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TRANSCRIPTION OF HUMAN ENDOGENOUS RETROVIRUSES DURING THE MENSTRUAL CYCLE SUGGESTS COORDINATED HORMONAL REGULATION

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ABSTRACT

Scattered among human and animal genomes are a class of repetitive genetic elements called endogenous retroviruses (ERVs), which are generally considered remnants of ancient viral infections. Because humans and chimpanzees share ERVs at similar genomic positions, evolutionists use these elements as another argument for common ancestry. From a creationist perspective, ERVs may have been created in strategic locations of the genome to perform essential functions, such as synchronized regulation of interspersed genetic elements. Since some human endogenous retroviruses (HERVs) contain putative steroid hormone-response elements, it would be reasonable that expression of such HERVs would be controlled by sex hormones, and might even demonstrate temporal patterns during the female menstrual cycle. Accordingly, we quantified the transcription dynamics of multiple HERV elements in peripheral blood leukocytes using SYBR Green-based RT-PCR in male and female human subjects. Preliminary data indicated that expression of HERVs indeed followed a temporal pattern in females. Moreover, transcription activity of ERV genes was strongly correlated with blood levels of progesterone. The same pattern was demonstrated for HERV-K elements and the syncytin-1 gene encoded by ERVWE1. These results suggest coordinated regulation of some ERV elements by progesterone in the female.

INTRODUCTION

> "If Charles Darwin reappeared today, he might be surprised to learn that humans are descended from viruses as well as from apes. Some 8% of human DNA represents fossil retroviral genomes... Darwin might be reassured that we share most though not all of these insertions with chimpanzees." (Weiss, 2006).

> "I've always said, if Darwin and Wallace decided to open a resort and spa in Cuba instead of going into science, if every fossil was still hidden… The second we found ERVs, common descent would have smacked us in the head like a sack full of doorknobs." (Smith, 2007).

Retroviruses are a family of viruses that inserts their DNA genome into that of the host cell. The viral DNA consists of long terminal repeats (LTRs) at both ends which control expression of three major genes in between: *gag*, *pol*, and *env*. The conserved *pol* gene encodes a reverse transcriptase, a hallmark of the *Retroviridae* family, while the *gag* and *env* genes encode viral structural proteins (Goff, 2001).

Endogenous retroviruses (ERVs) are cellular DNA sequences that are structurally similar to the genomes of retroviruses. They are highly repetitive elements interspersed throughout eukaryotic genomes, especially among vertebrate animals (Goff, 2001; Karp, 2008). Evolutionists assume that all ERVs were inserted into the genomes of cellular organisms as a consequence of retroviral infections of germ cells (Weiss, 2006; Smith, 2007), and this process is called endogenization. The fact that humans and primate species share similar ERVs at corresponding locations is claimed to be a result of random insertions in common ancestors (Polavarapu, *et al*, 2006). In addition, ERV elements have been thought to self-propagate at the cost of the host genome (Doolittle and Sapienza, 1980; Orgel and Crick, 1980), in accordance with Dawkin's contrivance of selfish genes (2006). While the concept of junk DNA in general is rapidly vanishing (The ENCODE Project Consortium, 2007 and 2012), mainstream biologists still regard ERVs and any movable genetic elements as remnants of troublesome foreign intruders (Zeh, *et al*, 2009; http://scienceblogs.com/erv/).

The theory of endogenization and common ancestry is not without flaws and inconsistencies, especially when one looks beyond primates. For example, humans, monkeys, mice, and sheep all use ERV genes in placental development. However, the placenta-forming ERVs of primates, rodents, and bovines are unrelated (Dunlap, *et al*, 2006; Dupressoir, *et al*, 2005; York, *et al*, 1991). If coincidence—or convergence in evolutionary terms--cannot explain positional homology of ERVs among humans and apes, neither should it be used to explain the concurrent use of different ERVs in placental development among members of the same mammalian class. In addition, endogenization cannot explain xenotropic ERVs, ERVs in species whose cells do not have a receptor for them (Coffin, *et al*, 1997). Third, many chimpanzee-specific ERVs appear to have integrated into the ancestral genome before the presumed human-chimp divergence, and there are no signs of ERV loss in the corresponding human loci (Polavarapu, *et al*, 2006).

There are two major creation models of ERVs. One accepts endogenization as the origin of all ERVs and suggests that ERVs selectively inserted into corresponding loci in unrelated genomes (Camp, 2001). This model relies upon the existence of "insertional hotspots". While it is true that retroviruses preferably target some genomic locations over others, this preference is weak and relative (Brady, *et al*, 2009; Fedoroff, 2012; Subramanian, *et al*, 2011), and therefore seems insufficient to explain the precise positional homology between multiple loci of human and primate ERVs (See Bonnaud, *et al*, 2005, for example). There is no known mechanism for precise sequence selection in non-homologous recombination between ERVs and host chromosomes.

Another creation model is that God created ERVs, at least some of them, as integral parts of cellular genomes at the beginning of life. Like other movable genetic elements, they may encode proteins and/or regulate neighboring genes (Fedoroff, 2012). A few ERV elements have been demonstrated to have functions that benefit the host organism, such as in placental development as mentioned above. There are also complex functional interactions between ERVs and host DNA sequences which cannot be explained by gradual addition and selection (Liu, 2006). ERVs were endowed with the ability of semiautonomous replication and transmission between cells, probably for recombinational repair or horizontal gene transfer, which could explain the origin of modern retroviruses ("exogenization" as proposed by Liu and Soper, 2009). Insertional polymorphism—the phenomenon that individuals of the same species may or may not have an ERV element at a specific genomic locus—may be caused by recent germ line infections or intracellular replicative transposition events. However, at least in humans, it is more often a result of ERV loss through homologous recombination (Hughes and Coffin, 2004).

To date, most reports of beneficial functions of ERVs concern only sporadic portions of stochastic ERV elements, which evolutionists claim to be results of "cooption" or "exaptation". For this reason, some view ERVs as parts of a "genetic scrap yard" (Karp, 2008). A key question needs to be addressed by creationists. Why did God choose ERVs to perform these functions? In other words, what are the advantages of using these repetitive elements with their unique structural genes and regulatory sequences?

The involvement of ERVs in reproduction of humans and animals is especially noteworthy. Multiple human endogenous retroviruses (HERVs) are known to be expressed in reproductive tissues (Muradrasoli, *et al*, 2006; Seifarth, *et al*, 2005). Placenta development in humans requires a cell fusion process induced by the *env* gene of two "fossilized" HERVs, ERVWE1 and HERV-FRD (Blaise, *et al*, 2003; Mallet, *et al*, 2004). Expression of HERV-Ks (the least degenerated category of HERV elements), is stimulated by female sex hormones in cultured breast cancer cells (Ono, *et al*, 1987; Wang-Johanning, *et al*, 2003). In addition, the LTR of some HERVs drives the expression of downstream cellular genes such as the placenta-specific pleiotrophin (Schulte, *et al*, 1996).

It is possible that common regulatory elements in the LTRs were designed for coordinated control of interspersed ERV genes and neighboring host genes in response to host signals such as sex hormones. Coordinated regulation would not only explain the function of certain ERV elements but would also argue for a general strategy in using ERV elements to organize and access genomic information. If ERVs are essential for genomic structure and function, positional homology of ERV elements between unrelated species would be out of necessity rather than as remnants of evolutionary accidents in common ancestors.

The purpose of this project was to seek signs of regulated activation of HERV genes in women during the menstrual cycle as the body undergoes systematic changes preparing for conception. Positive findings would support coordinated involvement of HERV elements in human reproduction. The dynamics of HERV transcription were investigated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Our results suggest cyclic hormonal regulation of HERV activity in reproductive-age women.

MATERIALS AND METHODS

Subjects and samples

Anticoagulated venous blood was drawn from six non-pregnant females of reproductive age (ages 17- 46), one pregnant female (age 37), four postmenopausal females (ages 55 – 83) and five males (ages 21 – 60). Multiple samples were taken from most subjects to study the dynamics of HERV transcription. The project was institutionally supervised for human subject protection. Donors were given informed consent before participation. Blood samples were drawn and handled by a trained professional and conformed to regulations of the Occupational Safety and Health Administration of the United States Department of Labor.

Extraction of RNA

Total cellular RNA was extracted either from whole blood or from purified peripheral blood mononuclear cells (PBMN cells). The RNA Blood Mini Kit (Qiagen) was used to extract RNA from whole blood (with heparin as an anticoagulant). Genomic DNA from whole blood samples was eliminated by on-column digestion with RNase I (Qiagen). PBMN cells were obtained by centrifugation on Histopaque (Sigma-Aldrich), and RNA was subsequently extracted using RNeasy Plus Mini Kit (Qiagen), which included a gDNA elimination column.

Reverse transcription

The Quantitect Reverse Transcription Kit (Qiagen) was used, which included a Wipeout buffer to further eliminate genomic DNA.

Quantitative PCR

The first step in quantitative PCR was to find a housekeeping gene that was stably transcribed during the menstrual cycle, which could subsequently be used as a reference to normalize HERV transcription. Transcription of four candidate genes, namely, the zeta polypeptide of the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ), glucose-6-phosphate dehydrogenase (G6PDH), ribosomal protein S18 (RPS18), and RNA polymerase II polypeptide A (RPII) were compared using two established computer algorithms, the GeNorm (http://medgen.ugent.be/~jvdesomp/genorm/) and Normfinder (http://www.mdl.dk/publicationsnormfinder.htm). Primers for the housekeeping genes were adopted from published sources (Radonić, *et al*, 2004; RTPrimerDB, 2002; Toegel, *et al*, 2007).

A pair of slightly degenerate primers targeting the *pol* gene of class II HERVs (HERV-K) has been shown to amplify HML-2 and HML-3 subtypes of HERV-K (Contreras-Galindo, *et al*, 2006), and was consequently used here. A pair of primers targeting the *env* gene of ERVWE1 (syncytin-1) with confirmed specificity was used (Nellaker, *et al*, 2006). Another pair of primers targets the *env* gene of HERV-K10 and related elements of the HML-2 subgroup (Wang-Johanning, *et al*, 2003).

Real time quantitative PCR was performed using the MiniOpticon system (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad). Primer concentrations and cycling conditions were optimized for each pair of primers. Results were analyzed using the Opticon Monitor 3 Analysis Software.

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.

Enzyme Immunoassay

Plasma levels of progesterone and estradiol were determined using enzyme immunoassay (kits from Cayman Chemical) according to manufacturer's instructions.

RESULTS

1. Selection of the reference gene

Due to unpredictable variances during RNA preparation and reverse transcription, quantification of transcription with real-time PCR is relative, expressed as a ratio between the target gene and a stably transcribed reference gene in each sample. Since our hypothesis concerns hormonal regulation of HERV genes, we needed a reference gene that is known to be unaffected by steroid sex hormones. The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was found to be irresponsive to 17β-estradiol (a female sex hormone) in dolphin fibroblast cultures (Spinsanti, *et al*, 2008). Glucose-6-phosphate dehydrogenase (G6PDH) and RNA polymerase II (RPII) were considered because they have been proven to be irresponsive to mitogens in a T-cell line (Radonić, 2004), and our samples are leukocytes or PBMN cells. Ribosomal protein S18 (RPS18) was considered because of its abundance and therefore presumed stability, and because it is a classical reference gene for quantitative PCR.

Four weekly samples of total leukocyte RNA from a woman with monthly menstrual cycles were used to evaluate the transcriptional stability of the above four candidate genes. A standard curve was simultaneously run for each candidate gene. Gene expression was compared using the geNorm and the NormFinder algorithms. The rank of stability according to both algorithms is YWHAZ, RPII, G6PDH, and RPS18 (Table 1). Therefore, YWHAZ was chosen as the reference gene for subsequent studies of HERV transcription.

Table 1. Summary of reference gene assessment

Algorithm output	YWHAZ	RPII	G6PDH	RPS18
GeNorm M value	0.450	0.461	0.532	1.082
NormFinder stability value	0.047	0.050	0.273	0.747

2. Comparison of HERV transcription between gender/age groups

There are three classes of HERVs. Class I HERVs are generally less intact than class II HERVs due to accumulation of mutations. There is only one member in class III, i.e., HERV-L. Most HERV elements expressed in reproductive tissues belong to class I. Among the best characterized elements of class I HERVs are the *env* genes of ERVWE1 and HERV-FRD (encoding syncytins 1 and 2), which are involved in placental development. Class II HERVs, also called HERV-Ks, include ten groups (HML1-10) of homologous elements (Nelson, *et al*, 2003).

Some HERV elements are known to contain putative progesterone-response elements in the LTR and respond to combined treatment of estradiol and progesterone—simulating the hormonal changes during the menstrual cycle—in cell cultures (Medstrand, *et al*, 1997; Ono, *et al*, 1987; Wang-Johanning, *et al*, 2003). To assess the effects of sex hormones on HERV activities *in vivo*, we first compared levels of HERV transcription in peripheral blood leukocytes (total leukocytes or purified mononuclear cells) between different gender/age groups. We used a pair of *pol*-based degenerate primers to detect transcripts of HERV-Ks, which mainly amplify the most intact HML-2 and 3 subgroups (Contreras-Galindo, *et al*, 2006). In addition, we used a pair of *env*-based primers—which are more locus-specific—to detect syncytin-1. These were published primers with characterized specificity.

RNA from total leukocytes and from purified mononuclear cells yielded similar results, allowing for pooling of data in statistical treatments. Transcription of HERVs in reproductive-age non-pregnant females, in males, and in postmenopausal women is compared in Table 2.

Table 2. Transcription of HERV-Ks and syncytin-1 in different gender/age groups

Group	Number of samples	HERV-K (Mean ±SD)	Syncytin-1 (Mean ±SD)
Reproductive-age females	33	0.011±0.025	0.028±0.025
Males	22	0.0040±0.0030	0.029±0.031
Postmenopausal females	4	0.0032±0.0017	0.0068±0.0058

Evidently, reproductive-age females showed higher transcription of HERV-K than males or postmenopausal females, but the differences are not statistically significant ($P = 0.10$ between males and reproductive-age females; $P = 0.071$ between reproductive and postmenopausal females) due to large variations among samples of each group. The expression levels of syncytin-1 are similar between males and reproductive-age females, but both are significantly higher than in postmenopausal females ($P = 0.00055$ between reproductive-age females and postmenopausal females, $P = 0.0049$ between males and postmenopausal females).

Transcription levels of HERV-K and syncytin-1 were determined in a 37-year-old pregnant female between 26 and 29 weeks of gestation. The numbers were comparable to those of non-pregnant women (data not shown).

3. Variation of HERV expression in females and in males

Expression of HERV genes varied significantly both between individuals and among serial samples of the same individual. Variances of HERV transcription levels among serial samples of the same subject are compared between reproductive-age women (6) and men (3) in Table 3. Transcription of both HERV genes varied more in reproductive-age females than in males, but the differences are not statistically significant due to large standard deviations.

Table 3. Variance of HERV transcription among serial samples of the same subject

Group	Samples per individual	Variance of HERV-K (Mean±SD)	Variance of Syncytin-1 (Mean± SD)
Reproductive-age females	3~6	0.00043±0.0010	0.00025±0.00033
Males	3~6	$2.7 \times 10^{-6} \pm 3.2 \times 10^{-6}$	$4.4 \times 10^{-5} \pm 4.2 \times 10^{-5}$

Maximum and minimum levels of HERV transcription in each subject are summarized in Table 4. There is a statistical difference between maximum and minimum levels of syncytin-1 transcription in reproductive-age women (P = 0.043) but not in men (P = 0.13). The mean range of variation of HERV-K transcription in reproductive-age women is more than an order of a magnitude, although the differences between maximum and minimum values are not statistically significant. The ranges of variation of HERV-K transcription in males are much narrower.

Table 4. Maximum and minimum levels of HERV transcription in each subject

Group	Samples per individual	HERV-K maximum (Mean±SD)	HERV-K minimum (Mean±SD)	Syncytin-1 maximum (Mean±SD)	Syncytin-1 minimum (Mean±SD)
Reproductive-age females	3~6	0.029±0.055	0.0022±0.0017	0.040±0.029	0.010±0.0032
Males	3~6	0.0024±0.0032	0.0010±0.00064	0.022±0.011	0.0079±0.0012

4. Patterns of HERV expression during the menstrual cycle

During the menstrual cycle, the ovaries produce two main steroid hormones, estradiol and progesterone. The concentrations of both hormones are low during the first half (the follicular phase) of the cycle and high during the second half (luteal phase) of the cycle. The levels of progesterone vary more than those of estradiol (Shier, *et al*, 2013).

Transcription of HERV genes demonstrates patterns in non-pregnant reproductive-age women. The patterns are similar between HERV-K and syncytin-1. There is typically a trough just before menstruation (luteal phase) and a peak during the follicular phase (Fig 1 A and B). A third pair of primers specifically targeting the *env* region of HERV-K10, which is a subgroup of HML-2, showed a similar pattern of transcription as that of the more broadly targeting HERV-K primers (Fig 1 C).

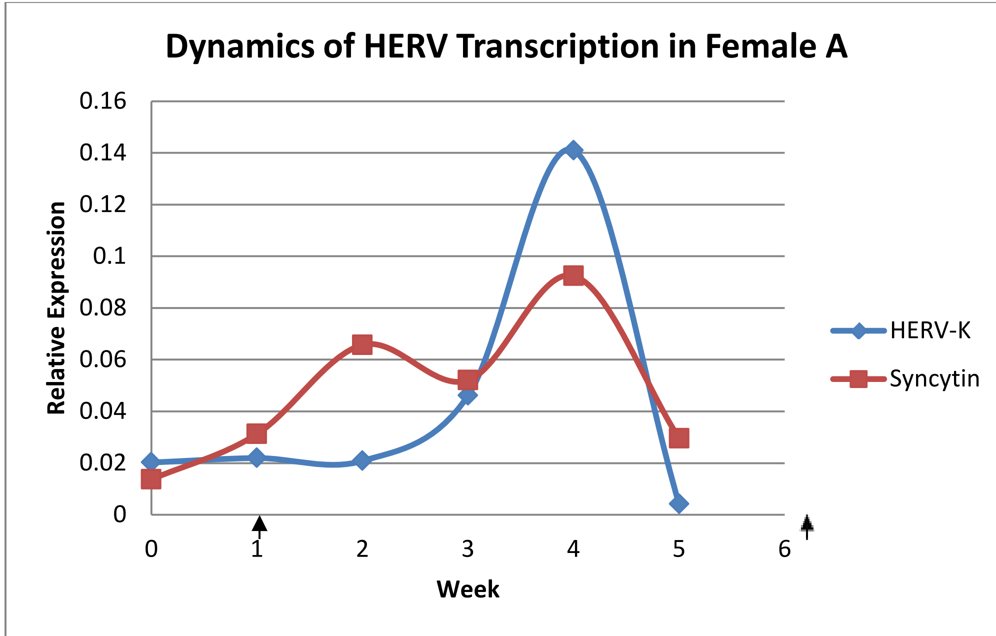
To study the correlation between HERV transcription and hormonal concentrations, plasma progesterone levels were titrated using an enzyme immunoassay in two cycling women. Transcription of both HERV-K and syncytin-1 genes were negatively correlated with plasma progesterone concentration with correlation coefficients varying between -0.53 and -0.94. The correlation between the inverse of progesterone concentration (1/[Progesterone]) and

transcription of HERV genes was stronger, with correlation coefficients between 0.71 and 0.97 (Fig 2).

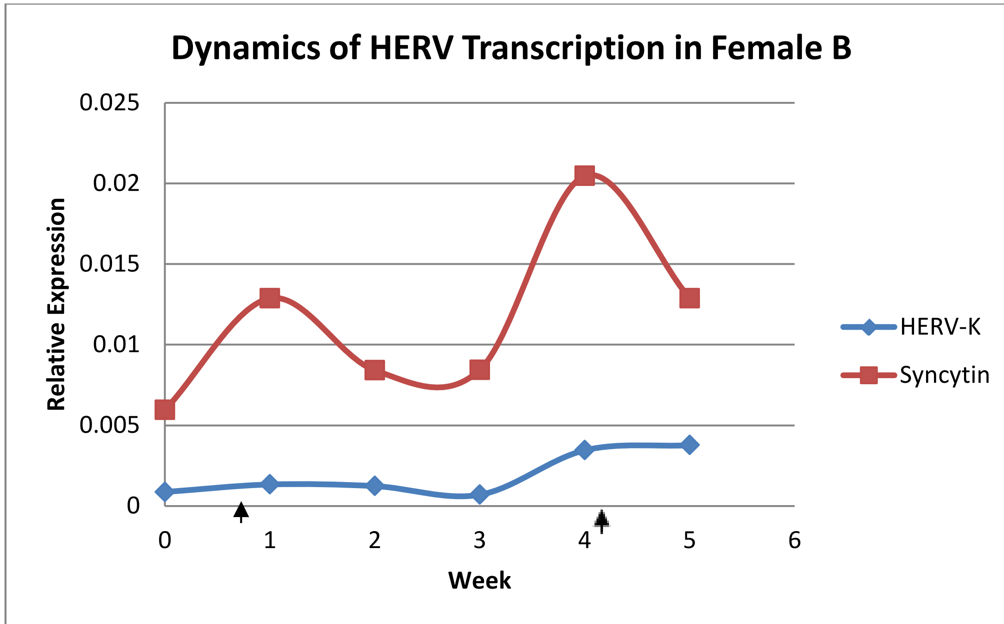
Plasma estradiol levels were titrated in one cycling woman. Similar negative correlations were found between transcription of HERV elements and plasma estradiol concentrations. Correlation coefficients were -0.83 for HERV-K and -0.77 for syncytin-1. Correlation coefficients with the inverse of estradiol concentrations were 0.89 for HERV-K and 0.96 for syncytin-1.

In view of the putative progesterone-response elements in the LTR of HERVs (Medstrand, *et al*, 1997; Wang-Johanning, *et al*, 2003), these patterns and correlations suggest that progesterone suppresses the transcription of HERV genes in peripheral leukocytes. Because blood levels of estrogens and progesterone are closely associated, the correlation between HERV transcription and estradiol may be non-causal, although we cannot rule out that estradiol is actually the major hormone acting on HERVs or both hormones regulate these elements directly.

(A)



(B)



(C)

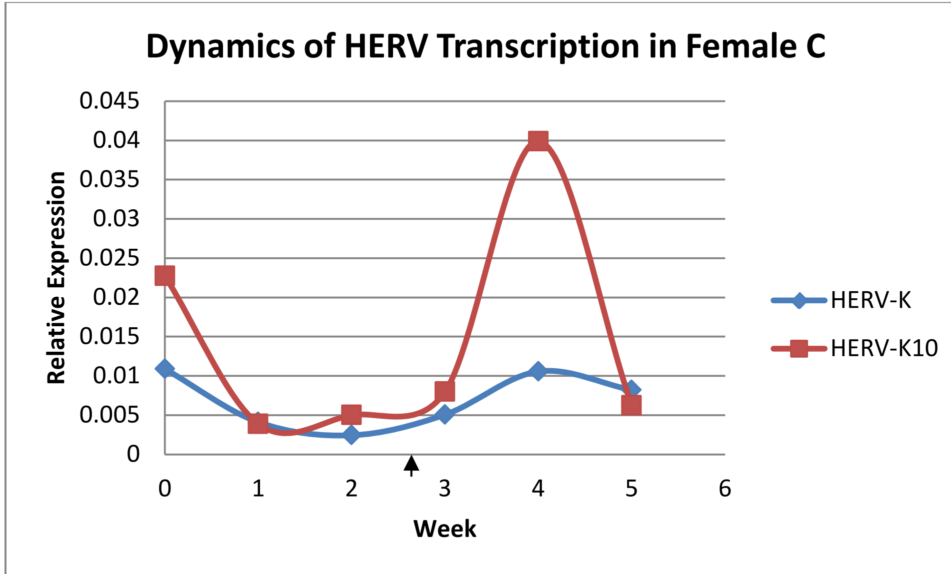
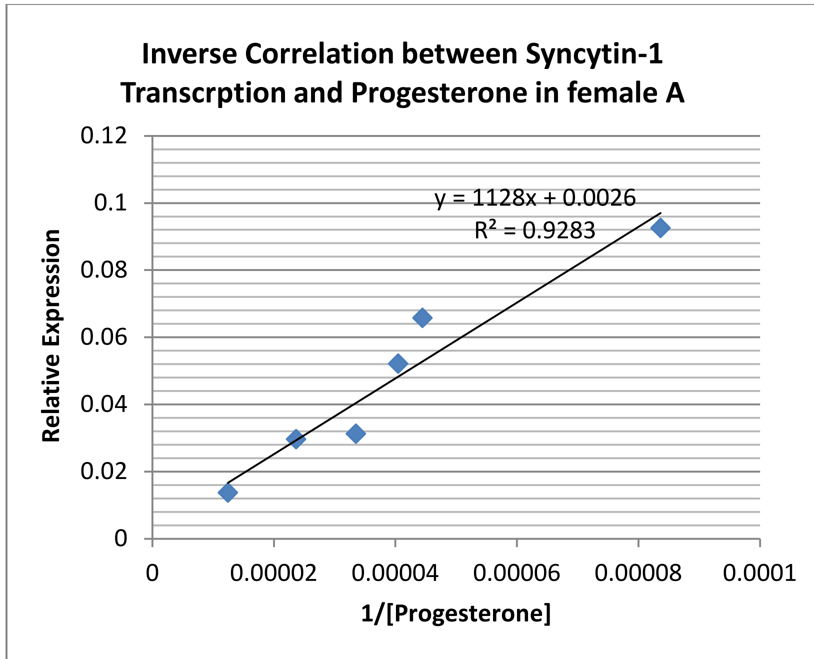


Figure. 1. Transcription of HERV during the menstrual cycle. (A), (B), and (C) each represents data from one reproductive-age, non-pregnant female. Levels of HERV expression were calculated as ratios between quantities of HERV transcripts and that of the YWHAZ housekeeping gene. Transcription of HERV-K elements as a class was quantified in all three subjects, while transcription of the syncytin-1 gene was quantified in subjects A and B only; and transcription of HERV-K10, a member of the HERV-K class, was quantified in subject C only. Arrows indicate beginning of menstruation.

(A)



(B)

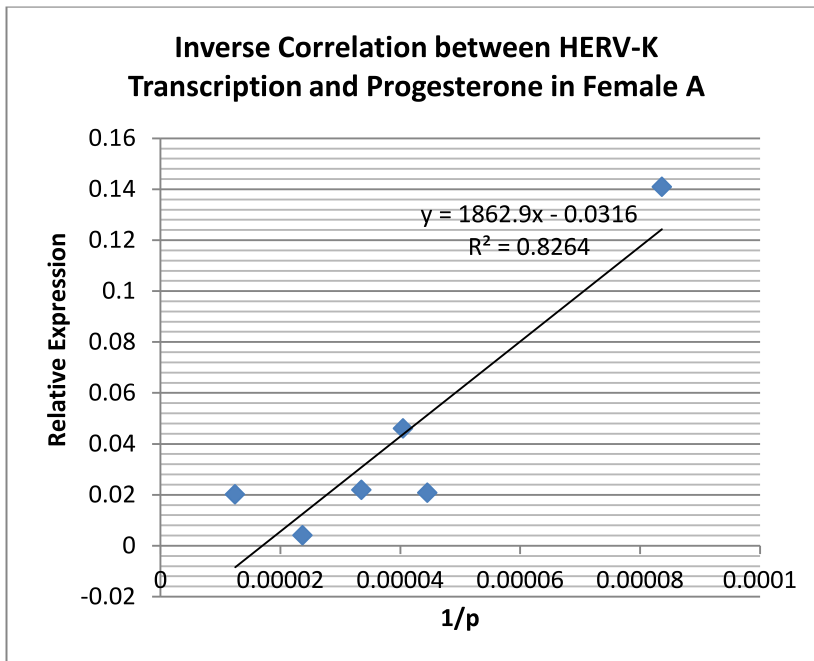


Figure. 2. Correlation between HERV transcription and plasma progesterone concentrations. (A) plots transcription of syncytin-1 against the inverse of progesterone concentration in female A; while (B) plots transcription of HERV-K against the inverse of progesterone concentration in the same subject. Levels of HERV expression were calculated as ratios between quantities of HERV transcripts and that of the YWHAZ housekeeping gene, while progesterone concentrations were titrated using the enzyme immunoassay.

DISCUSSION

We used well characterized PCR primers to study the transcription of representative members of class I and class II HERVs. In spite of the small number of subjects studied, the above results reveal interesting dynamics of HERV transcription. To our knowledge, this is the first comparison of HERV transcription between males and females, and between reproductive-age male/female and postmenopausal women. Indeed, this is the first report of the dynamics of HERV transcription *in vivo*. The pattern of expression during the menstrual cycle as well as the correlation between HERV expression and plasma hormonal concentrations suggest coordinated regulation of diverse HERV genes during the female reproductive cycle. Considering the fact that there are far more solo LTRs (solitary LTRs without accompanying ERV sequences) than full-length ERVs, and the hormone-response elements are likely in the LTRs, there should be more neighboring cellular genes than HERV genes whose transcription is influenced by sex-hormones through the LTRs. Coordinated gene expression implies a general purpose in the design of ERV elements in cellular genomes, contrary to the evolutionary concept of sporadic exaptation of exogenous viruses. This is corroborated by the recent ENCODE finding of a large number (507) of transcription start sites within LTR elements in the human genome (Djebali, *et al*, 2012).

As early as 1987, Ono and colleagues reported a stimulating effect of combined estradiol and progesterone treatment on the transcription of HERV-K in a breast cancer cell line using Northern blot analysis (Ono, *et al*, 1987). More recent studies with quantitative PCR confirmed this observation (Wang-Johanning, *et al*, 2003). We used peripheral leukocytes from healthy human subjects, mainly for convenience to obtain measurable quantities of cellular RNA. We hypothesized that sex hormones activate HERV transcription in peripheral leukocytes, just as in reproductive tissues. The negative correlation between plasma hormonal concentrations and HERV transcription thus came as a surprise. However, considering the different intracellular milieu between reproductive tissues and leukocytes, a different response of HERV elements to sex hormones should be understandable. Although the function of HERVs in leukocytes is unknown, their response to sex hormones may have to do with modulation of the immune system during pregnancy as the mother tolerates the semi-allogeneic embryo.

Another paradoxical finding was the lower expression of HERV genes in postmenopausal women. Declining function of HERV elements in senior females is not surprising, but cannot be explained by the suppressive effect of female sex hormones, which they lack. This implies that other factors (possibly androgens, gonadotropins, or non-hormonal factors) may regulate HERV expression in leukocytes.

Our finding of HERV activities in leukocytes and their association with sex hormones may open another door in reproductive immunology. The mechanisms of maternal tolerance of the semi-allogeneic embryo, along with other immunological changes during pregnancy, are still under investigation. Progesterone is known to modulate lymphocyte behavior and is believed to play a leading role in the immunology of pregnancy (Druckmann and Druckmann, 2005). On the other hand, the *env* protein of retroviruses, including that of an HERV, is proven to be immunosuppressive (Good, *et al*, 1990; Mangeney, *et al*, 2001). Involvement of HERVs in reproductive immunology has been suggested (Nelson, *et al*, 2003), but our findings provide the

first evidence of a link between progesterone and HERV activity. Research is now needed into the dynamics of HERV expression in lymphocytes as the cells respond to antigenic or mitogenic stimulants, as well as the effects of steroid hormones on HERV expression in lymphocyte cultures. Even though activation of HERV elements in leukocytes serves no recognized physiological function, such studies may still shed light on factors influencing ERV activities in the human body.

CONCLUSION

Transcription of multiple human endogenous retroviruses is correlated with levels of female sex hormones during the menstrual cycle in women. This suggests a collective and coordinated function of ERV elements in genomic regulation, as the cell responds to local or systemic signals such as hormones. This concept strongly suggests design of a highly integrated system, which is contrary to the evolutionary understanding of random cooption between cellular and viral genetic elements, and argues for the necessity of *in situ* creation of ERV elements in homologous loci among phylogenetically unrelated animals.

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