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BRIEF COMMUNICATION

Sustained correction of glycogen storage disease type II using adeno-associated virus serotype 1 vectors

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Glycogen storage disease type II (GSDII) is caused by a lack of functional lysosomal acid α-glucosidase (GAA). Affected individuals store glycogen in lysosomes beginning during gestation, ultimately resulting in fatal hypertrophic cardiomyopathy and respiratory failure. We have assessed the utility of recombinant adeno-associated virus (rAAV) vectors to restore GAA activity in vivo in a mouse model of GSDII ($Gaa^{-/-}$). A single systemic administration of a rAAV serotype 1 (rAAV1) vector to neonate animals resulted in restored cardiac GAA activity to 6.4 times the normal level (mean = 641 ± 190% of normal ($Gaa^{+/+}$) levels with concomitant glycogen clearance) at 11 months postinjection. Greater than 20% of normal levels of GAA activity were also observed in the diaphragm and quadriceps muscles. Furthermore, functional correction of the soleus skeletal muscle was also observed compared to age-matched untreated $Gaa^{-/-}$ control animals. These results demonstrate that rAAV1 vectors can mediate sustained therapeutic levels of correction of both skeletal and cardiac muscles in a model of fatal cardiomyopathy and muscular dystrophy.
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Muscular dystrophies are a group of diseases characterized by progressive muscle weakness and wasting. No corrective treatments are currently available and care is primarily palliative. Gene therapy provides an attractive therapeutic approach for the treatment of muscular dystrophies. A unique challenge for gene therapy-mediated treatment of muscular dystrophies is the need to achieve widespread correction of all affected tissues. In this work, we demonstrate long-term correction of cardiac, diaphragm, and skeletal muscles after a single intravenous administration of a recombinant adeno-associated virus serotype 1 vector in a murine model of glycogen storage disease type II.

Glycogen storage disease type II (GSDII; Pompe disease; MIM 232300) is an inherited disorder of glycogen metabolism, resulting from a lack of functional lysosomal acid α-glucosidase (GAA). GAA is responsible for cleaving α-1,4 and α-1,6 linkages of lysosomal glycogen, leading to the release of monosaccharides.[1,2] A deficiency of functional GAA results in massive accumulation of glycogen in lysosomal compartments of striated muscle, resulting in disruption of the contractile machinery of the cell. Affected individuals begin storing glycogen *in utero*, ultimately resulting in a variety of pathophysiological effects, the most significant of which are severe cardiomyopathy, respiratory insufficiency, and progressive skeletal myopathy. In its most severe form, clinical presentation of GSDII disease occurs within the first few months of life and culminates in cardio-respiratory failure and death within the first year of life.[3–5] Although there are no established treatments for GSDII, recombinant enzyme replacement therapy is currently being tested in phase I/II clinical trials and, additionally, gene therapy strategies are being intensely pursued. In particular, recombinant adeno-associated virus vector (rAAV)-mediated strategies have yielded the most encouraging results for the treatment of GSD type II.

AAV is a single-stranded DNA-containing, nonpathogenic human parvovirus that is being investigated as a potential gene therapy vector and is currently being assessed in phase I/II clinical trials.[6–9] In recent years, over 40 naturally occurring serotypes have been identified and, to-date, at least nine different AAV serotypes have been developed as candidate gene therapy vectors.[10–16] Several recent studies have highlighted distinct tissue tropisms for each AAV serotype, and several groups have shown that rAAV1-based gene therapy vectors are more efficient at transducing skeletal and cardiac muscle than serotype 2-based vectors.[10,12,14,17–19]

Previous studies demonstrated phenotypic correction of distinct skeletal muscles in mouse and quail models of GSDII disease using rAAV vectors.[20–22] The mouse model of GSDII displays storage of glycogen in all tissues with significant pathology in the heart and skeletal muscle tissues.[23] Fraites *et al* demonstrated that normal levels of GAA activity could be achieved in injected muscle of a GAA knockout mouse model ($Gaa^{-/-}$) after direct

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intramuscular or intracardiac administration of rAAV serotype 2 vector. Furthermore, they showed that intramuscular administration of a rAAV serotype 1 (rAAV1) vector lead to eight-fold normal levels of GAA activity in the injected *tibialis anterior* with concomitant clearance of glycogen, two weeks postinjection.[24]

In the current study, we demonstrate long-term, simultaneous correction of affected heart, diaphragm, and skeletal muscle tissues in a murine model of GSDII using rAAV1 vectors. Recent works have demonstrated widespread transduction in heart and skeletal muscle tissues from neonatal intraperitoneal injection of rAAV1 and rAAV6 vectors in hamster and mouse models, respectively.[25,26] While large-volume intraperitoneal injection is effective in small animal models, we sought to use a more clinically applicable method of intravenous injection. In this light, we administered 5×10^{10} particles of rAAV1 encoding the cytomegalovirus immediate-early promoter driving the human *GAA* gene (rAAV1-CMV-*GAA*) intravenously to 1-day-old (neonatal) $Gaa^{-/-}$ mice. Six and 11 months postinjection, tissues were isolated and assayed for GAA expression. As shown in Figure 1a, supraphysiologic levels of GAA expression were noted in the hearts and diaphragms of treated animals 6 months postinjection and were sustained in hearts out to 11 months, with an average of $641.27 \pm 190\%$ of normal levels. In addition, we observed diaphragm, quadriceps, and lung GAA enzyme activity levels, which were 23.73 ± 7.84, 24.27 ± 14.34, and $51.96 \pm 15.17\%$ of normal levels, respectively, 11 months postinjection. Notably, all the achieved levels were above the widely proposed therapeutic threshold of 20%.[2,4] Low levels of GAA activity were also noted in the soleus muscle and the liver.

To determine whether widespread GAA activity was due to vector-mediated transduction or transfer of active protein from other sites, total cellular DNA was isolated from heart, diaphragm, soleus, quadriceps, liver, and lung and analyzed for the presence of vector genomes by semiquantitative PCR. As shown in Figure 1b, an average of 2.44 ± 0.86 vector genomes per diploid cell were detected in transduced hearts, 11 months postinjection. Lower GAA activities noted in lung, diaphragm, quadriceps, and soleus tissues were likely due, at least in part, to lower levels of vector transduction, indicated by the lower number of vector genome copies per diploid cell. Together, these results suggest that the levels GAA activity noted in the heart, lung, diaphragm, and quadriceps are a direct result of rAAV-mediated transduction.

Interestingly, while a drop in GAA activity levels was noted in hearts and diaphragms from 6 months to 11 months postinjection, the mechanism for this decrease is unknown. The lower 11-month activity levels could not be attributed to a humoral immune response, as we did not detect any serum anti-GAA antibodies (background levels of anti-GAA antibodies as determined by ELISA, 26 weeks postinjection). In addition, there was no significant decline in the vector genome copy numbers in heart tissue (2.66 ± 0.5 vector genomes per diploid cell at 6 months postinjection as compared to 2.44 ± 0.86 at 11 months). Recent studies have indicated a significant gender bias for AAV8-mediated GAA activity.[27] In this study we did not have an even distribution of males and females ($n = 4$ females, 2 males at 6 months and $n = 4$ females, 1 male at 11 months) to make any statistical conclusions, but did not see an appreciable difference in the AAV1-mediated GAA activities between the male and female mice.

Although we noted very high levels of liver transduction (2.27 ± 0.11 vector genomes per diploid cell), only low levels of GAA activity could be detected in the liver. This result was expected, however, because the CMV promoter (which has been shown to be inactive in murine liver) was used to drive the vector-mediated transgene expression in this study.[28] Since approximately 10% of all expressed GAA is shunted to the secretory pathway and can be taken up by distal cells via a mannose-6-phosphate receptor-mediated pathway, it is probable that the low level of liver GAA activity was due to the uptake of secreted GAA protein.[2–5]

Of note, vector genomes could not be detected by PCR in gonads of male animals that were intravenously administered 5×10^{10} particles of rAAV-CMV-*GAA*, 6 months postinjection. While it is possible that the method of detection was not sensitive enough to detect very small copy numbers of vector, the absence of vector sequences or any GAA activity in heart or liver tissue from first generation progeny from parents that were both administered vector (data not shown), further suggest that vertical transmission of vector did not occur from intravenous administration of rAAV1 vector in the newborn.

In addition to the high levels of enzyme activity, as shown in Figure 1d, immunostaining of heart tissue from rAAV1-treated mice (using an anti-human GAA antibody) showed the presence of GAA in almost every cell, both at 6 and 11 months postinjection. GAA-positive cells could be found evenly distributed through the heart tissue as shown in the immunostained whole mount section. Furthermore, as shown in Figure 1c, we also noted a complete absence of accumulated glycogen in the hearts of treated animals, as determined by periodic acid-Schiff's reagent (PAS) staining. Pink staining, indicative of stored glycogen, is apparent in the untreated heart tissues whereas it is undetectable in the hearts of rAAV1-treated mice.

We have not noted any gross histologic abnormalities as a result of overexpression of GAA in cardiac tissues. While it has been suggested that overexpression ($\sim$9000% of normal) of GAA in heart could potentially be toxic, as determined histologically by the presence of irregular-shaped cells, several groups have demonstrated significant overexpression of GAA in heart in the absence of any notable toxicities.[27,29,30] Despite the absence of any noted histologic irregularities resulting from rAAV1-mediated GAA expression, the possibility of toxicity as a result of overexpression cannot be fully discounted and may potentially be associated with the noted decline of cardiac GAA activity from 6 to 11 months postinjection.

Having demonstrated complete restoration of GAA enzymatic activity in heart and partial restoration in other skeletal muscles, we next investigated whether systemic administration of a therapeutic rAAV1 vector could lead to phenotypic correction of skeletal muscle function. The soleus muscle was isolated from treated animals and was assessed for isometric force generation. As shown in Figure 2a, at the maximal stimulation frequency (200 Hz), we noted a marked improvement in

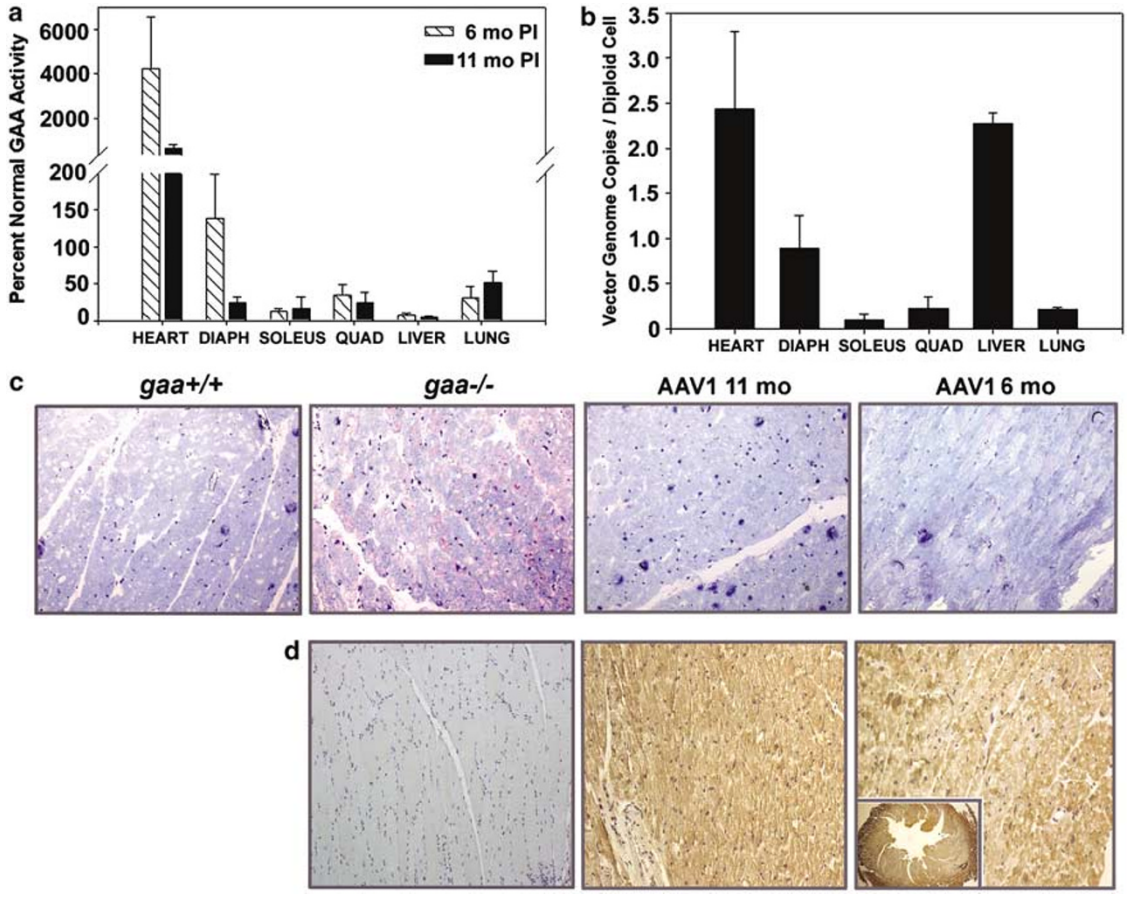

Figure 1 *Intravenous injection of rAAV1 results in simultaneous persistent therapeutic levels of GAA in heart, diaphragm, quadriceps, and lung. All animal procedures were performed in accordance with the University of Florida Institutional Animal Care and Use Committee guidelines. Recombinant AAV1-CMV-GAA was generated using the plasmid p43.2-GAA and packaged and purified as described previously.[24,33] $Gaa^{-/-}$ mice (1-day old) were administered 5×10^{10} particles (30 μl total volume) rAAV1-CMV-GAA intravenously via the superficial temporal vein as described previously.[34] Six ($n=6$) and 11 ($n=5$) months postinjection, heart, diaphragm, soleus, quadriceps, liver, and lung tissues were isolated from treated and age-matched untreated $Gaa^{-/-}$ ($n=3$) and normal C57BL6/129SvJ ($n=3$) mice. (a) Recombinant AAV1 can mediate sustained high levels of GAA in heart, diaphragm, and skeletal muscle. Assays for tissue GAA activity was performed as previously described.[24] Data are represented as percentage of normal levels of GAA in each tissue after subtraction of untreated $Gaa^{-/-}$ tissue levels. (b) Intravenous injection of rAAV1 leads to efficient infection of heart. At 11 months postinjection, heart, diaphragm, soleus, quadriceps, liver, and lung tissues were isolated from treated and age-matched untreated $Gaa^{-/-}$ mice. Vector genome copies were detected as described previously with the following modifications: total DNA was isolated from each tissue using a DNeasy kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocols.[35,36] A total of 1 μg DNA was used for PCR to simultaneously amplify a 697 bp fragment of the human GAA gene (using biotinylated primers 5′-TTGCGGACCAGTTCCTTCAG-3′ and 5′-CCGGTCTCGTTGGTGATGAAA-3′) and a 1087 bp fragment of the endogenous murine hypoxanthine guanine phosphoribosyl transferase gene (using biotinylated primers 5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′). PCR conditions were as follows: hot start denaturation at 94°C for 3 min followed by 30 cycles of 94°C denaturation for 1 min, 57°C annealing for 1 min, and 72°C for 2 min, followed by a 10-min extension at 72°C. (c) Intravenous injection of rAAV1 mediates complete clearance of glycogen in affected heart tissue. Six and 11 months postinjection, heart tissue from $Gaa^{-/-}$ mice administered rAAV1-CMV-GAA intravenously and untreated control $Gaa^{-/-}$ and $Gaa^{+/+}$ mice was fixed 2% glutaraldehyde in PBS, embedded in epon, sectioned, and stained with periodic acid-Schiff (PAS) by standard methods (Richard Allen, Kalamazoo, MI, USA).[23] (d) Intravenous injection of rAAV1 leads to global cardiac GAA. Six and 11 months postinjection, heart tissue from $Gaa^{-/-}$ mice administered rAAV1-CMV-GAA intravenously and untreated age-matched control $Gaa^{-/-}$ mice was fixed in 10% neutral buffered formalin, processed, and paraffin embedded by standard procedures. Sections (4 μm) were immunostained by the University of Florida Pathology Core using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) as per the manufacturer's protocols using a rabbit-anti-human GAA polyclonal antibody (1 : 10 000). Positive staining was visualized using 3,3′-diaminobenzidine (DAB) followed by counterstaining with Gill's hematoxylin (Vector Laboratories, Burlingame, CA, USA). Photographs were taken using a Zeiss light mircoscope, Olympus camera, and MagnaFire digital recording system. Magnification $\times 200$ for all sections except whole mount immunostained heart section (magnification $\times 25$).*

soleus muscle function with a contractile force of 18.96 ± 1.36 N/cm², as compared to similarly age-matched control $Gaa^{-/-}$ animals (14.43 ± 1.08 N/cm²) and even animals 5 months younger in age (17 ± 0.84 N/cm²). Though significant functional improvement was noted in soleus muscle ($P < 0.05$, compared to age-matched controls), the levels of GAA activity (average of 15.76% of normal levels) were less than the predicted amount that would completely eliminate clinical symptoms, suggesting that the level of functional improvement may not be a linear relationship with GAA activity. One potential explanation for the greater contractile force noted in the rAAV1-treated mice (compared to untreated controls) is that despite the lower levels of GAA activity, a small decrease in accumulated glycogen may have resulted in improved skeletal muscle function. As shown in Figure 2b, PAS staining of soleus muscle from rAAV1-treated mice shows a reduction in glycogen as compared

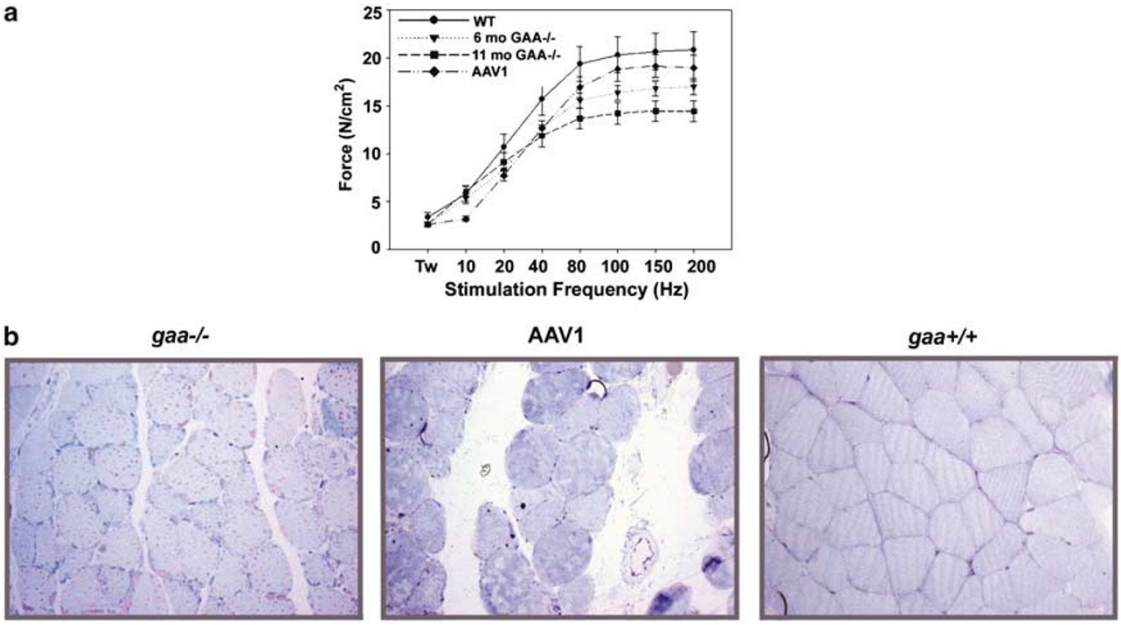

Figure 2 *Intravenous injection of rAAV1 leads to correction of soleus skeletal muscle function and reduction of stored glycogen. (a) Skeletal muscle contractile force was measured as described previously using intact soleus muscle isolated from treated Gaa$^{-/-}$ mice, untreated 6- and 12-month-old ($n=8$ and 4, respectively) Gaa$^{-/-}$ mice, and normal C57BL6/129SvJ mice ($n=5$).[24] (b) PAS staining of soleus muscle was performed using standard methods.[23] Magnification $\times 400$.*

to untreated mouse soleus. While previous studies have suggested that accumulated glycogen in skeletal muscle was resistant to clearance, recent work by Raben *et al*[31,32] have demonstrated a heterogeneous ability of *Gaa*$^{-/-}$ skeletal muscle to clear glycogen in response to GAA activity. In particular, complete clearance of glycogen can occur in skeletal muscle composed predominantly by type I fibers, such as the soleus muscle.

The success of this AAV-mediated gene therapy approach and the improved functional phenotype is due, in large part, to the correction of the metabolic defect. Also, since GAA is expressed from within the affected cells, the trafficking of the protein to the lysosome follows the usual pathway for lysosomal targeting unlike any therapeutic approach in which the protein is supplied to the cell surface. Intracellular delivery of GAA also potentially avoids antibody-mediated redistribution of therapeutic protein delivered in the circulation. Furthermore, therapeutic levels of GAA activity in the heart, diaphragm, and quadriceps are likely to have resulted in overall enhanced general health of the animal, which preserved the function of all affected tissues.

In this work, we demonstrate that simultaneous correction of the most severely affected tissues in GSDII, namely the heart, diaphragm, quadriceps, and soleus, can be achieved using rAAV1-based gene therapy vectors. Furthermore, we demonstrate that global cardiac transduction can be attained by simple systemic administration, rather than through invasive direct delivery approaches. These results demonstrate the potential for early intravenous administration of rAAV serotype vectors, which have the ability to escape the vasculature leading to systemic transduction of target tissues. This approach may be applicable for the treatment of many forms of cardiac and skeletal myopathy.

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