J Korean Soc Appl Biol Chem (2012) 55, 497–506
DOI 10.1007/s13765-012-2098-1

ORIGINAL ARTICLE

Development of a Lentiviral Vector and an Efficient Infection Method for Gene Therapy for p22phox-defective Chronic Granulomatous Disease

Young Mee Kim · Huh Jung Sik · Moonjae Cho · Kyung Sue Shin

Received: 3 May 2012 / Accepted: 30 May 2012 / Published Online: 31 August 2012

Abstract Chronic granulomatous disease (CGD) is caused by impaired antimicrobial activity in phagocytes due to the absence or malfunction of the respiratory burst NADPH oxidase. In a previous study, we found that 12 patients from 10 unrelated families on Jeju Island had an identical homozygous single-base C-to-T substitution in exon 1 (c.7C > T) of *CYBA*, which encodes p22phox. Autosomal recessive p22phox-defective CGD carrier-derived white blood cells were efficiently transduced by the elongation factor 1-alpha lentivirus constructs, as up to 90% of cells were green fluorescent protein (eGFP)-positive at 3 days post-transduction. pLL3.7-driven eGFP expression was stable for at least 4 weeks after transduction and persisted after CGD carrier-derived cells were immortalized by human telomerase reverse transcriptase (hTERT) and B lymphoma Mo-MLV insertion region 1 (Bmi-1). Upon macrophage-like differentiation of the transduced HL-60 cells by dimethyl sulfoxide, up to 28% of the cells had higher mean levels of superoxide production than undifferentiated cells, and lentivirus efficiently transduced cells and induced the expression of genes for extended periods.

Y. M. Kim · M. Cho (✉)
Department of Biochemistry, Jeju National University, Jeju 690-756, Republic of Korea
E-mail: moonjcho@jejunu.ac.kr

K. S. Shin (✉)
Department of Pediatrics, Jeju National University, Jeju 690-756, Republic of Korea
E-mail: kyungsue@jejunu.ac.kr

H. J. Sik
Department of Urology, Jeju National University School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea

M. Cho · K. S. Shin
Institute of Medical Science, Jeju National University, Jeju 690-756, Republic of Korea

Keywords chronic granulomatous disease · lentiviral vector · p22phox

Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder in which superoxide generation by the leukocyte NADPH oxidase is absent or markedly deficient. Because the superoxide-generating enzyme is essential for microbicidal activity in phagocytes, patients with CGD who lack the functional oxidase are susceptible to recurrent and often life-threatening bacterial and fungal infections. The disorder, which has a prevalence of approximately 1 in 500,000 individuals, results from mutations in any one of four essential subunits of the NADPH oxidase complexes (Kume and Dinauer, 2000).

Approximately two-thirds of cases of CGD are caused by defects in the X-linked gene encoding the larger subunit (gp91phox) of flavocytochrome b558, a plasma membrane heterodimer that is the redox center of the oxidase. A rare autosomal recessive form of CGD is caused by mutations in the gene encoding p22phox, the smaller subunit of flavocytochrome b558 (Roos et al., 2010).

Morbidity caused by infection or granulomatous complications remains a significant problem for many patients, particularly patients with X-CGD, and the overall mortality rate was recently estimated as approximately 2% per year (Petersen et al., 2002). According to a collection by the Korean College of Pediatric Clinical Immunology, the prevalence of CGD from 2001 to 2005 in Korea was 0.9 in 1,000,000 individuals. Most regions of Korea had a similar CGD prevalence (ranging from 0.4 to 1.7 per 1,000,000 individuals). The prevalence of CGD on Jeju Island was surprisingly high; 20.7 per 1,000,000 individuals. This is a 10–50-fold higher rate than that in other regions of Korea (Kim et al., 2009).

Recent work has revealed that retrovirus (RV)-based hematopoietic stem cell (HSC) gene therapy can provide significant clinical benefits to patients with severe combined immune deficiency (SCID) (Cavazzana-Calvo and Fischer, 2007). Unfortunately, RV therapy in both X-linked SCID and CGD has led to unanticipated adverse events due to long terminal repeat (LTR)-mediated protooncogene transcriptional activation (Hacein-Bey-Abina et al., 2003; Ott et al., 2006). Thus, activating RV insertions can lead to the emergence of clonal dominance and clonal fluctuation, which may eliminate or reduce the ongoing clinical benefit. These events have drawn attention to self-inactivating (SIN) lentiviral vectors as potential alternative delivery platforms for hematopoietic disorders. Lentiviral vectors offer the advantage of targeting nondividing cells and can efficiency target multipotent, nonhuman primate or human HSCs at a low viral copy number (Janssens et al., 2003; Michalkiewicz et al., 2007). The SIN design abolishes the transcriptional activity of the viral LTR in target cells and minimizes the risk of the emergence of replication-competent recombinants in the vector producer and target cells. Recently, third-generation human immunodeficiency virus (HIV)-1-based vectors, HIV 5'-LTR sequences were replaced by functionally homologous regions from either Rous sarcoma virus or cytomegalovirus (CMV). Further, there is less evidence for transcriptional silencing of internal promoters within integrated lentiviral vectors, and no bias for integration within transcription start sites has been observed (Brenner et al., 2003). Most work with lentivectors has been performed with nontherapeutic marker genes such as enhanced green fluorescent protein (eGFP) (Gao et al., 2001; Sirven et al., 2001; Ailles et al., 2002). It is important to demonstrate that the elongation factor 1-alpha (EF1α) lentivector efficiently transduces primitive cells with therapeutic genes of interest, such as the p22phox gene required for correcting AS-CGD. A previous study analyzed the clinical features and investigated the molecular genetic defects leading to CGD on Jeju Island (Kim et al., 2009). In addition, all CGD patients on Jeju Island had an identical mutation in *CYBA*. In the present study a novel lentiviral vector for CYBA gene-defective CGD, was developed. The transduction efficiency of the novel vector in immortalized myeloid cells of CGD carrier-derived white blood cells (WBC) and differentiated HL-60 (macrophage like) cells were determined. The expression of p22phox via HLV-1-based lentiviral delivery systems efficiently increased the production of superoxide upon stimulation.

Materials and Methods

Construction of vectors encoding p22phox. The lentiviral vector pLL3.7-EF1α-p22phox was constructed by replacing the U6 promoter in pLL37 with the EF1α promoter using *Xba*I and *Xho*I, after which human 22phox was inserted in the *Hpa*I and *Xho*I sites downstream of the EF1α promoter (Fig. 1).

Maintenance of cell culture and transfection. The human embryonic kidney cell line HEK 293T and human cervical adenocarcinoma cell line HeLa were maintained in Dulbeco modified eagles media (DMEM) (Gibco, Life Technologies Gmbh, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/mL, Gibco), and streptomycin (100 μg/mL, Gibco). The human premyeloid leukemia cell line HL60 was cultured in RPMI 1640 (containing 10% FBS, Gibco), supplemented with penicillin (100 U/mL, Gibco) and streptomycin (100 μg/mL, Gibco).

Vector production. The lentiviral generation procedure was as follows. Initially, 1.2×10^7 293 T cells grown in complete DMEM were seeded in 175-cm^2-tissue culture flasks on the day before transfection. Lentiviral vector DNA (45 μg) and packaging plasmids pMDG.2 carrying the envelope transgene (VSV-G) (11 μg) and pCMVΔ8.74 (Plasmid Factory, Germany) carrying the lentiviral transgene (gag-pol) (32 μg) were added to 5 mL of OPTI-MEM, filtered through a 0.22-μm filter, and combined with 5 mL of filtered OPTI-MEM supplemented with 1 μL of 10 mM polyethylenimine (PEI; Sigma, USA) transfection reagent. The transfection reaction was performed at room temperature for 20 min, during which the 293T cells were washed once with OPTI-MEM medium. The DNA/PEI complexes (10 mL) were subsequently added to the cells and incubated at 37°C/5% CO_2 for 4 h, followed by replacement of the medium with 14 mL of complete DMEM. Viral supernatant was harvested at 48 and 72 h post-transfection, filtered through a 0.22-μm filter, and concentrated by ultracentrifugation at 23,000 rpm for 2 h. Lentiviral pellets were resuspended in 100 μL OPTI-MEM, stored on ice for 20 min, and then snap frozen in aliquots at 70°C.

Virus titration. HeLa and HEK 293T cells were plated at 8×10^4 cells per well in 6-well plates in DMEM. On the following day, 2 μL of 8 mg/mL polybrene (Sigma) was added per well (1:1000 final dilution), and virus-containing supernatant was added at 1:10 to 1:10000 dilutions in a final volume of 2 mL. After 48 h of incubation, the viral titers were quantified by flow cytometric (FACSCalibur, BD Biosciences, USA) analysis of the eGFP reporter gene.

Transduction and calculation of the titer in transducing units. For transduction assays, HeLa or HEK 293T cells were seeded at 1×10^5 to 3×10^5 cells per well in 12-well plates one day before transduction. On day 1 post-transduction, the medium was removed and replaced with transduction medium consisting of 100 μL vector, 300 μL serum-free medium, and 4 μg/mL polybrene. After 3 h incubation, 1 mL of complete medium was added. On the same day, cells from three nontransduced wells were trypsinized and fixed to allow calculation of the rate of cell division. On day 3, cells were trypsinized, and GFP expression was analyzed by flow cytometry (FACSCalibur). Transduction titers (in transducing units per milliliter) were calculated using the percentage of GFP-positive cells, the difference in the rate of cell division between the day of transduction and the day of analysis, as well as the flow of the FACSCalibur (Woods et al., 2000). Multiple serial dilutions of vector-containing medium (1/10, 1/100, and 1/1,000) were used

for the transduction assays, and titers were calculated for linearly correlated values.

Infection with the hTERT and Bmi-1 constructs. The full-length human hTERT cDNA sequence was cloned by Reverse transcription polymerase chain reaction (RT-PCR) using RNA extracted from HeLa cells. Thermoscript RT-PCR (Invitrogen, USA) and *Themococcus kodakaraensis* (KOD) polymerase (TOYOBO, Japan) were used for the RT and PCR reactions, respectively. The forward primer, 5'-GGAATTCGCCGCGCGCT CCCCGCTGCCGAGCC-3', and reverse primer, 5'-GCTCTAG ATTAGTCCAGGATGGTCTTGAAGTCT-3', were designed to obtain the coding sequence of human hTERT flanked. PCR products were cloned between the multicloning sites of pCLXSN to generate pCLXSN-ACC- hTERT.

In addition, the full-length human *bmi-1* cDNA sequence was cloned by RT-PCR using RNA extracted from K562 cells. Thermoscript RT-PCR (Invitrogen) and KOD polymerase (TOYOBO) were used for the RT and PCR reactions, respectively. The forward primer, 5'-ACGCGTCGACCGCCATG CATCGAACAACGAGAAT-3', and reverse primer, 5'-CGGATC CTCAACCAGAAGAAGTTGCTG-3', were designed to obtain the coding sequence of human *bmi-1* flanked by a *Sal*I site (underlined), a Kozak consensus sequence at the 5'-end, and a *Bam*HI site (underlined) at the 3'-end. The *Sal*I-*Bam*HI segment of the PCR product was cloned between the *Xho*I and *Bgl*II sites of pM.

VSCV-puro to generate pCMV-puro-hBmi-1. The producers of the pCLXSN-ACC-hTERT and pCMV-puro-hBmi-1 retroviruses were obtained from Dr. Kyu-Kye Hwang, College of Veterinary Medicine, Jeju National University). One milliliter of producer cell culture fluid was added to isolated monocytes in the presence of polybrene (8 μg/mL). For combination retroviral infection, cells were sequentially transduced with LXSN-Bmi-1 and then with LXSH-hTERT (Okamoto et al., 2002; Saito et al., 2005). Stably transduced cells were maintained in RPMI containing 10% FBS.

Induction of differentiation. HL-60 cells were suspended in growth medium containing 1.3% DMSO (Sigma) for periods of up to 6 days, whereupon >96% of cells were viable and >90% of the cells exhibited the appearance of mature granulocytes (Collins, 1987).

Dihydrorhodamine 123 FACS (DHR 123) quantitation of superoxide production. The NADPH oxidase activity was quantified by DHR 123 (Sigma) flow cytometry (Teufelhofer et al., 2003). Differentiated granulocytic cells were washed twice and resuspended in 500 μL of hank's buffered salt solution (HBSS) (without Ca^{2+} and Mg^{2+}, Sigma) in duplicate: one sample was stimulated with phorbol myristate acetate (PMA), and the second sample was not stimulated. All samples were preincubated for 5 min in a 37°C culture incubator in the presence or absence of 20 μM PMA. Subsequently, 7.5 μL of DHR 123 (Sigma) were added to each sample and incubated for an additional 15 min at 37°C. Samples were then immediately placed on ice and analyzed by flow cytometry. Unstimulated cells incubated with DHR 123 served as negative controls. Superoxide production in the positive cells was determined by measuring the shift in FL-1 (green light) relative to the corresponding negative control, and the percentage of superoxide-producing cells was assessed by analyzing individual histograms. The relative percentage of functionally corrected cells compared to non-corrected cells was calculated as the ratio (PMA-stimulated transduced cells/PMA-stimulated cells) of their respective geometric mean fluorescence intensities (MFIs).

Isolation of white blood cells (WBCs). Human WBCs were isolated from buffy coats (from healthy donor blood). Buffy coats were diluted 1:1 with sterile PBS, layered onto a Ficoll-Paque™ Plus (GE Healthcare Bio-Sciences AB, Sweden) cushion at a ratio of 2:3, and centrifuged at 2000×g for 30 min with no braking. White blood cells (WBCs) were harvested from the Ficoll interface, diluted 1:1 with sterile phosphate buffered saline (PBS), and centrifuged at 1500×g for 5 min to pellet cells. The pellet was resuspended in PBS and centrifuged at 1000×g for 5 min. This step was repeated until the supernatant was clear, indicating the removal of platelets. After the final wash, the cell pellet was resuspended in RPMI 1640 containing 20% FBS and counted. Cells (1×10^6/mL) were added to tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 1 h, adherent cells were removed by washing with warm PBS and RPMI 1640 containing 20% FBS.

Results

Construction of lentiviral vector (pLL3.7 EF1α) carrying the therapeutic p22phox gene. The generation of an EF1α lentiviral vector carrying the therapeutic p22phox gene is illustrated in Fig. 1. To generate lentiviral particles, the PEI transfection procedure described in the methods was used. The packaging cell line was derived from 293T cells. The advantages of the 293T host cell line are its human origin, good transfectability, good adaptation to growth in suspension, and safe record in the production of lentiviral vectors. 293T cells were plated at 6×10^6 cells per well. The transfer plasmid (pLL3.7 EF1α) represents a further development of the previously described parental HIV-1-based vector pLL3.7. A 590-bp *Hpa*I-*Xho*I p22phox cDNA fragment was added to the pLL3.7 EF1α plasmid to generate the pLL3.7 EF1α-p22phox plasmid (Fig. 1A). In this vector, the p22phox gene is exclusively expressed by an internal EF1α promoter. Transcription of the p22phox coding sequence is regulated by the EF1α gene promoter (van Den Berg et al., 2009). Lentiviral particles were generated by transient cotransfection of the three plasmids into human embryonic kidney 293T cells. Figure 1B illustrates the procedure of lentivirus production in 293T packaging cells. pLL3.7 EF1α-p22phox lentiviral particles were generated by transient cotransfection of the specific transfer vector plasmid (pLL3.7 EFa-p22phox) with the two packaging plasmids (pMDG2 and pCMV-Δ8) into HEK 293T cells with PEI (Fig. 1B).

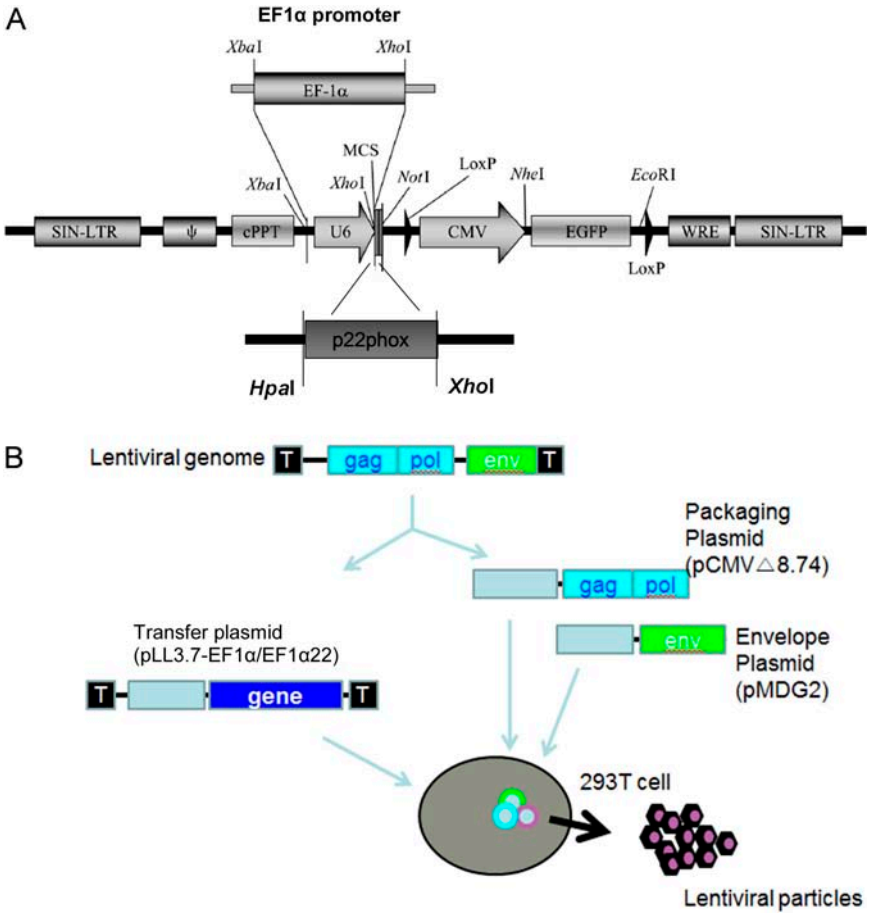

Fig. 1 Schematic diagram of lentiviral vector construction and transduction. (A) Linear map of the vectors. In the recombinant lentiviral vector, the U6 promoter in pLL37 was replaced with the EF1α promoter, and then the human p22phox gene was inserted in the *Hpa*I and *Xho*I sites downstream of the EF1α promoter. (B) Lentiviral vectors were produced by transient cotransfection of HEK293T cells with three plasmids employing PEI, as previously described in the materials and methods section.

Transduction of p22phox into cells by pLL3.7 EF1α. pLL3.7 EF1α can drive reproducible, stable, and long-term eGFP transgene expression in cell lines in the absence of drug-selective pressure. Therefore, the efficiency and efficacy of transgene expression in HEK 293T (data not shown) and HeLa cells were assessed (Fig. 2). The initial transduction efficiency was assessed using serial 10-fold dilutions of the virus and monitoring eGFP gene expression after 3 days by FACS analysis (Fig. 2). As a control for transduction efficiency, the expression of eGFP was measured. On day 3, cells transduced with different dilutions of the lentiviral vector were eGFP-positive according to microscopic analysis (Fig. 2A). In addition, flow cytometric analysis performed on day 3 revealed a peak shift in the histograms corresponding to the presence of eGFP-positive cells for all transduced cultures compared with the findings in nontransduced control cells (Fig. 2B). These findings correlated with the qualitative results obtained by direct visualization of the cells under a fluorescence microscope. These results indicate that 10-fold dilution transductions in HeLa cells were highly efficient, with 70–96% of cells displaying eGFP fluorescence (Fig. 2C).

Transduction of promyelocytic HL-60 cells by spin and polybrene infections. To increase the efficiency of infection with suspension cells, simultaneous seeding and infection followed by a spin method were utilized. The spin infection method and conditions were modified in the present study. Six days posttransduction, cells were analyzed by fluorescence microscopy (Fig. 3A) and flow cytometry. In all transduced cultures, a peak shift corresponding to the presence of eGFP-positive cells was observed (Fig. 3B and C). The results indicate that 20–30% of cells were eGFP-positive at 6 days posttransduction, indicating that HL-60 cells can be efficiently transduced with the pLL3.7 EF1α vector by using a spin infection method in the presence of polybrene.

hTERT/Bmi-1-infected CGD carrier-derived WBCs. Human primary cells are easy to isolate but difficult to study due to their limited life span. We first attempted to extend the life span of WBCs by using a retrovirus expressing Bmi-1 and hTERT. WBCs were obtained from the buffy coats of CGD carrier donors.

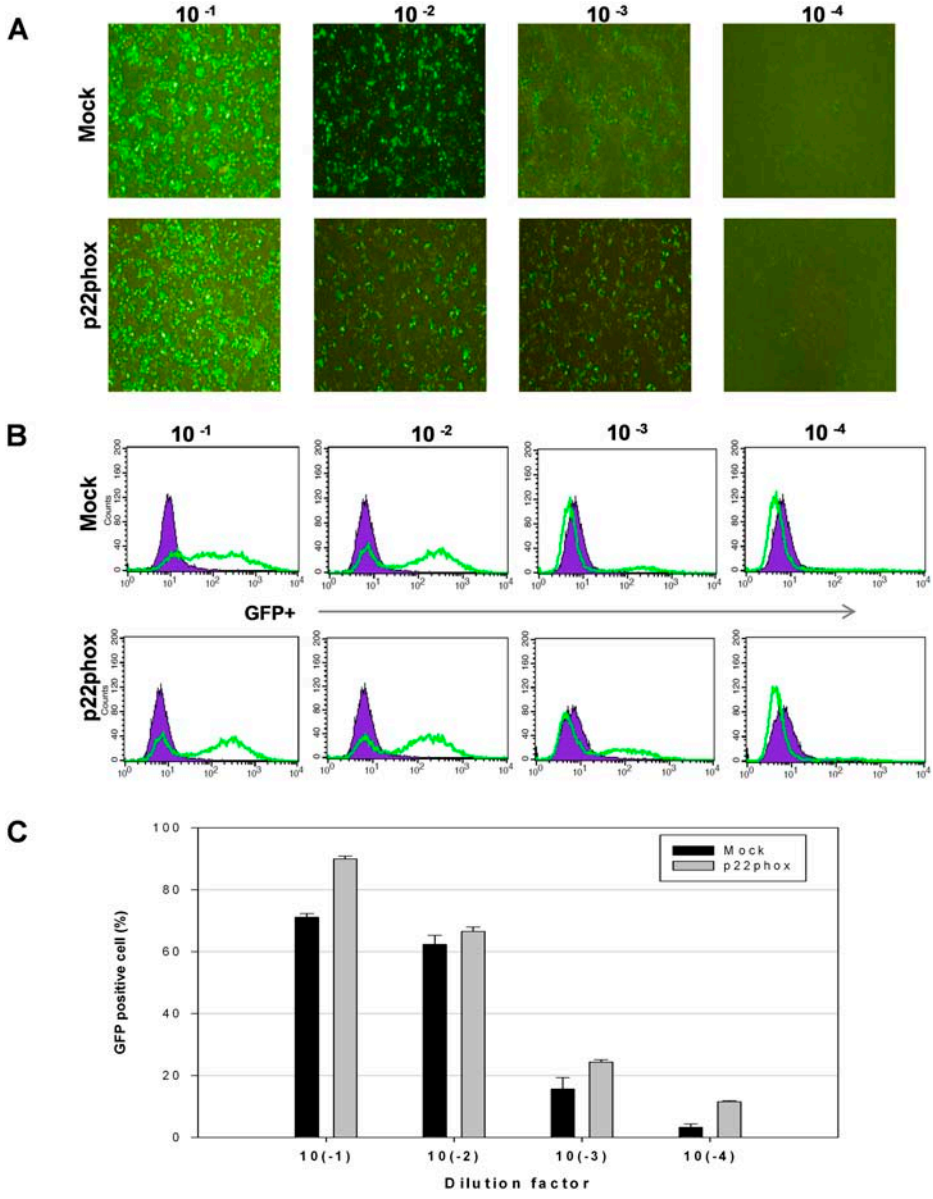

Fig. 2 Transducing efficiency in HeLa cells. (A) An expression plasmid for eGFP was added to verify the transduction efficiency, and expression was visualized by fluorescence microscopy (MOI=10^{-1}). (B) The relative efficiency of cell infection by lentiviral vector preparations was assessed by flow cytometry according to the percentages of eGFP-positive cells. (C) The percentage of infected cells as measured by flow cytometry. The results are presented as the mean ± SD of three independent experiments.

Mononuclear cells and granulocytes were isolated by the Ficoll-Paque method. Freshly isolated WBCs were prepared for infection with recombinant retroviruses carrying the Bmi-1 and hTERT genes and then transduced by the spin method in the presence of polybrene. The cells transduced with Bmi-1 and hTERT escaped replication crisis and proliferated continuously. During the subsequent culture, the cell proliferation rate accelerated, and the cells grew larger (Fig. 4A). Then, CGD carrier WBCs and immortalized (by Bmi-1/hTERT) WBCs were transduced by pLL3.7 EF1α and pLL3.7 EF1α-p22phox. Six days after transduction, the transduction efficiency was compared by FACS (Fig. 4B). The transduction efficiency of high-level $WBC^{hTERT+Bmi-1}$ cells was 40% higher than that of nonimmortalized WBCs (Fig. 4B). Next, the duration of transgene expression in a time course experiment was measured. eGFP expression was detectable at 3 days after transduction, and its expression persisted for up to 3 weeks, peaking on day 16 (Fig. 4C). As GFP is an indicator of transfection, the augmentation of superoxide production in response to pLL3.7 EF1α-p22phox22 infection was tested. pLL3.7 EF1α-p22phox-transduced $WBC^{hTERT+Bmi-1}$ cells on day 6 were analyzed by the DHR 123

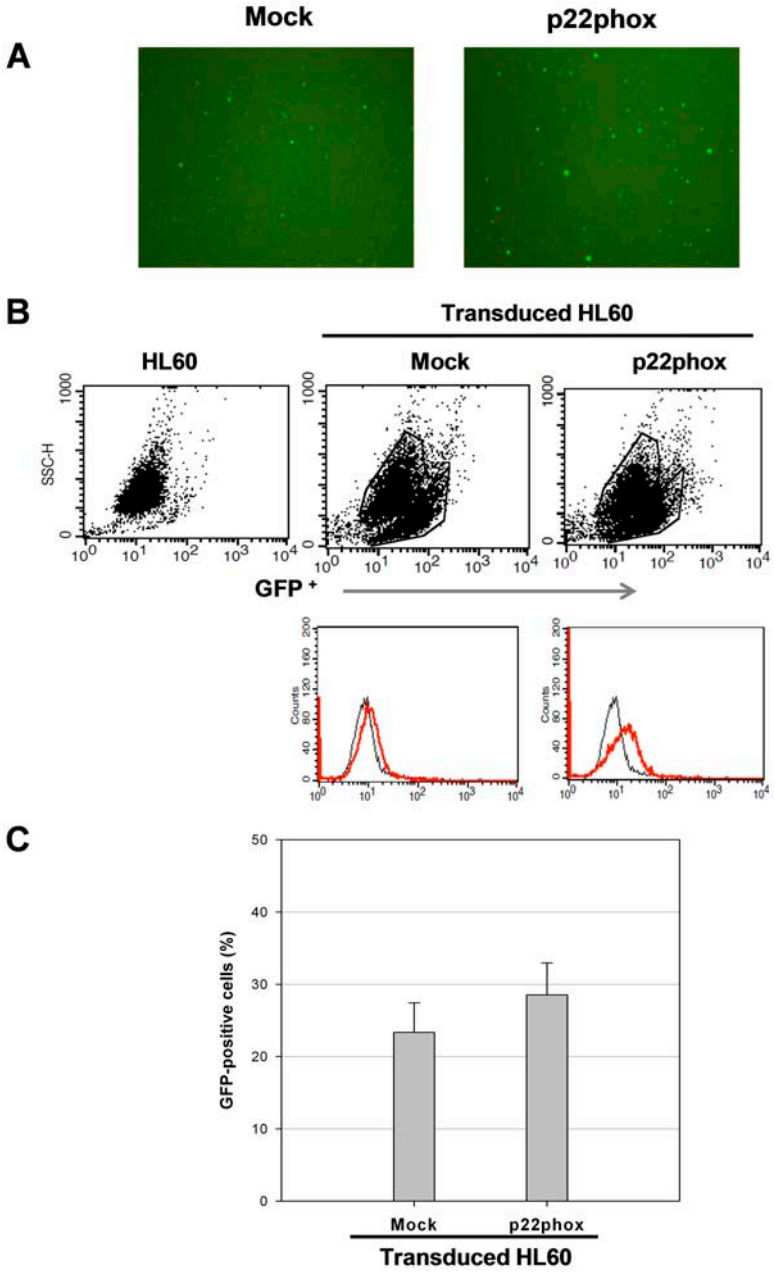

Fig. 3 Transduction efficiency of pLL3.7 EF1α and pLL3.7 EF1α p22phox lentiviral vectors in promyelocytic HL-60 cells. HL-60 cells were transduced with eGFP lentiviral vectors by spin infection in the presence of 8 μg/mL polybrene. (A) Cells were analyzed for eGFP (green fluorescence) by using a fluorescence microscope. (B) Representative flow cytometric analysis of lentiviral vector-mediated transduction of HL-60 cells. (C) Quantitative analysis of HL-60 cell transduction according to the proportion of eGFP-positive cells. The results are presented as the mean ± SD of three experiments.

assay after PMA stimulation. Transgene expression was significantly increased in CGD carrier-derived WBC$^{hTERT+Bmi\text{-}1}$ after PMA stimulation, as indicated by a significant shift in the fluorescent peak (Fig. 4D). Upon PMA stimulation, the number of DHR-positive cells increased by as much as 2-fold (Fig. 4D).

Analysis of superoxide generation before and after HL-60 cell differentiation induced by pLL3.7 EF1α and pLL3.7 EF1α-p22phox. In a previous report, differentiated HL-60 (dHL-60) cells, the differentiation of which is generally associated with the acquisition of NADPH oxidase, exhibited much higher levels of mRNA transcripts for the gp91phox and p47phox than ndHL-60 cells. NADPH oxidase-induced activation of p22phox required the interaction of the p47phox SH3 domains with their partner gp91phox (Teufelhofer et al., 2003).

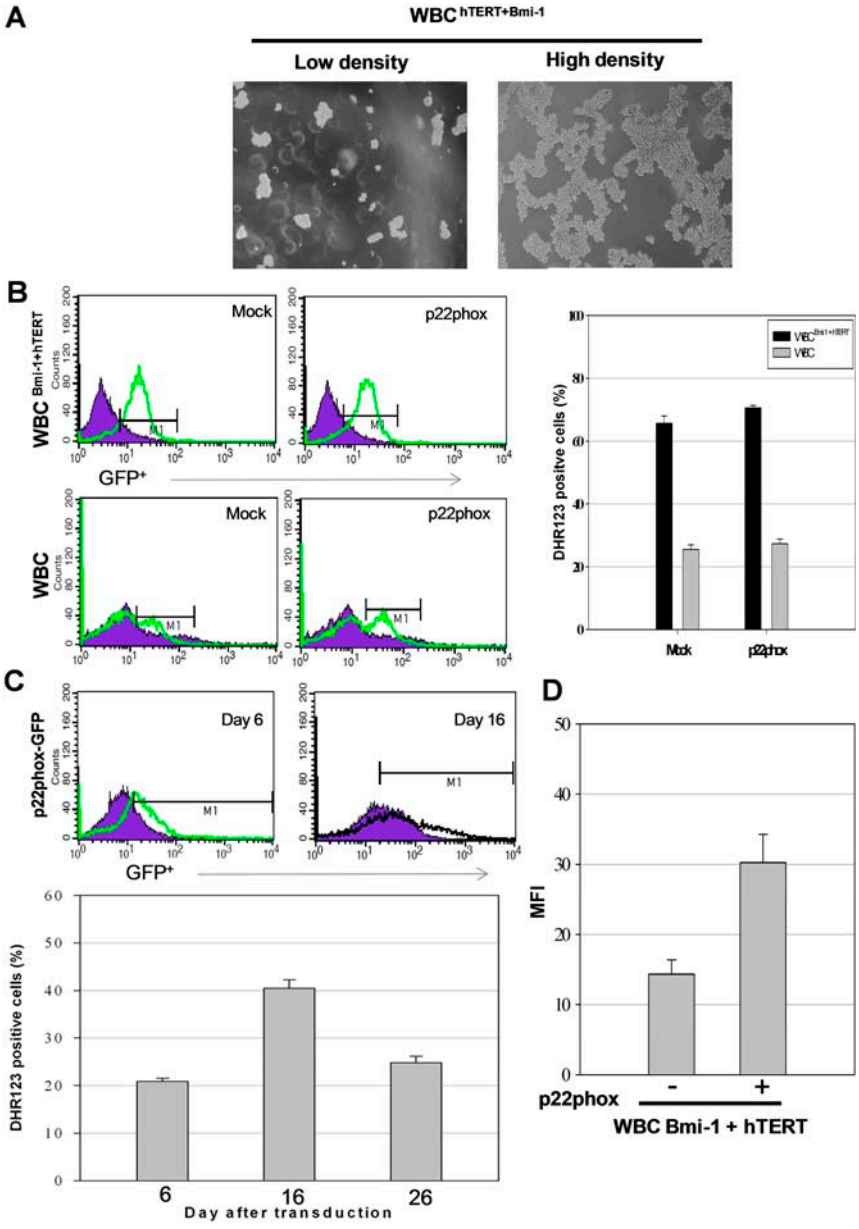

Fig. 4 Comparison of transgene expression in WBCs and WBC$^{hTERT+Bmi-1}$ cells. The transduction efficiency of each reaction was determined using fluorescence microscopy or FACS analysis, typically 6 days after transduction. (A) Phase-contrast photographs of CGD carrier-driven WBC$^{hTERT+Bmi-1}$ cells. (B) Transduction efficiency of the pLL3.7 EF1α and pLL3.7 EF1α p22phox lentiviral vectors in WBCs and WBC$^{hTERT+Bmi-1}$ cells. Cells were transduced using the spin infection method in the presence of polybrene in combination with the hTERT-Bmi-1 virus, and the transduction efficiency was measured by flow cytometry. (C) EGFP expression was regularly determined by FACS until 26 days after transduction. All experiments were repeated three times, and mean values are shown. (D) DHR analysis of oxidation in pLL3.7 EF1α p22phox-transduced WBC$^{hTERT+Bmi-1}$ cells. After DHR loading, cells were stimulated for 15 min with PMA and immediately analyzed by flow cytometry. The results are presented as the mean ± SD.

Changes in the superoxide release level profiles of these granulocyte-like cells were evaluated. HL-60 cells were transduced by spin infection (in the presence of polybrene) with the GFP-expressing vector pLL37 EF1α followed by differentiation for 6–7 days in RPMI supplemented with 1.3% DMSO. As expected, DMSO induced the differentiation of cells growing in suspension into adherent, clump-forming cultures (Fig. 5A). Moreover, the expression of the transgene persisted in approximately 40–50% of the differentiated cells for the entire observation period of 3 weeks. Taken together, these results indicate that the pLL3.7 EF1α vector facilitates efficient gene transfer into ndHL-60 and dHL-60 cells by using the spin infection method, and the EF1α promoter is functional in ndHL-60 and dHL-60 cells (Fig. 5B). PMA-triggered superoxide release was compared in ndHL-60 and

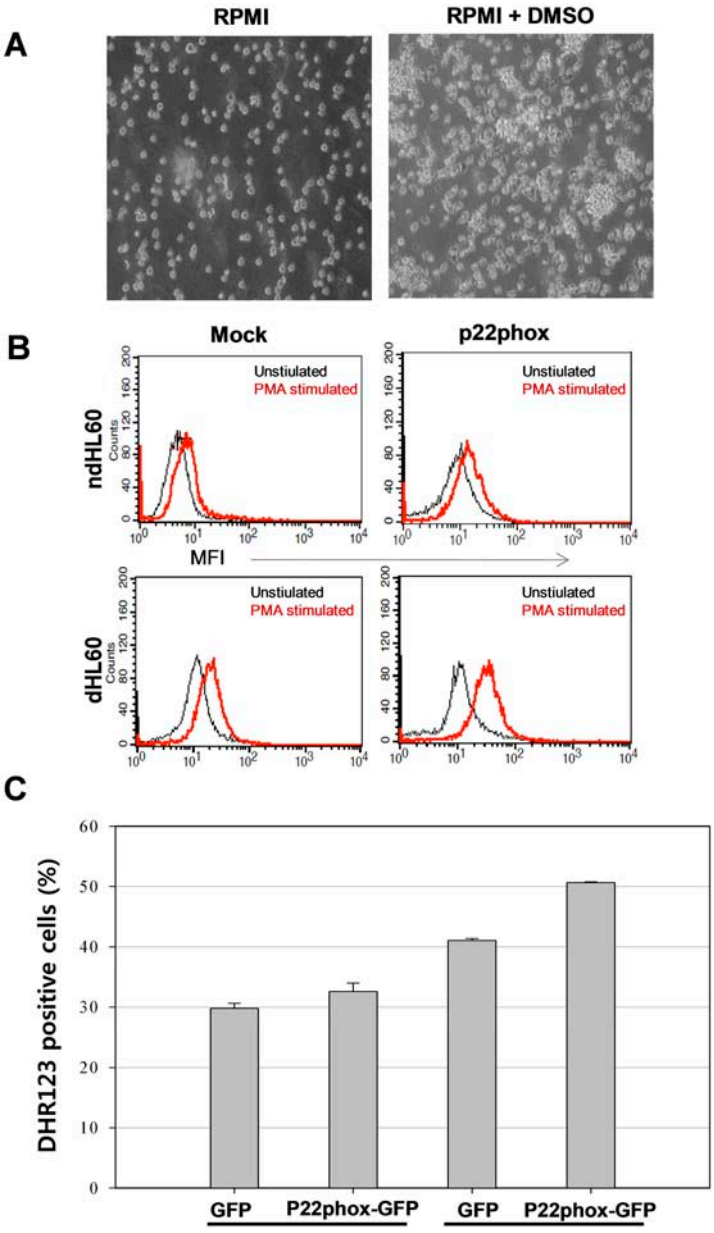

Fig. 5 Analysis of superoxide generation before and after pLL3.7 EF1α- and pLL3.7 EF1α-p22phox-induced differentiation of HL-60 cells. (A) Morphology of ndHL-60 and dHL-60 cells. Morphological changes in HL-60 cells induced by DMSO. (B) NADPH oxidase activity measured by intracellular superoxide production in ndHL-60 and dHL-60 cells in response to 20 μM PMA. (C) Average MFI (%) in ndHL-60 and dHL-60 cells in response to 20 μM PMA. All experiments were repeated three times, and the results are presented as the mean ± SD.

dHL-60 cells at 6 days after transduction by the DHR 123 assay. The MFI of p22phox-transduced differentiated HL-60 cells was approximately 10% higher after PMA stimulation (Fig. 5C).

Discussion

Lentiviral technology represents a powerful method for genetically modifying cells. In the present study, a pLL3.7 (HIV-1)-based vector was used to transduce the p22phox gene (Fig. 1A). Lentiviruses have been developed for gene transfer, particularly for applications in gene therapy. These vector systems have also been used to modulate gene function. The popularity of these vectors is in part due to their simplicity of design, ease of production, and efficiency of delivery to various cell types (Roe et al., 1993; Ho et al., 1996; Sutton et al., 1998).

In addition, we chose a pLL3.7 (HIV-1)-based vector to transduce the p22phox gene. The pLL3.7 vector is an attenuated,

SIN, HIV-1-based lentivector expressing the eGFP gene under the transcriptional control of the CMV immediate-early enhancer/promoter and EF1α promoter (Fig. 1). The U6 promoter in pLL3.7 was replaced by the EF1α promoter. The activity of the EF1α promoter was demonstrated to be high in primary human T lymphocytes isolated from adult peripheral blood. Using the CD34+ hematopoietic precursor cell line KG1a (Miyoshi et al., 1999), Ramezani and Hawley (2003) found that the highest levels of expression were directed by the EF1α promoters. The study results revealed that HIV-1-derived pLL3.7 EF1α vectors efficiently transduced HeLa cell lines, with up to 90% of cells expressing the transgene. When lentiviral vectors were added at an MOI of 10^{-1}, the percentage of eGFP-expressing cells exceeded 90% (Fig. 2A). Regarding transgene expression, due to their biological effect of inducing life-long infection, lentiviral vectors may be less prone to transgene silencing (Sirven et al., 2001). Therefore, lentiviral vectors may have further advantages over other viral vectors in addition to their likely benefit for transducing more primitive nondividing HSCs.

After 6 days of incubation with DMSO, 90% of the HL-60 cells were transformed into adherent macrophage-like cells (Fig. 5A). The DHR 123 assay data indicate that superoxide release was increased by 10% via DMSO stimulation for 6 days (Fig. 5B). This result suggests that differentiated macrophage-like cells are more permissive than undifferentiated cells regarding the replication of viruses. Granulocytic differentiation of the HL-60 myelocytic cell line provides a useful in vitro model for assessing cell transduction efficiency and superoxide generation. A previous report on dHL-60 cells, the differentiation of which is generally associated with the acquisition of NADPH oxidase, demonstrated much higher oxidation levels than ndHL-60 cells, in which small amounts of mRNA transcripts for the gp91phox and p47phox components were detected. NADPH oxidase activation of p22phox requires the interaction of the p47phox SH3 domains and with their partner gp91phox (Teufelhofer et al., 2003).

Saito et al. (2005) revealed that bmi-1 and hTERT can immortalize bovine dental follicle cells without affecting their differentiation potential, thus permitting the successful establishment of a cementoblast progenitor cell line from the immortalized cells. To prolong the life span of CGD carrier-derived WBCs, cells were immortalized with hTERT and Bmi-1. hTERT and Bmi-1 retroviruses were first used to transduce granulocytes. CGD carrier-derived WBCs and WBC $^{\text{bmi-1+hTERT}}$ cells were transduced by the pLL3.7EF1α lentiviral vector (Fig. 4). The transduction efficiency measured by eGFP expression was higher in $\text{WBC}^{\text{bmi-1+hTERT}}$ cells than in WBCs. $\text{WBC}^{\text{bmi-1+hTERT}}$ cells displayed increased superoxide production upon PMA stimulation. These results indicate that eGFP is expressed at high levels for up to 4 weeks after transduction of immortalized CGD carrier-derived cells, indicating persistent EF1α-driven p22phox expression during this period.

The present study describes the lentiviral-mediated transduction of CGD carrier-derived WBCs in an experimental setting to increase the efficiency of gene transfer and its compatibility with clinical constraints. Results indicate that highly efficient transduction can be achieved using nonconcentrated eGFP-expressing lentiviral vector stocks following the previously described techniques with modifications. Lentiviral vectors efficiently deliver and express genes for extended periods of 30 days.

Indeed, silencing of p91phox transgene expression in a CGD cell line has been reported after gene transfer using a recombinant pLL3.7EF1α lentiviral vector. Studies have therefore been initiated to replace the EF1α promoter with physiologic myeloid-specific regulatory elements. Further in vitro and animal model studies are needed to develop an optimal lentiviral vector for CGD gene therapy and a potential system for the p22phox gene. Although gene therapy has not yet cured CGD in patients, the field has made significant progress with the demonstration that gene therapy can provide clinical benefit to patients.

Acknowledgment This work was supported by a grant from Hyocheon Academic Research Fund of the Cheju National University in 2008.

References

Ailles L, Schmidt M, de Sio FRS, Glimm H, Cavalieri S, Bruno S et al. (2002) Molecular evidence of lentiviral vector-mediated gene transfer into human self-renewing, multi-potent, long-term NOD/SCID repopulating hematopoietic cells. *Mol Ther* **6,** 615–26.

Brenner S, Whiting-Theobald NL, Linton GF, Holmes KL, Anderson-Cohen M, Kelly PF et al. (2003). Concentrated RD114-pseudotyped MFGS-gp91phox vector achieves high levels of functional correction of the chronic granulomatous disease oxidase defect in NOD/SCID/â2-microglobulin–/–repopulating mobilized human peripheral blood CD34 cells. *Blood* **102,** 2789–97.

Cavazzana-Calvo M and Fischer A (2007) Gene therapy for severe combined immunodeficiency: are we there yet? *J Clin Invest* **117,** 1456.

Collins SJ (1987) The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* **70,** 1233.

Gao Z, Golob J, Tanavde VM, Civin CI, Hawley RG, and Cheng L (2001) High Levels of Transgene Expression Following Transduction of LongTerm NOD/SCIDRepopulating Human Cells with a Modified Lentiviral Vector. *Stem Cells* **19,** 247–59.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E et al. (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348,** 255–6.

Ho C, Vowels M, Lockwood L, and Ziegler J (1996) Successful bone marrow transplantation in a child with X-linked chronic granulomatous disease. *Bone Marrow Transplant* **18,** 213.

Janssens W, Chuah MKL, Naldini L, Follenzi A, Collen D, Saint-Remy JM et al. (2003) Efficiency of onco-retroviral and lentiviral gene transfer into primary mouse and human B-lymphocytes is pseudotype dependent. *Hum Gene Ther* **14,** 263–76.

Kim YM, Park JE, Kim JY, Lim HK, Nam JK, Cho M et al. (2009) Genetic Analysis of 10 Unrelated Korean Families with p22-phox-deficient Chronic Granulomatous Disease: An Unusually Identical Mutation of the CYBA Gene on Jeju Island, Korea. *J Korean Med Sci* **24,** 1045.

Kume A and Dinauer MC (2000) Gene therapy for chronic granulomatous disease. *J Lab Clin Med* **135,** 122–8.

Michalkiewicz M, Michalkiewicz T, Geurts AM, Roman RJ, Slocum GR, Singer O et al. (2007) Efficient transgenic rat production by a lentiviral vector. *Am J Physiol-Heart Circ Physiol* **293,** H881–94.

Miyoshi H, Smith KA, Mosier DE, Verma IM, and Torbett BE (1999) Transduction of human CD34 cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283,** 682–6.

Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K et al. (2002) Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* **295,** 354–61.

Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U et al. (2006) Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* **12,** 401–9.

Petersen JE, Hiran TS, Goebel WS, Johnson C, Murphy RC, Azmi FH et al. (2002) Enhanced cutaneous inflammatory reactions to Aspergillus fumigatus in a murine model of chronic granulomatous disease. *J Invest Dermatol* **118,** 424–29.

Ramezani A and Hawley RG (2003) Human immunodeficiency virus type 1based vectors for gene delivery to human hematopoietic stem cells. *Methods Mol Med* **76**, 467–92.

Roe TY, Reynolds TC, Yu G, and Brown P (1993) Integration of murine leukemia virus DNA depends on mitosis. *EMBO J* **12,** 2099.

Roos D, Kuhns DB, Maddalena A, Bustamante J, Kannengiesser C, de Boer M et al. (2010) Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (second update). *Blood Cells Mol Dis* **44,** 291–9.

Saito M, Handa K, Kiyono T, Hattori S, Yokoi T, Tsubakimoto T et al. (2005) Immortalization of cementoblast progenitor cells with Bmi1 and TERT. *J Bone Miner Res* **20,** 50–7.

Sirven A, Ravet E, Charneau P, Zennou V, Coulombel L, Guétard D et al. (2001) Enhanced transgene expression in cord blood CD34 -derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors. *Mol Therapy* **3,** 438–48.

Sutton RE, Wu HTM, Rigg R, Böhnlein E, and Brown PO (1998) Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J Virol* **72,** 5781–8.

Teufelhofer O, Weiss RM., Parzefall W, Schulte-Hermann R., Micksche M., Berger W et al. (2003) Promyelocytic HL60 cells express NADPH oxidase and are excellent targets in a rapid spectrophotometric microplate assay for extracellular superoxide. *Toxicol Sci* **76,** 376–83.

van Den Berg JM, Van Koppen E, Åhlin A, Belohradsky BH, Bernatowska E, Corbeel L et al. (2009) Chronic granulomatous disease: the European experience. *PLoS One* **4,** e5234.

Woods NB, Fahlman C, Mikkola H, Hamaguchi I, Olsson K, Zufferey R et al. (2000) Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* **96,** 3725–33.