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Human Endogenous Retrovirus-H and K Expression in Human Mesenchymal Stem Cells as Potential Markers of Stemness

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Keywords
Mesenchymal stem cells · Human endogenous retroviruses · NANOG · OCT-4 · SOX-2

Abstract
Objective: The human endogenous retroviruses (HERVs) are endogenous retroviruses that were inserted into the germ cell DNA of humans over 30 million years ago. Insertion of HERVs into the chromosomal DNA can influence a number of host genes in various modes during human evolution and their proviral long terminal repeats can participate in the transcriptional regulation of various cellular genes. Our aim was to evaluate the *pol* gene expression of HERV-K and HERV-H in mesenchymal stem cells (MSCs) in relation with the expression of stemness genes such as NANOG, OCT-4, and SOX-2. ***Methods:*** MSCs were isolated from bone marrow of healthy donors and expanded until the 5th passage in α-MEM with 10% fetal bovine serum. HERV-K, HERV-H *pol* gene, NANOG, OCT-4, SOX-2, and GAPDH expression was quantified by real-time PCR in MSCs during the expansion. ***Results:*** HERV-K and HERV-H expression was always higher at p1 compared to other passages and this difference reached a high statistical significance when passage p1 was compared with passage 3. In addition, NANOG, OCT-4, and SOX-2 expression at p1 was significantly higher than their expression at p3. Pearson's test demonstrated a strong correlation between the expression of HERV-K and HERV-H and the expression of NANOG, OCT-4, and SOX-2. ***Conclusions:*** Our findings showed that HERV-K and H were concurrently expressed with pluripotency biomarkers NANOG, OCT-4, and SOX-2.

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Introduction

The human endogenous retroviruses (HERVs) are endogenous retroviruses that were inserted into the germ cell DNA of humans over 30 million years ago [1]. They are able to modify the cell gene expression and their inserted long terminal repeats (LTRs) can act as alternative promoters to stimulate expressions of nearby genes and cause the activation of oncogenes or inactivation of tumor suppressor genes [2, 3]. HERV-H and K transcripts are expressed in embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) at higher levels than in differentiated cells [4, 5]. The core transcription factors, including pluripotency factors such as POU class 5 homeobox 1 (OCT-4), sex-determining region Y-box 2 (SOX-2), and NANOG homeobox (NANOG), occupy approximately 80% of the LTRs and regulate the expression of the 50 most highly expressed HERV-H proviruses. Furthermore, naïve ESCs and iPSCs are associated with elevated transcription of HERV-H, so their expression may be an indicator of pluripotency [5–7]. Moreover, LTR7/HERV-H is one of the most overexpressed transposable elements seeding NANOG and POU5F1 binding sites throughout the human genome [8].

Mesenchymal stem cells (MSCs) are adult stem cells with an extensive self-renewal capacity and multipotent cells [9, 10]. SOX-2, NANOG, and OCT-4 are three transcription factors able to maintain the stem cell pluripotency and self-renewal [10, 11]. Based on these considerations, our aim was to assess *pol* gene expression of HERV-K and HERV-H in MSCs and to investigate their possible role as pluripotency markers in MSCs at different passages by real-time PCR.

Materials and Methods

BM-MSC Isolation and Expansion

Human bone marrow (BM) samples were collected from the iliac crest of adult or pediatric Caucasian donors who underwent BM collection for a familiar allo-BM transplantation. The study was performed at the Pediatric Onco-Hematology Unit, Stem Cell Transplantation and Cell Therapy Division, City of Science and Health of Turin, Regina Margherita Children's Hospital, Turin, Italy, after the submission of written consent, in accordance with the ethics committee, which approved the collection of the samples and according to the Declaration of Helsinki.

When available, the bag of collection sets (Baxter Healthcare, Deerfield, IL, USA) left over at the end of the filtration of BM explants was used for hematopoietic stem cell transplantation. The bag was washed three times with 1× phosphate-buffered saline (Lonza, Versviers, Belgium), and the cells were collected and washed at 200 *g* for 10 min. An aliquot of whole BM was counted and plated directly in T25 or T75 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at 1×10^4 cells/cm^2. The culture medium was α-MEM (Biochrome, Berlin, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mmol/L of L-glutamine (Sigma-Aldrich), and penicillin/streptomycin 1X (Euroclone, Pero, MI, Italy). The culture was maintained at 37 °C in a 5% CO_2 atmosphere. After 5–7 days, non-adherent cells were removed, and the adherent cells were re-fed every 3–4 days. To expand the isolated cells, the adherent semi-confluent monolayer was detached with trypsin/EDTA (Sigma-Aldrich) for 5 min at 37 °C and expanded for several passages until they no longer reached confluence [9].

MSC Analysis and Characterization

The BM-MSCs used for this study were analyzed for viability, immunophenotype, and differential and proliferative potential to verify the MSC characteristics. Flow cytometry analysis was performed to analyze the immunophenotype of MSCs using the following antibodies: anti-CD90 fluorescein isothiocyanate (FITC), CD73 phycoerythrin (PE), CD34 FITC, CD14 FITC, CD45 FITC (Beckman Coulter), and CD105 PC-7 and CD146 APC (Miltenyi Biotec, Bologna, Italy). Details of the cytofluorimetric analysis are described below. To analyze multipotent capacity, MSCs isolated from BM were cultured in osteogenic (StemCell Technologies), adipogenic (StemCell Technologies), and chondrogenic (Lonza, Cologne, Germany) media for 21 days, according to the manufacturer's instructions. Briefly, 5,000 and 10,000 cells, for control samples and differentiation experiments, were seeded in a 6-well plate in osteogenic and adipogenic culture conditions, respectively. Osteogenic differentiation was demonstrated by the accumulation of crystalline hydroxyapatite on von Kossa staining, and adipogenic differentiation by the presence of intracellular lipid vesicles assessed with oil red O. MSC chondrogenic differentiation was achieved as previously described and was evaluated by Alcian blue staining, which identifies the presence of hyaluronic acid and sialomucin [9, 10].

Reverse Transcription and Relative Quantification by Real-Time PCR

Total RNA was extracted from MSCs using the automated extractor Maxwell (Promega, Madison, WI, USA) using a simple RNA Blood Kit protocol without modification. One microgram of total RNA was reverse-transcribed with 8 μL of buffer ×10, 4.8 μL of $MgCl_2$ 25 mM, 2 μL of ImProm-II (Promega), 1 μL of RNase inhibitor 20 U/L, 0.4 μL of random hexamers 250 μM (Promega), 2 μL of mix dNTPs 100 mM (Promega), and dd-water in a final volume of 20 μL. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min at 25 °C, 60 min at 42 °C, and 15 min at 70 °C for the inactivation of enzyme; the cDNAs were stored at –80° until use.

Relative quantification of the mRNA expression of selected genes was achieved by means of TaqMan amplification and normalization to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which was chosen as a reference gene, using the ABI PRISM 7500 real-time system (Life Technologies, Austin, TX, USA). The expression of HERV-K, H *pol* gene, NANOG, OCT-4, SOX-2, and GAPDH was quantified by real-time PCR. Approximately 100 ng of cDNA were amplified in a 20-μL total volume reaction containing 2.5 U of Ampli-Taq Gold DNA polymerase (Applied Biosystems), 1.25 mmol/L of $MgCl_2$, and 500 nmol of each specific primer, and

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200 nmol of specific probe were used: (KPOLF-5'-CCACTGTAGAGCCTCCTAAACCC-3') (KPOLR-5'-TTGGTAGCGGCCACTGATTT-3') and probe (KPOLP-6FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTAA-TAMRA); HERV-H primers (HPOLF-5'-TGGACTGTGCTGCCGCAA-3') (HPOLR-5'-GAAGSTCATCAATATATTGAATAAGGTGAGA-3') and probe (HPOLP-6FAM-TTCAGGGACAGCCCTCGTTACTTCAGCCAAGCTC-TAMRA); GAPDH specific primers (GAPDHF-5'-CCAAGGTCATCCATGACAAC-3') (GAPDHR-5'-GTGGCAGTGATGGCATGGAC-3') and probe (GAPDH-6FAM-TGGTATCGTGGAAGGA-3' MGB); NANOG primers (NANOGF-5'-GCCAGGATGGTCTCGATCTC-3') (NANOGR-5'-GGTGGCTCACGCCTGTAAAT-3') and probe (NANOGP-6FAM- TGACCTTGTGATCCACCCGCCTC–TAMRA); OCT-4 primers (OCT-4F-5'-ACCCACACTGCAGCAGATCA-3') (OCT-4R-5'-CACACTCGGACCACATCCTTCT-3') and probe (OCT-4P-6FAM-CCACATCGCCCAGCAGCTTGG–TAMRA), and SOX-2 primers (SOX-2F-5'-TGCGAGCGCTGCACAT-3') (SOX-2R-5'-GCAGCGTGTACTTATCCTTCTTCA-3') and probe (SOX-2P-6FAM-CCGGCGGAAAACCAAGAC-GCT–TAMRA). The established assays used the probe and primers designed by Primer Express™ software version 3.0 (Applied Biosystems).

The amplifications were in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was run in triplicate. Relative quantification of target gene expression in patients compared with normal samples was performed with the ΔΔCt method and the relative results are expressed in corresponding arbitrary units.

Statistical Analysis

Statistical analyses were performed using the Prism software (GraphPad Software version 5.01 for Windows). The Shapiro-Wilk test was used to verify the normality of the data distribution. Because the MSCs are heterogeneous cells which can express different levels of stemness markers on the basis of the donor age for instance, we compared the different gene expression between paired samples at the different passages by paired *t* test in each MSC batch during the expansion. Multivariate analysis was excluded because there was no homogeneity of variance between the groups. Pearson's test was used to analyze the correlation between HERV-H and HERV-K expression and the embryonic markers. All statistical tests were considered significant at $p < 0.05$, and highly significant at $p < 0.001$.

Results

For quantitative evaluation of *pol* gene expression of HERV-K, HERV-H, NANOG, OCT-4, and SOX-2, real-time PCR by the TaqMan system was used. The threshold value Ct, computed for each of the genes as the average of 3 determinations, was used to measure the amount of PCR products.

The study group enrolled 15 BM-MSCs from passage 1 to 5 (p1-p5). Because retro-transcription efficiency was equivalent in all samples, as suggested by GAPDH expression, the hypothesis that the strong variations of signal intensity between BM-MSCs could be related to differences in HERV-K, HERV-H *pol*, NANOG, OCT-4, and SOX-2 transcriptional levels was formulated.

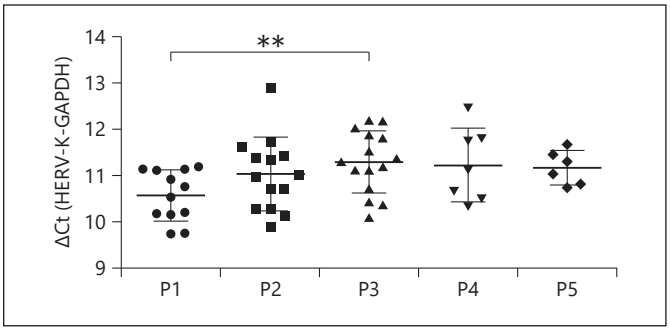

Fig. 1. Analysis by real-time quantitative RT-PCR of HERV-K gene expression at the different passages of the analyzed MSCs. ** $p < 0.01$.

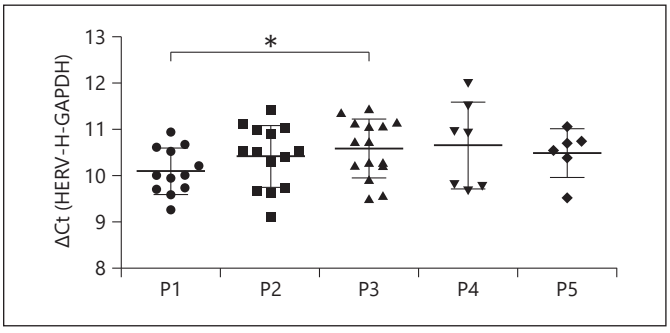

Fig. 2. Analysis by real-time quantitative RT-PCR of HERV-H *pol* gene expression at the different passages of the analyzed MSCs. * $p < 0.05$.

Relative Expression of HERV-K and HERV-H pol Gene in MSCs at Different Passages (p1-p5)

HERV-K expression was always higher at p1 than at other passages (Fig. 1) and its expression decreased among the passages analyzing the single MSC batch during the expansion. At p3 the expression was statistically lower ($p = 0.0071$) than p1. Conversely, no statistical significances were observed when passage p1 was compared with passages 2, 4, and 5 ($p = 0.1523$, 0.3328, and 0.4712, respectively). HERV-H followed a similar trend expression profile to that of HERV-K (Fig. 2). Moreover, this difference reached a statistical significance with $p = 0.049$ when passage p1 was compared with passage 3. Conversely, no statistical significance was reached when passage p1 was compared with passages 2, 4, and 5 ($p = 0.0744$, 0.4436, and 0.4278, respectively).

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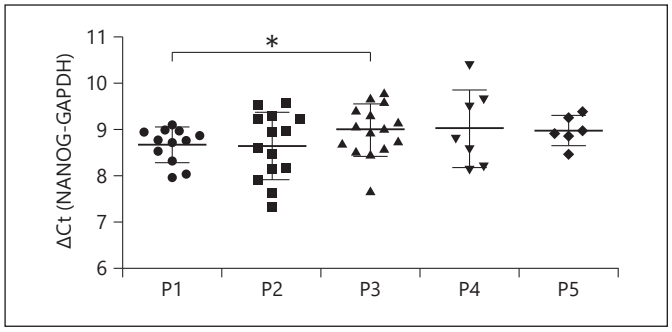

Fig. 3. Analysis by real-time quantitative RT-PCR of NANOG gene expression at the different passages of the analyzed MSCs. * $p < 0.05$.

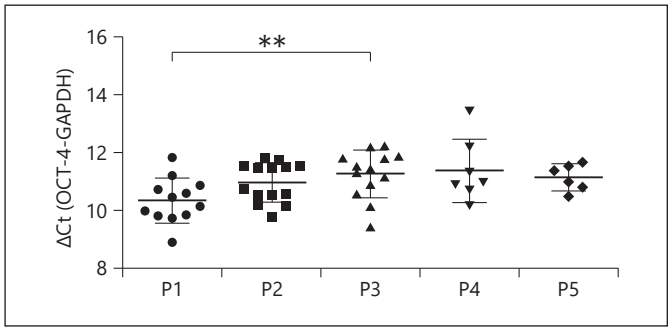

Fig. 4. Analysis by real-time quantitative RT-PCR of OCT-4 gene expression at the different passages of the analyzed MSCs. ** $p < 0.01$.

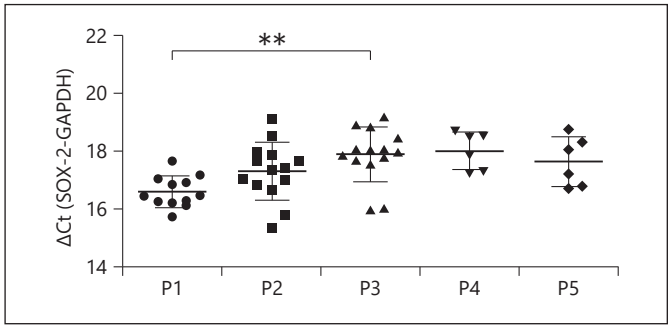

Fig. 5. Analysis by real-time quantitative RT-PCR of SOX-2 gene expression at the different passages of the analyzed MSCs. ** $p < 0.01$.

Relative Expression of NANOG, OCT-4, and SOX-2 in MSCs at Different Passages (p1-p5)

NANOG expression in each analyzed MSC batch decreased during the expansion after p1. The paired *t* test showed a statistically significant difference when NANOG expression was compared between p1 and p3 ($p = 0.0398$), as shown in Figure 3. *p* values calculated by the paired *t* test between NANOG expression at p1 and, respectively, at p2, p4, and p5 were 0.9572, 0.4828, and 0.2724.

Furthermore, OCT-4 expression at p1 was significantly higher than the expression at p3 ($p = 0.0151$), as shown in Figure 4. Conversely, no statistical significance was observed when passage p1 was compared with passages 2, 4, and 5 ($p = 0.1639$, 0.0821, and 0.142, respectively).

As shown in Figure 5, SOX-2 expression was also significantly higher at p1 than at p3 ($p = 0.0011$). No statistically significant differences were observed for SOX-2 expression between p1 and p2, p4, and p5 ($p = 0.1810$, 0.0054, and 0.0667, respectively).

Correlation Analysis of HERV-K and HERV-H Expression with Pluripotency Biomarkers NANOG, OCT-4, and SOX-2 Expression

Moreover, a correlation between the HERV-H and HERV-K *pol* gene expression levels, and the NANOG, OCT-4, and SOX-2 expression was analyzed. Pearson's test demonstrated a strong correlation between the expression of HERV-K and HERV-H and the expression of NANOG, OCT-4, and SOX-2 (Table 1). In particular, HERV-H was significantly correlated with: HERV-K expression at passage 2, 3, 4 and 5; NANOG expression at passage 2 and 3 with *p* values <0.0001, and at passage 4 with $p < 0.05$; OCT-4 expression at all passages, and SOX-2 at passage 3 and 5 with $p < 0.05$. HERV-K was also significantly correlated with NANOG and OCT-4 expression at passage 2, 3, 4, and 5.

The expression levels of NANOG, OCT-4, and SOX-2 were plotted among them. Pearson's test demonstrated a strong correlation between the expression of NANOG, OCT-4, and SOX-2 (data not shown), supporting the idea that an asynchrony between the genes was reached.

Discussion

The aim of our study was to evaluate the gene expression profile of HERV-H and HERV-K in MSCs at different passages. Our findings showed that these endogenous viruses were associated with the expression of the pluripotency biomarkers NANOG, OCT-4, and SOX-2. These results might suggest an important role of HERV *pol* genes in the MSC differentiation.

Recently, Santoni et al. [5] described a strong association between the HERV-H genomic location and H3K-4me3-modified histones, suggesting that HERV-H contributes to pluripotency in human ESCs and in some

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Table 1. Comparison of the expression levels of *pol* gene HERVs with pluripotency biomarkers NANOG, OCT4, and SOX calculated by Pearson's correlation test

Passage	Pearson's *r*	*p* value (two-tailed)	*p* value summary	Pearson's *r*	*p* value (two-tailed)	*p* value summary
HERV–H versus NANOG				*HERV–K versus NANOG*		
P1	0.1952	0.5889	ns	0.1206	0.74	ns
P2	0.9582	<0.0001	***	0.6999	0.0053	**
P3	0.8487	<0.0001	***	0.7135	0.0028	**
P4	0.8867	0.0078	**	0.883	0.0084	**
P5	0.6408	0.121	ns	0.7601	0.0473	*
HERV–H versus OCT-4				**HERV–K versus OCT-4**		
P1	0.289	0.3887	ns	0.2517	0.4554	ns
P2	0.8728	<0.0001	***	0.7599	0.0016	**
P3	0.8278	0.0005	***	0.6993	0.0078	**
P4	0.9036	0.0053	**	0.8917	0.007	**
P5	0.903	0.0053	**	0.9016	0.0055	**
HERV–H versus SOX2				**HERV–K versus SOX2**		
P1	0.8785	0.05572	ns	0.8877	0.05147	ns
P2	0.2668	0.3187	ns	0.2454	0.3977	ns
P3	0.563	0.0452	*	0.2877	0.3192	ns
P4	–0.04541	0.9319	ns	0.5596	0.2691	ns
P5	0.7578	0.0484	*	0.1625	0.5908	ns
HERV–H ve*rsu*s HERV-K						
P1	0.5853	0.0586	ns			
P2	0.7175	0.0039	**			
P3	0.5949	0.0193	*			
P4	0.7536	0.049	*			
P5	0.8608	0.0129	*			

* $p < 0.05$ (significant), ** $p < 0.001$ (highly significant), and *** $p < 0.0001$ (very highly significant). ns, not significant.

iPSCs. HERV-H expression was high in these pluripotent stem cells [5]. HERV-H transcripts are expressed in ESCs/iPSCs at higher levels than in differentiated cells. Approximately 80% of the LTRs belonging to the 50 most highly expressed HERV-H proviruses are occupied by core transcription factors involved in pluripotency, including OCT3/4, SOX-2, and NANOG [12]. In accordance with Santoni et al. [5], we demonstrated that HERV-H and HERV-K *pol* gene expression is correlated with NANOG, OCT-4, and SOX-2 expression. HERV-H and HERV-K can be exploited as a reliable marker of MSC cell pluripotency, as well as an indicator of the degree of "stemness." SOX-2 was more stably expressed during the differentiation than NANOG or OCT-4 expression and its expression did not correlate with that of HERV-H [5].

In accordance with our data, Fuchs et al. [4] demonstrated the same kinetics of HERV-K and OCT-4 and NANOG expression during reprogramming of the iPSCs in response to activation of the LTR5, and Ohnuki et al. [12] also demonstrated the same kinetics of HERV-H, OCT-4, and SOX-2 expression during reprogramming of the iPSC. HERV-H activation is inversely correlated with the DNA methylation status, and the activated copies are marked with transcriptionally active histone marks (H3K4me1/2/3, H3K9ac, H3K36me3, and H3K79me2), while the repressive marks (H3K9me3 and H3K27me3) are rare [13]. The pluripotency factors NANOG, OCT-4, KLF4, and LBP9/Tfcp2l1 all bind to active LTR7s of HERV-H and drive transcription of HERV-H-derived transcripts. Transactivation of LTR5_Hs/HERV-K by OCT-4 at hypomethylated LTR elements are also demonstrated. In hESCs, Göke et al. [14] found that the sequence-specific transcription factor NANOG regulates LTR7 expression. The dynamic expression pattern of ERV families may result from a combination of activating and silencing mechanisms that integrate ERV elements

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into the regulatory networks of early human embryos [15]. Moreover, this mechanism induces HERV-K expression during normal human embryogenesis, beginning with embryonic genome activation at the eight-cell stage, continuing through the stage of epiblast cells in preimplantation blastocysts, and ceasing during hESC derivation from blastocyst outgrowths [13, 15]. Grow et al. [16] also reported unequivocal experimental evidence demonstrating the presence of HERV-K viral-like particles and Gag proteins in human blastocysts, consistent with the idea that HERVs are functionally active during early human embryonic development.

In conclusion, our study demonstrates that human HERV-K and HERV-H elements show the same kinetics as pluripotency biomarkers NANOG, OCT-4, and SOX-2, and should be considered as new markers of stemness or differentiation in BM-MSCs.

Statement of Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of University of Turin research committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Disclosure Statement

The authors declare that they have no conflicts of interest.

Funding Sources

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