

ORIGINAL ARTICLE

Transgene optimization significantly improves SIN vector titers, gp91^{phox} expression and reconstitution of superoxide production in X-CGD cells

B Moreno-Carranza^{1,5}, M Gentsch^{2,5}, S Stein¹, A Schambach³, G Santilli⁴, E Rudolf¹, MF Ryser², S Haria⁴, AJ Thrasher⁴, C Baum³, S Brenner² and M Grez¹

¹Division of Applied Virology and Gene Therapy, Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt, Germany;

²Department of Pediatrics, University Clinic Carl Gustav Carus, Dresden, Germany; ³Department of Experimental Hematology, Hannover Medical School, Hannover, Germany and ⁴Molecular Immunology Unit, UCL Institute of Child Health and Great Ormond Street Hospital for Children NHS Trust, London, UK

Gene therapy has proven to be of potential value for the correction of inherited hematopoietic disorders. However, the occurrence of severe side effects in some of the clinical trials has questioned the safety of this approach and has hampered the use of long terminal repeat-driven vectors for the treatment of a large number of patients. The development of self-inactivating (SIN) vectors with reduced genotoxicity provides an alternative to the currently used vectors. Our initial attempts to use SIN vectors for the correction of a myeloid disorder, chronic granulomatous disease, failed due to low vector titers and poor transgene

expression. The optimization of the transgene cDNA (gp91^{phox}) resulted in substantially increased titers and transgene expression. Most notably, transgene optimization significantly improved expression of a second cistron located downstream of gp91^{phox}. Thus, optimization of the transgene sequence results in higher expression levels and increased therapeutic index allowing the use of low vector copy numbers per transduced cell and weaker internal promoters.

Gene Therapy (2009) 16, 111–118; doi:10.1038/gt.2008.143; published online 11 September 2008

Keywords: SIN vectors; sequence optimization; chronic granulomatous disease

Introduction

Gene transfer into hematopoietic stem cells has proven to be of therapeutic benefit for the treatment of inherited diseases affecting the immune system. In several phase I clinical trials, functional reconstitution of cellular and innate immunity has been achieved after reinfusion of gene-modified autologous stem cells into the patients.^{1–5} Despite these encouraging results, the occurrence of a lymphoproliferative disease in five patients treated for X-linked severe combined immunodeficiency has questioned the safety of this approach and has motivated intensive activities toward the understanding of vector–cell interactions.^{6–12} Until now, in all gene therapy trials aiming for the correction of immunodeficiencies, retroviral vectors derived from the murine leukemia virus (MLV) were used. In these vectors, the therapeutic gene is under the transcriptional control of the viral long terminal repeat, which normally contains strong enhancer activity. As untargeted integration into the host chromosome is a mandatory step in the

retrovirus life cycle, these types of vector are *per se* mutagenic. Indeed, the retroviral-mediated transcriptional activation of LMO2, a transcription factor essential for T-cell development, was one of the key events associated with the development of lymphoproliferative disease in the X-linked severe combined immunodeficiency trial.^{12,13}

In 2004, we initiated a gene therapy trial for the treatment of the X-linked form of chronic granulomatous disease (X-CGD), a severe life-threatening immunodeficiency affecting the capacity of phagocytic neutrophils to generate reactive oxygen species, which are essential for microbicidal activity.^{14–17} X-CGD patients are unable to eradicate infections caused by catalase-positive bacteria and fungi.¹⁸ Two young adults were treated with gene-modified autologous CD34+ cells resulting in the eradication of therapy refractory infections. However, an increase in gene-marked granulocytes was observed 5 months after transplantation, which was caused by retroviral-mediated transcriptional activation of MDS1/EV11, PRDM16 and SETBP1, genes involved in myeloid cell growth and differentiation.⁵ Although cell proliferation may have enhanced the therapeutic effect seen in our trial, the activation by retroviral insertional mutagenesis of well-known proto-oncogenes linked to the development of hematological abnormalities raised significant safety concerns.¹⁹ Despite these events, our trial demonstrated that gene therapy can provide significant

Correspondence: Dr M Grez, Institute for Biomedical Research, Georg-Speyer-Haus, Paul-Ehrlich-Strasse 42-44, 60596 Frankfurt, Germany.

E-mail: grez@em.uni-frankfurt.de

⁵These two authors contributed equally to this work.

Received 9 April 2008; revised 29 July 2008; accepted 14 August 2008; published online 11 September 2008

clinical benefit to CGD patients and emphasizes the need for a new generation of viral vectors with reduced genotoxicity.

The use of self-inactivating (SIN) vectors harboring deletions in the U3 enhancer/promoter region in combination with internal weaker cellular promoters may provide a safer alternative and create a therapeutic window for the treatment of CGD. However, these vectors have been associated with poor titers and low expression levels. In this study, we present a series of optimization steps leading to the generation of safety improved SIN vectors with high titers and enhanced transgene expression.

Results

Optimization of gp91^{phox} cDNA sequence results in increased viral titers

Retroviral vectors containing an optimized backbone architecture including deletion of enhancer and TATA box (SIN configuration)²⁰ were cloned for the expression of gp91^{phox} in hematopoietic cells (Figure 1). Initial attempts to generate high-titer SIN gammaretroviral vector expressing the wild-type version of gp91^{phox} failed because the titers of the virus produced by transient transfection protocols were low (<5 × 10⁵ TU/ml; Figure 2a), in contrast to the high titers achieved with similar vectors expressing enhanced green fluorescent protein (eGFP).²⁰ The inclusion of elements known to enhance titer and transgene expression such as WPRE did not increase titer (data not shown). Therefore, we considered optimization of the gp91^{phox} cDNA for which a complete new gp91^{phox} cDNA was synthesized. In addition to codon optimization, the GC content was

increased from originally 47% (wild-type gp91^{phox} cDNA) to 61%. Also putative polyA sites, internal consensus and cryptic splice donor sites and RNA instability motifs were removed (Supplementary Figure S1). The optimized gp91^{phox} cDNA (gp91s) was used to replace the native gp91^{phox} cDNA in the SIN gammaretroviral vector SERS11.SF.gp91.W to generate SERS11.SF.gp91s.W (Figure 1a). Inclusion of gp91s into the gamma SIN backbone led to a 10-fold increase in vector titer (Figure 2a), suggesting increased amounts of packagable viral genomic RNA. Indeed, 293T cells transfected with SERS11.SF.gp91s.W contained 4–6 times more genomic viral RNA than cells transfected with SERS11.SF.gp91.W (Figure 2b).

Higher reconstitution of superoxide production in X-CGD cells expressing gp91s

The myelomonocytic cell line X-CGD PLB-985²¹ was used to test for the reconstitution of superoxide production by SIN gammaretroviral vectors containing the optimized gp91^{phox} cDNA. X-CGD PLB-985 cells were transduced with SERS11.SF.gp91.W and SERS11.SF.gp91s.W at low multiplicity of infection (MOI) (<3). X-CGD PLB-985 cells transduced with the vector expressing the gp91s cDNA showed increased cell surface expression of the glycoprotein as indicated by the twofold increase in mean fluorescence intensity when compared with cells transduced with a similar vector containing the native gp91^{phox} cDNA (Figure 3a). Transduced cells were enriched by immunomagnetic selection to >95% gp91^{phox}-expressing cells and transgene copy numbers were estimated by quantitative PCR. Both populations were found to contain equal mean copy numbers per transduced cell (1–1.3 copies per cell each, data not shown). In agreement with the fluorescence-

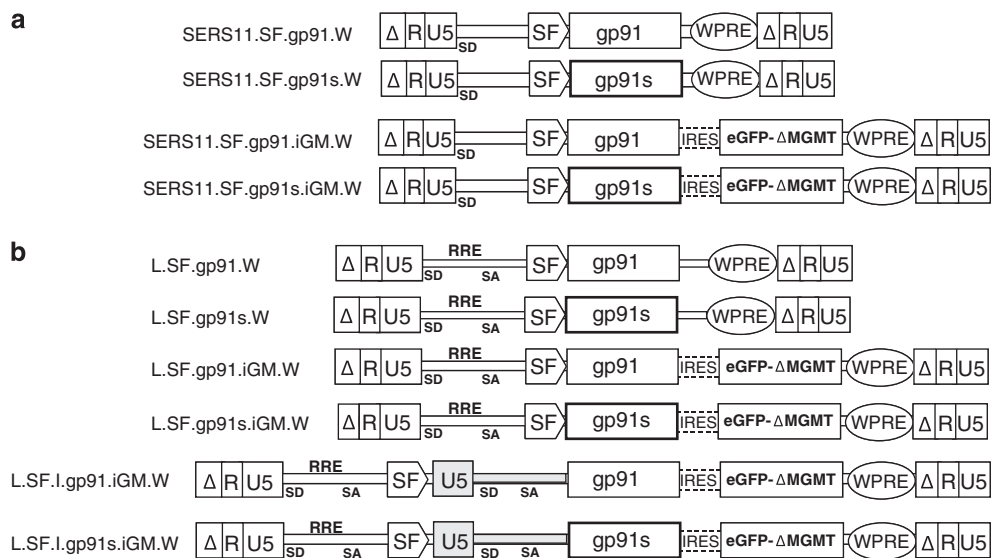


Figure 1 Schematic diagram of the vectors used in this study. The structures of the integrated proviral forms of monocistronic and bicistronic self-inactivating (SIN) gammaretroviral (a) and SIN lentiviral (b) vectors are shown. The following items are indicated: splice acceptor (SA) and splice donor (SD) sites within the 5'UTR, the open reading frames of wild-type gp91^{phox} (gp91) or synthetic gp91^{phox} (gp91s), the eGFP-ΔMGMT (enhanced green fluorescent protein-P144K mutant of canine O⁶-methylguanine-DNA methyltransferase-P144K) fusion separated by an EMCV-IRES site, the Rev responsive element (RRE), and the post-transcriptional regulatory element of the woodchuck hepatitis virus (WPRE). The SIN configuration is indicated by the deletion of the U3 (Δ) and the presence of an internal promoter (SF). SF denotes the enhancer and promoter elements derived from the long terminal repeat (LTR) of the spleen focus forming virus. The murine leukemia virus (MLV)-derived intron downstream of the internal SF promoter is boxed in gray.

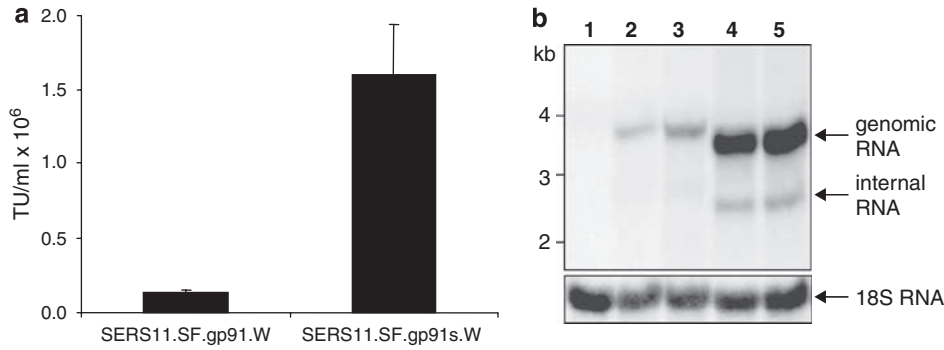


Figure 2 Optimization of gp91^{phox} cDNA leads to increased viral titers. (a) Titer of monocistronic self-inactivating (SIN) gammaretroviral vectors containing either wild-type or synthetic gp91^{phox}. Titers were determined on X-CGD PLB-985 cells by fluorescence-activated cell sorting (FACS) analysis with serial dilution of viral supernatants. The means and standard deviations of at least three independent experiments are shown. (b). 293T cells were transfected in duplicate with SERS11.SF.gp91.W (lanes 2 and 3) or SERS11.SF.gp91s.W (lanes 4 and 5) in the presence of the gag/pol and ecotropic env helper plasmids. After 48 h, total RNA was extracted from transfected cells, run on an agarose gel, blotted and hybridized against a WPRE probe. 18S RNA served as a loading control. Lane 1 contains RNA from untransfected cells. Internal RNA denotes the SF-driven gp91s transcript.

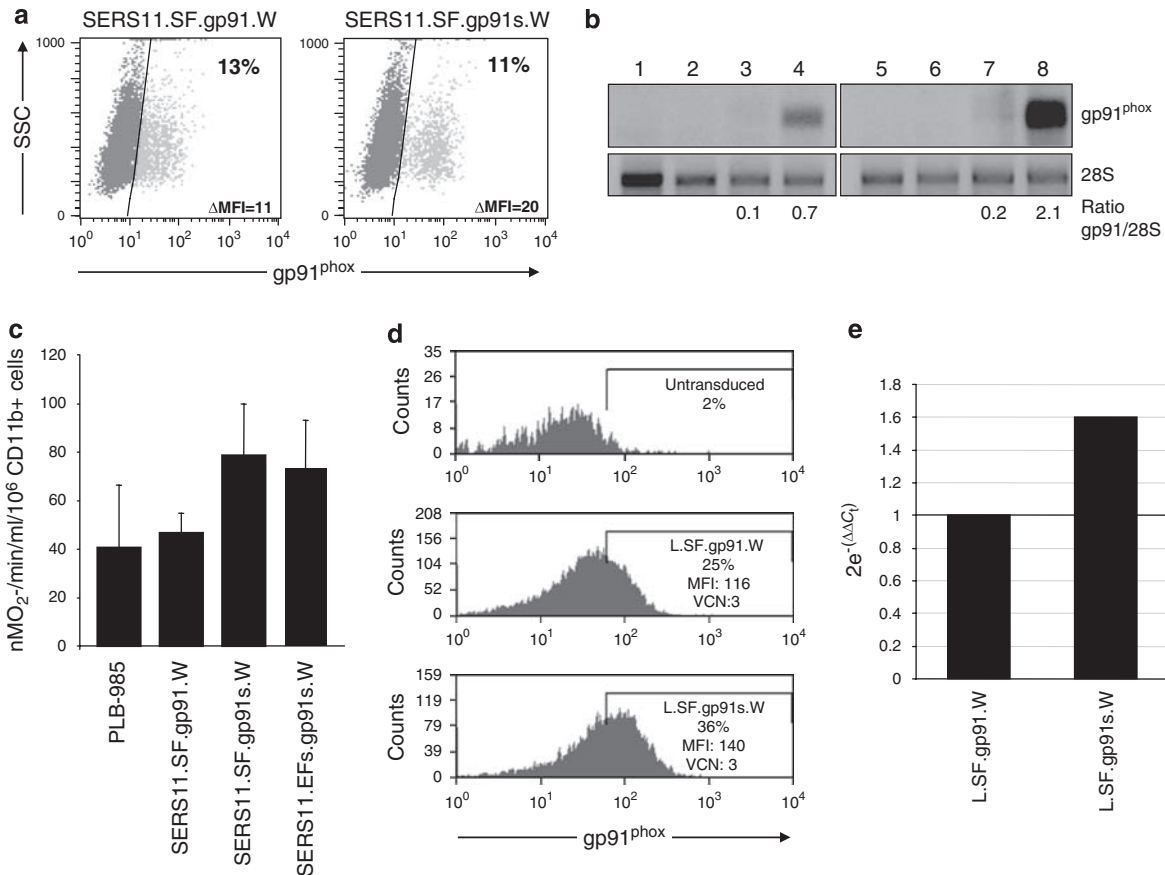


Figure 3 gp91^{phox} expression and reconstitution of superoxide production is improved in X-CGD PLB-985 and murine bone marrow cells expressing the synthetic gp91^{phox} cDNA. (a) Representative flow charts of X-CGD PLB-985 cells transduced with SERS11.SF.gp91.W or SERS11.SF.gp91s.W at equal multiplicity of infection (MOI). (b) Northern blot analysis of X-CGD PLB-985 cells (lanes 1 and 5), PLB-985 cells (lanes 2 and 6) and X-CGD PLB-985 cells transduced with either SERS11.SF.gp91.W (lanes 3 and 7) or SERS11.SF.gp91s.W (lanes 4 and 8) before (lanes 1–4) or after (lanes 5–8) dimethylsulfoxide (DMSO)-induced granulocytic differentiation. The hybridization probe was derived from the WPRE sequence. (c) X-CGD PLB-985 cells transduced with SERS11.SF.gp91.W, SERS11.SF.gp91s.W or SERS11.EFs.gp91s.W were sorted for gp91^{phox}-expressing cells and granulocytic differentiation was induced with DMSO for 6 days. Superoxide production was measured by the continuous cytochrome C reduction assay.²² (d) Representative histograms of murine Lin⁻ bone marrow cells transduced with the lentiviral vectors L.SF.gp91.W or L.SF.gp91s.W at an MOI of 10. (e) Relative quantification of gp91^{phox} transcripts by real-time RT-PCR in lin⁻-transduced cells. VCN, vector copy number.

activated cell sorting (FACS) data, almost a 10-fold increase in the levels of gp91^{phox} mRNA were detected by northern blotting in SERS11.SF.gp91s.W-transduced

X-CGD PLB-985 cells before and after granulocytic differentiation (Figure 3b). To test whether the increase in mRNA levels also results in higher superoxide

production, NADPH oxidase activity was assessed in transduced and immunomagnetically selected cells after dimethylsulfoxide induced granulocytic differentiation by the cytochrome C assay.²² Superoxide production in cells transduced with SERS11.SF.gp91s.W was twofold increased as compared with cells transduced with similar vectors expressing native gp91^{phox} (Figure 3c). This difference may underestimate the performance of the SERS11.SF.gp91s.W vector as immunomagnetic selection enriches for cells expressing high levels of gp91^{phox} and thus narrows the differences in the performance of the two vectors.

In further studies, we replaced the strong internal SFFV promoter by the short intronless version of the elongation factor 1 alpha promoter^{20,23,24} to test whether the gp91s expression levels driven by a weak internal promoter will also result in significant superoxide production levels. X-CGD PLB-985 cells were transduced at low MOI with SERS11.EFs.gp91s.W and cells expressing the transgene were isolated by immunomagnetic sorting. Copy number estimation revealed 1.3 proviral copies per transduced cell. After dimethylsulfoxide induced granulocytic differentiation, the SERS11.EFs.gp91s.W vector was as powerful in superoxide production as the SERS11.SF.gp91s.W vector (Figure 3c), indicating that high reconstitution of NADPH oxidase activity is possible from a weaker internal promoter if the optimized gp91^{phox} sequence is used.

SIN lentiviral vectors containing the native or the synthetic gp91^{phox} were also generated. These vectors were used to transduce lin⁻ bone marrow cells from X-CGD mice at an MOI of 10 and gp91^{phox} expression was evaluated by FACS 3–4 days after transduction. As observed previously for the SIN gammaretroviral vectors, the inclusion of the synthetic gp91^{phox} cDNA in these vectors resulted in higher expression efficiency as reflected by the increase in median fluorescence intensity and in the numbers of cells expressing detectable levels of gp91^{phox} (Figure 3d). Equal vector copy numbers were observed by real-time PCR in both populations arguing for a better performance of the gp91s expressing lentiviral vector. Similar to the results observed in cell lines, gp91^{phox} RNA levels were increased by 1.6-fold in cells expressing the synthetic version of gp91^{phox} (Figure 3e).

Introduction of gp91s in bicistronic vectors strongly increases transgene expression of the second cistron

In further work, we generated bicistronic SIN lentiviral and SIN gammaretroviral vectors encoding gp91^{phox} in the first cistron and a fusion of eGFP and the P144K mutant of canine O⁶-methylguanine-DNA methyltransferase (Δ MGMT, a kind gift from F Schuening) in the second cistron allowing selection of transduced cells and their detection by eGFP. Similar to our previous observations, replacement of eGFP in the SIN gammaretroviral vector SERS11.SF.GFP.W as well as in the SIN lentiviral vector RRL.PPT.SF.GFP.pre with the bicistronic cassette gp91^{phox}-IRES-eGFP- Δ MGMT resulted in rather low transgene expression levels when compared with the original eGFP expressing vectors (Figure 4a).

To increase transgene expression in our SIN lentiviral vectors, we initially introduced an MLV-based intron downstream of the internal SFFV promoter (L.SF.I.

gp91.iGM.W) and later replaced the open reading frame of the native gp91^{phox} by the synthetic gp91^{phox} (L.SF.I.gp91s.iGM.W). Although we did not achieve higher transgene expression with the intron containing vector in transduced K562 cells, transgene expression increased 1.6- to 2-fold after introduction of the optimized gp91s cDNA (Figure 4b). However, in other cell lines and most importantly in human primary CD34+ peripheral blood progenitor cells (PBPCs), incorporation of the MLV-based intron dramatically enhanced transgene expression of gp91^{phox} and eGFP- Δ MGMT in comparison to the unmodified lentiviral vector (Figures 4b–d). The introduction of the synthetic gp91^{phox} resulted in an even stronger increase in transgene expression also in the absence of the intron, showing that optimization of the first cistron has also a profound effect on expression from the non-optimized second cistron (Figures 4b–d). The combination of the optimized gp91^{phox} with the MLV intron in the lentiviral vector L.SF.I.gp91s.iGM.W did not increase transgene expression levels any further (Figures 4b and d).

As seen with the monocistronic vectors, vector titers were increased for bicistronic vectors containing an optimized gp91^{phox} about threefold (data not shown). Although transgene expression levels were lower in the non-optimized gammaretroviral vector when compared with the non-optimized lentiviral vector, they were similar in the gp91^{phox} optimized versions (Figure 4b). Quantitative PCR in CD34+ PBPC and K562 cells revealed almost equal vector copy numbers per transgene-positive cell for the vectors used (Figure 4c), confirming that the observed effects were not due to different copy numbers.

Discussion

Insertional oncogenesis caused by retroviral integration is a major concern in gene therapy. Most of the actual vectors in gene therapy trials are long terminal repeat-driven vectors and were derived from MLV because of their well-known biological properties, their innate property to integrate into the genome of the host cells, the simplicity of vector construction and the robust titers achieved in stable packaging systems. However, intensive studies on retroviral integration have revealed that integration of gammaretroviruses is not a random process but occurs semi-randomly with an exquisite preference for promoter-proximal regions in gene-dense regions of the genome.^{25–28} From both promoter-proximal and distal positions, transcriptional activation of cellular genes has been observed in a series of *in vitro* and *in vivo* approaches, including human gene therapy trials leading to clonal imbalance and eventually malignant transformation.^{11,12,29–32} Although insertional mutagenesis cannot be avoided at present, the effects of retrovirus integration on cellular gene expression and physiology can be significantly lowered by modifications within the retroviral backbone. Among others the inclusion of insulators in the viral backbone has been shown to drastically reduce the effects of the viral enhancer on cellular gene expression.^{33,34} Similarly, SIN vectors, in which the viral enhancers are deleted, are significantly less mutagenic than their long terminal repeat-driven counterparts.^{35–37} Depending on the cargo,

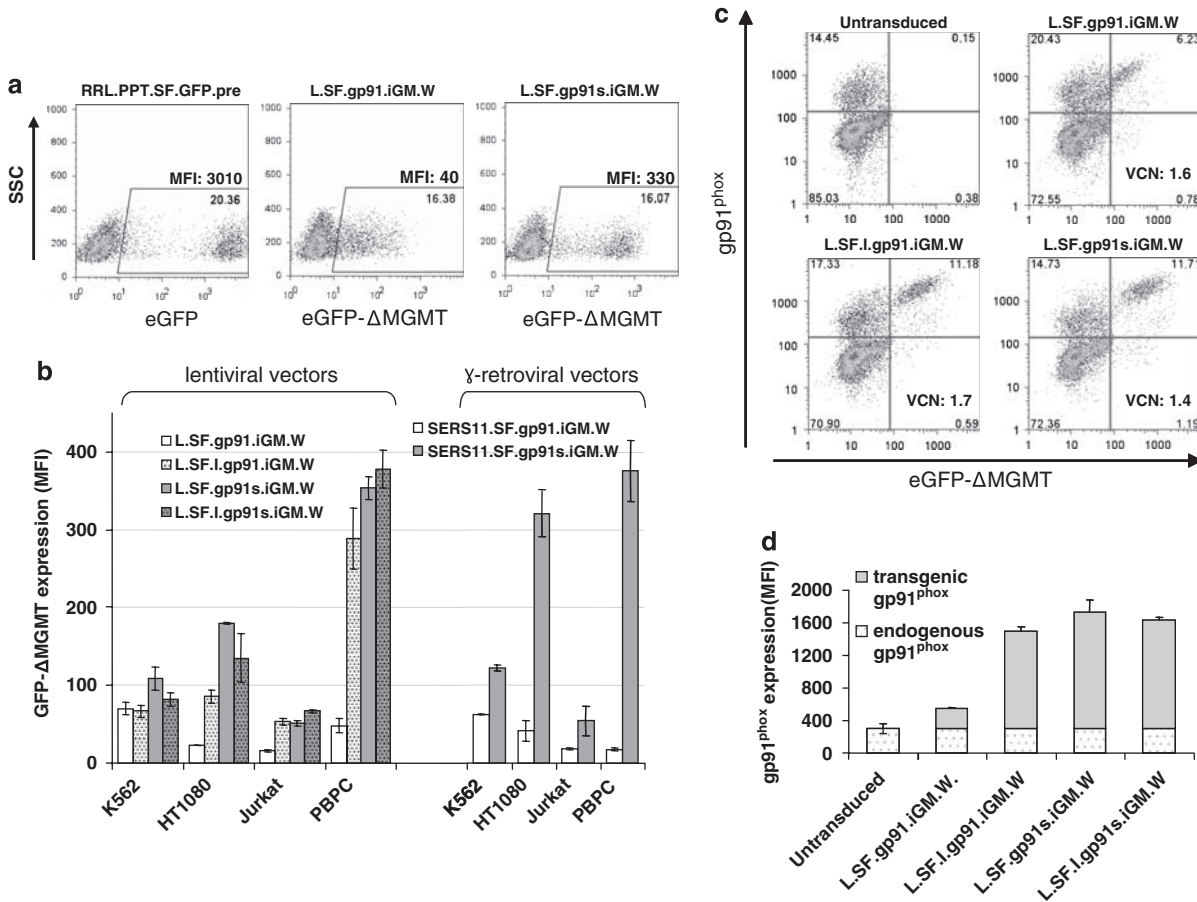


Figure 4 Optimization of gp91^{phox} and introduction of a murine leukemia virus (MLV)-based intron enhances transgene expression of both cistrons in bicistronic vectors. (a) Representative flow charts of primary human CD34+ peripheral blood progenitor cells (PBPCs) transduced with lentiviral vectors containing enhanced green fluorescent protein (eGFP) only (RRL.PPT.SF.GFP.pre), native gp91^{phox} (L.SF.gp91.iGM.W) or synthetic gp91^{phox} (L.SF.gp91s.iGM.W), analyzed 5 days after transduction. (b) eGFP-ΔMGMT median fluorescence intensity (MFI) of cell lines and PBPC transduced with the indicated bicistronic retroviral vectors was measured by flow cytometry 5 days after transduction. Means and standard deviations of at least three independent experiments are shown. To avoid multicopy integrations, cells were transduced with different virus dilutions and only those samples with low (<20%) transduction rates were chosen for comparative analyses. Vector copy number (VCN) determination in transduced K562 cells and CD34+ PBPC revealed VCNs of 1.2–1.3 copies per transgene-positive K562 cell, and 1.4–1.7 copies per transgene-positive human CD34+ PBPC. (c) Representative flow cytometric analyses of human CD34+ PBPC transduced with the indicated self-inactivating (SIN) lentiviral vectors and stained for gp91^{phox} at day 12 of *ex vivo* culture. VCNs per transgene-positive cell are shown. (d) MFI of gp91^{phox} in transduced human CD34+ PBPC. The dotted parts of the bars indicate endogenous gp91^{phox} expression, whereas retroviral vector-driven expression is indicated by the gray parts of the bars. Means and standard deviations of at least two independent experiments are shown. Transduction of cells was performed with diluted lentiviral vectors at a multiplicity of infection (MOI) of 0.8.

however, the SIN configuration often results in lower titers and lower transgene expression. Indeed, our efforts to generate SIN gammaretroviral and lentiviral vectors for clinical application failed at the beginning because titers and transgene expression were below the therapeutic threshold required for a clinical intervention. We reasoned that the cDNA for gp91^{phox} could be one of the causes of this problem as similar vectors with other transgenes did not show such a drastic reduction in titer and transgene expression.²⁰ The synthesis of a new gp91^{phox} cDNA resulted in a significant increase in vector titers and gene expression levels. In our case, this improvement seems to be mainly due to improved mRNA stability and/or transport, as higher gp91^{phox} mRNA levels were observed in 293T producer cells as well as in X-CGD PLB-985 cells transduced with vectors containing gp91s. Most significantly, we could also detect a similar increase in eGFP-ΔMGMT expression from the

second cistron that was not codon-optimized. Similar observations have been made for vectors containing factor VIII and factor IX cDNAs, in which codon optimization of the transgene resulted in a 10-fold increase in viral titers and improved expression levels.^{38,39} Interestingly, the extent of increased transgene expression after cDNA optimization or introduction of an intron varies tremendously between different cell lines, which underlines the importance to test a vector on target cells rather than cell lines.

The use of SIN vectors with improved titers and transgene expression levels has important advantages for gene therapy. First, there is no necessity for a strong internal promoter to drive transgene expression. The use of a weak promoter or even a cell-type-specific promoter generating physiologically adequate levels of the therapeutic protein may be sufficient for a clinical benefit and might further reduce the incidence of

insertion tumorigenesis. Second, there is no necessity for multiple copies of the vector genome in the target cell. The better performance of SIN vectors with optimized transgenes should allow for a therapeutic effect with only one or two vector copies per transduced cell.

Materials and methods

Cell lines

The human X-CGD PLB-985 cell line was derived from the PLB-985 myelomonocytic leukemia cell line by disrupting the *gp91^{phox}* gene by homologous recombination.²¹ The non-adherent growing K562, Jurkat and PLB-985 cells and their derivatives were grown in RPMI complemented with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 µg/µl streptomycin). HT1080 cells were cultured in Dulbecco's modified Eagle's medium with identical supplements as stated above.

Retroviral vectors and vector production

The gammaretroviral vector backbone was derived from the SERS11.SF.GFP.W vector.²⁰ The eGFP gene was substituted either with the wild-type *gp91^{phox}* cDNA or the optimized *gp91s* cDNA. For construction of bicistronic vectors, the eGFP cassette in SERS11.SF.GFP.W or in RRL.PPT.SF.GFP.pre⁴⁰ was exchanged by a cassette containing the native *gp91^{phox}* cDNA in the first cistron and a eGFP-canine O⁶-methylguanine-methyltransferase-P144K (Δ MGMT) fusion, analogous to that described in Choi *et al.*⁴¹ in the second cistron. In vector L.SF.gp91.iGM.W the 5'UTR sequence from the MFGS-*gp91^{phox}* vector⁴² (including the U5 region, an intron and further 280 bp of 5'UTR) was inserted downstream of the SFFV promoter to generate L.SF.I.gp91.iGM.W. In vector L.SF.I.gp91s.iGM.W, the native *gp91^{phox}* cDNA was exchanged with that of *gp91s*.

For monocistronic lentiviral vectors, the native *gp91^{phox}* cDNA or the optimized *gp91^{phox}* sequence were subcloned into the pHR-SIN SEWe lentiviral vector⁴³ to generate L.SF.gp91.W and L.SF.gp91s.W, respectively. Cell-free viral supernatants were generated by transient co-transfection on HEK293T cells as described elsewhere.⁴³

Isolation and ex vivo transduction of primary hematopoietic progenitor cells

Murine bone marrow cells were harvested from the femurs and tibias of *gp91^{phox}* knockout mice at approximately 10 weeks of age. Lin⁻ progenitors were isolated using a StemSep mouse progenitor enrichment cocktail and negative selection columns following the manufacturer's instructions (StemCell Technologies, London, UK). Lin⁻ cells were cultured in StemSpan SFEM serum-free medium (StemCell Technologies) supplemented with 1% penicillin/streptomycin and cytokines (100 ng/ml murine stem cell factor, 100 ng/ml mFlt3L, 100 ng/ml hIL-11 and 20 ng/ml mL-3). Cells were transduced at an MOI of 10. At 3–4 days after transduction, 2×10^5 transduced lin⁻ cells were stained with the fluorescein isothiocyanate (FITC)-conjugated murine anti-human *gp91^{phox}* monoclonal 7D5 and PE-labeled anti-Sca-1 antibody (Insight Biotechnology Ltd, London, UK) and analyzed by flow cytometry.

Human CD34+ cells were purified from the apheresis product of granulocyte colony-stimulating factor-mobilized peripheral blood of healthy volunteers after informed consent (protocol no. EK221102004, approved by the Institutional Review Board of the University Clinic Dresden) using MACS MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of peripheral blood CD34+ progenitor cells (CD34+ PBPC) was >98%. CD34+ PBPCs were cultured in X-VIVO10 with 1% human serum albumin (HSA); 50 ng/ml FLT3 ligand, 20 ng/ml stem cell factor, 10 ng/ml thrombopoietin and 10 ng/ml IL3. Transduction of human CD34+ PBPC was performed on RetroNectin (Takara, Shiga, Otsu, Japan)-precoated 48-well plates (1×10^5 CD34+ PBPC per well) 72 h after initiation of culture using different dilutions of virus vector containing media and 5 µg/ml protamine sulfate.

Cells were analyzed by flow cytometry with a FACS Calibur (BD Biosciences, San Jose, CA, USA). EGFP- Δ MGMT expression was analyzed 3, 5 and 9 days after transduction, whereas *gp91^{phox}* expression was measured 3 days after transduction of K562 and PLB-985 cells or 9 days after transduction of CD34+ cells. For *gp91^{phox}* detection, cells were either stained with FITC-7D5 or stained indirectly using an unlabeled 7D5 followed by Cy5-conjugated rat anti-mouse antibody (Dianova, Hamburg, Germany). CD34+ PBPC cells were permeabilized prior to 7D5 staining. Healthy human PBPCs produce native *gp91^{phox}* starting by day 7 after initiation of culture. For comparison of *gp91^{phox}* transgene expression levels in healthy PBPC, native *gp91^{phox}* expression of non-transduced cells was subtracted.

Immunomagnetic selection of *gp91^{phox}*-expressing cells and cytochrome C assay

gp91^{phox}-expressing cells were incubated with the cytochrome *b558*-specific 7D5 antibody⁴⁴ and incubated for 20 min at room temperature. After washing with phosphate-buffered saline, the cells were resuspended in phosphate-buffered saline containing RAM-IgG1 FITC Ab followed by incubation at room temperature for 15 min. After several wash steps, the cells were incubated with anti-FITC MicroBeads and applied to the separation column according to manufacturer's instructions (Miltenyi Biotec GmbH). Cytochrome C assays were carried out according to Mayo and Curnutte²² using a Spectra MAX 340 reader (Molecular Devices, Sunnyvale, CA, USA) and the SOFTmax Version 2.02 PRO software.

Determination of viral vector copy number by real-time quantitative PCR

Genomic DNA was extracted from transduced cells using the DNeasy kit (Qiagen GmbH, Hilden, Germany). Real-time quantitative PCR to determine lentiviral copy number was performed using an ABI 7000 Sequence Detection System (ABI; Applied Biosystems, Warrington, UK). The primer sequences for WPRE were as follows: forward, 5'-TGGATTCTGCGCGGA-3'; reverse, 5'-GAAGGAAGTCCGCTGGATT-3'. The WPRE probe sequence was 5'-FAM-CTTCTGCTACGTCCCTTCGGC CCT-TAMRA-3'. The mouse titin gene (*Ttn*) was used

as an endogenous two-copy gene control. The Ttn primers sequences were as follows: forward, 5'-AAAAC GAGCAGTGACCTGAGG-3'; reverse, 5'-TTCAGTCAT GCTGCTAGCGC-3'. The Ttn probe sequence was 5'-FAM-TGCACGGAATCTCGTCTCAGTC-TAMRA-3'. For human CD34+ PBPC and human cell lines, the phenol sulfotransferase gene (STP) served as an internal control. The STP primer sequences were as follows: forward, 5'-GGTGCCCTTCCTTGAGTTCA-3'; reverse, 5'-CCCCT TGCACCCAGGAC-3'; probe, 5'-FAM-CCCCAGGGATT CCCTCAGGTGTGT-TAMRA-3'. The percentage of transgene-positive cells as determined by flow cytometry was used to correct for vector copy number in transduced cells.

RNA extraction and real-time PCR analysis

Total RNA was obtained using the RNeasy kit (Qiagen GmbH). After DNase treatment, RNA was reverse transcribed using 200 U M-MLV reverse transcriptase (Invitrogen Corporation, Paisley, UK) following the manufacturer's instructions. Real-time PCR was performed using the probe and primers described above. The relative quantification of RNA transcripts was performed following the Livak method ($\text{ratio} = 2^{-\Delta\Delta C_t}$).⁴⁵

Northern blot

Analysis of 3–7.5 μg total RNA by northern blot was accomplished using the NorthernMax Kit according to the instructions of the manufacturer (Ambion Incorporation, Cambridgeshire, Paisley, UK). A ³²P-labeled WPRE fragment was used for hybridization. After several washings, the membrane was exposed to an X-ray film (Kodak BioMax MS Film; Sigma-Aldrich, Steinheim, Germany) for 4 h at -80°C . The 18S or 28S rRNA bands were used to assess for equal amounts of loaded RNA.

Acknowledgements

This study was supported by grants from the CGD Research Trust (J4G/04B/GT) to MG and AJT, the Deutsche Forschungsgemeinschaft to SB (BR 2057/3-2), CB, AS and MG (SPP1230) and to CB and AS (excellence cluster REBIRTH) and from the Bundesministerium für Bildung und Forschung to CB, AS and MG (TreatID). AS was further supported by a personal stipend of the Else-Kröner Memorial Foundation. The Georg-Speyer-Haus is supported by the Bundesministerium für Gesundheit and the Hessisches Ministerium für Wissenschaft und Kunst.

References

- 1 Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A *et al*. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* 2002; **296**: 2410–2413.
- 2 Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nussbaum P *et al*. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000; **288**: 669–672.
- 3 Gaspar HB, Björkregren E, Parsley K, Gilmour KC, King D, Sinclair J *et al*. Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther* 2006; **14**: 505–513.

- 4 Gaspar HB, Parsley KL, Howe S, King D, Gilmour KC, Sinclair J *et al*. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 2004; **364**: 2181–2187.
- 5 Ott MG, Schmidt M, Schwarzwaelder K, Stein S, Siler U, Koehl U *et al*. Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. *Nat Med* 2006; **12**: 401–409.
- 6 Baum C, Dullmann J, Li Z, Fehse B, Meyer J, Williams DA *et al*. Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* 2003; **101**: 2099–2114.
- 7 Baum C, Fehse B. Mutagenesis by retroviral transgene insertion: risk assessment and potential alternatives. *Curr Opin Mol Ther* 2003; **5**: 458–462.
- 8 Baum C, Kustikova O, Modlich U, Li Z, Fehse B. Mutagenesis and oncogenesis by chromosomal insertion of gene transfer vectors. *Hum Gene Ther* 2006; **17**: 253–263.
- 9 Baum C, von Kalle C, Staal FJ, Li Z, Fehse B, Schmidt M *et al*. Chance or necessity? Insertional mutagenesis in gene therapy and its consequences. *Mol Ther* 2004; **9**: 5–13.
- 10 Cavazzana-Calvo M, Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? *J Clin Invest* 2007; **117**: 1456–1465.
- 11 Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E *et al*. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2003; **348**: 255–256.
- 12 Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; **302**: 415–419.
- 13 McCormack MP, Rabbitts TH. Activation of the T-cell oncogene LMO2 after gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2004; **350**: 913–922.
- 14 Heyworth PG, Cross AR, Curnutte JT. Chronic granulomatous disease. *Curr Opin Immunol* 2003; **15**: 578–584.
- 15 Segal AW. The NADPH oxidase and chronic granulomatous disease. *Mol Med Today* 1996; **2**: 129–135.
- 16 Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine (Baltimore)* 2000; **79**: 170–200.
- 17 Anderson-Cohen M, Holland SM, Kuhns DB, Fleisher TA, Ding L, Brenner S *et al*. Severe phenotype of chronic granulomatous disease presenting in a female with a *de novo* mutation in gp91-phox and a non familial, extremely skewed X chromosome inactivation. *Clin Immunol* 2003; **109**: 308–317.
- 18 Winkelstein JA, Marino MC, Johnston Jr RB, Boyle J, Curnutte J, Gallin JI *et al*. Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)* 2000; **79**: 155–169.
- 19 Naldini L. Inserting optimism into gene therapy. *Nat Med* 2006; **12**: 386–388.
- 20 Schambach A, Mueller D, Galla M, Versteegen MM, Wagemaker G, Loew R *et al*. Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. *Gene Therapy* 2006; **13**: 1524–1533.
- 21 Zhen L, King AA, Xiao Y, Chanock SJ, Orkin SH, Dinanuer MC. Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. *Proc Natl Acad Sci USA* 1993; **90**: 9832–9836.
- 22 Mayo LA, Curnutte JT. Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. *Methods Enzymol* 1990; **186**: 567–575.
- 23 Kostic C, Chiodini F, Salmon P, Wiznerowicz M, Deglon N, Hornfeld D *et al*. Activity analysis of housekeeping promoters using self-inactivating lentiviral vector delivery into the mouse retina. *Gene Therapy* 2003; **10**: 818–821.

- 24 Thornhill SI, Schambach A, Howe SJ, Ulaganathan M, Grassman E, Williams D *et al*. Self-inactivating gammaretroviral vectors for gene therapy of X-linked severe combined immunodeficiency. *Mol Ther* 2008; **16**: 590–598.
- 25 Aiuti A, Cassani B, Andolfi G, Mirolo M, Biasco L, Recchia A *et al*. Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy. *J Clin Invest* 2007; **117**: 2233–2240.
- 26 Bushman FD. Retroviral integration and human gene therapy. *J Clin Invest* 2007; **117**: 2083–2086.
- 27 Deichmann A, Hacein-Bey-Abina S, Schmidt M, Garrigue A, Brugman MH, Hu J *et al*. Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J Clin Invest* 2007; **117**: 2225–2232.
- 28 Schwarzwaelder K, Howe SJ, Schmidt M, Brugman MH, Deichmann A, Glimm H *et al*. Gammaretrovirus-mediated correction of SCID-X1 is associated with skewed vector integration site distribution *in vivo*. *J Clin Invest* 2007; **117**: 2241–2249.
- 29 Du Y, Jenkins NA, Copeland NG. Insertional mutagenesis identifies genes that promote the immortalization of primary bone marrow progenitor cells. *Blood* 2005; **106**: 3932–3939.
- 30 Kustikova OS, Geiger H, Li Z, Brugman MH, Chambers SM, Shaw CA *et al*. Retroviral vector insertion sites associated with dominant hematopoietic clones mark ‘stemness’ pathways. *Blood* 2007; **109**: 1897–1907.
- 31 Metais JY, Dunbar CE. The MDS1-EVII gene complex as a retrovirus integration site: impact on behavior of hematopoietic cells and implications for gene therapy. *Mol Ther* 2008; **16**: 439–449.
- 32 Modlich U, Kustikova OS, Schmidt M, Rudolph C, Meyer J, Li Z *et al*. Leukemias following retroviral transfer of multidrug resistance 1 (MDR1) are driven by combinatorial insertional mutagenesis. *Blood* 2005; **105**: 4235–4246.
- 33 Emery DW, Yannaki E, Tubb J, Stamatoyannopoulos G. A chromatin insulator protects retrovirus vectors from chromosomal position effects. *Proc Natl Acad Sci USA* 2000; **97**: 9150–9155.
- 34 Ryu BY, Evans-Galea MV, Gray JT, Bodine DM, Persons DA, Nienhuis AW. An experimental system for the evaluation of retroviral vector design to diminish the risk for proto-oncogene activation. *Blood* 2008; **111**: 1866–1875.
- 35 Modlich U, Bohne J, Schmidt M, von Kalle C, Knoss S, Schambach A *et al*. Cell-culture assays reveal the importance of retroviral vector design for insertional genotoxicity. *Blood* 2006; **108**: 2545–2553.
- 36 Zychlinski D, Schambach A, Modlich U, Maetzig T, Meyer J, Grassman E *et al*. Physiological promoters reduce the genotoxic risk of integrating gene vectors. *Mol Ther* 2008; **16**: 718–725.
- 37 Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C *et al*. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 2006; **24**: 687–696.
- 38 Radcliffe PA, Sion CJ, Wilkes FJ, Custard EJ, Beard GL, Kingsman SM *et al*. Analysis of factor VIII mediated suppression of lentiviral vector titres. *Gene Therapy* 2008; **15**: 289–297.
- 39 Wu Z, Sun J, Zhang T, Yin C, Yin F, Van Dyke T *et al*. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* 2008; **16**: 280–289.
- 40 Schambach A, Baum C. Vector design for expression of O⁶-methylguanine-DNA methyltransferase in hematopoietic cells. *DNA Repair (Amst)* 2007; **6**: 1187–1196.
- 41 Choi U, DeRavin SS, Yamashita K, Whiting-Theobald N, Linton GF, Loktionova NA *et al*. Nuclear-localizing O⁶-benzylguanine-resistant GFP-MGMT fusion protein as a novel *in vivo* selection marker. *Exp Hematol* 2004; **32**: 709–719.
- 42 Brenner S, Ryser MF, Choi U, Whiting-Theobald N, Kuhlisch E, Linton G *et al*. Polyclonal long-term MFGS-gp91phox marking in rhesus macaques after nonmyeloablative transplantation with transduced autologous peripheral blood progenitor cells. *Mol Ther* 2006; **14**: 202–211.
- 43 Demaison C, Parsley K, Brouns G, Scherr M, Battmer K, Kinnon C *et al*. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of immunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Hum Gene Ther* 2002; **13**: 803–813.
- 44 Nakamura M, Murakami M, Koga T, Tanaka Y, Minakami S. Monoclonal antibody 7D5 raised to cytochrome b558 of human neutrophils: immunocytochemical detection of the antigen in peripheral phagocytes of normal subjects, patients with chronic granulomatous disease, and their carrier mothers. *Blood* 1987; **69**: 1404–1408.
- 45 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; **25**: 402–408.

Supplementary Information accompanies the paper on Gene Therapy website (<http://www.nature.com/gt>)