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Design and assessment of safety-oriented retroviral vectors for the gene transfer of an active glucocerebrosidase gene in human hematopoietic CD34+ progenitor cells

Research Article

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Summary

Quantitative or qualitative deficiency of the lysosomal enzyme glucocerebrosidase results in the accumulation of its substrate, glucocerebroside, in macrophages, leading to the pathology of Gaucher disease. For more than two decades of research, gammaretroviral vectors have been used for gene transfer of the glucocerebrosidase gene for the correction of the enzyme's deficit. It has been shown, even on clinical level, that the design of efficient as well as safe gammaretroviral vectors, aiming mainly at eliminating "insertional mutagenesis", is an imperative need. The present study aims at the design and assessment of the efficiency of six new and safety-oriented gammaretroviral vectors for the gene transfer of the glucocerebrosidase gene into human hematopoietic progenitor cells. All vectors are Self-Inactivating and bear sequences for the improvement of transcriptional termination; either the WPRE ("Woodchuck hepatitis virus Post-transcriptional Regulatory Element") or the 2xSV40 USE (two copies of "Simian Virus 40 Upstream Sequence Element"). It is shown that these new gammaretroviral vectors are efficient in transferring an active wild type glucocerebrosidase gene into a CD34+ human primary hematopoietic progenitor cell population. All six vectors showed increased enzyme activity, as compared to the untransduced cell population, albeit with differences amongst them, partly due to differences in the transduction efficiency.

I. Introduction:

The autosomal recessive (Fried, 1973) deficiency of the lysosomal enzyme glucocerebrosidase (GC) leads to the clinical entity of the most prevalent lysosomal storage disease, Gaucher disease (GD) (Brady et al., 1965). Approximately 300 mutations of the glucocerebrosidase gene (*GBA*, MIM *606463, GenBank accession no. J03059) have been described, which lead to quantitatively or functionally insufficient glucocerebrosidase (Hruska et al., 2008). The clinical results occur from the substrate accumulation in the lysosomes and mainly from the accumulation of the glycosphingolipid glucocerebroside (Brady, 1997). Glucocerebroside is the main product of the hydrolysis of the complex glycosphingolipids and an intermediate in their synthetic pathway. Although glucocerebrosidase is expressed in all nucleated cells, glucocerebroside catabolism is maximum in monocytes / macrophages, setting these cells in the epicenter of the pathology of Gaucher disease. Phagocytosis of aged blood cells seems to be the main source of accumulating glucocerebroside, which gives macrophages the characteristic morphology ("Gaucher cells") (Beutler and Grabowski, 2001). Predominance of Gaucher cells in organs results in organomegaly and organ dysfunction (hepatomegaly, splenomegaly) and cytopenia due to substitution of normal blood cells in the bone marrow (anemia with fatigue, leucopenia with susceptibility to infections, thrombocytopenia with bleeding diathesis) (Beutler and Grabowski, 2001).

The classical classification divides Gaucher disease into non-neuronopathic (type 1) and neuronopathic (types 2 and 3) forms. Enzyme replacement therapy (ERT) is the gold standard for Gaucher type 1 treatment; however it is not a permanent cure and the high cost raises moral and practical / financial issues and generally restricts the availability to developed countries (Beutler, 2006; Kesselman et al., 2006). Even the newest approach of the first plant cell–based recombinant ERT, taliglucerase alfa (Hollak, 2012) is still expected to be of high cost. ERT does not overcome the blood brain barrier (Xu et al., 1996) and there is no evidence that it can reverse, stabilize or slow down the progression of neurological involvement (Vellodi et al., 2009); as a result it is saved for all patients with Gaucher type 3 and patients with Gaucher type 1 fulfilling certain clinical and laboratory criteria (Martins et al., 2009; Vellodi et al., 2009).

Allogeneic stem cell transplantation has been successful in the past for patients who survived the procedure (Peters and Steward, 2003; Ringden et al., 1995). However, the considerable mortality (Kumar, 2007), especially in patients with notable organ dysfunction, in other words in those patients who need it more, and the negative effect in development (Beutler, 1991; Storb et al., 1991; Sullivan and Reid, 1991) prevent its routine clinical use for a disease with an existent effective enzyme replacement therapy despite the much lower cost.

The central pathogenic role of macrophages has led to the research approach of gene transfer of a wild-type glucocerebrosidase gene into macrophages through transduction of hematopoietic stem cells (HSCs). Optimism from positive *in vitro* and preclinical results (Correll et al., 1992; Fink et al., 1990; Kohn et al., 1991; Nolta et al., 1990; Nolta et al., 1992; Ohashi et al., 1992) paved the way for a clinical study with a gammaretroviral vector. In this sole study for patients with type 1 Gaucher disease the vector was detectable only in a very small percentage of cells – and in no case for more than three months – with no detectable clinical result.

These results raised questions not only about possible vector and transduction procedure modifications, but also about the need of partial myeloablation before autologous transplantation of transduced HSCs, so that sufficient engraftment of the genetically corrected HSCs is achieved (Dunbar et al., 1998).

The rationale of genetic correction of HSCs and autologous transplantation of patients with type 1 Gaucher disease has been reinforced by the generation of a murine model for the disease, which has proven possible to successfully treat or prevent both with allogeneic bone marrow transplantation and with retroviral transduction and transplantation of bone marrow cells (Enquist et al., 2006). Furthermore, partial myeloablation in this animal model has been shown sufficient for a therapeutic outcome, even with less than 10% wild-type cell engraftment (Enquist et al., 2009). This evidence supports the further development of glucocerebrosidase gene transfer vectors for the correction and consecutive autologous transplantation of HSCs in type 1 Gaucher patients.

Clinically valuable gene transfer vectors are expected to be both efficient and safe. Leukemogenesis caused by insertional mutagenesis in 5 out of 10 patients in the clinical trial for SCID-X1(Hacein-Bey-Abina et al., 2008; Howe et al., 2008; Shaw and Kohn, 2011) accentuated the importance of safety in the design of gammaretroviral vectors. The likelihood of proto-oncogene activation by promoter / enhancer sequences of the gammaretroviral U3 region of the LTRs seems to be an important risk factor. The risk is further enhanced by the evolutional selection of weak polyadenylation signals in the gammaretroviral 3'LTRs, which allow for downstream readthrough transcription with hazardous consequences, if the integration site happens to be upstream of a proto-oncogene (Furger et al., 2001; Zaiss et al., 2002).

This study presents six new gammaretroviral vectors with a SIN (self-inactivating) design together with a weak cellular internal promoter to drive the GC cDNA with the aim of minimizing the proto-oncogene activation risk and improving polyadenylation. SIN vectors have been shown to have a reduced gene transfer mutagenic potential (Montini et al., 2009; Zychlinski et al., 2008); a SIN gammaretroviral vector using one of the internal promoters of this study is already in a clinical trial for SCID-X1 gene therapy (Children's Hospital Boston, 2010-). In the present study it is shown that these new gammaretroviral vectors are efficient in transferring an active wild-type glucocerebrosidase gene copy into a CD34+ human primary hematopoietic progenitor cell population. All six vectors showed increased enzyme activity, as compared to the untransduced cell population, albeit with differences amongst them, partly due to differences in transduction efficiency.

II. Materials and Methods:

A. Vector plasmids

All the vector plasmids are SIN gammaretroviral vectors based on the gammaretroviral (Mo-MLV) backbone SERS11, as previously described (Schambach et al., 2006d). The 600 bp Woodchuck Posttranscriptional Regulatory Element (bPRE* (Schambach et al., 2006a), abbreviated as WPRE here) was included into the 3'UTR as a *BamH*I / *Hind*III fragment where indicated. Alternatively, a 2xSV USE (Upstream Polyadenylation Enhancer) was inserted into the EcoRI site of the ΔU3 region (Schambach et al., 2007) to improve polyadenylation. The cDNA of the human glucocerebrosidase gene was amplified via PCR using primers 5' GlucoC age 5'-CCACCGGTGCCACCATGGAGTTTTCAA

GTCCTTCCA-3' (restriction sites underlined) and 3' GlucoC sal 5'-CCGTCGACGCGGCCATCTCAGTTATCTA CTGGCGACGCCACAGGTA-3'. An IRES-GFP cassette driven by the EMCV (Encephalomyocarditis Virus) internal ribosomal entry site was added for co-expression of a fluorescent marker where indicated. Three different promoters were used: (a) The strong retroviral promoter / enhancer of the U3 region of the SFFV LTR, which is 400 bp in size; (b) the EF1α promoter (1200 bp, containing an intron); and (c) the EF1α short version (EFS, 250 bp) lacking an intronic sequence (Schambach et al., 2006b; Schambach et al., 2006c). All vector modules were confirmed by sequencing.

B. Vector production

Gammaretroviral vector suspensions were produced with cotransfection of a) the vector plasmid, b) the RD114/TR expressing plasmid phCMV-RD114/TR (Sandrin et al., 2002) and c) the retroviral gag-pol expressing plasmid pcDNA3.MLVg/p (Voelkel et al., 2010).

Gammaretroviral vectors were produced in the packaging cell line Phoenix-gp (Kinsella and Nolan, 1996). Cells are kept in DMEM with 4.5 g/l glucose (DMEM+GlutaMAX-I, GIBCO®) and 10% fetal calf serum (GIBCO®).

The day before transfection, $5x10^6$ Phoenix-gp cells (Kinsella and Nolan, 1996) are plated on a 10 cm dish. On the day of transfection the medium is changed to "transfection medium" (DMEM+Glutamax-I supplemented with 10% fetal calf serum, 1% sodium pyruvate (GIBCO®), 100 U/ml penicillin - 100 μg/ml streptomycin (Penicillin G sodium – Streptomycin sulfate, GIBCO®) supplemented with 25 mM chloroquine (Sigma–Aldrich, St. Louis, MO)). Cells are transfected with 5 μg of the transfer vector, 5 μg of the RD114/TR expressing plasmid phCMV-RD114/TR (Sandrin et al., 2002) and 6 μg of the retroviral gag-pol expressing plasmid pcDNA3.MLVg/p (Voelkel et al., 2010) using the calcium phosphate precipitation method with the Calcium Phosphate Transfection Kit (SIGMA®, Missouri, USA). The plasmids are diluted in 450 μl water and supplemented with 50 μl of a 2.5 M $CaCl_2$ solution (final concentration 250 mM). The solution is added and mixed with "air-bubbling" in 500 μl 2XHEPES-Buffered Saline, pH 7,05 (50 mM HEPES, 280 mM NaCl, 1,5 mM Na_2HPO_4), incubated at room temperature for 20 minutes and added in the Phoenix-gp cells immediately after the medium has been changed to "transfection medium" with chloroquine. The medium is changed to just "transfection medium" twice, 7-8 hours (8 ml) and 24 hours (7 ml) after the transfection. Supernatant containing viral particles is collected 36 hours after the transfection, filtered (0.40 μm pore size) and stored at -80°C until usage.

C. Isolation of CD34+ cells

Mononuclear cells are isolated from human umbilical cord blood using Lymphoprep™ (Ficoll-Isopaque) d=1.077 g/ml (Axis-Shield PoC AS, Oslo, Norway) density gradient separation. CD34+ enriched population of cells is then isolated with positive selection with the use of LS MACS® Selection Columns and the CD34 MicroBead Kit, human (Miltenyi Biotec®, Gladbach, Germany). Briefly, the isolated mononuclear cells are magnetically labeled with monoclonal mouse anti-human CD34 antibodies conjugated to MicroBeads, passed through an LS MACS® Selection Column placed in the magnetic field of a MACS® Seperator (Miltenyi Biotec®), so that the CD34 negative cells are washed away, and the magnetically retained CD34 positive fraction is eventually eluted after the column has been removed from the magnetic field. Isolated cells are stored in DMEM+GlutaMAX-I supplemented

with 20% fetal calf serum and 10% dimethylsulfoxide (SIGMA®, Missouri, USA), gradually frozen in Nalgene® Cryo 1°C Freezing Container to -80°C and moved at -196°C the next day until usage.

D. Transduction of primary cells

Transduction of CD34+ population of primary cells is performed after 48-hour cytokine prestimulation. $6x10^5$ cells per 35 mm well are seeded in 6-well suspension culture plates (Greiner Bio-One GmbH) in 2 ml serum-free medium: Biowhittaker™ X-VIVO 15 (Cambrex Bio Science Verviers, Verviers, Belgium) supplemented with 1% Bovine Serum Albumine (StemCell Technologies Inc., Ohio, USA), 100 U/ml penicillin - 100 μg/ml streptomycin, 2mM L-glutamine (GIBCO®), 10^{-6} M 2-mercaptoethanol (ICN Biomedicals INC) supplemented with the following human recombinant growth factors (all from PeproTech EC Ltd): 100 ng/ml stem cell factor (SCF), 100 ng/ml Flt-3 ligand (FL) and 50 ng/ml thrombopoietin (TPO).They are cultured at 37°C, 5% CO_2, >95% humidity for 48 hours. After this period, cells are pooled and $2.4x10^5$ cells are reseeded on 35 mm wells (6-well plates, Greiner Bio-One GmbH) in 2 ml of the same serum-free medium with the same cytokine concentrations. In order to increase transduction efficiency, the wells have been previously coated with recombinant fibronectin fragment CD-296 (Hanenberg et al., 1996) (Retronectin®, Takara Bio Inc, Otsu, Shiga, Japan) at a concentration of 9 μg/cm^2 and preloaded with vector by centrifugation (Kuhlcke et al., 2002); 2 ml of virus-containing supernatant are thawed and placed on a fibronectin-coated well and the plate is centrifuged at 4°C, 1000g for 90 minutes. The plates are returned in the incubator directly after the cell seeding. Untransduced cells used as a control population are treated the same way with Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DMEM+GlutaMAX-I, GIBCO®) supplemented with 10% fetal calf serum (GIBCO®) substituting for viral supernatant during the centrifugation.

E. Enzyme assay (on suspension culture)

Cells are centrifuged and resuspended and lysed in an aqueous solution of 50 mM citric acid / 1 M sodium phosphate pH 6.0, 0.2% Triton® X-100, 10 mg/ ml sodium taurocholate by three freeze / thaw cycles (ethanol-dry ice bath / 37°C water bath). After sedimentation of the lysed membranes by centrifugation, aliquots of the lysate are incubated for 40 min at 37°C with or without 400 μM of the glucocerebrosidase inhibitor conduritol B epoxide (Enquist et al., 2006) (Sigma–Aldrich, St. Louis, MO). Glucocerebrosidase activities are determined fluorimetrically after 2 hours of incubation at 37°C with 15 mM 4-methylumbelliferyl-D-glucuronide trihydrate (Sigma–Aldrich, St. Louis, MO).

The reaction is stopped with the addition of a solution of glycine / NaOH at final concentrations of 25 mM each. In all assays control (untransduced) cells were analyzed, and the level in these was set as 1.

F. BCA (bicinchoninic acid) Protein assay

The protein content of the lysates was measured with the BCA™ Protein Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). Protein (kit bovine serum albumin) standards are made and 10 μl of all the standards and all the lysates are incubated with 200 μl of a BCA A+B (50:1) reagents' mix for 30 minutes at 37°C. After the solutions reach room temperature, the absorption at 562 nm is measured on a spectrophotometer (Eppendorf® Biophotometer). The protein standards are used for the calibration / creation of a standard

curve, in order to correlate the samples' absorption with protein content values.

G. Colony assay and Colony PCR

48 hours after preloading cells are seeded in methylcellulose for granulocytic / monocytic colony growth. Cytokine-free methylcellulose (MethoCult® H4230, StemCell Technologies Inc.) is diluted with IMDM (PAA Laboratories GmbH, Pasching, Austria) (80 ml methylcellulose, 20 ml IMDM) and supplemented with GM-CSF, IL-3 and SCF at a concentration of 10 ng/ml each. Cells are retrieved from the wells of the 6-well plates and added to the methylcellulose at a concentration of 350 cells/ml. 1 ml is seeded on a 3-cm plate and incubated at 37°C, 5% CO_2, >95% humidity. 40 to 50 individual conlonies of at least 50 cells each are picked after 10-12 days; each one is added at a 25 μl aliquot of lysis buffer (aqueous solution of 105 mM KCl, 14 mM TrisHCl pH 8.3, 2.5 mM $MgCl_2$, 0.3 mg/ml Gelatin, 0.45% IGEPAL CA-630, 0.45% Tween 20) supplemented with 60 μg/ml Proteinase K (SIGMA®, Missouri, USA), mixed and incubated for cells to be lysed at 56°C for 1 hour and 95°C for 15 minutes.

PCR for the detection of the integrated glucocerebrosidase cDNA was performed on these colonies with the use of these primers (ordered from Invitrogen™):

5'- ACTGGAACCTTGCCCTGAAC -3' (forward)

5'- ACCACAACAGCAGAGCCATC -3' (reverse)

and this program:

1. 94°C for 5 minutes
2. 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72 °C for 90 seconds
3. 72 °C for 5 minutes

at concentrations of 1x PCR Buffer, 0.9 mM (each) dNTPs, 1.5 mM $MgCl_2$, 1.2 μM each primer, 5 units Platinum™ *Taq* DNA Polymerase (all from Invitrogen™) with a substrate of 7 μl of cell lysate (a mean of 2 μg of DNA) in a total reaction volume of 25 μl.

These primers were designed, so that they give a PCR product of 607 bp when they amplify on the genomic glucocerebrosidase DNA and a PCR product of 238 bp when they amplify on the glucocerebrosidase cDNA, which is integrated in the vectors used.

PCR detection of the human actin gene was used as a positive control.

H. eGFP expression analysis with Flow Cytometry

Cell analysis for eGFP was performed in a BD FACSCalibur™ (BD Biosciences, Franklin Lakes, NJ) with the CellQuest (Becton Dickinson) and FlowJo (Tree Star Software, San Carlos, CA) software. A gate was set on a homogenous population, as determined by scatter characteristics, and at least 10000 gated events on a healthy population were counted.

I. Vector titration

All vector titrations were performed on the human fibroblast cell line HT1080. HT1080 are seeded on tissue culture polystyrene 24-well plates (Multiwell™, Becton Dickinson Labware, NJ, USA) at a number of 50000 cells per well, diluted in 1 ml Dulbecco's modified Eagle's medium with 4,5 gr/l glucose (DMEM+GlutaMAX-I, GIBCO®) supplemented with 10% fetal calf serum (GIBCO®). 3, 10, 30 and 100 μl of virus are added the next day, after the cells have already divided once, and the cells are analyzed with flow cytometry for eGFP expression 4 to 5 days after the addition of the virus.

III. Results

A. Vector design

The design of the new gammaretroviral vectors targets to the minimization of the proto-oncogene activation risk. All six vectors were designed as Self-Inactivating (SIN) vectors, which have been shown to have a reduced gene transfer mutagenic potential (Montini et al., 2009; Zychlinski et al., 2008), through a U3 deletion leading to LTRs with no promoter / enhancer sequences. This approach aims at the prevention of nearby proto-oncogene activation from the promoter / enhancer sequences of the LTR U3 region of the integrated viral vector (Trobridge, 2011); furthermore, it deals with the phenomenon of transcriptional interference from these promoter sequences, which suppresses the transgene expression from an internal promoter after integration.

The same phenomenon of transcriptional interference restricts the transcription of the full-length transcript during viral vector production in a packaging cell line. In order to enhance the full-length transcript synthesis in the packaging cell line, the promoter used in the 5' LTR consists of a combination of the SV40 enhancer (Dean et al., 1999) and the U3 region of the RSV LTR (Yankulov et al., 1994). This combination has been shown to reduce transcriptional interference from the internal promoter and lead SIN gammaretroviral vectors to increased titers (Schambach et al., 2006d).

Pseudotyping with the chimeric protein RD114/TR was used during vector production, which has been shown to lead both lentiviral (Sandrin et al., 2002) and gammaretroviral – including SIN (Schambach et al., 2006b; Schambach et al., 2006d) – vectors to high transduction efficiency during transduction of a CD34+ human primary hematopoietic progenitor cell population.

The vectors contain the coding sequence of the glucocerebrosidase gene and / or the eGFP reporter gene. Their transcription is driven by one of the following promoters, which have been shown to drive notable transgene expression in hematopoietic cells:

a) The promoter / enhancer of the U3 region of the SFFV LTR, which has been shown to drive very potent transgene expression in hematopoietic cells, both in established cell lines and in a CD34+ human primary hematopoietic progenitor cell population (Schambach et al., 2006b) ("SFFV U3").

b) A sequence from the human EF1α gene, which includes the gene promoter and expands downstream, including the first intron of the gene (Schambach et al., 2006b) ("EF1α promoter"). EF1α is one of the most abundant cellular proteins (Uetsuki et al., 1989). The EF1α promoter is able to drive remarkable transgene expression levels (Taboit-Dameron et al., 1999), especially in a CD34+ human primary hematopoietic progenitor cell population (Schambach et al., 2006b). It is the most potent cellular promoter known so far for transgene expression in hematopoietic cells (Schambach and Baum, 2007). The first intron of the gene was characterized from the very beginning of the use of the EF1α promoter in gene transfer as an important sequence for high transgene expression (Zaiss and Chang, 2000).

c) The 5' fragment of the above sequence, which includes the promoter of the EF1α gene without its first intron (Schambach et al., 2006b; Schambach et al., 2006c) ("EFS promoter" – EF1α Short promoter). This promoter has been also extensively validated in preclinical safety tests and is currently used in a clinical study for treatment of SCID-X1 (Children's Hospital Boston, 2010-).

The safety design was further enhanced by two viral elements, which have been shown to reduce readthrough transcription and enhance the transcriptional termination through polyadenylation (Higashimoto et al., 2007;

Schambach et al., 2007):

a) A 600-bp sequence of Woodchuck Hepatitis Virus (WHV) called WPRE (WHV Post-transcriptional Regulatory Element), inserted in the vectors' 3' UTR.

b) A 44-bp sequence of Simian Virus 40 (SV40) called SV40 USE (SV40 Upstream Sequence Element), inserted in two tandem repeats (2xSV40 USE) in the SIN deletion site of the 3' U3. This addition has been shown to enhance polyadenylation and therefore named upstream polyadenylation enhancer (USE).

These sequences have been shown to enhance not only the safety of gammaretroviral vectors, but also their efficiency, exhibiting a positive effect both on their titer and on the transgene expression, with their transcriptional termination enhancing role being important (Higashimoto et al., 2007; Schambach et al., 2006a; Schambach et al., 2007). Transgene expression enhancement might also correlate to increased translational efficiency due to polyadenylation improvement (Colgan and Manley, 1997). Two vector groups were used, one bearing the WPRE and another bearing the 2xSV40 USE. Each group includes three vectors, each with one of the abovementioned internal promoters **(Figure 1)**. In order to have a rough titer estimate, two reporter vectors with the EFS promoter and 2xSV40 USE were used; one with eGFP and another with GC-eGFP in the transcriptional unit.

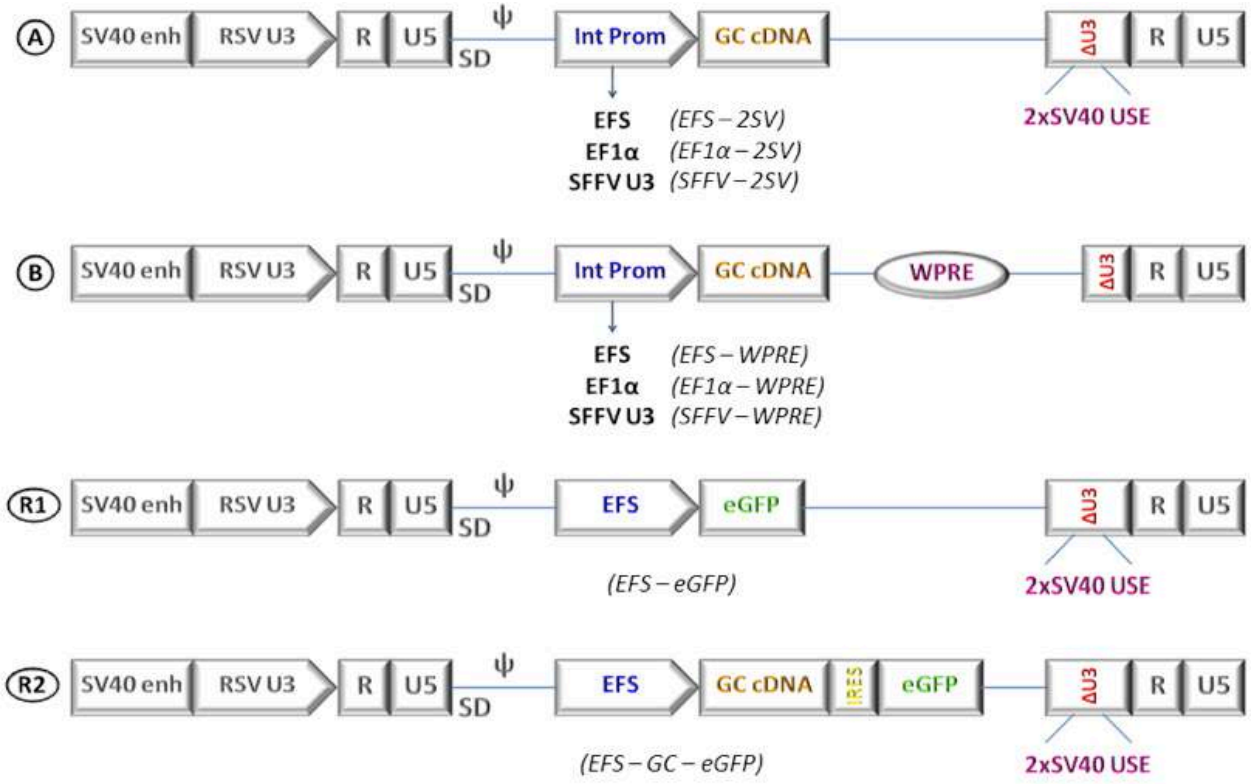

Figure 1: Six new gammaretroviral vectors and the two reporter vectors. Six new SIN gammaretroviral vectors were constructed; three with 2xSV40 USE (group A) and three with WPRE (group B). The two reporter vectors (R1, R2) use EFS as an internal promoter. The shapes indicate the direction of the transcription. The short names of the vectors are indicated in parentheses. SV40 enh: SV40 enhancer, Int Prom: internal promoter ψ: packaging signal, SD: splice donor site, ΔU3: U3 region with SIN deletion, IRES: Encephalomyocarditis Virus Internal Ribosomal Entry Site.

B. Successful CD34+ hematopoietic cell transduction

All six vectors proved efficient in transferring an active glucocerebrosidase transgene in a CD34+ cell population isolated from fresh human umbilical cord blood, leading to increased glucocerebrosidase enzyme activity in the cell population **(Figure 2).** The enzyme assay showed large differences in the enzyme activity resulting from the transgene activity; the enzyme activity of the transduced cells was 2.8 to 159 times the endogenous glucocerebrosidase enzyme activity of a similar untransduced cell population.

The titer of the eGFP-bearing vectors on HT1080 cells was $1.2x10^5$ tu/ml for EFS-eGFP and $0.8x10^5$ for EFS-GC-eGFP. Flow cytometry of CD34+ cells 48 hours after transduction with these vectors showed transduction efficiency of 78% (range ±8%) with EFS-eGFP and 44% (range ±18%) with EFS-GC-eGFP.

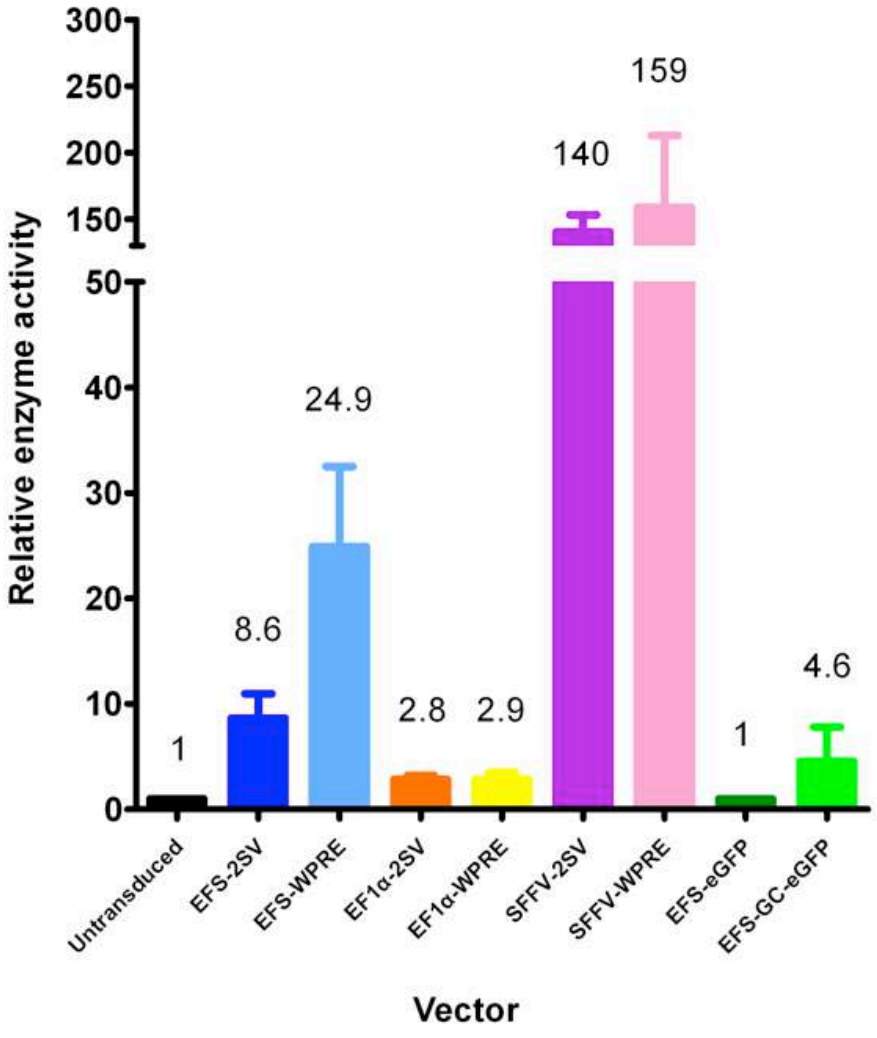

Figure 2: Six efficient glucocerebrosidase gammaretroviral vectors. All six gammaretroviral vectors were proven efficient in transferring an active glucocerebrosidase transgene in CD34+ primitive primary human hematopoietic cells. Mean values and ranges of three independent experiments of the relative enzyme activity compared to an untransduced cell population are shown.

C. Enzyme activity differences are related to transduction efficiency differences

In order to investigate whether differences in the enzyme activity derived from the transgene activity are related to differences in the transduction efficiency of the vectors, colonies of transduced cell populations were studied. After transduction and culture, single colonies were picked and separately investigated with PCR for the presence of the glucocerebrosidase transgene (colony PCR). The percentages of the colonies of each population bearing the transgene **(Figure 3)** indicate that differences in the transduction efficiency are involved in the differences in the enzyme activity **(Figure 2)**.

It is noteworthy, that all vectors result in populations with enzyme activity above physiological levels. This fact is suggestive of a curative potential, since only a small percentage of physiological enzyme activity has been shown to be sufficient for clinical correction of the Gaucher type 1 model (Enquist et al., 2009). Furthermore, lysosomal enzyme overexpression has been previously shown to be of therapeutic significance in the case of involvement of the central nervous system (Biffi et al., 2004); glucocerebrosidase supraphysiological expression could possibly prove important in the case of neuronopathic Gaucher disease.

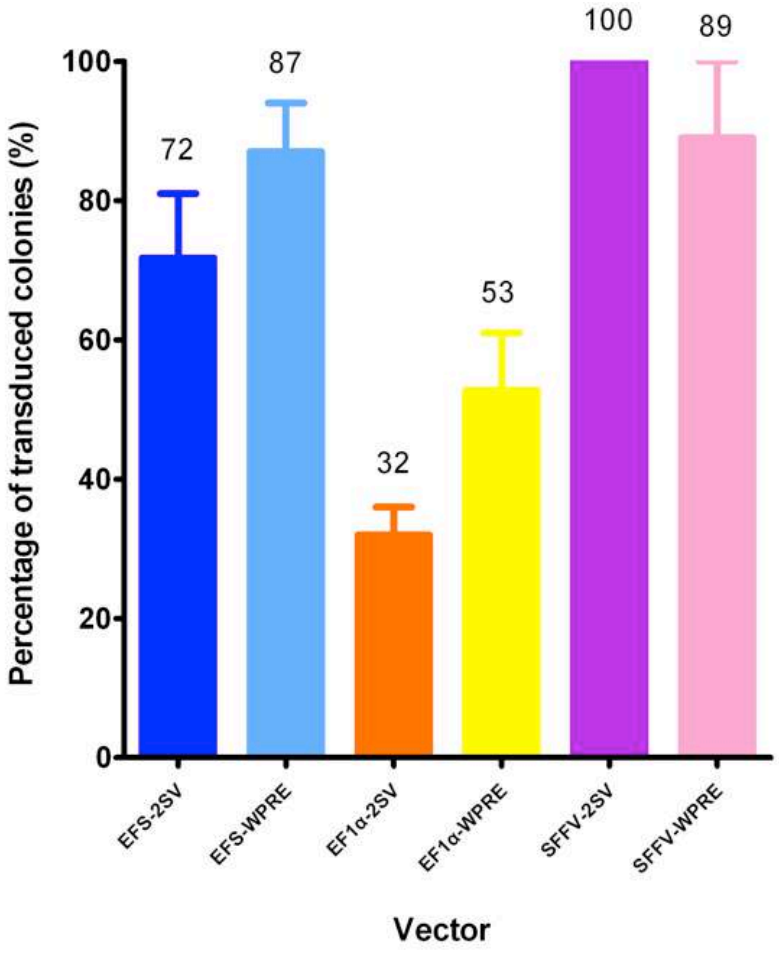

Figure 3 Enzyme activity differences are related to transduction efficiency differences. Mean values and ranges of three independent experiments are shown.

IV. Discussion

Gaucher disease is an inherited enzyme disorder, for which enzyme replacement therapy (ERT) has changed the clinical course for the majority of the patients. Patients with non-neuronopathic Gaucher disease, who represent the vast majority of cases, have gained access to a treatment, which improves clinical manifestations and the quality of life (Futerman et al., 2004), while new medications have either been added or are under investigation. Drug treatment, however, doesn't always have satisfactory results, while it cannot deal with cases of sever pulmonary or bone involvement (Elstein and Zimran, 2009). Furthermore, ERT requires intravenous infusions every second week. A very important parameter is the huge cost of ERT, reaching an annual amount of circa 100 000 euro per patient (Connock et al., 2006; Kesselman et al., 2006). This raises both practical / financial (Kesselman et al., 2006) and moral (Beutler, 2006) issues.

Autologous transplantation of genetically corrected HSCs would offer patients with type 1 Gaucher disease a permanent cure. The potential of treatment with a small percentage of cells with physiological levels of glucocerebrosidase enzyme activity and the potential therapeutic transplantation under partial myeloablation, as described in the Gaucher disease type I murine model (Enquist et al., 2009), outline the therapeutic frame of the perspective of gene therapy of the disease.

In this study, six new gammaretroviral vectors have been designed and used for gene transfer of a wild-type glucocerebrosidase gene in a CD34+ human primary hematopoietic progenitor cell population. Experience with gammaretroviral vectors has shown that they have to be designed with provision of efficiency as much as of safety (Hacein-Bey-Abina et al., 2008; Trobridge, 2011). The vectors used in this study were designed, so that they offer a theoretically safer approach to the gene transfer of glucocerebrosidase compared to previous preclinical (Enquist et al., 2006) and clinical approaches (Dunbar et al., 1998), incorporating both a SIN design and viral elements, that have been shown to reduce readthrough transcription and enhance the transcriptional termination through polyadenyliosis.

All six gammaretroviral vectors have proven efficient in the gene transfer of an active glucocerebrosidase gene in a CD34+ human primary hematopoietic progenitor cell population. Human EF1α promoter, with and without the first intron of the gene, and the U3 area of the LTR of SFFV led to potent transgene expression, being consistent with their previously observed high transgene expression efficiency in hematopoietic cells (Baum et al., 1995; Schambach et al., 2006b; Woods et al., 2001). Glucocerebrosidase enzyme activity in the transduced cell population increased to 2.8 to 159 times the endogenous enzyme activity. Glucocerebrosidase expression in supraphysiological levels suggests a possible clinical advantage during gene transfer; even low transduction efficiency could probably lead to clinical efficacy. Differences in the observed transgene expression could be attributed to possible differences in: a) the promoter ability to drive transcription (promoter strength), b) the transduction efficiency (percentage of transduced cells), c) the number of integrated transgene copies (copy number).

Previous comparative transduction of a CD34+ human primary hematopoietic progenitor cell population with SIN lentiviral vectors of similar design with different vectors, taking transduction differences under consideration, has shown that among these three vectors, SFFV U3 drives the highest transgene expression, followed by the EF1α promoter and leaving EFS promoter in the last

place (Schambach et al., 2006b) (while lenti- and gammaretroviral vectors with the same internal promoter led to similar transgene expression levels). In that same study, the vector titers were variable with different promoters: EFS promoter led to 6 times the EF1α promoter titer (in lentiviral vectors) and SFFV U3 to 5 times the EFS titer (in gammaretroviral vectors). Thus, it is obvious, that the enzyme activity differences in the transduced cell populations are expectedly related not only to differences in the promoters' transcriptional strength, but also to differences in the vector titers. Furthermore, with intron-containing vectors, such as a vector with EF1α, splicing is expected to have a negative effect on the titer (Schambach et al., 2006b).

The titer differences are reflected at the transduction efficiency differences, as they were observed through colony PCR **(Figure 3)**. It has been previously shown, that with low percentages, up to 30%, of gammaretrovirally transduced cells the majority of the transduced cells integrates only one transgene copy, while at higher percentages the increase in the transduction efficiency results in a logarithmic increase of the transgene copy number, leading, for example, in a mean of 3 copies per cell, when 60% of the cells of a CD34+ human primary hematopoietic progenitor cell population are transduced (Kustikova et al., 2003). In the present study, the majority of the vectors – apart from EF1α-2SV – are expected to have integrated in high copy numbers, depending on the transduction efficiency. Of note is that the transduction efficiency of SFFV-2SV, although it is certainly high, it has been obviously overestimated due to technical limitations.

EFS vectors have resulted in higher transduction efficiency than EF1α vectors and, therefore higher titers (Kustikova et al., 2003). This titer difference and the resulting higher copy number surpass the higher transcriptional strength of the EF1α promoter (Schambach et al., 2006b), resulting in higher transgene expression in the cell population transduced with EFS vectors.

It is noteworthy that the effects of the incorporation of sequences improving the posttranscriptional mRNA processing, such as WPRE and 2xSV40 USE, depend on the vector context and are hence not absolutely predictable (Schambach et al., 2000). In the EF1α vector context the two sequences lead to similar transgene expression levels (**Figure 2**). Given the higher transduction efficiency of EF1α-WPRE (**Figure 3**), it is readily concluded that, compared to EF1α-2SV, EF1α-WPRE is produced at higher titers, but leads to lower transgene expression per integrated copy.

In conclusion, this study presents six new gammaretroviral vectors, which are successfully used for the gene transfer of an active wild-type glucocerebrosidase transgene in a CD34+ human primary hematopoietic progenitor cell population. The vectors show differences in transgene expression related to differences in the promoters' transcriptional strength, the transduction efficiency and the number of integrated copies per cell. Enzyme activity above physiological levels highlights the potential of the vectors used in this study to lead to clinically effective levels of glucocerebrosidase activity; only a small percentage of physiological enzyme activity has been shown enough to cure the Gaucher type 1 model (Enquist et al., 2009). Further studies of these vectors for gene transfer on preclinical and clinical level need to make provision for limitation of the transduction efficiency at low levels, below 30% (Kustikova et al., 2003), with concurrent laboratory confirmation of integration at low copy numbers, around 1 copy per cell, so that the expected insertional mutagenesis risk is minimized (Baum et al., 2003).

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