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RESEARCH ARTICLE

Antibody formation and mannose-6-phosphate receptor expression impact the efficacy of muscle-specific transgene expression in murine Pompe disease

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Abstract

Background Lysosomal storage disorders such as Pompe disease can be more effectively treated, if immune tolerance to enzyme or gene replacement therapy can be achieved. Alternatively, immune responses against acid α-glucosidase (GAA) might be evaded in Pompe disease through muscle-specific expression of GAA with adeno-associated virus (AAV) vectors.

Methods An AAV vector containing the MHCK7 regulatory cassette to drive muscle-specific GAA expression was administered to GAA knockout (KO) mice, immune tolerant GAA-KO mice and mannose-6-phosphate deficient GAA-KO mice. GAA activity and glycogen content were analyzed in striated muscle to determine biochemical efficacy.

Results The biochemical efficacy from GAA expression was slightly reduced in GAA-KO mice, as demonstrated by higher residual glycogen content in skeletal muscles. Next, immune tolerance to GAA was induced in GAA-KO mice by co-administration of a second AAV vector encoding liver-specific GAA along with the AAV vector encoding muscle-specific GAA. Antibody formation was prevented by liver-specific GAA, and the biochemical efficacy of GAA expression was improved in the absence of antibodies, as demonstrated by significantly reduced glycogen content in the diaphragm. Efficacy was reduced in old GAA-KO mice despite the absence of antibodies. The greatest impact upon gene therapy was observed in GAA-KO mice lacking the mannose-6-phosphate receptor in muscle. The clearance of stored glycogen was markedly impaired despite high GAA expression in receptor-deficient Pompe disease mice.

Conclusions Overall, antibody formation had a subtle effect upon efficacy, whereas the absence of mannose-6-phosphate receptors markedly impaired muscle-targeted gene therapy in murine Pompe disease.

Keywords acid α-glucosidase; acid maltase; adeno-associated virus; antibody formation; gene therapy; glycogen storage disease type II; Pompe disease

Introduction

Glycogen storage disease type II (GSD-II; Pompe disease; MIM 232 300) results from the inherited deficiency of lysosomal acid α-glucosidase

(GAA; acid maltase; EC 3.2.1.20). The disease is characterized by the massive accumulation of lysosomal glycogen in striated muscle with an accompanying disruption of cellular functions. Although enzyme replacement (ERT) has shown promise in patients with both infantile-onset and late-onset Pompe disease, no curative therapy is available. Efforts to develop gene therapy in Pompe disease are justified by the limitations of ERT, which include the requirement for frequent intravenous infusions of high levels of GAA to achieve efficacy and the risk of neutralizing antibodies [1]. High level GAA replacement (40–100 mg/kg/dose) reduced the glycogen content of heart and skeletal muscle in immune tolerant GAA-knockout (KO) mouse models [2,3]. Similarly, doses of 20–40 mg/kg were required to improve clinical endpoints in clinical trials of ERT in patients with Pompe disease [4,5]. The amount of GAA required to achieve efficacy is approximately 30–100-fold greater than the doses used for ERT in other lysosomal disorders [6].

Pompe disease patients who lacked any residual GAA protein by western blot analysis of skin fibroblasts are deemed cross-reacting immune material negative (CRIM-negative). CRIM-negative Pompe disease subjects produced anti-GAA antibodies and demonstrated markedly reduced efficacy from ERT [1,7]. In the first pilot study of ERT using Chinese hamster ovary cell-derived recombinant GAA, both CRIM-negative Pompe disease subjects produced the highest titers of anti-GAA antibodies and demonstrated markedly reduced efficacy from ERT [1]. The similarity with regard to neutralizing antibody response in GAA-KO mice and in CRIM-negative Pompe disease patients could be linked to the lack of residual GAA protein expression. If GAA deficiency is caused by an underlying null mutation(s), the immune system is likely to react to introduced GAA by forming neutralizing antibodies. Neutralizing antibodies occurred in CRIM-negative Pompe disease patients, and reduced the efficacy of ERT in those subjects.

Wide variability in the response in different organs to ERT has been observed in mice with Pompe disease, with liver and heart muscle taking up GAA more efficiently than skeletal muscle [8]. Barriers to the uptake of GAA in muscle fibers include not only the endothelial barrier, but also the endomysium. Type II myofibers were resistant to correction by ERT compared to type I myofibers, which explained the resistance of the quadriceps and gastrocnemius to over-expression from the liver-specific transgene [3]. Poor uptake of GAA by skeletal muscle was also linked to the low abundance of the cation-independent mannose-6-phosphate receptor in skeletal muscle compared to the heart [2,9,10].

The availability of GAA-KO mice has aided the development of gene therapy with adeno-associated virus (AAV) vectors because the immune responses in GAA-KO mice to GAA are similar to those of CRIM-negative Pompe disease patients. GAA-KO mice feature no residual GAA expression, and therefore respond to recombinant human (h)GAA with antibody formation and very low efficacy [2]. GAA-KO mice resemble CRIM-negative patients with regard to the response to ERT by forming high-titer antibodies and failing to demonstrate efficacy [2,11]. Liver-expressing transgenic GAA-KO mice express very low levels of GAA only in the liver, mimicking untreated CRIM-positive patients by not forming antibodies in response to recombinant GAA; therefore, these mice have been termed tolerant GAA-KO mice. The levels of GAA in cardiac and skeletal muscles for tolerant GAA-KO mice remain extremely low, and glycogen accumulates to high levels in striated muscle of tolerant GAA-KO mice, just as it does in CRIM-positive patients with Pompe disease [2]. A third strain of GAA-KO mice features an additional KO of the cation-independent mannose-6-phosphate receptor [double (D)KO], and might result in a CRIM-negative phenotype with impaired receptor-mediated uptake of recombinant GAA [12]. Thus, the DKO mouse could be useful when evaluating the complicating factor of deficient mannose-6-phosphate receptor upon ERT in Pompe disease.

Major obstacles to therapy in Pompe disease have been identified over the last several years. Consequently, we have more thoroughly investigated the effects of antibody formation, age and mannose-6-phosphate receptor availability upon the response to muscle-targeted gene therapy in GAA-KO mice.

Materials and methods

Preparation of AAV 2/8 vector

Briefly, 293 cells were transfected with the vector plasmid, the AAV packaging plasmid p5E18-VD 2/8 [13], and pAdHelper (Stratagene, La Jolla, CA, USA). The liver-specific regulatory cassette in pAAV-LSPhGAApA (subcloned from pAV-LSP-cFIX, courtesy of Dr Inder Verma, Salk Institute, La Jolla, CA, USA; sequence available upon request) contains a thyroid hormone-binding globulin promoter sequence downstream from ywo copies of a α1-microglobulin/bikunin enhancer sequence, and previously achieved long-term efficacy in hemophilia B mice within an AAV vector encoding coagulation factor IX [14]. The AAV-MHCK7hGAApA vector plasmid contains the muscle specific regulatory cassette from αMHCKChAP [15] (provided by Dr Stephen Hauschka, University of Washington, Seattle, WA, USA) and expressed high level human GAA in the heart and skeletal muscle [16]. The pAAV-CBhGAApA vector plasmid contains the cytomegalovirus early immediated enhancer and chicken β-actin promoter to drive human GAA expression [17]. Cell lysate was harvested 48 h following infection and freeze-thawed three times, and isolated by sucrose cushion pelleting followed by two cesium chloride gradient centrifugation steps. AAV stocks were dialysed against three changes of Hanks buffer, and aliquots were stored at $-80\,^{\circ}$C. The number of vector DNA containing-particles was determined by DNase I digestion, DNA extraction, and Southern blot analysis. All viral vector stocks were handled according to Biohazard

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Safety Level 2 guidelines published by the NIH (Bethesda, MD, USA).

In vivo analysis of AAV vector

All mice were genotyped to confirm GAA-KO and transgene status. Tolerant GAA-KO micew were genotyped as described previously [18]. Muscle-specific cation-independent mannose-6-phospate receptor KO mice were generated using tissue specific promoter (creatine kinase; CK) and the cre/loxP conditional KO system as described previously [12]. The muscle-specific mannose-6-phosphate KO mice were crossed with GAA-KO mice to generate DKO mice. GAA-KO genotyping was achieved using a polymerase chain reaction (PCR)-based assay to determine the deletion of exon 6 in GAA-KO mice. Genomic mouse DNA (100 ng) was used as template in a PCR reaction (35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) using the primers: exon 5 forward (5′-CCTTTCTACCTGGCACTGGAGGAC-3′), exon 7 reverse (5′-GGACAATGGCGGTCGAGGAGTA-3′) and neomycin forward (5′-CCTCGTGCTTTACGGTATCGC-3′). This genotyping was continued until GAA KO mice colony was successfully established. Mouse CI-MPR Genotyping was performed with a PCR-based assay to determine the presence of the *loxP* site in *M6P/IGF2R* intron 9. One hundred nanograms of mouse genomic DNA was used as template in a PCR reaction (35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) using the primers INT9F2 (5′-CCTTCCCTCCAGGCCGTTAC-3′) and INT9R1 (5′-AGGTCTCCATCTGAGTACC-3′). Routine genotyping of this colony was continued until the muscle-CI-MPR-KO mouse colony was successfully established. Mouse Cre genotyping performed with a PCR-based assay was used to determine the presence of C*re* recombinase expressed in specific tissue. Every DKO colony litter was genotyped using 100 ng of mouse genomic DNA as template in a PCR reaction (35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min) using the primers: *Cre* forward (5′- ATGTCCAATTTACTGACCG-3′), *Cre* reverse (5′- CGCCGCATAACCAGTGAAA-3′). Mouse testing positive for murine creatine kinase (MCK)-*Cre* were used for experimentation.

The glycogen storage in the heart and skeletal muscles of DKO mice was equivalent to that observed in GAA-KO mice (not shown). The vector stocks were administered intravenously (via the retroorbital sinus) in 3-month-old and 15-month-old GAA-KO mice, and to 3-month-old tolerant GAA-KO and DKO mice. At the indicated time points post-injection, plasma or tissue samples were obtained and processed as described below. All animal procedures were carried out in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines.

Rotarod testing was performed as described previously [19]. GAA activity and glycogen content were analyzed as described [20]. $p < 0.05$ indicated a significant difference between the observed values for each group of GAA-KO mice following AAV vector administration and the control group of phosphate-buffered saline-injected GAA-KO mice.

Western blotting of hGAA was performed as described previously [19] using the hGAA monoclonal antibody (courtesy of Genzyme Corp., Framingham, MA, USA). An enzyme-linked immunosorbent assay (ELISA) was performed as described previously [19]. All samples yielded absorbance values that were within the linear range of the assay at this dilution.

Statistical analysis

Comparison of two groups was assessed by a homoscedastic Student's *t*-test. The significance of differences between multiple groups was tested using a two-sided Wilcoxon rank sum test for continuous variables, and was conducted with Stata 10 (StataCorp LLC, College Station, TX, USA). $p < 0.05$ was considered statistically significant.

Results

The most efficient approach to gene therapy in Pompe disease might be the expression of GAA in directly transduced myofibers because the main therapeutic target is striated muscle. The question of whether immune tolerance would impact the efficacy of muscle-targeted gene therapy within muscle was further investigated by administration of an AAV2/8 vector expressing muscle-restricted GAA, AAV-MHCK7hGAA, to GAA-KO mice. We previously demonstrated that AAV-MHCK7hGAA provoked antibody formation, but not cytotoxic T lymphocyte (CTL) responses, and achieved long-term biochemical correction in GAA-KO mice [16].

AAV2/8-MHCK7hGAA was administered to mice that featured immune tolerance to human GAA or its absence. AAV2/8-MCHK7hGAA [7×10^{11} vector particles (vp)] was administered to transgenic GAA-KO mice that were immune tolerant to GAA due to the liver-specific expression of low levels of GAA, termed tolerant GAA-KO mice [18], and to GAA-KO mice. In both strains of Pompe disease mice, the vector significantly elevated GAA activity in the heart and the majority of skeletal muscles analyzed compared to untreated mice (Figure 1A; $p \leq 0.01$ for each muscle). Only in the gastrocnemius of tolerant GAA-KO did AAV2/8-MHCK7hGAA fail to significantly elevate GAA activity compared to untreated GAA-KO mice. Furthermore, AAV2/8-MCHK7hGAA significantly lowered glycogen content in the heart and skeletal muscle of both tolerant GAA-KO and GAA-KO mice (Figure 1B; $p \leq 0.01$ for each muscle), with the exception of the gastrocnemius in GAA-KO mice ($p = 0.06$). However, the glycogen content of the gastrocnemius was significantly elevated in GAA-KO mice in comparison with tolerant GAA-KO mice following AAV2/8-MHCK7hGAA administration. The presence of higher GAA activity in the muscle of

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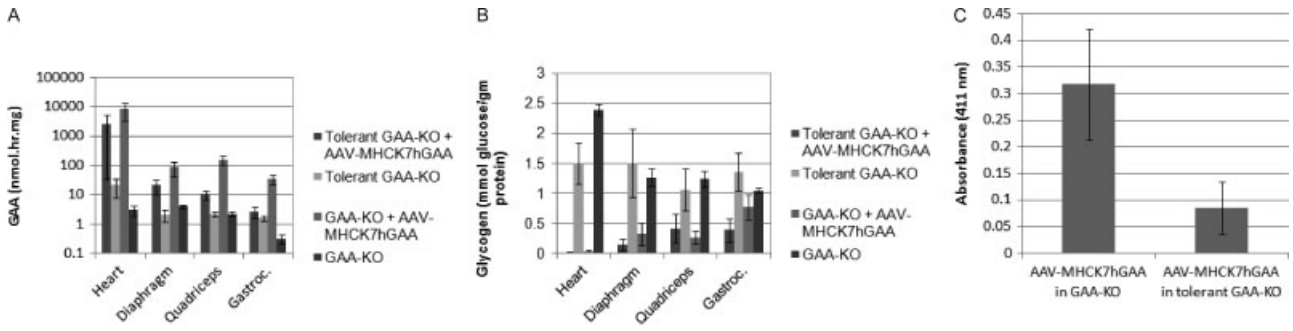

Figure 1. Biochemical correction of striated muscle following the administration of AAV2/8-MCHK7hGAA to GAA-KO or tolerant GAA-KO mice. GAA activity and glycogen content in the indicated striated muscles, 18 weeks following AAV2/8 vector administration. AAV2/8-MHCK7hGAA (7×10^{11} vp) was administered intravenously at 3 months of age to GAA-KO mice ($n = 7$) and to tolerant GAA-KO mice ($n = 5$). Sham-treated GAA-KO mice ($n = 4$) and tolerant GAA-KO mice ($n = 4$) were negative controls. Data are the mean ± SE. (A) GAA activity in the heart, diaphragm, quadriceps, and gastrocnemius. (B) Glycogen content for striated muscles of GAA-KO mice in (A). (C) ELISA for anti-GAA IgG1 at 18 weeks following vector administration

GAA-KO mice following vector administration ($p = 0.007$ for each muscle) in comparison with tolerant GAA-KO mice did not result in lower glycogen content in GAA-KO mice. Tolerant GAA-KO mice did not form anti-GAA antibodies, whereas GAA-KO mice uniformly produced antibodies in response to AAV-MCHK7hGAA administration (Figure 1C). These data demonstrated that immune responses impacted the reduction of glycogen storage in the skeletal muscle of Pompe disease mice.

The question of whether eliminating immune tolerance would impair efficacy in GAA-KO mice was investigated by administration of an AAV2/8 vector that ubiquitously expressed GAA, AAV-CBhGAA, simultaneously with AAV-MHCK7hGAA. This experiment evaluated whether adding a second vector that expressed GAA but provoked CTL, in the form of AAV-CBhGAA, would affect the efficacy from AAV-MHCK7hGAA. AAV-CBhGAA (1×10^{11} vp) was administered simultaneously with AAV-MHCK7hGAA (7×10^{11} vp) in GAA-KO mice to provoke immune responses against GAA. Administration of both vectors significantly elevated GAA activity in the heart, gastrocnemius, and extensor digitorum longus (EDL) compared to untreated GAA-KO mice (Figure 2A; $p < 0.005$ for each). However, efficacy was low in GAA-KO mice following the administration of both vectors, as reflected by a lack of significantly reduced glycogen content in the gastrocnemius and EDL muscles. By contrast, the administration of AAV-MHCK7hGAA alone in tolerant GAA-KO mice significantly reduced glycogen content in the heart and all skeletal muscles (Figure 2B). Moreover, even though the GAA activity was significantly elevated in the gastrocnemius of GAA-KO mice treated with both vectors compared to tolerant mice treated with only AAV-MHCK7hGAA (Figure 2A; $p = 0.006$), the glycogen content of gastrocnemius trended lower in the latter group of mice (Figure 2B; $p = 0.05$). Only GAA-KO mice formed antibodies following the administration of AAV-CBhGAA, whereas tolerant GAA-KO mice did not form antibodies (Figure 2C). These data confirmed the criticality of immune tolerance to GAA with regard to correction of GAA deficiency because simply adding another vector that expressed GAA ubiquitously at higher levels did not enhance the correction of glycogen storage in the skeletal muscle of Pompe disease mice.

Histomorphological analysis performed 18 weeks following vector administration revealed an improved clearance of glycogen vacuoles in the gastrocnemius of AAV-MHCK7hGAA-treated tolerant GAA-KO mice compared to GAA-KO mice treated with AAV-MHCK7hGAA and AAV-CBhGAA (Figures 3A to 3C). CTL was not expected in mice following the administration of AAV-CBhGAA because this immune response has been observed within the first 3 weeks following vector administration [21]. The size of vacuolated glycogen accumulations was reduced in AAV-MHCK7hGAA-treated tolerant GAA-KO mice. The reductions in glycogen vacuoles in multiple tissues of GAA-KO mice in the absence of antibody formation supported the role of immune tolerance in modulating the efficacy of gene therapy in Pompe disease.

The data presented above were obtained in two different GAA-KO mouse strains; moreover, tolerant GAA-KO mice express GAA in liver that notably increased GAA activity and decreased glycogen content in the heart in the absence of vector administration compared to sham-treated GAA-KO mice (Figure 1). To further investigate the effects of immune tolerance upon efficacy in a single strain of mice with Pompe disease, GAA-KO mice were treated with an AAV vector, AAV2/8-LSPhGAA, that induced immune tolerance to hGAA in GAA-KO mice [11,17]. GAA-KO mice were treated with both AAV2/9-MHCK7hGAA (1×10^{11} vp) and AAV2/8-LSPhGAA (1×10^{11} vp), or with AAV2/9-MHCK7hGAA (1×10^{11} vp) alone. AAV-MHCK7hGAA was pseudotyped as AAV9 to increase the transduction of striated muscle, as demonstrated previously [16]. Indeed, GAA expression was similarly elevated for both groups of vector-treated mice compared to sham-treated GAA-KO mice (Figure 4A–4B). However, the glycogen content was significantly reduced in the diaphragm of dual vector-treated GAA-KO mice compared to GAA-KO mice that were treated with AAV2/9-MHCK7hGAA alone (Figure 4C; $p = 0.05$). The diaphragm is a critical

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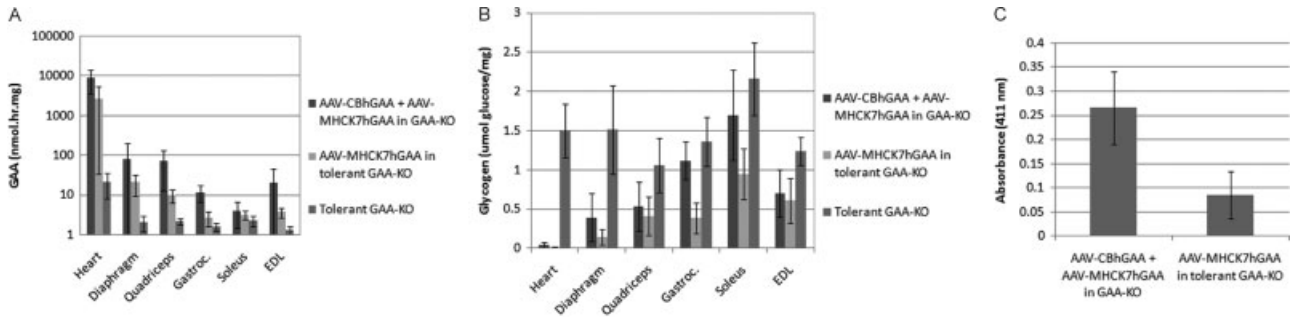

Figure 2. Biochemical correction of striated muscle following the administration of AAV-CBhGAA and AAV2/8-MCHK7hGAA to GAA-KO mice or AAV2/8-MCHK7hGAA to tolerant GAA-KO mice. GAA activity and glycogen content in the indicated striated muscles, 18 weeks following AAV2/8 vector administration. AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp) were administered intravenously at 3 months of age to GAA-KO mice ($n = 4$) and AAV2/8-MHCK7hGAA (7×10^{11} vp) was administered to tolerant GAA-KO mice ($n = 5$). Sham-treated tolerant GAA-KO mice were negative controls ($n = 4$). Data are the mean ± SE. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in (A). (C) ELISA for anti-GAA IgG1 at 18 weeks following vector administration

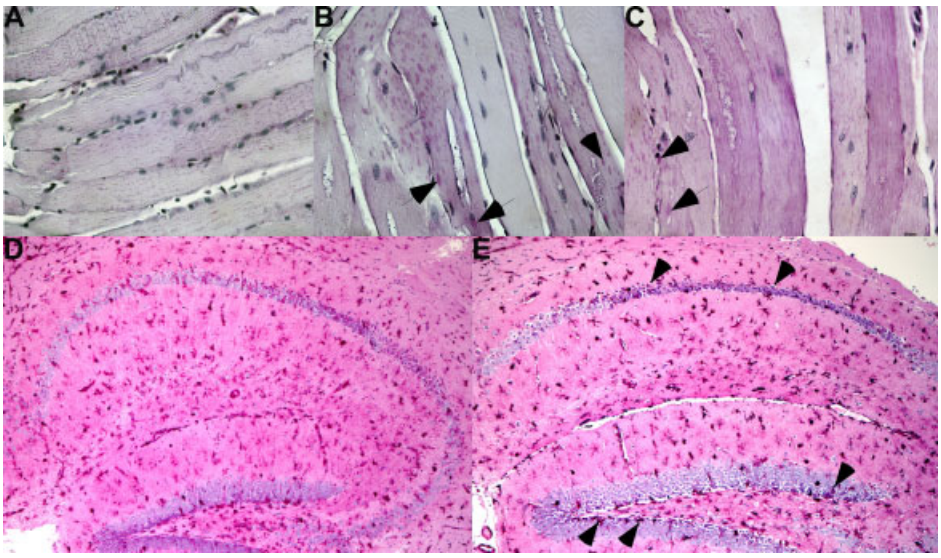

Figure 3. Glycogen staining of gastrocnemius muscle. Periodic acid-Schiff staining of tissues 18 weeks following the intravenous administration of vectors to GAA-KO mice. (A) Gastrocnemius following the administration of dual vectors, both AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp). (B) Gastrocnemius following the administration of AAV2/8-MHCK7hGAA (7×10^{11} vp). (C) Gastrocnemius for sham-treated, age-matched GAA-KO mouse sample shown for comparison. (D) Hippocampus following the administration of dual vectors, both AAV2/8-CBhGAA (1×10^{11} vp) and AAV2/8-MHCK7hGAA (7×10^{11} vp). (E) Hippocampus for sham-treated, age-matched GAA-KO mouse sample shown for comparison. Glycogen-filled vacuoles representing accumulated lysosomal glycogen indicated (arrows). Focal areas of glycogen staining indicated (arrowheads). Original magnification for gastrocnemius: ×400. Original magnification for hippocampus: ×100

therapeutic target in Pompe disease, and therefore the improved correction of glycogen storage in that muscle is an important marker for efficacy [22]. Evidence for a more global correction of glycogen storage was demonstrated in the brain of GAA-KO mice treated with both AAV2/9-MHCK7hGAA and AAV2/8-LSPhGAA, which trended towards decreased glycogen content in the cerebrum ($p = 0.08$) compared to AAV2/9-MHCK7hGAA-treated GAA-KO mice (Figures 3D to 3E). The basis for improved efficacy from dual vector treatment was revealed by western blot analysis of plasma, which revealed the presence of circulating 110 kDa GAA precursor that could be taken up through mannose-6-phosphate receptors by untransduced cells (Figure 5A). Furthermore, the presence of transgene expression in the liver was detected only dual-vector treated mice (Figure 5B). The presence of increased GAA in the quadriceps confirmied that liver expression from AAV2/8-LSPhGAApA was responsible for improved biochemical correction in dual vector-treated mice, through secretion of GAA from the liver and receptor-mediated uptake in skeletal muscle (Figure 5B).

The absence of antibody formation in GAA-KO mice treated with both AAV2/9-MHCK7hGAA and AAV2/8-LSPhGAA demonstrated that immune tolerance had been established by hepatic expression of GAA (Figure 6A). Further evidence for efficacy was demonstrated by increased Rotarod latency in dual vector-treated GAA-KO

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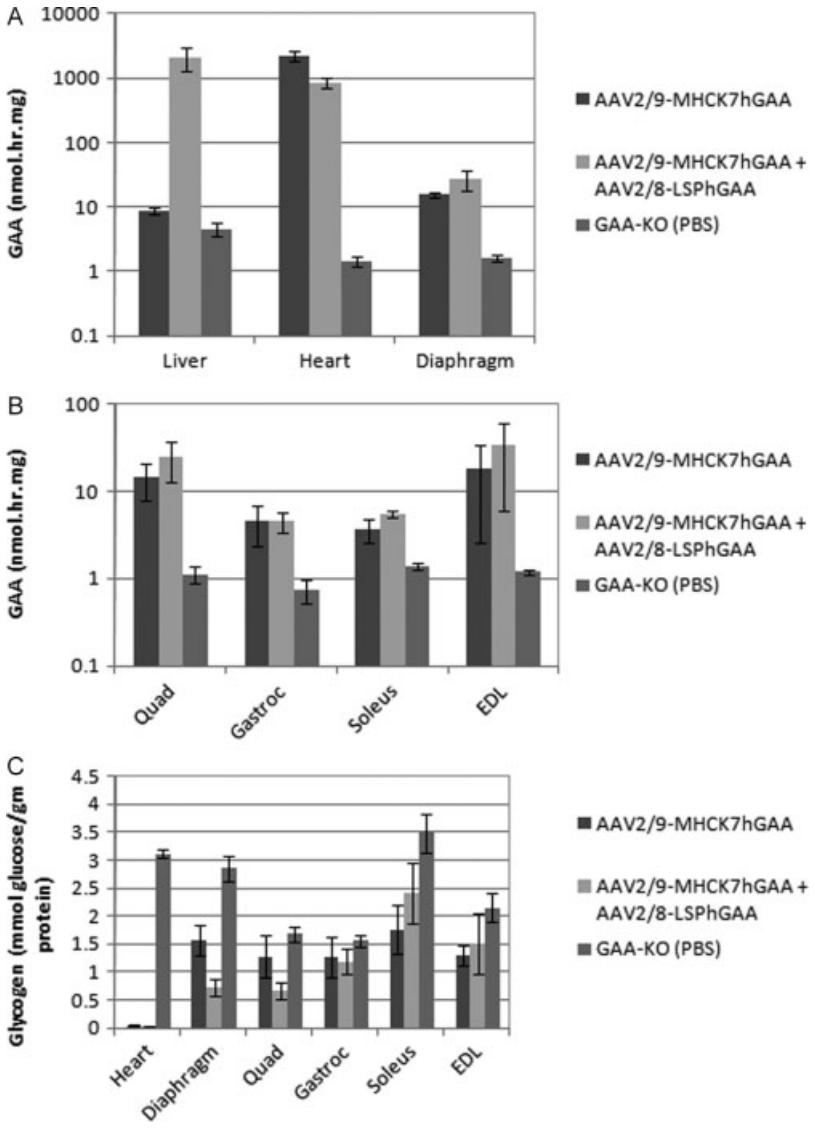

Figure 4. Biochemical correction of striated muscle following the administration of AAV2/9-MHCK7hGAA ± AAV2/8-LSPhGAA. GAA activity and glycogen content in the indicated striated muscle following AAV vector administration. Either AAV2/9-MHCK7hGAA (1×10^{11} vp; $n = 4$), or both AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp; $n = 6$) were administered to GAA-KO mice at 3 months of age, and tissues were analyzed 18 weeks later. One mouse treated with both vectors died of unknown causes before the end of the experiment. Mock-treated GAA-KO mice were negative controls ($n = 4$). Data are the mean ± SE. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in (A)

mice compared to GAA-KO mice that were treated with AAV2/9-MHCK7hGAA alone (Figure 6B).

The effectiveness of dual vector administration was evaluated in a challenging model for gene therapy, namely older GAA-KO mice. The intractability of advanced Pompe disease to biochemical correction has been emphasized by ERT and gene therapy experiments [23–26]. Although GAA activity was elevated by dual vector administration in the heart and diaphragm (Figure 7A), glycogen content was reduced only in the heart of these old GAA-KO mice, in contrast to GAA-KO mice treated at a young age where glycogen storage was also reduced in skeletal muscles (Figure 7B versus Figure 4B). Thus, treatment of advanced Pompe disease remains a significant obstacle for the development of new therapy in Pompe disease.

The role of receptor-mediated uptake and lysosomal targeting of GAA was investigated in DKO mice that were deficient in both GAA and GAA uptake, which were generated by crossing GAA-KO mice with muscle-specific cation-independent mannose-6-phosphate receptor KO mice. The AAV8-pseudotyped vectors, AAV-MHCK7hGAA (1×10^{11} vp/mouse) and AAV-LSPhGAA (2×10^{10} vp/mouse) were administered simultaneously in an attempt to prevent the formation of anti-GAA antibodies observed following AAV-MHCK7hGAA with fewer vector particles of AAV-LSPhGAA; however, anti-GAA antibodies were detected in all mice following dual vector administration (not shown). Nonetheless, significantly elevated GAA activity was observed in the muscles of mice following vector administration compared to untreated GAA-KO mice

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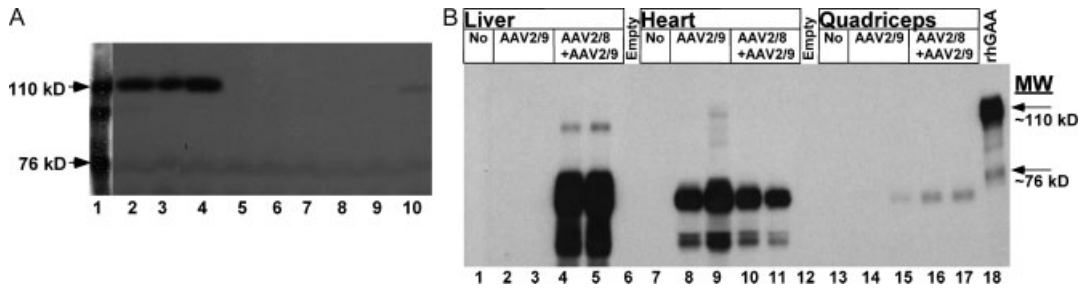

Figure 5. Western blot detection of GAA. Each lane represents an individual mouse. (A) Plasma samples from GAA-KO mice (lanes 2–4) following the administration of dual vectors, AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp); tolerant GAA-KO mice (lanes 5–7) following the administration of AAV2/9-MHCK7hGAA (1×10^{11} vp); and sham-treated GAA-KO mice (lanes 8–10). Comparison with recombinant human GAA standard (lane 1) revealed the 110 kDa precursor GAA was present in dual vector treated GAA-KO mice (lanes 2–4), whereas no GAA was detected in tolerant mice treated with AAV2/9-MHCK7hGAA (lanes 5–7) or in GAA-KO mice treated with AAV2/9-MHCK7hGAA (not shown). (B) Detection of GAA in the indicated tissues, following the administration of AAV2/9-MHCK7hGAA (AAV2/9), dual vectors AAV2/8 + AAV2/9), or no vector (No). The 110 kDa precursor GAA was present in the liver of dual vector treated GAA-KO mice, whereas processed GAA of approximately 70 kDa was present in the heart and skeletal muscle of both vector-treated groups

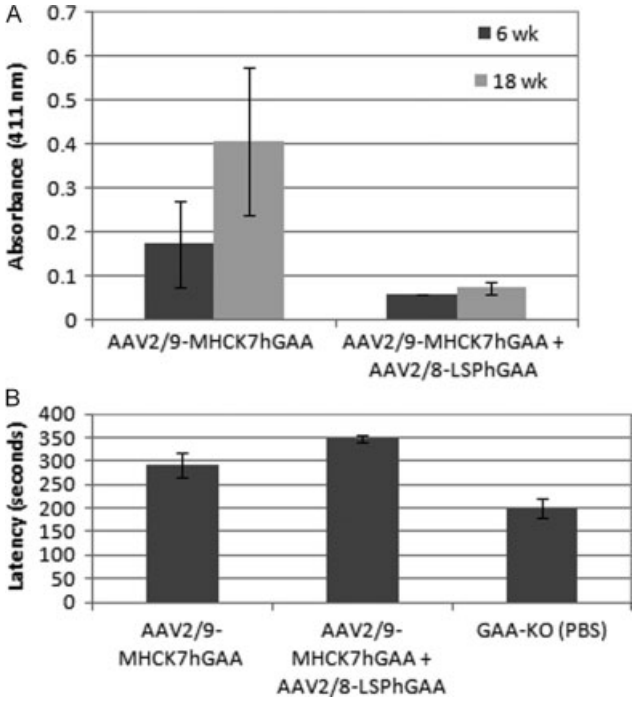

Figure 6. Humoral response and muscle function testing following AAV vector administration. Mean and standard error are shown. (A) ELISA for anti-GAA IgG1 at 6 and 18 weeks following vector administration. (B) Rotarod latency at 6 months of age. GAA-KO mice were treated with AAV2/8-LSPhGAA and AAV2/9-MHCK7hGAA ($n = 5$), AAV/2/9-MHCK7hGAA ($n = 4$), or no vector (GAA-KO; $n = 4$)

(Figure 8A). As expected, DKO mice had equivalently elevated glycogen content in striated muscle to that observed in GAA-KO mice (not shown). Surprisingly, no significant difference in GAA activity between DKO and GAA-KO striated muscle was observed following vector administration, except in the gastrocnemius muscle that contained significantly higher GAA activity in DKO mice compared to GAA-KO mice (Table 1). The latter, unexpected result was attributed to random variation because uptake and expression of GAA would not be adversely affected by the presence of mannose-6-phosphate receptor. Consistent with expectations, glycogen was significantly increased in the majority of muscles from DKO mice compared to GAA-KO mice following vector administration (Figure 8B). The only exception was for the quadriceps, which demonstrated approximately equal glycogen content in both DKO and GAA-KO mice (Table 1). The role of the mannose-6-phophate receptor was implicated not only in the uptake of GAA, but also in the intracellular trafficking to lysosomes by these data.

Discussion

Three factors involved in the response to muscle-targeted gene therapy were evaluated in Pompe disease mice: antibody formation, age, and mannose-6-phosphate receptor availability. Each of these has been implicated in previous research, either preclinical or clinical, as impacting GAA replacement therapy in Pompe disease [3,7,24]. Of these factors, antibody formation was least relevant, apparently due to antibodies being limited to the extracellular space such that muscle transduction was unaffected. Suppression of antibody formation did not improve the response of old GAA-KO mice to gene therapy. Mannose-6-phosphate receptor availability was implicated as a critical factor in the response to gene therapy because glycogen clearance was dependent upon mannose-6-phosphate receptor expression despite the presence of markedly elevated muscle GAA activity.

The advantages of muscle-restricted expression with an AAV vector have been demonstrated in mouse models of muscular dystrophy. AAV vectors evaded cellular immune responses against foreign proteins expressed in muscle in normal mice, possibly because dendritic cells were transduced inefficiently [27]; however, cellular immune responses were demonstrated to ubiquitously expressed foreign proteins in mice with muscular dystrophy. Dystrophin-deficient mdx mice had increased immune

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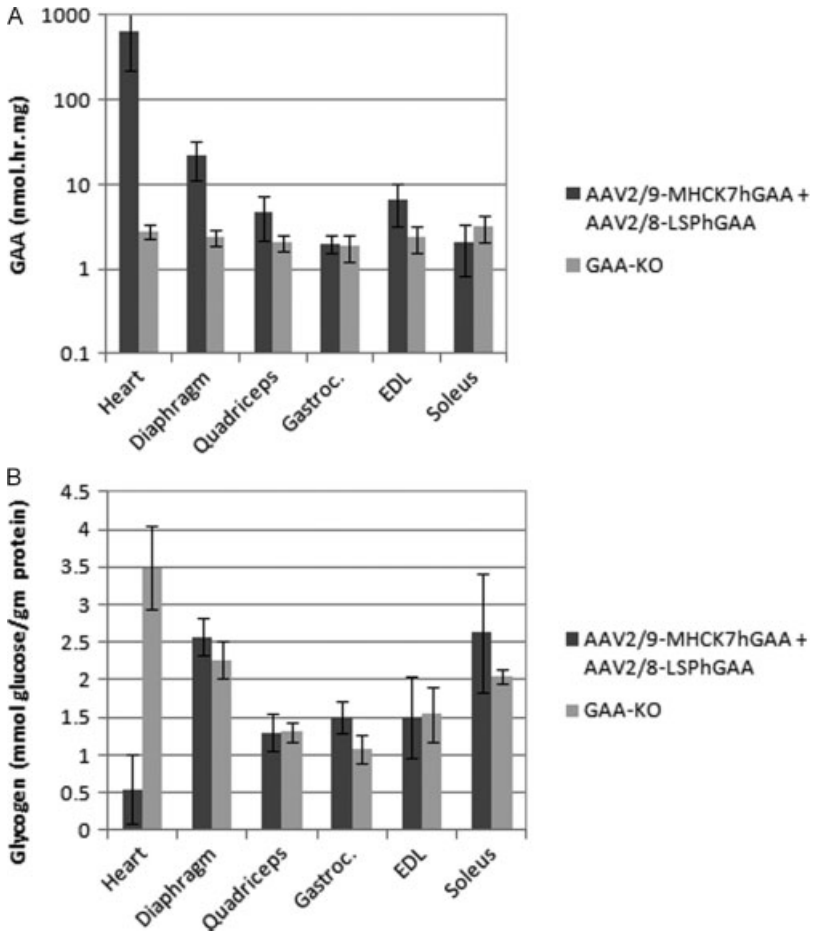

Figure 7. Biochemical correction of striated muscle following the administration of AAV2/9-MHCK7hGAA ± AAV2/8-LSPhGAA to old Pompe disease mice. GAA activity and glycogen content in the indicated striated muscles following AAV vector administration. AAV2/8-LSPhGAA (1×10^{11} vp) and AAV2/9-MHCK7hGAA (1×10^{11} vp) were injected intravenously at 15 months of age and tissues were analyzed 12 weeks later ($n = 7$). Three mice died of unknown causes, most likely age-related, before the end of the experiment. Sham-treated, 11-month-old mice were analyzed as controls for old GAA-KO mice ($n = 3$). Data are the mean ± SD. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in (A)

responses to β-galactosidase expression driven by a cytomegalovirus (CMV) regulatory cassette compared to nondeficient mice, and substitution of an MCK regulatory cassette/enhancer delayed the onset of the immune reaction in mdx mice [28]. The lymphocytic infiltration in response to MCK regulatory cassette-driven β-galactosidase was predominantly $CD4^+$ lymphocytes in mdx mice [28], as was the lymphocytic infiltrative response to MCK-driven GAA in Pompe disease mice [21]. Substitution of an MCK regulatory cassette/enhancer for a CMV regulatory cassette/enhancer in an AAV vector encoding γ-sarcoglycan reduced the immune response and increased levels of transgene expression in γ-sarcoglycan-deficient mice; moreover, CTL assays were positive against LacZ and not against γ-sarcoglycan, when each was expressed with the CMV regulatory cassette [29,30].

Investigating the role of antibody formation in determining the efficacy of muscle-targeted gene therapy was inspired by knowing the relevance of cell-mediated responses to gene therapy in Pompe disease mice. We described the avoidance of a cellular immune response against muscle-targeted gene therapy in GAA-KO mice by muscle-restricted expression of GAA with an AAV vector pseudotyped as AAV2/6 (AAV-MCKhGAA) [21]. By contrast to the analogous vector containing the hybrid CB regulatory cassette (AAV-CBhGAA), which expressed GAA only transiently, AAV-MCKhGAA expressed GAA at high levels 6 weeks after intramuscular administration [21]. The presence of anti-GAA antibodies did not prevent the correction of glycogen content in the skeletal muscle of GAA-KO mice, presumably related to the stable transduction of individual myofibers. Glycogen content was significantly reduced in the injected gastrocnemius with AAV-MCKhGAA, whereas it was not reduced with AAV-CBhGAA. Similarly, an improved vector containing the MHCK7 regulatory cassette to drive GAA transduced skeletal muscle efficiently but provoked anti-GAA antibody formation without preventing glycogen clearance [16]. However, it remained unclear whether antibody formation also impaired the response to muscle-specific GAA expression in GAA-KO mice, and current

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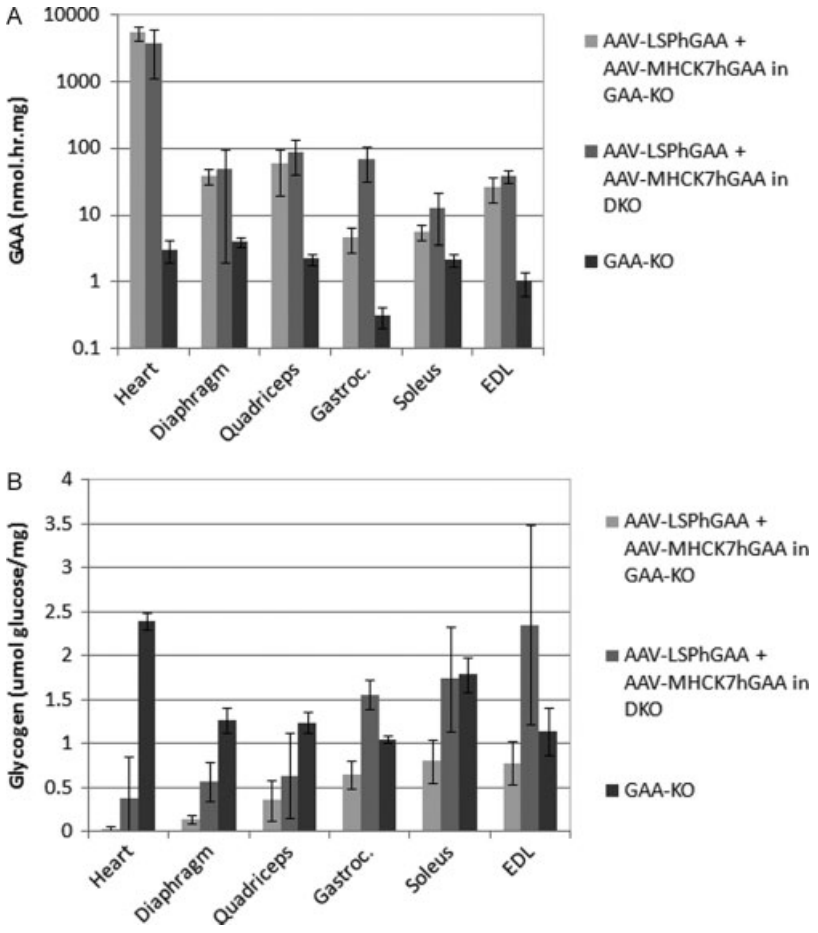

Figure 8. Impaired biochemical correction of striated muscle in the absence of mannose-6-phosphate receptor expression following the administration of AAV2/8-MHCK7hGAA and AAV2/8-LSPhGAA. GAA activity and glycogen content in the indicated striated muscle following AAV vector administration. AAV2/8-MHCK7hGAA (7×10^{11} vp) and AAV2/8-LSPhGAA (2×10^{10} vp) were injected intravenously at 3 months to DKO mice ($n = 5$) and to GAA-KO mice ($n = 4$), and tissues were analyzed 18 weeks later. Mock-treated GAA-KO mice were negative controls (GAA-KO; $n = 4$). Data are the mean ± SE. (A) GAA activity in the heart, diaphragm, quadriceps, gastrocnemius, soleus and EDL. (B) Glycogen content for striated muscles of GAA-KO mice in (A)

Table 1. Comparison of dual vector administration in DKO and GAA-KO mice

Muscle	GAA decrease in DKO, versus GAA-KO (%)	GAA (*p*, DKO versus GAA-KO)	Glycogen increase in DKO, versus GAA-KO (%)	Glycogen (*p*, DKO versus GAA-KO)
Heart	32	0.3	1300	0.20
Diaphragm	−26	0.7	410	0.008
Quadriceps	−49	0.4	180	0.3
Gastrocnemius	−1400	0.01	240	0.00006
Soleus	−120	0.2	220	0.02
EDL	−50	0.08	303	0.03

studies indicate that the impact of antibody formation is subtle.

Previous studies had suggested that the transduction of skeletal muscle following systemic delivery of myotrophic AAV vectors encoding GAA could circumvent the obstacles to enzyme replacement therapy and liver-targeted gene therapy in Pompe disease, including anti-GAA antibodies and mannose-6-phosphate receptor down-regulation in mature skeletal muscle [2,3]. AAV2/6 vectors transduced striated muscle with high efficiency following intravenous administration in mice, if the vector is co-administered with vascular endothelium growth vector (VEGF) to increase vascular permeability [31]. At higher particle numbers an AAV2/6 vector containing the CMV regulatory cassette to drive β-galactosidase expression effectively transduce striated muscle in the absence of VEGF, and the proportion of transduced myofibers was much reduced compared to the level achieved by VEGF co-administration [31]. An AAV2/6 vector containing an abbreviated MCK regulatory cassette/enhancer (CK6 regulatory cassette) transduced skeletal muscle with lower efficiency compared to an analogous vector containing a CMV

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regulatory cassette/enhancer; however, the MCK-driven β-galactosidase expression persisted longer than CMV-driven expression. In contrast to abbreviated expression with the CMV regulatory cassette, the CK6 regulatory cassette prolonged the expression of dystrophin in mdx mice for at least 6 weeks and enhanced the efficacy of muscle-targeted GAA expression [32]. These studies implied that muscle-specific transgene expression would more stably achieve biochemical correction in Pompe disease. However, the current study has revealed that direct muscle transduction failed to circumvent the limitation posed by low mannose-6-phophate receptor expression.

Muscle-targeted gene therapy has distinct advantages for gene therapy in Pompe disease, including stable transduction in the absence of cell-mediated immune responses against GAA. Remaining issues that should drive future research include reducing the vector dose requirements, which remain high. Importantly, the limitations posed by reduced mannose-6-phosphate receptor availability in type II myofibers should be addressed by new therapeutic strategies.

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References

1. Amalfitano A, Bengur AR, Morse RP, *et al*. Recombinant human acid α-glucosidase enzyme therapy for infantile glycogen storage disease type II: Results of a phase I/II clinical trial. *Genet Med* 2001; **3:** 132–138.
2. Raben N, Danon M, Gilbert AL, *et al*. Enzyme replacement therapy in the mouse model of Pompe disease. *Mol Genet Metab* 2003; **80:** 159–169.
3. Raben N, Fukuda T, Gilbert AL, *et al*. Replacing acid alpha-glucosidase in Pompe disease: recombinant and transgenic enzymes are equipotent, but neither completely clears glycogen from type II muscle fibers. *Mol Ther* 2005; **11:** 48–56.
4. Kishnani PS, Corzo D, Nicolino M, *et al*. Recombinant human acid α-glucosidase: Major clinical benefits in infantile-onset Pompe disease. *Neurology* 2007; **68:** 99–109.
5. Kishnani PS, Nicolino M, Voit T, *et al*. Chinese hamster ovary cell-derived recombinant human acid alpha-glucosidase in infantile-onset Pompe disease. *J Pediatr* 2006; **149:** 89–97.
6. Desnick RJ. Enzyme replacement and enhancement therapies for lysosomal diseases. *J Inher Metab Dis* 2004; **27:** 385–410.
7. Kishnani PS, Goldenberg PC, DeArmey SL, *et al*. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab* 2010; **99:** 26–33.
8. Raben N, Jatkar T, Lee A, *et al*. Glycogen stored in skeletal but not in cardiac muscle in acid alpha-glucosidase mutant (Pompe) mice is highly resistant to transgene-encoded human enzyme. *Mol Ther* 2002; **6:** 601–608.
9. Winkel LP, Van den Hout JM, Kamphoven JH, *et al*. Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow-up. *Ann Neurol* 2004; **55:** 495–502.
10. Zhu Y, Li X, Kyazike J, *et al*. Conjugation of mannose 6-phosphate-containing oligosaccharides to acid alpha-glucosidase improves the clearance of glycogen in pompe mice. *J Biol Chem* 2004; **279:** 50336–50341.
11. Sun B, Bird A, Young SP, *et al*. Enhanced response to enzyme replacement therapy in pompe disease after the induction of immune tolerance. *Am J Hum Genet* 2007; **81:** 1042–1049.
12. Wylie AA, Pulford DJ, McVie-Wylie AJ, *et al*. Tissue-specific inactivation of murine M6P/IGF2R. *Am J Pathol* 2003; **162:** 321–328.
13. Gao GP, Alvira MR, Wang L, *et al*. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Nat Acad Sci USA* 2002; **99:** 11854–11859.
14. Wang L, Takabe K, Bidlingmaier SM, Ill CR, Verma IM. Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc Natl Acad Sci USA* 1999; **96:** 3906–3910.
15. Salva MZ, Himeda CL, Tai PW, *et al*. Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. *Mol Ther* 2007; **15:** 320–329.
16. Sun B, Young SP, Li P, *et al*. Correction of multiple striated muscles in murine Pompe disease through adeno-associated virus-mediated gene therapy. *Mol Ther* 2008; **16:** 1366–1371.
17. Franco LM, Sun B, Yang X, *et al*. Evasion of immune responses to introduced human acid alpha-glucosidase by liver-restricted expression in glycogen storage disease type II. *Mol Ther* 2005; **12:** 876–884.
18. Raben N, Lu N, Nagaraju K, *et al*. Conditional tissue-specific expression of acid alpha-glucosidase (GAA) gene in the GAA knockout mice: implications for therapy. *Hum Mol Genet* 2001; **10:** 2039–2047.
19. Sun B, Zhang H, Franco LM, *et al*. Efficacy of an adeno-associated virus 8-pseudotyped vector in glycogen storage disease type II. *Mol Ther* 2005; **11:** 57–65.
20. Amalfitano A, McVie-Wylie AJ, Hu H, *et al*. Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-alpha-glucosidase. *Proc Natl Acad Sci USA* 1999; **96:** 8861–8866.
21. Sun B, Zhang H, Franco LM, *et al*. Correction of glycogen storage disease type II by an adeno-associated virus vector containing a muscle-specific promoter. *Mol Ther* 2005; **11:** 889–898.
22. Mah C, Fraites TJ Jr, Cresawn KO, *et al*. A new method for recombinant adeno-associated virus vector delivery to murine diaphragm. *Mol Ther* 2004; **9:** 458–463.
23. Raben N, Jatkar T, Lee A, *et al*. Glycogen stored in skeletal but not in cardiac muscle in acid alpha-glucosidase mutant (Pompe) mice is highly resistant to transgene-encoded human enzyme. *Mol Ther* 2002; **6:** 601–608.
24. Xu F, Ding E, Migone F, *et al*. Glycogen storage in multiple muscles of old GSD-II mice can be rapidly cleared after a single intravenous injection with a modified adenoviral vector expressing hGAA. *J Gene Med* 2005; **7:** 171–178.
25. Sun B, Zhang H, Bird A, *et al*. Impaired clearance of accumulated lysosomal glycogen in advanced Pompe disease despite high-level vector-mediated

transgene expression. *J Gene Med* 2009; **11:** 913–20.
26. Ziegler RJ, Bercury SD, Fidler J, *et al*. Ability of adeno-associated virus serotype 8-mediated hepatic expression of acid alpha-glucosidase to correct the biochemical and motor function deficits of presymptomatic and symptomatic Pompe mice. *Hum Gene Ther* 2008; **19:** 609–621.
27. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* 1998; **72:** 4212–4223.
28. Yuasa K, Sakamoto M, Miyagoe-Suzuki Y, *et al*. Adeno-associated virus vector-mediated gene transfer into dystrophin-deficient skeletal muscles evokes enhanced immune response against the transgene product. *Gene Ther* 2002; **9:** 1576–1588.
29. Cordier L, Hack AA, Scott MO, *et al*. Rescue of skeletal muscles of gamma-sarcoglycan-deficient mice with adeno-associated virus-mediated gene transfer. *Mol Ther* 2000; **1:** 119–129.
30. Cordier L, Gao GP, Hack AA, *et al*. Muscle-specific promoters may be necessary for adeno-associated virus-mediated gene transfer in the treatment of muscular dystrophies. *Hum Gene Ther* 2001; **12:** 205–215.
31. Gregorevic P, Blankinship MJ, Allen JM, *et al*. Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* 2004; **10:** 828–834.
32. Hauser MA, Robinson A, Hartigan-O'Connor D, *et al*. Analysis of muscle creatine kinase regulatory elements in recombinant adenoviral vectors. *Mol Ther* 2000; **2:** 16–25.