Liver-Directed Recombinant Adeno-Associated Viral Gene Delivery Rescues a Lethal Mouse Model of Methylmalonic Acidemia and Provides Long-Term Phenotypic Correction

Nuria Carrillo-Carrasco,^{1*} Randy J. Chandler,^{1,2*} Suma Chandrasekaran,¹ and Charles P. Venditti¹

Abstract

Methylmalonic acidemia is a severe metabolic disorder caused by a deficiency of the ubiquitously expressed mitochondrial enzyme, methylmalonyl-CoA mutase (MUT). Liver transplantation has been used to treat a small number of patients with variable success, and whether liver-directed gene therapy might be employed in such a pleiotropic metabolic disorder is uncertain. In this study, we examined the therapeutic effects of hepatocyte-directed delivery of the *Mut* gene to mice with a severe form of methylmalonic acidemia. We show that a single intrahepatic injection of recombinant adeno-associated virus serotype 8 expressing the *Mut* gene under the control of the liver-specific thyroxine-binding globulin (TBG) promoter is sufficient to rescue *Mut*^{−/−} mice from neonatal lethality and provide long-term phenotypic correction. Treated *Mut*^{−/−} mice lived beyond 1 year of age, had improved growth, lower plasma methylmalonic acid levels, and an increased capacity to oxidize [1-¹³C]propionate *in vivo*. The older treated mice showed increased *Mut* transcription, presumably mediated by upregulation of the TBG promoter during senescence. The results indicate that the stable transduction of a small number of hepatocytes with the *Mut* gene can be efficacious in the phenotypic correction of an inborn error of organic acid metabolism and support the rapid translation of liver-directed gene therapy vectors already optimized for human subjects to patients with methylmalonic acidemia.

Introduction

METHYLMALONYL-CoA MUTASE (MUT) (EC 5.4.99.2) is a mitochondrial enzyme that catalyzes the conversion of L-methylmalonyl-CoA into succinyl-CoA in the mitochondrial matrix (Fenton *et al.*, 2001). This reaction is essential for the metabolism of propionyl-CoA, an important intermediate in the degradation of isoleucine, valine and odd-chained fatty acids. Deficiency of MUT causes isolated methylmalonic acidemia (OMIM #251000), a severe disorder of intermediary metabolism associated with multisystemic disease and lethal metabolic instability (Oberholzer *et al.*, 1967; Stokke *et al.*, 1967). Patients with complete absence of enzyme activity are classified as *mut*⁰ and have a poor prognosis for long-term survival despite aggressive nutritional and medical management (Matsui *et al.*, 1983; van der Meer *et al.*, 1994; Nicolaides *et al.*, 1998; de Baulny *et al.*, 2005; Dionisi-Vici *et al.*, 2006; Hörster *et al.*, 2007).

Elective liver (Kayler *et al.*, 2002; Nyhan *et al.*, 2002; Hsui *et al.*, 2003; Kaplan *et al.*, 2006b; Kasahara *et al.*, 2006) and/or combined liver–kidney transplantation (van 't Hoff *et al.*, 1998; Nagarajan *et al.*, 2005) has been used to treat a subset of *mut*⁰ patients. Although solid organ recipients do not experience peripheral metabolic correction after transplantation (van 't Hoff *et al.*, 1998; Nagarajan *et al.*, 2005; Kaplan *et al.*, 2006b), they have improved growth and protection from intermittent metabolic crises. These observations suggest that gene and cell therapy directed toward the liver might have therapeutic effects in the treatment of methylmalonic acidemia. However, the variability in the selection of candidates for liver transplantation, timing of the surgery, lack of standard monitoring pre- and postoperatively, and uniqueness in individual biochemical genetic phenotypes of recipient patients have confounded the ability to clearly establish the efficacy of liver transplantation as a treatment for methylmalonic acidemia. Experimental studies will be needed to carefully address this important clinical issue.

¹Organic Acid Research Section, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health Bethesda, MD 20892.

²Institute for Biomedical Sciences, George Washington University, Washington, DC 20052.

*N.C.C. and R.J.C. contributed equally to this study.

We have previously reported the temporal correction of a murine model of *mut*⁰ methylmalonic acidemia by adenovirus-mediated gene therapy (Chandler and Venditti, 2008) and described long-term correction in the same model, using a recombinant adeno-associated virus serotype 8 (rAAV8) vector that expressed the *Mut* gene from the enhanced chicken β -actin (CBA) promoter (Chandler and Venditti, 2010). In wild-type mice, the *Mut* enzyme is present ubiquitously (Chandler *et al.*, 2007a) but is most highly expressed and active in the liver (Wilkemeyer *et al.*, 1993), an organ that plays a central role in methylmalonyl-CoA metabolism. The rAAV8-CBA-m*Mut*-treated *Mut*^{-/-} mice showed evidence of transgene expression in numerous organs, including the liver, skeletal muscle, and heart, with significant levels of *Mut* protein detected in the liver (Chandler and Venditti, 2010). Although the contribution of *Mut* enzymatic activity from each tissue to the corrected phenotype was not discernable, the sustained improvement in the *Mut*^{-/-} mice in our earlier rAAV experiments might largely be explained by expression of the *Mut* transgene in hepatocytes.

In this study, we explore the therapeutic efficacy of liver-directed gene therapy as a potential treatment for methylmalonic acidemia. In addition to targeting a cell type established to be central in the pathology of methylmalonic acidemia (Hayasaka *et al.*, 1982; Krahenbuhl *et al.*, 1991; Chandler *et al.*, 2009; de Keyser *et al.*, 2009), selective expression of the *Mut* gene in the liver might help minimize transgene-directed immune responses (Franco *et al.*, 2005) and afford rapid translation to the development of an rAAV8-MUT vector similar to those given to human subjects with hemophilia (Manno *et al.*, 2006) and α_1 -antitrypsin deficiency (Brantly *et al.*, 2009). An rAAV8 vector that expresses the *Mut* gene from the liver-specific thyroxine-binding globulin (TBG) promoter (Hayashi *et al.*, 1993; Xiao *et al.*, 1998) was constructed and delivered to neonatal *Mut*^{-/-} mice. Hepatic expression of the viral *Mut* transgene completely rescues *Mut*^{-/-} mice from neonatal lethality, restores growth, and affords long-term survival and yet, exactly as is observed in methylmalonic acidemia patients who have received liver transplants, it does not completely correct biochemical abnormalities. Expression of the *Mut* gene in a small percentage of stably transduced cells accounts for the clinical effects and is higher in older mice, likely because of senescence upregulation of the transgene from the TBG promoter. The results of our experiments have therapeutic implications for other disorders of intermediary metabolism, particularly inborn errors of organic acid metabolism.

Materials and Methods

Construction and production of rAAVs

The expression construct, p-AAV8-CI-TBG-RGB, was provided by the University of Pennsylvania Vector Core (Philadelphia, PA). The plasmid contains transcriptional control elements that include a cytomegalovirus enhancer, the TBG promoter, and the rabbit globin polyadenylation signal. These elements are flanked by inverted terminal repeat sequences from AAV serotype 2. Murine methylmalonyl-CoA mutase and enhanced green fluorescent protein (GFP) cDNAs were cloned into pAAV2/8.CI.TBG.RBG (see Supplementary Fig. 1 at www.liebertonline.com/hum). The expression vector pAAV2/8.CI.CB7.m*Mut*.RBG has been described and

contains the cytomegalovirus enhancer/chicken β -actin promoter driving the expression of the murine methylmalonyl-CoA mutase cDNA (Chandler and Venditti, 2010). The vector genomes were packaged into an AAV8 capsid, purified by cesium chloride gradient purification, and titered by quantitative PCR as previously described (Hayashi *et al.*, 1993; Gao *et al.*, 2002).

Animal studies

Murine experiments were approved and performed according to the regulations and standards of the National Human Genome Research Institute (NHGRI, Bethesda, MD) Animal Care and Use Committee (ACUC). The mice used harbor a deletion of exon 3 in the *Mut* gene. This mutation abolishes the production of mature RNA, protein, and detectable enzymatic activity (Chandler *et al.*, 2007a). Mice homozygous for this mutation (*Mut*^{-/-}) display a severe methylmalonic acidemia phenotype that is lethal in the newborn period and is accompanied by progressive elevation of methylmalonic acid (MMA) levels to 2000 μ M (normal range, 5–10 μ M) at the time of death. Heterozygous animals (*Mut*^{+/-}) appear normal and have biochemical profiles identical to those of wild-type animals, and were used as controls throughout the study. Immediately before injection, 2×10^{11} or 4×10^{11} vector genome copies (GC) of either the pAAV2/8.CI.TBG.GFP.RBG or pAAV2/8.CI.TBG.m*Mut*.RBG vector were diluted to a total volume of 20 μ l with phosphate-buffered saline. Hepatic injections were performed on nonanesthetized neonatal mice, typically within several hours of birth, using a 32-gauge needle and transdermal approach as previously described (Chandler and Venditti, 2010).

Expression analysis

Livers from untreated mice and mice injected in the neonatal period with pAAV2/8.CI.TBG.GFP.RBG were collected at 60 days of life, embedded, frozen immediately on dry ice, and stored at -80°C . Frozen liver sections (thickness, 8 μ m) were placed onto glass slides, stained with 4',6-diamidino-2-phenylindole (DAPI), and mounted with Fluoro-Gel aqueous mounting medium (Electron Microscopy Sciences, Hatfield, PA). Random slides from each liver section were evaluated by confocal microscopy (510 NLO Meta; Carl Zeiss, Oberkochen, Germany) with fluorescein isothiocyanate (FITC) and DAPI filters for the same microscope and camera settings and using the threshold level from untreated control mice as a reference for liver background fluorescence, as previously described (Wang *et al.*, 2010b). The total number of cells was calculated with software and manually validated. GFP-labeled cells were quantified manually by careful examination of 13 randomly selected sections by three independent observers. Each observer evaluated 680 cells and the results were averaged as the number of GFP-positive nuclei per total nuclei visualized. The GFP intensity in each image was also determined.

Quantitative real-time PCR analysis

Mut RNA expression in liver was calculated for untreated *Mut*^{-/-} mice ($n = 3$), and for *Mut*^{+/-} mice ($n = 3$) and *Mut*^{-/-} mice treated with 2×10^{11} GC of rAAV8-TBG-m*Mut*, 90 days ($n = 3$) and 270 days ($n = 3$) after injection. Total RNA from

frozen livers was extracted, using an RNeasy mini kit (Qiagen, Valencia, CA). Residual DNA was digested with DNA-free (Ambion, Austin, TX). *Mut* and *Serpina7* (TBG) transcripts were quantified by real-time PCR, in triplicate, using TaqMan gene expression assays with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4352932E), murine methylmalonyl-CoA mutase (Mm00485312_m1), and thyroxine-binding globulin (Mm00626105_m1) probes, all from Applied Biosystems (Foster City, CA), and analyzed with an Applied Biosystems 7500 real-time PCR system. Expression of *Mut* and *Serpina7* was normalized on the basis of liver GAPDH transcript levels.

Vector genome copy number

Genome copy (GC) number was measured by quantitative real-time PCR analysis as described previously. A standard curve was prepared, using serial dilutions of a plasmid carrying the murine *Mut* cDNA. Genomic DNA was extracted from murine liver samples and 100 ng of DNA was used to determine the genome copy number of rAAV at 90 days ($n=2$) and 270 days ($n=3$) of life.

Western blotting

Tissue samples were homogenized with a 2-ml Tenbroeck tissue grinder (Wheaton Science International, Millville, NJ) in tissue protein extraction reagent (T-PER; Pierce Protein Research Products/Thermo Scientific, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with 35 mg of clarified liver extract and transferred to nylon membranes. Western blot analysis was performed with a rabbit polyclonal antibody against murine Mut at a 1:500 dilution (Chandler *et al.*, 2007b) and mouse monoclonal anti-OxPhos (oxidative phosphorylation) complex III core II antibody (A-11143; Invitrogen, Carlsbad, CA) at a 1:4000 dilution; incubation was for 3 hr. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1858415; Pierce Protein Research Products/Thermo Scientific) at a 1:10,000 dilution or HRP-conjugated goat anti-mouse antibody (1858413; Pierce Protein Research Products/Thermo Scientific) at a 1:30,000 dilution was incubated for 1 hr. Signal was detected with SuperSignal West Pico chemiluminescent substrate (34080; Pierce Protein Research Products/Thermo Scientific).

Metabolic measurements

Blood samples were obtained by retro-orbital sinus plexus sampling, using a sterile glass capillary tube at 24, 60, 90, 120, 180, 270, and 360 days of life, for measurement of plasma methylmalonic acid (MMA) levels. Samples were immediately centrifuged and plasma was collected, diluted in water, and frozen at -80°C until measurements were performed. Plasma MMA levels were quantified by gas chromatography-mass spectrometry with stable isotopic internal calibration as previously described (Marcell *et al.*, 1985; Allen *et al.*, 1993).

In vivo propionate oxidation studies

Mut^{+/-}, untreated *Mut*^{-/-}, and treated *Mut*^{-/-} mice were injected intraperitoneally with 200 μg of sodium [$1\text{-}^{13}\text{C}$]propionate (Cambridge Isotope Laboratories, Andover, MA). [$1\text{-}^{13}\text{C}$]propionate oxidation was measured by collecting expired gas, using an adaptation of a method developed to study

propionate oxidation in patients with methylmalonic and propionic acidemia, as previously described (Chandler and Venditti, 2010). Mice were placed into a respiratory chamber that contained a CO_2 probe to allow the direct measurement of CO_2 generated by each animal. An aliquot of expired air was removed from the chamber at each time point for analysis of ^{13}C enrichment in CO_2 . The isotope ratio ($^{13}\text{C}/^{12}\text{C}$) of the collected breath was measured with a gas isotope ratio mass spectrometer (Metabolic Solutions, Nashua, NH). The percent dose metabolized was calculated as follows: percent dose metabolized = total ^{13}C excreted (mmol)/dose (mmol) $\times 100$.

Statistical analyses

In all instances, p values were considered significant if the value was less than 0.05. Differences in survival between treated groups were analyzed by χ^2 test. The weights between treated and untreated mice and differences in metabolite levels were assessed by two-sided, two-tailed unpaired Student t test. The Kruskal-Wallis test was used to determine the statistical significance of differences in measured propionate oxidation rates between groups at 25 min.

Results

Twenty-one *Mut*^{-/-} mice received a single intrahepatic injection of either 2×10^{11} GC ($n=15$) or 4×10^{11} GC ($n=6$) of rAAV8-TBG-mMut at birth. In the untreated mutant group, 94% perished before 21 days of life and only one of the untreated mice was alive by 72 days of life. In contrast, all but one of the *Mut*^{-/-} mice that received 2×10^{11} GC of AAV8-TBG-mMut ($n=15$), and all that received 4×10^{11} GC of AAV8-TBG-mMut ($n=6$), were alive at 21 days of life after injection. After weaning on day 24, 93% (14 of 15) of the 2×10^{11} GC-treated mutants were alive (Fig. 1). One year after

FIG. 1. Long-term survival of *Mut*^{-/-} mice after liver-directed gene therapy. Survival (in days) of untreated *Mut*^{-/-} mice ($n=58$) and those treated with either 2×10^{11} GC ($n=15$) or 4×10^{11} GC ($n=6$) of rAAV8-TBG-mMut in the neonatal period. Mice treated with 4×10^{11} GC of AAV8-TBG-mMut ($n=6$) had 84% survival (5 of 6) by 360 days of life compared with 53% (8 of 15) for those treated with 2×10^{11} GC of AAV8-TBG-mMut. Both treated groups had significantly increased survival at 24, 60, and 100 days and beyond ($*p<0.01$) compared with the group of untreated *Mut*^{-/-} mice.

injection, 53% (8 of 15) of the $Mut^{-/-}$ mice that received 2×10^{11} GC of rAAV8-TBG-mMut and 83% (5 of 6) of the $Mut^{-/-}$ mice that received 4×10^{11} GC were still alive. In the group that received the dose of 2×10^{11} GC ($n = 15$) one animal died soon after birth, another was found dead at 30 days, one was killed at 90 days to harvest tissue, and another two were reported sick and were killed at 90 days of life. Another two $Mut^{-/-}$ mice from this group were found dead after their cage accidentally flooded at about 270 days of life. Postmortem examination of the treated $Mut^{-/-}$ mice that perished during the study period revealed no evident cause of death or gross abnormalities in any organ on necropsy.

rAAV8-TBG-mMut-treated $Mut^{-/-}$ mice achieved and maintained 60–80% of body weight when compared with their $Mut^{+/-}$ littermates (Fig. 2). Treated $Mut^{-/-}$ mice were significantly greater in size than untreated $Mut^{-/-}$ mice on days 24 and 60 ($p < 0.01$), and close to the size of sex-matched,

FIG. 2. Improved growth parameters for mice treated with rAAV8-TBG-mMut. Weights were calculated as a percentage of sex-matched $Mut^{+/-}$ littermates. Error bars represent plus and minus 1 standard deviation. Treated $Mut^{-/-}$ mice showed improved growth compared with the group of untreated $Mut^{-/-}$ mice at 24 and 60 days (* $p < 0.01$). Results for the treated $Mut^{-/-}$ group compared with the treated $Mut^{+/-}$ group were significantly different at all points ($p < 0.01$). Top: Appearance at 1 year of age of a $Mut^{-/-}$ mouse treated with rAAV8-TBG-mMut (right) and an age- and sex-matched treated $Mut^{+/-}$ littermate (left).

heterozygous treated littermates at all time points (Fig. 2, top). There were no differences between the weights of $Mut^{-/-}$ mice treated with 2×10^{11} or 4×10^{11} GC of rAAV8-TBG-mMut (data not presented). Although the older rAAV8-TBG-mMut-treated $Mut^{-/-}$ mice were slightly smaller than heterozygous controls, they were otherwise vigorous and appeared grossly normal. Although formal testing has not been performed, these animals appeared clinically well, with no obvious neurological or behavioral phenotypes (see Supplementary Video 1 at www.liebertonline.com/hum).

A single intrahepatic injection of rAAV8-TBG-mMut (2×10^{11} GC) at birth produced detectable levels of Mut mRNA in the liver of $Mut^{-/-}$ mice. When compared with the Mut RNA expression of untreated $Mut^{+/-}$ mice ($n = 3$), $Mut^{-/-}$ mice killed at 90 days had 13% of transcript levels ($n = 3$) and mice killed at 270 days of life had 46% of transcript levels ($n = 3$) (Fig. 3A). An immunoblot showed immune-reactive Mut protein in some of these liver samples (Fig. 3C). Transgene copy numbers in genomic DNA extracted from the livers of treated mice averaged 0.05 and 0.06 per diploid genome at 90 days ($n = 2$) and 270 days ($n = 3$) of life, respectively. *Serpina7* (TBG) mRNA, derived from the endogenous genomic locus, had a 2.4-fold increase in expression at 270 days compared with 90 days of life (Fig. 3B).

To approximate the amount of hepatic correction achieved in our treated $Mut^{-/-}$ mice, we injected 2×10^{11} GC of rAAV8-TBG-GFP and evaluated the liver of mice 60 days after a neonatal intrahepatic injection. Clusters of cells accounting for approximately 9% of hepatocytes visualized were GFP positive (Fig. 3D). Attempts to perform immunohistochemistry with anti-Mut antibodies were unsuccessful.

A decrease in plasma MMA concentration was used as an indirect measure of increased Mut enzymatic activity. Plasma MMA concentrations were assayed at 24, 60, 90, 120, 180, 270, and 360 days of life. $Mut^{-/-}$ mice treated with rAAV8-TBG-mMut at 2×10^{11} GC had significantly lower plasma MMA levels compared with untreated $Mut^{-/-}$ mice (Fig. 4A). Treated $Mut^{-/-}$ mice had mean plasma MMA levels of $709 \mu M$ ($n = 11$) and liver Mut mRNA expression of 13% ($n = 3$) at 90 days of life. At 270 days of life, the treated mice had a mean plasma MMA concentration of $379 \mu M$ ($n = 8$) and liver Mut mRNA expression of 46% ($n = 3$). There was no difference in MMA concentrations between the 2×10^{11} and 4×10^{11} GC-treated groups (data not presented). Overall, plasma MMA levels in treated $Mut^{-/-}$ mice remained elevated 50 to a 100 times above the levels of $Mut^{+/-}$ mice ($5\text{--}10 \mu M$).

Whole-animal propionate oxidation capacity, which is dependent on the functional activity of methylmalonyl-CoA mutase, was measured in an *in vivo* assay. $Mut^{-/-}$ mice at 1 year posttreatment with either 2×10^{11} GC of rAAV8-TBG-mMut ($n = 4$) or 2×10^{11} GC of rAAV8-CBA-mMut ($n = 4$) were injected with sodium [$1\text{-}^{13}C$]propionate. The latter group of mice was included as a comparator for long-term, whole-body correction. The *in vivo* metabolism of this tracer involves a series of enzymatic reactions, including methylmalonyl-CoA mutase, and results in oxidation of [$1\text{-}^{13}C$]propionate to $^{13}CO_2$ via the Krebs cycle. Assuming normal activity of other enzymatic reactions, an increase in sodium [$1\text{-}^{13}C$]propionate oxidized to $^{13}CO_2$ by treated mice relative to untreated $Mut^{-/-}$ mice indicates an increase in methylmalonyl-CoA mutase activity. As can be seen in Fig. 4B, $Mut^{+/-}$ mice ($n = 12$)

FIG. 3. Increased expression of methylmalonyl-CoA mutase in the liver of mice treated with a single intrahepatic injection of AAV8-TBG-mMut (2×10^{11} GC). (A) Liver *Mut* mRNA expression was determined by quantitative PCR. Treated *Mut*^{-/-} mice had significantly higher mRNA expression: 13% at 90 days of life ($n = 3$; $p < 0.01$) and 46% at 270 days of life ($n = 3$; $*p < 0.01$), compared with untreated *Mut*^{+/+} mice ($n = 3$). Untreated *Mut*^{-/-} mice had less than 0.5% of the *Mut* mRNA expression of *Mut*^{+/+} mice. (B) The expression of *Serpina7* (TBG) was assayed by quantitative PCR in treated *Mut*^{-/-} mice. The mice at 270 days ($n = 3$) had, on average, 2.4-fold increased *Serpina7* RNA levels compared with younger animals ($n = 3$). (C) Immunoblotting showed that one of two treated *Mut*^{-/-} mice studied at 90 days had hepatic Mut protein levels similar to that of *Mut*^{+/+} mice. No Mut protein was detected in untreated *Mut*^{-/-} mice. (D) rAAV-mediated transgene expression in the liver. Shown is GFP expression and localization in liver sections of mice killed 60 days after injection of rAAV8-TBG-GFP at 2×10^{11} GC (A–C) compared with control mice (D–F). GFP expression (A and D) and nuclear DNA (B and E) images were merged (C and F), using LSM Image Browser software (Carl Zeiss). The white arrow indicates an area where the nuclear contour is preserved with green signal present. Color images available online at www.liebertonline.com/hum.

metabolize approximately $69.4 \pm 4.3\%$ of [$1\text{-}^{13}\text{C}$]propionate into $^{13}\text{CO}_2$ in 25 min whereas untreated *Mut*^{-/-} mice ($n = 9$) oxidize $12.6 \pm 2.2\%$ of the dose, with flat enrichment kinetics. At 1 year of age, rAAV8-TBG-mMut-treated *Mut*^{-/-} mice ($n = 4$) showed an increased capacity to metabolize [$1\text{-}^{13}\text{C}$]propionate and could convert approximately $32.5 \pm 8.1\%$ of the injected dose into $^{13}\text{CO}_2$ in 25 min, which is not significantly different ($p = 0.20$) from the $39.8 \pm 9.4\%$ range observed in a group of mice treated with 2×10^{11} GC of rAAV8-CBA-mMut.

Discussion

The successful outcomes seen in some patients with methylmalonic acidemia who have received liver transplants led us to perform liver-directed gene delivery experiments in a mouse model that faithfully replicates the severest phenotype of methylmalonic acidemia. We selected the TBG promoter because it has been used in rAAVs that have successfully corrected liver-specific metabolic disorders in mice, such as ornithine transcarbamylase deficiency (Moscioni

FIG. 4. Increase in methylmalonyl-CoA mutase activity in mice treated with 2×10^{11} GC of AAV8-TBG-mMut. **(A)** Plasma methylmalonic acid (MMA) levels at 24, 60, 90, 120, 180, 270, and 360 days of life in untreated *Mut*^{-/-} mice and *Mut*^{-/-} mice treated with 2×10^{11} GC of rAAV8-TBG-mMut. The mean plasma MMA levels of untreated *Mut*^{-/-} mice were 1,361 μ M at 24 days ($n = 13$) and 1,120 μ M at 60 days ($n = 6$). The mean plasma MMA levels of treated *Mut*^{-/-} mice were considerably lower at 703 μ M in mice 24 days old ($n = 12$; $*p < 0.01$) and 528 μ M in mice 60 days old ($n = 11$; $*p < 0.01$). Plasma MMA levels in older mice stabilized between 345 and 465 μ M after 90 days ($n = 4-8$ at each point). The plasma MMA levels in unaffected *Mut*^{+/+} mice ranged from 5 to 10 μ M. **(B)** *In vivo* [$1-^{13}$ C]propionate oxidation in *Mut*^{-/-} mice 1 year after treatment with 2×10^{11} GC of rAAV8-TBG-mMut or 2×10^{11} GC of rAAV8-CBA-mMut. Mice were injected with 200 mg of sodium [$1-^{13}$ C]propionate and the percentage of dose oxidized was measured at 25 min. rAAV8-TBG-mMut-treated *Mut*^{-/-} mice ($n = 4$) oxidized $32.5 \pm 8.1\%$ of the injected tracer, compared with $12.6 \pm 2.2\%$ by untreated *Mut*^{-/-} mice ($n = 9$) ($*p < 0.01$). rAAV8-CBA-mMut-treated *Mut*^{-/-} mice ($n = 4$) oxidized $39.8 \pm 9.4\%$ of the injected tracer (p value not significant vs. rAAV8-TBG-mMut-treated mice). Untreated *Mut*^{+/+} mice ($n = 12$) oxidized $69.4 \pm 4.3\%$ of the injected tracer at the same time point. Error bars surround the 95% confidence intervals.

et al., 2006) and apolipoprotein deficiency (Leberherz *et al.*, 2007), and has been extensively studied in nonhuman primates (Wang *et al.*, 2010a). The use of a hepatotropic rAAV serotype 8 (Gao *et al.*, 2002) further enhanced delivery of the transgene to the liver. *Mut* RNA levels in rAAV8-TBG-mMut-treated *Mut*^{-/-} mice were consistent with the GFP expression pattern in the liver seen after treatment with rAAV8-TBG-GFP (Fig. 3), suggesting that the correction of approximately 9% of hepatocytes is responsible for maintaining long-term metabolic stability and phenotypic correction in treated *Mut*^{-/-} mice.

Gene therapy with either 2×10^{11} GC of rAAV8-TBG-mMut or 2×10^{11} GC of rAAV8-CBA-mMut resulted in comparable phenotypic and metabolic correction of *Mut*^{-/-} mice. For example, at 60 days the growth effects were similar between the treated mutant groups ($72 \pm 18\%$ [Fig. 2] vs. $81 \pm 15\%$; $p = 0.20$), as were the plasma MMA levels ($528 \pm 133 \mu$ M [Fig. 4B] vs. $511 \pm 160 \mu$ M; $p = 0.77$). Even 1 year after injection, *Mut*^{-/-} mice treated with 2×10^{11} GC of rAAV8-TBG-mMut had a propionate oxidative capacity that was equivalent to that observed in mice treated with 2×10^{11} GC of rAAV8-CBA-mMut (Fig. 4B). The hepatic RNA and protein expression seen at later times in *Mut*^{-/-} mice treated with 2×10^{11} GC of rAAV8-TBG-mMut establish that the TBG promoter can effectively direct long-term expression in a small population of transduced cells, as the GFP reporter experiments show.

Endogenous TBG (*Serpina7*) transcript levels increased over time, suggesting there was an increase in the promoter-

driven expression of *Mut* from the rAAV-TBGmMut transgene in our treated mice. Such an observation would be consistent with a prior report that established TBG as a senescence-upregulated protein in rodents (Savu *et al.*, 1991). We therefore quantified the expression of TBG RNA at 90 and 270 days of life and found an approximate 2.4-fold increase in the levels of RNA at the later time point (Fig. 3B). Because mice at both the 90- and 270-day time points had similar genome copy levels of the rAAV8-TBG-mMut vector, the most plausible explanation for the apparent increase in *Mut* transcript levels at 270 days compared with 90 days in the rAAV8-TBG-mMut-treated *Mut*^{-/-} mice would be transcriptional upregulation from the TBG promoter in the AAV vector, as occurs at the endogenous locus. Further studies will be required to evaluate more thoroughly whether promoter regulation may be used to modulate the effects of gene therapy in methylmalonic acidemia, but these data support the use of an rAAV configured with a liver-specific promoter, especially if such a vector could facilitate a physiological, long-term increase in the expression of a therapeutic viral transgene.

Complete biochemical correction of methylmalonic acidemia, in humans or mice, is unlikely. Patient studies have clearly, and repeatedly, documented failure of metabolites to normalize in liver (Kaplan *et al.*, 2006a) and/or combined liver-kidney transplant recipients (van 't Hoff *et al.*, 1998; Nagarajan *et al.*, 2005). In the gene therapy experiments presented here, we evaluated plasma MMA levels to indirectly measure *Mut* activity, not as a definitive parameter to

establish that the disease phenotype had been corrected. Numerous metabolic changes have been documented in methylmalonic acidemia, but the role(s) that each plays in the pathology of this heterogeneous disease remains unclear. AAV8-TBG-mMut-treated *Mut*^{-/-} mice display substantially increased concentrations of MMA but appear grossly normal, suggesting that a small amount of Mut enzymatic activity can provide long-term phenotypic correction and demonstrating that stably transduced cells persist in the setting of an abnormal metabolic milieu.

The data presented here examine the efficacy of liver-directed gene therapy in a tractable experimental animal model and help clarify some of the confounding issues that have surrounded liver transplantation for methylmalonic acidemia patients. Our results clearly demonstrate the efficacy of this approach and are consistent with the suggestion offered by some authors to perform a liver transplantation as soon as possible as a treatment for this disorder (Morioka *et al.*, 2007). Because the stable transduction of a small number of hepatocytes appears sufficient to obtain therapeutic effects in a mouse model that replicates a severe form of the condition, liver-directed gene therapy would likely benefit patients with methylmalonic acidemia and, possibly, other inborn errors of organic acid metabolism. Last, our vector has been harmonized in design with those currently being used in human clinical trials and is similar to one that has been tested in nonhuman primates (Wang *et al.*, 2010a). This should facilitate the translation of our murine studies to a clinical gene therapy trial for methylmalonic acidemia.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Charles P. Venditti

Organic Acid Research Section

Genetics and Molecular Biology Branch

National Human Genome Research Institute

National Institutes of Health

Building 49, Room 4A18

Bethesda, MD 20892

E-mail: venditti@mail.nih.gov

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