Long-term Rescue of a Lethal Murine Model of Methylmalonic Acidemia Using Adeno-associated Viral Gene Therapy

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Methylmalonic acidemia (MMA) is an organic acidemia caused by deficient activity of the mitochondrial enzyme methylmalonyl-CoA mutase (MUT). This disorder is associated with lethal metabolic instability and carries a poor prognosis for long-term survival. A murine model of MMA that replicates a severe clinical phenotype was used to examine the efficacy of recombinant adeno-associated virus (rAAV) serotype 8 gene therapy as a treatment for MMA. Lifespan extension, body weight, circulating metabolites, transgene expression, and whole animal propionate oxidation were examined as outcome parameters after gene therapy. One-hundred percent of the untreated *Mut*$^{-/-}$ mice (n = 58) died by day of life (DOL) 72, whereas >95% of the adeno-associated virus–treated *Mut*$^{-/-}$ mice (n = 27) have survived for ≥1 year. Despite a gradual loss of transgene expression and elevated circulating metabolites in the treated *Mut*$^{-/-}$ mice, the animals are indistinguishable from unaffected control littermates in size and activity levels. These experiments provide the first definitive evidence that gene therapy will have clinical utility in the treatment of MMA and support the development of gene therapy for other organic acidemias.

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INTRODUCTION

Methylmalonic acidemia (MMA) is a severe autosomal recessive inborn error of intermediary metabolism characterized by intermittent metabolic instability, multiorgan pathology, growth retardation, and a poor prognosis for long-term survival.[1–6] The disorder exhibits genetic heterogeneity and can be caused by deficient enzymatic activity of methylmalonyl-CoA mutase (MUT) or defective intracellular transport, processing, and metabolism of cobalamin.[7] MUT is an important mitochondrial enzyme in propionyl-CoA metabolism and converts L-methylmalonyl-CoA into succinyl-CoA, a Krebs cycle intermediate. A block at this enzymatic step results in elevated plasma levels of methylmalonic acid as well the accumulation of other propionyl-CoA-derived metabolites such as 2-methylcitrate.[8] The etiology of the many medical problems that patients with MMA suffer is not well understood. However, the wide spectrum of severity that can be seen in patients who harbor missense mutations suggests that restoring a very low level of enzyme activity would provide substantial clinical benefit.

Currently, the main treatment for affected patients is dietary restriction of propiogenic amino acids to reduce circulating metabolites. Liver[9–15] and/or combined liver/kidney[16,17] transplantation has been performed in a limited fashion in an attempt to improve metabolic stability through the provision of organ-specific enzymatic activity. Although this approach has been effective, and even curative, for other metabolic disorders,[18] the clinical utility of solid organ transplantation as a standard treatment for MMA is unclear given the small number of patients that have undergone the procedure.[14,15,19] The need for new and widely available therapies for MMA is underscored by a recent multicenter European long-term patient study, which described an overall 46% (n = 52) mortality for patients with MMA resulting from MUT deficiency by 30 years of age.[6]

We have previously developed a murine model of MMA that replicates the severest clinical phenotype of MMA seen in patients.[20] Affected animals display a 100–200-fold increase in plasma methylmalonic acid concentrations and most perish within the first few days of life.[20,21] Recently, we reported that direct hepatic, but not intramuscular, injection of an adenovirus that expressed the *Mut* gene under the control of a cytomegalovirus promoter could only partially rescue the *Mut*$^{-/-}$ mice.[22] A liver-directed approach was initially selected because the hepatocytes of *Mut*$^{-/-}$ mice and MMA patients manifest morphological changes and display a severe secondary electron transport chain defect that is likely contributory to the pathology of the disease.[21] Additionally, we had demonstrated that robust correction of human *MUT*$^{-/-}$ hepatocytes was feasible with this vector.[23] However, in the surviving adenoviral-treated *Mut*$^{-/-}$ mice, the *Mut* levels steadily declined, and most animals died between 1 and 3 months after treatment. These results suggested that persistent expression would be needed to provide long-term amelioration of the lethal phenotype in this murine model of MMA.

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Recombinant adeno-associated viruses (rAAVs) have been successfully used as gene delivery vehicles in numerous animal models of human disease and have yielded long-term transgene expression without vector-related toxicity. Furthermore, clinical trials using rAAV have involved hundreds of subjects, supporting the safety of these vectors for use in humans.[24] Over 100 different natural AAV serotypes have been isolated from a variety of species and some display striking tissue tropism.[25] Several studies have demonstrated that rAAV serotype 8 (rAAV8) vectors can transduce mouse hepatocytes with high efficiency when delivered via the portal vein or intraperitoneal injection in the neonatal period.[26–28] Similarly, rAAV8 vectors can efficiently transduce skeletal muscle,[29,30] a tissue that makes a significant contribution to the circulating metabolite pool in patients with MMA.[20]

In this report, we describe the therapeutic efficacy of a rAAV vector as a new gene therapy treatment for MMA and apply a novel stable isotope metabolic method to monitor the function of Mut after gene therapy. The treated *Mut*$^{-/-}$ mice have reduced circulating metabolites, are phenotypically indistinguishable from their unaffected *Mut*$^{+/-}$ mice littermates and show sustained enzymatic activity 1 year after treatment with the rAAV8 vector. Our results provide the first evidence that systemic gene delivery using rAAV should be useful as a treatment for patients with MMA and other organic acidemias, disorders that currently lack definitive therapy. This gene delivery platform and metabolic monitoring technique should be immediately translatable to a human gene therapy trial for MMA.

RESULTS

Gene therapy rescues the lethal *Mut*$^{-/-}$ phenotype

Mut$^{-/-}$ newborn mice received a direct hepatic injection with either 1 or 2 × 10^{11} vector genome copies (GCs) of rAAV8-mMut. All *Mut*$^{-/-}$ mice (n = 27) injected in the neonatal period with rAAV8-mMut survived until day of life (DOL) 90 (**Figure 1**).

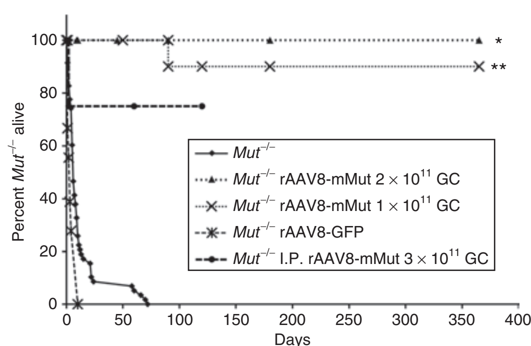

Figure 1 Rescue of *Mut*$^{-/-}$ mice. Survival in days between the untreated *Mut*$^{-/-}$ mice (n = 58), intrahepatic-injected rAAV8-GFP 1 or 2 × 10^{11} GC *Mut*$^{-/-}$ mice (n = 18), intrahepatic-injected rAAV8-mMut 1 × 10^{11} GC (n = 10) or 2 × 10^{11} GC (n = 17) *Mut*$^{-/-}$ mice, and i.p. rAAV8-mMut 3 × 10^{11} GC injected (n = 4) *Mut*$^{-/-}$ mice. All three groups of rAAV8-mMut-treated *Mut*$^{-/-}$ mice exhibited a significant improvement in survival relative to untreated and rAAV8-GFP-treated *Mut*$^{-/-}$ mice. The intrahepatic-injected rAAV8-mMut-treated *Mut*$^{-/-}$ mice show significantly improved survival at 24, 60, and 100 days and beyond compared to the untreated and rAAV8-GFP-treated *Mut*$^{-/-}$ mice (*$P < 10^{-22}$ for rAAV8-mMut 1 × 10^{11} GC and **$P < 10^{-22}$ for rAAV8-mMut 2 × 10^{11} GC at the 100-day time point). GC, genome copy; GFP, green fluorescent protein; i.p., intraperitoneal.

A single *Mut*$^{-/-}$ mouse from the rAAV8-mMut 1 × 10^{11} GC group perished at DOL 92 following a blood collecting procedure; a full necropsy was performed, and no abnormalities were observed. Ninety-six percent (26/27) of the *Mut*$^{-/-}$ mice treated with rAAV8-mMut have survived beyond a year. In contrast, 100% of the untreated mutants (n = 58) perished by DOL 72, with >90% of this group dying by DOL 24. In another control group, newborn *Mut*$^{-/-}$ mice (n = 18) received a direct hepatic injection with either 1 or 2 × 10^{11} GC of rAAV8 containing green fluorescent protein (GFP) complementary DNA driven by the same chicken β-actin promoter/enhancer (**Figure 1**). None of the rAAV8-GFP-treated *Mut*$^{-/-}$ mice survived beyond DOL 3. Untreated *Mut*$^{-/-}$ mice and rAAV8-GFP-treated *Mut*$^{-/-}$ animals were found dead or cannibalized; the exact causes of death were undetermined. To determine whether direct hepatic injection was necessary to rescue *Mut*$^{-/-}$ mice, newborn *Mut*$^{-/-}$ mice (n = 4) received an intraperitoneal injection of 3 × 10^{11} GC rAAV8-mMut. Three out of the four (75%) of these mice survived and are still alive at DOL 120 (**Figure 1**). Other than mild hepatomegaly, the *Mut*$^{-/-}$ mice

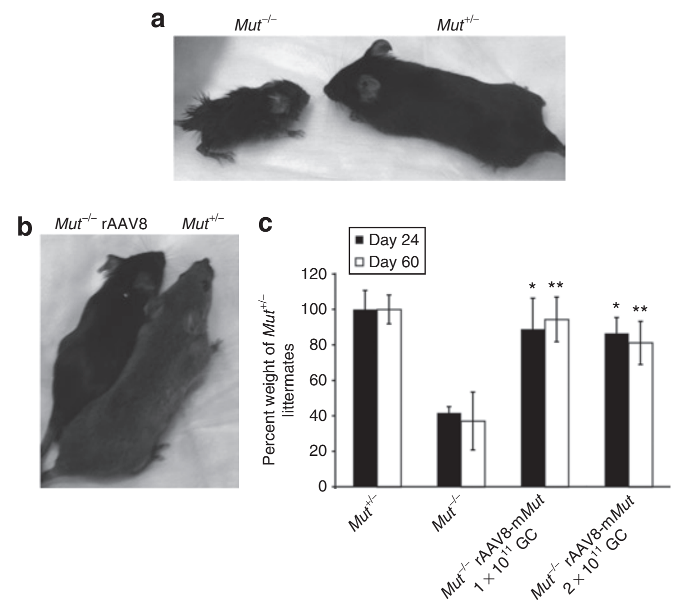

Figure 2 Growth and phenotypic correction after rAAV8-mMut gene therapy. (**a**) An untreated *Mut*$^{-/-}$ mouse (left) compared to a *Mut*$^{+/-}$ littermate at day of life 55 to illustrate the relative difference in size and appearance. The *Mut*$^{-/-}$ mouse is severely growth retarded and has achieved only 40% of the *Mut*$^{+/-}$ littermate weight. Shortly after this photo was taken, the *Mut*$^{-/-}$ mouse became lethargic and perished. (**b**) A *Mut*$^{-/-}$ mouse (left, neck flexed) that received a single intrahepatic injection of rAAV8-mMut 2 × 10^{11} GC aside a *Mut*$^{+/-}$ littermate (right, neck extended) at day of life 120. The treated *Mut*$^{-/-}$ animal is similar in size to a control *Mut*$^{+/-}$ littermate. (**c**) Growth correction between untreated *Mut*$^{-/-}$ mice, intrahepatic rAAV8-mMut-treated *Mut*$^{-/-}$ mice, and untreated age-, diet-, and gender-matched *Mut*$^{+/-}$ littermates. The graph depicts the percent weight at day of life 24 and 60 of *Mut*$^{+/-}$ diet- and gender-matched littermates (n = 25, 25) compared to untreated *Mut*$^{-/-}$ mice (n = 6, 3) or *Mut*$^{-/-}$ mice treated via an intrahepatic injection of rAAV8-mMut 1 × 10^{11} GC (n = 10, 10) or rAAV8-mMut 2 × 10^{11} GC (n = 17, 17) at birth. The rAAV8-mMut-treated *Mut*$^{-/-}$ mice at both doses showed significant growth improvement compared to the untreated *Mut*$^{-/-}$ mice (*$P < 10^{-6}$ for day 24, **$P < 0.01$ for day 60). Error bars represent plus and minus one standard deviation. GC, genome copy.

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(n = 4) treated by rAAV8-mMut gene therapy and sacrificed for tissue harvesting on DOL 90 or at 1 year had no gross pathologic changes noted on dissection.

Correction of growth retardation following gene therapy

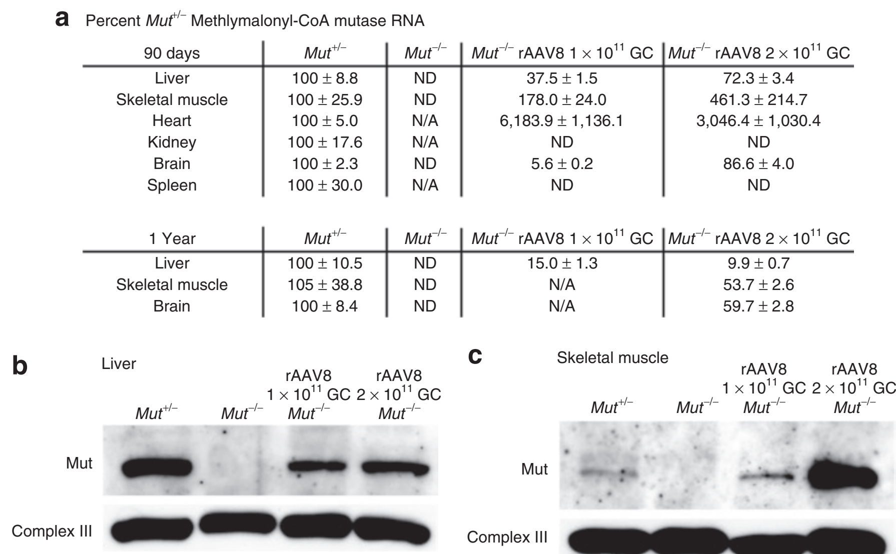
Mut$^{-/-}$ mice were indistinguishable from their *Mut*$^{+/-}$ littermates at birth, but those rare mutants that escaped neonatal lethality were grossly abnormal. The small number of untreated *Mut*$^{-/-}$ mice that survived to DOL 24 (6/58) and 60 (3/58) were severely growth retarded, weighing <40% of sex-matched *Mut*$^{+/-}$ littermates (**Figure 2a**). *Mut*$^{-/-}$ mice treated with an intrahepatic injection of either 1 or 2 × 10^{11} GC of rAAV8-mMut were grossly indistinguishable in size and behavior compared to controls (**Figure 2b**) and achieved body weights that were similar to their *Mut*$^{+/-}$ sex-matched littermates on DOL 24 and 60 (**Figure 2c**).

Expression of Mut after treatment with rAAV8-mMut

Mut expression in liver and muscle samples from treated *Mut*$^{-/-}$ mice at DOL 90 and 1 year of life was analyzed using quantitative PCR (qPCR) to measure mRNA levels and by western blotting to examine protein content. The *Mut*$^{-/-}$ mice at DOL 90 injected with either 1 or 2 × 10^{11} GC of rAAV8 expressed 38 and 72% of the endogenous *Mut* mRNA levels found in the liver of untreated *Mut*$^{+/-}$ animals (**Figure 3a**), whereas the level of *Mut* mRNA in the lower limb skeletal muscle of treated animals exceeded the endogenous *Mut* transcript levels measured to control heterozygotes (**Figure 3a**). Even greater expression was noted in the hearts of the treated *Mut*$^{-/-}$ mice compared to untreated *Mut*$^{+/-}$ mice, consistent with previous observations of highly efficient transduction of cardiac and skeletal myocytes by rAAV8 vectors.[30] *Mut* mRNA was variably detected in whole brain extracts and not detected in the kidney or spleen. Western blotting showed Mut protein in both the liver and the skeletal muscle of the treated *Mut*$^{-/-}$ mice at levels that paralleled those observed in the qPCR experiments on DOL 90 (**Figure 3b**). Mice studied at longer times showed persistent expression of the *Mut* transgene. The levels of *Mut* mRNA in the liver and skeletal muscle of treated *Mut*$^{-/-}$ mice 1 year after injection diminished but was still readily detectable by qPCR (**Figure 3a**), but not by western analysis.

Gene therapy restores Mut function and activity

Several parameters reflective of Mut enzymatic function were examined in the treated *Mut*$^{-/-}$ mice. Circulating metabolites were measured and taken to reflect whole-body Mut enzymatic activity because all the mice ingested a precursor unrestricted diet. The plasma methylmalonic acid concentrations in the treated *Mut*$^{-/-}$ mice were significantly lower than in untreated *Mut*$^{-/-}$ mice at both time points measured (**Figure 4a**). The untreated *Mut*$^{-/-}$ mice had mean plasma methylmalonic acid concentrations of 1,342 and 1,120 μmol/l on days 24 and 60. No untreated *Mut*$^{-/-}$ mice survived beyond day 72; therefore, plasma methylmalonic acid levels after day 60 from this group could not be obtained. Both the 1 and 2 × 10^{11} GC groups of treated *Mut*$^{-/-}$ mice had mean plasma methylmalonic acid concentrations between 440 and 540 μmol/l at the day 24 and 60 time points. Metabolites in these two groups of treated *Mut*$^{-/-}$ mice were also measured at 90, 120, 180, and 360 days with the mean methylmalonic acid levels at these time points ranging from 365 to 596 μmol/l (**Figure 4a**). As observed in humans with MMA whom have received liver or



a Percent *Mut*$^{+/-}$ Methlymalonyl-CoA mutase RNA

90 days	*Mut*$^{+/-}$	*Mut*$^{-/-}$	*Mut*$^{-/-}$ rAAV8 1 × 10^{11} GC	*Mut*$^{-/-}$ rAAV8 2 × 10^{11} GC
Liver	100 ± 8.8	ND	37.5 ± 1.5	72.3 ± 3.4
Skeletal muscle	100 ± 25.9	ND	178.0 ± 24.0	461.3 ± 214.7
Heart	100 ± 5.0	N/A	6,183.9 ± 1,136.1	3,046.4 ± 1,030.4
Kidney	100 ± 17.6	N/A	ND	ND
Brain	100 ± 2.3	ND	5.6 ± 0.2	86.6 ± 4.0
Spleen	100 ± 30.0	N/A	ND	ND

1 Year	*Mut*$^{+/-}$	*Mut*$^{-/-}$	*Mut*$^{-/-}$ rAAV8 1 × 10^{11} GC	*Mut*$^{-/-}$ rAAV8 2 × 10^{11} GC
Liver	100 ± 10.5	ND	15.0 ± 1.3	9.9 ± 0.7
Skeletal muscle	105 ± 38.8	ND	N/A	53.7 ± 2.6
Brain	100 ± 8.4	ND	N/A	59.7 ± 2.8

Figure 3 Methylmalonyl-CoA mutase expression after rAAV8-mMut treatment. (**a**) Quantitative PCR analysis of *Mut* expression in tissues. The level of *Mut* mRNA (plus or minus one standard deviation) detected in the various tissues from *Mut*$^{+/-}$ controls was set at 100% and used as a comparator. GAPDH was independently examined for normalization. ND equals none detected (<1%), N/A equals not analyzed. The treated *Mut*$^{-/-}$ mice show significant expression in the liver, muscle, heart, and brain after neonatal gene delivery at 90 days that diminished after 1 year. (**b**) Western analysis of liver (top) and lower limb skeletal muscle (bottom) total extracts were prepared from *Mut*$^{+/-}$ (day 90), untreated *Mut*$^{-/-}$ (day 45), and *Mut*$^{-/-}$ mice (day 90) that had received either 1 × 10^{11} GC or 2 × 10^{11} GC of rAAV8-mMut and analyzed by western blotting. The same membranes were probed with either anti-methylmalonyl-CoA mutase antibody (labeled Mut) or an anti-ubiquinol-cytochrome c oxidoreductase antibody (labeled complex III) to control for loading and mitochondrial content. Immunoreactive Mut enzyme is present in all lanes, except those from the untreated *Mut*$^{-/-}$ mice. The mitochondrial loading control shows approximately the same intensity in each sample. GC, genome copy.

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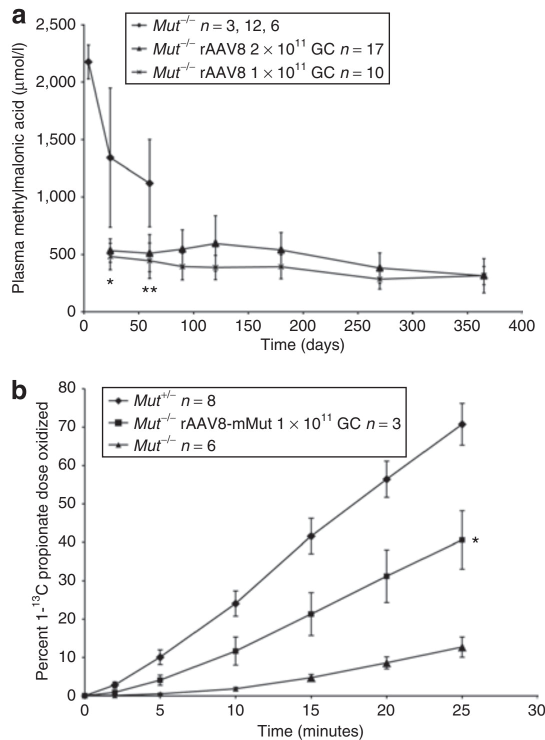

Figure 4 Metabolic improvements after rAAV8-mMut treatment. (**a**) Plasma methylmalonic acid levels (µmol/l) were measured at time points of 24, 90, 120, 180, and 360 days after birth in the rAAV8-mMut-treated *Mut*$^{-/-}$ mice as an indication of Mut activity. Three groups are presented: untreated *Mut*$^{-/-}$ mice, *Mut*$^{-/-}$ mice treated with 1 × 10^{11} GC rAAV8-mMut, and *Mut*$^{-/-}$ mice treated with 2 × 10^{11} GC rAAV8-mMut. Untreated and treated *Mut*$^{+/-}$ mice have plasma methylmalonic acid levels between 5 and 10 µmol/l, and are not depicted in this graph. The numbers in each group are presented in the graph. Error bars represent plus and minus one standard deviation. The rAAV8-mMut-treated mutant mice show a significant reduction in plasma methylmalonic acid levels compared to the untreated *Mut*$^{-/-}$ mice at all time points (*P < 0.001 on day 24, **P < 0.01 on day 90). (**b**) 1-^{13}C-propionate oxidation 1 year after rAAV8-mMut treatment. Two hundred micrograms of 1-^{13}C-sodium propionate was injected intraperitoneally into *Mut*$^{+/-}$ (n = 8), 1 × 10^{11} GC rAAV8-mMut-treated *Mut*$^{-/-}$ (n = 3), or untreated *Mut*$^{-/-}$ (n = 6) mice. ^{13}C enrichment in expired CO_2 was measured and used to determine the percent of the administered 1-^{13}C-propionate dose that was oxidized. Error bars surround the 95% confidence intervals. The rAAV8-mMut-treated *Mut*$^{-/-}$ mice show a significant increase in the ability to oxidize 1-^{13}C-propionate compared to the untreated *Mut*$^{-/-}$ mice at 25 minutes (*P < 0.01). GC, genome copy.

combined liver–kidney transplants, plasma metabolites were not normalized[13,16,17] and remained 50–100-fold increased over the level seen in unaffected *Mut*$^{+/-}$ mice (5–10 µmol/l).

To examine whether the long-term survival and ameliorated metabolite levels observed in the treated *Mut*$^{-/-}$ mice corresponded with increased whole-body enzyme activity, we developed a novel *in vivo* propionate oxidation assay. *Mut*$^{-/-}$ mice at 1 year after treatment were injected with 1-^{13}C-sodium propionate and the subsequent metabolism of this tracer through the Mut reaction, into the Krebs cycle, with eventual oxidation into $^{13}CO_2$ was determined. As can be seen in **Figure 4b**, *Mut*$^{+/-}$ mice metabolize ~70% of 1-^{13}C-propionate into $^{13}CO_2$ in 25 minutes. Untreated *Mut*$^{-/-}$ mice convert ~10% of the dose, with very flat enrichment kinetics. At 1 year of age, the treated *Mut*$^{-/-}$ mice show a

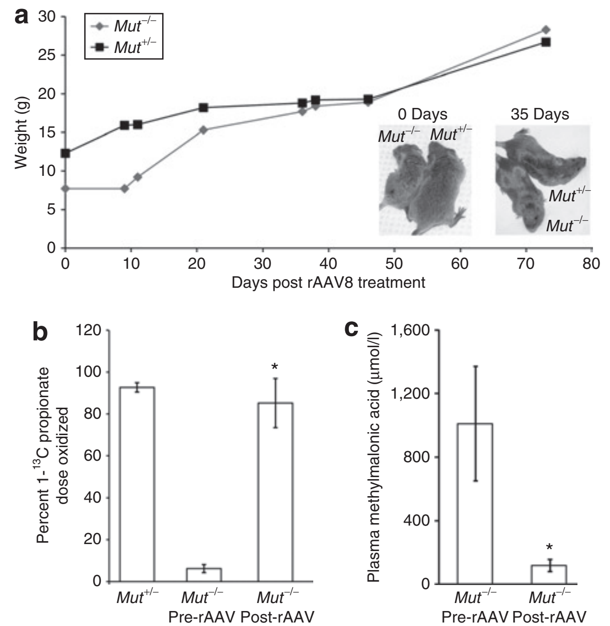

Figure 5 rAAV8-mMut treatment in 20-day-old *Mut*$^{-/-}$ mice. (**a**) A *Mut*$^{-/-}$ mouse was treated on day of life (DOL) 20 with 3 × 10^{11} GC rAAV8-mMut delivered intraperitoneally (i.p.), and serial weights were measured in parallel with a control *Mut*$^{+/-}$ littermate. The treated *Mut*$^{-/-}$ mouse achieved the control weight 40 days after treatment. (**b**) Two hundred micrograms of 1-^{13}C-propionate was injected i.p. into *Mut*$^{-/-}$ mice (labeled *Mut*$^{-/-}$ pre-rAAV, n = 3) or control *Mut*$^{+/-}$ littermates (labeled *Mut*$^{+/-}$, n = 3) on DOL 20 prior to receiving 3 × 10^{11} GC rAAV8-mMut. The study was repeated 10 days after the viral injection and propionate oxidation at 20 minutes in the treated *Mut*$^{-/-}$ mice (labeled *Mut*$^{-/-}$ post-rAAV, n = 3) achieved levels that were significantly greater than the untreated mutants (*P < 0.01) and equivalent to the controls. Error bars surround the standard deviation. (**c**) Plasma methylmalonic acid levels in response to rAAV8 treatment on DOL 20 in *Mut*$^{-/-}$ mice (n = 3). Before (pre-rAAV; mean 1,011 µmol/l) was significantly more than after (post-rAAV; mean 118 µmol/l). *P < 0.05. Error bars surround the standard deviation. GC, genome copy.

markedly increased capacity to oxidize 1-^{13}C-propionate and on average, can convert ~40% of the injected dose into $^{13}CO_2$.

rAAV8-mMut rescues postneonatal *Mut*$^{-/-}$ mice

As an extension of rAAV8-mMut gene therapy beyond the immediate neonatal period, three rare untreated *Mut*$^{-/-}$ mice that survived until DOL 20 received a single intraperitoneal injection of 3 × 10^{11} GC of rAAV8-mMut. At the time of injection, the animals were hypoactive, runted (**Figure 5a**) and showed an impaired ability to produce $^{13}CO_2$ from 1-^{13}C-propionate. When studied 10 days after receiving the rAAV8-mMut, the mice had an improved clinical appearance (**Figure 5a**), fully restored propionate oxidation (**Figure 5b**) and displayed a tenfold reduction in plasma methylmalonic acid concentrations (**Figure 5c**).

DISCUSSION

The experiments undertaken in the present report were designed to test the efficacy of rAAV-mediated gene therapy in a murine model of MMA, a prototypical organic acidemia. Our earlier studies in *Mut*$^{-/-}$ mice[22] and the demonstration that liver transplantation appears beneficial for a subset of MMA patients[15] led to the

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selection of adeno-associated virus serotype 8 as a gene delivery vector. The observed results are striking: a single intrahepatic injection of rAAV8-mMut delivered in the neonatal period was sufficient to uniformly rescue treated $Mut^{-/-}$ mice from certain death for over a year. The effects of gene therapy extended beyond immediate mortality and allowed the treated $Mut^{-/-}$ mice to gain weight, thrive, and reproduce. Limited pathological investigations have been performed on the treated mutants at older times and will be the subject of future studies, particularly to examine whether renal, hepatic, central nervous system, or pancreatic changes are present and if they have functional consequences for the treated mice. The treated $Mut^{-/-}$ animals were also able to tolerate a liberalized diet in the face of elevated circulating metabolites, which were greatly diminished compared to the untreated $Mut^{-/-}$ group, but still significantly increased compared to heterozygous controls. The $Mut^{-/-}$ mice, both treated and untreated, did not receive a precursor-restricted diet, commonly employed to treat patients[31] that likely would have further decreased methylmalonic acid levels in the treated animals. Complete restoration of plasma metabolites to normal in the treated mice was not expected because patients with MMA who have received replacement liver and kidney combined transplantation procedures also display persistent MMA and methylmalonic aciduria.[16,17] Also, there is no evidence to suggest that free methylmalonic acid can be efficiently metabolized, even when delivered exogenously to a wild-type host.

Consistent with many previous studies, the rAAV8-mMut vector produced persistent expression in the liver and muscle that was readily detected at 90 days after therapy at the mRNA and protein level, and at 1 year through mRNA expression and *in vivo* propionate oxidation. Because the mice were treated at the time of birth, the rapid growth of the liver and subsequent dilution in the number of transduced cells by cell division likely explains the relative diminution of Mut expression over time.[32] The cohort of treated $Mut^{-/-}$ mice, which is >25, have survived to 1 year and beyond, demonstrating that even low levels of Mut expression are sufficient to provide metabolic homeostasis, and prevent morbidity and mortality. Furthermore, although formal testing has not been performed, the mutant animals appeared clinically well, with no obvious neurological or behavioral phenotypes. rAAV-based gene vectors have previously shown promising proof-of-principle correction in other mouse models of metabolic disease[33] and now includes a pleiotropic disorder of organic acid metabolism.

The hereditary MMAs, as well as other inborn errors of metabolism that lack conventional therapy, are included in routine newborn screening panels used by many states and countries.[34] There has been a vigorous and public debate on the inclusion of these disorders in the list of conditions for which screening is offered. In this report, we have demonstrated that a single injection of rAAV8-mMut was sufficient to cure the lethal phenotype of Mut deficiency in a murine model of MMA that closely replicates the human condition. Our studies are the first to demonstrate that MMA, and by extension other organic acidemias, might be treated by gene therapy with a safe and effective vector. This conclusion offers strong support for the continued and expanded screening of infants for disorders of intermediary metabolism and to the application of gene therapy to humans with MMA.

MATERIALS AND METHODS

Murine model of MMA. The targeted *Mut* allele harbors a deletion of exon 3 in the *Mut* gene. This exon encodes the putative substrate-binding pocket in the Mut enzyme. The *Mut* allele does not produce mature RNA, protein, or enzymatic activity.[20] $Mut^{-/-}$ mice on a mixed (C57BL/6 × 129SV/Ev × FvBN) background exhibit a semipenetrant neonatal lethal phenotype with most mice perishing in the early neonatal period.[21] Coat colors are variable in these mice due to parental strain contributions. $Mut^{-/-}$ mice display massively elevated methylmalonic acid concentrations in the plasma that progressively rises to the 2 mmol/l range until death occurs. $Mut^{+/-}$ animals have biochemical parameters identical to $Mut^{+/+}$ wild-type animals and were used as controls throughout.

rAAV8 construction, production, and delivery. The University of Pennsylvania Vector Core provided the expression vector, p-AAV2-CI-CB7-RBG. The vector contains transcriptional control elements from the cytomegalovirus enhancer/chicken β-actin promoter, cloning sites for the insertion of a complementary DNA, and the rabbit β-globin polyA signal.[35] Terminal repeats from AAV serotype 2 flank the expression cassette. Either the murine *Mut* (mMut*)* or GFP was cloned into pAAV2/8.CI.CB7.RBG and packaged into rAAV8, purified by cesium chloride centrifugation, and titered by qPCR as previously described.[26] pAAV2/8.CI.CB7.EGFP.RBG had a titer of 2.25×10^{13} GC/ml and pAAV2/8.CI.CB7.mMut.RBG had a titer of 4.13×10^{13} GC/ml. Animal studies were reviewed and approved by the National Human Genome Research Institute Animal User Committee. Hepatic injections were performed on nonanesthetized neonatal mice, typically within several hours after birth. Viral particles were diluted to a total volume of 20 microliters with phosphate-buffered saline immediately before injection and were delivered into the liver parenchyma using a 32-gauge needle and transdermal approach, as previously described.[22]

Quantitative real-time PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA), and DNase digested was preformed using DNA-free (Ambion, Austin, TX). qPCR was accomplished with TaqMan gene expression assays [mouse GAPD (4352932E) and murine *Mut* (Mm00485312_m1) from Applied Biosystems, Foster City, CA]. Samples were analyzed in an Applied Biosystems 7500 fast real-time PCR system, in accordance with the manufacturer's protocol. All samples were analyzed in triplicate. Three individual mouse tissue samples were used to determine the 100% comparator $Mut^{+/-}$ *Mut* mRNA expression level.

Western blotting. Tissue samples were homogenized with a 2-ml Tenbroeck tissue grinder (Wheaton, Millville, NJ) in T-PER (Pierce Biotechnology, Rockford, IL) tissue protein extraction buffer in the presence of Halt (Pierce Biotechnology) protease inhibitor cocktail. Twenty micrograms of clarified extract were used in western analysis and probed with affinity-purified, rabbit polyclonal antisera raised against the murine Mut enzyme.[23] Complex III Core II was used as a loading control and was also detected by immunoblotting [mouse monoclonal anti-OxPhos Complex III (ubiquinol-cytochrome c oxidoreductase) Core II antibody, Invitrogen SKU# A-11143]. The anti-mutase antibody was used at a dilution of 1:750, and the anti-Complex III Core II antibody was used at a dilution of 1:2,000. Horseradish peroxidase–conjugated anti-rabbit IgG (NA934; GE Healthcare Life Sciences, Piscataway, NJ) or rabbit anti-goat IgG (sc-2768; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody and was visualized with chemiluminescence detection (Pierce Biotechnology).

Metabolic studies. Plasma was isolated from blood collected by orbital bleeding. The samples were immediately centrifuged, and the plasma was removed, diluted in water, and stored at −80 °C in a screw-top tube for later analysis. Methylmalonic acid was analyzed by gas chromatography–mass spectrometry with stable isotopic internal calibration to measure methylmalonic acid as previously described.[36,37] *In vivo* 1-^{13}C-propionate oxidation was determined by collecting expired gas from

mutant, control, and treated mice after the animals were injected by the intraperitoneal route with 200 micrograms of 1-^{13}C-sodium propionate, using an adaptation of a method developed to study propionate oxidation in patients with methylmalonic and propionic acidemia.[38] The 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 ($^{13}C/^{12}C$) of the expired gas was determined with a gas isotope ratio mass spectrometer (Metabolic Solutions, Nashua, NH). The percent dose metabolized at each time point was calculated as % dose metabolized = total ^{13}C excreted (mmol/dose (mmol) × 100%).

Statistical analyses. In all instances, P values were considered significant if the value was <0.05. Differences in the survival between treated groups were analyzed using a χ^2 test. The weights between treated and untreated mice, and differences in metabolite levels were assessed using a two-sided, two-tailed unpaired Student's *t*- test. The Kruskal–Wallis test was used to determine the statistical significance in measured propionate oxidation rates between groups.

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REFERENCES

1. Fenton, WA, Gravel, RA and Rosenblatt, DS (2001). Disorders of propionate and methylmalonate metabolism. In: Scriver, CR, Sly, WS, Childs, B, Beaudet AL, Valle, D, Kinzler, KW *et al.* (eds). *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. McGraw-Hill: New York. pp. 2165–2192.
2. Matsui, SM, Mahoney, MJ and Rosenberg, LE (1983). The natural history of the inherited methylmalonic acidemias. *N Engl J Med* **308**: 857–861.
3. van der Meer, SB, Poggi, F, Spada, M, Bonnefont, JP, Ogier, H, Hubert, P *et al.* (1994). Clinical outcome of long-term management of patients with vitamin B12-unresponsive methylmalonic acidemia. *J Pediatr* **125**(6 Pt 1): 903–908.
4. Nicolaides, P, Leonard, J and Surtees, R (1998). Neurological outcome of methylmalonic acidaemia. *Arch Dis Child* **78**: 508–512.
5. de Baulny, HO, Benoist, JF, Rigal, O, Touati, G, Rabier, D and Saudubray, JM (2005). Methylmalonic and propionic acidaemias: management and outcome. *J Inherit Metab Dis* **28**: 415–423.
6. Hörster, F, Baumgartner, MR, Viardot, C, Suormala, T, Burgard, P, Fowler, B *et al.* (2007). Long-term outcome in methylmalonic acidurias is influenced by the underlying defect (mut0, mut-, cblA, cblB). *Pediatr Res* **62**: 225–230.
7. Fenton, WA and Rosenblatt, DS (2001). Inherited disorders of folate and cobalamin transport and metabolism. In: Scriver, CR, Sly, WS, Childs, B, Beaudet AL, Valle, D, Kinzler, KW *et al.* (eds). *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. McGraw-Hill: New York. pp. 3897–3933.
8. Ando, T, Rasmussen, K, Wright, JM and Nyhan, WL (1972). Isolation and identification of methylcitrate, a major metabolic product of propionate in patients with propionic acidemia. *J Biol Chem* **247**: 2200–2204.
9. Nyhan, WL, Gargus, JJ, Boyle, K, Selby, R and Koch, R (2002). Progressive neurologic disability in methylmalonic acidemia despite transplantation of the liver. *Eur J Pediatr* **161**: 377–379.
10. Chakrapani, A, Sivakumar, P, McKiernan, PJ and Leonard, JV (2002). Metabolic stroke in methylmalonic acidemia five years after liver transplantation. *J Pediatr* **140**: 261–263.
11. Kayler, LK, Merion, RM, Lee, S, Sung, RS, Punch, JD, Rudich, SM *et al.* (2002). Long-term survival after liver transplantation in children with metabolic disorders. *Pediatr Transplant* **6**: 295–300.
12. Hsui, JY, Chien, YH, Chu, SY, Lu, FL, Chen, HL, Ho, MJ *et al.* (2003). Living-related liver transplantation for methylmalonic acidemia: report of one case. *Acta Paediatr Taiwan* **44**: 171–173.
13. Kaplan, P, Ficicioglu, C, Mazur, AT, Palmieri, MJ and Berry, GT (2006). Liver transplantation is not curative for methylmalonic acidopathy caused by methylmalonyl-CoA mutase deficiency. *Mol Genet Metab* **88**: 322–326.
14. Kasahara, M, Horikawa, R, Tagawa, M, Uemoto, S, Yokoyama, S, Shibata, Y *et al.* (2006). Current role of liver transplantation for methylmalonic acidemia: a review of the literature. *Pediatr Transplant* **10**: 943–947.
15. Morioka, D, Kasahara, M, Horikawa, R, Yokoyama, S, Fukuda, A and Nakagawa, A (2007). Efficacy of living donor liver transplantation for patients with methylmalonic acidemia. *Am J Transplant* **7**: 2782–2787.
16. van't Hoff, WG, Dixon, M, Taylor, J, Mistry, P, Rolles, K, Rees, L *et al.* (1998). Combined liver-kidney transplantation in methylmalonic acidemia. *J Pediatr* **132**: 1043–1044.
17. Nagarajan, S, Enns, GM, Millan, MT, Winter, S and Sarwal, MM (2005). Management of methylmalonic acidaemia by combined liver-kidney transplantation. *J Inherit Metab Dis* **28**: 517–524.
18. Meyburg, J and Hoffmann, GF (2005). Liver transplantation for inborn errors of metabolism. *Transplantation* **80**(1 suppl.): S135–S137.
19. Leonard, JV, Walter, JH and McKiernan, PJ (2001). The management of organic acidaemias: the role of transplantation. *J Inherit Metab Dis* **24**: 309–311.
20. Chandler, RJ, Sloan, J, Fu, H, Tsai, M, Stabler, S, Allen, R *et al.* (2007). Metabolic phenotype of methylmalonic acidemia in mice and humans: the role of skeletal muscle. *BMC Med Genet* **8**: 64.
21. Chandler, RJ, Zerfas, PM, Shanske, S, Sloan, J, Hoffmann, V, DiMauro, S *et al.* (2009). Mitochondrial dysfunction in mut methylmalonic acidemia. *FASEB J* **23**: 1252–1261.
22. Chandler, RJ and Venditti, CP (2008). Adenovirus-mediated gene delivery rescues a neonatal lethal murine model of mut(0) methylmalonic acidemia. *Hum Gene Ther* **19**: 53–60.
23. Chandler, RJ, Tsai, MS, Dorko, K, Sloan, J, Korson, M, Freeman, R *et al.* (2007). Adenoviral-mediated correction of methylmalonyl-CoA mutase deficiency in murine fibroblasts and human hepatocytes. *BMC Med Genet* **8**: 24.
24. Mueller, C and Flotte, TR (2008). Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther* **15**: 858–863.
25. Wu, Z, Asokan, A and Samulski, RJ (2006). Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* **14**: 316–327.
26. Gao, GP, Alvira, MR, Wang, L, Calcedo, R, Johnston, J and Wilson, JM (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* **99**: 11854–11859.
27. Sarkar, R, Tetreault, R, Gao, G, Wang, L, Bell, P, Chandler, R *et al.* (2004). Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* **103**: 1253–1260.
28. Inagaki, K, Piao, C, Kotchey, NM, Wu, X and Nakai, H (2008). Frequency and spectrum of genomic integration of recombinant adeno-associated virus serotype 8 vector in neonatal mouse liver. *J Virol* **82**: 9513–9524.
29. Louboutin, JP, Wang, L and Wilson, JM (2005). Gene transfer into skeletal muscle using novel AAV serotypes. *J Gene Med* **7**: 442–451.
30. Wang, Z, Zhu, T, Qiao, C, Zhou, L, Wang, B, Zhang, J *et al.* (2005). Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nat Biotechnol* **23**: 321–328.
31. Touati, G, Valayannopoulos, V, Mention, K, de Lonlay, P, Jouvet, P, Depondt, E *et al.* (2006). Methylmalonic and propionic acidurias: management without or with a few supplements of specific amino acid mixture. *J Inherit Metab Dis* **29**: 288–298.
32. Cunningham, SC, Dane, AP, Spinoulas, A, Logan, GJ and Alexander, IE (2008). Gene delivery to the juvenile mouse liver using AAV2/8 vectors. *Mol Ther* **16**: 1081–1088.
33. Alexander, IE, Cunningham, SC, Logan, GJ and Christodoulou, J (2008). Potential of AAV vectors in the treatment of metabolic disease. *Gene Ther* **15**: 831–839.
34. Green, NS, Rinaldo, P, Brower, A, Boyle, C, Dougherty, D, Lloyd-Puryear, M *et al.*; Advisory Committee on Heritable Disorders and Genetic Diseases in Newborns and Children (2007). Committee Report: advancing the current recommended panel of conditions for newborn screening. *Genet Med* **9**: 792–796.
35. Daly, TM, Okuyama, T, Vogler, C, Haskins, ME, Muzyczka, N and Sands, MS (1999). Neonatal intramuscular injection with recombinant adeno-associated virus results in prolonged beta-glucuronidase expression *in situ* and correction of liver pathology in mucopolysaccharidosis type VII mice. *Hum Gene Ther* **10**: 85–94.
36. Marcell, PD, Stabler, SP, Podell, ER and Allen, RH (1985). Quantitation of methylmalonic acid and other dicarboxylic acids in normal serum and urine using capillary gas chromatography-mass spectrometry. *Anal Biochem* **150**: 58–66.
37. Allen, RH, Stabler, SP, Savage, DG and Lindenbaum, J (1993). Elevation of 2-methylcitric acid I and II levels in serum, urine, and cerebrospinal fluid of patients with cobalamin deficiency. *Metabolism* **42**: 978–988.
38. Barshop, BA, Yoshida, I, Ajami, A, Sweetman, L, Wolff, JA, Sweetman, FR *et al.* (1991). Metabolism of 1-13C-propionate *in vivo* in patients with disorders of propionate metabolism. *Pediatr Res* **30**: 15–22.

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