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Alterations in gene expression in human umbilical cord blood CD34+ hematopoietic progenitor cells after lentiviral vector transduction

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Summary. *Aim:* Transplantation of genetically modified human umbilical cord blood (UCB)-derived CD34+ hematopoietic stem/progenitor cells has emerged as a promising therapy for various malignant and non-malignant hematologic disorders. In turn, lentiviral vectors have been considered as suitable gene delivery vehicles for hematopoietic progenitor cells. However, their safety/risk profiles need to be further assessed. This study aimed to analyze the proviral-genomic integration sites and gene expression in human UCB-derived CD34+ cells transduced with a third generation HIV-1-based, vesicular stomatitis virus G glycoprotein-pseudotyped, GFP-tagged self-inactivating lentiviral vector. *Materials and Methods:* CD34+ cells, isolated from UCB of healthy full-term newborns, were transduced with the lentiviral vector. Proviral-genomic integration sites were analyzed by linear amplification-mediated polymerase chain reaction and DNA sequencing. Differential gene expression was analyzed by cDNA microarray. *Results:* Seven integration sites were identified. Two genes were up-regulated and six down-regulated, all by more than 2-fold, in the lentiviral vector-transduced CD34+ cells. The two up-regulated genes were IGSF4 (ImmunoGlobin Super Family member 4) and HEC (Highly Expressed in Cancer, rich in leucine heptad repeats), which are involved in leukemia and cell division. *Conclusions:* Given the importance of some of the differentially expressed genes detected in this study, the safety/risk profiles of lentiviral vectors as gene delivery vehicles for UCB-derived hematopoietic stem/progenitor cells warrant particular attention and further investigations.

Key words: lentiviral vector, genetic modification, umbilical cord blood CD34+ cells, integration sites, differential gene expression

«Alterazioni nell'espressione genica nelle cellule ematopoietiche progenitrici CD34+ del cordone ombelicale umano dopo trasduzione con vettore lentivirale»

Riassunto. *Scopo:* Il trapianto di cellule ematopoietiche staminali/progenitrici geneticamente modificate CD34+ derivate da cordone ombelicale umano si è rivelato come promettente terapia per diverse patologie ematologiche sia maligne che non. A sua volta, i vettori lentivirali sono stati considerati come trasportatori appropriati per i geni delle cellule progenitrici ematopoietiche. Tuttavia i loro profili di sicurezza/rischio necessitano di essere accertati. Questo studio ha l'obiettivo di analizzare sia i siti di integrazione genomici provirali che l'espressione genetica nelle cellule CD34+ derivate da cordone ombelicale umano trasdotte attraverso una terza generazione di virus basati su HIV-1, virus della stomatite vescicolare pseudo tipizzato glicoproteina G, vettore lentivirale autoinattivante marcato GFP. *Materiali e metodi:* Le cellule CD34+, isolate dal cordone ombelicale umano di neonati nati a termine in salute, sono state trasdotte con il vettore lentivirale. I siti di integrazione genomici provirali sono stati analizzati attraverso una amplificazione lineare mediata da catena di reazione polimerica (PCR) e sequenziamento del DNA. La differente espressione genetica è stata analizzata mediante cDNA microarray. *Risultati:* sono stati identificati sette siti di integrazione. Due geni erano

sovraregolati e sei sottoregolati, tutti per più di due volte, nelle cellule CD34+ trasdotte con vettore lentivirale. I due geni sovraregolati erano IGSF4 (Super Famiglia Immunoglobina 4) e HEC (Altamente espresso nel cancro, ricco in leucina ripetuta 7 volte), che sono coinvolti nella leucemia e nella divisione cellulare. *Conclusioni:* Data l'importanza di alcuni dei geni espressi in modo differenziale scoperti in questo studio, i profili di sicurezza/rischio di vettori lentivirali come trasportatori genetici per le cellule ematopoietiche staminali/progenitrici derivate dal cordone ombelicale umano, necessitano di particolare attenzione e di ulteriori indagini.

Parole chiave: vettore lentivirale, modificazione genetica, cellule di cordone ombelicale CD34+ , siti di integrazione, espressione genetica differenziale

Introduction

Hematopoietic stem/progenitor cells are capable of self-renewal and multipotency (1). Transplantation of genetically modified autologous or allogeneic hematopoietic stem/progenitor cells represents a promising cell replacement therapy for various hematologic and non-hematologic disorders (2). Although bone marrow is the first identified source of hematopoietic stem cells, umbilical cord blood (UCB) has emerged as an alternative to bone marrow as one of the most common sources of CD34+ hematopoietic stem/progenitor cells (3-5) since the successful treatment of a 5-year-old boy suffering from Fanconi anemia by transplantation of UCB cells isolated from his newborn sister with a normal karyotype was reported in the New England Journal of Medicine in 1989 (6). Relatively speaking, UCB-derived stem/progenitor cells can tolerate a higher degree of human leukocyte antigen (HLA) disparity and thereby may result in lower incidence of graft-versus-host disease when used in cell replacement therapy (7,8).

Efficient *ex vivo* gene delivery is fundamental to the success of UCB hematopoietic progenitor cell-based replacement therapy (9). To date, retroviral vectors derived from Moloney murine leukemia virus (MoMLV) have been most commonly used in the delivery of corrective genes to hematopoietic stem cells. However, retroviral vectors are inefficient in transducing non-dividing or slowly dividing cells (10) while hematopoietic stem/progenitor cells are generally quiescent (11). Moreover, retroviral vectors are associated with the risk of insertional mutagenesis. In the French trial on severe combined immunodeficiency (SCID) gene therapy, consecutively 5 out of 10 subjects developed leukemia after receiving retroviral vector-transduced hematopoietic cells (12-14). Because of this incidence, the US Food and Drug Administration (FDA) suspended all similar trials using retroviral vectors (15), while the UK likewise had to stop the world's only active SCID trial at the time (13). In another gene therapy trial aimed at treating X-linked chronic granulomatous disease (X-CGD), 2 subjects developed myelodysplasia, as a result of insertional activation of the ecotropic viral integration site, after receiving retrovirus modified autologous hematopoietic stem cells, and one of them died from severe sepsis 27 months after therapy (16).

Given their capacity for effectively transducing not only actively dividing but also non-dividing quiescent cells (17), lentiviral vectors are considered as more suitable vehicles than retroviral vectors when it comes to delivering corrective genes to hematopoietic cells. Several *ex vivo* studies have demonstrated efficient transduction of primitive human blood-derived CD34+ hematopoietic progenitor cells by third generation vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped human immunodeficiency virus type 1 (HIV-1)-based self-inactivating lentiviral vectors (18-21). Preclinical studies have demonstrated the therapeutic potential of UCB-derived hematopoietic stem/progenitor cells after lentiviral vector transduction (22, 23). In our previous study, we also demonstrated that HIV(VSV) lentiviral vectors do not significantly influence the gene expression of human CD34+ cord blood cells (24). However, like retroviral vectors, lentiviral vectors also bear a potential risk of insertional mutagenesis. Schmidt and colleagues observed multiple vector copies (5.6±3.3, n=12) present in the human hematopoietic cells transduced with len-

tiviral vectors and detected one integration site in a known tumor suppressor gene BRCA1, suggesting an association of increased insertional mutagenesis risk of lentiviral vectors in hematopoietic cells (25). Apparently, the safety/risk profiles of lentiviral vector-transduced UCB-derived hematopoietic stem/progenitor cells still need more thoroug assessment before more clinical trials are performed. The present study was conducted: 1) to analyze the viral integration sites in primitive human UCB CD34+ after transduction with HIV(VSV) lentiviral vectors expressing humanized *Renilla reniformis* green fluorescence protein (GFP); 2) to determine the effect of this lentiviral vector on gene expression in the transduced cells.

Materials and methods

CD34+ cell isolation and culture

Umbilical cord blood samples were collected from healthy full-term newborns into heparin tubes. Buffy coats were prepared by Ficoll-Hypaque gradient centrifugation. Mononuclear CD34+ hematopoietic progenitor cells were isolated using the Diamond CD34+ Isolation Kit from Miltenyi Biotec Shanghai Office (Shanghai, China) as instructed by the manufacturer. The isolated cells were cultured in RPMI-1640 (Life Technologies, Grand Island, NY, USA) supplemented with serum substitute BIT9500, stem cell factor, IL-3 (Kirin Brewery Co., Ltd, Tokyo, Japan) as previously described (19). The protocols for cord blood sample collection, CD34+ cell isolation and virus manipulation were reviewed and approved by the Institutional Review Board of Jilin University. Written informed consent was obtained from all cord blood donor mothers.

Vector production and titration

An HIV(VSV) lentiviral vector system was used. The construction of the four plasmids, including pCSCDF-ChG-PRE, pMDLg/p.RRE, pRSV-rev and pMD.g, is outlined in reference (19). HIV(VSV) lentiviral vector generation and titration were carried out as previously described in reference (19). Briefly, human embryonic kidney 293T cells were seeded onto 10-cm dishes at 2×10^6 cells/dish. Next day, cells were co-transfected with the four plasmids by the calcium phosphate precipitation method. After an initial culture for 12 h, DNA precipitate-containing medium was replaced with fresh culture medium. Conditioned media were collected 48 and 72 h after transfection and pooled. After passing through 0.22 mm filters (Millipore, Bedford, MA, USA), the media were concentrated at 50,000 g for 2 h. Viral stocks were made by diluting the resultant viral pellet from ultracentrifugation in serum-free Iscove's modified Dulbecco's medium and stored at -80°C until use. Viral titers of the stocks were determined in HeLa cells by flow cytometry.

Vector transduction

Cord blood CD34+ cells were seeded onto 24-well plates at 1×10^4 cells/ml in RPMI-1640 supplemented with 10% BIT 9500 for 12 h and transduced with the viral stocks generated above at a multiplicity of infection (MOI) of 0.5. Two hours after transduction, cells were washed with PBS and cultured in RPMI-1640 supplemented with 10% BIT 9500, 25 ng/ml SCF, 10 ng/ml IL-3 for two days. The fluorescence signal of GFP was checked under a fluorescence microscope and the transduction efficiency was assessed by flow cytometry using one replicate culture. The remaining cells were cultured for 5 more days and then subjected to DNA and RNA extraction for use in other analyses.

Viral integration analysis

This was done by linear amplification-mediated polymerase chain reaction (LAM-PCR) and DNA sequencing. In step 1, the long terminal repeat (LTR) vector-genome junctions were pre-amplified through a linear PCR (50 cycles: 95°C for 1 min, 60°C for 45 sec, 72°C for 1 min) with biotinylated LTR primers LTR-U5-1 ([BiotinTEG]-GAGCTCTCTGGCTAACTAGG-3') and LTR-U5-2 ([BiotinTEG]-GAACCCACTGCTTAAGCCTCA-3') respectively. PCR products were purified using the μMACS Streptavidin Kit from Miltenyi Biotec Shanghai Office as instructed by the manufacturer. In step 2, complementary double strands of DNA were synthesized by hexanucleotide priming using the synthesized single-strand DNA in

step 1. Products were purified as in step 1. In steps 3 and 4, purified double-strand DNAs were digested by restriction enzyme TasI (New England BioLabs Inc., Ipswich, MA, USA). The linker cassette with a known sequence was ligated to the genomic end of the fragments with unknown sequences using T4 DNA ligase. In step 5, the double-strand DNA composing the linker cassette, genomic DNA and vector DNA was denatured. The resultant single-strand DNA was amplified by nested PCR using 2 sets of linker- and vector-specific primers: viral LTR II (5'-AGC TTG CCT TGA GTG CTTCA-3') and linker cassette 1 (5'-GACCCGGGAGATCTGAATTC-3'); viral LTR III (5'-AGT AGT GTGTGCCCGTCTGT-3') and linker cassette 2 (GATCTGAATTCAGTG-GCACAG-3'). In step 6, the nested PCR products were separated on and purified from 2.5% agarose gels (Fig. 1) and cloned into plasmid pCRII-TOPO using TOPO TA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Positive clones were sequenced using the ABI BigDye automated sequencing kit from Applied Biosystems China (Beijing, China). Lentiviral integration was analyzed by comparing sequences of these clones with known sequences derived from the BLAST (Basic Local Alignment Search Tool) database.

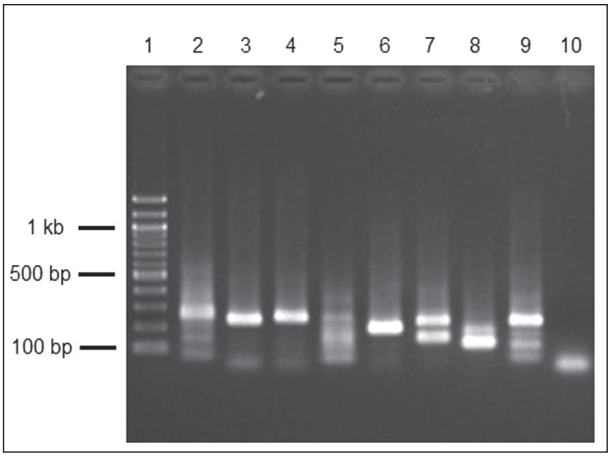

Figure 1. Photograph of LAM-PCR products after ethidium bromide staining on an agarose gel. Lane 1: 100 bp DNA marker; lanes 2-9: LAM-PCR products of various sizes; lane 10: negative control.

Differential expression analysis

Total RNA was isolated from control CD34+ cells and HIV(VSV) lentiviral vector-transduced CD34+ cells with the RNeasy Mini Kit from QIAGEN China (Shanghai, China). RNA to cDNA conversion was achieved through reverse transcription using a SuperScript® III first-strand synthesis system kit (Life Technologies, Inc.) according to the manufacturer's instructions. The cDNAs reversely transcribed from RNA samples extracted from the control and virus-transduced CD34+ cells were labeled with Cye3 and Cye5 (GE Life Science), respectively. Fluorescently labeled cDNAs were subsequently hybridized to microarray chips comprising 23,000 human genes as reported previously (26, 27). Briefly, labeled probes were mixed with microarray hybridization solution version 2 (GE Life Science) and formamide (Sigma) to a final concentration of 50%. After hybridization for 14-16 h at 42°C, the slides were washed in 2× SSC and 1% SDS for 10 min at 55°C, washed in 0.2×SSC and 0.1% SDS for 10 min at 55°C, then washed in 0.1×SSC for 1 min at room temperature. Following washing, hybridization signals were scanned at ~540 nm (green) for the control and ~630 nm (red) for the experimental channel respectively, using the Array Scanner Generation III (GE Life Science). The intensity of each hybridization signal was evaluated photometrically by the Array Vision computer program and normalized to the averaged signals of housekeeping genes. The Cye5:Cye3 ratio for each sample was calculated by averaging spots. A cut-off value for each expression level was automatically calculated according to the background fluctuation, as previously described (27). A normalized Cye5:Cye3 ratio greater than 2 and less than 0.5 was the criterion for up-regulation and down-regulation respectively.

Statistical analysis

For gene ontology analyses, we only considered classes significant when they were represented by at least 3, a fold increase by more than 3 or decrease by less than 0.33, and a P value of less than 0.05. The results were corrected for multiple testing errors within each data set/system combination with Bonferroni's meth-

od. For gene expression analysis, the 1-way ANOVA test with Bonferroni's multiple comparison post-hoc test was used to assess the statistical significance of differences among all samples ($P < 0.05$). In all graphs, the mean ± standard deviations are indicated.

Results

Pre-viral integration

After the LAM-PCR products were cloned into plasmid pCRII-TOPO, 24 positive clones were picked up and viral integrations were detected in 13 of these 24 clones. Table 1 presents the sequences of these integrations. Seven integration sites were detected: two on chromosome 7, two on chromosome 10, one each on chromosomes 3, 6 and 21. False positive integration was detected in three of the 13 clones, possibly due to primer annealing.

Alterations in the host genome after viral transduction

To determine the effect of the HIV(VSV) lentiviral vector on gene expression in human UCB-derived hematopoietic stem/progenitor cells, differential expression was performed on the vector-transduced and naïve UCB CD34+ cells by cDNA microarray using a Chip of 23,000 genes. As shown in Fig. 2, for comparisons of expression levels, the relative expression

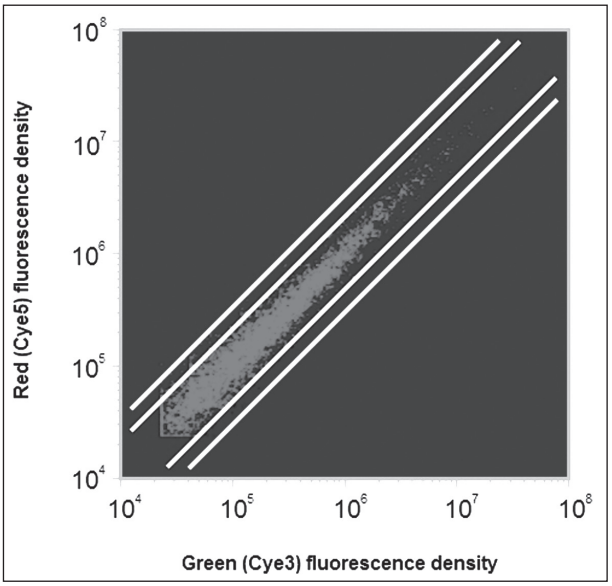

Figure 2. Representative scatter plots of cDNA microarray analysis. Lentiviral vector-transduced CD34+ cells (labeled with Cy5) and naïve CD34+ cells (labeled with Cy3) were labeled and hybridized by the cDNA microarray.

Table 1. Proviral integration sites and flanking sequences in the genome of human UCB-derived CD34+ hematopoietic cells.

Integration ID	Colony #	Viral fragment	Host fragment	Host genome sequence	Localization
1	1	149 bp	37 bp	AATTCCATGTGCCAATAATGAAACTTAGAGTTCTTTG	Human chr 6
2	2	244 bp	132 bp	AATTGAAAGGAGAAATAAATGAGGGCTCAGTTTCCGGT	Human chr 7
3	10	135 bp	23 bp	CACACTGATTAAACTATTAAATT	Human chr 10
4	13, 14, 15	204 bp	92 bp	AATTAACCTCACTGTGTATCAAACTGGTGGCACAACCA	Human chr 3
5	16, 22, 23	236 bp	207 bp		HIV-1 genome
6	17	173 bp	61 bp	ATGACCTTCATCATTTTTTATGGATTAAGAGACTGATA	Human chr 7
7	18	158 bp	46 bp	AATTATCTGGGCGTGGTGGCGGGCACCTGTAATCCCAGT	Human chr 21
8	20, 21	139 bp	27 bp	AATTATTATGTTATAGAACTATAAAAG	Human chr 10

Table 2. Differentially expressed genes in human cord blood CD34+ hematopoietic progenitor cells after lentiviral transduction.

Gene name	Abbreviation	Cye5/Cye3 ratio
Immunoglobulin super family, member 4	IGSF4	2.70
Highly expressed in cancer, rich in leucine heptad repeats	HEC	2.36
Heterogeneous nuclear ribonucleoprotein A0	HNRPA0	0.34
Zinc finger protein 205	ZNF205	0.37
Filamin A, alpha (actin-binding protein-280)	FLNA	0.38
CDC-like kinase 3	CLK3	0.41
LIM domain binding protein 1	LDB1	0.44
Actin, alpha 1, skeletal muscle	ACTA1	0.46

of each gene was recorded in one of three categories: (a) up-regulated in lentiviral vector-transduced cells (Cy5:Cy3 signal ratio > 2.0); (b) down-regulated in lentiviral vector-transduced cells (Cy5:Cy3 ratio < 0.5); (c) unchanged in naïve or transduced cells. An accurate report of up-regulated and down-regulated results is given in Table 2, two genes being up-regulated and six down-regulated in HIV(VSV) lentiviral vector-transduced cells as compared with naïve cells. The two up-regulated genes were IGSF4 (immunoglobulin super family member 4) and HEC (highly expressed in cancer, rich in leucine heptad repeats). The 6 down-regulated genes include transcription factors, cell cycle regulating kinases and skeletal proteins. Based on normalized Cye5/Cye3 ratios, the difference between virally transduced cells and naïve cells was higher than 2 fold for all these differentially expressed genes.

Discussion

Genetically modified UCB CD34+ multipotent hematopoietic cells are more valuable than naïve cells in cell replacement medicine. But successful genetic modification relies on efficient delivery and stable expression of the therapeutic gene in hematopoietic cells with a long-term repopulating ability to achieve enduring correction. Lentiviral vectors are capable of stably integrating the genome of both actively dividing and mitotically quiescent cells, thereby offering a suitable means of gene delivery for UCB-derived hematopoietic stem/progenitor cells. However, before lentiviral vectors can be used in clinical settings, their safety and risk profiles have to be more thoroughly assessed.

It is known that successful correction of defective genes in viral vector-mediated genetic modification requires integration of adequate functional copies of transgenes into target cells. However, integration of therapeutic vectors at excessive or wrong sites may activate proto-oncogenes, resulting in tumorigenesis. In the present study, through LAM-PCR and DNA sequencing analysis, we detected a total of seven integration sites in the genome of primary UCB-derived CD34+ cells after transduction with HIV(VSV) lentiviral vectors. These integrations were located in chromosomes 3, 6, 7, 10 and 21. In a previous study, Di Nunzio and Piovani analyzed RD114-TR-pseudotyped, HIV-1-derived lentiviral vectors for their ability to transduce human cord blood CD34(+) hematopoietic stem/progenitor cells. Potential changes in the CD34(+) cell transcription profile and phenotype on transduction with RD114-TR-pseudotyped vectors were comparatively investigated by microarray analysis, compared with a standard VSV-G-based packaging system. The biology of repopulating hematopoietic stem cells and their progeny was not affected by transduction with RD114-TR-pseudotyped lentiviral vectors (28). Nevertheless, further investigations are warranted to make sure that genetic modification of human hematopoietic stem cells is safe and efficient.

To determine the adverse effects of lentiviral vectors on blood hematopoietic stem/progenitor cells, we performed cDNA microarray on naïve and HIV(VSV) lentiviral vector-transduced CD34+ cells isolated from UCB from normal full-term newborns. Out of 23,000

human genes, 2 were up-regulated and 6 down-regulated (Table 2). Although the difference between virally transduced cells and naïve cells was higher than 2 fold for all these differentially expressed genes, according to the corresponding Cye5/Cye3 ratio, the vector-mediated up-regulation of IGSF4 and HEC may pose more serious safety risks. The 6 down-regulated genes included transcription factors, cell cycle regulating kinases and skeletal proteins. To our knowledge, none of these down-regulated genes was associated with tumorigenesis or tumor suppressor activity.

In contrast to tumor suppressor activity, tumorigenic effects were later demonstrated for IGSF4: remarkably increased (30-fold) ectopic expression of IGSF4 was detected in adult T-cell leukemia (ATL) patients (29) and primary ATL cells with high TSLC1 expression were shown to cause more tumor formation and aggressive infiltration of various organs in NOD/SCID mice (30). IGSF4 is now regarded as a novel cell surface marker for adult T-cell leukemia/lymphoma (31). Given the reported involvement of IGSF4 in leukemia and the lentivirus induction of IGSF4 up-regulation in UCB-derived hematopoietic stem/progenitor cells observed in the present study, together with the occurrence of leukemia in the French SCID trial involving the use of viral vectors, there is an urgent need for more extensive and intensive assessment of the safety/risk of lentiviral vectors as gene delivery vehicles for UCB-derived hematopoietic stem/progenitor cells.

HEC is a novel nuclear protein, to date, and the function of this newly identified nuclear protein has not been well defined, but the currently available literature indicates that HEC may modulate M phase progression, partially, through the regulation of proteasome-mediated degradation of cell cycle regulatory proteins (32). Since insertional oncogene activation is a potential risk of viral vectors and oncogene activation results in increased cell division in an uncontrolled manner, our observation of more than 2-fold up-regulation of HEC gene expression in primary human UCB-derived hematopoietic stem/progenitor cells after HIV(VSV) lentiviral vector transduction in this study may suggest a likelihood of potential insertional mutagenesis in lentivirus transduced hematopoietic stem/progenitor cells.

In summary, we detected a total of seven integration sites in primary CD34+ cells isolated from human UCB samples from healthy full-term newborns after transduction with a HIV(VSV) lentiviral vector. We also demonstrated that out of 23,000 genes IGSF4 and HEC were up-regulated. To our best knowledge, lentiviral vector induction of up-regulation of IGSF and HEC has never been reported in the literature.

Conclusions

Given the importance of some of the differentially expressed genes detected in this study, IGSF4 in particular, in insertional mutagenesis and leukemia, the safety/risk profiles of lentiviral vectors as gene delivery vehicles for UCB-derived hematopoietic stem/progenitor cells warrant particular attention and further investigations.

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