# Expression of HERV-HX2 in Cancer Cells and Human Embryonic Stem Cells

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## ABSTRACT

**The endogenous retrovirus-like elements (HERVs) found on several human chromosomes are somehow involved in gene regulation, especially during the transcription level. HERV-H, located on chromosome Xp22, may regulate gastrin-releasing peptide receptor (GRPR) in connection with diverse diseases. By suppression subtractive hybridization screen on SV40-immortalized lung fibroblast (WI-38 VA-13), we discovered that expression of HERV-HX2, a clustered HERV-H sequence on chromosome X, was upregulated in immortalized lung cells, compared to that of normal cells. Expression of HERV-HX2 was then analyzed in various cell lines, including normal somatic cells, cancer cells, SV40-immortalized cells, and undifferentiated and differentiated human embryonic stem cells. Expression of HERV-HX2 was specifically upregulated in continuously-dividing cells, such as cancer cells and SV40-immortalized cells. Especially, HERV-HX2 in HeLa cells was highly upregulated during the S phase of the cell cycle. Similar results were obtained in hES cells, in which undifferentiated cells expressed more HERV-HX2 mRNA than differentiated hES cells, including neural precursor and endothelial progenitor cells. Taken together, our results suggest that HERV-HX2 is upregulated in cancer cells and undifferentiated hES cells, whereas downregulated as differentiation progress. Therefore, we assume that HERV-HX2 may play a role on proliferation of cancer cells as well as differentiation of hES cells in the transcriptional level.**

**(Key words : HERV-HX2, Proliferation, Differentiation, Gene regulation, Cell cycle)**

## INTRODUCTION

The human genome contains many families of endogenous retrovirus-like elements (HERVs) (Benveniste and Todaro, 1974), among which HERV-H is one of the most abundant. The majority of HERV-H elements (800~900 copies) contain deletions of the *pol* and *env* regions, but a fraction (50~100 copies) are full-length elements (Mager and Henthorn, 1984; Hirose *et al.*, 1993; Wilkinson *et al.*, 1993). HERV-H has two long terminal repeats (LTRs), a potential primer binding site that is homologous to histidine tRNA, a polypurine tract upstream of the 3′ LTR, and flanking 5-bp cellular direct repeats (Mager and Henthorn, 1984; Mager and Freeman, 1987).

Although the exact biological function of these proviruses is still unclear, their viral proteins can regulate adjacent cellular genes (Kitamura *et al.*, 1994; Venables *et al.*, 1995; Sauter *et al.*, 1995). These specific regulatory sequences localize to the U3 regions of the LTRs, ranging from 150 to 1,200 bases in different viruses. These sequences contain signal structures essential for transcription initiation, such as promoters, enhancers, transcription factor binding sites (Majors, 1990; Kato *et al.*, 1990; Feuchter *et al.*, 1992), and polyadenylation signals (Goodchild *et al.*, 1992). For instance, the LTR sequence of the HERV-E family is involved in tissue-specific expression of human salivary amylase. Insert of a HERV element into upstream of three amylase complex genes is related to a switch of expression from pancreatic to parotid (Samuelson *et al.*, 1988; Ting *et al.*, 1992). Several studies showed that HERV-H occupies specific locations on human chromosomes. *In situ* hybridization studies have localized HERV-H sequences on chromosomes 1 p and 7 q (Fraser *et al.*, 1988); HERV-H sequences were also shown to be inserted downstream of the HERV-K 5' LTR in human chromosomes 10, 19, and Y (Lapuk *et al.*, 1999).

Previously, we used a suppression subtractive hybridization process to identify genes that were differentially expressed in immortalized lung fibroblasts mediated by SV40 (Choi *et al.*, 2006). Expression of HERV-H clone HX2 (HERV-HX2), located on chromosome

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Xp22, was highly upregulated in the immortalized lung cells. In the present study, we report that HERV-HX2 expression is upregulated in cancer cell lines, and undifferentiated hES cells as well as SV40-immortalized cell lines, but not in normal cells or differentiated hES cells. These results suggest that HERV-HX2 may be involved in proliferation and differentiation processes of cells.

## MATERIALS AND METHODS

### Cell Culture

The cell lines WI-38, RWPE-1, CCD-18Co, Hs 677.St, AGS, K-562 and SVG p12 were purchased from the American Type Culture Collection (Manassas, VA, USA). WI-38 VA-13, DU-145, KM1214, CCD-986sk, WM-266-4, NCI-H596, HeLa, SK-N-SH, A172, and WI-26 VA-4 were obtained from the Korean Cell Line Bank (Seoul, Korea). WI-38, CCD-18Co, WM-266-4 and SVG p12 were propagated in minimal essential media containing Earle's salts, 2 mM L-glutamine, and 0.1 mM nonessential amino acids (EMEM), supplemented with 1 mM sodium pyruvate. WI-38 VA-13, AGS, DU-145, NCI-H-596, HeLa, SK-N-SH, and A172 were cultured in RPMI-1640; Hs 677.St, K-562, KM1214, CCD-986sk, and WI-26 VA-4 were maintained in Dulbecco's modified Eagle's Medium (DMEM); RWPE-1 was cultured in keratinocyte serum-free medium; all media were supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml). All cell lines were cultured at 37℃ in a humidified 5.0% $CO_2$ incubator. Undifferentiated human embryonic stem cells (CHA-hES3 and CHA-hES4) and differentiated hES cells (neural precursor cells and endothelial progenitor cells) were kindly provided by Dr. Choi and Dr. Chung at CHA Stem Cell Institute.

### Preparation of Total RNA

Total RNA was extracted from the cell lines using TRI REAGENT® (Molecular Research Center; Cincinnati, Ohio, USA). A260 and A260/A280 of the samples were determined using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.; Waltham, MA, USA). The integrity of the extracted RNA was verified by electrophoresis on a 1.0% agarose gel. The RNA was treated with DNase I (Roche; Germany) to eliminate residual genomic DNA.

### Reverse Transcription PCR Analysis

To validate differential expression among the cell lines, we performed reverse transcription polymerase chain reaction (RT-PCR). Full-length cDNA was synthesized using the SuperScript™ First-Strand Synthesis System (Invitrogen; Carlsbad, CA, USA). Briefly, 1 μg of total RNA was converted to cDNA in a total volume of 21 μl using random hexamers. All cDNAs were normalized to levels of 18S rRNA. PCR amplification was carried out in a total volume of 25 μl, using 1 μl of RT samples as templates and specific primers designed in 20 mers (Table 1). PCR was performed under the following conditions: 300 seconds (sec) at 94℃ for initial denaturation, followed by 35 cycles of 94℃ for 30 sec (denaturation), 55℃ for 30 sec (annealing), and 72℃ for 30 sec (extension), with a final extension step at 72℃ for 300 sec. Aliquots (7 μl) of the PCR products were analyzed on 1% agarose gels. Primers were designed used for PCR are listed in Table 1.

**Table 1. Sequences of primer pairs**

| Gene name | Primer sequences (5'~3') | Annealing Tm (℃) | Size (bp) |
|---|---|---|---|
| 18S rRNA | 5′-TACCTACCTGGTTGATCCTG-3′<br>5′-GGGTTGGTTTTGATCTGATA-3′ | 55℃ | 243 |
| HERV-HX2 (RT-PCR) | 5′-ATCCTGTTGTGGAGTTTGAG-3′<br>5′-TTCTACAAACCCATCTGACC-3′ | 55℃ | 451 |
| HERV-HX2 (qRT-PCR) | 5′-ACGAAACTGTAAGCCGGACC-3′<br>5′-CACGCCAAGCTAGGTCCCAAA-3′ | 55℃ | 255 |

Tm: Temperature; bp: base pairs; RT: reverse transcription; qRT: quantitative real time

### Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using a SYBR Green I kit (Qiagen; Hilden, Germany); comparing gene expression to those of housekeeping genes provided relative quantification. PCR amplification was conducted as follows: 600 sec of initial denaturation at 94℃, followed by 40 PCR cycles of a 10 sec denaturation at 94℃, a 20 sec annealing at 55℃, and a 30 sec elongation at 72℃. All PCR was performed in a Rotor-gene 3000 (Corbett Research; Sydney, Australia). All plates were analyzed in duplicate, based on which average copy numbers were calculated. Primers used for PCR are listed in Table 1.

### HeLa Cell Synchronization

HeLa cells were cultured in RPMI 1640 supplemented with 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml). For G1/S boundary synchronization, we added 2 mM thymidine to the cells (Sigma; St. Louis, MO, USA) for 18 hours, washed them with PBS, and cultured them with fresh medium without thymidine for 8 hours. To mediate G1/S phase arrest, 2

mM thymidine was added again for 18 hours, after which the cells were given fresh medium and harvested every hour for the next 15 hours, as described (Fang *et al.*, 1998; Stewart and Fang, 2005).

## RESULTS

Previously, our team had screened for differentially-expressed genes (DEGs) in SV40-immortalized lung fibroblasts compared to normal lung cells (Choi *et al.*, 2006). Among the DEGs, we identified that HERV-HX2, a clustered HERV-H sequence on chromosome X, was more highly expressed in continuously-dividing cells than in normal cells. To analyze the cell-specific expression pattern of HERV-HX2, we conducted RT-PCR analysis in five normal cell lines, nine cancer cell lines, and three immortalized cell lines. The normal cells were WI-38 (lung), RWPE-1 (prostate), CCD-18Co (colon), Hs 677.St (stomach), and CCD-986sk (skin); the cancer cells were NCI-H596 (lung adenosquamous), DU-145 (prostate adenocarcinoma), KM1214 (colon carcinoma), AGS (stomach adenocarcinoma), WM-266-4 (skin melanoma), HeLa (cervix adenocarcinoma), K562 (chronic myelogenous leukemia, CML), SK-N-SH (brain neuroblastoma), and A172 (brain glioblastoma); the immortalized cells were WI-38 VA-13 (lung), WI-26 VA-4 (lung), and SVG p12 (brain). Our results revealed a pattern: HERV-HX2 was overexpressed in cancer cells and immortalized cells, but not in normal cells (Fig. 1). Moreover, expression levels were much higher in undifferentiated hES cells than in their differentiated


**Fig. 1. Expression pattern of HERV-HX2 in various cell lines.** 1: Normal lung cells (WI-38), 2: SV40-immortalized lung cells (WI-38 VA-13), 3: Normal prostate cells (RWPE-1), 4: Prostate adenocarcinoma cells (DU-145), 5: Normal colon cells (CCD-18Co), 6: Colon carcinoma cells (KM1214), 7: Normal stomach cells (Hs 677.St), 8: Stomach cancer cells (AGS), 9: Normal skin cells (CCD-986sk), 10: Skin tumor cells (WM-266-4), 11: Lung carcinoma cells (NCI-H596), 12: Cervical adenocarcinoma cells (HeLa), 13: Erythroleukemia cells (K562), 14: Brain neuroblastoma cells (SK-N-SH), 15: Brain glioblastoma cells (A172), 16: SV40-immortalized brain cells (SVG p12), 17: SV40-immortalized lung cells (WI-26 VA-4).

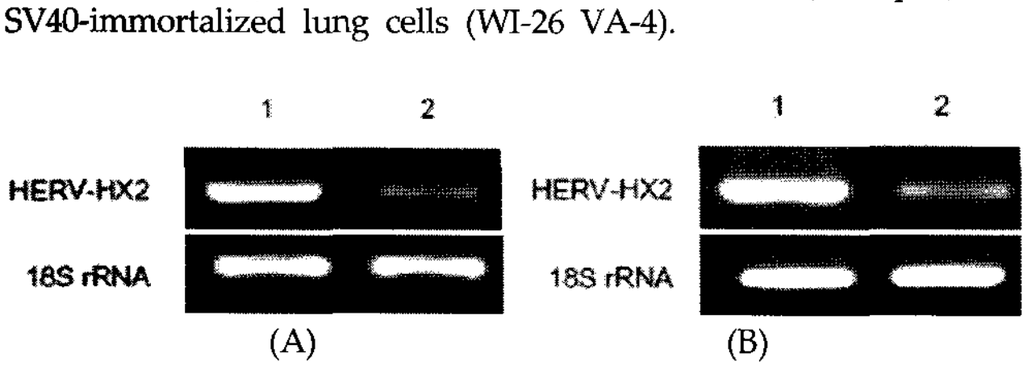


**Fig. 2. Expression pattern of HERV-HX2 in human ES cells and differentiated cells.** (A) 1: CHA-hES4 cells, 2: Neural precursor cells. (B) 1: CHA-hES3 cells, 2: Endothelial progenitor cells.

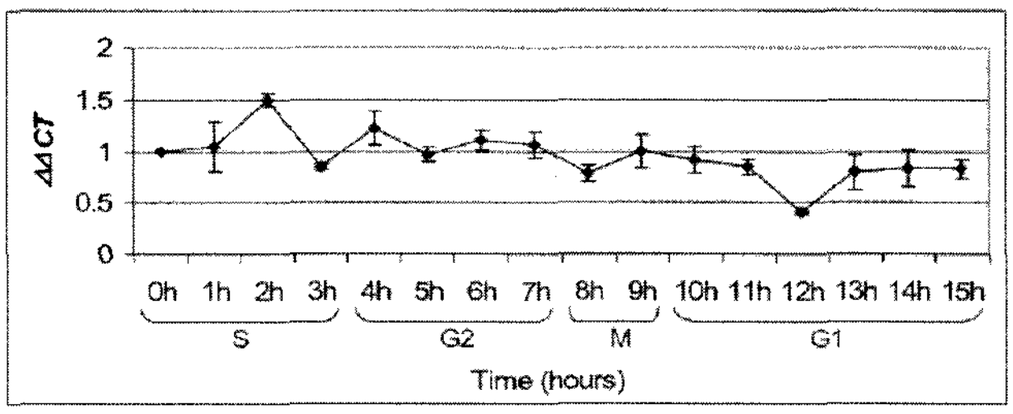


**Fig. 3. Expression pattern of HERV-HX2 in synchronized HeLa cells.** qRT-PCR data were analyzed using the comparative $C_t$ method. Differential expression of HERV-HX2 at different times, as compared to a housekeeping gene (18S rRNA) was calculated as follows: $\Delta C_t = C_{t\ \text{HERV-HX2}} - C_{t\ 18S\text{rRNA}}$. These values were used to calculate HERV-HX2 (0h):1~15h ratios: $\Delta(\Delta Ct) = 2^{-(\Delta Ct\ \text{each time} - \Delta Ct\ 0h)}$. Distinguishable upregulation was observed at 2 h, and downregulation was observed at 12 h.

cells, such as neural precursor cells (Fig. 2 (A)) and endothelial progenitor cells (Fig. 2 (B)).

To observe whether HERV-HX2 expression has relevance to the cell cycle, we synchronized HeLa cells and determined whether HERV-HX2 expression changed with respect to time (*i.e.*, a specific point in the cell cycle). Interestingly, HERV-HX2 was upregulated during S phase and downregulated during G1 phase. Specifically, after we rendered the G1-S arrest on the HeLa cells, their expression of HERV-HX2 increased dramatically for two hours (corresponding to S phase); it then began to decrease, to the point where we observed only slight changes of expression during G2 and M phase. This decrease persisted for nine hours, until the G1 phase began. The expression at 12 hours reached a minimum and then the expression level began to recover after 13 hours (Fig. 3).

## DISCUSSION

Cell immortality has been studied for many years, but its nature or the underlying mechanism is still not clearly understood. Reportedly, maintenance of telomeres and telomerase activity, a balance between oncogenes and tumor suppressor genes, and infection of viral oncogenes are all involved in the immortalization processes (Blagosklonny, 2003). During immortalization processes, cells acquire genetic alterations that override normal senescence processes. Alterations that inhibit tumor suppressor genes in the p53 and pRb pathways are common in cancer (Sherr and McCormick, 2002). Tumor suppressor genes might be inactivated by deletion of alleles, methylation of promoter regions, mutations in genes that induce premature translational termination, or destabilization of mRNAs (Leal *et al.*, 2008).

The importance of HERV-H expression is still far from clear, but emerging evidence suggests that HERV-

H elements are transcriptionally activated in human placenta (Kitamura *et al.*, 1994), germ cell tumors, bladder carcinomas (Wilkinson *et al.*, 1990), several tumor cell lines (Lower *et al.*, 1995; Sauter *et al.*, 1995), and peripheral blood mononuclear cells (Medstrand *et al.*, 1992). HERV-H LTR elements are expressed in several normal tissues (brain, prostate, testis, kidney, placenta, skeletal muscle, spleen, thymus, and uterus) as well as cancer cells (Sin *et al.*, 2006). A recent report indicated that HERV-H-X was highly upregulated (almost 25-fold) in colon cancer tissue compared to normal tissue (Liang *et al.*, 2007).

Shiraish *et al.* identified three HERV-H sequences (HERV-HX1, HERV-HX2, and HERV-HX3) within the telomere of the short centromeric arm, clustered within a 300-kilobase region near the gastrin releasing peptide receptor (GRPR) locus on the X chromosome (Xp-22) (Shiraishi *et al.*, 1996). GRP is a mammalian bombesin-like peptide that is widely expressed in the central nervous system. GRP interacts with GRPR to regulate its biological effects on behavior, digestion, and metabolism. GRPR-deleted transgenic mice exhibit increased locomotor activity and social interaction, as demonstrated by increased sniffing, mounting, and approach behaviors without increased aggression (Wada *et al.*, 1997). GRPR has also been implicated in various psychiatric conditions; for example, possessing eight translocations of the GRPR gene in Xp22 increases the risk of multiple exostoses or autism (Ishikawa-Brush *et al.*, 1997), and two cases of paranoid schizophrenia exhibited overlapping deletions in Xp22.3 (Milunsky *et al.*, 1999). These observations also indicate that HERV-H may be relevant in psychiatric illnesses, in addition to its current associations with multiple sclerosis (Christensen *et al.*, 2000), brain diseases (Johnston *et al.*, 2001), and various cancers (small intestine, bone marrow, bladder, and cervix) (Stauffer *et al.*, 2004).

In the present study, we analyzed the upregulated expression pattern of HERV-HX2 in cancer cells and SV40-immortalized human cells. In addition, the expression level of HERV-HX2 was abundant in embryonic stem cells. HERV-HX2 was downregulated as the hES cells differentiated to neural precursor and endothelial progenitor cells. This suggests that HERV-HX2 may not only involve in proliferation but also in differentiation. In aspect that sequence of HERVs contains signal structures related with transcriptional activity (Majors, 1990; Kato *et al.*, 1990; Feuchter *et al.*, 1992), HERV-HX2 possesses the potential to be involved in controlling the mechanism of proliferation as well as differentiation in the transcription level. However, it is too early to conclude that HERV-HX2 directly regulates the proliferation or differentiation. So far we assume that HERV-HX2 has indirect influence by regulating another gene expression which is involved in differentiation. Pattern of HERV-HX2 expression in cell cycle was carried out by the HeLa cell synchronization experiment. HeLa cells were arrested at the G1/S boundary by double-thymidine treatment, resulting in cell synchronization upon release of the block. The cells were induced to re-enter the cell cycle (at which point they all entered S phase), harvested periodically over the next 15 hours (Stewartt *et al.*, 2005), and examined for HERV-HX2 expression. Expression of HERV-HX2 was upregulated during S phase and downregulated during G1 phase, while the expression did not fluctuate significantly during G2 and M phases.

In summary, our data showed that expression of HERV-HX2, a clustered HERV-H sequence on chromosome X, is upregulated in continuously-dividing cells, particularly during the S phase of the cell cycle, suggesting that HERV-HX2 may play a role in proliferation as well as immortalization. Moreover, Fig. 3 shows the possibility that transcriptional regulation of HERV-HX2 might participate in differentiation of hES cells by regulating some gene expression. Further studies need to be carried out to determine the connection of HERV-HX2 regulation in the cell cycle and differentiation.

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