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Elements of lentiviral vector design toward gene therapy for treating mucopolysaccharidosis I

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ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a lysosomal disease caused by α-L-iduronidase (IDUA) deficiency and accumulation of glycosaminoglycans (GAG). Lentiviral vector encoding correct IDUA cDNA could be used for treating MPS I. To optimize the lentiviral vector design, 9 constructs were designed by combinations of various promoters, enhancers, and codon optimization. After *in vitro* transfection into 293FT cells, 5 constructs achieved the highest IDUA activities (5613 to 7358 nmol/h/mg protein). These 5 candidate vectors were then tested by injection (1×10^7 TU/g) into neonatal MPS I mice. After 30 days, one vector, CCEoIDW, achieved the highest IDUA levels: 2.6% of wildtype levels in the brain, 9.9% in the heart, 200% in the liver and 257% in the spleen. CCEoIDW achieved the most significant GAG reduction: down 49% in the brain, 98% in the heart, 100% in the liver and 95% in the spleen. Further, CCEoIDW had the lowest transgene frequency, especially in the gonads (0.03 ± 0.01 copies/100 cells), reducing the risk of insertional mutagenesis and germ-line transmission. Therefore, CCEoIDW is selected as the optimal lentiviral vector for treating MPS I disease and will be applied in large animal preclinical studies. Further, taken both *in vitro* and *in vivo* comparisons together, codon optimization, use of EF-1α promoter and woodchuck hepatitis virus posttranscriptional response element (WPRE) could enhance transgene expression. These results provided a better understanding of factors contributing efficient transgene expression in lentiviral gene therapies.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease that leads to neurodegeneration, mental retardation and death in early age. MPS I results from deficiency of α-L-iduronidase (IDUA, E.C.3.2.1.76), which degrades the glycosaminoglycans (GAG). The neurological pathology of MPS I is extremely difficult to treat, because the blood–brain-barrier (BBB) blocks the entry of enzyme into the brain [1–3]. Currently, MPS I is treated by hematopoetic stem cell transplantation (HSCT) and often in conjunction with enzyme replacement therapy (ERT). However, HSCT has a high rate of mortality (10–15%) and severe morbidity. While ERT alone is thought to have negligible therapeutic impacts at the brains, a high-dose infusion of IDUA can lead to significant increase of enzyme activity in the brain cortex [4]. Given that a small amount of enzyme is sufficient to degrade GAG storage [5–7], it should be possible to achieve improved neurological outcomes. However, high dose treatment is not feasible for clinical application due to the immune response against human recombinant enzyme [8].

Previously, gene therapy with different vectors in animal models has been used to treat MPS I disease, including retrovirus [9,10], lentivirus [8,11,12], adeno-associated virus [3,13–15], Sleeping Beauty transposon [16] and minicircles [17]. It has been shown that injection of lentiviral vector into MPS I mice can achieve metabolic correction and neurological improvements [8,11,12]. However, it is difficult to apply the dose (1.65 to 4.5×10^8 TU/g) used into clinical trials. Therefore, optimization of lentiviral vector design is essential for advancing lentiviral gene therapy protocols.

Promoters and enhancers are essential vector components for optimization of transgene expression. It has been shown that the human phosphoglycerate kinase 1 (PGK) promoter leads to moderate transgene expression of lentiviral vector [18–20]. Lentiviral vector with the human elongation factor 1α (EF-1α) promoter has been used for treating Fabry disease in a murine model [21]. Additionally, a hybrid promoter consisting of the enhancer of the murine cytomegalovirus (CMV) *immediate-early* gene and human EF-1α promoter was shown to achieve high transgene expression of lentivital vector [22]. However, the effects of different promoters on transgene expression are still not elucidated. Moreover, the use of woodchuck hepatitis virus (WHV) posttranscriptional response element (WPRE) has been found to enhance transgene expression and titers of therapeutic vectors [23–25]. The enhancing ability of WPRE depends on target cells, the type of

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viral vector context and its sequence [26–29]. However, WHV X protein is implicated in the development of liver tumors [30], which raises the safety concern about use of WPRE in vectors for gene therapy. Herein, we designed constructs with full-length WPRE, truncated WPRE (tWPRE) and depleted WPRE for a side-by-side comparison. In this study, an initial *in vitro* screening of 9 plasmids identified 5 candidates with the highest IDUA transgene expression. Then, the efficacy of these 5 candidate lentiviral vectors in neonatal MPS I mice was comparatively evaluated. This allowed us to determine which lentiviral constructs yielded the highest IDUA levels and the most efficient GAG reduction *in vivo*.

2. Materials & methods

2.1. Plasmid construction

The human IDUA cDNA generated by reverse transcription PCR from total mRNA of an unaffected individual was inserted into the multi-cloning sites of pHIV-CS (CMV promoter upstream of 5′ LTR). The IDUA expression was under the control of PGK promoter and named as pCPGKID. Then, codon optimization of IDUA cDNA sequence was performed, resulting in what we named the oIDUA sequence. Similar techniques were used to generate similar variants with different promoters (hybrid promoter named as CE, PGK and EF-1α) and variants of WPRE, resulting in 8 more plasmids as followed: pCEFIDW, pCEFoIDW, pCPGKoIDW, pCEoIDW, pCEFoID-tWPRE, pCEFoID, pCEID and pCCEIDW. All plasmids were confirmed by sequencing.

2.2. In vitro plasmid transfection

For each transfection, 25 μg of candidate plasmid and 25 μg of HIV CMVeGFP plasmid were mixed with 133 μL 2.5 M $CaCl_2$ (25 °C) and 1.33 mL RNase/DNase free sterile H_2O. After adding 1.33 mL of 2× HEPES buffered saline (pH 7.1), 7 mL serum free medium was added to the mixture. Then, the HEK 293FT cells were incubated with this transfection solution for 4 to 6 h (37 °C, 5% CO_2). After removing the transfection solution, cells were incubated with 9 mL of 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO) for another 48 h. Finally, cells and medium were collected by centrifuge and processed for biochemical assays.

2.3. Lentiviral vector production

The candidate plasmid was co-transfected with three additional helper plasmids, pLp1 (gag/pol), pLp2 (Rev) and VSVG envelope plasmid into HEK 293FT cells [31]. Vector-containing medium was collected 24, 40 and 64 h after transfection and concentrated. After ultracentrifugation at 7000 rpm overnight (4 °C), the vector pellet was resuspended in 40 mg/mL of lactose/PBS buffer and was stored at −80 °C. The titer of vector preparations was determined by QPCR.

2.4. MPS I mice and injection

MPS I knockout mice (*idua*−/−), a kind gift from Dr. Elizabeth Neufeld, UCLA, were generated by insertion of neomycin resistance gene into exon 6 of the 14-exon IDUA gene on the C57BL/6 background [32]. The colony was housed in a pathogen-free facility on a 12-hour light/dark cycle. Newborn mice (1–2 days old) were injected with concentrated lentiviral vector through the superficial temporal vein. All mouse care and handling procedures were in compliance with the rules of the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

2.5. IDUA enzyme assay

IDUA enzyme assay was conducted as previously described [33]. IDUA activity was determined by a fluorometric assay using 4-methylumbelliferyl α-L-iduronide (4-MU iduronide) (Glycosynth, Cheshire, UK) as the substrate, which was diluted with sodium formate buffer (0.4 M, pH 3.5). Then, 25 μL aliquots (360 μM) of substrate were mixed with 25 μL aliquots of tissue homogenates (diluted with 0.2% bovine serum albumin in phosphate buffered saline). The mixture was incubated at 37 °C for 30 min, and 200 μL glycine carbonate buffer (pH 10.4) was added to stop the reaction. IDUA catalyzed the cleavage of the non-fluorescent substrate (4-MU iduronide) and released a fluorescent product (4-MU). 4-Methylumbelliferone (4-MU) (Sigma-Aldrich, St. Louis, MO) was used to generate the standard curve. The resulting fluorescence was measured with excitation at 355 nm and emission at 460 nm. IDUA enzyme activity was expressed in units (nmol converted to product per hour) per mg protein as determined with a Pierce protein assay kit (Fisher, Waltham, MA) or per mL plasma. Then, IDUA enzyme activity was adjusted by Michaelis–Menten equation as described previously [33]. All reactions were performed in triplicate.

2.6. Tissue GAG assay

Tissue GAG assays were conducted as described previously [4]. The supernatants of tissue homogenates were treated by proteinase K (ProK) (NEB, Ipswich, MA) with the ratio of 3(Pro K):1(sample), incubated at 55 °C overnight, and boiled for 10 min to inactivate the enzyme. Samples were incubated with 2.5 μg RNase (Sigma-Aldrich, St. Louis, MO) and 250 U DNase (Sigma-Aldrich, St. Louis, MO) at room temperature overnight. After boiling for 10 min to inactivate the enzymes, GAG levels were determined by the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor, Carrickfergus, UK). Tissue GAG levels were expressed as μg GAG/mg protein.

2.7. Quantitative PCR

DNA was extracted from tissues with QIAGEN DNA mini kit (QIAGEN # 51306). Primers (forward primer: 5′-CGACTGGTGAGTACGCCAAA-3′; reverse primer: 5′- CGCACCCATCTCTCTCCTTCT-3′) and the probe (5′-FAM-ATTTTGACTAGCGGAGGC-TAMRA-3′) targeted the lentiviral psi (Ψ) packaging signal region. Amplicon size was 61 bp. Each reaction contained 2× TaqMan universal PCR master mix (Life Technologies, Carlsbad, CA), primers (0.008 nmol each), TaqMan probe (0.008 nmol), and 100 ng of sample DNA in a final volume of 10 μL. Real-time PCR was also performed using the mouse apolipoprotein B (ApoB) gene as an internal control, using the following primers (forward primer, 5′-CGTGGGCTCCAGCATTCTA-3′; reverse primer, 5′-TCACCAGT CATTTCTGCCTTTG-3′) and probe (5′-FAM-CCTTGAGCAGTGCCCGACCA TTC-TAMRA-3′). Amplification conditions were 2 min at 50 °C and 10 min at 95 °C for the first cycle, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard curve was established from a series of genomic DNA mixtures derived from plasmid with Ψ packaging signal region sequence (1copy per genome). Unknown samples were run in triplicate, while standards and internal controls were performed in duplicate.

2.8. Statistical analysis

Data were represented as mean ± standard errors. For evaluation of differences between samples, Student's T test for comparisons between paired samples and one-way analysis of variance (ANOVA) for comparisons between three or more samples were performed. Statistical significant level was set at $p < 0.05$. Data analysis was conducted with SAS 9.3 (Cary, NC).

3. Results

3.1. Design of 9 lentiviral constructs

A plasmid CPGKID was constructed using the human PGK promoter to drive expression of human IDUA cDNA in the genome of a self-