α-Galactosidase A-Tat Fusion Enhances Storage Reduction in Hearts and Kidneys of Fabry Mice

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The protein transduction domain from human immunodeficiency virus (HIV) Tat allows proteins to penetrate the cell membrane. Enhanced cellular uptake of therapeutic proteins could benefit a number of disorders. This is especially true for lysosomal storage disorders (LSDs) where enzyme replacement therapy (ERT) and gene therapy have been developed. We developed a novel recombinant lentiviral vector (LV) that engineers expression of α-galactosidase A (α-gal A)-Tat fusion protein for correction of Fabry disease, the second-most prevalent LSD with manifestations in the brain, kidney and heart. *In vitro* experiments confirmed mannose-6-phosphate independent uptake of the fusion factor. Next, concentrated therapeutic LV was injected into neonatal Fabry mice. Analysis of tissues at 26 wks demonstrated similar α-gal A enzyme activities but enhanced globotriaosylceramide (Gb3) reduction in hearts and kidneys compared with the α-gal A LV control. This strategy might advance not only gene therapy for Fabry disease and other LSDs, but also ERT, especially for cardiac Fabry disease.

INTRODUCTION

Fabry disease is a lysosomal storage disorder (LSD) caused by a deficiency of α-galactosidase A (α-gal A; EC 3.2.1.22) activity (1), one of the lysosomal hydrolases. It is the second-most prevalent LSD and a model for the development of therapy for single gene defects. A defect in α-gal A activity leads to the systemic accumulation of glycosphingolipids, mainly globotriaosylceramide (Gb3), resulting in cardiac, renal and cerebrovascular disease. In early childhood, angiokeratomas, acroparesthesia, hypohidrosis and corneal opacities are common symptoms in patients with typical Fabry disease. In cardiac Fabry disease, which is an atypical variant of Fabry disease reported in 3% to 4% of patients with left ventricular

hypertrophy (2,3), manifestations are primarily confined to the heart. Enzyme replacement therapy (ERT) for Fabry disease is available and some improvements in clinical and pathological manifestations have been shown. That said, ERT requires permanent and frequent infusions and more seems to slow progression of the disorder than anything else. As such, ERT seems to be less effective in more advanced Fabry patients (4–6). Gene therapy could obviate one of these issues at least as it has the potential to cure the disorder by a single intervention. We have been focused on developing gene therapy for Fabry disease (7–11).

Metabolic cooperativity, wherein α-gal A can be taken up through mannose-6-phosphate (M6P) receptors (7), enables

ERT and makes gene therapy for Fabry disease a real possibility. If we could further improve the efficacy of α-gal A uptake into target cells or differential tissues relevant to the disorder, enhanced therapeutic outcomes may occur. Xia *et al*. showed that the protein transduction domain (PTD) from HIV Tat improved uptake and biodistribution of the lysosomal enzyme β-glucuronidase (12). They also demonstrated that Tat allowed for M6P-independent uptake of β-glucuronidase *in vitro* (12). Further studies by Orii *et al*. found less rapid clearance of this Tat fusion form from circulation in rats (13). Additionally, Lee *et al*. demonstrated that the Tat PTD improved intracellular delivery of glucocerebrosidase *in vitro*; another lysosomal hydrolase that is implicated in Gaucher disease (13,14). Although the uptake mechanism of the Tat PTD is not fully understood, it is thought to occur by the reverse micelle model (15) wherein the positive charge of Tat interacts with the negative charge of the phospholipid membrane.

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We have already shown in Fabry mice that direct neonatal injection of recombinant LVs engineering expression of human α-gal A demonstrated significant long-term therapeutic potential (10). Here we hypothesized that an α-gal A-Tat fusion protein would further improve uptake of α-gal A secreted from vector transduced cells after intravenous injection of recombinant lentiviral vector (LV) in α-gal A-deficient mice.

MATERIALS AND METHODS

Lentiviral Vector (LV) Production and Transductions

pHR'cPPT-EF1α-α-gal A-WPRE-SIN (LV/α-gal A), and control recombinant LV encoding enGFP (LV/enGFP) have been described previously (11). Primers from 1 to 6, indicated in Supplementary Table 1, were used to generate the α-gal A-Tat fusion protein by mutagenesis using the QuickChange Kit (Stratagene, La Jolla, CA, USA). Primer pair 1,2 was used to introduce the TATGGCAGGAA sequence at the carboxy-terminal end (between residue 429 and the stop codon) of the α-gal A cDNA. Primer pairs 3,4 and 5,6 were used to add GAAGCGGAGAC and AGCGACGAAGA sequences, respectively. The resultant plasmid was labeled LV/α-gal A-Tat (Figure 1).

Vesicular stomatitis virus-glycoprotein pseudotyped (VSV-g) LVs were generated by transient transfection as before (10). Virus supernatants were harvested after 48 h and concentrated at 50,000*g* for 2 h. The p24 antigen levels of concentrated viral supernatants were determined by an HIV-1 p24 ELISA (PerkinElmer, Waltham, MA, USA).

HeLa and 3T3 cells (ATCC, Manassas, VA, USA) were transduced with p24 level-matched LV/α-gal A or LV/α-gal A-Tat in the presence of 8 µg/mL of protamine sulfate.

RT-PCR for α-Gal A and α-Gal A-Tat mRNA Sequences

Total RNA was isolated from transduced and non-transduced (NT) 3T3 cells using the TRIzol reagent (Invitro-

α-gal A-Tat

Figure 1. Schematic of alignment of Tat PTD domain at the carboxy-terminus of α-galactosidase A.

gen, Carlsbad, CA, USA). First-strand cDNA was synthesized by the Super-Script First-Strand Synthesis System (Invitrogen). Primers used to amplify the specific α-gal A or α-gal A-Tat sequence are indicated in Supplementary Table 2. Cycle steps: denaturation at 94°C, 30 s; annealing at 55°C, 30 s; extension at 72°C, 90 s.

Functional Expression of α-Gal A-Tat and Uptake Assays

Transduced and NT HeLa cells were seeded (4×10^6 cells/10-cm dish) in 10 mL of DMEM (Sigma Aldrich, St Louis, MO, USA) supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Supernatant was harvested 20 h later and filtered using a 0.45 µm filter (Millipore, Billerica, MA, USA). α-Gal A activity was measured using a fluorometric assay as before (10). Supernatant corresponding to α-gal A activity of

1,000 nmol/hr/mL was overlaid onto Fabry mouse fibroblasts, which were derived from the lower-limb muscle, and incubated in the presence or absence of 1.5 mM M6P. After 3 h, α-gal A activity in cell lysates of the Fabry fibroblasts was measured.

In vivo Study

α-Gal A-deficient mice (Fabry mice) (8) were bred at the University Health Network (UHN) (Toronto, Ontario, Canada). Animal experiments were performed under protocols approved by the UHN Animal Care Committee. Numbers of animals used in all parts of both Experiments are demarcated in Figures 2 and 3.

In Experiment 1, concentrated LVs (2 µg/mL p24 levels) or PBS was administrated to neonatal Fabry mice through the superficial temporal vein in a volume of 100 µl as previously described (10). In Experiment 2, one-quarter dose
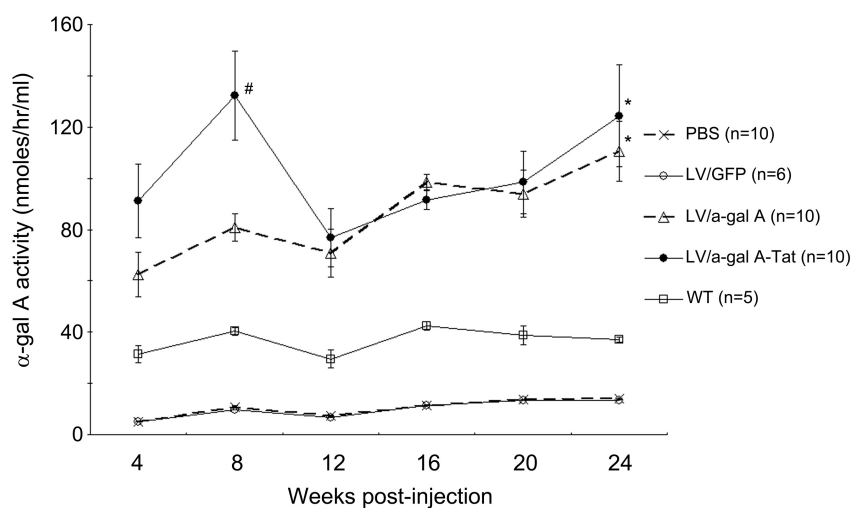

Figure 2. Plasma α-gal A activity in high-dose LV-injected Fabry mice from Experiment 1. *$P < 0.001$ compared with PBS-injected group. #$P < 0.05$ compared with LV/α-gal A-Tat-injected group.
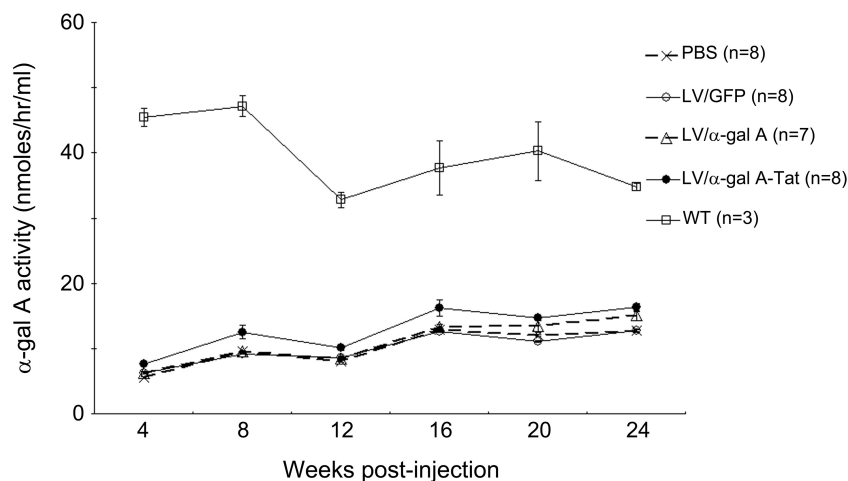
Figure 3. Plasma α-gal A activity in low-dose LV-injected Fabry mice from Experiment 2.

(0.5 μg/mL p24 levels) of concentrated LVs was used. In both experiments, concentrated LV/enGFP-injected animals and C57Bl/6 mice (wild-type; WT) were used as controls. Whole blood was collected every 4 wks. Plasma was isolated and α-gal A activity was measured as above. Twenty-six wks after LV administration, mice were euthanized to evaluate α-gal A activity in extracts of organs from treated and control mice. Gb3 levels were analyzed by high performance liquid chromatography (HPLC) (11). Insufficient bone marrow cells could be harvested to evaluate both α-gal A specific activity and Gb3 levels; therefore, we analyzed only α-gal A specific activity for those samples.

Statistics

In vivo data are presented as mean ± SEM. Statistical analyses were performed using a two-sample Student *t* test. *P* values < 0.05 were considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

RT-PCR for α-Gal A and α-Gal A-Tat

Figure 4A demonstrates that similar levels of the α-gal A specific band were obtained in LV/α-gal A and LV/α-gal A-Tat transduced 3T3 cells, and that the Tat specific band was obtained in just the LV/α-gal A-Tat transduced cells.

α-Gal A Activity in the Supernatant of Transduced HeLa Cells and Enzyme Uptake Assays

We analyzed α-gal A activity of the supernatant from pooled transduced (LV/α-gal A or LV/α-gal A-Tat) and NT HeLa cells. The supernatant of both transduced cell populations showed higher α-gal A activity than that of NT cells (Figure 4B). There was no significant difference in α-gal A activity between α-gal A and α-gal A-Tat supernatant. This implies that the Tat PTD attached to the α-gal A enzyme did not dramatically impair α-gal A enzyme activity.

Next, we examined the uptake of α-gal A and α-gal A-Tat enzyme activity into fibroblasts from Fabry mice. In the absence of added M6P, higher α-gal A activity was detected in α-gal A-Tat treated group compared with α-gal A treated group (*P* < 0.05) (Figure 4C). While α-gal A activity was completely inhibited by added M6P in the LV/α-gal A treated group, α-gal A activity was only partially diminished by added M6P in LV/α-gal A-Tat treated group (see Figure 4C). Taken together, the α-gal A-Tat fusion protein allowed for M6P receptor-independent uptake *in vitro*.

Increased α-Gal A Activity in the Plasma of Treated Fabry Mice

To determine the systemic efficacy of the α-gal A-Tat fusion protein, we injected LV/α-gal A and LV/α-gal A-Tat into neonatal Fabry mice. In a high-dose LV-injection experiment (Experiment 1), plasma α-gal A activities increased significantly (*P* < 0.001) in the both LV/α-gal A and LV/α-gal A-Tat group compared with PBS-injected group (see Figure 2). In fact, plasma α-gal A activities in the therapeutically treated group were more than twice those of the WT mice. No statistically significant differences of enzyme activity (except at 8 wks, *P* < 0.05) were observed between LV/α-gal A and LV/α-gal A-Tat-injected group.

In a low-dose LV-injection experiment (Experiment 2), we used one-quarter of the LV used in Experiment 1. We could not detect any difference of α-gal A activity in the plasma of the therapeutically treated group compared with PBS-injected group (see Figure 3). Our previous study also demonstrated this LV dose-dependent α-gal A expression (10).

α-Gal A Activity and Gb3 Levels in the Organs of Treated Fabry Mice

Twenty-six wks postinjection, we euthanized the animals and determined α-gal A activity in extracts of various organs from the treated and control mice. Dramatic differences were observed in some organs examined in comparison to control values for both LV/α-gal A and LV/α-gal A-Tat (Table 1). In Experiment 1, there were no significant differences in α-gal A activity in most organs between LV/α-gal A and LV/α-gal A-Tat treated group even though we found the tendency of α-gal A activity to be higher in LV/α-gal A-Tat group in liver, heart, kidney, bone marrow and lung (Table 1). Also, in Experiment 2 (low-dose injections), we did not observe any differences in α-gal A activity in any organs assessed (data not shown).

In Experiment 1 (high-dose), Gb3 levels in most organs in the therapeutically treated groups were reduced compared
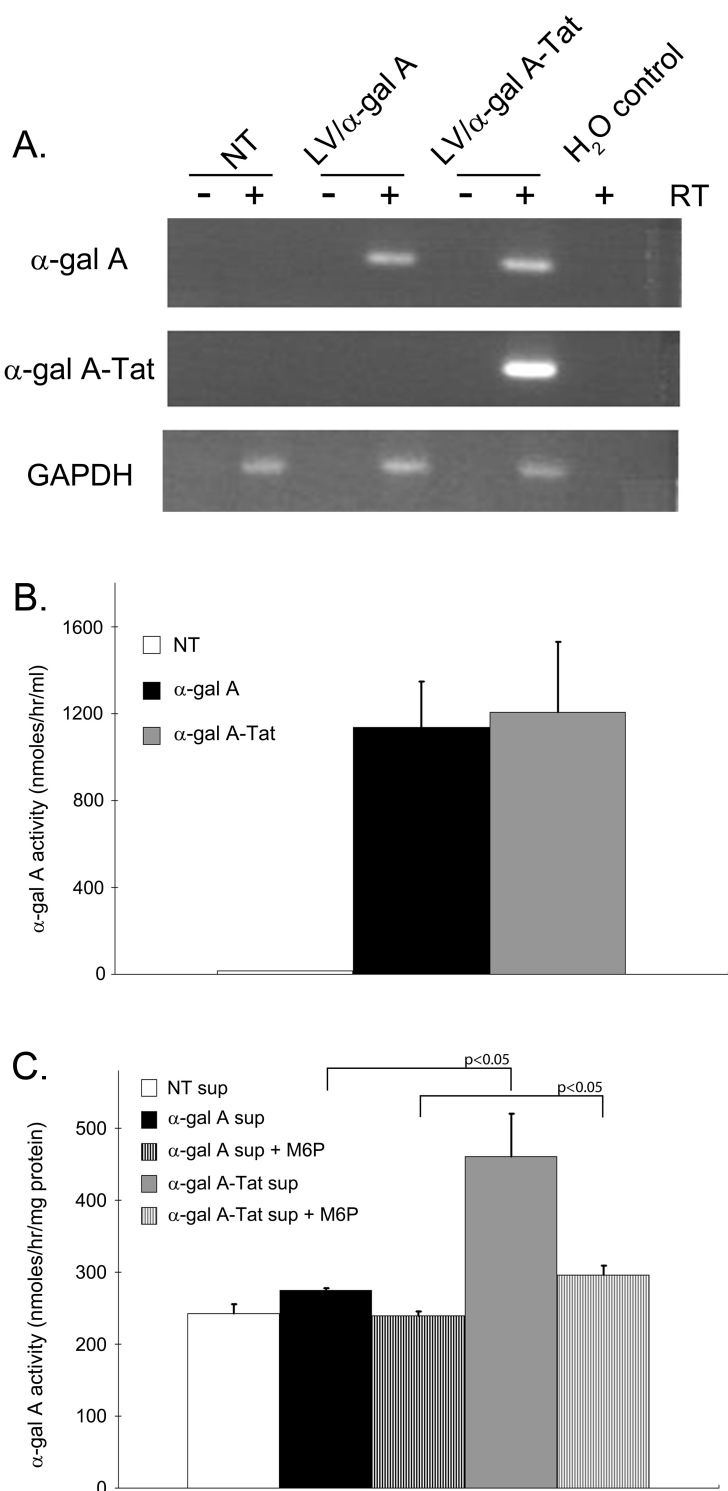
Figure 4. α-Gal A and Tat mRNA expression and α-gal A enzyme activity. (A) Similar levels of α-gal A and Tat mRNA expression in transduced 3T3 cells were confirmed by RT-PCR. (B) α-Gal A activity secreted from transduced HeLa cells. (C) Uptake of secreted α-gal A and α-gal A/Tat in Fabry mouse fibroblasts with or without M6P. NT, non-transduced; RT, reverse transcriptase; sup, supernatant; M6P, mannose-6-phosphate. α-Gal A activity assays were measured in triplicates. Values are mean ± SD.

with control groups. Significant differences in Gb3 levels in the heart and kidney between the LV/α-gal A and LV/α-gal A-Tat treated groups were observed (Table 2). Moreover, even though we used one-quarter of the LV stock for injections in Experiment 2, there were still statistically significant differences in Gb3 levels in heart and liver between both treated groups (Table 3).

DISCUSSION

We have previously shown that neonatal injection of recombinant LVs into Fabry mice encoding the α-gal A cDNA led to sustained systemic expression of the therapeutic transgene (10) and reductions in Gb3 storage. This functional outcome occurred from low levels of enzyme produced continuously in contrast to a single bolus of enzyme given in ERT. Here we sought to determine functionally whether differences in α-gal A uptake could be mediated by the addition of an α-gal A-Tat fusion protein utilizing the same delivery method. Indeed, we have first demonstrated that such a fusion protein is produced and enzymatically functional and can be taken up into cells independent of the mannose-6-phosphate receptor *in vitro*. We also demonstrate that the α-gal A-Tat fusion protein decreased Gb3 levels in some organs more effectively than α-gal A itself in LV-injected Fabry mice. Though we saw increased plasma and organ α-gal A activity in LV/α-gal A and LV/α-gal A-Tat group compared with control groups, for the most part there were no statistically significant differences in plasma and organ α-gal A activity between therapeutically treated groups. However, a greater reduction of Gb3 levels in the heart and kidney of LV/α-gal A-Tat treated mice was detected compared with LV/α-gal A treated mice in the high-dose LV-injected experiment. Differences here may be more pronounced because the Gb3 assay examines a cumulative effect on substrate accumulation/reduction over time, whereas the enzyme assay examines a veritable snapshot of hydrolase

Table 1. Organ α-gal A activity 26 weeks postinjection (Experiment 1)

	α-Gal A specific activity (nmol/hr/mg protein)						
	Liver	Spleen	Heart	Kidney	BM[a]	Brain	Lung
PBS (n = 10)	36 ± 3	135 ± 5	12 ± 1	29 ± 3	53 ± 4	33 ± 1	ND[b]
LV/enGFP (n = 6)	26 ± 4	111 ± 12	13 ± 2	26 ± 4	58 ± 4 (n = 3)	29 ± 3	117 ± 4 (n = 3)
LV/α-gal A (n = 10)	255 ± 26	328 ± 36	31 ± 2	29 ± 2	66 ± 4 (n = 9)	31 ± 1	113 ± 16 (n = 7)
LV/α-gal A-Tat (n = 10)	310 ± 24	238 ± 34[c]	35 ± 3	33 ± 3	75 ± 5	30 ± 1	121 ± 18 (n = 7)
WT[d] (n = 10)	421 ± 22	803 ± 53	89 ± 5	249 ± 10	1481 ± 21	713 ± 26	385 ± 65 (n = 3)

[a]BM, bone marrow.
[b]ND, not done.
[c]$P < 0.05$ vs LV/α-gal A group.
[d]WT, wild type.

activity at the point of lysate production. Moreover, greater reductions of Gb3 levels in the heart at 26 weeks were also observed with LV/α-gal A-Tat using the low-dose LV injection. Heart, brain and kidney are the major organs affected in Fabry disease; therefore this result is valuable for Fabry patients.

Comparisons between our data from Experiment 1 (high dose) and Experiment 2 (low dose) may thus indicate a differential sensitivity of those organs to therapy in this disorder, though the current study was really not designed to address this in detail. Table 2 shows that some differences could still be seen for the heart and kidney whereas no difference with WT enzyme-mediated effects were observed for the liver. Thus, the high dose LV injection in this case was likely enough to clear Gb3 in the liver without the PTD. This also would seem to indicate that the heart and kidney are still undergoing Gb3 reductions mediated by the continual secretion of enzyme at this systemic dosing level, whereas the liver may have reached a point in Experiment 1 where further Gb3 reduction was incremental and not distinguishable by this assay. In this scenario, the data in Table 3 also aligns well. Here heart and liver Gb3 levels are reduced, perhaps indicating their relative sensitivity to therapy, while possible insufficient enzyme was taken up in total into the kidney for catalytic differences to be impacted.

Table 2. Organ Gb3[a] levels 26 weeks postinjection (Experiment 1)

	Gb3 levels (nmol/mg protein)				
	Liver	Spleen	Heart	Kidney	Lung
PBS	13.4 ± 0.8 (n = 7)	18.4 ± 1.8 (n = 4)	8.8 ± 1.0 (n = 6)	29.8 ± 1.5 (n = 7)	59.1 ± 6.5 (n = 7)
LV/enGFP	15.4 ± 2.0 (n = 5)	11.9 ± 1.3 (n = 4)	5.1 ± 0.5 (n = 3)	28.6 ± 7.1 (n = 5)	8.3 ± 1.4[b] (n = 3)
LV/α-gal A	1.0 ± 0.1 (n = 8)	2.8 ± 1.2 (n = 6)	1.3 ± 0.1 (n = 7)	17.2 ± 1.3 (n = 9)	12.4 ± 2.2 (n = 7)
LV/α-gal A-Tat	0.9 ± 0.2 (n = 8)	3.0 ± 0.6 (n = 8)	0.8 ± 0.1[c] (n = 6)	8.2 ± 1.7[d] (n = 9)	10.2 ± 0.7 (n = 7)
WT[e]	0.6 ± 0.2 (n = 5)	1.3 ± 0.1 (n = 5)	0.2 ± 0.1 (n = 4)	4.9 ± 1.6 (n = 5)	2.3 ± 0.6 (n = 3)

[a]Gb3, globotriaosylceramide.
[b]Little sample to analyze because of technical issues.
[c]$P < 0.05$.
[d]$P < 0.01$ vs LV/α-gal A group.
[e]WT, wild type.

Table 3. Organ Gb3[a] levels 26 weeks postinjection (Experiment 2)

	Gb3 levels (nmol/mg protein)				
	Liver	Spleen	Heart	Kidney	Lung
LV/enGFP	20.9 ± 2.9 (n = 7)	63.2 ± 1.8 (n = 6)	17.0 ± 1.4 (n = 6)	37.0 ± 3.0 (n = 5)	48.0 ± 0.7 (n = 6)
LV/α-gal A	7.5 ± 1.1 (n = 6)	34.0 ± 2.8 (n = 6)	15.8 ± 2.0 (n = 6)	30.2 ± 1.3 (n = 5)	44.1 ± 3.4 (n = 4)
LV/α-gal A-Tat	1.9 ± 0.3[b] (n = 8)	29.8 ± 2.0 (n = 7)	10.0 ± 0.8[c] (n = 6)	30.3 ± 1.9 (n = 5)	47.5 ± 2.9 (n = 7)

[a]Gb3, globotriaosylceramide.
[b]$P < 0.01$ vs LV/α-gal A group.
[c]$P < 0.05$.

The cell membrane prevents peptides and proteins from getting into cells. The Tat motif has the potential to circumvent this barrier. A further advantage of using Tat PTD is low toxicity to cells (16) even though the full-length Tat peptide itself is toxic (17). Along those lines, no differences were observed in our study in the health of any injected or control animal.

In conclusion, this study demonstrated for the first time that neonatal injection of LV encoding α-gal A-Tat fusion protein can reduce Gb3 accumulation more effectively in the heart and kidney of Fabry mice compared with LV/α-gal A treated group. This study may advance not only gene therapy but also ERT for Fabry disease, especially cardiac Fabry disease.

ACKNOWLEDGMENTS

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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