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# Establishment of Modified Retroviral Vector Targeting X-Linked Severe Combined Immunodeficiency

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**Abstract**

Gene therapy targeting hematopoietic stem cells has been proposed as a potential therapy for numerous genetic disorders affecting hematopoiesis. Moloney murine leukemia retroviral vectors are now widely used for clinical gene transfer into hematopoietic progenitors and progeny. However, maintaining expression of therapeutic genes inserted via moloney murine leukemia virus (MoMLV)-based vectors has proven to be more difficult than previously expected. In this study, an MND-IL-2R vector containing IL-2Rcγ cDNA to treat X-linked severe combined immunodeficiency (X-SCID) was constructed from an MND vector that was modified by substituting the myeloproliferative sarcoma virus (MPSV) enhancer for that of MoMLV, deleting the negative control region located in the long terminal repeat (LTR) as an enhancer, and replacing the primer binding site (PBS) of MoMLV with the PBS of the endogenous murine retrovirus dl587rev. This vector was transduced into human CD34+progenitor cells with comparable efficiency to that of the MoMLV-based vector. The use of this newly created vector may be advantageous for gene therapy of X-SCID.
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**Key words**: Gene therapy, X-linked severe combined immunodeficiency, hematopoietic stem cells, retrovirus vector

## Introduction

Retroviral vectors based on the Moloney murine leukemia virus (MoMLV) are the most commonly used and studied vectors for gene therapy targeting hematopoietic stem cells (HSCs)[1]. However, despite the reasonable efficiency of in vitro transduction into hematopoietic stem cells, long-term expression of the transgene has not yet been achieved with retroviral vectors in the majority clinical studies.

X-linked severe combined immunodeficiency (X-SCID) is the most common form of severe combined immunodeficiency and is characterized by an early block in T and natural killer lymphocyte differentiation. This disorder is caused by mutations of genes encoding the γ-cytokine receptor subunit of interleukin-2, -4, -7, -9 and -15 receptors[2,3]. Infection by bacteria, viruses or fungi can be fatal to those with this deficiency, unless allogeneic hematopoietic stem cell transplantation (HST) is successfully performed. However, before HST can be performed, HLA-iden-

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tical donors are necessary, and even after this procedure, some patients continue to exhibit persistent deficiencies in T-cell function[4]. This has prompted several investigators to develop gene therapy techniques to treat X-SCID by gene transfer into hematopoietic stem cells[5,6]. Clinical studies of gene therapy using retroviral vectors have recently begun in patients with X-SCID. Therapeutic gene transfer into hematopoietic stem cells has resulted in not only in vitro data, but also clinical evidence of immune function and has increased our understanding of gene therapy itself[7,8]. In this study, we constructed a retrovirus vector with MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted) in order to improve gene therapy of X-SCID, and we evaluated the transduction efficiency of this vector into human CD34+hematopoietic progenitors.

## Materials and Methods

### Retroviral vector construction

The XbaI fragment containing the IL-2Rcγ cDNA sequence was isolated from pSRG1/IL-2R (kindly supplied by Dr. Asao) and inserted into pGEM7zf to produce pGEM7zf/IL-2R. PG1 is based on the MoMLV originating from the LN vector constructed by A. Dusty Miller[9]. To construct PG1-IL-2R, the IL-2 Rcr fragment was digested with XhoI and ApaI. MND-IL-2R plasmid was constructed by insertion of the PCR amplified IL-2Rcγ fragments into pMND-MFG[10]. Briefly, using pGEM7zf/IL-2R as a template, the 220 bp 5' fragment starting at the initiation codon and the 920 bp 3' fragment were separately amplified by PCR. The oligonucleotide primers were 5'-AGAGCCAAGCGACATGTTGAAGCC-3' and 5' CAATTCATGTAGTCGACATTGAA-3' for the 5' fragment and 5'-TTGTGTTCAATCTCGAGTACATG-3' and 5'-GGGCTACAGGATCCTGGGGAAGA-3' for the 3' fragment. The two amplified fragments were inserted into the NcoI and ClaI sites of pMND-MFG by triple ligation. The detailed protocol will be provided upon request.

### Establishment of retroviral packaging cells

Two types of high-titer amphotropic virus-producing cells (GP Am12/PG1-IL-2R, GP Am12/MND-IL-2R) were created by multiple infection with ecotropic viral supernatants from transient ecotropic virus-producing BOSC 23 cells[11]. Briefly, BOSC 23 cells were first transfected by calcium phosphate coprecipitation of the recombinant viral constructs pPG1-IL-2R and pMND-IL-2R. Ecotropic viral supernatants were collected from the transfected BOSC 23 cells. Amphotropic GP Am12 cells[12] were made by repeated (10 times) transduction with a mixture of viral supernatants (5 m*l*) and standard medium (5 m*l*). Expression of the IL-2Rcγ in the GPAm12 cells was confirmed using flow cytometry analysis, as described below.

### FACS analysis

GP Am12 cells were washed twice and resuspended in phosphate-buffered saline. Cells were stained in 200 μ*l* with biotin conjugated anti IL-2Rcγ (human TUGH4) antibody (Pharmingen) on ice for 30 minutes, and excess antibody was removed by washing. Cells were sequentially stained with conphycoerythrin (PE)-conjugated streptavidin (Pharmingen). CD34+progenitors were additionally stained with fluorescein isothiocyanate (FITC)-anti-human CD34 antibody (Pharmingen).

### Transduction of human CD34+cell

Human CD34+hematopoietic progenitors from cord blood were isolated with a Dynabeads M-450 CD34 kit, and transduced three times with viral supernatant from GP Am12/PG1-IL-2R and GP Am12/MND-IL-2R cells in the presence of CH296, interleukin IL-3, IL-6, SCF, TPO and Flt3 for 3 consecutive days.

### Clonogenic assay and detection of retroviral vector in colonies by PCR

To evaluate the transduction efficiency, transduced CD34+progenitors were plated in methylcellulose containing 50 ng/m*l* of SCF, 10 ng/m*l* of GMCSF, 10 ng/m*l* IL-3, and 3 units/m*l* of EPO (MethoCult™ GF H4434) for committed progenitors. After ten days, the colonies were isolated and analyzed individually by PCR for the presence of the retroviral vector and were subjected to southern blot analysis[13].

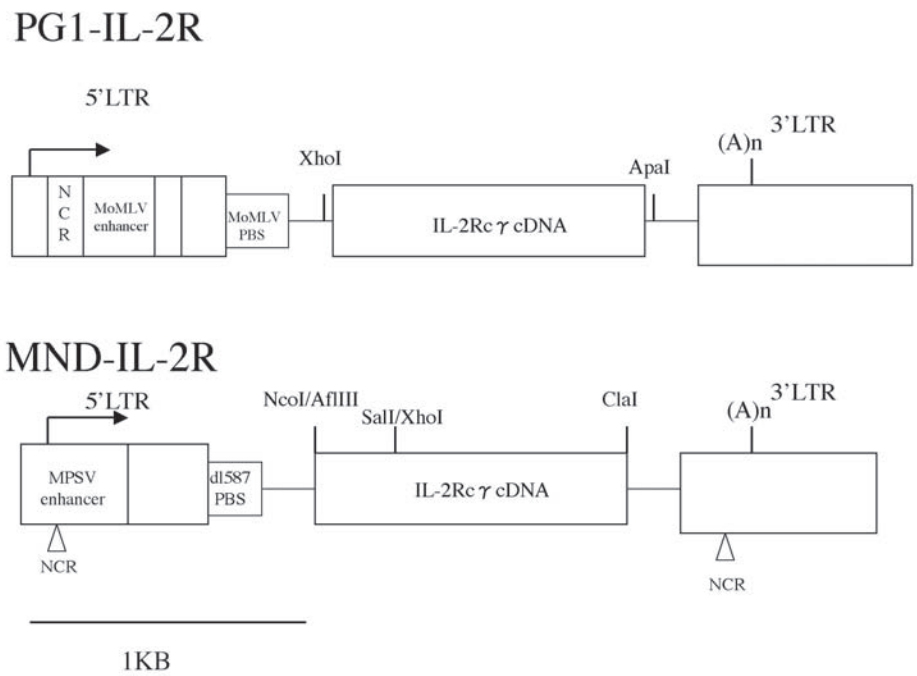


Fig. 1 Diagram of retroviral vectors

Vectors were constructed so that LTRs drive IL-2Rcγ genes in both constructs. NCR; negative control region, PBS; primer binding site, dl587PBS; endogenous murine retrovirus dl587rev, (A) n; polyadenylation signal.

The MND-LTR was modified by replacing the MoMLV enhancer with the MPSV enhancer, deleting the NCR and replacing the PBS of the MoMLV with that of the endogenous murine retrovirus dl587rev.

## Results

### Vector producing cells

As shown in **Fig. 1**, two types of vector plasmids (pPG1-IL-2R, pMND-IL-2R) were constructed. Vectors were designed such that the long terminal repeat (LTR) drives IL-2Rcγ. The 5' LTR of PG1-IL-2R was the Mo-MLV LTR and that of MND-IL-2R was modified by substituting the MPSV enhancer for that of MoMLV before deleting the negative control region and replacing the PBS of the MoMLV to that of the endogenous murine retrovirus dl587rev. Two types of high-titer amphotropic virus-producing cells (GP Am12/PG1-IL-2R, GP Am12/MND-IL-2R) were created by transduction with ecotropic viral supernatants from BOSC 23 cells. Since both vectors lacked a drug-selectable marker, a direct measurement could not be made for the viral titer of packaging cells. Stable high expression of IL-2Rcγ both on the GPAm12 cells was confirmed by flow cytometory (**Fig. 2**).

### Expression of IL-2 Rcγ on CD34+cells

Human CD34+hematopoietic progenitor cells were transduced with PG1-IL-2R and MND-IL-2R vectors for three days. When CD34+cells were maintained in the presence of IL-3, IL-6, SCF, TPO and Flt 3, progenitors began to differentiate and expression of CD34 was reduced. On the forth day, 19.5% of cultured cells with growth factors were double positive for anti-CD34 and anti-TUGH4 antibodies, while double-positive cells increased to 27.4% and 27.2% after transduction with the IL-2Rcγ transgene via the PG1-IL-2R and MND-IL-2R vectors (**Fig. 3**).

### Clonogenic assay and detection of retroviral vector in colonies by PCR

The transduced CD34+progenitor cells were plated onto methylcellulose and hematopoietic colonies were detected. Transduction efficiency into GM-colonies was 32% for the MND-IL-2R vector and 34% for the PG1-IL-2R vector, as determined by PCR-southern blot analysis in two separate experiments (**Fig. 4**) (**Table 1**).

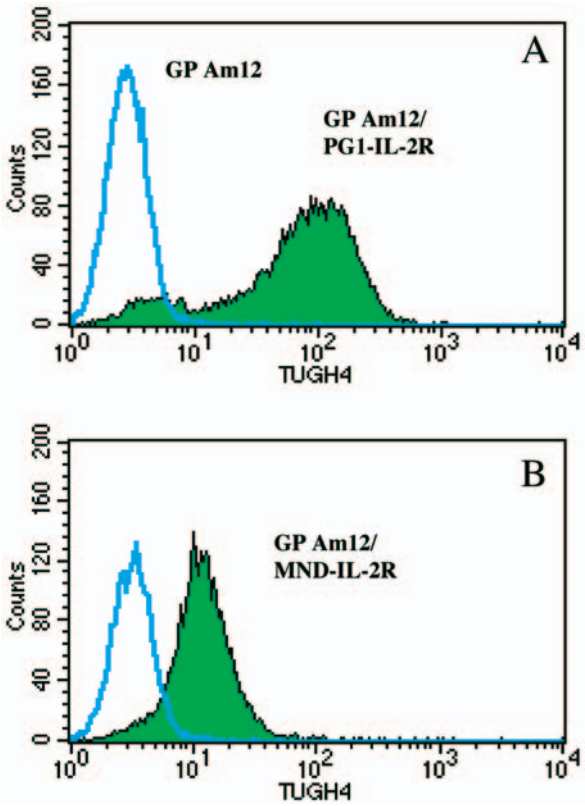


Fig. 2 FACS profile of retroviral–packaging cells
Two types of high-titer amphotropic virus-producing cells (GP Am12/PG1-IL-2R, GP Am12/MND-IL-2R) were prepared. GP Am12/PG1-IL-2R (A); GP Am12/MND-IL-2R (B). The y axis represents the number of examined cells, and the x axis shows expression of TUGH4.

## Discussion

HSCs are capable of self-renewal to maintain a long-term supply of progeny and are capable of differentiation into multiple hematopoietic lineages. These cells are therefore regarded as an ideal target for permanent cures via gene therapy[14]. In order to achieve gene transfer into HSCs, retroviral vectors based on Mo-MLV vectors are currently the most commonly used tool. Despite considerable success in murine models, most of the clinical trials conducted to date using MoMLV-based vectors have yielded mixed results. Even with reasonable transduction efficiency into human CD34+cells using Mo-MLV vectors, expression of the therapeutic gene is seen in a limited number of transduced cells and gradually decreases to undetectable levels. In retroviral vector systems, expression of the inserted gene is driven by the LTR as an enhancer. It is now known that the LTR of MoMLV is often subject to silencing in vitro and in vivo[15,16] and this phenomenon is partially due to several negative factors in the LTR.

In order to overcome these problems with MoMLV-LTR, an MND vector was constructed with a modified LTR. Firstly, MoMLV enhancer repeats were replaced with those from a related virus, the myeloproliferative sarcoma virus, which has greater

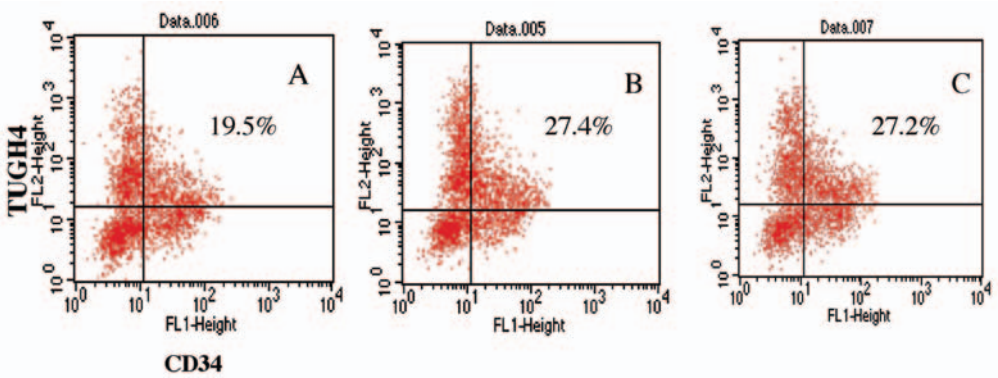


Fig. 3 Expression of IL-2Rcγ on CD34+cells
Non-transduction (A); Transduction with PG1-IL-2R vector (B); Transduction with MND-IL-2R vector (C).
CD34+cells are maintained with growth factors in the presence of CH296, progenitors start to differentiate and expression of CD34+is decreased on the forth day. The proportion of double-positive cells increased to 27.4% and 27.2% after insertion of the IL-2Rcγ transgene via the PG1-IL-2R and MND-IL-2R vectors, while this was 19.5% in non-transduced control.

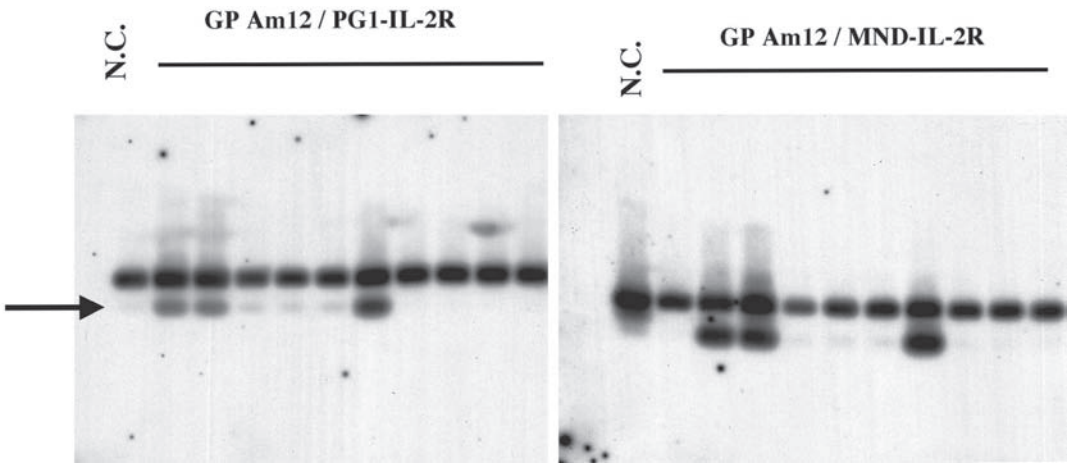


Fig. 4 Transduction efficiency by GM-colonoy assay
N.C.; non-transduced cell-derived colony as negative control.
An arrow indicates the band derived from transgene IL-2cγ cDNA.
Transduced CD34+progenitors were plated on methylcellulose and after ten days, colonies were isolated and individually analyzed by PCR with oligonucleotide primers (5'-TCCAAGTGCAA-TTCATGTAC-3' plus 5'-CGGAGCAGTCACACTCTAAG-3') for the presence of retroviral vector and subject to southern blot analysis using a fragment of IL-2cγ cDNA as a probe. The upper band corresponds to the genomic IL-2cγ band, and the lower band (~800bp) results from the integrated vector. These figures (left; GM conolies derived from CD34+cells transduced with GPAm12/PG1-IL-2R, right; those with GPAm12/MND-IL-2R) show representative data of PCR-southern blot analysis of a colony.

Table 1 Evaluation of retroviral transduction efficiency into GM-colonies

| | PG1-IL-2R | MND-IL-2R |
|---|---|---|
| EXP. 1 | 10/30 | 9/30 |
| EXP. 2 | 7/20 | 7/20 |
| Total | 34% (17/50) | 32% (16/50) |

transcriptional activity in HSCs[17]. Secondly, the negative control region of MoMLV-LTR was deleted to prevent binding of the YY-1 protein, which can downregulate LTR-directed expression[18]. Thirdly, the MoMLV PBS region was substituted with the PBS from dl587rev virus[19] in order to prevent binding of the repressor binding protein, which interferes with LTR-directed transcription[20]. This vector was reported to show a higher frequency of expression in embryonic cartinoma (F 9)[10] and in murine HSC in vitro, and a longer expression in murine HSC in vivo than the standard MoMLV vector[21].

In the present study, these modified retroviral vector systems were applied to create the new vector plasmid for gene therapy for the most promising target disease, X-SCID with the objective of attaining a higher transduction efficiency into human CD34+hematopoietic progenitors. Transduction efficiency into hematopoietic progenitors showed more than 30% in colony assay, whereas the enhancement of expression of IL-2 Rcγ on CD34+cells was approximately 10% in FACS analysis. This discrepancy might be caused by the low intensity of IL-2Rcγ on CD34+cells or lower affinity to IL-2 Rcγ of TUGH4 antibody.

A new vector plasmid, pMND-IL-2R, was constructed and a high-titer amphotropic virus-producing cell population, GP Am12/MND-IL-2R, was established. Our initial goal that this newly created retroviral vector could be transduced into human CD34+progenitor cells as efficiently as the MoMLV-based vector was clearly confirmed. The use of this newly created vector may be advantageous for gene therapy of X-SCID.

Unfortunately, two patients in a gene therapy clinical trial for X-SCID have since developed acute lymphoblastic leukemia (T-cell type) as a serious adverse event[22]. These events are thought to have been caused by retroviral vector insertion into the LMO-2 gene region on chromosome 11, which regulates the differentiation of lymphoid and myeloid

lineages[23]. Currently, we investigators who participated in the studies of gene transfer into hematopoietic stem cells have a responsibility to the patients to elucidate how these adverse events occurred. Our ultimate goal is to apply this vector system to an in vivo animal model and to confirm not only the efficacy of this vector, but also the clinical safety of gene therapy by determining whether these events recur in such a model.

## References


1. Marcel A, Grausz J: The TWC worldwide gene therapy enrollment report, end 1996. Hum Gene Ther 1997; 8 : 775–780.
2. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW, Leonard WJ: lnterleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell 1993; 73: 147–157.
3. Sugamura K, Asao H, Kondo M, Tanaka N, Ishii N, Nakamura M, Takeshita T: The common gamma-chain for multiple cytokine receptors. Adv Immunol 1995; 59: 225–277.
4. Haddad E, Landais P, Friedrich W, Gerritsen B, Cavazzana-Calvo M, Morgan G, Bertrand Y, Fasth A, Porta F, Cant A, Espanol T, Muller S, Veys P, Vossen J, Fischer A: Long-term immune reconstitution and outcome after HLA-nonidentical T-cell-depleted bone marrow transplantation for severe combined immunodeficiency: a European retrospective study of 116 patients. Blood 1999; 94: 2923–2930.
5. Candotti F, Johnston JA, Puck JM, Sugamura K, O' Shea JJ, Blaese RM: Retroviral-mediated gene correction for X-linked severe combined immunodeficiency. Blood 1996; 87: 3097–3102.
6. Taylor N, Uribe L, Smith S, Jahn T, Kohn DB, Weinberg K: Correction of interleukin-2 receptor function in X-SCID lymphoblastoid cells by retrovirally mediated transfer of the gamma-c gene. Blood 1996; 87: 3103–3107.
7. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova J-L, Bousso P, Deist FL, Fisher A: Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000; 288: 669–672.
8. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A: A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2003; 348: 255–256.
9. Miller A, Rosman GJ: Improved retroviral vectors for gene transfer and expression. Biotechniques 1989; 9 : 980–990.
10. Challita PM, Skelton D, el-Khoueiry A, Yu XJ, Weinberg K, Kohn DB: Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. J Virol 1995; 69: 748–755.
11. Pear WS, Nolan GP, Scott ML, Baltimore D: Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci USA 1993; 90: 8392–8396.
12. Markowitz DG, Goff SP, Bank A: Safe and efficient ecotropic and amphotropic packaging lines for use in gene transfer experiments. Trans Assoc Am Physicians 1988; 101: 212–218.
13. Xu L, Stahl SK, Dave HP, Schiffmann R, Correll PH, Kessler S, Karlsson S: Correction of the enzyme deficiency in hematopoietic cells of Gaucher patients using a clinically acceptable retroviral supernatant transduction protocol. Exp Hematol 1994; 22: 223–230.
14. Medin JA, Migita M, Karlsson S: Gene therapy of enzyme and immunedeficiencies in the hematopoietic system. Cell therapy (Morstyn G, Sheridan WP, eds), 1996; pp 386–413, Amgen, California.
15. Chalita P-M, Kohn DB: Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. Proc Natl Acad Sci USA 1994; 91: 2567.
16. Lange C, Blankenstein T: Loss of retroviral gene expression in bone marrow reconstituted mice correlates with down-regulation of gene expression in long-term culture initiating cells. Gene Ther 1997; 4 : 303–308.
17. Bowtell DD, Cory S, Johnson GR, Gonda TJ: Comparison of expression in hemopoietic cells by retroviral vectors carrying two genes. J Virol 1988; 62: 2464–2473.
18. Tsukiyama T, Niwa O, Yokoro K: Mechanism of suppression of the long terminal repeat of Moloney leukemia virus in mouse embryonal carcinoma cells. Mol Cell Biol 1989; 9 : 4670–4676.
19. Colicelli J, Goff SP: Isolation of an integrated provirus of Moloney murine leukemia virus with long terminal repeats in inverted orientation: integration utilizing two U3 sequences. J Virol 1988; 62: 633–636.
20. Petersen R, Kempler G, Barklis E: A stem cell-specific silencer in the primer-binding site of a retrovirus. Mol Cell Biol 1991; 11: 1214–1221.
21. Halene S, Wang L, Cooper RM, Bockstoce DC, Robbins PB, Kohn DB: Improved expression in hematopoietic and lymphoid cells in mice after transplantation of bone marrow transduced with a modified retroviral vector. Blood 1999; 94: 3349–3357.
22. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A: A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2003; 16: 255–256.
23. Rabbits TH, Bucher K, Chung G, Grutz G, Warren A, Yamada Y: The effect of chromosomal translocations in acute leukemia: the LMO2 paradigm in transcription and development. Cancer Res 1999; 59: 1794–1798.


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