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**METHODS: ORIGINAL ARTICLE**

# HIV Tat Domain Improves Cross-correction of Human Galactocerebrosidase in a Gene- and Flanking Sequence-dependent Manner

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**Krabbe disease is a devastating neurodegenerative lysosomal storage disorder caused by a deficiency of β-galactocerebrosidase (GALC). Gene therapy is a promising therapeutic approach for Krabbe disease. As the human brain is large and it is difficult to achieve global gene transduction, the efficacy of cross-correction is a critical determinant of the outcome of gene therapy for this disease. We investigated whether HIV Tat protein transduction domain (PTD) can improve the cross-correction of GALC. Tat-PTD significantly increased (~6-fold) cross-correction of GALC through enhanced secretion and uptake in a cell-culture model system. The effects of Tat-PTD were gene and flanking amino acids dependent. Tat-fusion increased the secretion of α-galactosidase A (α-gal A), but this did not improve its cross-correction. Tat-fusion did not change either secretion or uptake of β-glucocerebrosidase (GC). Tat-PTD increased GALC protein synthesis, abolished reactivity of GC to the 8E4 antibody, and likely reduced mannose phosphorylation in all these lysosomal enzymes. This study demonstrated that Tat-PTD can be useful for increasing cross-correction efficiency of lysosomal enzymes. However, Tat-PTD is not a mere adhesive motif but possesses a variety of biological functions. Therefore, the potential beneficial effect of Tat-PTD should be assessed individually on each lysosomal enzyme.**

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## Introduction

Krabbe disease (globoid cell leukodystrophy) is a demyelinating disease caused by a genetic deficiency of the lysosomal enzyme β-galactocerebrosidase (GALC).[1] In the infantile form of this disease, neurological symptoms progress rapidly and most patients die before 2 years of age.[2] The clinical and pathological manifestations are almost exclusively confined to the central nervous system and peripheral nerve. The deterioration of the nervous system is postulated to be the result of the dysfunction of myelin-forming cells (oligodendrocytes and Schwann cells). Currently, treatment to Krabbe disease is limited to hematopoietic stem cell transplantation.[3] Previous studies in the mouse model of Krabbe disease (twitcher mouse), including those from our group, suggested that brain-targeted gene therapy is a promising treatment approach for this disease.[4–11] However, the broad distribution of neuropathology, and relatively large size of the human brain, preclude clinical use of current gene transfer techniques, which genetically correct only a small number of cells. A phenomenon, referred to as cross-correction, whereby lysosomal enzymes can be secreted from enzyme-producing cells and taken up by surrounding enzyme-deficient cells provides an important basis for a strategy to overcome this problem.[12] However, our previous work showed that the cross-correction of GALC is not efficient.[5] The goal of the present study is to improve the efficacy of cross-correction of GALC and thus to enhance the therapeutic effect of gene therapy for Krabbe disease.

Cross-correction can be improved by either increasing the secretion of the therapeutic enzyme from gene-transduced cells or increasing the uptake of the enzyme by enzyme-deficient cells. Previous studies have shown that heterogeneous secretion signals can increase secretion of β-glucuronidase (GUSB) and acid α-glucosidase.[13,14] On the other hand, it has been reported that the protein transduction domain (PTD) from HIV Tat can improve biodistribution and/or uptake of GUSB,[15,16] β-glucocerebrosidase (GC),[17] and α-galactosidase A (α-gal A).[18] In this study, we tested whether heterogeneous secretion signals and Tat-PTD can improve cross-correction of GALC and investigated potential functions of Tat-fusion in the secretion/uptake system.

## Results

### HIV Tat-PTD improved cross-correction of GALC

To facilitate detection and purification of GALC, human influenza hemagglutinin (HA) and hexa-histidine (His) tags were fused to the C-terminus of the GALC gene (GALC-HA, **Figure 1**). These tags did not change the expression and secretion of GALC in transfected 293T cells compared with native GALC (data not shown). This was consistent with the observations in our previous study, in which 3 tandem c-myc-epitope in C-terminus did not affect lysosomal targeting and

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intracellular catalytic activity of GALC.[5] Thus, GALC-HA, instead of native GALC, was used as control throughout the experiments in this study.

To test whether Tat-PTD can improve uptake of GALC, Tat-PTD was fused to the C-terminus of the GALC gene (GALC-TatHA, **Figure 1**). Uptake efficiency was assessed *in vitro* by feeding enzyme-deficient cells with conditioned media from transiently transfected 293T cells. We found significantly increased (~6-fold) intracellular GALC activity in Krabbe patient's fibroblasts that were fed with GALC-TatHA–containing medium compared with GALC-HA (**Figure 2a**). Similar results were obtained when an uptake study was performed in twitcher mouse-derived Schwann cells (TwS1),[19] which area disease relevant cell type (**Figure 2a**).

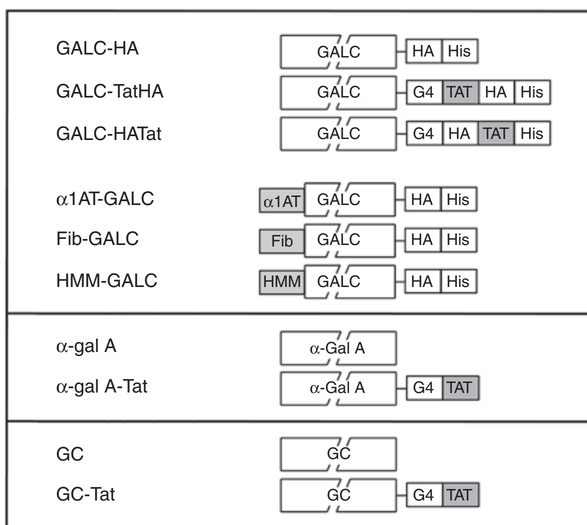


**Figure 1 Schematic representations of the constructs.** G4, 4-glycine spacer; HA, human influenza hemagglutinin epitope tag; His, hexa-histidine tag; HMM, an artificial secretion signal; TAT, HIV Tat protein transduction domain; α1AT and Fib, secretion signals from α1-antitrypsin and fibronectin respectively.

To understand the underlying mechanism for this increased uptake of GALC-TatHA, we tested the conditioned media from the transfected 293T cells. GALC protein was detected by antibody to HA tag. We found that GALC activity and protein levels in conditioned media were significantly increased in GALC-TatHA–transfected 293T compared with GALC-HA–transfected cells (**Figure 2b**). This suggested that the increased protein amount of GALC-TatHA in conditioned medium contributed to the improved cross-correction. Intracellular GALC expression was also increased in GALC-TatHA–transfected 293T cells (**Figure 2c**). In both conditioned media and cell lysates, the changes of GALC activity were proportional to that of protein levels, suggesting that enzyme activity per molecule is not compromised by Tat-fusion. To further determine whether Tat-PTD itself contributed to the increased uptake, GALC-TatHA–containing conditioned medium was diluted to obtain the same GALC activity as that of GALC-HA–containing medium, and their uptake rates were compared. Tat-PTD led to moderately but significantly increased uptake (about 1.4-folds) compared with GALC-HA (**Figure 2d**). Taken together, these data indicate that GALC Tat-fusion results in improved cross-correction through both increased secretion of the fusion protein from gene-transduced cells and enhanced uptake of the enzyme by recipient cells.

### Tat-PTD caused increased protein synthesis of GALC

The increased GALC protein in both cell lysates and conditioned medium of GALC-TatHA–transfected 293T cells (**Figure 2b,c**) suggested that Tat-PTD upregulates GALC protein expression. This was not seen in other lysosomal enzymes with Tat-fusion in previous studies.[17,20] We therefore studied the potential mechanism for the increased expression of GALC-Tat.

To confirm that the effect of Tat-PTD on increased expression of GALC-TatHA is not a cell line–specific phenomenon, we retrovirally introduced GALC-HA and GALC-TatHA into a twitcher mouse fibroblast cell line, Tw2.[5] GALC protein in cell lysate and conditioned medium were significantly increased

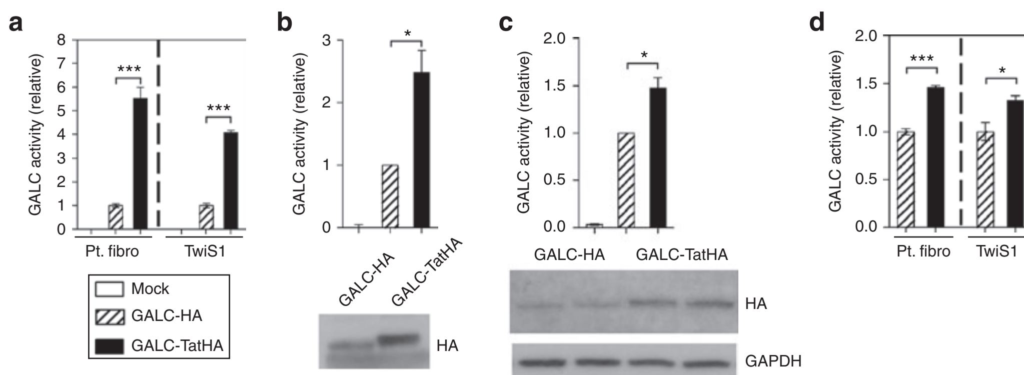


**Figure 2 HIV Tat-fusion improves cross-correction of GALC.** (**a**) GALC activities in lysates of Krabbe patient's fibroblasts (Pt. Fibro) and twitcher mouse Schwann cells (TwS1) incubated with conditioned media of 293T cells that were transfected with empty vector (mock), GALC-HA and GALC-TatHA ($n = 3$). (**b**) GALC in conditioned media of transfected 293T cells assessed by enzyme assay (upper, $n = 3–4$) and western blot analysis (lower). (**c**) GALC in lysates of transfected 293T cells assessed by enzyme assay (upper, $n = 4–5$) and western blot analysis (lower). (**d**) GALC activities in the lysates of Krabbe patient's fibroblasts (Pt. Fibro) and twitcher Schwann cells (TwS1) incubated with GALC-HA- or GALC-TatHA–containing conditioned media that have the same GALC activity ($n = 3$). Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ determined by Mann–Whitney test. GALC, β-galactocerebrosidase; HA, human influenza hemagglutinin epitope tag.

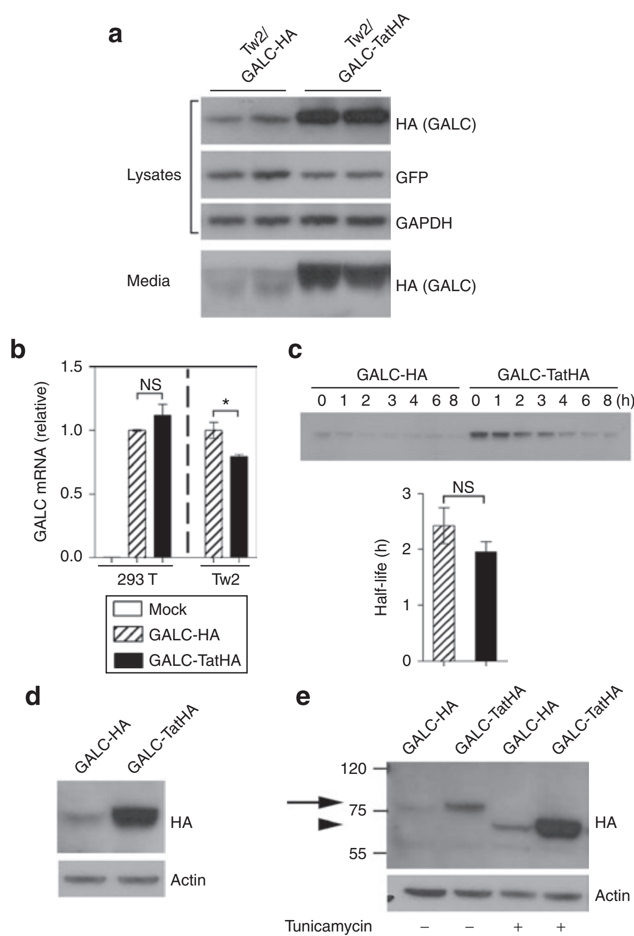


**Figure 3 Tat-PTD increases protein expression of GALC.** (**a**) Western blot analysis of GALC protein levels in the lysates and conditioned media of twitcher mouse fibroblasts (Tw2) stably expressing GALC-HA or GALC-TatHA. (**b**) mRNA levels of human GALC in transfected 293T cells and the retrovirus-infected Tw2 cells assessed by quantitative RT-PCR ($n = 3$). (**c**) Half-life of GALC protein in Tw2 cells expressing GALC-HA or GALC-TatHA assessed by cycloheximide chase assay. The representative western blot was shown (upper). Half-life was calculated using protein levels quantified by densitometry (lower, $n = 3$). (**d**) Western blot analysis of GALC protein in 293T cells that were treated with brefeldin A for 2 days after transfection. (**e**) Western blot analysis of GALC in 293T cells that were treated with tunicamycin for 2 days after transfection. Arrow and arrowhead indicate GALC with and without N-linked glycosylation. The molecular weight standards were shown on the left. Data are presented as mean ± SEM. *$P < 0.05$, determined by (**b**) Mann–Whitney test and (**c**) *t*-test. GALC, β-galactocerebrosidase; HA, human influenza hemagglutinin epitope tag; PTD, protein transduction domain.

in Tw2 expressing GALC-TatHA compared with GALC-HA (**Figure 3a**). The expression of GFP, which occurs through internal ribosome entry site (IRES) element, was not increased, confirming that the increased expression of GALC-Tat is not due to higher gene transduction efficiency (**Figure 3a**).

Real-time RT-PCR results showed that Tat-PTD did not increase transcription level of GALC in both transiently transfected 293T cells and retrovirus-infected Tw2 cells (**Figure 3b**), suggesting that the increased GALC-Tat expression is regulated at the protein level.

Half-life of GALC-TatHA protein in retrovirus-infected Tw2 cells, assessed by cycloheximide chase assay, was unchanged compared with GALC-HA (**Figure 3c**), suggesting that Tat-fusion did not change intracellular protein stability. Most lysosomal enzymes are synthesized as precursors in the rough endoplasmic reticulum (ER), where the signal peptide is cleaved and the precursors undergo N-linked glycosylation and carbohydrate processing, which continues in the Golgi apparatus. After acquiring the mannose 6-phosphate (M6P) marker, enzymes are either transported to the lysosomes to be converted to their mature forms, or enter the constitutive secretory pathway.[21] To evaluate the protein synthesis in the ER without confounding contributions from secretion and processing in different compartments, transiently transfected 293T cells were treated with brefeldin A, which specifically blocks transport of newly synthesized proteins from ER to Golgi, and thus also blocks secretion. Western blot analysis showed massive increase of GALC-TatHA compared with GALC-HA, suggesting increased protein synthesis in the ER (**Figure 3d**). In addition, comparing the apparently increased molecular weight of GALC-TatHA to that of GALC-HA (**Figures 2b** and **3a**,**d**) leads us to ask whether Tat-PTD caused increased glycosylation. To test this, we treated transfected 293T cells with tunicamycin to inhibit N-linked glycosylation of newly synthesized GALC proteins. The inhibition of N-glycosylation did not eliminate the difference in protein mobility between GALC-TatHA and GALC-HA (**Figure 3e**), suggesting that the slower mobility is not due to increased glycosylation. Massive increase of GALC-Tat protein in tunicamycin-treated cells also indicated that the effect of Tat-PTD on increased GALC protein is not via modification of N-linked glycosylation.

Taken together, these data suggested that Tat-PTD increases expression of GALC fusion protein mainly through increased translation efficiency.

### Effect of Tat-PTD on improving cross-correction is gene and flanking residues dependent

To study whether the role of Tat-PTD on improved cross-correction is GALC-specific or universal for other lysosomal enzymes, we tested the effect of Tat-fusion on α-gal A and GC.

Compared with native α-gal A, α-gal A-Tat had decreased α-gal A activity in cell lysates (**Figure 4a**) but significantly increased α-gal A activity in conditioned media (**Figure 4b**) of transfected 293T cells. Protein levels analyzed by western blot were consistent with enzyme activities (**Figure 4c**). In brefeldin A–treated 293T cells, α-gal A protein levels were similar between native α-gal A and α-gal A-Tat (**Figure 4d**), suggesting that protein synthesis of α-gal A was not changed by Tat-PTD. Uptake study was performed on Fabry patient's fibroblasts. Despite significantly increased secretion of α-gal A-Tat, intracellular α-gal A activity in recipient cells that were fed with α-gal A-Tat–containing medium was not significantly different from native α-gal A (**Figure 4e**). Compared with native α-gal A, half-life of α-gal A-Tat in conditioned medium at 37 °C was decreased by 29% ($n = 2$), suggesting that lower stability of α-gal A-Tat may contribute, at least in part, to failed improvement of cross-correction relative to significantly increased secretion from gene-transduced cells.

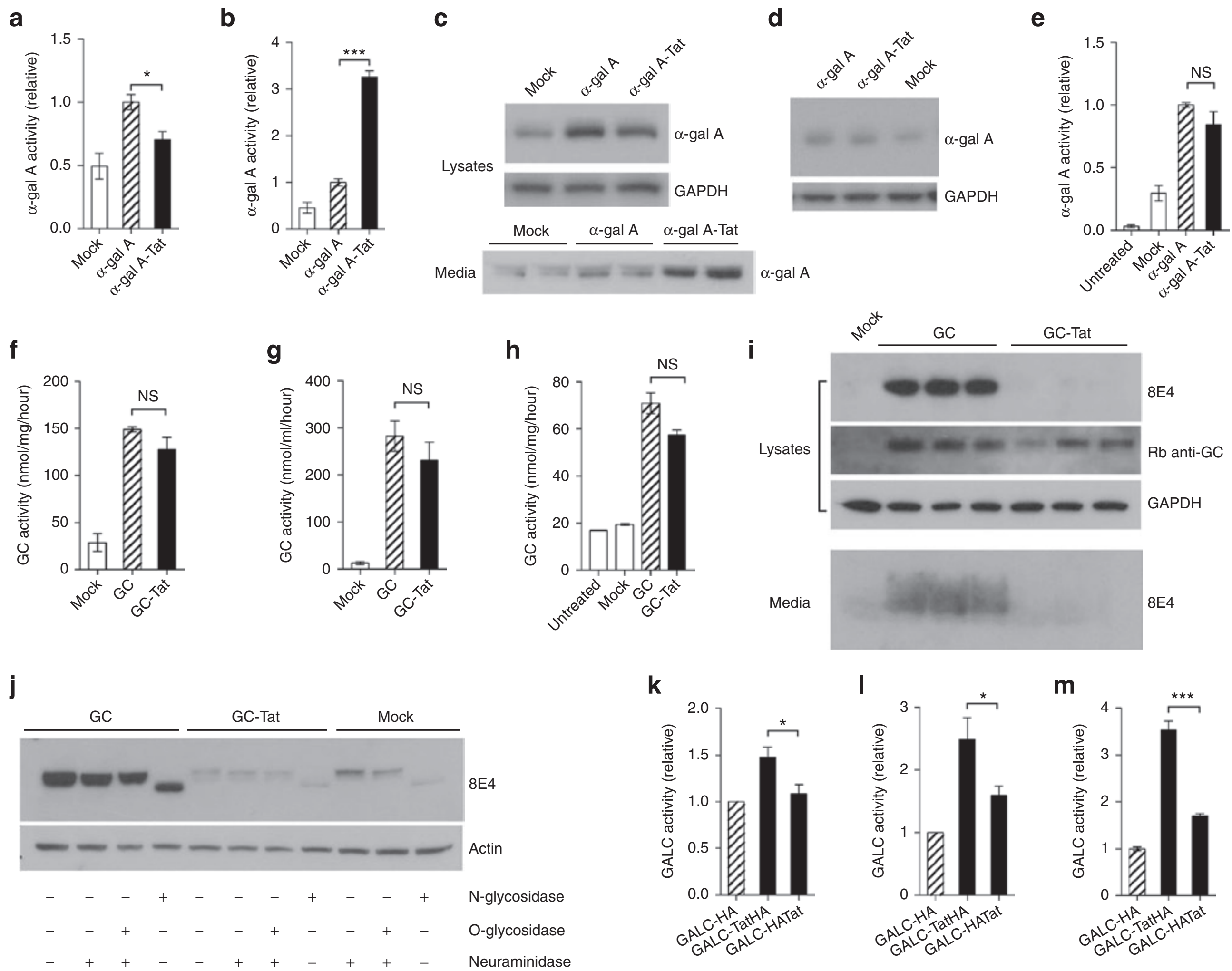


**Figure 4 Effect of Tat-PTD on improving cross-correction is gene- and flanking residues dependent.** (**a**–**e**) Effects of Tat domain in α-gal A. (**a**) α-gal A activities in the lysates of transfected 293T cells ($n = 4$–5). (**b**) α-gal A activities in the conditioned media of transfected 293T cells ($n = 4$–5). (**c**) Western blot analysis of α-gal A protein in the lysates and media of transfected 293T cells. (**d**) Western blot analysis of α-gal A in 293T cells treated with brefeldin A after transfection. (**e**) Intracellular α-gal A activities of Fabry patient's fibroblasts that were fed with conditioned media from 293T cells transfected with various constructs ($n = 5$–9). (**f**–**j**) Effects of Tat domain in GC. (**f**) GC activities in the lysates of transfected 293T cells ($n = 3$). (**g**) GC activities in the conditioned media of transfected 293T cells ($n = 3$). (**h**) Intracellular GC activities of Gaucher patient's fibroblasts that were fed with conditioned media from 293T cells ($n = 3$). (**i**) GC protein in the cell lysates and conditioned media of transfected 293T cells analyzed by western blot with 8E4 or rabbit polyclonal antibody to GC. (**j**) Western blot analysis of GC in the lysates of transfected 293T cells after deglycosylation with neuraminidase, O-glycosidase and PNGase F. (**k**–**m**) Comparison of GALC-TatHA and GALC-HATat. (**k**) GALC activities in the lysates of transfected 293T cells ($n = 4$–5). (**l**) GALC activities in the conditioned media of transfected 293T cells ($n = 3$–4). (**m**) Intracellular GALC activities of Krabbe patient's fibroblasts loaded with media from transfected 293T ($n = 3$). Data are presented as mean ± SEM. *$P < 0.05$, ***$P < 0.001$ determined by (**a**,**b**,**e**,**k**–**m**) Mann–Whitney test and (**f**–**h**) *t*-test. GALC, β-galactocerebrosidase; GC, β-glucocerebrosidase; HA, human influenza hemagglutinin epitope tag; PTD, protein transduction domain; α-gal A, α-galactosidase A.

Tat-fusion to GC did not change expression, secretion, and uptake compared with native GC (**Figure 4f**–**h**). Interestingly, when we analyze GC protein by western blot, we found that GC-specific monoclonal antibody 8E4[22] could not recognize GC-Tat in either cell lysates or conditioned media (**Figure 4i**). A polyclonal antibody could detect GC-Tat (**Figure 4i**). One possible situation that could change immunogenicity is altered glycosylation of the protein that can mask the epitope.[23] To test whether the lost of reactivity to 8E4 is caused by altered glycosylation of GC-Tat, we treated cell lysates of GC- or GC-Tat–transfected 293T cells with different types of glycosidase (neuraminidase, O-glycosidase, and PNGase F) before western blotting. Removal of sugar moieties did not increase immunogenicity of GC-Tat to 8E4 over the background level (*i.e.*, endogenous GC in 293T cells) (**Figure 4j**).

The above results showed that the effect of Tat-PTD is highly gene specific. To further test whether the effects of Tat-PTD are influenced by flanking amino acid residues, we made another construct GALC-HATat and compared it with GALC-TatHA. The only difference between these two constructs is the relative position of the 9-amino-acid HA tag and Tat-PTD (see **Figure 1**). The effects of Tat-PTD in the secretion/uptake system were changed significantly by this minor

modification. Compared with GALC-TatHA, GALC-HATat led to significantly lower expression, secretion, and intracellular activity of recipient cells (Figure 4k–m). Uptake of GALC-HATat was much less inhibited by M6P relative to GALC-TatHA (Table 1).

The major findings of Tat-PTD in GALC, α-gal A, and GC are summarized in Table 1. The only consistent effect of Tat on these enzymes was that the endocytosis of fusion proteins is less inhibited by M6P (Table 1). This suggested that Tat-PTD caused less M6P content in these enzymes (see discussion below).

**Table 1** Summary of the effects of Tat-fusion on cross-correction

| Enzymes | Expression in 293T | Secretion by 293T | Uptake by fibroblast[a] | Inhibition of uptake by M6P | |
|---|---|---|---|---|---|
| | | | | Native | Tat-fusion |
| GALC | ↑ | ↑↑ | ↑↑ | 56.2% | 20.2%[b]; 2.9%[c] |
| α-Gal A | ↓ | ↑↑ | NS | 96.3% | 83.6% |
| GC | NS | NS | NS | 51.5% | 42.1% |

*Abbreviations*: GALC, β-galactocerebrosidase; GC, β-glucocerebrosidase; NS, the difference is not significant; α-gal A, α-galactosidase A; ↑/↓, Tat-fusion causes increased or decreased enzyme activities compared with native enzymes; ↑↑, More than twofold increment.
[a]Patient's fibroblasts with deficiency of a given lysosomal enzyme.
[b]GALC-TatHA. [c]GALC-HATat.

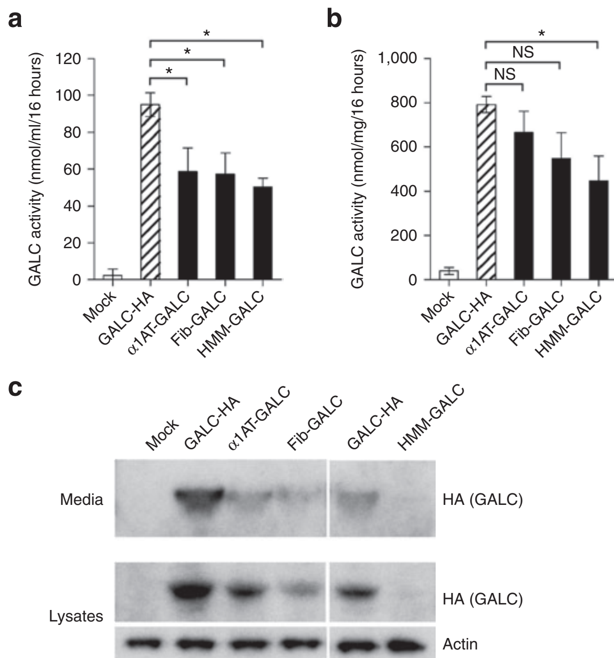


**Figure 5** Heterogeneous secretion signals decrease secretion and expression of GALC. (**a**) GALC activities in the conditioned media of 293T cells transfected with constructs with various secretion signals ($n$ = 3). (**b**) GALC activities in the lysates of transfected 293T cells ($n$ = 3). (**c**) Western blot analysis of GALC protein in the conditioned media and cell lysate of 293T cells. Data are presented as mean ± SEM. *$P$ < 0.05, determined by one-way analysis of variance followed by Bonferroni's multiple comparison test. GALC, β-galactocerebrosidase.

### Heterogeneous secretion signal did not increase the secretion of GALC

It has been reported that the secretion of some lysosomal enzymes can be increased by the use of "stronger" heterogeneous secretion signals.[13,14] To test whether this approach can increase the secretion of GALC, we replaced the secretion signal of GALC by that of α1-antitrypsin (α1AT), fibronectin (Fib), or a synthetic secretion signal (HMM),[24] which were shown to enhance secretion of chimeric proteins[14,24,25] (Figure 1). We analyzed GALC activity and protein level in the conditioned media of transiently transfected 293T cells. All three heterogeneous signal peptides tested did not increase but rather decreased secretion of GALC compared with the original signaling peptide (Figure 5a,c). Overall, heterogeneous secretion signals led to decreased GALC protein levels in homogenates of transfected 293T cells (Figure 5b,c), indicating that the decreased secretion of chimeric GALC is due, at least in part, to decreased protein expression. Previously, we showed that replacement of ATG surrounding sequences with ones that fit better the Kozak rule did not increase but rather decreased GALC expression in NIH3T3 cells.[5] Thus, it is likely that the sequences in the secretion signal of human GALC are stringent, and any changes may compromise its expression.

## Discussion

Because of the limited gene transfer efficiency achieved by current gene therapy techniques, gene therapy in lysosomal storage disorders is in fact an analogue of enzyme-replacement therapy (*i.e.*, gene-transduced cells work as a pump to supply the therapeutic enzyme to surrounding untransduced cells). Thus, cross-correction efficiency is a critical determinant of the outcome of gene therapy approaches in lysosomal storage disorders. Our study showed that Tat-fusion significantly improved cross-correction of GALC through both increased secretion and uptake, suggesting the potential usefulness of this approach to gene therapy for the treatment of Krabbe disease. Tat-PTD increased up to sixfold cross-correction efficiency in enzyme-deficient fibroblasts and Schwann cells after overnight incubation with conditioned medium. Although *in vitro* and *in vivo* situations may not be directly comparable, one can expect that Tat-fusion to human GALC would significantly improve the cross-correction effect *in vivo*, in which gene-transduced cells constantly secrete enzyme within the brain. Previously, Tat has been tested in mouse GALC.[20] Tat-fusion increased the secretion rate and M6P receptor–independent uptake of mouse GALC. Our study further confirmed these Tat effects in human GALC. However, it should be taken into consideration that Tat-PTD may induce the production of vector-specific antibodies and thus may compromise the therapeutic effects *in vivo*. An earlier study reported by Elliger *et al.* suggested that maternal antibodies from animals that received AAV-GUSB-Tat crossed the placenta and led to a resistance to GUSB-Tat treatment in their pups.[13] Further investigations will be conducted in twitcher mouse to explore whether Tat-fusion to human GALC could increase therapeutic efficacy of brain-targeted gene therapy for Krabbe disease.

In contrast to GALC, cross-correction of α-gal A and GC was not improved by Tat. Previously, it has been reported that Tat causes higher uptake of these two enzymes *in vitro*.[17,18] The discrepancy between these studies and ours may be explained by the use of different experimental systems. For example, in the GC-Tat study by Lee *et al.*,[17] cell extracts instead of conditioned media of gene-transduced cells were used to feed recipient cells. The constant experimental conditions (expression vector, transient expression system, and feeding condition) in our study led to a conclusion that the role of Tat in cross-correction is gene dependent. Our results further demonstrated that the effects of Tat-PTD are sensitive to surrounding amino acid residues. The location of the HA tag significantly influenced the expression, secretion, and uptake of GALC-Tat. Absorptive endocytosis through binding of the positively charged Tat-PTD to cell surface proteoglycans contributes to the uptake of Tat-fused proteins.[16,26,27] The global structure and flanking residues in C-terminus of the proteins may influence the topography of Tat-PTD and thus affect its binding capacity to cell membrane. Different susceptibility of individual enzymes to the potential adverse effect of Tat-PTD (*e.g.*, decreased stability of α-gal A-Tat) may also contribute to various effects of Tat-PTD on cross-correction.

The only consistent effect of Tat on GALC, α-gal A, and GC was that the rate of M6P-dependent endocytosis is decreased in these fusion proteins (**Table 1**). This not only suggested that Tat-PTD may confer alternative binding/internalization mechanism other than M6P receptor-mediated endocytosis but also suggested a possibility that Tat-PTD caused less M6P content in these enzymes. M6P terminal residues are recognized in the Golgi by M6P receptors that mediate the sorting of the majority of lysosomal enzymes from the secretory pathway and deliver them to lysosomes.[21,28] Absent or reduced M6P results in increased secretion of these enzymes. However, GC is targeted to lysosomes through a M6P receptor–independent pathway,[29] and thus mannose phosphorylation status does not influence its secretion. The fact that Tat-fusion results in increased secretion of GALC and α-gal A (**Figures 2b** and **4b**) but not GC (**Figure 4g**) further supports that Tat-PTD causes decreased mannose phosphorylation. More direct evidence suggesting this possibility has come from the decreased phosphorylation rate of GUSB-Tat protein.[16] The reduced mannose phosphorylation might be a consequence of suboptimal recognition of Tat-fused proteins by UDP-N-acetylglucosamine 1-phosphotransferase, which recognizes conformation-dependent protein structures of lysosomal enzymes in which lysine residues are the major determinants.[30,31]

We assume that the reduced M6P is the major contributor for the increased secretion of Tat-fused GALC, α-gal A, and GUSB[13] that utilize M6P receptors for their lysosomal targeting. From a therapeutic viewpoint, the increased secretion is advantageous. However, the reduced M6P decreases M6P receptor–dependent endocytosis of fusion enzymes. The impact of the reduced M6P combined with the increased cell surface adherence on the uptake of Tat-fused enzymes will be cell type specific, depending on preferential uptake system in each cell type. In fact, in an enzyme infusion study, GUSB-Tat had a higher uptake than native GUSB in the renal tubular cells but not in other cell types.[16]

Our study also showed other previously unreported functions of Tat-PTD. Tat-PTD led to significantly increased protein synthesis of GALC but not that of α-gal A and GC. It also led to 8E4 antibody failed to recognize GC in the western blot. The mechanisms by which Tat-PTD causes these alterations are unclear. However, it is possible that the effect of Tat-PTD on upregulated protein synthesis of GALC is through altering the mRNA structure of the gene, which is a determinant of translation efficiency.[32] In addition, HIV envelop protein gp120 is a heavily glycosylated protein with carbohydrates accounting for about 50% of its molecular weight. Rapid modification of these glycans is an important viral defense mechanism for immune evasion of HIV.[33] Although *in vitro* deglycosylation did not restore the reactivity of GC to 8E4, we cannot exclude the possibility that Tat-PTD may modulate glycosylation and its modification in fusion proteins through an unidentified mechanism. Taken together, our data suggested that Tat-PTD is not a mere adhesive motif; it actively collaborates with the fused protein to exert a variety of biological functions.

In conclusion, our study demonstrated that Tat-fusion can significantly improve cross-correction efficiency of human GALC and thus will be a useful approach in gene therapy for Krabbe disease. As the effect of Tat-PTD on cross-correction is gene and flanking sequence dependent, the effect of Tat-PTD on a specific lysosomal enzyme should be investigated carefully to determine whether it would be beneficial.

## Materials and methods

*Plasmid construction.* All the plasmids used in this study were derived from the pMMP retroviral vector backbone in which the gene of interest is driven by the long terminal repeat, and downstream GFP is expressed through IRES (internal ribosome entry site). The constructs were shown in **Figure 1**. Replacement of secretion signals and Tat-fusion were made using synthesized complementary oligonucleotides or PCR-based mutagenesis. All the modified sequences were verified by DNA sequencing. Detailed procedure and information are available upon request.

*Cell culture and treatments.* 293T cells, fibroblasts obtained from patients with Krabbe disease, Fabry disease (DMN84.41), or Gaucher disease (DMN83.102), and spontaneously immortalized fibroblasts (Tw2)[5] and Schwann cells (TwS1)[19] derived from twitcher mice were cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

*Gene transduction.* For transient gene transduction, 293T cells were transfected with plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. Conditioned media and cells were harvested and analyzed 2 days later. Culture media were filtered through 0.45 μm filter and were directly used for enzyme assay, western blot, or uptake study. For stable gene transduction, plasmids were transfected into an ecotropic retroviral packaging cell line, Phoenix (eco), and the virus-containing medium was used to infect Tw2 cells in the presence of 8 μg/ml polybrene overnight.

*Uptake study.* Conditioned media were harvested from transfected 293T cells as described above. Enzyme-deficient cells were incubated with conditioned media overnight in the presence or absence of 5 mmol/l M6P (Sigma, St Louis, MO). Then, cells were treated with 0.25% trypsin to eliminate extracellular enzymes and harvested and rinsed with phosphate-buffered saline. The cells were lysed and analyzed for enzyme activity as described below.

*Lysosomal enzyme assays.* All the enzyme activities were determined by fluorimetric assays. For GALC activity, the cell pellets were suspended in distilled water and sonicated, and the lysates were used for enzyme activity assay as described previously.[34] For α-gal A or GC enzyme activity, the cells were lysed in 0.2% triton in saline and were sonicated. The lysates were centrifuged at 14,000 rpm for 15 minutes at 4 °C, and the supernatants were used for enzyme assay as described.[34] For enzyme activities in conditioned media from 293T cells, 10 µl of culture media harvested as described above were directly used for enzyme assays. Protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL).

*Western blot.* Western blot analysis was performed as described previously.[34] Cell lysates containing ~25 µg total protein or 12 µl of conditioned media were loaded in each lane. Primary antibodies used were mouse monoclonal antibodies to HA tag (Covance, Alice, TX); GC (8E4),[22] and β-actin (Sigma); rabbit polyclonal antibodies to GFP (Abcam, Cambridge, MA), human α-gal A (Shire Human Genetic Therapies, Cambridge, MA) and GC (Santa Cruz Biotechnology, Santa Cruz, CA); and goat polyclonal antibody to GAPDH (Santa Cruz Biotechnology).

*Quantitative RT-PCR.* Real-time RT-PCR for human GALC was performed using predesigned SYRB green primers (Takara, Osaka, Japan) as described previously.[35] 18s rRNA was used as internal control and detected using TaqMan probe and primers (Applied Biosystems, Foster City, CA).

*Cycloheximide chase assay.* Tw2 cells stably expressing GALC-TatHA or GALC-HA were treated with 50 µg/ml cycloheximide (Sigma) for 0–8 hours, and the cells were lysed in 1× sample buffer at the indicated time points and were analyzed by western blot.

*Brefeldin A and tunicamycin treatment.* 293T cells were transfected as described above. The cells were incubated with lipid/DNA complex for 6 hours, then the medium was replaced by growth media containing brefeldin A (Sigma) at 10 µg/ml or tunicamycin (Sigma) at 1 µg/ml. Vehicles were used for controls. Two days after, cells were lysed directly in 1× sample buffer and were analyzed by western blot.

*Deglycosylation of cell lysates.* 293T cells transfected with native GC or GC-Tat were lysed in phosphate-buffered saline containing 0.1% SDS and protease inhibitor cocktail Complete (Roche, Indianapolis, IN), sonicated and were centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatants were denatured by heating at 98 °C for 5 minutes. The lysates containing ~20 µg total protein were treated with 20 milliunits of neuraminidase (Roche) and/or 1 milliunits of O-glycosidase (Roche) in 50 mmol/l phosphate buffer (pH 6.8) in the presence of 1% NP40 at 37 °C overnight. Neuraminidase is required to remove the terminal sialic acid that prevents the functioning of O-glycosidase. For N-glycosidase treatment, the samples were treated with 750 units of PNGase F (New England Biolabs, Ipswich, MA) in G7 buffer in the presence of 1% NP40 at 37 °C overnight. The samples were then analyzed by western blot.

*Statistical analysis.* Data were presented as mean ± SEM. Statistical significance was determined by the Student's *t*-test or Mann–Whitney *U*-test (for ratio data). One-way ANOVA followed by Bonferroni's multiple comparison test was used for multiple comparisons.

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