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A comparative evaluation of gene transfer into blood cells using the same retroviral backbone for independent expression of the EGFP and ΔLNGFR marker genes

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ABSTRACT

Background and Objectives. **Retroviral vectors are widely used to deliver foreign genes to hematopoietic stem cells (HSC). Improvement of marking protocols needs reporter genes to allow rapid detection and efficient selection of transduced cells. The great potential of EGFP and ΔLNGFR as reporter systems prompted us to compare them simultaneously, using the same retroviral backbone and the same gene transfer procedures.**

Design and Methods**. The EGFP and ΔLNGFR coding sequences were separately cloned into the MFG retroviral backbone. A cloning strategy assuring that both genes utilize the same ATG as the start codon was adopted. Marker gene expression, viral titers, transduction efficiency, and vector stability were evaluated in expanded amphotropic packaging clones and human hematopoietic cell lines by flow cytometry and PCR analysis. Vectors were also tested for their ability to transduce CD34$^+$ peripheral blood cells.**

Results. **A significantly larger number of MFG-ΔLNGFR packaging clones were obtained that produced high viral titers. A direct correlation between viral titer and marker gene expression in packaging clones was demonstrated for both constructs. Similar expression kinetics and absence of *in vitro* toxicity in transduced cells were also observed for both constructs. Successful infection of CD34$^+$ cells was achieved even after a short time of exposure to recombinant viruses.**

Interpretation and Conclusions. **Our results demonstrate that EGFP and ΔLNGFR marker genes are equally useful for a rapid, specific and non-toxic detection of transduced cells. The MFG-EGFP construct appears useful to optimize gene transfer protocols *in vitro*. On the other hand, the MFG-ΔLNGFR construct, for making possible a more efficient selection of high titer producer clones, as well as for safety and adaptability to the *in vivo* use, is more suitable for clinical applications.**

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Gene marking studies on hematopoietic stem cells (HSC) were initially undertaken to identify the source of tumor relapse and to investigate the contribution of infused bone marrow cells to long-term hematopoietic recovery in patients receiving autologous bone marrow transplantation as cancer therapy.[1-4] Advances in marking technology have broadened this application to the assessment of marrow purging effectiveness[5] and to the monitoring of survival of genetically modified cells of the donor in adoptive immunotherapy.[6-8] Furthermore, important information about the consequences of *ex vivo* manipulation of HSC on long-term recovery and survival of stem cells and on gene transfer efficiency can be obtained by gene marking *in vitro*,[9] in animal models[10-13] and in humans.[14-17]

Retroviral vectors, based on the Moloney murine leukemia virus (MoMLV) are widely used for gene delivery into HSC for their capacity of stable genomic integration into all cell lineages.[18]

The adoption of marker genes that can be rapidly monitored and used for efficient selection of transduced cells will facilitate the improvement of gene marking protocols. To date the neomycin phosphotransferase (*neo*) gene has been the most widely used marker in clinical studies.[1-4,14-17] However, the deployment of *neo* is hampered by non-specific toxicity of neomycin and by potential immunoclearence of transduced cells.[19] Furthermore, selection is time-consuming. Cytochemical markers, such as β-galactosidase,[20,21] also have important limitations. In fact, the presence of endogenous activity in some cell types, the need to use fluorogenic substrates, and the potential for passive transfer of the enzymatic activity to untransduced neighboring cells make this marker quite unreliable.

Several alternatives including the genes coding for the enhanced green fluorescent protein (EGFP) and the truncated version of the low-affinity nerve growth factor receptor (ΔLNGFR) have been recently proposed. The EGFP is a red–shifted, humanized variant of the GFP protein, with improved fluorescent inten-

sity, obtained by genetic engineering.[22-24] EGFP protein is well expressed in human cells and its fluorescence activity requires no substrates, cofactors or additional gene products, so that transduced cells can be immediately scored by fluorescence microscopy and/or by flow cytometry.

The cell-surface marker ΔLNGFR is a defective, nonfunctional form of the LNGFR (p75NGFR),[25-27] truncated in its intracytoplasmatic domain.[8,28] ΔLNGFR is not recognized by the human immune system, being constitutively expressed on the surface of human nervous cells and mature B lymphocytes but not on hematopoietic stem cells.[25-27] This marker has been already approved for clinical application in bone marrow transplantation studies.[8] Cells expressing ΔLNGFR can be rapidly detected and easily selected by fluorescence activated cell sorting (FACS) or immunomagnetic sorting.[12,28-31] The present study was directed at comparing EGFP and ΔLNGFR as marker genes for HSC tracking, while evaluating also the potential adaptability of the two marker systems for operational procedures that may be adopted in the clinical setting. To do this, we developed two MoMLV-derived retroviral vectors, differing only for the primary sequence of their marker gene. This paper describes the vector construction and generation of amphotropic MFG-ΔLNGFR- and MFG-EGFP-producer cell lines. The correlation between virus yield and intensity of marker gene expression in packaging clones was investigated. Transduction efficiency, short- and long-term cell toxicity and marker gene expression were evaluated for both vectors in different cell lines and in primary human hematopoietic stem cells.

Design and Methods

Progenitor cells and cell lines

Peripheral blood (PB) mononuclear cells were obtained, by leukapheresis, from myeloma patients after stem cells mobilization with granulocyte-colony stimulating factor (G-CSF) and cyclophoshamide.[32] $CD34^+$ cells were isolated using the Ceprate LC stem cell concentrator (CellPro Inc., Bothell, WA, USA) as previously described.[33] Briefly, leukapheresis products were incubated with a biotinylated anti-CD34 monoclonal antibody (MoAb) (clone 12.8). The samples were then passed through an avidin column. $CD34^+$ cells were retained within the column and then released by agitation. The entire procedure was performed according to the manufacturer's instructions. The purity of the $CD34^+$ fraction was usually greater than 95% (median 96%, range: 92-98.5%), as assessed by staining with a phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (clone 8G12, Becton Dickinson, San Josè, CA, USA).

Cells were cryopreserved at -80°C in RPMI 1640 medium (RPMI + Glutamax, GIBCO-BRL, Paisley, Scotland), 10% DMSO and 4% human serum albumin (Farma-Biagini, Lucca, Italy) until use.

Amphotropic GP+*env*Am12 packaging cells,[34] NIH3T3 murine fibroblasts, K562 (human chronic myelogenous leukemia), and Raji (human Burkitt's lymphoma) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MA, USA); the ecotropic Bosc23 packaging cell line[35] was kindly provided by Dr. F. Tatò (University of Rome, Italy) whereas the NB4 cell line (human acute promyelocytic leukemia) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). K562, Raji and NB4 cells were grown in RPMI 1640 medium, whereas NIH3T3 and packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL). All culture media were supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO-BRL), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO-BRL). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Vector construction

Both MFG-EGFP and MFG-ΔLNGFR constructs derived from the pMFGnlsLacZ retroviral vector[36] by replacing the nlsLacZ-encoding NcoI/BamHI fragment with the EGFP or the ΔLNGFR marker gene, respectively.

A similar cloning strategy was adopted to assure that both genes utilized the same MoMLV envelope ATG as the start codon.

The EGFP coding sequence was obtained from pEGFP-1 (Clontech, Palo Alto, CA, USA) by digestion with NcoI/NotI restriction enzymes. The 724bp resulting fragment was inserted into NcoI/BamHI sites of the MFG vector backbone after fill-in of the BamHI and NotI recessed extremities, by sticky-blunt ligation, using standard cloning procedures (Figure 1). The modified ΔLNGFR coding sequence was obtained from ptLNGFR (kindly provided by C. Bordignon, Milan, Italy) by polymerase chain reaction (PCR) using the following primers: 5'-GAG-

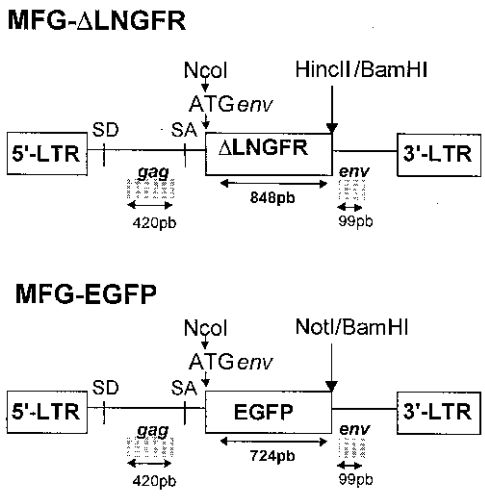

Figure 1. Schematic representation of the MFG-ΔLNGFR and MFG-EGFP retroviral vectors. LTR, long terminal repeat; SD and SA, splice donor and acceptor sites, respectively; ATG, env start codon; ΔLNGFR, intracitoplasmatically truncated low affinity nerve growth factor receptor; EGFP, enhanced green fluorescent protein; *gag* and *env* indicate the presence of part of *gag* and *env* sequences.

GCGGGC**C**ATGGGGGCAGGTGCCACCGGC-CGCGC**A**ATGGAC-3' and 5'-CATGCCTGCAGGTC-GACTCTAGAG-3', mapping within the ΔLNGFR gene. The forward primer contains two silent mutations (bases in bold type), which were intentionally introduced to create an NcoI site encompassing the first ATG of the ΔLNGFR coding sequence, and to suppress the one localized at the second ATG. PCR was performed by 12 minutes denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 2 min at 72°C and a final 10 min extension at 72°C. The PCR product was subsequently cut with NcoI/HincII restriction enzymes, and the 848pb resulting fragment was cloned into NcoI/BamHI sites of the MFG backbone, after fill-in of the BamHI recessed extremity, by sticky-blunt ligation, using standard cloning procedures (Figure 1). The correct insertion of both marker genes was confirmed by restriction endonuclease mapping and automated sequencing of the resulting vectors.

Generation of EGFP and ΔLNGFR amphotropic packaging clones and viral titers

Packaging clones were generated using a cross-infection protocol. Briefly, vector DNAs were transfected into the ecotropic Bosc23 packaging cell line, by calcium-phosphate co-precipitation (Calcium Phosphate Transfection System, Life Technologies, GIBCO-BRL). After 48 hours, virus-containing supernatants were harvested and used to transduce the amphotropic GP+*env*Am12 cells in the presence of 8 μg/mL polybrene (Sigma, St. Louis, MO, USA). Six days after infection, ΔLNGFR-Am12 expressing cells were selectively enriched to high purity by magnetic cell sorting using a mouse anti-human LNGFR MoAb (clone 8211, Boehringer Mannheim, Mannheim, Germany) and magnetic beads coupled to a rat anti-mouse IgG1 antibody (miniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). EGFP-Am12 expressing cells were selected by sterile FACS sorting. To generate MFG-EGFP and MFG-ΔLNGFR producer clones, different cells subsets were sorted by FACS from each mixed population on the basis of their increasing range of fluorescence intensity; clones were finally obtained from each cell subset by limiting dilution in 96-well plates.

Viral titers were estimated by transducing NIH3T3 cells with different dilutions of retrovirus-containing supernatants in the presence of 8 μg/mL polybrene. Cells were analyzed by flow cytometry 48 hours after infection. Supernatant dilutions were plotted against the percentage of fluorescent cells and titers were calculated from the volumes corresponding to the linear slope of the regression line, according to the reported formula:[37]

$$\text{viral titer} = \frac{\text{NIH3T3 cell n°} \times \text{\% of fluorescent cells} \times \text{dilution factor}}{\text{infection volume (mL)}}$$

Cell culture supernatants were harvested from confluent producer clones 48 hours after replenishment of the medium, passed through a 0.45-μm filter (Costar, Cambridge, MA, USA) and frozen in aliquots at –80°C until use.

Transduction of hematopoietic cell lines and progenitor cells

All infections were performed using supernatants collected from the best producer clones for each vector, the EE4 for the MFG-ΔLNGFR vector and the GF20C for the MFG-EGFP, producing recombinant retroviruses at titers of $1\text{-}3\times10^7$ infectious units per mL of culture supernatant (IU/mL) and $1\text{-}3\times10^6$ IU/mL, respectively. Target cell number and infection volumes were kept constant for both vectors in all infection experiments.

Transduction of cell lines was performed in 24-well plates (Nunc, Roskilde, Denmark) by exposing target cells (3×10^5) to 1 mL of viral supernatant diluted 1:2 with fresh media and 8 μg/mL polybrene final concentration. After 1 hour at 37°C, cells were spun at 1,800x g for 2 hours and 30 min at 32-35°C. Supernatant was removed after a further 20 hours at 37°C. Cells were rinsed in phosphate-buffered saline (PBS) and cultured in fresh appropriate media for 3 more days to allow gene expression. Viability of the transduced cells was evaluated using the trypan blue exclusion method (Sigma) 24 hours after transduction. The percentage of cycling cells ($S/G_2/M$) was determined by propidium iodide (PI) staining and subsequent analysis by flow cytometry using the Multicycle elaboration program (Phoenix Flow Systems, San Diego, CA, USA). Cryopreserved CD34-enriched PB cells were cultured at an initial cell concentration of 2.5×10^5/mL in RPMI Glutamax-1, supplemented with 10% FCS and 1% penicillin/streptomycin in the presence of IL-1β (3 ng/mL), IL-3 (100 ng/mL), IL-6 (100 ng/mL), Stem Cell Factor (SCF, 10 ng/mL), Flt3-ligand (50 ng/mL) and G-CSF (10 ng/mL). All growth factors were purchased from Peprotech, Rocky Hill, NJ, USA. Infections were performed in fibronectin-coated 12-well tissue culture plates (BIOCOAT, Becton Dickinson, Bedford, MA, USA). Briefly, after 72 hours of prestimulation, progenitor cells (6×10^5) were exposed to 2 mL of viral supernatant diluted 1:2 with fresh media, containing the cytokine cocktail as in the prestimulation phase and 8 μg/mL polybrene final concentration. After 1 hour at 37°C, cells were spun at 1,800xg for 4 hours at 32-35°C, and put back in culture for another 2 hours. Supernatant was then removed, cells were rinsed in PBS and cultured in fresh appropriate media supplemented with the usual cytokine cocktail for 3 more days to allow gene expression. Cell viability was evaluated just after thawing, after prestimulation and 24 hours after transduction.

A mock infection (no vector-containing medium) was always performed in parallel under identical culture conditions.

Flow cytometry

Flow cytometric analysis was performed using a Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon ion laser tuned at 488nm. Data were analyzed using the System II Vers.3.0 software. To assess cell-surface ΔLNGFR expression, transduced cells were incubated with the unconjugated anti-hLNGFR MoAb in the presence of FCS as blocking reagent. Cells were washed twice in PBS and stained with a fluorescein

isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 antibody (Harlan Sera-Lab, Loughborough, Leicestershire, England). To assess EGFP expression, cells were directly analyzed by flow cytometry. Isotypic control IgG1 antibodies (FITC- and PE-labeled, from Dako A/S, Glostrup, Denmark) were used in all experiments to set the quadrant markers so that the quadrant defining negative FITC or PE fluorescence contained at least 99% of the isotype control cells. Different compensations were performed for ΔLNGFR- and EGFP-transduced cells. FACS sorting was performed according to light scatter and fluorescence emission using a FACStar flow cytometer equipped with an argon-ion laser (488nm) (Becton Dickinson, Palo Alto, CA, USA).

Progenitor cell assays

Infected and mock-infected progenitor cells were added to methylcellulose medium containing a mixture of recombinant cytokines, including rh-SCF, rh-IL-3, rh-IL-6, rh-erythropoietin (rhEpo), rh-G-CSF and rh-GM-CSF (MethoCult GFH4434, Stem Cell Technologies, Vancouver, BC, Canada), at 1-2×10^3 cells/mL final concentration. The medium (1 mL) was subsequently plated in duplicate in 6-well plates (Nunc) and incubated at 37°C in a 95% humidified atmosphere containing 5% CO_2. Colonies (≥50 cells) were scored under an inverted microscope after 14 days of culture. To detect EGFP-transduced CFU-C (colony forming unit-cell) an inverted fluorescence microscope was also used (Fluovert Fu, Leitz, Wetzlar, Germany). Prior to analysis by flow cytometry, colonies of each plate were pooled, washed several times in PBS and resuspended in lysis buffer (NH_4Cl, $KHCO_3$, EDTA, pH 7.3) to eliminate the confounding factor represented by erythrocytes.

Statistical analysis

The Mann-Whitney test was used to determine whether the values of viral titers for the MFG-ΔLNGFR packaging clones differed significantly ($p<0.05$) from the viral titers for the MFG-EGFP packaging clones.

PCR for ΔLNGFR and EGFP detection

Genomic DNA was extracted following standard procedures from infected and mock-infected packaging clones, hematopoietic cell lines, and pooled colonies from methylcellulose cultures. The ΔLNGFR-specific detection was performed with a forward primer mapping within the ΔLNGFR gene (5'-CCGCTGCGCCTACGGCTA-3') and a reverse primer localized in the vector backbone, upstream from the 3'LTR (5'-AAACTAGAGCCTGGACCACTGATATC-3'). EGFP detection was performed with a forward primer (5'-ACGGCATCGCAGCTTGGATACACG-3') mapping into the MFG backbone, downstream to the splice acceptor (SA) site, and with the same reverse primer used for ΔLNGFR. In both cases amplification conditions were: 12 min denaturation at 94°C, followed by 40 cycles of 1 min at 94°C, 1min at 60°C, 1 min at 72°C and a final 10 min extension at 72°C. The expected size of PCR products was 586pb and 852pb for ΔLNGFR and EGFP, respectively. Amplification of the human β-globin gene was carried out with specific primers as a control for DNA extraction.

Results

Construction of MFG-ΔLNGFR and MFG-EGFP retroviral vectors

To compare EGFP and ΔLNGFR genes as selectable markers for HSC tracking, two MFG-based retroviral vectors were constructed, whose structure differed only for the primary sequence of the inserted marker gene. The complete coding sequence of both ΔLNGFR and EGFP genes was cloned downstream to the MFG splice acceptor site (SA),[38,39] under the transcriptional control of the 5'MoMLV long terminal repeat (LTR) (Figure 1). A similar cloning strategy was adopted to assure that both genes utilized the same MoMLV envelope ATG as the start codon. The two constructs contained no selectable markers other than ΔLNGFR or EGFP, so that transduction efficiency had to be evaluated by flow cytometry.

Selection of packaging clones: comparison of viral titers

Two polyclonal populations of stable amphotropic producer cells were generated for both MFG-EGFP and MFG-ΔLNGFR vectors, as described in the Material and Methods section. Cell subsets, characterized by increasing levels of marker gene expression, were sorted over the entire fluorescence distribution curve by FACS.

Variability in marker gene expression was higher for the EGFP than ΔLNGFR clones (Figures 2A and 3A). Viral titration was performed on twenty different clones for each vector. Comparison between the two groups of clones showed a significant difference in viral titer ($p = 0.037$): in fact, the MFG-ΔLNGFR clones produced recombinant retroviruses at titers ranging from 5×10^5 to 3×10^7 IU/mL (mean value: 5.6×10^6 IU/mL; median value: 1.77×10^6), whereas the MFG-EGFP clones were less productive, with titers ranging from 2×10^3 to 3×10^6 IU/mL (mean value: 0.77×10^6 IU/mL; median value: 0.57×10^6).

Nevertheless, focusing the analysis only on those clones which showed the highest marker gene expression for each vector (six clones for the MFG-ΔLNGFR and six for the MFG-EGFP) the difference in viral titer was not statistically significant ($p = 0.22$).

The highest-titer clones for each vector were cultured for 40 days. Cell phenotype and proviral sequences (Figure 4) were maintained during the entire culture period, without any appreciable loss of viral yield. Interestingly, a direct correlation between the intensity of marker gene expression in packaging clones and transduction efficiency on NIH3T3 could be observed for both vectors (Figures 2 and 3).

Expression kinetics and transduction efficiency in human cell lines

Three different blood-derived human cell lines, K562, NB4 and Raji were initially employed to evaluate transduction efficiency of both vectors. Infections were performed with supernatants collected from the best producer clone for each vector, producing recombinant retroviruses at titers of 1-3×10^7

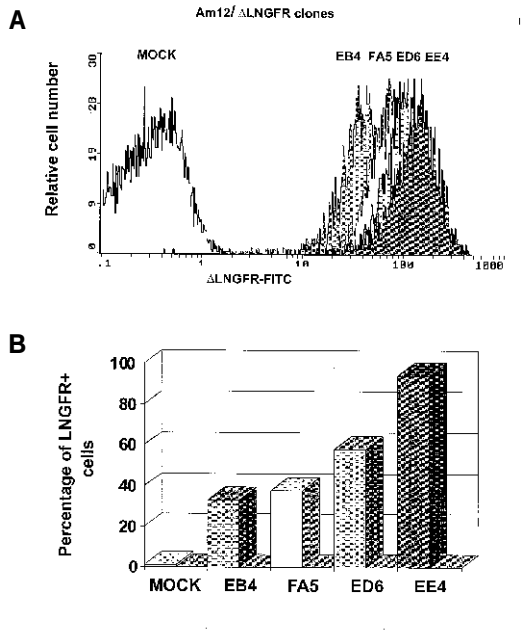

Figure 2. ΔLNGFR expression and transduction efficiency of Am12 / MFG-ΔLNGFR clones. MFG-ΔLNGFR Am12 producer clones were obtained by FACS selection and subsequent limiting dilution from a pool of Am12 / MFG-ΔLNGFR+ immunomagnetic sorted cells. (A) Flow cytometric analysis of ΔLNGFR cell surface expression in four representative clones. (B) Gene transfer efficiency following transduction of NIH3T3 cells.

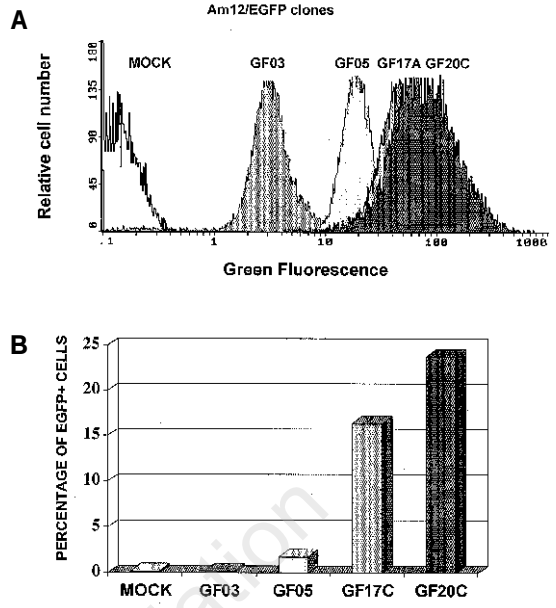

Figure 3. EGFP expression and transduction efficiency of Am12 / MFG-EGFP clones. MFG-EGFP Am12 producer clones were obtained by FACS selection and subsequent limiting dilution from a pool of Am12 / MFG-EGFP+ FACS sorted cells. (A) Flow cytometric analysis of EGFP expression in four representative clones. (B) Gene transfer efficiency following transduction of NIH3T3 cells.

IU/mL (MFG-ΔLNGFR) and 1-3×10^6 IU/mL (MFG-EGFP).

To assess the effect of viral vector concentration on gene transfer efficiency, cell lines were initially infected at different multiplicities of infection (MOI) (30 for the MFG-ΔLNGFR and 3 for the MFG-EGFP), keeping constant the target cell number and infection volumes for both vectors. Gene transfer efficiency was evaluated after 3 days, as the percentage of EGFP or ΔLNGFR positive cells by flow cytometric analysis. Viability after transduction was similar in all cell lines for both vectors (mean value: 90±3.6%) and was only slightly inferior to the viability of the untransduced control cells (97±3%). Transduction efficiency varied significantly between the different cell lines, ranging from 9% (Raji) to 71% (K562) for the MFG-ΔLNGFR vector, and from 1.5% (Raji) to 23% (K562) for the MFG-EGFP vector (Figure 5). To investigate whether this variability was related to the cell cycle status at the moment of infection, the percentage of cycling cells ($S/G_2/M$) was evaluated immediately before transduction and shown to be similar for all tested cell lines (K562 median 63%, NB4 median 54%, Raji median 49%). To investigate whether the lower infectivity observed for the MFG-EGFP vector relative to the MFG-ΔLNGFR was due to the one log difference in viral titer, transduction experiments were repeated, by infecting K562 and

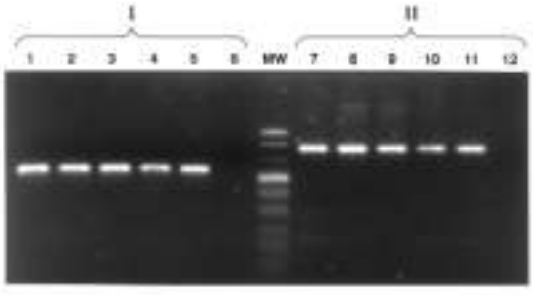

Figure 4. ΔLNGFR (I) and EGFP (II) sequence detection by PCR on EE4 (MFG-ΔLNGFR) and GF20C (MFG-EGFP) packaging clones (lanes 2 and 8), K562 transduced cells (lanes 3 and 9) and pooled colonies from methylcellulose cultures (lanes 4, 5, 10 and 11 as two representative examples). Positive controls (lane 1 and 7) correspond to amplified DNA from pMFG-ΔLNGFR and pMFG-EGFP plasmid vectors, respectively. Negative controls (lane 6 and 12) correspond to amplified DNA from mock-infected K562 cells.

NB4 cells at the same MOI for both vectors. To this end, the supernatant from the MFG-ΔLNGFR was diluted 1:10, and used to infect target cells in parallel with the MFG-EGFP supernatant. Under these conditions no difference in transduction efficiency

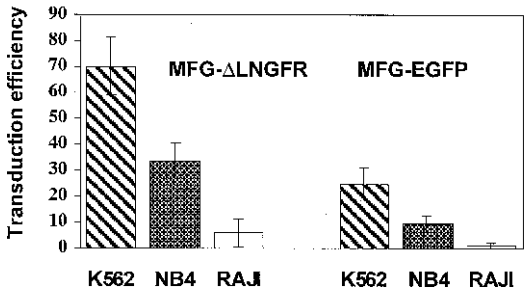

Figure 5. Gene transfer efficiency in different cell lines. Cells were exposed for 24h to 1:2 diluted retrovirus-containing supernatants collected from the EE4 (MFG-ΔLNGFR) or the GF20C (MFG-EGFP) packaging clones. Gene transfer efficiency was assessed 3 days later by flow cytometric analysis. Data representative of three sets of experiments are shown.

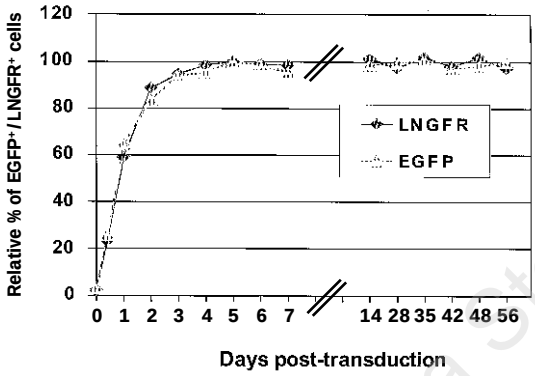

Figure 6. Flow cytometric analysis of EGFP and ΔLNGFR expression kinetics in K562 transduced cells. The relative percentages of transduced cells expressing each marker gene at different time intervals from infection are shown. Absolute percentage of ΔLNGFR+ cells at 120h (referred as 100%): 67%; Absolute percentage of EGFP+ cells at 120h (referred as 100%): 30%.

was observed between the two supernatants (data not shown).

The expression kinetics of EGFP and ΔLNGFR was evaluated in K562 transduced cells by flow cytometry at different time intervals from infection. As shown in Figure 6, a similar, rapid expression kinetics was observed for both marker genes. Moreover, the relative percentage of ΔLNGFR and EGFP expressing cells was already elevated at 24 hours after infection, reaching the 95% value at 72 hours in both cases. No ΔLNGFR or EGFP signal could be detected immediately after transduction.

Proviral sequences (Figure 4) and marker gene expression (Figure 6) were maintained in both transduced populations for two months without any detrimental effect to the cell growth.

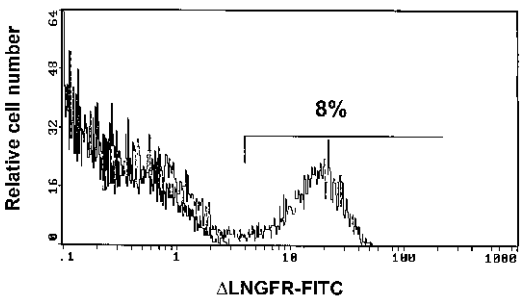

Figure 7. Flow cytometric analysis of ΔLNGFR expression in CD34-enriched PB cells, at 72 hours from transduction. The corresponding profile for mock-transduced cells (white area) is overlaid for comparison. The percentage of ΔLNGFR+ cells is indicated.

Gene transfer into CD34-enriched mobilized PB cells

Cryopreserved, clonogenically competent (CFU-C median 234, CFU-GM median 172, BFU-E median 59), CD34+-enriched PB cells were stimulated in cytokine-supplemented growth media for 72 hours. Subsequently, cells were exposed to MFG-ΔLNGFR and MFG-EGFP vector-containing supernatants at MOIs of 15 and 1.5, respectively, for a short time period. Viability of both freshly thawed and prestimulated CD34+ cells was 93% ± 4%.

A fourfold higher transduction efficiency was obtained with the MFG-ΔLNGFR vector (Figure 7), as could be anticipated on the basis of the higher viral concentration of the MFG-ΔLNGFR supernatant. Viability of the transduced cells was 85±3.4% for the MFG-ΔLNGFR vector and 80±4.5% for the MFG-EGFP, as assessed by trypan blue exclusion test 24 hours after transduction. Clonogenic assays performed 3 days after transduction gave rise to similar numbers of CFU-C for both infected and mock infected cells (median CFU-C for mock infected cells, 133; median CFU-C for MFG-EGFP infected cells, 152; median CFU-C for MFG-ΔLNGFR infected cells, 140). Marker gene expression and proviral sequences (Figure 4) were still detected in pooled myeloid and erythroid colonies from each transduced population two weeks after seeding in methylcellulose cultures (see Figure 8 for a representative GFP example).

Discussion

The use of EGFP and ΔLNGFR as marker genes for HSC tracking has been already described by several groups.[28-31,40-42] This is the first study comparing, in parallel, the gene transfer ability into blood cells of two MFG-based retroviral vectors expressing EGFP or ΔLNGFR as marker genes. In order to obtain an unbiased comparison, a similar cloning strategy was adopted to generate two constructs differing only for the primary sequence of the inserted marker gene. In particular, the study took into account some vari-

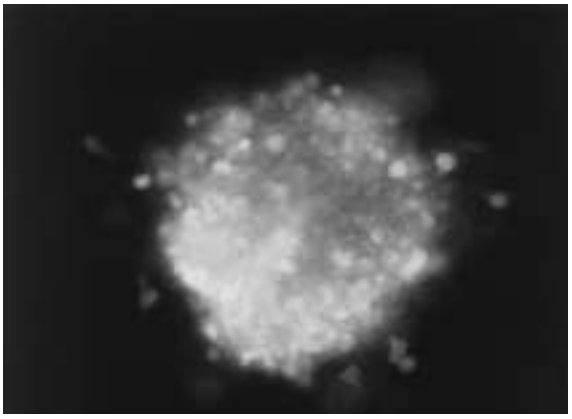

Figure 8. Fluorescence microscopic image of a representative EGFP+BFU-E methylcellulose colony.

ables that could have an impact on the clinical use of the vectors, including production and selection of packaging clones, correlation between marker gene expression and viral titer, short- and long-term cell toxicity and transgene expression.

One of the most critical constraints of gene transfer into HSC is represented by poor transgene expression in transduced cells.[43] It has been reported that the MFG-based vectors, which differ from the standard N2 and LN vectors for the presence of an additional SA site, may provide augmented short and long-term expression of foreign genes.[38,39] To exploit the transductional advantages of this vector, ΔLNGFR and EGFP coding sequences were separately cloned into the viral backbone, downstream to the SA site, with their start codon placed exactly at the position of the Moloney envelope ATG (Figure 1).

We were able to isolate different packaging clones for both vectors (Figures 2A and 3A); this is an important prerequisite for any preclinical or clinical study. At variance with others groups,[44] we did not find any difficulty in generating stable packaging clones producing high titer GFP-containing retroviral vectors. Contrasting results presented in the literature could be explained by 1) the source of the packaging cell lines used for propagation of the retroviral vectors 2) the type of GFP variant employed for monitoring gene transfer. In this regard it is noteworthy that, while the expression of both wild-type GFP and humanized S65T-GFP variant is toxic for the PA317 packaging cells line harbouring the amphotropic receptor,[44] no toxicity was found for other packaging cell lines such as amphotropic GP+*env*Am12 or gibbon ape leukemia virus (GALV) PG13 cells, expressing the EGFP gene.[11,40-42]

The variation in the range of the relative fluorescence intensity was lower for the MFG-ΔLNGFR clones than for the MFG-EGFP ones as assessed by flow cytometric analysis (Figures 2A and 3A). This could be due to the fact that Am12/ΔLNGFR-transduced cells were initially selected using an immunomagnetic procedure. Conceivably, an enrichment of cells expressing the surface marker at the highest intensity might have occurred during this procedure.

The success of retrovirus-mediated gene therapy and marking protocols is dependent on a source of high-titer virus stock. In this regard, the identification of the best producer clones is a laborious and time-consuming procedure that could be facilitated by the presence of selectable marker genes in the vector, as in our case.

A direct correlation between the intensity of marker gene expression in packaging clones, measured as relative fluorescence intensity, and transduction efficiency on NIH3T3 was observed for both constructs (Figure 2 and 3). This suggests the feasibility of recovering high-titer clones simply by screening cells with the highest ΔLNGFR or EGFP expression. It follows that the immunomagnetic procedure used to enrich ΔLNGFR+Am12 cells could have contributed to select cells not only exhibiting maximal expression of the marker gene, but also exhibiting maximal viral yield. This hypothesis is supported by the fact that, overall, the viral titers for the MFG-ΔLNGFR clones differ significantly from the viral titers for the MFG-EGFP clones. Such a difference is no longer evident when the comparison is restricted to clones exhibiting maximal expression of the marker gene.

A negative selection effect due to toxicity of the transgene product was ruled out since high-titer MFG-EGFP and MFG-ΔLNGFR producer clones were cultured for 40 days without phenotypic changes or loss of viral yield.

As for gene transfer in human target cells, short-term experiments in K562 cells showed similar expression kinetics for both EGFP and ΔLNGFR marker genes, confirming that they can both be used for a sensitive and specific evaluation of transduced cells as early as 72 hours post-infection (Figure 6). In fact, at variance with previous reports on the use of ΔLNGFR, we did not observe a decrease in the percentage of ΔLNGFR positive cells in the early days after transduction.[45] Viability of the cell lines, as assessed at 24 hours from transduction, was similar for both vectors, and only slitghtly inferior to the viability of the uninfected control cells, confirming the low toxicity of the transduction procedure. Initial infection experiments showed a higher transduction efficiency for the MFG-ΔLNGFR vector compared to that of the MFG-EGFP (Figure 5). To verify whether the observed difference was related to the different MOI (30 for the MFG-ΔLNGFR vector and 3 for the MFG-EGFP), to the difference in vector structure, or to the signal detection systems used for the gene transfer evaluation (intrinsic fluorescence versus indirect immunostaining procedure), vectors were tested under normalized conditions of viral concentration. No significant difference in transduction efficiency was observed in this case. Our results confirm the strict dependence of gene transfer efficiency on virus concentration in supernatants.[46,47]

Long-term experiments showed the persistence of both EGFP and ΔLNGFR expression in transduced cells for 2 months of culture confirming the stability of both vectors once integrated and the absence of any toxicity related to the transgene products (Figure 6).

Successful vector integration following retroviral entry is dependent on mitosis and is limited by the

rate of intracellular decay of internalized vectors.[47] Since retroviruses half-lives is generally short, being in the range of 4-7 hours at 37°C, it is most likely that transduction of cells that are in S or in G_2/M phases will result in vector integration. In our experiments, transduction efficiency was apparently unrelated to the cell cycle status and therefore likely to be dependent on other biological parameters such as amphotropic receptor density on the cell membrane.[48]

Gene transfer was also performed into cryopreserved progenitor cells to mimic clinical gene therapy procedures. Both vectors were able to transfer genes into CD34+ peripheral blood cells even after short-term of exposure to recombinant viruses, suitable for clinical experiments (Figure 7 and Figure 8).[3,16] As for the cell lines, viability of the CD34+ transduced cells[49,50] was similar for both vectors.

In summary, this study demonstrates that EGFP and ΔLNGFR are equally useful for rapid, specific and non-toxic detection of successfully transduced cells; both marker genes show similar expression kinetics without any toxicity for cell growth, even after a long time in culture.

The insertion of these markers in a retroviral vector simplifies the screening of high titer packaging clones, due to the direct correlation between marker gene expression and viral titer. ΔLNGFR can be considered a more suitable marker than EGFP for clinical applications since it avoids the problems linked to the expression of an heterologous protein[26,27] and is amenable to easy selection on a clinical scale.[28,29] Quite significantly in this regard is our observation that higher titer producer clones are more easily obtained with the MFG-ΔLNGFR vector. On the other hand, EGFP appears more useful in gene transfer protocols optimization. In fact, immediate detection of EGFP transduced cells is possible, without any immunostaining procedure.

In conclusion, the information obtained *in vitro* by using the MFG-EGFP vector can be reliably transposed to the safer MFG-ΔLNGFR for its subsequent employment *in vivo*, since the two constructs have an almost identical structure and a similar behavior in transduced cells.

Contributions and Acknowledgments

IG wrote the paper and formulated the design of the study with the contribution of GP and FR. IG carried out vector construction, DNA transfection experiments and infection assays on blood cells with the contribution of RB. DM performed the isolation of the packaging clones and the flow cytometric analysis of transduced cells with the contribution of AC. RB produced and titered the virus stocks, and performed the PCR assays. AC carried out the CD34+ cells purification and clonogenic assays. RB, DM and AC were responsible for analysis of data, drafting and critical revising the article. GP and FR were responsible for funding, interpretation of results and final approval of the version to be submitted.

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Disclosures

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Potential implications for clinical practice

- A correlation was found between viral titer and marker gene expression in packaging clones for both the MFG-ΔLNGFR and MFG-EGFP vectors. This will greatly facilitate and accelerate the screening of the best producer clones for clinical use.
- Both EGFP and ΔLNGFR marker genes are equally useful for rapid, specific and non-toxic detection of transduced cells. Nevertheless, EGFP, because of its natural fluorescence, allowing direct detection of the transduced cells, seems to be more useful for *in vitro* optimization of gene marking protocols. ΔLNGFR is preferable for clinical applications for its safety. Furthermore, it is amenable to an easier selection of high titer packaging clones, by a procedure based on magnetic beads, widely employed in clinical practice.

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