C. The OCT4 gene was amplified using a touchdown PCR: 95°C 15”, touch down (with annealing temperatures decreasing from 61°C to 58°C in 30 cycles) 45”, 78°C 5” for 50 cycles.

4. Quantification of mRNA

Quantities of transcripts were calculated using a modified Pfaffl method (Pfaffl, 2001). Amplification efficiencies were determined with standard curves using serial dilutions of cDNA templates. The ratio between the quantity of the target transcript (T) and that of the reference transcript (R) is calculated for each sample as:

$$\frac{T}{R} = \frac{E_R^{Ct_R}}{E_T^{Ct_T}}$$

where:

E_R and E_T are the efficiencies of the primers for the reference and the target genes, respectively. Ct_R and Ct_T are the threshold cycle numbers of the reference and the target genes, respectively.

5. Plasmid and constructs

In order to analyze the response of retroviral LTRs to sex hormones, entire LTRs were cloned into luciferase reporter plasmids. A solo LTR5HS at 12q13.13 between exons 5 and 6 of the SLC4A8 gene that drives expression of antisense RNA (Gogvadze et al. 2009) was amplified from genomic DNA of T47D cells using CloneAmp HiFi PCR Premix (Clontech). The primers were 5'-ACTGAAGAGGTAGGGGCACT-3' and 5'-GCTAGGCATGGGGTTATGAA-3'. Similarly, A reversely oriented solo LTR13A in the first intron of the IPTR3 gene (Inositol 1,4,5-Trisphosphate Receptor Type 3 at 6p21.31, NCBI Reference Sequence NG_027729.1) was amplified using primers 5'-GTGGGGCAGGTGACTATCAA-3' and 5'-GTTGGCACAGGGTGGATTCT-3'. PCR products were verified with restriction fragment analyses. The amplicons were cloned into pMetLuc2-Reporter (Clontech) using the In-Fusion HD Cloning Plus Kit (Clontech), creating pMetLuc2-5HS.

Mutant and wild-type LTR5HS from HERVK-con cloned into pGL3 were kindly donated by Dr. Joanna Wysocka. In the mutant, the OCT4 motif was replaced with a NotI site (Grow et al. 2015).

6. Transfections and reporter assays

pMetLuc2 encodes a secreted Metridia luciferase, while pGL3 encodes an intracellular firefly luciferase under the con. For luciferase reporter assays, T47D cells were seeded in 12-well plates and treated with estradiol (10 nM) for 24 hours followed by a combination of estradiol (10 nM) and progesterone (1 µM) for 15 hours. Immediately after progesterone was added, cells were transfected with 2.4 µg of the reporter plasmid and 2.4 µg of a control plasmid that constitutively expressed a secreted alkaline phosphatase (pSEAP2-Control) or Metridia luciferase (pMetLuc2-

Control). The Xfect Transfection Reagent (Clontech) was used. Activity of secreted Metridia luciferase and alkaline phosphatase were quantified using the Ready-To-Glow™ Dual Secreted Reporter Vector Kit (Clontech). Activity of intracellular firefly luciferase was quantified using the Steady-Luc Firefly HTS Assay Kit (Biotium).

7. siRNA knockdown

Small interfering RNA against the human OCT4 (POU5F1) gene was purchased from Origene, along with the transfection reagent, siTran 1.0. T47D cells were cultured in 12-well plates. siRNA or random control RNA was transfected at 20 nM in the medium using 10 µL of siTran per well. The same wells were transfected twice in consecutive days. Estradiol was added to 10 nM immediately after the second transfection (day 2), and progesterone was added on day 3, 15 hours before harvest. Total cell RNA was harvested 48 hours after the second transfection on day 4. Knockdown efficiency was evaluated with quantitative PCR using primers for OCT4 mRNA.

8. Activation of HERVK in primary mammary epithelial cells

For activating HERVs in primary mammary epithelial cells, cells were cultured in 12-well plates, and treated with decitabine (10 µM) for 2 days, with medium change every day and fresh decitabine added. When cells were 60% confluent, 1 µg of pcDNA₊OCT4 (a gift from Derrick Rossi) was used to transfect each well using siTran 1.0 (Origene) and CombiMag (OZBiosciences). For hormone treatments, estradiol was added to 10-nM after transfection. After 24 hours of estradiol treatment, cells were treated with a combination of estradiol (10 nM) and progesterone (10 nM or 100 nM) for 14-15 hours. Total cell RNA was harvested using RNeasy Plus Mini Kit (Qiagen). Reverse transcription and analysis of the expression of the HERVK10 *env* gene by quantitative PCR were carried out as described above.

9. Electrophoresis mobility shift assay (EMSA)

We performed Electrophoresis Mobility Shift Assays (EMSA) using the Odyssey Infrared EMSA Buffer Kit (LI-COR) to study the interaction between hormones and OCT4 in T47D cells. Crude DNA-free nuclear extract from the T47D breast cancer cell line was used as a source of PR and OCT4. The cells were treated with estradiol (10 nM) for 39 hours. Nuclear extract was prepared using the EpiQuik Nuclear Extraction Kit II (Epigentek). A 27-mer oligonucleotide containing the progesterone-response element (TTAAGGGCGGTGCAAGATGTGCTTTGT, corresponding to nucleotide 186-212 of LTR5HS, with the core sequence underlined) was synthesized and labeled with IRDye700 at the 5' end (IDT). Another version of the probe with the cytosine of the CG pair methylated (TTAAGGGC^mGGTGAAGATGTGCTTTGT) was labelled with IRDye800. A 25-mer oligonucleotide containing the octamer-binding site (ATTGTCTATGATGCAAGACCTTTG, corresponding to 681-705 of LTR5HS, with the octamer-binding site underlined) was synthesized and labelled with IRDye800 at the 5' end. Binding reactions were assembled for a total reaction volume of 20 µL, containing 10 mM Tris, 100 mM KCl, 3.5 mM dithiothreitol, 1 µL of LightShift™ Poly (dI·dC), 500 nM progesterone, 10% glycerol, 50 fmole of each labeled probe, and 4 µg of nuclear extract (unless otherwise indicated). The reaction was incubated at room temperature for 20 minutes. For competition experiments, the unlabeled oligonucleotide was added before

labeled probes and incubated at room temperature for 7-8 minutes. For supershifting, 1 μ L of undiluted mouse monoclonal antiPR (Fischer, MA1412) or rabbit monoclonal anti-OCT4 (Boster, M00174) was added to the reaction and incubated for 20 minutes at room temperature or 10 minutes at room temperature followed by 1 hour on ice. Normal mouse plasma and normal rabbit serum served as controls for mouse antiPR and rabbit anti-OCT4. Concentration of dithiothreitol was reduced to 2 mM for antiPR and mouse plasma, and 1.5 mM for anti-OCT4 and rabbit serum. Polyacrylamide gel electrophoresis was carried out using non-denaturing Mini-PROTEAN TBE Gels (BIO-RAD). Oligonucleotide bands were visualized using an Odyssey CLx scanner.

10. Co-immunoprecipitation and Western blotting

Pierce Co-Immunoprecipitation Kit (Thermo Scientific) was used. Ten micrograms of rabbit anti-OCT4 or rabbit antiPR monoclonal antibodies (Boster) were linked onto AminoLink Plus Coupling Resin. Rabbit serum was used as negative control. Linked antibodies were allowed to react with nuclear extract from T47D cells stimulated sequentially with estradiol and progesterone. After washing resin with phosphate-buffered saline, proteins were eluted, separated with an Any kD™ Mini-PROTEAN® TGX™ Precast Protein Gel, and transferred onto a nitrocellulose membrane using the Mini Trans-Blot® Cell (Bio-Rad). Progesterone receptor proteins were detected using rabbit antiPR and the Enhanced Chemiluminescent Reagent Kit (Boster).

11. Statistical analysis

All quantitative data were analyzed with the Student's t-test. Statistical significance is defined at $P < 0.05$.

RESULTS

1. Female sex hormones enhance HERVK expression in T47D cells through nuclear receptors

Although the T47D breast cancer cell line has been known to express human endogenous retroviruses in response to female sex hormones, the underlying mechanisms of hormonal induction have not been studied (Golan et al. 2008; Patience et al. 1996; Ono et al. 1987).

According to a protocol established by Ono et al., we treated T47D cells with estradiol for 24 hours followed by a combination of estradiol and progesterone treatment for 13-15 hours. Using quantitative reverse-transcription PCR, we analyzed the expression of various HERV elements. Consistent with previous findings, we were able to demonstrate hormonal activation of HERVK genes in the cell line (Fig. 1A and 1B, $P < 0.05$). The effect of progesterone is dose-dependent (Fig. 1C). Either an estrogen antagonist, fulvestrant, or a progesterone antagonist, mifepristone, can block the enhancing effect of the hormones. Because these drugs work on nuclear receptors, it is evident that estradiol and progesterone activate HERVK through nuclear receptors.

Testosterone or cortisol had no effect on the expression of HERVK *env* (data not shown).

Patience et al. (1996) reported that only HERVK10-like sequences were discovered in retroviral particles produced by hormone-stimulated T47D cells. Wang-Johanning et al. (2001) failed to detect expression of ERV3 or HERV-E4-1 in breast tumor tissues. However, well-known HERV elements involved in human

reproduction are mostly class I HERVs, not HERVKs. To study the expression of representative class I HERVs in response to female sex hormones, we quantified the transcripts of syncytin-1 and syncytin-2, which are *env* genes of ERV and ERVFRD, respectively, and the pleiotrophin gene, which is a cellular gene whose expression in trophoblasts is driven by a truncated ERV (Ball et al. 2009; Schulte, et al. 1996). All three genes are known to be involved in placental development, and therefore may be influenced by female sex hormones. Using quantitative PCR, we found appreciable expression of all three genes in T47D cells. Consistent with the findings of Patience et al., expression of none of these genes were altered by hormonal treatments (data not shown).

2. Female sex hormones regulate host gene expression through solo LTRs

Gogvadze et al. (2003) reported that two solo LTRs, both belonging to the LTR5HS subgroup, drove expression of downstream host DNA sequences. Both reside in introns on the noncoding strand, initiating transcription of antisense RNA against the host gene. One of them is located between exons 5 and 6 of a gene named Solute Carrier Family 4 Member 8 (SLC4A8), and the other is located between exons 23 and 24 of a gene called Intraflagellar Transport 172 (IFT172). Quantification of the LTR-driven transcripts in the brain showed a difference between human male and female samples, as well as a difference between the human samples and a chimpanzee sample (Gogvadze et al. 2003).

The T47D cells provide a model to investigate the effect of female sex hormones on gene expression driven by solo LTRs. Using primers designed by Gogvadze et al., we indeed found a stimulating effect of hormonal treatment on LTR-driven expression of downstream host sequences, specifically antisense RNA against exons of SLC4A8 and IFT172 (Fig. 1D, $P < 0.01$ for SLC4A8). Mifepristone, a progesterone receptor antagonist, blocked the effect of female sex hormones completely.

We found in T47D cells that the LTR5HS in SLC4A8 is a stronger promoter/enhancer than the LTR5HS in IFT172 (Fig. 1D). This is interesting because Gogvadze et al. (2009) reported higher expression of the IFT172 antisense RNA in normal testes and seminoma (germ cell tumor of testis) specimens. It indicates that promoter/enhancer activities of different versions of LTR5HS may be tissue-specific and/or gender-specific.

3. LTR5HS drives expression of reporter genes and responds to sex hormones in T47D cells

In order to investigate the molecular mechanisms of the action of steroid hormones on the HERV LTR, we cloned LTR5HS and LTR13A upstream of a luciferase reporter gene. The LTR5HS was amplified from the SLC4A8 intron at 12q13.13, as described previously, while LTR13A was amplified from the first intron of a gene called Inositol 1,4,5-Trisphosphate Receptor Type 3 (IPTR3) at 6p21.31. Upon transfection into T47D cells, we found that estradiol and progesterone upregulated the promoter/enhanced activity of LTR5HS but not that of LTR13A (Fig. 2A). In the absence of progesterone, the effect of both LTRs on the expression of the reporter gene was comparable to that of the vector alone.

Using LTR5HS from HERVK-con (Lee and Bieniasz 2007), Grow et al. (2015) found that the promoter/enhancer activity of

Figure 1. Effect of estradiol and progesterone on HERVK and adjacent genes in T47D cells. **A.** Effect of hormones and antagonists on the expression of HERVK10 *env* gene. Cells were treated with estradiol (10 nM) and progesterone (1000 nM) in the absence or presence of antagonists. Total RNA was reverse transcribed and quantitative RT-PCR was performed using primers targeting the *env* gene of HERVK10 elements. Transcript quantity was normalized against that of the YWHAZ housekeeping gene using the Pfaffl method. * denotes statistical significance compared with untreated control. ** denotes significant difference compared with sample without antagonists. **B.** Similar to A except using degenerate primers targeting the *pol* gene of all HERVK elements. **C.** Similar to A except using a gradient of progesterone concentrations. **D.** Similar to A except using primers targeting transcripts of host DNA downstream of solo LTRs, and the progesterone concentration was 100 nM. Bottom of D show locations of LTRs and PCR primers (adapted from Gogvadze et al. 2003). Quantitative PCR was performed in triplicates or duplicates.

LTR5HS was dependent on an OCT4 motif which is located about 500 bp downstream of the conserved androgen-response element/progesterone response element/glucocorticoid response element (Manghera and Douville 2013). With the plasmids constructed by Grow et al., we demonstrated that responsiveness to sex hormones is completely abolished if the OCT4 motif is deleted (Fig. 2B).

We also discovered a surprising effect of DNA methylation on the responsiveness of LTR5HS to sex hormones. If the plasmids were grown in the Stellar Competent Cells (Clontech), we failed to see the effect of sex hormones on LTR5HS. The above mentioned effects were only visible when the plasmids were grown in a non-methylating strain of *Escherichia coli* (Stellar Competent Cells, dam⁻/dcm⁻). Indeed, we observed a repressive effect of hormones when the OCT4 motif was mutated (Fig. 2C). Even though bacterial methylation of DNA is very different from that of eukaryotic

cells, this prompted us to look into the interplay between DNA methylation and the action of hormones.

4. Knocking down OCT4 reduces expression of HERVK elements

To further investigate the role of OCT4 in activation of HERVK elements, we used specific siRNA to knockdown OCT4 expression in T47D cells. OCT4-specific siRNA reduced the expression of HERVK10 *env* for about 70%, although transfection with random duplex RNA also significantly reduced expression of *env* (Fig. 3A). Similarly, expression driven by the solo LTR in the SLC4A8 gene was inhibited by OCT4 knockdown, although the difference between specific siRNA and random duplex RNA did not reach statistical significance (data not shown).

Figure 2. Effect of estradiol and progesterone on HERVK LTRs in transiently transfected T47D cells. **A.** LTR5HS and LTR13A were cloned upstream of the gene for secreted Metridia luciferase in pMetLuc2 (Clontech). The plasmid construct was cotransfected with pSEAP2-Control that constitutively expresses a secreted alkaline phosphatase. Luciferase activity was normalized against alkaline phosphatase activity. **B.** LTR5HS and LTR5HS-ΔOCT4 were cloned upstream of the gene for firefly luciferase in pGL3 (Promega). The constructs were kindly donated by Dr. Rajini Srinivasan of Stanford University. The constructs were cotransfected with pMetLuc2-Control that constitutively expresses a secreted Metridia luciferase. Intracellular firefly luciferase activity was normalized against the secreted Metridia luciferase. **C.** Similar to B, except the plasmid constructs were harvested from an E coli strain that methylates its DNA, and pSEAP2-Control was cotransfected as an internal control. * denotes statistical significance compared with untreated control. **D.** LTR5HS was gel-purified from methylated and unmethylated pMetLuc2-LTR5HS and subjected to digestion with methylation-sensitive restriction enzymes PspGI (lanes 2 and 3) and BclI (lanes 4 and 5). Lanes 2 and 4 are digests of unmethylated LTR, while lanes 3 and 5 are digests of methylated LTR.

5. DNA demethylation and overexpression of OCT4 activate HERVK expression in primary mammary epithelial cells

It has been long recognized that sex hormones do not stimulate expression of HERV genes in normal mammary epithelium (Wang-Johanning et al. 2003). In T47D cells, we observed a slight but statistically significant stimulating effect of sex hormones on the expression of OCT4 (Figure 3B). So we hypothesized that transfection of primary epithelial cells with an OCT4-expressing plasmid may achieve an effect similar to hormonal treatment of T47D cells. In addition to overexpression of OCT4, we also treated the primary cells with decitabine, an inhibitor of DNA methyltransferase, since DNA demethylation is known to activate HERV-K in HEK293 cells (Grow et al. 2015).

As expected, we found both OCT4 and decitabine enhanced expression of the HERVK10 *env* gene in primary mammary epithelial cells. Moreover, we observed a synergistic effect of the two treatments (Figure 3C, $P < 0.05$).

6. Methylated progesterone-response element (PRE) binds progesterone receptor with higher affinity

The progesterone-response element in LTR5HS contains a CpG pair (nucleotide 193-194). To study the effect of cytosine methylation, double-stranded 27-mer oligonucleotide probes (nucleotide 186-212) were synthesized for electrophoresis mobility shift assays (EMSA). In one version, the cytosine residue of the CpG pair on both strands were methylated and the probe was labeled with IRDye800 at the 5' ends. Another version of the probe, labeled with IRDye700, was not methylated. Because the fluorophores emit light at different wavelengths, binding of progesterone receptors onto both probes can be carried out in the same tubes to compare the binding affinity of each probe.

As can be seen in Fig. 4 A, B, and C, more of the methylated probe was shifted while more of the unmethylated probe remained unbound, indicating that the methylated probe has a higher protein-binding affinity. Protein-binding of both probes could be

Figure 3. OCT4 mediates the effect of progesterone in T47D cells. **A.** Effect of OCT4-specific siRNA and random duplex RNA on the expression of the *env* gene of HERVK10. Cells were treated with female sex hormones in the presence of siRNA against OCT4 or random duplex RNA. Quantitative RT-PCR was carried out in a manner similar to that in Fig. 1. * indicates significant difference compared with random RNA. **B.** Effect of female sex hormones on the expression of OCT4 in T47D cells. Cells were treated with sex hormones and antagonists as described in Fig. 1. OCT4 transcript was quantified with quantitative RT-PCR. *denotes significant difference compared with untreated control cells. **D.** Effect of decitabine treatment and OCT4 overexpression on the expression of HERVK10 *env* in primary mammary epithelial cells. Decitabine was used at 10 μ M for four days. pcDNA_OCT4 was transfected into cells 48 hours prior to RNA harvest. Treatment with female sex hormones was essentially the same as with T47D cells, except progesterone was used at 100 nM. Average of two independent experiments.

competitively inhibited with an unlabeled, unmethylated version of the PRE probe, but the unmethylated probe was more easily competed off, suggesting sequence-specific binding of both probes and the higher-affinity binding of the methylated probe. The irrelevant OCT4 probe did not inhibit protein-binding of the labeled PRE probes to the same extent, further indicating that the interaction between proteins and the PRE probes was sequence-specific.

Protein-binding kinetics demonstrated preferential binding of PR protein to methylated DNA. In this experiment, 50 fmoles of each probe was allowed to react with increasing concentrations of nuclear extract. As shown in Fig. 4 D, the methylated probe bound low concentrations of proteins more tightly, while the unmethylated probe only bound proteins when the concentrations of nuclear

extract were high. A plot of bound fractions of the fluorophores against concentrations of nuclear extract revealed distinct binding kinetics of the two probes (Fig. 4E). The methylated probe approached a second-order polynomial kinetics and was saturated within the range of the experiment, while the unmethylated probe demonstrated a third order polynomial kinetics and was not saturated within the range of the experiment.

To confirm that the shifted band contained progesterone receptors, a mouse monoclonal antibody that binds both isoform A and isoform B of progesterone receptors was used in supershifting experiments. The antibody consistently supershifted the DNA-protein complex, as seen in Fig. 4F. Significantly, a rabbit monoclonal antibody against OCT4 also supershifted the complex, indicating that OCT4 was also in the complex.

Fig. 4. Binding of methylated and unmethylated progesterone-response element (PRE) to progesterone receptors in nuclear extract of T47D cells. A, B, and C compare binding affinities of methylated (green) and unmethylated (red) PRE probes as revealed by competition with an unlabeled PR probe. Numbers above each column indicate the amount of unlabeled PR probe in the reaction. Unlabeled OCT4 probe served as a control for nonspecific binding. D and E show binding-kinetics of the probes using different concentrations of nuclear extract (The probes were **not** combined as in A, B, and C). F shows supershifting using antibodies against OCT4 and PR (See Materials and Methods). Rabbit serum (RS) and mouse serum (MS) served as controls for nonspecific shifting effect.

Figure 5. Co-immunoprecipitation of progesterone receptor and OCT4 transcription factor. Rabbit anti-OCT4 was used to pull down progesterone receptor. AntiPR was used as a positive control and rabbit serum was used as a negative control. Precipitates and flow-through were analyzed by Western Blot using rabbit antiPR as the primary antibody and HRP-conjugated goat anti-rabbit IgG. Signals were detected with enhanced chemiluminescent peroxidase substrate reagents.

7. Co-immunoprecipitation

To further investigate potential interactions between the progesterone receptors and the OCT4 transcription factor, rabbit anti-OCT4 was linked to resin and incubated with nuclear extract from T47D cells that have been treated sequentially with estradiol and progesterone. After washing with phosphate buffered saline, bound proteins were eluted and subjected to Western Blot analysis. As seen in Fig. 5, the rabbit monoclonal anti-OCT4 was able to pull down isoform B of the progesterone receptor (119 KD). The higher molecular weight protein (~220 KD) which was also present in the flow-through but not the serum control was presumably a non-specific signal from the nuclear extract.

DISCUSSION

While there has been some research on the activation of HERVs in early embryos (Grow et al. 2015, for example), our project focuses on hormonal regulation of these elements. Our major findings are: Class II HERVs are regulated by female sex hormones in T47D cells through nuclear receptors; sex hormones regulate cellular sequences through a class of solo LTRs; the effect of progesterone on HERVK expression is partly mediated by OCT4; DNA methylation has an overall effect of suppressing HERVK expression but enhances the binding of progesterone receptors on DNA.

Of particular interest is the requirement of two transcription factor binding motifs in the LTR for progesterone to take effect, the palindromic progesterone-response element (GGTGCAAGATGTTCT) and the OCT4-binding element

(ATGCAAAG). Only LTR5HS of the HML-2 subgroup of HERVK contain both elements. There are two other types of LTRs within HML-2, LTR5a and LTR5b, which do not respond to progesterone because they lack the octamer and have poorly conserved PREs. It seems that only a narrow category of HERMK (HML-2) respond to sex hormones. LTR5HS was originally thought to be human-specific, but a close examination found some LTR5HS elements are shared between human and chimpanzee, and there is at least one chimpanzee-specific solo LTR5HS (Buzdin et al. 2003). All LTR5HS elements should respond to progesterone because their sequences are highly conserved and all contain identical PRE and the octamer. However, they may not affect transcription of downstream sequences, depending on the presence or absence of transcription termination signals.

Our findings offer direct support to the notion that the HERVs were created in the human genome to simultaneously regulate genes that are scattered throughout the genome. In addition, these elements are interwoven into a complex network of regulatory factors in the human body including sex hormones, hormone receptors, and other transcription factors such as OCT4. DNA methylation has been considered a major mechanism by which the cell silences the HERV sequences. While this is still largely true, we just found that methylation of certain cytosine residues within HERVs actually facilitates their upregulation by progesterone. Instead of simply silencing HERVs, some cells seem to have delicate mechanisms to modulate their expression up or down according to their need.

HERVKs are the most intact class of ERVs in the human genome. Based on positional polymorphism within human genomes, transposition of HERVKs may have occurred in the recent past (Belshaw et al. 2005; Hughes and Coffin 2004). We are unclear about the effects of the individual HERVK genes on the cell, but full-length HERVKs may have been designed as units with abilities of regulated expression, moving around in the genome (transposition), packaging into viral particles, and even transmission between cells and hosts. However, most HERVs are so degenerate that some of these abilities may have been lost. The significance of hormone-regulated, LTR-driven transcription of host DNA still awaits further studies.

The role of HERVs in carcinogenesis has been a subject of much speculation (Nelson et al. 2003). Our preliminary findings indicate that activation of HERVs comes after OCT4 expression and DNA hypomethylation, therefore it is more likely a result, rather than the cause, of carcinogenesis. The role of OCT4 in carcinogenesis is still debated, even though it is active in tumor-initiating cells (Wang and Herlyn 2015). Clarification of the potential interactions and collaborations between hormones and OCT4 in breast cancer cells may shed light on fundamental questions in cancer biology.

The indication that progesterone acts partially through OCT4 sheds light on a long-standing puzzle: ERVs are not expressed in normal breast tissues with or without hormonal treatment, but are expressed in some breast cancer cells, especially in response to progesterone (Wang-Johanning et al. 2001). It also explains the association between ERV activity and breast cancer prognosis (Liu et al. 2014; Zhao et al. 2011). In addition, the link between progesterone and OCT4 provides a theoretical basis for the therapeutic effect of

progesterone antagonists/modulators in some breast cancer cases. Further research in this field may improve our understanding of breast cancer pathogenesis, guide treatment of breast cancer with hormones, and even lead to new anticancer approaches based on suppression of OCT4.

If the progesterone receptor prefers methylated DNA, or mediates different effects when bound to methylated and unmethylated DNA, it will affect expression of ERVs in germline cells and during early embryonic development, when DNA is demethylated and remethylated. The finding will also have profound implications in hormone physiology and hormone therapies, as demethylating agents may affect the potency of progesterone receptor modulators. Additionally, oncologists will have to watch for consequences of endocrine dysregulation and HERV activation when demethylating agents are used in chemotherapy.

CONCLUSION:

Coordinated regulation of human endogenous retroviruses and solo LTR elements suggest that they were designed to mediate the action of hormones and/or other physiological signals to regulate expression of interspersed genes. Interactions of ERV elements and DNA methylation enzymes, hormone receptors, and other transcription factors such as OCT4 indicate that HERVs are integral parts of the human genome. At least some of them must have been created at the beginning of mankind. Structural and positional homology of HERVs between humans and animals are more likely due to functional necessity rather than common descent.

REFERENCES

- Ball, M., M. Carmody, F. Wynne, P. Dockery, A. Aigner, I. Cameron, J. Higgins, S.D. Smith, J.D. Aplin, and T. Moore. 2009. Expression of pleiotrophin and its receptors in human placenta suggests roles in trophoblast life cycle and angiogenesis. *Placenta* 30, no. 7:649-653.
- Belshaw, R., A. L. A. Dawson, J. Woolven-Allen, J. Redding, A. Burt, and M. Tristem. 2005. Genomewide screening reveals high levels of insertional polymorphism in the human endogenous retrovirus family HERV-K(HML2): implications for present-day activity. *Journal of Virology* 79, no. 19: 12507-12514.
- Bergman J. 1999. Did God make pathogenic viruses? *Journal of Creation* 13, no. 1:115-125.
- Buzdin, A., S. Ustyugova, K. Khodosevich, I. Mamedov, Y. Lebedev, G. Hunsmann, and E. Sverdlov. 2003. Human-specific subfamilies of HERV-K (HML-2) long terminal repeats: three master genes were active simultaneously during branching of hominoid lineages. *Genomics* 81, no. 2:149-156.
- Fabich, A. 2015. Do endogenous retroviruses (ERVs) support common ancestry? Retrieved February 16, 2018 from <https://answersingenesis.org/biology/microbiology/endogenous-retroviruses-common-ancestry/>
- Gogvadze, E., E. Stukacheva, A. Buzdin, and E. Sverdlov. 2009. Human-specific modulation of transcriptional activity provided by endogenous retroviral insertions. *Journal of Virology* 83, no. 12:6098-6105.
- Golan, M., A. Hizi, J. H. Resau, N. Yaal-Hahoshen, H. Reichman, I. Keydar, and I. Tsarfaty. 2008. Human Endogenous Retrovirus (HERV-K) Reverse Transcriptase as a Breast Cancer Prognostic Marker. *Neoplasia* 10:521-533.
- Grow, E. J., R. A. Flynn, S. L. Chavez, N. L. Bayless, M. Wossidlo, D. J. Wesche, L. Martin, C. B. Ware, C. A. Blish, H. Y. Chang, R. A. Reijo Pera, and J. Wysocka. 2015. Intrinsic retroviral reactivation in human

- preimplantation embryos and pluripotent cells. *Nature* 522:221-225.
- Hughes J. F. and J. M. Coffin. 2004. Human endogenous retrovirus K solo-LTR formation and insertional polymorphisms: Implications for human and viral evolution. *Proceedings of the National Academy of Sciences of the United States of America* 101, no. 6:668–1672.
- Lee, Y. N. and P. D. Bieniasz. 2007. Reconstitution of an infectious human endogenous retrovirus. *PLoS Pathogens* 3, no. 1:e10.
- Liu, T., B. Sun, X. Zhao, Y. Li, Q. Gu, X. Dong, and F. Liu. 2014. OCT4 expression and vasculogenic mimicry formation positively correlate with poor prognosis in human breast cancer. *International Journal of Molecular Sciences* 15:19634-19649.
- Mackey, E., O. Mueller, D. Chadwick, B. Thompson, P. Taylor, and Y. Liu. 2013. Transcription of human endogenous retroviruses during the menstrual cycle suggests coordinative hormonal regulation. In *Proceedings of the Seventh International Conference on Creationism*, ed. M. Horstemeyer. Pittsburg, PA: Creation Science Fellowship.
- Manghera, M., and R. N. Douville. 2013. Endogenous retrovirus-K promoter: a landing strip for inflammatory transcription factors? *Retrovirology* 10:16.
- Mitchell, E. 2015. Endogenous retroviruses: key to mammalian brain development? Retrieved February 16, 2018 from <https://answersingenesis.org/biology/microbiology/endogenous-retroviruses-key-to-mammalian-brain-development/>.
- Nelson, P. N., P. R. Cargenie, J. Martin, H. Davari Ejtehad, P. Hooley, D. Roden, S. Rowland-Jones, P. Warren, J. Astley, and P. G. Murray. 2003. Demystified Human endogenous retroviruses. *Molecular Pathology* 56:11-18.
- Ono, M., M. Kawakami, and H. Ushikubo. 1987. Stimulation of expression of the human endogenous retrovirus genome by female steroid hormones in human breast cancer cell line T47D. *Journal of Virology* 61:2059-2062.
- Patience, C., G. R. Simpson, A. A. Colletta, H. M. Welch, R. A. Weiss, and M. T. Boyd. 1996. Human endogenous retrovirus expression and reverse transcriptase activity in the T47D mammary carcinoma cell line. *Journal of Virology* 70, no. 4:654–2657.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29:e45.
- Schulte, A. M., S. Lai, A. Kurtz, F. Czubyko, A.T. Riegel, and A. Wellstein. 1996. Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ-line insertion of an endogenous retrovirus. *Proceedings of the National Academy of Sciences of the United States of America* 93, no. 25:14759–14764.
- Toufaily, C., A. G. Lokossou, A. Vargas, É. Rassart, and B. Barbeau. 2015. A CRE/AP-1-like motif is essential for induced syncytin-2 expression and fusion in human trophoblast-like model. *PLoS One* 10, no. 3:e0121468.
- Wang Y-J and M. Herlyn. 2015. The emerging roles of Oct4 in tumor-initiating cells. *American Journal of Physiology - Cell Physiology* 309: C709–C718.
- Wang-Johanning, F., A. R. Frost, G. L. Johanning, M. B. Khazaeli, A. F. LoBuglio, D. R. Shaw, and T. V. Strong. 2001. Expression of human endogenous retrovirus K envelope transcripts in human breast cancer. *Clinical Cancer Research* 7, no. 6:1553-1560.
- Wang-Johanning, F., A. R. Frost, B. Jian, L. Epp, D. W. Lu, G. L. Johanning. 2003. Quantitation of HERV-K *env* gene expression and splicing in human breast cancer. *Oncogene* 22:1528–1535.
- Weiss, R. 2006. The discovery of endogenous retroviruses. *Retrovirology* 3:67.
- Zhao, J., Ki. Rycaj, S. Geng, M. Li, J. B. Plummer, B. Yin, H. Liu, X. Xu, Y. Zhang, Y. Yan, S. A. Glynn, T. H. Dorsey, S. Ambs, G. L. Johanning, L. Gu, F. Wang-Johanning. 2011. Expression of human endogenous retrovirus type K envelope protein is a novel candidate prognostic marker for human breast cancer. *Genes & Cancer* 2:914-922.

THE AUTHORS

Yingguang Liu holds a Bachelor of Medicine from Shandong Medical University, a Master of Medicine from Shanghai Medical University, and a Doctor of Philosophy from Ohio University. He was a professor of science in Maranatha Baptist University and is currently an associate professor of microbiology in Liberty University College of Osteopathic Medicine.

Tam Nguyen earned his BS in chemistry from the University of Notre Dame and is currently a second-year medical student at Liberty University College of Osteopathic Medicine.