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Optimized lentiviral transduction of erythroid precursors from healthy adults and patients with myelodysplastic syndromes

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Lentivectors, derived from human immunodeficiency virus-1 (HIV-1), represent a novel investigational and therapeutic tool for targeting hematopoietic progenitor cells. We describe a new protocol whereby we achieved a highly efficient lentiviral transduction of erythroid precursor cells originating from the bone marrow of healthy adults and patients with myelodysplastic syndromes (MDS). CD34$^+$ stem cells from healthy subjects were cultured with erythropoietin, IL-3 and stem cell factor, and thereby expanded approximately 300-fold. When these cultures were transduced with a lentiviral vector expressing GFP as a reporter gene, 70% glycophorin$^+$ cells were GFP$^+$. Although proliferation and levels of transduction were reduced in cultures of CD34$^+$ stem cells from patients with myelodysplastic syndromes, 50% of glycophorin$^+$ cells became GFP$^+$, amongst which 30% were sideroblastic erythroid precursors. This study demonstrates that lentiviral vectors are capable of efficiently transducing MDS precursors and offers new perspectives to investigate the influence of specific genes on normal erythroid differentiation. This may eventually help to correct defects in patients suffering from myelodysplastic syndromes.
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Introduction

Gene transfer in human hematopoietic progenitor and stem cells (HSCs) has much potential, both for the understanding of fundamental mechanisms of hematopoiesis and treatment of its related disorders. In the last few years, lentiviral vectors have emerged as excellent candidates for gene transduction because they can integrate into slowly or non-dividing cells such as HSCs.[1] Oncoretroviral vectors, derived from Moloney murine leukemia virus, for instance, require passage through mitosis in order to be able to integrate into target cells.[2] Until now, lentiviral transduction has mainly been carried out on murine hematopoietic cells,[3] or on hematopoietic cells from human cord blood.[4–7]

We describe a new protocol whereby we achieved a highly efficient lentiviral transduction of erythroid precursor cells from the bone marrow of healthy adults and of patients with low risk myelodysplastic syndrome (MDS) (sideroblastic (SA) and refractory (RA) anemia). These syndromes are characterized by a deficient clonogenic growth and an excessive apoptotic rate of multipotent and primitive erythroid progenitors, leading to ineffective hematopoiesis.[8–11] The study of low-risk myelodysplastic syndromes is difficult, due to reduced survival of these cells *in vitro*, inefficient amplification in culture and the lack of animal models. We report here a new approach to investigate these syndromes using lentiviral vectors. Other groups have already reported efficient transduction of erythroblasts with murine oncoretroviruses derived from the Molony murine leukemia virus,[12,13] but in these studies CD34$^+$ cells were obtained from human cord blood and transduction was only obtained for a fraction of dividing cells, since these vectors require cells undergoing mitosis in order to integrate.[2] We therefore chose lentivectors to transduct dysplastic erythroid precursors with inefficient and aborted *in vitro* proliferation, and we were able to demonstrate that constructs with an optimized promoter (cPPT-PGK) constitute excellent candidates for the transduction of these MDS cells.

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Materials and methods

Cytokines and antibodies

Erythropoetin (Eprex; Cilag, Schaffhausen, Switzerland), IL-3, stem cell factor, thrombopoetin, Flt-3 ligand (all from Peprotech EC, London, UK) were used for cell cultures at 2 U/ml, 20 ng/ml, 20 ng/ml, 10 ng/ml, and 25 ng/ml, respectively. Anti-glycophorin (Dako Diagnostics, Zug, Switzerland), anti-CD71, anti-CD34 and anti-CD45 antibodies (all from BD Biosciences, Heidelberg, Germany) were used for flow cytometric analysis. Corresponding isotype control antibodies were also from BD Biosciences.

Vectors

Production of HIV-derived vectors pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein was achieved by transient cotransfection of three plasmids into 293T cells.[5,14] VSV G was expressed from pMDG. The HIV-derived packaging construct used was pCMV 8.91.[15] The HIV vector plasmids used in this study contain the self-inactivating modification (SIN), as previously described[16] and the woodchuck hepatitis transcriptional element (WPRE).[17] The PGK (phosphoglycerate kinase) and EF-1 (human elongation factor 1) HIV vector plasmids were a gift from D Trono and were described previously.[5] The cPPT-PGK vector plasmid was a gift[18] from L Naldini. The HIV vector plasmids are derived from the original pHR′ backbone and express GFP as a reporter.[5,14] Vector titers were determined by transduction and flow cytometry analysis of GFP expression in HeLa cells, as previously described.[5] Titers were between 2×10^7 and 6×10^7 HeLa transducing units (TU) per milliliter.

Purification and culture of CD34$^+$ cells

Bone marrow samples were obtained from six adult patients with normal bone marrow and from nine patients with low-risk myelodysplastic syndrome (three RA, six SA), according

to institutional guidelines. After Ficoll–Paque gradient centrifugation, CD34+ cells were isolated with magnetic beads as previously described.[5,19] The percentage of purified CD34+ cells was 85 ± 10%; contamination with glycophorin+ cells was <2% in all cases. For cell culture, 2.5×10^4 cells were seeded in 96-round well plates and grown in IMDM medium (Gibco BRL, Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (Seromed; Biochrom, Berlin, Germany), erythropoietin/IL-3/stem cell factor (E/I/S) or thrombopoietin/Flt3-ligand/stem cell factor (T/F/S). Cell proliferation was assessed by counting cells after trypan blue dye exclusion staining. Flow cytometry was performed as described[5,20] after labeling with various antibodies and with 7-amino-actinomycin (Sigma, Buchs, Switzerland) to exclude apoptotic and dead cells.

Transduction of CD34+ cells

Two different methods were employed.

2.5×10^4 purified CD34+ cells were seeded in 96-well plates in 100 μl of IMDM + thrombopoietin, as described.[5] After overnight prestimulation the vector was added and the volume adjusted to 200 μl with IMDM + thrombopoietin. After 24 h, cells were washed and cultured in IMDM + 10% FCS + E/I/S or T/F/S.

2.5×10^4 purified CD34+ cells were seeded in 96-well plates in 200 μl of IMDM + 10% FCS, supplemented with E/I/S or T/F/S. The vector was added after 1, 3 or 7 days of culture.

Immunofluorescence analysis

Cells were stained with the appropriate antibodies, fixed with paraformaldehyde 2%, and a cytospin prepared. Images were made as described previously.[21]

Cell sorting and staining for iron

After 12 days of culture, cells were labeled with anti-glycophorin, then sorted with a cell sorter (FACStar, CMU, Geneva University, Geneva, Switzerland). Subsequently, cytospins were prepared and stained for mitochondrial iron with Prussian blue.

Results and discussion

In vitro generation of erythroid precursors and mature erythrocytes

CD34+ cells obtained from six normal human adult bone marrow samples were cultured for 20 days in E/I/S. Cells were amplified 96 ± 20-fold (s.d. for standard deviation) after 10 days and 329 ± 56-fold (s.d.) after 20 days; longer periods of culture did not result in any further increase in cell number. At day 20, 88 ± 9% of these cells were glycophorin+/CD45− erythroid cells, 10–30% of which lost the CD71 antigen. Accordingly, all stages of erythroblast differentiation were observed, including that of enucleated mature erythrocytes. This protocol generated a higher amount of erythroid cells than previously reported,[5] which involved a preliminary culture in T/F/S before induction in E/I/S (data not shown). Alternatively, CD34+ cells were cultured for 7 days in T/F/S, followed by E/I/S, as reported previously.[5] Whereas cell proliferation was similar to cells cultured from the beginning in E/I/S (AF = 315 ± 151-fold), the yield of glycophorin+/CD45− cells was only 53 ± 6%.

Optimizing transduction efficiency in normal CD34+ cells

In order to increase the relatively weak transduction levels of erythroid precursors reported so far,[5] we evaluated three complementary approaches using the culture conditions which gave rise to the highest percentage of glycophorin+/CD45− cells, ie culture containing E/I/S from culture start: (1) transducing the CD34+ cells at different times of culture (1, 3 and 7 days) (Figure 1a), (2) using multiplicities of infection (MOIs) from 1 to 10 (Figure 1b); and (3) testing three different lentiviral constructs (Figure 2). The HIV vector plasmids differed in their internal promoter. EF1α, PGK[5] and a construct with the PGK-promoter and a central polypurine tract (PGK-cPPT vector) were used.[18] This latter sequence has recently been shown to play an important role in nuclear import[22] thereby increasing transduction efficiency in HSCs.[18,23] A maximum transduction was achieved with the PGK–cPPT vector at a MOI of 10 when the HSCs were transduced after 3 days of culture in E/I/S (Figure 1). At a similar MOI, we obtained 68 ± 6% GFP-expressing glycophorin+/CD45− cells with the PGK-cPPT vector compared to 38 ± 8% with the PGK vector alone and 44 ± 4% with the vector EF1α (values obtained from four independent experiments). Interestingly, GFP expression with the PGK-cPPT construct was also much stronger than with the PGK promoter (median GFP intensity of 317 ± 64 *vs* 35 ± 15), and slightly superior to the EF1-α promoter (median GFP intensity 273 ± 18) (Figure 2a).

The morphology of the transduced cells, as evaluated by Giemsa staining and by immunofluorescence microscopy, showed normal features (Figure 2b).

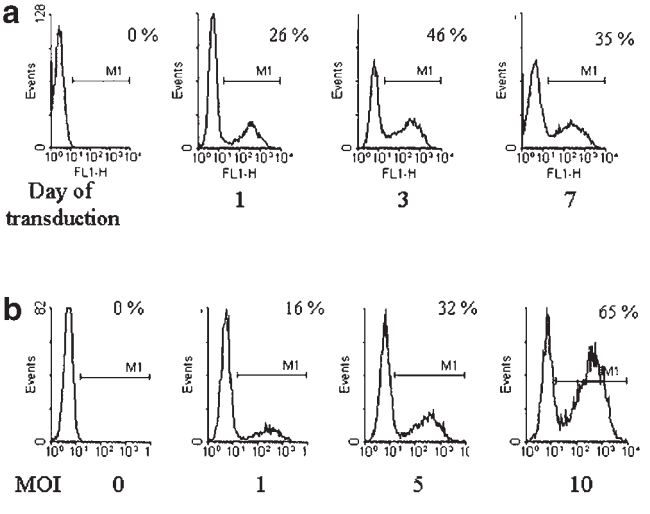

Figure 1 Efficient lentiviral transduction of normal human CD34+ cells obtained from adult bone marrow. 2.5×10^4 CD34+ cells were cultured for 20 days in E/I/S. Cells were transduced (a) with the EF1α vector at different time points of culture (days 1, 3 or 7), (b) with the cPPT-PGK vector at different MOIs (1 to 10).

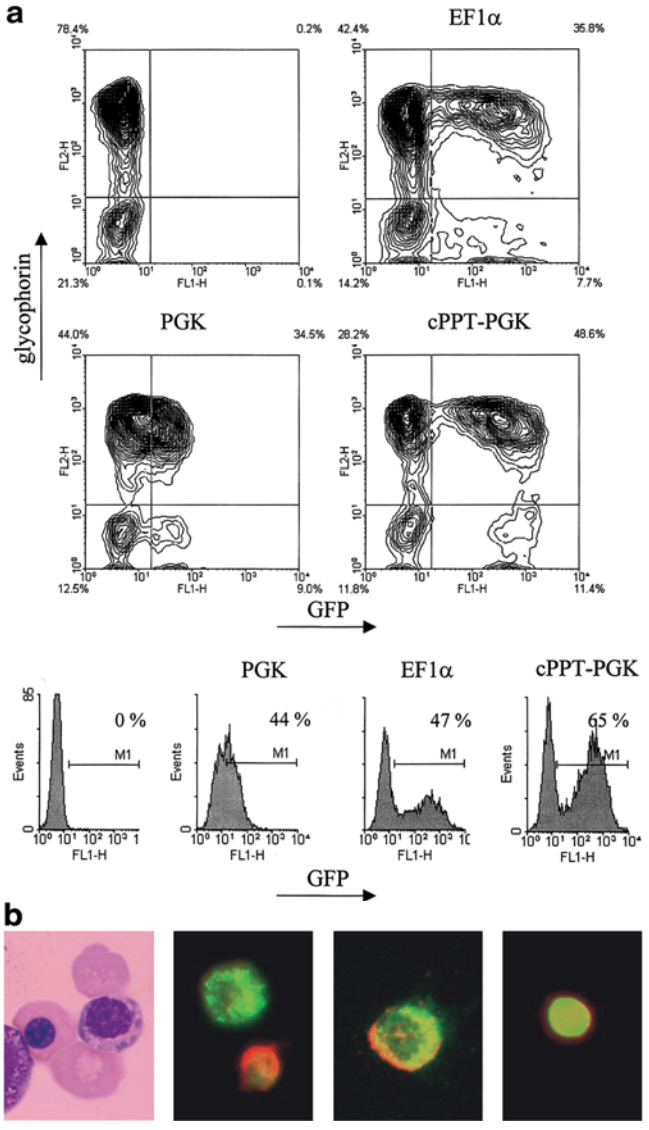

Figure 2 Transduction with the cPPT–PGK vector is more efficient than with the EF1α or the PGK vector. 2.5×10^4 CD34$^+$ cells were cultured for 20 days in E/I/S. Cells were transduced (a) with different viral constructs: the EF1α vector, the PGK vector or the cPPT–PGK vector. Flow cytometry was performed after labeling cells with anti-glycophorin monoclonal antibody (mAb) coupled to PE, anti-CD45 coupled to APC and with 7-AAD to exclude dead and apoptotic cells. The histograms depict the analysis after gating on glycophorin$^+$/CD45$^-$ cells. Results are representative of three independent experiments. (b) A Giemsa staining (left) demonstrates the normal morphology of the transduced cells. Immunofluorescence microscopy performed after 20 days of culture shows a transduced non-erythroid and a non-transduced mature RBC, a transduced erythroid progenitor and a transduced mature RBC, respectively.

Culture and transduction of CD34$^+$ cells from patients with low-risk myelodysplastic syndromes (MDS)

CD34$^+$ cells were purified from the bone marrow of six patients with SA and three patients with RA and cultured in E/I/S. Proliferation was low compared to normal adult CD34$^+$ cells (mean 27 ± 26-fold) and differentiation into

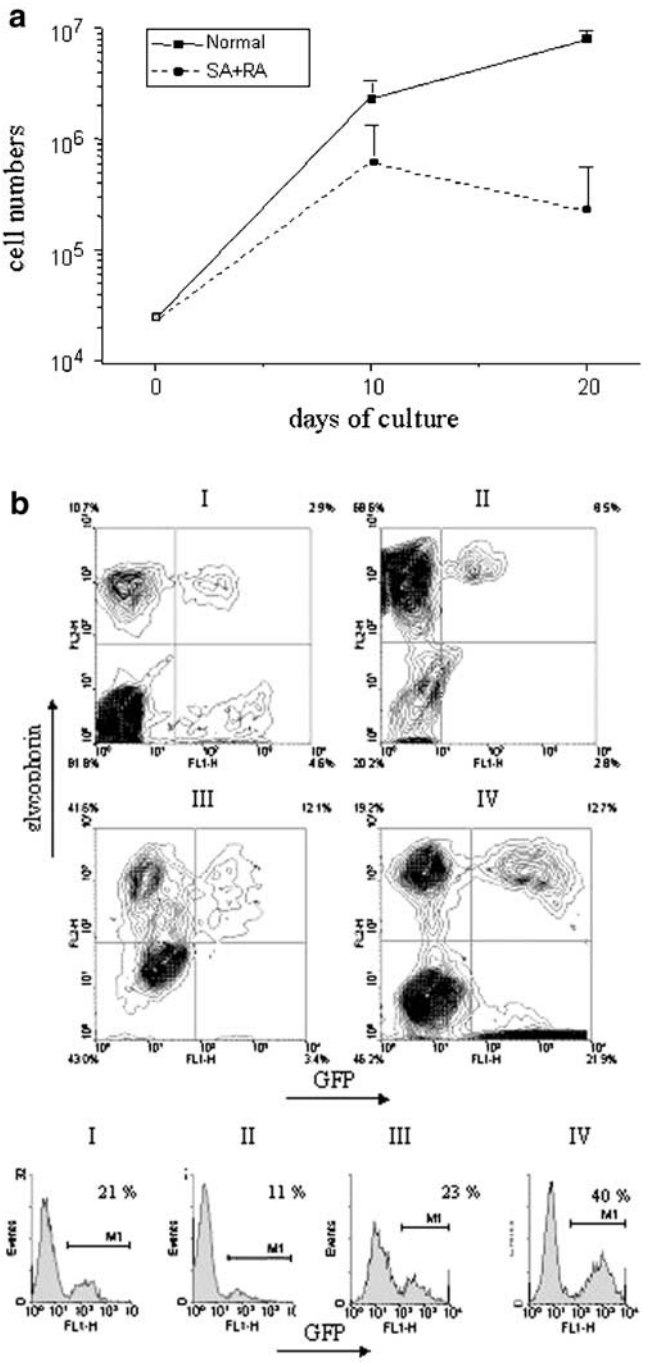

Figure 3 Transduction of CD34$^+$ cells from the bone marrow of patients with low-risk MDS. 2.5×10^4 CD34$^+$ cells from MDS patients or from healthy individuals were cultured for 20 days in E/I/S. (a) Cell proliferation was estimated by counting cell numbers after trypan blue dye staining. Data are representative of six independent experiments (for normal and for MDS). Error bars represent s.d. (b) Cells from MDS patients were transduced with the EF1α vector (patients I and II) or the cPPT–PGK vector (patients III and IV) after 3 days of culture (MOI of 10) and analyzed by flow cytometry at day 10. Cells were labeled with anti-glycophorin mAb coupled to PE, anti-CD45 mAb coupled to APC and with 7-AAD to exclude dead and apoptotic cells. The dot blots depict results from four patients responding to erythropoietin; the histograms show the corresponding results after gating on glycophorin$^+$/CD45$^-$ cells.

glycophorin$^+$/CD45$^-$ cells was variable and lower than in healthy subjects (mean 34 ± 8% s.e.m., standard error of the mean) in agreement with previous reports[24,25] (Figure 3a). Cells from MDS patients were transduced with either EF1α or cPPT-PGK vectors, using the optimal conditions described above. In two patients no glycophorin$^+$ cells were obtained and >80% cells were apoptotic when tested after 10 days of culture. Moreover, cell cultures from these patients in T/F/S followed by E/I/S also resulted in a very low proliferation and negligible differentiation into erythroid cells (data not shown). In the seven patient cultures responding to E/I/S, we were able to achieve efficient transduction of 46 ± 9% (s.e.m.) in the erythroid precursors (Figure 3b). The cPPT–PGK vector which was used for cultures from four patients once again gave superior yields than the EF1α vector (59% *vs* 40%).

In order to demonstrate that precursors from the myelodysplastic clone were transduced and not only residual normal precursors, we searched for the presence of sideroblasts in transduced erythroid precursors from MDS patients. A FISH analysis for the detection of a chromosomal abnormality would have been uninformative since all samples exhibited a normal karyotype. We therefore isolated glycophorin$^+$/GFP$^+$ cells after 12 days of culture in E/I/S by cell sorting from three patients with MDS (three patients with SA) (Figure 4a) and stained them for mitochondrial iron (Figure 4b), this being the standard technique for detecting sideroblasts. In each of the three sorted patient samples tested, we detected sideroblastic erythroid precursors (mean 34% ± 9% s.e.m.). Together, these experiments demonstrate that lentiviral vectors are able to efficiently transduce MDS precursors.

In conclusion, our study demonstrates that optimized lentiviral vectors are excellent candidates for the transduction of erythroid precursors of normal adults and of patients suffering from myelodysplastic syndromes. Furthermore, these vectors offer new opportunities for studying the effect of specific genes implicated in the regulation of erythropoiesis. Transduction of dysplastic or deficient HSCs with genes regulating erythroid proliferation and differentiation might therefore constitute a novel and potent tactic to study the complex dysregulations occurring in myelodysplastic syndromes. New perspectives for the treatment for these frequent disorders may be discovered as a result of these studies.

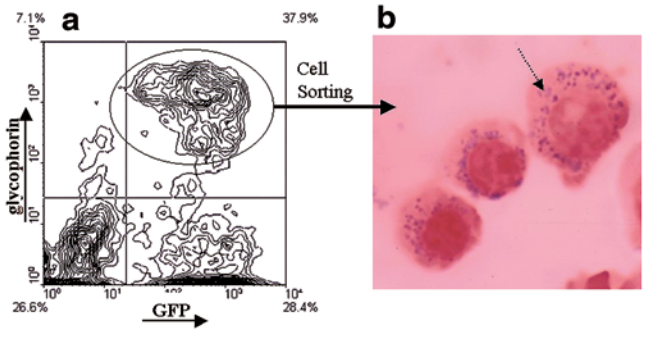

Figure 4 Lentiviral transduction of sideroblastic precursors from patients with low-risk MDS. (a) Erythroid precursors from patients with sideroblastic anemia (SA) were obtained after culture of CD34$^+$n E/I/S. At day 3 of culture, precursors were transduced with cPPT–K vector and at day 12, glycophorin$^+$/GFP$^+$ cells were sorted with a cell sorter (black arrow). (b) Glycophorin$^+$/GFP$^+$ cells were fixed and stained for the presence of mitochondrial iron, the hallmark of sideroblasts (dotted arrow). One representative result of three experiments is shown.

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