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Optimization of a retroviral vector for transduction of human CD34 positive cells

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Human stem and progenitor cells have recently become objects of intensive studies as an important target for gene therapy and regenerative medicine. Retroviral vectors are among the most effective tools for genetic modification of these cells. However, their transduction efficiency strongly depends on the choice of the *ex vivo* transduction system. The aim of this study was to elaborate a system for retroviral vector transduction of human CD34 positive cells isolated from cord blood. The retroviral vector pMINV EGFP was chosen for transduction of two human erythroblastoid cell lines: KG-1a (CD34 positive) and K562 (CD34 negative). For vector construction, three promoters and two retroviral vector packaging cell lines were used. To optimize the physicochemical conditions of the transduction process, different temperatures of supernatant harvesting, the influence of centrifugation and the presence of transduction enhancing agents were tested. The conditions elaborated with KG-1a cells were further applied for transduction of CD34 positive cells isolated from cord blood. The optimal efficiency of transduction of CD34 positive cells with pMINV EGFP retroviral vector (26% of EGFP positive cells), was obtained using infective vector with LTR retroviral promoter, produced by TE FLY GA MINV EGFP packaging cell line. The transduction was performed in the presence of serum, at 37ºC, with co-centrifugation of cells with viral supernatants and the use of transduction enhancing agents. This study confirmed that for gene transfer into CD34 positive cells, the detailed optimization of each element of the transduction process is of great importance.

Keywords: retroviral vector, $CD34^+$ cells, transduction optimization

INTRODUCTION

Human stem cells have recently become the object of intensive studies as a very promising therapeutic target for gene therapy (Dropulic, 2005; Kaiser, 2005). Retrovirus-mediated gene transfer is widely used for this purpose, because retroviral vectors efficiently carry out gene transfer to many cell types and can stably integrate into their genomes, resulting in long term gene expression, that also persists in daughter cells (Grignani *et al.*, 1998; MacNeil *et al.*, 1999; Schmidt *et al.*, 2005). However, investigators developing clinically effective retroviral vectors face numerous problems.

Retroviral vectors require cell division to integrate into the host genome, as the preintegration nucleoprotein complex is unable to cross an intact nuclear membrane. Therefore, the transduction efficiency also depends on the proliferative status of target cells. As the requirement for mitosis is a crucial condition for efficient retroviral transduction, stem cells, usually quiescent, are a really demanding target for retroviral vector manipulations (MacNeil *et al.*, 1999; Schiedlmeier *et al.*, 2000).

Abbreviations: c.f.u., colony forming unit; DLR, Dual Luciferase Reporter Assay; DMEM, Dulbecco modified Eagle's medium; DMSO, dimethyl sylfoxide; EGFP, enhanced green fluorescence protein; FCS, foetal calf serum; GaL V, gibbon ape leukemia virus; HSV-TK, *Herpes simplex* virus thymidine kinase; PBS, phosphate-buffered saline; SCF, stem cell factor; TC, tissue culture.

Retroviral vectors can be packaged into different viral envelopes, which consist of envelope proteins (Env), recognizing specific receptors used for entry into the target cell: on $CD34^+$ cells these are PiT 1 and PiT 2 receptors. The PiT 2 (amphotropic) receptor level is low both on human and murine $CD34^+$ cells, which implies the low level of transduction with vectors produced by the commonly used packaging cell lines (Thomsen *et al.*, 1998). The PiT 1 receptor level on $CD34^+$ cells is much higher. It has been reported that the use of vectors with the gibbon ape leukemia virus (GaL V) Env proteins which are recognized by the PiT 1 receptor, allows for higher transduction efficiency of human haematopoietic $CD34^+$ cells (Thomsen *et al.*, 1998; MacNeil *et al.*, 1999).

Improvements can also be made in the titer (number of colony forming units; c.f.u./mL) of retroviral vector containing supernatants, by choosing an appropriate packaging cell line combined with strategies to maximize the production of particular vector components, which may result in retroviral packaging cells releasing large numbers of infectious viral particles (MacNeil *et al.*, 1999; Schiedlmeier *et al.*, 2000). Major improvements are also required in methods for enhancing the interactions between the retrovirus and its target cells.

The aim of this study was to prepare a retroviral vector and to establish its optimal transduction conditions into human $CD34^+$ cells. The retroviral vector pMINV EGFP, carrying the enhanced green fluorescence protein (*EGFP*) reporter gene was used for transduction of KG-1a ($CD34^+$) and K562 ($CD34^-$) cells. The effect of a range of factors and conditions on cell transduction process was evaluated. The promoter activity tests (Dual Luciferase Reporter Assay, DLR assay) were performed in order to obtain the highest possible transferred gene expression in the target cells. Two different packaging cell lines, producing different retroviral envelope proteins were examined: TE FLY GA cells, which produce GaL V envelope protein, and TE FLY A cells producing amphotropic envelope protein. Because some reports indicated that a simultaneous infection with two different vectors targeting both amphotropic and GaL V receptors gave better transduction effects than with either of the vectors alone (MacNeil *et al.*, 1999; Grabarczyk *et al.*, 2002), we also investigated whether the simultaneous use of both kinds of vectors has an impact on the transduction efficiency.

Among the other factors which may have a strong influence on target cell transduction efficiency, we tested different physicochemical conditions such as: serum presence, co-localization of target cells with the virus present in a supernatant by centrifugation, the temperature of the transduction process and the presence of transduction supporting factors. These were: polycationic agents (Polybrene, protamine sulfate), lipid agents (Dotap, Lipofectamine 2000) and a bacterial agent (Pansorbin).

The optimized transduction conditions, established for the $CD34^+$ KG-1a leukemic cell line, were subsequently used for transduction of $CD34^+$ cells isolated from human umbilical cord blood. However, for the efficient transduction of $CD34^+$ cells with pMINV EGFP vector, some additional minor modifications of the transduction process were necessary. Those experiments indicate the necessity of individual optimization for each particular retroviral transduction system.

MATERIALS AND METHODS

Cell lines. KG-1a (ATCC: CCL-246.1) (CD34 positive), K562 (ATCC: CCL-243) (CD34 negative) human erythroblastoid cell lines were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 50 IU/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine and 1 mM Na-pyruvate (RPMI 1640 complete medium), in 25 cm^2 culture tissue (TC) flasks (Falcon, UK). HeLa (ATCC: CCL-2) human epithelial carcinoma cell line and 293 (ATCC: CCL-1573) human kidney embryonic cell lines were also cultured in RPMI 1640 complete medium.

Packaging cell lines used: murine amphotropic packaging fibroblast cell line PA317 (ATCC: CRL-9078), and human rhabdomyosarcoma packaging cell lines: TE FLY GA with GaLV envelope protein expression (recognizing PiT 1 receptor), and TE FLY A with amphotropic A-MLV envelope protein expression (recognizing PiT 2 receptor). Both human packaging cell lines were kindly provided by Dr. O. Cohen-Haguenauer from the Laboratory of Gene Transfer and Molecular Oncology (Paris, France). Packaging cell lines were cultured in Dulbecco Modified Eagle's medium supplemented with 10% FCS, 50 IU/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine, (DMEM complete medium), in 25 cm^2 TC flasks, at 37°C in 95% air, 5% CO_2 humidified atmosphere. The culture media used were purchased from Gibco, BRL.

Umbilical cord blood CD34 positive cells. Cord blood was collected *ex vivo* from the delivered placenta from normal full-term deliveries taking place at the Chair of Obstetrics and Gynecology of Wroclaw Medical University. Written informed consent was obtained from the patients before labor and delivery. Protocols for sampling human umbilical cord blood were approved by the Commission of Bioethics at Wroclaw Medical University. Umbilical cord blood samples were collected by the umbilical vein puncture with a 17-gauge needle, connected

to a closed system collection bag (Maco Pharma, France) containing citrate dextrose anticoagulant.

CD34 positive ($CD34^+$) cells were isolated by a positive selection procedure, with the use of Rosettesep (Stem Cell Technologies, USA), according to the manufacturer's instruction. Briefly, the blood sample was incubated with Rosettesep in proportion: 1 mL of blood : 50 μL of Rosettesep, for 20 min at room temperature, and then diluted twice with PBS/2% FCS. The mixture was overlaid on the Lymphoflot (Biotest, Germany), and centrifuged (350 × *g*, 20 min, room temp.). After separation, $CD34^+$ cells were collected and suspended in RPMI 1640 medium supplemented with 20% FCS and 10% DMSO, and kept frozen in liquid nitrogen until further use.

$CD34^+$ cells were cultured at the concentration 1×10^6 cells/mL in the Myelocult medium supplemented with growth/proliferation promoting factors: rhSCF (50 ng/mL) and rhIL-3 (20 ng/mL) all from Stem Cell Technologies (USA). The transduction was performed after 48–72 h of culture. For DLR assay, cells were taken after 48–72 h of culture („early" $CD34^+$ cells; $CD34^+$e) or 5 days of culture („late" $CD34^+$ cells; $CD34^+$l).

Plasmids for DLR assay. Plasmid pGL3/Promoter Vector encodes firefly luciferase under the control of the SV-40 promoter and enhancer. The pGL3/Basic without the promoter activity encodes firefly luciferase. The pRL-TK Vector contains the *Herpes simplex* virus thymidine kinase (HSV-TK) promoter region upstream of the *Renilla* luciferase gene. All plasmids were purchased from Promega (Madison, WI, USA). All promoter sequences were obtained from retroviral vectors by restriction cleavage. LTR and PGK promoters were from pMSCV vector (Clontech, USA, cat. no. K1062-1) and CMV promoter from pQCXIN vector (Clontech, USA, cat. no. 9134-1).

DLR activity assay. Dual Luciferase Reporter Assay, DLR assay (Promega, USA), provides immediate sequential quantitation of both activities of firefly (*Photinus pyralis)* and sea pansy (*Renilla reniformis)* luciferases in a single cell culture sample. K562 and KG-1a cells were plated on 24-well TC plates, 5×10^4 cells/well (Costar, USA) in 1 mL of culture medium at 37°C. After 24 h of culture all cell samples were transfected with plasmids with firefly luciferase activity: pGL3/Promoter (SV40), pGL3/Bassic, pGL3/CMV, pGL3/PGK, pGL3/LTR (1.8 μg DNA each), in the presence of pRL-TK plasmid (0.2 μg DNA) with the *Renilla* luciferase activity. Transfections were performed in the presence of Lipofectamine 2000 for 4 h at 37°C. Then cells were washed carefully, resuspened in RPMI 1640 complete medium and cultured for 48 h. The measurement of DLR activity was conducted according to the manufacturer's protocol in Tadiran TD 20/20 luminometer.

Retroviral vector construction. The retroviral pMINV vector was constructed on the basis of pMSCV 2.1 vector (Clontech, USA). The neomycin resistance gene (*neo*^{r}) from pMSCV 2.1 was deleted together with the coding sequence for phosphoglycerate kinase *pgk–neo*^{r}. After dephosphorylation the deleted fragment was replaced by the IRES–*neo*^{r} sequence derived from retroviral vector pSAMEN (kindly provided by Dr. R. G. Hawley, Toronto, Canada). In order to obtain those fragments, pMSCV 2.1 vector was digested with *Bam*HI and *Bgl*II enzymes and pSAMEN was digested with *Bam*HI enzyme. pMINV-EGFP vector was constructed on the basis of pMINV. The EGFP cDNA was obtained from pEGFP reporter vector (Clontech, USA, cat. no. 6084-1) by restriction cleavage with *Xho*I and *Not*I enzymes and inserted into the *Hpa*I position of pMINV. All restriction enzymes were purchased from Promega (Promega Inc., WI, USA).

Producer cell lines. PA317 cells, transfected by electroporation with pMINV-EGFP, resulted in amphotropic retroviral packaging cell line PA MINV EGFP. The electroporation was performed with the use of Eurogentec electroporator (Belgium) (300 V voltage, 100 ms time constant). After 24 h incubation, complete DMEM medium supplemented with 1 mg/mL geneticin (G418, Sigma, USA) was added. Medium was changed every 48 h for 14 consecutive days. After 14 days the viral supernatant was collected for the „*ping–pong*" transduction of the packaging human TE FLY GA and TE FLY A cell lines. The selection of highly effective packaging cell clones was performed by dot blot hybridization with radiolabeled EGFP-specific probe. Packaging cell line TE FLY A MINV EGFP supernatant was further used for transduction of K562 and KG-1a cells. Packaging cell line TE FLY GA MINV EGFP supernatants were used for transduction of K562 and KG-1a cells as well as $CD34^+$ cells isolated from umbilical cord blood.

Preparation of retrovirus-containing supernatants. TE FLY A MINV EGFP and TE FLY GA MINV EGFP cells were trypsinized and plated in 25 cm^2 culture flasks. After obtaining approximately full culture confluence, the complete DMEM medium was changed for Opti-MEM medium (GIBCO BRL, Germany) supplemented with 5% FCS and cells were cultured for further 24 h at 37°C, or in 32°C for TE FLY GA MINV EGFP producing cells, and 24 h at 37°C for TE FLY A MINV EGFP producing cells. Alternatively, some supernatants were harvested in the absence of FCS in the culture medium. After collection, supernatants were centrifuged three times (800 × *g*, 10 min, room temp.). When necessary, the dilution of the virus samples with the appropriate fresh medium was done. The virus-containing supernatant volumes used for transduction ranged

from 50 μL to 1000 μL in final 1000 μL volume. The functional titers of the virus produced by TE FLY GA MINV EGFP and TE FLY A MINV EGFP cells were evaluated on the 293 cell line and confirmed on HeLa cells by the end point dilution method, as described by Coffin *et al*. (1997), and were established as 10^5–10^6 c.f.u./mL for each cell line.

Transduction enhancing agents. The preliminary transduction experiments were performed according to the standard protocol, using polycationic agent Polybrene (8 μg/mL; hexadimethrine bromide, Sigma, Germany) as a transduction supporting agent. In further experiments, other agents were tested. Polycationic agent protamine sulfate (Sigma, Germany) was used at the concentration of 5 μg/mL. Pansorbin (Calbiochem, UK) dilution was prepared by incubation of 5 μl Pansorbin with 5 mL of viral supernatant at 4°C for 2 h (Darling *et al.,* 2000). Two liposomal transfection agents: Dotap (Roche, Germany) and Lipofectamine 2000 (Gibco BRL, Germany), were used in dilution of 1 μL/mL.

Transduction of K562 and KG-1a cells. K562 and KG-1a cells were seeded in 24 well TC plates (Costar, USA), 2×10^5 cells per well in 250 μL of culture medium and incubated at 37°C. After 24 h of culture transduction was performed using the supernatants from packaging cell lines TE FLY GA MINV EGFP and TE FLY A MINV EGFP, added separately or in a mix (ratio 1:1). Transductions were performed in different combinations: at different temperatures, with or without co-centrifugation of cells and viral particles (800 × *g*, 60 min, 32°C), in the presence or absence of serum in culture medium and in the presence of transduction enhancing agents applied separately or in various combinations.

After 24 h of culture the transduction medium was replaced with fresh RPMI 1640 complete medium. The transduction efficiency was estimated as a percentage of the EGFP positive cells by flow cytometric analysis on FACS*Calibur* apparatus (Becton–Dickinson, USA). The registered data were analyzed with the Cell Quest® software (Becton–Dickinson, USA).

Transduction of CD34⁺ cells. $CD34^+$ cells were plated in 96-well TC plates, 5×10^4 cells per well (Costar, USA) in 50 μL of RPMI 1640 complete medium. After 24 h of culture, cells were transduced with supernatants from packaging cell lines: TE FLY GA MINV EGFP and TE FLY A MINV EGFP, used separately or in a mix (ratio 1:1). The transduction was performed at different temperatures, with or without co-centrifugation of cells and virus-containing supernatants (800 × *g*, 60 min, 32°C), in the presence or absence of serum in culture medium and in the presence of transduction enhancing agents applied separately or in various combinations.

After 72 h of culture, transduction medium was replaced with fresh RPMI 1640 complete medium. The transduction efficiency was evaluated as a percentage of EGFP positive cells by flow cytometry, using FACS*Calibur* apparatus and the registered data were analyzed with Cell Quest® software.

Statistical analysis. The results obtained were analyzed by *Statistica*® (version 5.5 A) software.

RESULTS

The optimization of the process of human $CD34^+$ cell transduction with pMINV retroviral vector was performed. Studies included choice of vector promoter and vector packaging cell line, and further checking of various physicochemical factors and conditions of the transduction process.

Viral vector promoter choice

Three promoters used in retroviral gene transfer protocols: LTR and PGK promoters derived from pMSCV vector and CMV promoter from pQCXIN vector were tested using Dual Luciferase Reporter Assay (DLR). Experiments were performed on KG-1a ($CD34^+$), K562 ($CD34^-$), and cord blood $CD34^+$ cells. In $CD34^+$ positive cells, both KG-1a cell line and cells isolated from cord blood, the highest activity was observed for the LTR promoter. In K562 cells the highest activity was observed for the CMV promoter (Fig. 1).

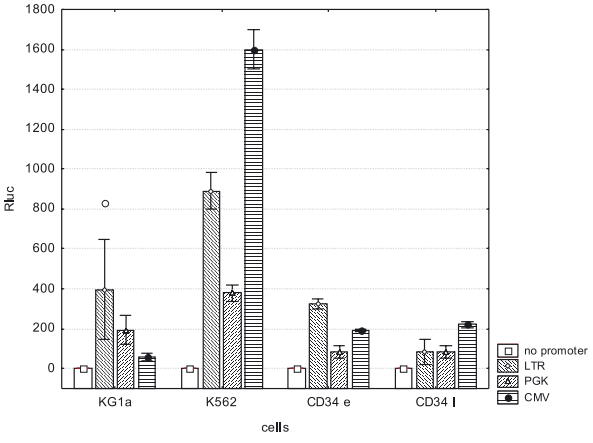

Figure 1. Comparison of promoter activities in the DLR assay.
Results are expressed as the relative luciferase activity (Rluc) of the pGL3/Promoter control vector. Data were calculated from independent transfections with pGL3/LTR (left-striped boxes), pGL3/PGK (right-striped boxes) and pGL3/CMV (horizontally striped boxes). Control transfection with pGL3/Bassic with no promoter activity is represented as open boxes. $CD34^+$ e, „early" (after 48–72 h of culture) CD34 positive cells, $CD34^+$ l, „late" (after 5 days of culture) CD34 positive cells. Data represent the mean from three independent experiments ±S.D.

Therefore, for pMINV EGFP vector construction the LTR promoter was chosen.

Transduction of KG-1a and K562 cells with pMINV EGFP retroviral vector

Viral vector packaging cell line selection

K562 and KG-1a cells were infected with viral vector containing supernatants from TE FLY A MINV EGFP or TE FLY GA MINV EGFP cells (both with titer 10^5–10^6 c.f.u./mL), or with a mixture of supernatants from both cell lines (ratio 1:1). Experiments were performed according to the standard protocol using Polybrene as the transduction supporting agent.

The highest transduction efficiencies for both K562 and KG-1a cells were obtained with virus-containing supernatant from TE FLY GA MINV EGFP cells. The synergistic or additive effect of transduction with mixed supernatans from both packaging cell lines was not observed (Fig. 2). Similar results were obtained at different transduction conditions: in the presence or absence of serum and with or without co-centrifugation of cells with virus-containing supernatants (not shown).

The effect of FCS presence on vector-containing supernatant activity

The transduction efficiency of supernatants collected from TE FLY GA MINV EGFP packaging cell line in the presence or absence of FCS in the culture medium were also evaluated. The presence of serum in the medium resulted in higher transduction efficiency for all transduction conditions tested (Fig. 3).

Temperature of virus-containing supernatant harvesting

The transduction efficiency of virus-containing supernatants from packaging cell lines TE FLY GA MINV EGFP and TE FLY A MINV EGFP grown at two different temperatures (32°C and 37°C) were compared. As it was reported by other investigators, an increased half-life of viral particles harvested at 32°C may result in higher transduction efficiency (Kotani *et al.*, 1994; Kaptein *et al.*, 1997). However, we did not observe any significant influence of virus-containing supernatant harvesting temperature on the transduction results (not shown).

Co-centrifugation of cells and virus-containing supernatants

Co-localization of target cells and viral vector particles during centrifugation may increase gene transduction efficiency (Kotani *et al.*, 1994; Grignani *et al.*, 1998; Lee *et al.*, 1998). K562 and KG-1a cells were incubated with TE FLY GA MINV EGFP virus-containing supernatant with centrifugation (800 × ***g***, 60 min), or without centrifugation. Centrifugation resulted in higher transduction efficiency for both cell lines tested (Fig. 3).

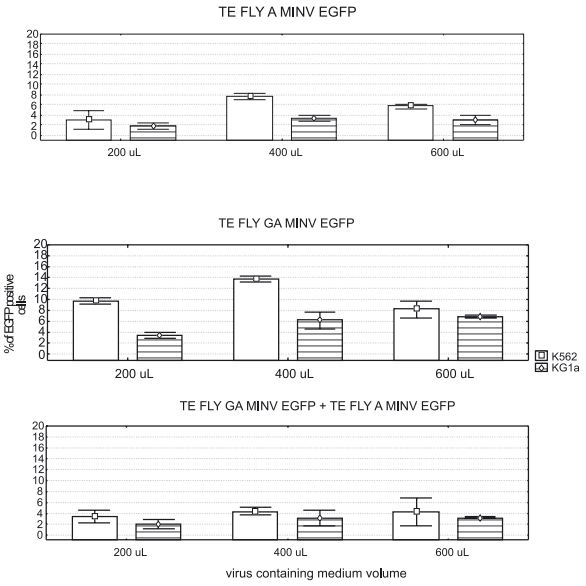

Figure 2. Effect of K562 and KG-1a cell transduction with virus-containing supernatants from packaging cell lines TE FLY A MINV EGFP, TE FLY GA MINV EGFP or a mixture of both media (ratio 1:1).
Virus-containing media were used at three different volumes: 200 μL, 400 μL, 600 μL; at 37°C, in the presence of Polybrene. Data are means from six independent experiments for each cell line ±S.D.

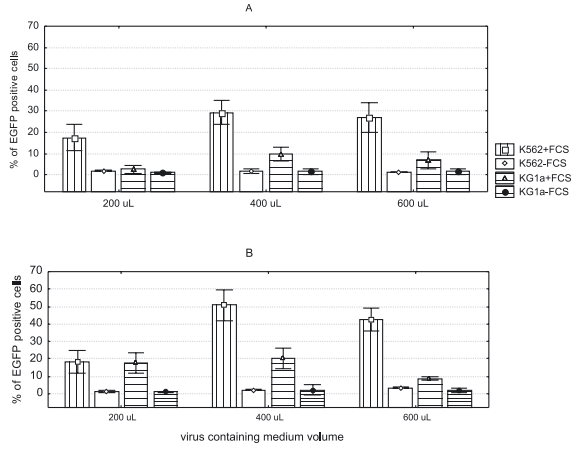

Figure 3. Effect of serum presence and co-centrifugation on K562 and KG-1a cell transduction efficiency.
Supernatants from TE FLY GA MINV EGFP packaging cell line were used at three different volumes: 200 μL, 400 μL and 600 μL. Cells were cultured at 37°C in the presence of Polybrene. [+ FCS], serum presence; [– FCS], serum absence. **A**. Transduction performed without centrifugation of target cells with virus-containing supernatants. **B**. Transduction performed with centrifugation of target cells with virus-containing supernatants. The data are means from six independent experiments for both cell lines ±S.D.

Volume of the virus-containing supernatant used for transduction

To find the optimal dose of infecting viral vector used for transduction, different volumes of TE FLY GA MINV EGFP packaging cell line supernatant, with titer 10^5–10^6 c.f.u./mL, were tested, at 50 up to 1000 μL, in 1000 μL of final volume. Transduction was performed in the presence of Polybrene at 37°C. For transduction of KG-1a cells the ratio: 2:5 (400 μL of supernatant in 1000 μL of culture medium) was found to be the most effective (Fig. 4). For K562 cells the highest outcome was obtained when undiluted virus-containing supernatant was applied. However, to standardize transduction conditions for further experiments, the 2:5 ratio for both above cell lines was chosen.

Transduction enhancing agents

The effects of transduction enhancing agents on K562 and KG-1a cell transduction efficiency were examined. Apart from the widely used protocol using Polybrene, other transduction enhancing agents were tested, such as Pansorbin, protamine sulfate and two liposomal agents: Dotap and Lipofectamine 2000. The agents were tested separately or in different combinations (Fig. 5).

For KG-1a cells, Polybrene and Pansorbin gave the highest transduction level of 16% and 13% of transduced cells, respectively. Even better results were obtained when these agents were combined with lipid factors. Polybrene and Dotap used together gave 28% transduction efficiency (Fig. 5).

For K562 cells the transduction efficiency was much higher. The presence of Pansorbin and Polybrene gave 53% and 51% of EGFP-positive cells, respectively. However, the combination of Polybrene with lipid factors such as Lipofectamine 2000 and Dotap gave worse transduction effects (43% and 45%, respectively) (Fig. 5). Further combinations of Polybrene with Dotap and Pansorbin or Lipofectamine 2000 and Pansorbin also did not increase the transduction efficiency (not shown).

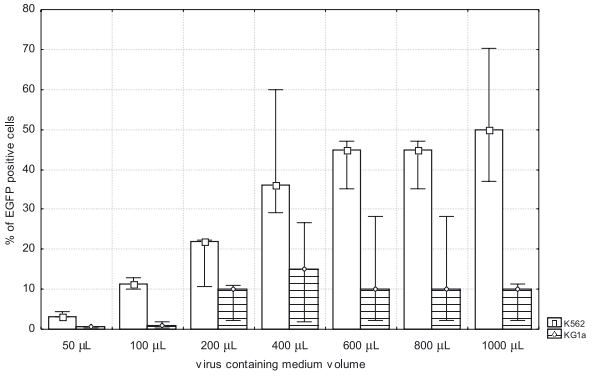

Figure 4. Effect of different volumes of virus-containing media on transduction efficiency of K562 and KG-1a cells.
Experiments were performed at 37°C in the presence of Polybrene. The data are means from six independent experiments for each cell line ±S.D.

Transduction of CD34⁺ cord blood cells with pMINV EGFP retroviral vector

The protocol established with pMINV EGFP retroviral vector for KG-1a cells, was applied for umbilical cord blood CD34⁺ cell transduction. After some further modifications the optimal conditions of transduction were elaborated.

First, activities of three different promoters: LTR, PGK and CMV were evaluated with the DLR assay. Similarly as it was previously found for CD34⁺ positive KG-1a cells, LTR appeared to be the most effective, especially in „early" CD34⁺ cells (see Fig. 1). Therefore, LTR retroviral promoter was applied for further vector constructions.

Virus-containing supernatants from TE FLY A MINV EGFP and TE FLY GA MINV EGFP packaging cells or a mixture of both were used for CD34⁺ cell transduction. Use of TE FLY GA MINV EGFP supernatant (10^5–10^6 c.f.u./mL) resulted in the highest transduction efficiency (Fig. 6). When transduction was performed with a mixture of supernatants, a strong competitive effect appeared. Hence, TE FLY GA MINV EGFP packaging cell line was chosen for vector-producing supernatant production.

During optimization of CD34⁺ transduction process, the protocol previously established for KG 1 cells was applied. Transduction was performed with 2:5 ratio of virus-containing supernatant to culture medium, in the presence of serum, at 37°C, with co-centrifugation of cells with virus-containing supernatant. The choice of transduction enhancing agents was the only factor that had to be modified, as for transduction of CD34⁺ cells Lipofectamine 2000 appeared the most effective (Fig. 7). The use of

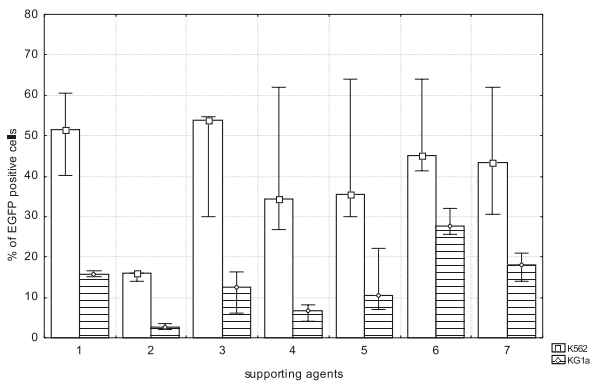

Figure 5. Effect of transduction enhancing agents on K562 and KG-1a cell transduction efficiency.
1, Polybrene; 2, protamine sulfate; 3, Pansorbin; 4, Lipofectamine 2000; 5, Dotap; 6, Dotap + Polybrene; 7, Lipofectamine 2000 + Polybrene.

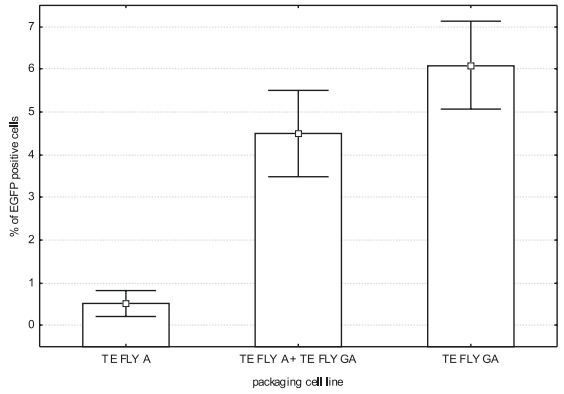

Figure 6. Effect of transduction of CD34⁺ cells with supernatants from packaging cell lines TE FLY A MINV EGFP, TE FLY GA MINV EGFP and a mixture of both (ratio 1:1).
Virus-containing supernatants were used from TE FLY A MINV EGFP cells, from TE FLY GA MINV EGFP cells and also from mixed virus-containing media from TE FLY A MINV EGFP and TE FLY GA MINV EGFP cells, at 37°C, in the presence of Polybrene. The data are means from six independent experiments ±S.D.

Lipofectamine 2000 resulted in 26% of EGFP positive cells *versus* 8% and 10% for Pansorbin and Dotap, respectively. The lowest transduction level was obtained for the standard protocol with Polybrene (about 4% of EGFP positive cells).

DISCUSSION

Attempts of genetic modifications of stem cells with retroviral vectors have achieved limited success, partly because of low gene transfer efficiency (Thomsen *et al.*, 1998; Hong *et al.*, 2004). There are many factors which may affect the transduction process, such as viral backbone, promoter activity and packaging cell line choice, combined with transduction conditions and the presence of transduction enhancing agents (Liu *et al.*, 2000; Hong *et al.*, 2004; Schmidt *et al.*, 2005).

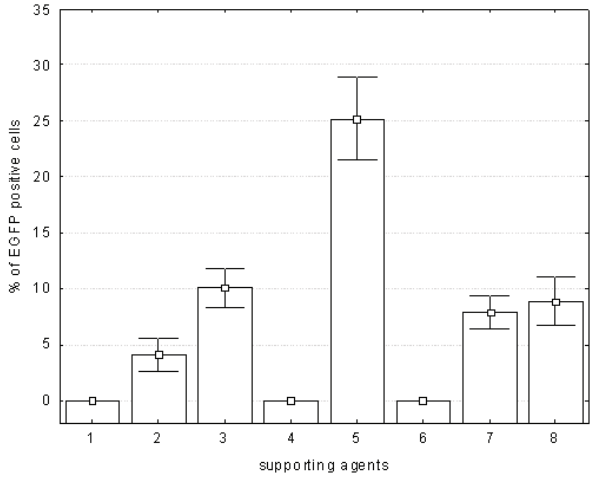

Figure 7. Effect of transduction enhancing agents on transduction efficiency of human CD34⁺ cells.
1, Control; 2, Polybrene; 3, Dotap; 4, Dotap + Polybrene; 5, Lipofectamine 2000; 6, Lipofectamine 2000 + Polybrene; 7, Pansorbin; 8, Pansorbin + Polybrene. Data are means from five independent experiments ±S.D.

Efforts to optimize the transduction process have adapted different strategies. New vector constructs have been designed for more efficient transgene expression. These were packaged into different vector-packaging cell lines, with specific target cell tropisms. Also culture conditions for producing high number of viral particles were improved. The probability of target cell infection by retroviral particles can be increased by incubation with transduction enhancing agents or by low-speed centrifugation of retrovirus-containing medium with target cells. It is necessary to balance between a retroviral vector half-life time, temperature conditions, the receptor presence on the target cells and the use of transduction enhancing agents (Chuck *et al.*, 1996; Lee *et al.*, 1998; Darling *et al.*, 2000; Liu *et al.*, 2000; Hong *et al.*, 2004).

In this study we attempted to optimize retroviral gene transfer into CD34⁺ cells. The retroviral vector pMINV EGFP carrying the *EGFP* reporter gene, derivative of MSCV virus, was constructed and further chosen for the experiments. The MSCV vector backbone was claimed to be the best for genetic modifications of CD34⁺ cells (Hawley *et al.*, 1994; Kim *et al.*, 2000).

The choice of effective promoter for viral backbone construction has a strong influence on transduction efficiency and on further transgene expression in target cells (Byk *et al.*, 1998; Salmon *et al.*, 2000; Woods *et al.*, 2001). Byk *et al.* (1998) found that CMV and PGK promoters were active in haematopoietic cells, whereas Salmon observed low activity of CMV and high activity of EF1-α promoter in CD34⁺ cells (Salmon *et al.*, 2000). When choosing the promoter, we examined the activity profile of three commonly used promoters in CD34 positive and CD34 negative model cell lines. There was also a question, whether the promoter activity changes with the CD34⁺ cell differentiation level. Among the promoters analyzed, the LTR promoter showed the best activity in CD34⁺ cells. Moreover, this activity was much higher in less differentiated "early" CD34⁺ cells than it was in „late" CD34⁺ cells.

Retroviral vectors are packaged into infectious viral particles in a retroviral packaging cell line. The species- and cell type-specificity of the retroviral vector particle is mainly determined by the type of envelope protein produced in the packaging cell line. For the experiments presented here, the human rhabdomyosarcoma packaging cell lines were

chosen. For final $CD34^+$ cell transduction, the best results were obtained with use of packaging cell line TE FLY GA MINV EGFP, producing GAL V envelope protein.

Many investigators indicated the possibility that simultaneous infection with vectors targeting both amphotropic (PiT 2) and GaL V (PiT 1) receptors may give higher transduction yield than use of any of these vectors alone (MacNeil *et al.*, 1999; Grabarczyk *et al.*, 2002). However, the data obtained by us did not reveal any positive effect of such a manipulation.

It has been also reported by Kaptein *et al.* (1997) and by Kotani *et al*. (1994) that transduction efficiency increases when the retroviral superntants are harvested at 32°C, due to higher stability of viral particles at this temperature. They suggested that it might prolong the retrovirus half-life even fourfold. These observations concerned the PA 317 mouse packaging cell line. In our experiments with human packaging cell lines, no significant differences in viral vector titer harvested at 37°C and 32°C were observed, which was in agreement with the data of Forestell *et al.* (1995). For many gene therapy applications, the effective titer of retroviral vectors is a limiting factor of transduction efficiency. Purification and concentration of retrovirus from packaging cell line supernatant by filtration or by centrifugation, help to overcome this problem. Based on the observation that viral supernatant filtration after harvesting reduced viral titer by about a half (Reeves & Cornetta, 2000), we decided to use centrifugation for removing cell debris from the virus-containing supernatant (Kotani *et al.*, 1994; Lee *et al.*, 1998).

An important condition, required for successful retroviral transduction, is a contact between viral particles and target cells. It has been shown that in a static transduction system the majority of viral particles do not contact the target cells (Chuck *et al.*, 1996). Therefore, centrifugation of virus-containing supernatants with target cells significantly increases transduction efficiency, due to the contact of target cells with the vector (Darling *et al.*, 2000; Liu *et al.*, 2000). This was also our experience; the centrifugation improved the transduction efficiency approx. 2-fold as compared to static conditions (see: Fig. 3).

Some protocols use polycationic or biological agents for enhancing retroviral vector efficiency. Liu *et al.* (2000) found that use of Lipofectamine significantly (about 10-fold) enhanced the transduction efficiency of K562 and $CD34^+$ cells. The effect of the transduction enhancement by cationic or biological reagents is due to their ability to form complexes with virions, which can settle under gravity in culture (Darling *et al.*, 2000). Some other researchers also suggested that cationic lipids may reduce electrostatic repulsion between virus and target cell (Liu *et al.*, 2000). However, it is important to remember that cationic lipids can exert toxic effect on cells (Liu *et al.*, 2000).

The elaborated transduction protocol allowed us to obtain the relatively high transduction efficiency of $CD34^+$ cells (26% of EGFP positive cells). This protocol will be applied for human $CD34^+$ cell transduction with therapeutic genes, such as growth factors or multidrug resistance genes. However, we emphasize the need for careful optimization of transduction conditions.

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