Rescue of enzyme deficiency in embryonic diaphragm in a mouse model of metabolic myopathy: Pompe disease

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Summary

Several human genetic diseases that affect striated muscle have been modeled by creating knockout mouse strains. However, many of these are perinatal lethal mutations that result in death from respiratory distress within hours after birth. As the diaphragm muscle does not contract until birth, the sudden increase in diaphragm activity creates permanent injury to the muscle causing it to fail to meet respiratory demands. Therefore, the impact of these mutations remains hidden throughout embryonic development and early death prevents investigators from performing detailed studies of other striated muscle groups past the neonatal stage. Glycogen storage disease type II (GSDII), caused by a deficiency in acid α-glucosidase (GAA), leads to lysosomal accumulation of glycogen in all cell types and abnormal myofibrillogenesis in striated muscle. Contractile function of the diaphragm muscle is severely affected in both infantile-onset and late-onset individuals, with death often resulting from respiratory failure. The knockout mouse model of GSDII survives well into adulthood despite the gradual weakening of all striated muscle groups. Using this model, we investigated the delivery of recombinant adeno-associated virus (rAAV) vectors encoding the human *GAA* cDNA to the developing embryo. Results indicate specific high-level transduction of diaphragm tissue, leading to activity levels up to 10-fold higher than normal and restoration of normal contractile function. Up to an estimated 50 vector copies per diploid genome were quantified in treated diaphragms. Histological glycogen staining of treated diaphragms revealed prevention of lysosomal glycogen accumulation in almost all fibers when compared with untreated controls. This method could be employed with disease models where specific rescue of the diaphragm would allow for increased survival and thus further investigation into the impact of the gene deletion on other striated muscle groups.

Key words: In utero, rAAV, Gene therapy, GSDII, Acid alpha-glucosidase, Diaphragm

Introduction

Knockout mouse models are useful tools for studying the effects of specific gene deletions on biochemical, cellular, and organ functions and interactions. Mouse models created with mutations in the gene encoding mitochondrial trifunctional protein (MTP) and muscle-specific genes, such as sarco(endo)plasmic reticulum Ca^{2+}-ATPase (*Serca1*), and in the gene encoding the cytoskeletal protein Cypher, are viable throughout embryonic development, but succumb soon after birth because of respiratory failure. The authors propose that the effect of the deficient protein in diaphragm muscle is hidden until birth when the diaphragm is first needed for respiration. The sudden, intense exertion causes the muscle to fail (Ibdah et al., 2001; Pan et al., 2003; Zhou et al., 2001). Unfortunately, the effects on muscle function and pathology cannot be studied later in life because of early respiratory insufficiency and mortality. Selective rescue of a single gene defect can be accomplished by gene transfer using viral vectors. If the diaphragm were selectively transduced to allow vector-derived protein expression, then the mice may survive long enough to allow for further evaluation. Such a 'vector-transgenic' would provide specific rescue in the neonate, allowing for comprehensive examination of the postnatal consequences known to be of developmental importance in the context of adult disease.

Glycogen storage disease type II (GSDII) or Pompe disease is a rare autosomal recessive disorder caused by a defect in the gene encoding acid α-glucosidase (GAA) (Hirschhorn and Reuser, 2000; Raben et al., 2002). In the lysosome, the 110 kDa precursor form of GAA is successively cleaved at both termini to produce 95, 76 and 67 kDa peptides of which the 76 and 67 kDa isoforms are catalytically active (Hoefsloot et al., 1990; Wisselaar et al., 1993). GAA reduces lysosomal glycogen and maltose to glucose by hydrolyzing α-1,4 and α-1,6 linkages at acid pH. In the absence of GAA, glycogen accumulates within the lysosomes and cytoplasm of all cells. Pathophysiology of the disease is limited to striated muscle, where accumulated glycogen causes disruption of the contractile apparatus, eventually leading to muscle weakness (Hers, 1963; Pompe, 1932). The age of onset, as well as the severity and progression of muscle weakness, is usually dependent on the level of residual GAA activity. Mutations leading to no detectable active GAA protein cause infantile-onset GSDII. This form of the disease manifests as severe

cardiac and skeletal myopathies, resulting in death before 1 or 2 years of age due to cardiorespiratory failure. Individuals diagnosed with late-onset GSDII typically have residual levels of GAA activity. These patients show less cardiac involvement, and instead suffer from respiratory and proximal muscle weakness with death eventually resulting from respiratory insufficiency (Hirschhorn and Reuser, 2000).

The knockout mouse model used in these studies was produced by interrupting exon 6 of the mouse *GAA* cDNA with a neomycin cassette (Raben et al., 1998). In this mouse model, glycogen is found highly concentrated in heart, skeletal muscle, diaphragm and liver as early as 3 weeks of age. By 18 months, the mice exhibit extreme muscle wasting, resulting in kyphosis, anterior and posterior weakness, and difficulty breathing (Raben et al., 1998; Raben et al., 2000).

Currently, there is no widely accessible treatment for GSDII. Enzyme replacement therapy (ERT) has progressed significantly in the treatment of lysosomal storage disorders and is currently being assessed for the treatment of GSDII in a Phase I/II clinical trial (http://clinicaltrials.gov/ct/show/NCT00053573) (Amalfitano et al., 2001; Eng et al., 2001; Kakkis et al., 2001; Van den Hout et al., 2001). The premise of ERT is based on the uptake of circulating enzyme through mannose 6-phosphate receptor-mediated endocytosis.

We, and others, have shown the potential of viral vectors in treating GSDII (Chen and Amalfitano, 2000). Direct intramuscular, intracardiac or portal vein injections of replication-defective, E1-deleted adenoviral vectors containing the human *GAA* cDNA (rAd-*GAA*) into animal models of the disease resulted in production of therapeutic levels of GAA and corresponding reduction of intracellular glycogen stores (Amalfitano et al., 1999; Ding et al., 2001; Ding et al., 2002; Martin-Touaux et al., 2002; Pauly et al., 1998; Pauly et al., 2001; Tsujino et al., 1998). Cross-correction resulting from uptake of secreted enzyme by distant organs, such as heart and skeletal muscle, was achieved through high-level rAd-*GAA* transduction of the liver in GAA-deficient animals. Regrettably, anti-GAA antibody titers increased, resulting in a 100-fold reduction in the initial level of GAA enzyme (Ding et al., 2002). Clinical applications of gene replacement strategies using recombinant adenoviral vectors are currently limited because of toxicity and transient expression due to host immune responses.

A newer class of gene therapy vectors, based on the adeno-associated virus (AAV), has become a popular vehicle for delivering genes to cells for the treatment of several diseases. Two recent studies demonstrated the use of recombinant AAV vectors encoding the human *GAA* cDNA for gene replacement in deficient human cells and in two animal models (Fraites et al., 2002; Lin et al., 2002). Lin et al. injected rAAV directly into the pectoral muscle of the Japanese quail model and demonstrated expression of GAA, correlating with a decrease in intracellular glycogen content and an increase in muscle performance as measured by wing motion (Lin et al., 2002). Our group showed the effectiveness of intramyocardial and intramuscular delivery of a similar vector to the exon 6-knockout mouse model of GSDII (Fraites et al., 2002). Both delivery methods established that near normal levels of GAA activity could be achieved, and, in the case of the direct intramuscular injections, the increase in GAA activity correlated with an increase in muscle function.

Our objective was to deliver rAAV vectors encoding human *GAA* to the developing mouse model of GSDII in an effort to prevent the disease state in target organs. However, there is very limited information on murine in utero gene delivery. The first murine in utero vector transduction experiments were conducted using recombinant adenoviral vectors. Pioneer studies involved using adenovirus to deliver the *lacZ*, *CFTR* or Factor IX gene into the amniotic fluid of fetal *Cftr*$^{-/-}$ mice at 15 days post-coitum (pc), three-quarters of the way through the gestation period. Expression of the transgene was detected in the liver, epidermis, lung and gastrointestinal tract (Cohen et al., 1998; Douar et al., 1997; Holzinger et al., 1995; Larson et al., 1997; Schneider et al., 1999). Several in utero *CFTR*-treated knockout mice were rescued from lethal intestinal obstruction (Cohen et al., 1998). Other studies using adenovirus were directed toward liver transduction via intrahepatic, intraperitoneal, yolk sac vessel or retro-orbital injections to midgestation mice. These studies involved the delivery of the luciferase or *lacZ* reporter gene under transcriptional control of the cytomegalovirus (CMV) promoter (Lipshutz et al., 1999a; Lipshutz et al., 1999b; Lipshutz et al., 1999c; Schachtner et al., 1999). When the animals reached a month of age, only minimal transgene expression was detected after a gradual decline in activity (Lipshutz et al., 1999b). Other gene therapy studies in adult mice indicated that CMV transcriptional activity was inactivated a few weeks after initial expression (Loser et al., 1998). It is not known whether expression in the liver after in utero vector delivery decreases because of promoter inactivation, because of an immunologic response to adenoviral transduction, or because of a dilution of vector genomes as the cells of the liver divide during fetal development.

Even less is known about in utero delivery of AAV to the developing murine fetus. To date, three published manuscripts and a handful of abstracts address this issue. Similar CMV-*lacZ* or CMV-luciferase expression cassettes were used in the context of rAAV. In cases where the vector was delivered intrahepatically or intraperitoneally, expression was detected mainly in the liver or the luminal wall of the peritoneal cavity, respectively. This activity dropped significantly over time (Lipshutz et al., 2000; Schneider et al., 2002). However, studies involving intramuscular injections of vector containing CMV-driven *lacZ* or human factor IX expression cassettes indicated that the level of transgene expression was maintained throughout the course of the experiment (Mitchell et al., 2000; Schneider et al., 2002).

We treated *Gaa*$^{-/-}$ mice in utero with a genetic therapy that would prevent glycogen accumulation and maintain normal muscle function. We used in utero delivery of recombinant AAV to introduce the human *GAA* cDNA into *Gaa*$^{-/-}$ diaphragm muscle at an early stage in development. Active GAA protein was produced in the diaphragm and prevented glycogen from accumulating and causing long-term irreversible damage. This method of in utero vector delivery could be extended to other knockout models such as *Mtpa*$^{-/-}$, *Serca1*$^{-/-}$ and *Cypher*$^{-/-}$, where expression of the normal gene product specifically in the diaphragm would permit the mice to survive the neonatal stage and allow investigators to study the disease pathology in other muscle types (Ibdah et al., 2001; Pan et al., 2003; Zhou et al., 2001).

Materials and methods

Construction and preparation of viral vectors

The plasmid pCI-*GAA* containing the human *GAA* cDNA, minus the 5′ untranslated region (UTR), under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter, was described previously (Fraites et al., 2002; Pauly et al., 1998). The plasmid p43.2-h*GAA*3.1 was created by cloning the CMV-h*GAA* expression cassette (from pCI-*GAA*) into p43.2, between two AAV serotype 2 inverted terminal repeats (ITRs). The 3′ UTR of the h*GAA* cDNA was then removed to create p43.2-h*GAA*2.8. The truncated h*GAA* cDNA was also cloned into UF12, downstream of the chicken β-actin promoter plus the CMV enhancer (CBA), to produce pTR-CBA-h*GAA*2.8 (Xu et al., 2001). The rAAV reporter plasmid, pTR-CBA-Luc, was constructed by a similar method of replacing the IRES-GFP cassette in UF12 with the firefly luciferase cDNA from pGL3 (Promega, Madison, WI). Cells transfected with p43.2-h*GAA*2.8 and pTR-CBA-h*GAA*2.8 were analyzed for the production of enzymatically active GAA protein in the absence of the 3′ UTR. Highly purified rAAV serotype 2 (rAAV2-CMV-h*GAA*, rAAV2-CBA-h*GAA* and rAAV2-CBA-Luc) and serotype 1 (rAAV1-CMV-h*GAA*) vectors were generated using methods previously described (Zolotukhin et al., 2002). Virus was analyzed by dot-blot to determine the particle titer, and by infectious center assay to quantify infectious titer.

In utero viral delivery

Animal procedures were performed in accordance with the guidelines of the University of Florida Institutional Animal Care and Use Committee. On day 15 of gestation, pregnant females were anesthetized using 0.03 mL/gm total body weight of 20 mg/mL Avertin (tribromoethanol in tert-amyl alcohol diluted in PBS) administered intraperitoneally. A midline laparotomy was performed on each pregnant female, with the abdominal wall being retracted to expose the peritoneal cavity. Each horn of the uterus was exposed individually onto a pre-warmed saline-moistened sponge and up to 10 μL of saline, beads or virus was injected into each fetus. A preloaded Hamilton syringe, bearing a 33-gauge needle with a beveled end and side pore (Hamilton Company, Reno, NV), was inserted through the uterine wall into the fetal liver or peritoneal cavity. After the injections, the abdominal muscle layer was sewn using 5-0 prolene and the skin layer was closed using 5-0 vicryl. Ampicillin (2.4 μL/gm body weight of 0.1 g/mL stock) and Buprenorphine (0.1 mg/kg) were administered after the surgery to control infection and pain. Mothers were monitored until they regained consciousness, after which they were returned to the colony and permitted to proceed with the pregnancy. Newborn pups were kept with their mothers for 1 month before weaning.

Perfusion, necropsy, histology and electron microscopy

After animals were perfused with PBS to remove excess blood, organs were successively removed from the animal using sterile surgical utensils, first beginning with skeletal muscle removed from lower extremities, then gonad, spleen, kidney, liver, diaphragm, lung, heart, tongue and brain. The tissues were snap frozen in liquid nitrogen and stored at –80°C until analyzed by activity assays, western analysis and rAAV genome copy number.

Tissues isolated for electron microscopy and histology were taken after first perfusing the mice with PBS for 5 minutes, followed by 5 minutes of fixative (2% paraformaldehyde/1% glutaraldehyde in PBS, pH 7.4). Skeletal muscle, liver, diaphragm and heart were removed, dissected into very small cubes and stored overnight in 2% glutaraldehyde. They were rinsed in 0.1 M sodium cacodylate buffer and incubated at 4°C in 2% osmium tetroxide in cacodylate buffer for 1 hour. They were then rinsed twice in cacodylate buffer, dehydrated in a series of graded alcohol solutions, rinsed in 100% propylene oxide and embedded in TAAB resin (Marivac, Halifax, Canada). All other reagents were purchased from Electron Microsopy Sciences (Fort Washington, PA). Thick sections (1 μm) were stained with Schiff's reagent, followed by Toluidine Blue, and photographed using light microscopy. Thin sections (0.1 μm) were stained with lead citrate and uranyl acetate, and photographed with a Zeiss EM10 transmission electron microscope at 80 kV.

Biochemical assays

Luciferase expression assay

The Luciferase Assay System (Promega, Madison, WI) was used to quantify the expression of luciferase. The samples were prepared by homogenization in 300 μL of water. Then 20 μL of the supernatant, and 100 μL of luciferase assay substrate diluted in assay buffer, was added to a glass test tube and incubated at room temperature for 20 minutes. The intensity of light emitted from the reaction was detected using the Monolight® 2010 luminometer (BD Biosciences, Mississauga, ON). Luciferase expression was reported as relative light units per μg protein, as determined by *DC* Protein Assay (Bio-Rad, Hercules, CA).

Acid α-glucosidase activity assay

GAA naturally cleaves the α1,4 bond of glycogen, and in this fluorimetric assay converts synthetic substrate 4-methylumbelliferyl-α-D-glucopyranoside (4-MUG; Calbiochem-Novabiochem, San Diego, CA) to 4-methylumbelliferone (4-MU) and glucose. Snap frozen tissues were homogenized in water, and cell pellets were resuspended in water and lysed by three freeze/thaw cycles. Lysates were centrifuged and 20 μl of clarified supernatant was added to each well in triplicate of a black 96-well plate. The reaction was initiated by the addition of 40 μl of substrate solution [2.2 mM 4-MUG in 0.2 M sodium acetate (pH 3.6)] and was incubated for 1 hour at 37°C before the reaction was stopped with 200 μl of 0.5 M sodium carbonate (pH 10.7). Fluorescence (360 nm/460 nm) was then measured using an FLx800 Microplate Fluorescence Reader (Biotek Instruments, Winooski, VT). GAA specific activity was quantified in nmoles of substrate hydrolyzed (nmoles 4-MUG/hr/mg protein), based on a standard curve of 4-MU concentrations and standardized by protein concentration determination by *DC* Protein Assay (Bio-Rad, Hercules, CA).

Acid α-glucosidase staining of tissues

GAA was detected in fixed tissues by cytochemical staining using the synthetic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-Gluc; Calbiochem-Novabiochem, San Diego, CA), which when cleaved releases a blue product. After washing with PBS, X-Gluc stain (0.25 mM potassium ferricyanide, 0.25 mM potassium ferrocyanide, 1 mM magnesium chloride, 1 mg/mL X-Gluc in PBS reduced to pH 3.6) was added and the samples incubated at room temperature overnight. The tissues were photographed using a digital camera attached to a dissecting microscope.

Western blotting

Rabbit polyclonal antiserum was raised against placentally derived human GAA as previously described (Pauly et al., 1998). The antiserum was used for western blotting to detect hGAA protein. A total of 5 μg of protein from tissue homogenates was applied to Novex® 8% Tris-Glycine gels (Invitrogen Life Technologies, Carlsbad, CA) and separated at 125 V for approximately 2 hours. After transfer to nitrocellulose filters, blots were probed with a 1:1000 dilution of primary antibody followed by a 1:5000 dilution of peroxidase-labeled anti-rabbit IgG, and detected using the ECL+Plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). Human placental GAA was included on each blot as a positive control.

Quantification of genome copies by quantitative-competitive PCR

Competitor plasmid construct, p43.2-h*GAA*2.8-5′del, was created in which approximately 350 nucleotides from the 5′ end of the *GAA* gene were removed. The 5′ primer (5′-GCTAGCCTCGAGAATTC-3′) was located in the multiple cloning site after the CMV promoter of p43.2,

and the 3′ primer (5′-CGGTTCTCAGTCTCCATCAT-3′) was positioned beginning at nucleotide 514 of the h*GAA* coding sequence. These primers were designed to amplify 595 nucleotides of rAAV-CMV-h*GAA* genomic DNA and 239 bp of the p43.2-h*GAA*2.8-5′del competitor template.

Total DNA was isolated from snap-frozen specimens using the DNeasy® tissue kit (Qiagen, Valencia, CA). An RNase digestion step was included to remove any mRNA species that may contaminate the QC-PCR. Reactions were arranged by adding 200 ng of total DNA, competitor plasmid DNA (ranging from 0 to 10^8 copies), 20 pmol of each primer, and water to Ready-To-Go™ PCR beads (Amersham Biosciences, Piscataway, NJ). The reaction contained 1.5 mM MgCl in a total volume of 25 μL according to the manufacturer's suggestions. Samples were subjected to 30 cycles of denaturation at 95°C for 30s seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, using a RoboCycler® Gradient 96 thermocycler (Stratagene, La Jolla, CA).

QC-PCR samples were separated on a 2% agarose gel and photographed using the Eagle Eye™ II imaging system (Stratagene, La Jolla, CA). The amplified products were quantified using Imagequant™ software (Amersham Biosciences, Piscataway, NJ). Intensities of products from amplified genomic rAAV-CMV-h*GAA* and competitor plasmid DNA were plotted on the same graph using SigmaPlot 2001 software (SPSS, Chicago, IL). The point where both lines crossed was considered the point of equal amplification. Given that the amount of competitor and sample template is equal at this point, we approximated the number of vector genome copies present in the sample. Data were reported as vector genome copies/diploid cell after converting from vector genome copies/200 ng DNA using a conversion factor of 5 pg DNA/diploid nucleus.

In vitro assessment of diaphragm contractile function

Mice were anesthetized via intraperitoneal (IP) injection of sodium pentobarbital (65 mg/kg). After reaching a surgical plane of anesthesia, the diaphragm was surgically excised, with the ribs and central tendon attached, and placed in a cooled dissecting chamber containing Krebs-Henseleit solution equilibrated with a 95% O_2/5% CO_2 gas mixture. A single muscle strip (3-4 mm in width) was cut from the ventral costal diaphragm parallel to the connective tissue fibers. Segments of the rib and central tendon were used to attach the strip to two lightweight Plexiglas clamps. The muscle strip was vertically suspended between the two lightweight Plexiglas clamps, connected in series to a force transducer (Model FT03, Grass Instruments, West Warwick, RI) in a water-jacketed tissue bath (Radnoti, Monrovia, CA) containing Krebs-Henseleit solution equilibrated with a 95% O_2/5% CO_2 gas mixture (bath, ~37±0.5°C; pH, ~7.4±0.05; osmolality, ~290 mOsmol). Transducer outputs were amplified and differentiated by operational amplifiers, and underwent A/D conversion for analysis using a computer-based data acquisition system (Polyview, Grass Instruments).

In vitro contractile measurements began with empirical determination of the optimal length (L_o) of the muscle for isometric tetanic tension development. The muscle was field-stimulated (Model S48, Grass Instruments) along its entire length with platinum electrodes. Using a micrometer, muscle length was progressively increased until maximal isometric twitch tension was obtained. Once the highest twitch force was achieved, all contractile properties were measured isometrically at L_o. The force-frequency relationship was examined using previously described methods (Brooks and Faulkner, 1988; Fraites et al., 2002; Staib et al., 2002).

Results

Abnormal glycogen accumulation is evident in several tissue types by 1 month of age in the *Gaa*$^{-/-}$ mouse

By 1 month of age, glycogen inclusions are apparent in the heart, skeletal muscle, liver and diaphragm of the *Gaa*$^{-/-}$ mouse (Fig. 1). Fetal, neonatal and 1-month-old tissues from *Gaa*$^{-/-}$ and normal C57B6/129-SvJ controls were stained with Schiff's reagent, which specifically detects glycogen, to determine the earliest visible difference in glycogen content between knockout and normal strains. Using this staining method, there were no apparent differences in intracellular glycogen content between *Gaa*$^{-/-}$ and normal fetal or neonatal heart, skeletal muscle, liver or brain (data not shown). By using a combination of histology and electron microscopy, we found significant differences in glycogen content in the heart, skeletal muscle, liver and diaphragm of 1-month-old *Gaa*$^{-/-}$ mice when compared with normal animals (Fig. 1A-H). A punctuate staining pattern was seen in all *Gaa*$^{-/-}$ tissues (Fig. 1A-D), indicative of centralized lysosomal glycogen. Glycogen was observed in high proportions within the myofibers of all *Gaa*$^{-/-}$ striated muscle cell types: cardiac, skeletal muscle and diaphragm (Fig. 1A-C). This is characteristic of GSDII muscle pathology.

Analysis at the cellular level using electron microscopy revealed abnormal glycogen deposition within various tissues of *Gaa*$^{-/-}$ mice. We examined heart, skeletal muscle, diaphragm and liver of 1-month-old *Gaa*$^{-/-}$ and normal (C57B6/129-SvJ) mice by electron microscopy (Fig. 1I-P). Even at this early age, enormous glycogen inclusions were seen crowding muscle fibers of *Gaa* knockout heart, skeletal muscle and diaphragm (Fig. 1I-K). Massive amounts of glycogen were also identified in knockout liver (Fig. 1L). Deposits of glycogen were rarely observed among normal tissues (Fig. 1M-P). Some glycogen was found associated with lysosome-like membrane structures, whereas in other cases, deposits were seen grouped in the cytoplasm without defined membrane structures. Most aggregates of glycogen observed in *Gaa*$^{-/-}$ skeletal muscle were associated with what appeared to be cellular debris (Fig. 1J). Glycogen seemed to take on different forms among knockout tissues. For instance, in the heart, the glycogen seemed dense (Fig. 1I), whereas it was more dispersed in skeletal muscle and liver (Fig. 1J,L). This could be an artifact caused by fixation differences among tissues, with dense glycogen indicating better preservation of the tissue.

Localization of fluorescent beads to the liver after in utero hepatic injection

We focused on in utero hepatic delivery of rAAV with the aim of achieving high-level gene expression of GAA in the liver. GAA produced in the liver could be secreted and dispersed via the circulation to target tissues such as the heart, diaphragm, and skeletal muscle. In the target tissue, the protein would be escorted to the lysosome by mannose 6-phosphate receptor-mediated endocytosis. Localization of the injected medium after in utero hepatic injections was investigated using 10 μL of 0.1% (w/v) 30 nm fluorescent beads. The beads were introduced by injecting through the uterine wall and into the red-pigmented liver of a 15 pc CD-1 fetus. Fluorescent beads were found localized in the liver at the site of injection (data not shown). This was important as the diameter of the fluorescent beads (30 nm) and rAAV (approximately 25 nm) are similar. From these results, we were confident we could successfully deliver rAAV to the liver of the developing murine fetus, and that the fetus could be carried to term.

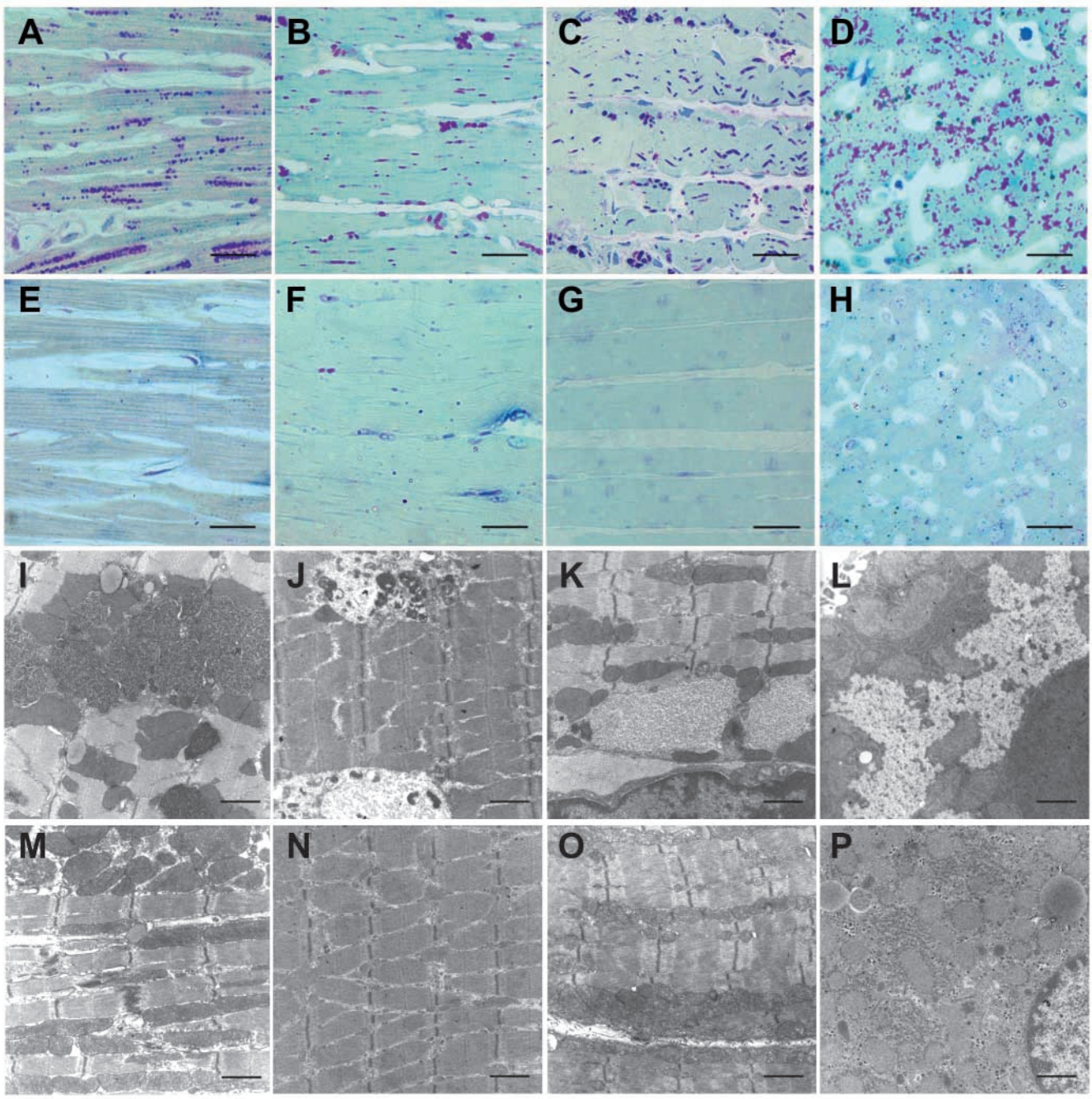

Fig. 1. Large glycogen inclusions are observed in 1-month-old *Gaa*$^{-/-}$ tissues. (A-D) Histological sections of 1-month-old *Gaa*$^{-/-}$ heart (A), skeletal muscle (B), diaphragm (C) and liver (D) were stained for glycogen with PAS. Similar tissues from age-matched normal C57B6/129-SvJ animals were included as controls (E-H). (I-P) Electron micrographs of single cells from *Gaa*$^{-/-}$ (I-L) and normal (M-P) heart (I,M), skeletal muscle (J,N), diaphragm (K,O) and liver (L,P). Scale bars: 20 μm (A-H); 500 μm (I-P).

Survival study of *Gaa*$^{-/-}$ in utero injections

Our group has injected a total of 294 *Gaa*$^{-/-}$ fetuses at 15 days gestation from 50 timed-pregnant females, of which 167 fetuses were brought to term leading to a surgery survival rate of 60.5%, compared with 100% normal birth rate. Of the 148 injected mice allowed to reach a weaning age of 3 weeks, 108 remained. This indicated a post-birth survival rate of 73.0%, a rate similar to animals of this strain not treated in utero. Most of these deaths were due to maternal neglect or cannibalization, which is normally seen in this and other knockout strains.

High-level transduction of diaphragm muscle through in utero delivery of rAAV serotype 2 to the liver and peritoneal cavity

We delivered rAAV containing the luciferase reporter gene driven by the chicken β-actin promoter plus the CMV enhancer (CBA) to fetal liver. Several reports demonstrate the use of the

CBA promoter in hepatic gene transfer studies for high-level transduction (Daly et al., 2001; Song et al., 2001; Xiao et al., 1998; Xu et al., 2001). The level of luciferase expression was determined in several tissue types 1 month after in utero hepatic delivery of 3×10^7 infectious particles of rAAV2-CBA-Luc to *Gaa*$^{-/-}$ fetuses on day 15 of gestation. Expression levels were highest in the diaphragm and liver (Fig. 2A,B), whereas no significant expression was detected in kidney, spleen, skeletal muscle, gonad, lung, heart, brain or tongue of 1-month-old *Gaa*$^{-/-}$ vector-treated mice (data not shown). In Fig. 2, luciferase expression levels of individual samples are shown by black circles, whereas averaged activity values of saline and rAAV2-CBA-Luc-treated tissues are indicated by white and gray bars, respectively. More than 100-fold higher luciferase expression was detected in rAAV in utero-treated diaphragms compared with in saline-treated mice. As the liver was the site of delivery, we were surprised to find drastically lower luciferase expression in rAAV2-CBA-Luc-treated livers compared with treated diaphragms. This could be attributed to a dilution effect when nonintegrated or episomal forms of the vector are dispersed to daughter cells as the liver divides during development. It is likely that high-level diaphragmatic transduction occurred through intraperitoneal exposure to the rAAV2 vector.

In utero transduction of diaphragm muscle leads to production of normal levels of enzymatically active GAA protein in *Gaa*$^{-/-}$ mice

We injected *Gaa*$^{-/-}$ fetuses at 15 days gestation with 2×10^8 infectious particles of rAAV2-CBA-h*GAA*, 1×10^9 infectious particles of rAAV2-CMV-h*GAA*, 3×10^7 infectious particles of rAAV2-CBA-Luc, or saline. Four C57B6/129-SvJ normal mice, four saline and rAAV2-CBA-Luc-treated *Gaa*$^{-/-}$ negative controls, eight rAAV2-CBA-h*GAA* (numbered as animals 1-8) and four rAAV2-CMV-h*GAA* (1-4) treated *Gaa*$^{-/-}$ mice were sacrificed at 1 month of age to isolate liver, kidney, spleen, skeletal muscle, gonad, diaphragm, lung, heart, brain and tongue for GAA activity assays. Again, h*GAA* vector-treated diaphragms yielded the highest enzyme activity (Fig. 3A), whereas levels in other tissues tested did not reach significance (data not shown). In Fig. 3A, black circles indicate individual enzyme values, and averaged values within experimental groups are shown as bars with C57B6/129-SvJ normal, rAAV2-CBA-h*GAA*-treated and rAAV2-CMV-h*GAA*-treated diaphragms being designated as white, light gray and dark gray bars, respectively. Average GAA enzyme activity in normal diaphragm was 23.6 nmol 4-MUG/hour/mg protein, and this level was reached in animals rAAV2-CBA-h*GAA*-2 (26.2 nmol 4-MUG/hour/mg protein) and rAAV2-CMV-h*GAA*-1 (27.3 nmol 4-MUG/hour/mg protein), whereas higher than normal levels were observed in rAAV2-CMV-h*GAA*-3 and rAAV2-CMV-h*GAA*-4 (44.5 and 40.0 nmol 4-MUG/hour/mg protein, respectively). On average, the rAAV2-CBA-h*GAA*-treated group reached almost 25% of normal GAA activity, whereas the rAAV2-CMV-h*GAA* group surpassed normal levels. The rAAV2-CMV-h*GAA* group attained higher levels than the rAAV-CBA-h*GAA* group possibly because the CMV-treated group received five times more vector, although differences in promoter strength cannot be excluded.

To determine which isoform of hGAA protein was being detected enzymatically, western analysis of diaphragm extract from the same 1-month-old rAAV2-CBA-h*GAA* and rAAV2-CMV-h*GAA* in utero-treated animals, as well as *Gaa*$^{-/-}$ untreated and normal diaphragm, was performed using a polyclonal antibody specific for hGAA. GAA purified from human placenta was used as a control to show the predominant isoforms: 95 kDa precursor and 76 and 67 kDa processed forms (Fig. 3B,C; lane 1). A cross-reacting protein possibly the heavy chain of IgG (about 50 kDa) was detected in all samples, but served as a loading control. Endogenous murine GAA in normal diaphragm extracts was not detected by this antibody, as it is specific against hGAA (Fig. 3B,C; lane 2). As expected, no signal was detected from untreated *Gaa*$^{-/-}$ diaphragm (Fig. 3B,C; lane 3). Those animals expressing detectable levels of hGAA by enzyme assay, rAAV2-CMV-h*GAA*-1 and rAAV2-CMV-h*GAA*-2, and rAAV2-CBA-h*GAA*-1, rAAV2-CBA-h*GAA*-3 and rAAV2-CBA-h*GAA*-4, revealed the presence of the catalytically active 76 kD processed form (Fig. 3B, lanes 4, 5; Fig. 3C, lanes 4, 6 and 7). Protein levels detected by

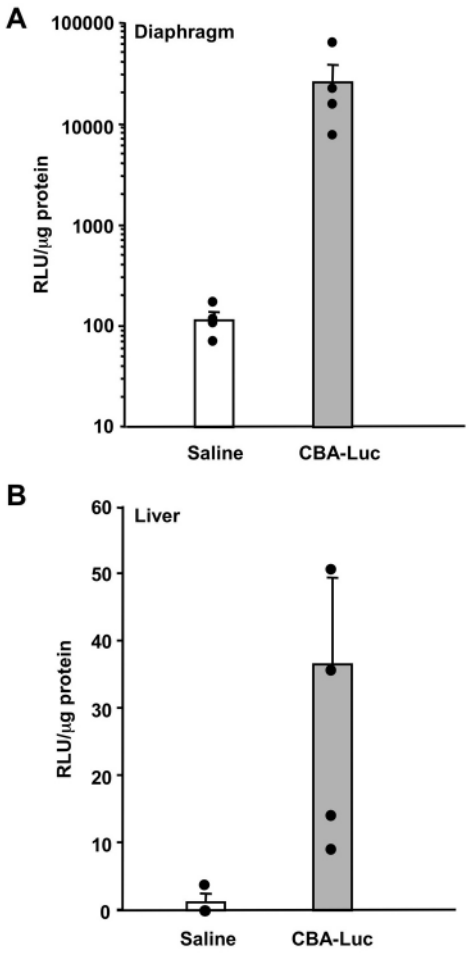

Fig. 2. Luciferase expression detected in the diaphragm and liver after hepatic in utero delivery of rAAV2-CBA-Luc. At 15 days gestation, *Gaa*$^{-/-}$ fetuses were injected in the liver with 3×10^7 infectious particles of rAAV2-CBA-Luc. Animals were sacrificed at 1 month and assayed for luciferase expression. The highest levels of luciferase activity were detected in diaphragm (A) and liver (B). Black circles reflect values of individual samples; white and gray bars represent averages from saline-treated and rAAV2-treated samples, respectively. Diaphragm expression is plotted on a log scale.

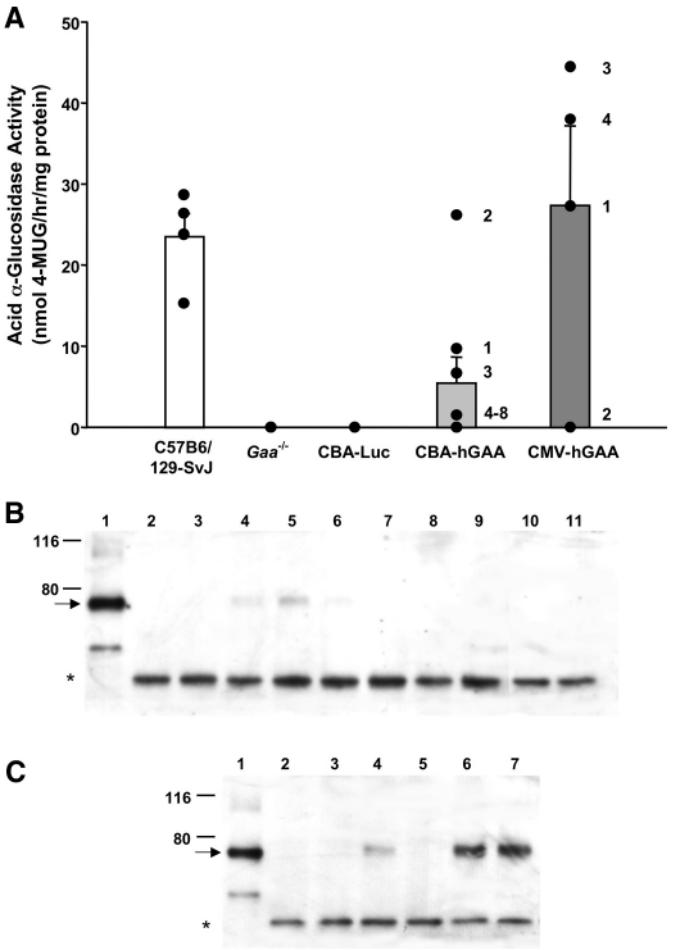

Fig. 3. Normal levels of GAA expression are achieved in *Gaa*$^{-/-}$ diaphragm after in utero rAAV2-h*GAA* transduction. 15 day pc *Gaa*$^{-/-}$ fetuses were injected with saline (n=4), 3×10^7 infectious particles (i.p.) of rAAV2-CBA-Luc (n=4), 2×10^8 i.p. rAAV2-CBA-h*GAA* (n=8) or 1×10^9 i.p. rAAV2-CMV-h*GAA* (n=4). (A) GAA expression was assayed from 1-month-old diaphragms of normal C57B6/129-SvJ mice and in utero-treated *Gaa*$^{-/-}$ mice. Black circles represent individual values and bars show averaged values within experimental groups (C57B6/129-SvJ, white bar; rAAV2-CBA-h*GAA*, light gray bar; rAAV2-CMV-h*GAA*, dark gray bar). Animals within treated groups are assigned numbers to simplify explanations in the text. (B,C) Protein extracts from same animals as shown in A were separated by SDS-PAGE and the blots probed with a polyclonal antibody against human placental GAA. Normal C57B6/129-SvJ (B,C; lane 2), untreated *Gaa*$^{-/-}$ (B,C; lane 3), rAAV2-CBA-h*GAA*-treated *Gaa*$^{-/-}$ (B, lanes 4-11) and rAAV2-CMV-h*GAA*-treated *Gaa*$^{-/-}$ (C, lanes 4-7) diaphragms are shown. GAA purified from human placenta was included as a positive control showing predominant isoforms of 95 kDa precursor, and 76 and 67 kDa processed forms (B,C; lane 1). Arrows indicate the 76 kDa mature form. The asterisk designates a cross-reacting protein of unknown origin.

western analysis were consistent with the relative levels of measured enzymatic activity presented in Fig. 3A.

Higher level expression achieved using rAAV serotype 1

Based on previous experimentation, we discovered that rAAV serotype 1 is highly effective at transducing muscle tissue when delivering the human *GAA* cDNA to the *Gaa*$^{-/-}$ mouse (Fraites et al., 2002). In addition, we purified serotype 1 vector that was higher in titer than the serotype 2 vector used in this study (8.14×10^{12} particles/ml of rAAV1-CMV-h*GAA* compared with 9.4×10^{10} particles/ml of rAAV2-CMV-h*GAA*). We sought to determine whether in utero delivery of 8.14×10^{10} particles of rAAV1-CMV-h*GAA* to *Gaa*$^{-/-}$ fetuses at 15 days gestation could result in a higher level of transduction than 1×10^9 infectious particles of rAAV2-CMV-h*GAA*. After allowing the vector-treated pups to reach 1 month of age, they were sacrificed to isolate liver, kidney, spleen, skeletal muscle, gonad, diaphragm, lung, heart, brain and tongue for GAA activity assays. Once again, GAA activity was detected only in diaphragm. No other tissues tested expressed a significant level of GAA activity (data not shown). In several cases, diaphragmatic transduction with rAAV serotype 1 resulted in almost 10-fold higher GAA activity, surpassing both normal controls as well as rAAV serotype 2 in utero-treated *Gaa*$^{-/-}$ animals (Fig. 4B).

We examined GAA expression in the diaphragm by cytochemical staining using 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-Gluc), a synthetic substrate similar to X-Gal, used to detect GAA. This staining procedure identified cells expressing GAA activity higher than normal levels, as no blue cells were observed after X-Gluc staining of normal C57B6/129-SvJ diaphragms (data not shown). After fixation, we immersed half of each of the in utero-treated diaphragms into the X-Gluc solution overnight and photographed them to document the level of GAA expression. Fig. 4A shows one stained untreated diaphragm and eight stained rAAV1-CMV-h*GAA* in utero-treated diaphragms (numbered 1-8). Some showed significant blue staining (1,2,5,6 and 8), whereas others were indistinguishable from the untreated *Gaa*$^{-/-}$ control (3,4 and 7).

The level of GAA activity determined from the other half of the diaphragm indicated that the amount of staining was relative to the level of activity (Fig. 4B). The diaphragms with the highest intensity of staining reached over 100 nmol 4-MUG/hour/mg protein (rAAV1-CMV-h*GAA*-2, rAAV1-CMV-h*GAA*-6 and rAAV1-CMV-h*GAA*-8), with one yielding 824 nmol 4-MUG/hour/mg protein (rAAV1-CMV-h*GAA*-2); those with intermediate staining attained normal levels of approximately 20 nmol 4-MUG/hour/mg protein (1 and 5); and those that lacked staining had no detectable GAA activity (rAAV1-CMV-h*GAA*-3, rAAV1-CMV-h*GAA*-4 and rAAV1-CMV-h*GAA*-7).

By western analysis, we discovered that the 76 kDa mature form of GAA was responsible for the observed activity (Fig. 4C). The intensity of the 76 kDa band observed in each of the in utero-treated diaphragms was consistent with what was determined by activity assay and X-Gluc staining, with the exception of rAAV1-CMV-h*GAA*-5. Although an intermediate level of X-Gluc staining was observed in rAAV1-CMV-h*GAA*-5 (Fig. 4A), GAA activity analysis revealed that only 18 nmol 4-MUG/hour/mg protein of active protein was present (Fig. 4B). Correlating with the activity assay, western analysis indicated a very low level of mature enzyme was present. However, there was a predominant band of a molecular weight higher than the 95 kDa intermediate form visible in the placental control lane (Fig. 4C, lane 5). This was likely to be the 110 kDa precursor

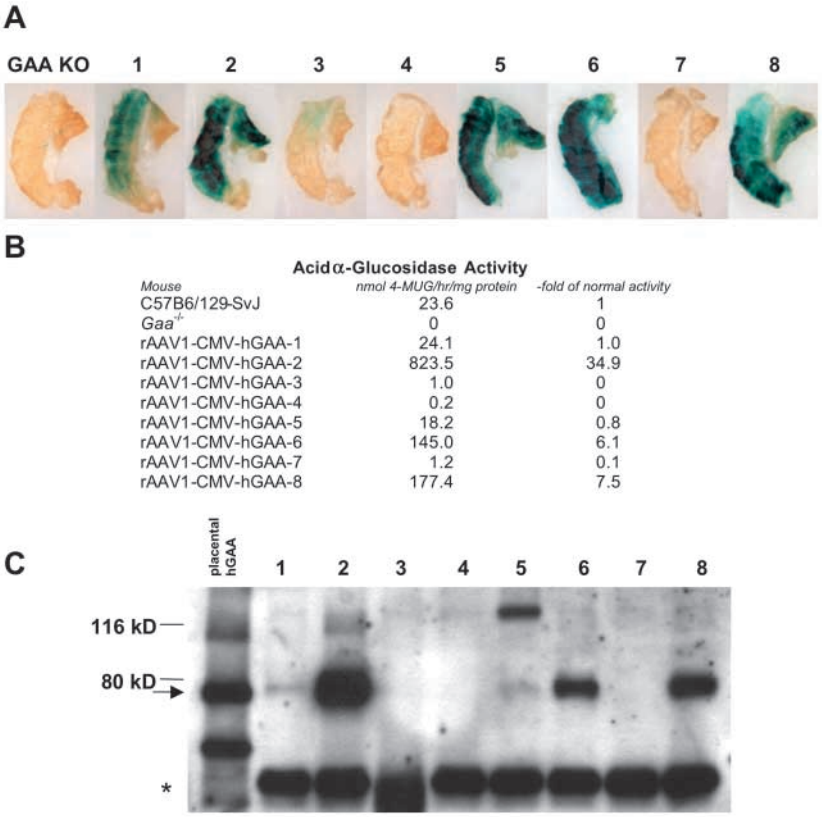

Fig. 4. Higher than normal levels of GAA expression with serotype 1. Diaphragms harvested from 1-month-old *Gaa*$^{-/-}$ mice treated in utero with 8.14×10^{10} particles of rAAV1-CMV-h*GAA* (1-8) were assayed for GAA expression by: (A) X-Gluc staining, (B) GAA activity assay and (C) western analysis. Expression levels ranged from nothing to vastly greater than normal. In the western analysis, human placental GAA was included as a positive control, showing predominant isoforms of 95 kDa precursor, and 76 and 67 kDa processed forms, with the arrow indicating the 76 kDa mature form. The unknown species detected at the bottom of the blot serves as a loading control (asterisk).

form of the protein. This partially explains why the X-gluc staining of this diaphragm did not correlate with the activity assay. The higher molecular weight species detected by western analysis may be able to enzymatically cleave the X-Gluc substrate more efficiently than 4-MUG, which was used in this activity assay. The 110 kDa precursor form of GAA exhibits low level activity on particular substrates, but this activity increases as the protein is further processed.

It is not known why the predominant species in rAAV1-CMV-h*GAA*-5 was the precursor form when in every other treated tissue the mature form prevailed. The presence of a similar sized precursor was also detected in other rAAV1-CMV-h*GAA* treated diaphragms, but the ratio of the precursor to the mature form was reversed. We conclude that this animal experienced an error in processing that prevented the precursor from being efficiently cleaved into its mature form. Further studies are necessary to determine what kind of processing error was responsible.

Prevention of lysosomal glycogen accumulation in GAA$^{-/-}$ mice treated in utero with rAAV2-h*GAA*

As rAAV-h*GAA* in utero-treated *Gaa*$^{-/-}$ animals were exposed to vector-produced hGAA enzyme at an early stage in development, we wanted to determine whether lysosomal glycogen accumulation associated with GSDII and observed in this animal model was prevented in the treated animals. For this purpose, periodic acid-Schiffs (PAS) reagent was used to stain intracellular glycogen deposits of normal C57B6/129-SvJ, untreated *Gaa*$^{-/-}$ and rAAV1-CMV-h*GAA*-treated *Gaa*$^{-/-}$ diaphragm sections from 1-month-old mice (Fig. 5A-C). At this early stage in development, glycogen inclusions were evident in the diaphragm of untreated *Gaa*$^{-/-}$ mice. Numerous pink-stained glycogen-filled lysosomes scattered the field of the untreated *Gaa*$^{-/-}$ diaphragm (Fig. 5B). Lysosomes swollen with undegraded glycogen were found both at the cell periphery and among the fibers of the microtubes. Conversely, all of the myfibers of the normal and vector-treated *Gaa*$^{-/-}$ diaphragms were free of stain, making it impossible to differentiate between the two (Fig. 5A,C). These findings were confirmed by electron microscopy analysis. Extremely large lysosomes full of glycogen were present among the muscle fibers of untreated *Gaa*$^{-/-}$ diaphragm (Fig. 5E), but were not seen in normal tissue (Fig. 5D) or in treated samples expressing normal levels of GAA (Fig. 5F). However, we were not successful in transducing the diaphragm to act as a factory for producing secreted GAA to treat other tissues. For instance, heart tissue from the same animal whose diaphragm is pictured in Fig. 5C had significant PAS-positive material (Fig. 5I). Control heart tissue from C57B6/129-SvJ and untreated *Gaa*$^{-/-}$ mice was also included (Fig. 5G,H).

Level of GAA expression in diaphragm following in utero delivery of rAAV1-CMV-h*GAA* depends on vector genome copy number

Quantitative-competitive PCR was performed to determine vector genome copy number among 1-month-old *Gaa*$^{-/-}$ mice after in utero delivery of rAAV1-CMV-h*GAA*. The tissues assayed were from the same diaphragms previously described in Fig. 4. Total DNA was extracted from treated and untreated diaphragms, and 200 ng of DNA was mixed with increasing copies of plasmid competitor DNA. PCR was performed using a pair of primers that detected both the rAAV1-CMV-h*GAA* vector (595 bp product) and the CMV-h*GAA* 3′ deleted plasmid competitor (239 bp product) at equal efficiency.

The 595 bp rAAV1-CMV-h*GAA* amplified product was detected in each treated diaphragm sample, rAAV1-CMV-h*GAA*-1 through rAAV1-CMV-h*GAA*-8, but to varying levels (data not shown). All samples indicated the presence of vector genomes whether or not GAA protein was detected by staining, enzyme assay or western analysis (Fig. 4A-C). The 595 bp rAAV1-CMV-h*GAA* amplified product was not detected in untreated *Gaa*$^{-/-}$ animals (Fig. 6). Densitometry was performed to more accurately determine vector genome copy number

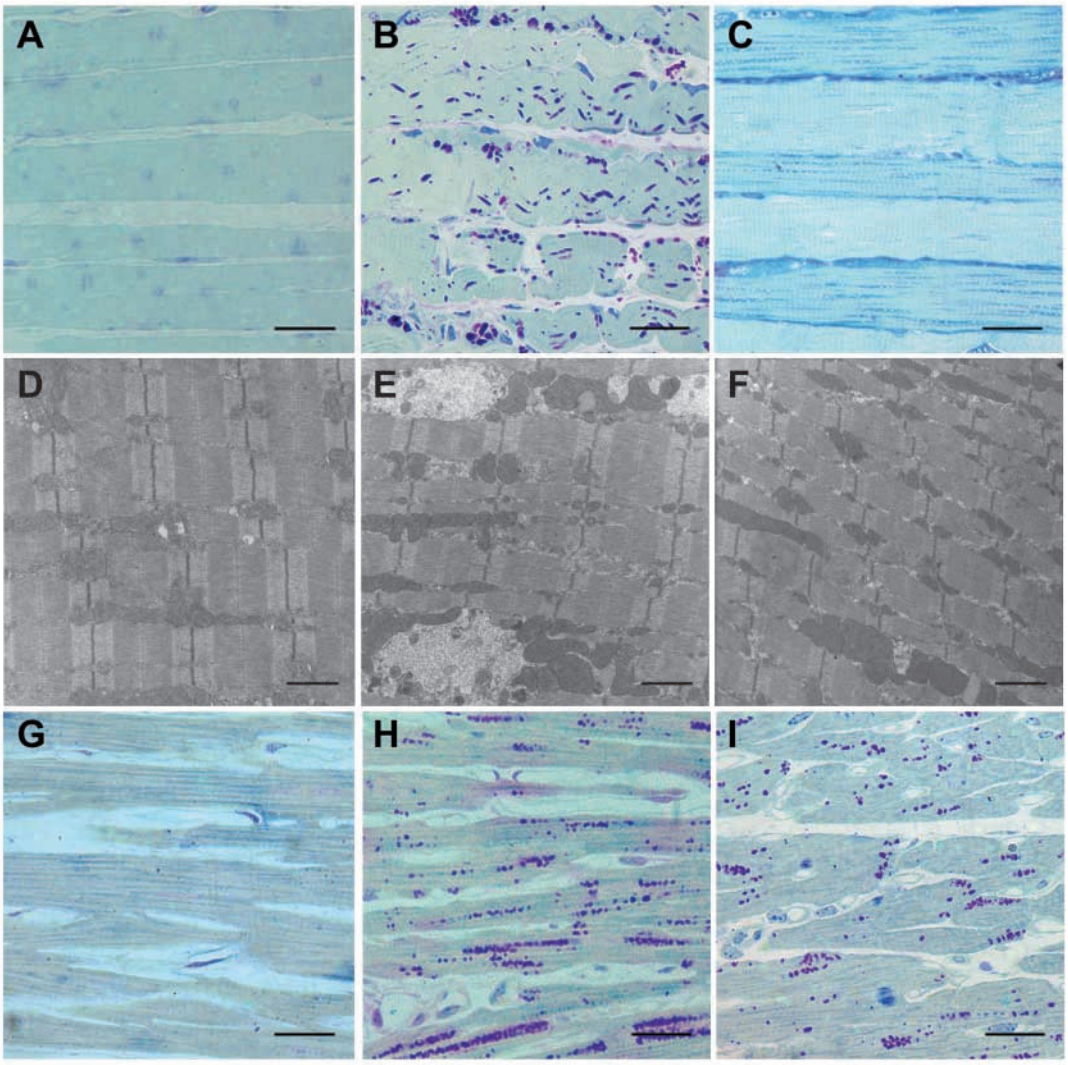

Fig. 5. *Gaa*$^{-/-}$ diaphragms transduced with rAAV1-CMV-h*GAA* are free of glycogen deposits. Epon-embedded diaphragms from normal C57B6/129-SvJ (A), untreated *Gaa*$^{-/-}$ (B) and *Gaa*$^{-/-}$ mice injected with 8.14×10^{10} particles of rAAV1-CMV-h*GAA* in the liver in utero (C) were sectioned and stained with PAS to determine intracellular glycogen content. Electron micrographs were taken of similar tissues from normal (D), untreated (E) and in utero-treated (F) diaphragms to identify glycogen deposits at the cellular level. Representative micrographs from three different vector-treated diaphragms are pictured in C and F. Heart tissue from the rAAV1-CMV-h*GAA*-treated animal whose diaphragm is pictured in C was stained with PAS to show that other deficient tissues did not benefit from transduction of the diaphragm (I). Heart tissue from normal C57B6/129-SvJ (G) and untreated *Gaa*$^{-/-}$ (H) mice was included as controls. Scale bars: 20 μm (A-C,G-I); 500 μm (D-F).

present within 200 ng of diaphragm DNA, and this value was converted into vector copies/diploid genome based on a conversion factor of 5 pg total DNA/cell. Representative quantitative-competitive PCR experiments for diaphragm samples rAAV1-CMV-h*GAA*-1, rAAV1-CMV-h*GAA*-2 and rAAV1-CMV-h*GAA*-7 are summarized in Fig. 6. Listed beside each sample number, is the vector copy number reported as estimated vector copies per diploid genome, the GAA activity value as previously determined, and the raw data from each QC-PCR experiment. Control reactions were completed in which β-actin was amplified from 200 ng DNA from each sample. This showed that the amount of DNA added to each QC-PCR reaction was relatively the same (data not shown).

The general trend indicated by the QC-PCR experiments was that as the copy number of vector genomes per diploid cell increased, the resulting GAA activity also increased. Diaphragm from rAAV1-CMV-h*GAA*-2, which had the highest level of GAA activity (824 nmol 4-MUG/hour/mg protein), contained an estimated 50 vector copies per diploid genome. Diaphragm rAAV1-CMV-h*GAA*-1, which had a normal level of GAA activity (24 nmol 4-MUG/hour/mg protein), and over 10-fold less activity than rAAV1-CMV-h*GAA*-2, contained only a slightly lower copy number, at 20 estimated vector copies per diploid genome. Even sample rAAV1-CMV-h*GAA*-7, which had minimal GAA activity (1.2 nmol 4-MUG/hour/mg protein), was found to contain significant vector genomes with 2.5 estimated vector copies per diploid genome detected. Even though significant levels of rAAV1-CMV-h*GAA* vector genome copies were present in all treated diaphragms, there was an optimal threshold of genome copies that must be present in order to produce a detectable level of GAA.

We also performed QC-PCR on the four livers from the treated mice in which the diaphragms exhibited high GAA activity (rAAV1-CMV-h*GAA*-2, rAAV1-CMV-h*GAA*-5, rAAV1-CMV-h*GAA*-6 and rAAV1-CMV-h*GAA*-8). Three of the livers tested had positive amplification signals, and were found to contain, on average, 0.1 estimated vector copies per diploid genome (data not shown). Even though it was uncertain whether the livers sampled in this experiment were actually the lobes directly injected, this indicated that there were vector genomes present in most of the livers tested. For the most accurate representation of vector genome copies in the liver, QC-PCR should be performed on DNA representative of the entire liver.

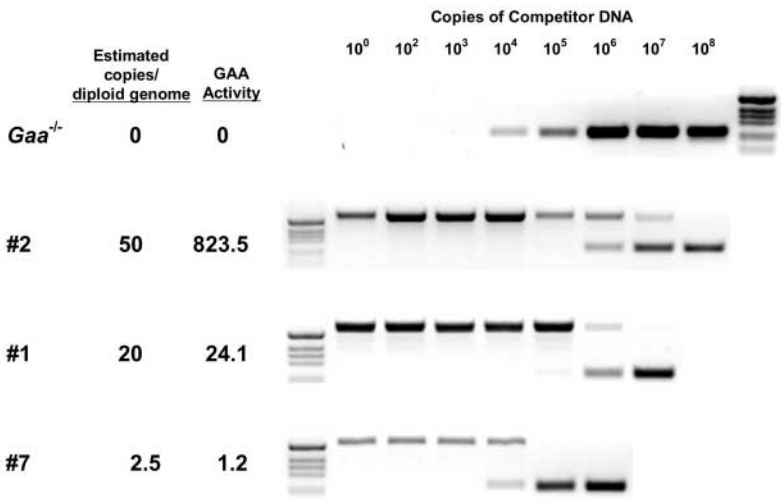

Fig. 6. Vector is detected in in utero rAAV1-CMV-h*GAA*-transduced diaphragms by QC-PCR. Quantitative-competitive PCR was performed to determine vector genome copies in in utero vector-treated and untreated diaphragm samples that were previously analyzed in Fig. 4. PCR was performed after mixing increasing amounts of competitor DNA (10^0 to 10^8 copies) and 200 ng of total DNA isolated from the diaphragms of *Gaa*$^{-/-}$ untreated mice or *Gaa*$^{-/-}$ mice in utero-treated with 8.14×10^{10} particles of rAAV1-CMV-h*GAA*. Representative samples (3 of the 8 assayed) are listed, with their respective values representing estimated vector copies per diploid genome and average GAA activity. Raw QC-PCR data from each diaphragm is shown on the far right.

Diaphragmatic transduction following in utero delivery of rAAV1-CMV-h*GAA* results from intraperitoneal exposure to the vector

To determine whether intraperitoneal exposure of rAAV-h*GAA* after hepatic in utero injections was the source of diaphragmatic transduction, we performed several intraperitoneal in utero injections. We delivered 8.14×10^{10} particles rAAV1-CMV-h*GAA* to 15 day pc *Gaa*$^{-/-}$ fetuses via the intraperitoneal cavity and harvested the diaphragms from three animals (1-3) at 1 month of age. The tissues were assayed by X-Gluc staining, GAA activity, western analysis and QC-PCR. Each diaphragm was positive to a varying extent for X-Gluc staining (data not shown), GAA activity and vector genomes (Table 1).

At least normal levels of GAA activity were achieved in all treated samples (Table 1). Western analysis confirmed the presence of the 76 kD mature form of GAA (data not shown). QC-PCR was used to analyze rAAV1-CMV-h*GAA*-1, rAAV1-CMV-h*GAA*-2 and rAAV1-CMV-h*GAA*-3 diaphragms for vector genome copy number (Table 1). Every intraperitoneal in utero-treated diaphragm was positive for vector genomes, resulting in 1 to 100 estimated vector copies per diploid genome. There were some discrepancies between the relative level of GAA activity and the vector genome copy number among these samples. For instance, rAAV1-CMV-h*GAA*-2 exhibited significantly higher GAA activity than rAAV1-CMV-h*GAA*-3, but rAAV1-CMV-hGAA-3 contained several more vector genome copies per diploid genome. This could be due to unequal transduction over the entire diaphragm muscle. Protein for GAA activity assays and western analysis was isolated from a different part of the diaphragm than that that was used to isolate DNA for QC-PCR. Nevertheless, all in utero intraperitoneal-treated *Gaa*$^{-/-}$ diaphragms resulted in higher than normal levels of GAA activity, and all were positive for rAAV1-CMV-h*GAA* vector genomes.

Table 1. Biochemical and genomic analysis of diaphragms after intraperitoneal in utero delivery of rAAV1-CMV-h*GAA*

	GAA activity assay (nmol 4-MUG/ hour/mg protein)	QC-PCR (estimated vector genome copies/ diploid genome)
Gaa$^{-/-}$ (*n*=4)	0	0
Wild type C57B6/129-SvJ (*n*=4)	23.6	N/A†
rAAV1-CMV-h*GAA*-1*	32.6	1
rAAV1-CMV-h*GAA*-2*	559.0	15
rAAV1-CMV-h*GAA*-3*	154.1	100

*Intraperitoneal in utero delivery of 2×10^8 infectious particles of rAAV-CMV-h*GAA*.
†N/A, not applicable.

In utero delivery of rAAV2-CBA-h*GAA* preserves diaphragm muscle contractile force in *Gaa*$^{-/-}$ mice

Many patients suffering from GSDII succumb to respiratory insufficiencies associated with diaphragm muscle weakness. We determined that near-normal diaphragm muscle function can be preserved in 6-month-old *Gaa*$^{-/-}$ mice when treated in utero with rAAV2-CMV-h*GAA*. Using isometric force-frequency relationships as an index of contractile function, we tested the contractile properties of diaphragm muscle strips from age-matched knockout and normal mice (Fig. 7). At 6 months postpartum, impairment of contractile function was observed in *Gaa*$^{-/-}$ diaphragms, as evidenced by their decreased developed tensions over a range of stimulation frequencies (circles). By contrast, 6-month-old *Gaa*$^{-/-}$ mice treated in utero with 2×10^8 infectious particles of rAAV2-CBA-h*GAA* did not exhibit the same functional pathology and maintained near-normal contractile properties (triangles) compared with normal controls (squares). The same diaphragm strips used to study contractile function were also assayed for GAA expression by X-Gluc staining and revealed several X-Gluc positive myofibers (data not shown). GAA activity in the diaphragms of these rAAV2-CBA-h*GAA*-treated 6-month-old *Gaa*$^{-/-}$ animals was 84.0, 74.0 and 7.6 nmol 4-MUG/hour/mg protein. Western analysis revealed the presence of the mature form of GAA (data not shown). These studies showed that long-term expression of GAA can be achieved after in utero delivery of rAAV-h*GAA*.

Analysis of immunological response to vector-derived human *GAA* following in utero delivery of rAAV1 and rAAV2 vectors

To determine immunological response to vector-derived human

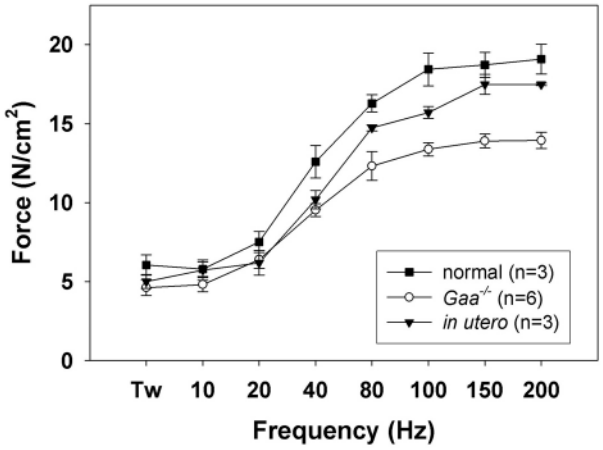

Fig. 7. In utero delivery of rAAV2-CBA-h*GAA* preserved diaphragm muscle contractile force in *Gaa*$^{-/-}$ mice. A dose of 2×10^8 infectious particles of rAAV2-CBA-h*GAA* was delivered in utero to *Gaa*$^{-/-}$ mice (black triangles; *n*=3). Animals were sacrificed and diaphragm muscle strips were prepared 6 months postpartum. In vitro isometric force-frequency relationships were obtained and compared with those of age-matched, untreated normal C57B6/129-SvJ (black squares; *n*=3) and *Gaa*$^{-/-}$(white circles; *n*=6) mice.

GAA, serum from *Gaa*$^{-/-}$ animals treated in utero with rAAV-h*GAA* was analyzed by ELISA. Treated animals tested at one month of age (*n*=22) exhibited very little (*n*=4) or no (*n*=18) antibody response to vector-derived human GAA. Of the group assayed at 6 months of age, 5 out of 10 *Gaa*$^{-/-}$ animals treated with rAAV-h*GAA* in utero exhibited extremely high levels of antibody production. The 5 remaining 6-month-old animals were negative for antibody production against vector-derived human GAA. It is likely that those animals not producing antibodies, expressed vector-derived GAA prior to the maturity of their immune system. Conversely, those animals producing high levels of GAA antibodies were most likely tolerized against the human GAA protein. These hypotheses were supported by experiments involving the challenge of untreated (*n*=7) and rAAV1-CMV-h*GAA* (*n*=8) in utero-treated *Gaa*$^{-/-}$ mice with 4 doses of recombinant human GAA over a period of 4 weeks. The majority of the group not exposed to GAA by in utero delivery of rAAV-GAA (*n*=6) died as a result of anaphylactic shock, whereas the majority of in utero-treated mice were tolerant to recombinant human GAA (*n*=7).

Discussion

We have demonstrated in utero delivery of recombinant adeno-associated vectors to a knockout mouse model of human disease. In addition, we show efficient in utero transfer of the therapeutic gene encoding acid α-glucosidase to the diaphragm of the mouse model of GSDII. This is a crucial finding in the study of GSDII, as diaphragm is one tissue affected in both mild late-onset and severe early-onset disease. Furthermore, this approach to specific expression in the diaphragm is useful in the study of other inherited myopathies where knockout mouse models are perinatal lethal due to respiratory failure. Selectively delivering rAAV encoding the normal gene to the embryonic diaphragm could prevent these mice from dying soon after birth and at the same time allow investigators to analyze heart and skeletal muscle disease pathology.

Limited work has been reported on the in utero delivery of rAAV. Two groups reported high-level gene expression in several tissues on the first day of life after liver-targeted delivery of rAAV2 to the mouse at day 15 of gestation (Lipshutz et al., 2001; Schneider et al., 2002). The authors also reported a decrease in expression as development of the animal continues, with low-level expression being maintained after 1 month of age. The current hypothesis for loss of expression is that as cells divide throughout development, the unintegrated vector genomes are dispersed to daughter cells and the effective dose of copies per cell dramatically decreases over time. This hypothesis also explains why a steady level of expression is maintained after the initial phases of development, and after target cells differentiate. A dilution effect similar to that observed in the liver is not seen in the diaphragm because, even though transduced embryonic myoblasts of the diaphragm may be diluted after cell division, these myoblasts fuse to create multi-nucleated myotubes, thereby reversing the dilution effect. However, other possibilities may explain why liver is difficult to transduce in utero. For example, rAAV2 may not transduce embryonic liver with high efficiency because a majority of the cells in the fetal liver are hematopoietic progenitors. Cells of hematopoietic lineage are not easily transduced with rAAV2 (Srivastava, 2002). This may be because these cells do not express high enough levels of the appropriate rAAV receptors, although there are many other possible reasons. Our study focused on expression analysis at one month of age, after early development and after the initial dilution of vector genomes has already occurred.

We found that expression was concentrated to the diaphragm of in utero hepatic-injected mice and that very little to no expression was found in the liver, the site of injection. We believe that during these hepatic injections, vector was released into the peritoneal cavity, allowing for direct transduction of the diaphragm. Delivery of the human *GAA* gene under transcriptional control of the CMV promoter by rAAV serotype 2 resulted in levels of GAA expression reaching and exceeding normal levels in diaphragm. Diaphragms transduced with the same expression cassette, but by rAAV serotype 1 and using almost a hundred times more total viral particles, expressed GAA at even higher levels, reaching over 10-fold above normal levels. Chemical staining of diaphragms treated with rAAV1-CMV-h*GAA* showed that transduction of the entire muscle can be achieved by this delivery method. In both cases, the predominant form of GAA detected by western analysis was the 76 kDa mature form. In diaphragms in which GAA expression reached normal or above normal levels, most of the fibers were clear of lysosomal glycogen deposits and normal muscle structure was preserved.

The diaphragm of animals treated with a lower dose of rAAV2-CBA-h*GAA* were assayed for contractile function at 6 months of age. Significant improvement was observed when these force-frequency relationships were compared with those of untreated age-matched controls. Diaphragms from adult animals treated with rAAV2-CMV-h*GAA* and rAAV1-CMV-h*GAA* are similarly expected to show significant functional improvement, and possibly preservation of normal contractile function, as these tissues reached normal or above normal

levels of GAA expression after in utero transduction. The studies in adult animals are ongoing in the laboratory.

QC-PCR experiments, used to quantify vector genomes present in a particular tissue, indicated that up to an estimated 50 vector copies per diploid genome could be achieved in diaphragm when serotype 1 vectors were used. This correlated with GAA activity levels reaching over 10-fold higher than those observed in normal diaphragm. Livers from hepatic in utero-treated mice were assayed for the presence of vector DNA by the same method. On average, 0.1 estimated vector copies per diploid genome were detected. This correlated with the undetectable enzymatic activity. This result supports the hypothesis that upon direct delivery of vector to the liver, unintegrated vector genomes are diluted as they are dispersed to daughter cells by repeated cell divisions during liver development.

Results from adult mouse diaphragmatic transduction experiments showed very limited transduction of muscle fibers by either direct intramuscular injection, or by tail vein administration followed by clamping of the vena cava below the diaphragm (Decrouy et al., 1997; Petrof et al., 1995; Petrof, 1998). The transduction levels attained in diaphragm muscle presented in this study surpassed other published results several fold. We showed that transduction of the entire diaphragm muscle was possible through either in utero hepatic delivery or direct intraperitoneal delivery of rAAV. We found that intraperitoneal delivery of rAAV1-CMV-h*GAA* resulted in diaphragm transduction in all of the treated mice tested. GAA activity in all cases reached normal, or above normal, levels. We believe in utero intraperitoneal injection of rAAV-*GAA* to be an efficient delivery method to achieve high-level diaphragm transduction, and it should be explored further as a possible treatment method of GSDII. In addition, this technique will be helpful for those studying other skeletal myopathies in which the knockout mouse models are perinatal lethal because of respiratory distress caused by lack of specific proteins being produced in the diaphragm (Ibdah et al., 2001; Pan et al., 2003; Zhou et al., 2001).

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