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AAV Gene Therapy Corrects OTC Deficiency and Prevents Liver Fibrosis in Aged OTC-Knock Out Heterozygous Mice

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Abstract

Ornithine transcarbamylase (OTC) deficiency is an X-linked disorder of the urea cycle. Hemizygous males and heterozygous females may experience life-threatening elevations of ammonia in blood and brain, leading to irreversible cognitive impairment, coma, and death. Recent evidence of acute liver failure and fibrosis/cirrhosis is also emerging in OTC-deficient patients. Here, we investigated the long-term consequences of abnormal ureagenesis in female mice heterozygous (Het) for a null mutation in the OTC gene. Two-month-old Het OTC knockout (KO) mice received a single dose of self-complementary adeno-associated virus (AAV) encoding a codon-optimized human OTC gene at 1×10^{10}, 3×10^{10}, or 1×10^{11} vector genome copies per mouse. We compared liver pathology from 18-month-old treated Het OTC-KO mice, age-matched untreated Het OTC-KO mice, and WT littermates, and assessed urinary orotic acid levels and vector genome copies in liver at 4, 10, and 16 months following vector administration. Het OTC-KO female mice showed evidence of liver inflammation and the eventual development of significant fibrosis. Treatment with AAV gene therapy not only corrected the underlying metabolic abnormalities, but also prevented the development of liver fibrosis. Our study demonstrates that early treatment of OTC deficiency with gene therapy may prevent clinically relevant consequences of chronic liver damage from developing.

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Authors' contributions: L.W., P.B., H.M., M.L.B., and J.M.W. conceived this study. L.W., P.B., and J.M.W. designed the experiments. L.W., P.B., Z.H., E.P., H.Y., and J.W. performed the experiments. L.W., J.M.W., P.B., H.M., and M.L.B. wrote and edited the manuscript.

Keywords

Liver fibrosis; OTC deficiency; AAV; gene therapy

1. Introduction

Ornithine transcarbamylase (OTC) deficiency, an X-linked recessive disorder, is the most common inherited defect of ureagenesis.[1] The prevalence of OTC deficiency is estimated to be 1/63,000.[2] Most symptomatic patients with OTC deficiency are hemizygous males; however, approximately 20% of female carriers of OTC mutations also present with symptoms.[3] Clinical findings range from vomiting and lethargy to coma and death, resulting from marked elevations of ammonia in blood and brain. The onset and severity of OTC deficiency clinical symptoms are influenced by the consequences of the mutation on enzyme activity. A complete deficiency of OTC enzyme activity (found in approximately half of hemizygous males) usually leads to severe and life-threatening hyperammonemia within the first week of life.[4] Patients with partial OTC deficiency, including hemizygous males with missense mutations and some heterozygous (Het) females, depending on their pattern of X-chromosome inactivation, present after birth, with the first symptoms often occurring in later childhood or even adulthood.

The chronic metabolic dysregulation associated with OTC deficiency has been ascribed to various types of liver pathology. In fact, acute liver failure has only been reported in a minority of patients with OTC deficiency.[5–7] While histopathological findings in liver samples from neonates were non-specific,[8, 9] samples from late-onset OTC deficiency patients showed thin fibrous septa with portal-to-portal bridging fibrosis, suggesting that chronic exposure to defective ureagenesis is damaging.[8] Liver samples from OTC deficiency Het female children and adults have shown some histologic abnormalities, including piecemeal necrosis and stellate portal fibrosis, suggesting that liver pathology is not limited to hemizygous males.[9] We have previously reported a subject with Het OTC deficiency who presented with hepatocellular carcinoma.[10]

Liver fibrosis has also been observed in other urea cycle disorders (UCDs), such as argininosuccinic aciduria, carbamoylphosphate synthetase deficiency, and arginase deficiency,[8, 11–13] and early-onset UCDs are associated with hepatocellular damage and liver dysfunction.[1, 14]

The OTC knockout (KO) mouse, generated in our laboratory through the deletion of exons 2–3, closely mimics the severe neonatal-onset form of OTC deficiency in humans. In addition, it provides an authentic animal model of the disorder to supplement the existing *spf* and *spf*^{ash} models, which resemble patients with late-onset OTC deficiency that retain partial OTC enzyme activity.[15, 16] In this study, we characterized the ureagenesis and liver histopathology of Het OTC-KO female mice at different ages and investigated if adeno-associated virus (AAV)-mediated gene therapy could correct or prevent these abnormalities.

2. Materials and methods

2.1 AAV vector

The methods for constructing scAAV8.TBG.hOTCco, a self-complementary AAV8 vector that encodes the codon-optimized human OTC gene driven by the liver-specific thyroid hormone-binding globulin promoter, have been previously described.[17] The vector was produced by the Penn Vector Core at the University of Pennsylvania as previously described.[18] The genome titer in genome copies/mL (GC/mL) of AAV vectors was determined using real-time polymerase chain reaction (PCR). All of the vectors used in this study passed the endotoxin assay using the QCL-1000 Chromogenic LAL test kit (Cambrex Bio Science, East Rutherford, NJ).

2.2 Mouse model and animal studies

Ingenious Targeting Laboratory, Inc. (Stony Brook, NY) produced a mouse model in which exons 2–3 of the OTC gene were replaced with a neomycin cassette. A targeting vector was designed with a 4.6 kb long homology arm and a 2.79 kb short homology arm to replace a 2.79 kb region in OTC exons 2–3 with a neomycin cassette. Exon 3 contains the Ser-Thr-Arg-Thr-Arg motif that is essential for carbamoyl phosphate binding. The vector was linearized and electroporated into iTL BA1 (C57BL/6×129/SvEv) hybrid embryonic stem cells. G418 resistant clones were selected for expansion and initial confirmation using PCR. Secondary confirmation was performed using Southern blot. Whereas wild-type (WT) mice have a 7.2 kb ApaI fragment, successfully disrupted clones have a 13.5 kb fragment. Correctly targeted clones were injected in C57BL/6 blastocysts; chimeras with a high percentage of agouti color were mated to WT C57BL/6 mice to generate F1 Het offspring. Affected OTC-KO male pups die within 24 hours of birth, but OTC-KO Het females are fertile and were genotyped at weaning. Therefore, the colony is maintained by mating Het females with WT C57BL/6 males in a University of Pennsylvania facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and assured by the Public Health Service. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. In gene therapy studies, eight-week-old OTC-KO Het females received a single tail vein injection of AAV vector at three doses: 1×10^{11}, 3×10^{10}, or 1×10^{11} GC/mouse. Untreated OTC-KO Het and WT littermates served as controls. Mice were sacrificed at 6, 12, and 18 months of age, and liver samples were subsequently harvested for analyses. All mice were on Laboratory Rodent Diet 5001 provided by LabDiet (Animal Specialties & Provisions, Quakertown, PA).

2.3 Sirius Red staining to detect and quantify liver fibrosis

Sirius Red staining was performed on paraffin-embedded liver sections. Sections were deparaffinized and stained in a solution of 0.1% (w/v) Direct Red (Sigma-Aldrich, St. Louis, MO) for 90 min, 4% (w/v) picric acid (Sigma-Aldrich) for 90 min, and then washed twice with 0.01 N HCl. Sections were dehydrated through an ethanol and xylene series and coverslipped. For quantification, a representative image was taken at low magnification (4x) from each liver section and the percentage of fibrosis area stained with Sirius Red was determined by thresholding (i.e., selecting the red stain in each image) using ImageJ

software (Rasband W. S., National Institutes of Health, USA; http://rsb.info.nih.gov/ij/). The mean percentage of stained area for each group is presented.

2.4 Fat staining by Oil Red O

Frozen liver sections were stained with Oil Red O to identify lipids. Cryosections were fixed in 4% paraformaldehyde for 5 min, stained with hematoxylin for 10 s, rinsed thoroughly in water, and incubated with 0.25% Oil Red O in 60% isopropanol for 15 min. After a final rinse in water, sections were embedded in an aqueous mounting medium.

2.5 OTC enzyme activity staining and OTC immunostaining

Sliced liver tissue (2 mm) was fixed, embedded, sectioned (9 μm), and mounted onto slides for histochemical staining of OTC enzyme activity as previously described.[19] Immunofluorescence for OTC expression was performed on frozen liver sections, as previously described.[20]

2.6 Measuring urinary orotic acid

Urine samples were collected before vector administration and at various time points (24 h and 1, 2, 4, 8, 24, 44, 50, and 70 weeks) after vector administration for orotic acid analysis as previously described.[21]

2.7 Quantifying vector genomes in liver

Liver DNAs were extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Detection and quantification of vector genomes in extracted DNA were performed by real-time PCR (TaqMan Universal Master Mix, Applied Biosystems, Foster City, CA) as described previously.[17, 22]

2.8 Statistical analyses

Statistical analyses were performed with GraphPad Prism 6.03 (La Jolla, CA) for Windows. Tukey's multiple comparisons test was used to compare variations between three or more groups. The Mann-Whitney test was used to compare two groups. Group averages are presented as mean ± standard deviation (STD).

3. Results

3.1 Characterization of OTC-KO Het mice

OTC-KO Het females matured to adulthood and survived at least 18 months without obvious clinical sequelae. Compared to WT female littermates, they had slightly reduced liver OTC enzyme activity (Figure 1A), with significantly elevated plasma ammonia (Figure 1B), increased urinary orotic acid levels (Figure 1C), and lower body weight (Figure 1D). Both OTC immunostaining (Figure 1E) and OTC enzyme activity staining (Figure 1G) of OTC-KO Het liver samples showed mosaic patterns of positive cells compared to WT females (Figure 1F and 1H), which is expected due to random X-chromosome inactivation. [23, 24] Overall, these results are consistent with the expected OTC deficiency symptoms in Het females.

3.2 Liver fibrosis in aged OTC-KO Het mice

Abnormal liver pathology, such as fibrosis, has been observed in UCD patients, including OTC deficient hemizygotes and Het carriers.[8, 9] Indeed, collagen staining with Sirius Red on liver sections from an 11-year-old OTC deficiency patient showed excess collagen deposition bridging adjacent portal areas to central veins (Figure 2I) compared to that of a normal 21-year-old female (Figure 2J).

To investigate if OTC-KO Het mice develop liver pathology, we sectioned liver samples collected from OTC-KO Het mice and WT littermates at various ages (6, 12, and 18 months old) and stained them with Sirius Red. We did not detect any abnormal findings in 6- or 12-month-old OTC-KO Het mice (Figure 2E and 2F); collagen staining was indistinguishable from their WT littermates (Figure 2A and 2B). However, liver fibrosis was evident in 18-month-old OTC-KO Het mice (Figure 2G and H), with intensified collagen staining and bridging; age-matched WT animals showed no pathology (Figure 2C and D). Morphometric quantification showed a statistically significant increase in fibrosis of OTC-KO Het mice (Figure 2K).

The livers of OTC-KO Het mice had other pathological abnormalities. They often demonstrated an irregular pattern of fat deposits, as evidenced by Oil Red O staining of liver sections (Figure 3B and D), compared to the typical peri-central staining pattern seen in WT mice (Figure 3A and C). We also observed frequent lymphocytic infiltrates on liver sections from 18-month-old Het mice stained with haemotoxylin and eosin (Figure 3F) that were not seen in WT mice (Figure 3E). Serum transaminases in 18-month-old Het mice were about two-fold higher than the levels in age-matched WT mice (Figure 3G). Overall, the abnormal liver pathologies observed in OTC-KO Het mice show similarities to the livers of OTC deficient patients, but which have not been reported in other OTC deficient mouse models, such as *spf* or spf^{ash}.

3.3 AAV8 gene therapy corrects ureagenesis and prevents liver fibrosis in aged OTC-KO Het mice

We then tested if gene therapy could correct ureagenesis and prevent liver fibrosis. Two-month-old OTC-KO Het mice received a single tail vein injection of a self-complementary AAV8 vector encoding a codon-optimized human OTC gene at 1×10^{11}, 3×10^{10}, or 1×10^{10} GC/mouse. One week following vector treatment, mice in all three dosage groups had normal urinary orotic acid levels, which remained steady throughout the 16-month study (Figure 4). We harvested liver samples from 18-month-old AAV8-treated mice 16 months after vector administration and compared them to age-matched untreated Het mice and WT littermates. OTC-KO Het mice treated with any of the three vector doses showed normal liver histology that was similar to WT mice; by contrast, the untreated OTC-KO Het animals had fibrosis throughout the liver (Figure 5). OTC enzyme activity staining in liver sections from AAV8-treated mice showed dose-correlated increases in the abundance of functional OTC enzyme (Figure 6). Vector GC in liver were dose correlated and stable for the first 10 months post vector administration (Figure 7), suggesting efficient gene transfer. A statistically significant two-fold reduction in vector GC in liver was observed between four and 16 months post-vector administration for the high- and mid-dose groups (Figure 7A and

7B), although it was not statistically different for the low-dose group (Figure 7C). In conclusion, AAV gene therapy efficiently and stably corrected the ureagenesis defect in OTC-KO Het mice, and prevented the development of liver fibrosis.

4. Discussion

Our OTC-KO mouse provides a new and authentic animal model for studying OTC deficiency. Unlike the existing *spf* or *spf*^{ash} mice, which have residual OTC enzyme activity and can live to adulthood and breed, OTC-KO hemizygous mice closely resemble the neonatal-onset form of OTC deficiency. Affected male pups have no detectable OTC protein or activity in liver, have elevated plasma ammonia (Wang L et al., manuscript in preparation), and perish within 24 hours of birth. Thus, these male mice can be used to evaluate novel therapies to treat the neonatal onset hyperammonemic crisis in OTC deficiency and to study their impact on survival.

This study focuses on the characterization of OTC-KO Het mouse and its potential use as a model to study liver pathology in OTC deficient female carriers. Urine orotic acid in OTC-KO Het is consistently elevated, even more consistent than it is in hemizygous *spf*^{ash} males. One possible reason is in those OTC-negative hepatocytes, the complete lack of OTC enzyme activity leads to excess accumulation of carbamoyl phosphate in the mitochondria which would be converted to orotic acid when exits to cytosol. While the elevation of orotic acid seems more severe in OTC-KO Het mice than in human OTC carriers, both symptomatic and asymptomatic carriers show orotic aciduria under protein loading test.[25] Het female patients have variable clinical expression, ranging from being asymptomatic to having recurrent episodes of life-threatening hyperammonemic coma.[26] Even asymptomatic OTC deficiency heterozygotes, the largest group of UCD patients, have subtle cognitive deficits and are at risk for learning disabilities and attention/executive function deficits.[10, 27, 28] Histopathology analyses of samples from liver biopsies, or from explanted livers at the time of transplant, detected liver pathology, including fibrosis and frank cirrhosis, in some female carriers.[8, 9] However, the limited availability of tissues for detailed analyses has precluded an accurate analysis of the risks and pathogenesis of liver disease in patients with OTC deficiency.

In this study, we evaluated the livers from OTC-KO Het mice to determine if they develop liver pathology that can be prevented by gene therapy. Through the analyses of liver samples from OTC-KO Het mice at different ages, we found age-correlated liver fibrosis that became evident at 18 months, but not earlier (i.e., at 6 or 12 months; Figure 2). Surprisingly, even modest doses of gene therapy corrected the metabolic derangements in OTC-KO Het mice (Figure 4), and prevented the development of liver fibrosis (Figure 5). We have previously demonstrated inverse zonation of hepatocyte transduction with AAV8 vectors between mice and non-human primates.[29] In mice, AAV8 predominantly transduces hepatocytes near central veins and yields lower transduction levels in hepatocytes in periportal regions. The periportal transduction pattern in nonhuman primates, and likely in humans, would favor AAV gene therapy of urea cycle disorders, since the urea cycle is most active in periportal areas.

Liver analyses indicated that gene transfer is long-lasting, which is desirable for treating metabolic diseases. We observed a two-fold reduction in vector GC between 10 and 16 months post-vector administration (Figure 7). Compared to our previous gene therapy study, vector GC in OTC-KO Het females were about 10-fold lower than they were in male OTC *spf*^{ash} mice treated with the same doses of this vector at similar time points.[17] This is consistent with previous reports on gender differences in AAV gene transfer in murine liver.[6, 30, 31]

What was surprising about these results was the dramatic effect that even low levels of gene transfer had on liver pathology. The actual transduction of hepatocytes in the low-dose female OTC-KO Het mice was hard to quantify, because the endogenous enzyme creates a mosaic pattern (Figure 6). Histological analysis revealed that a large proportion of the cells in the low dose-treated animals was OTC negative (Figure 6). It is unclear how fibrosis is prevented with only a modest increase in OTC expression in some hepatocytes over a background of around 50% of cells expressing normal enzyme. It is possible that this incremental increase in enzyme activity in some cells diminishes pathogenic metabolites in adjacent non-OTC-expressing cells. We hypothesize that the long-term accumulation of high-energy charged carbamoyl phosphate in the mitochondria of OTC-negative cells in untreated OTC deficiency patients and mice could react with protein or nucleic acids, which could lead to mitochondrial damage and the release of reactive oxygen species into the cell, triggering apoptosis or cell damage. OTC enzyme expression from transduced cells could enhance the conversion of mitochondrial ornithine and carbamoyl phosphate to citrulline, diminishing the accumulation of this putatively toxic metabolite. How this may affect neighboring non-OTC-expressing cells is unclear.

As new therapies improve the quality and longevity of life in OTC deficiency patients, chronic consequences of the disease are likely to emerge. For instance, long-term liver fibrosis is a risk factor for the development of cirrhosis and hepatocellular carcinoma.[32] Our study suggests that, in addition to alleviating acute hyperammonemia and its potential brain injury, gene therapy could prevent liver disease in OTC deficiency patients.

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Abbreviations

OTC	ornithine transcarbamylase
Het	heterozygous
UCD	urea cycle disorder
KO	knockout

AAV	adeno-associated virus
GC	genome copies
PCR	polymerase chain reaction
WT	wild type
STD	standard deviation

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Highlights

- OTC-KO Het mice can serve as a model to study liver pathology in OTC-deficient female carriers.
- Aged OTC-KO Het mice develop liver fibrosis.
- AAV8 gene therapy corrects ureagenesis and prevents liver fibrosis in aged OTC-KO Het mice.
- Early treatment of OTC deficiency with gene therapy may prevent what could become clinically relevant consequences of chronic liver damage.

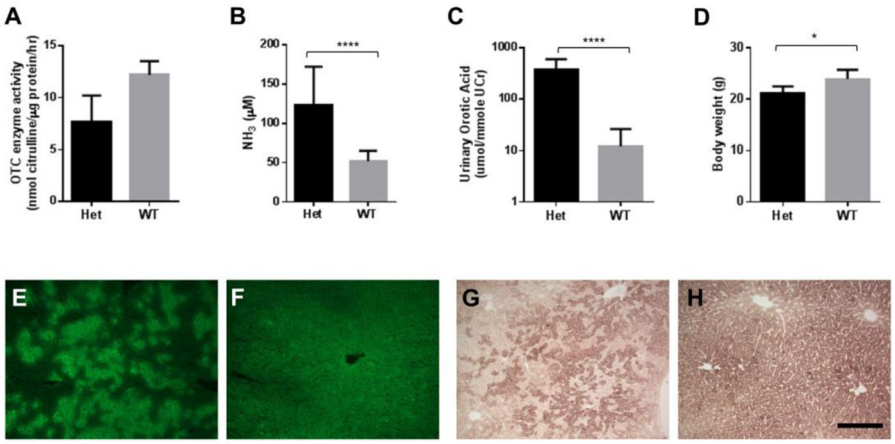

Figure 1. Characterization of adult OTC-KO Het mice

(A) OTC enzyme activity in liver (Het, n=3; WT, n=4). (B) Plasma ammonia (Het, n=15; WT, n=10). (C) Urinary orotic acid (Het, n=34; WT, n=13). (D) Body weight of 3-month-old Het and WT mice (n=5). (E, F) OTC immunofluorescent staining on liver sections of 6-month-old Het and WT mice, respectively. (G and H) OTC histochemical staining on liver sections of 6-month-old Het and WT mice, respectively. Scale bar is 200 μm. Means ± STD are shown. * $p<0.05$; **** $p<0.0001$ (Mann-Whitney test).

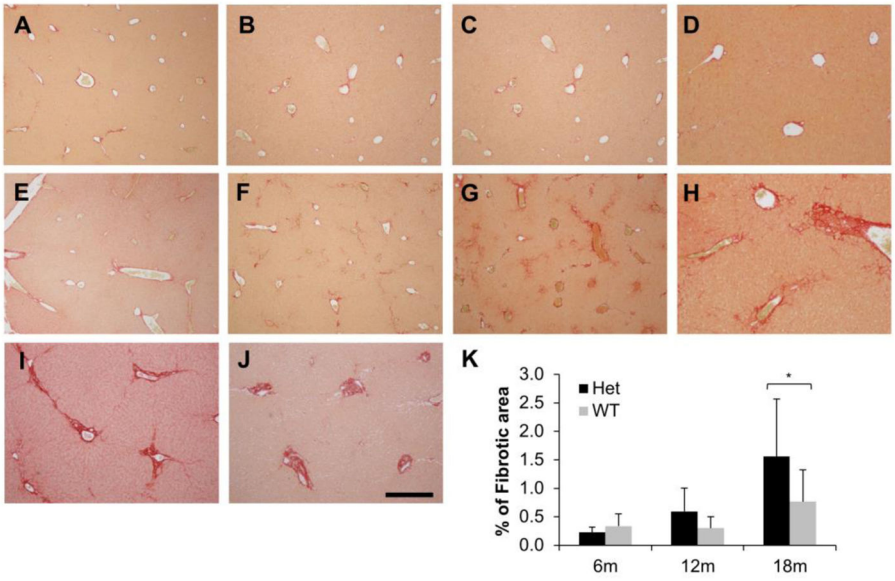

Figure 2. Development of liver fibrosis in aged OTC-KO Het mice and in an OTC deficiency patient

Collagen staining with Sirius Red on liver sections from: (A) 6-month-old WT mouse, (B) 12-month-old WT mouse, (C, D) 18-month-old WT mouse, (E) 6-month-old Het mouse, (F) 12-month-old Het mouse, (G, H) 18-month-old Het mouse, (I) 11-year-old OTC deficiency patient, and (J) normal 21-year-old female. Representative pictures are shown. Scale bar is 500 μm for all except for (D) and (H), where it is 200 μm. (K) Quantification of liver fibrosis area in 6-, 12-, and 18-month-old Het and WT mice. Means ± STD are shown (n=4–19). * p<0.05 (Mann-Whitney test).

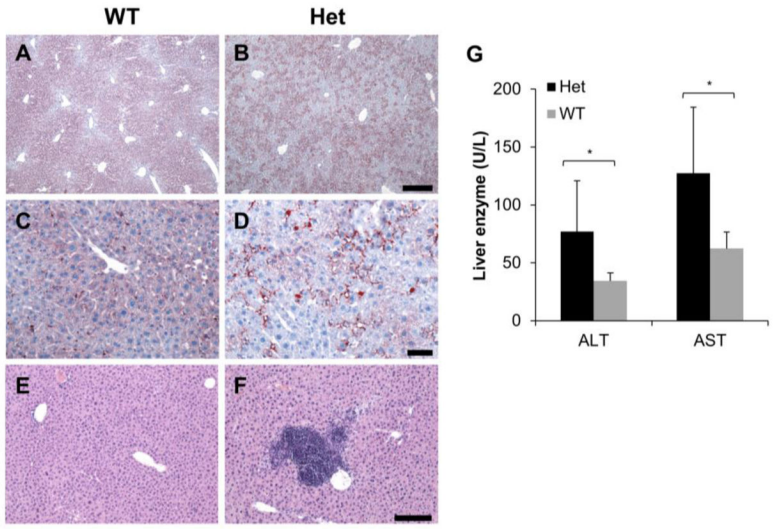

Figure 3. Abnormal fat staining and lymphocytic infiltrate in the liver of OTC-KO Het mice

Oil-Red-O staining for lipids in liver from a WT (A, C) and OTC-KO Het mouse (B, D), showing representative images at low and high magnification, respectively. (E, F) Haemotoxylin and eosin staining on liver sections from a WT and OTC Het mouse, respectively. The liver from the OTC-KO Het mouse shows a lymphocytic infiltrate, which we frequently observed in these animals. (G) Liver enzyme levels in 18-month-old Het and WT mice. Scale bar is 300 μm (A, B), 50 μm (C, D), and 150 μm (E, F). ALT, alanine aminotransferase; AST, aspartate aminotransferase. Means ± STD are shown (Het, n=8; WT, n=5). * p<0.05 (Mann-Whitney test).

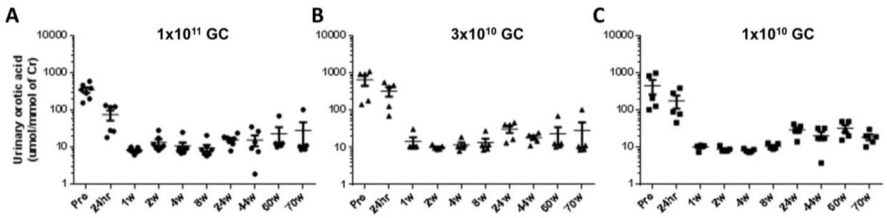

Figure 4. Robust and sustained normalization of urinary orotic acid in OTC-KO Het mice by AAV gene therapy

Urinary orotic acid levels in AAV8-treated OTC-KO Het mice normalized within one week following vector treatment and remained within the normal range through the experiment. Vector doses for each group were: (A) 1×10^{11}, (B) 3×10^{10}, and (C) 1×10^{10} GC/mouse. Means ± STD are shown (n=5).

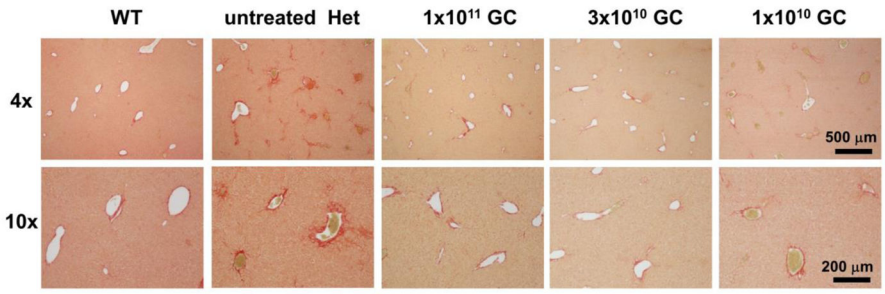

Figure 5. Prevention of liver fibrosis in OTC-KO Het mice by AAV gene therapy

Collagen staining with Sirius Red on liver samples harvested from 18-month-old AAV8-treated OTC-KO Het mice (16 months post-vector administration at the doses of 1×10^{11}, 3×10^{10}, and 1×10^{11} GC/mouse) compared to untreated OTC-KO Het mice and WT littermates. Representative pictures taken at 4x (scale bar is 500 μm) and 10× (scale bar is 200 μm) magnifications of the groups are shown (n=5).

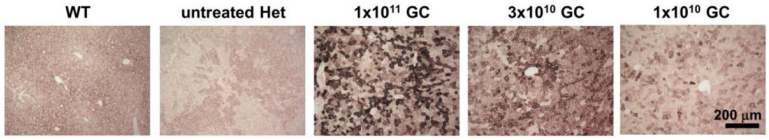

Figure 6. Long-term and high levels of OTC enzyme activity in the liver of OTC-KO Het mice following AAV gene therapy

Two-month-old OTC-KO Het mice received a single tail vein injection of AAV8 vector at the doses of 1×10^{11}, 3×10^{10}, and 1×10^{11} GC/mouse. Liver samples were harvested 16 months later and stained for OTC enzyme activity. A representative picture of each group is shown (n=5; scale bar is 200 μm).

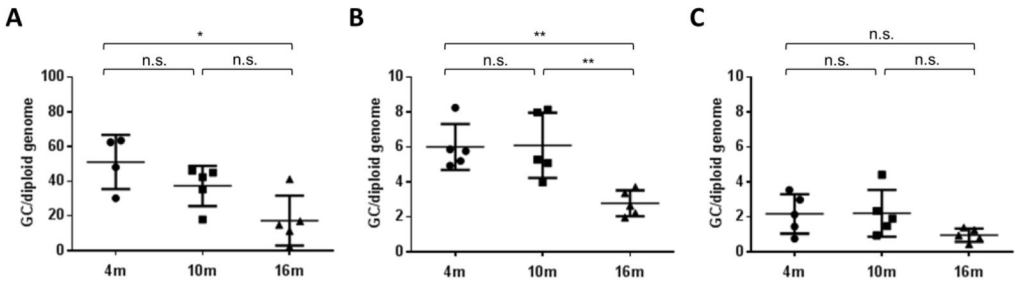

Figure 7. Dose-correlated vector GC in the liver of AAV8-treated OTC-KO Het mice at different time points post vector administration

Two-month-old OTC-KO Het mice received a single tail vein injection of AAV8 vector at the doses of 1×10^{11} (A), 3×10^{10} (B), and 1×10^{10} (C) GC/mouse. Liver samples were harvested 4, 10, and 16 months later for vector GC analysis by real-time PCR. Means ± STD are shown (n=5). * $p<0.05$ (Tukey's test). n.s., not significant.