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Helper-dependent adenoviral vectors for liver-directed gene therapy of primary hyperoxaluria type 1

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Abstract

Primary hyperoxaluria type 1 (PH1) is an inborn error of liver metabolism due to deficiency of the peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) which catalyzes conversion of glyoxylate into glycine. AGT deficiency results in overproduction of oxalate which ultimately leads to end-stage renal disease and death. Organ transplantation as either preemptive liver transplantation or combined liver/kidney transplantation is the only available therapy to prevent disease progression. Gene therapy is an attractive option to provide an alternative treatment for PH1. Towards this goal, we investigated helper-dependent adenoviral (HDAd) vectors for liver-directed gene therapy of PH1. Compared to saline controls, AGT-deficient mice injected with an HDAd encoding the AGT under the control of a liver-specific promoter showed a significant reduction of hyperoxaluria and less increase of urinary oxalate following challenge with Ethylene Glycol (EG), a precursor of glyoxylate. These studies may thus pave the way to clinical application of HDAd for PH1 gene therapy.

Keywords

Helper-dependent adenoviral vectors; gene therapy; primary hyperoxaluria type 1

INTRODUCTION

The liver is an attractive organ for gene therapy and has long been appreciated that hepatocyte gene therapy with long-term expression provides a major opportunity for treatment or perhaps even cure for many human disorders.[1] Primary hyperoxaluria type 1 (PH1), caused by deficiency of the liver peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT), is considered a good disease candidate for gene therapy. PH1 is estimated to account for about 1% of pediatric cases of end-stage renal failure[2, 3] and to

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CONFLICT OF INTEREST

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occur in 1:120,000 live births in Europe.[4] The lack of AGT activity results in a severe disease with overproduction of oxalate, which forms insoluble calcium salts that accumulate in the kidney resulting in nephrocalcinosis and urolithiasis. Calcium oxalate stone deposition occurs also in several other organs causing myocarditis, arrhythmias, stroke, and peripheral vascular occlusions. In most patients, the first symptoms (e.g. renal colic, asymptomatic gross hematuria) occur before the age of 5 years and approximately 50% of patients present with end stage renal disease at the time of diagnosis.[5, 6] Combined liver-kidney transplantation which replaces the biochemically defective organ is currently the only available treatment for most patients with PH1. Following liver transplantation, the rate of endogenous oxalate synthesis drops to normal levels as expected based on pattern of AGT expression.[7, 8] Before the use of liver-kidney transplantation, 80% of the patients died by the age of 20 years.[5, 9] Alternative, less invasive approaches, such as small molecule chaperones[10, 11] and oral administration of bacteria to degrade oxalate[12-14] have been investigated. However, it is unknown whether these approaches will be effective enough to avoid liver and kidney transplantation which remains the only long-term treatment for patients with PH1. Organ transplantation is far from being an ideal treatment. Short-term peritransplant and long-term morbidities associated with lifelong immunosuppression continue to be significant problems.[15, 16] Hepatocyte transplantation, which is a less invasive transplantation strategy, is unlikely to be successful because the number of engrafted hepatocytes required for correction of PH1 is highly likely to be beyond the capacity of this procedure.[17] In addition, long-term persistence of transplanted hepatocytes has not been demonstrated so far and hepatocyte transplantation would still require lifelong immunosuppression.

Given the limitations of current therapy and the severity of the disease, PH1 is an attractive target for gene therapy which has been previously investigated in two preclinical studies.[18, 19] The first study showed transient correction of hyperoxaluria in the PH1 mouse model using a first generation adenoviral (FGAd) vector.[18] However, FGAd vectors are not suitable for long-term correction of inherited diseases because they express low levels of viral genes that elicit an immune response against the transduced cells resulting in loss of transgene expression.[20] A second study showed sustained correction of hyperoxaluria and expression of AGT in transduced livers of PH1 mice transduced with serotypes 5 or 8 of adeno-associated viral (AAV) vectors.[19]

In contrast to FGAd, helper-dependent adenoviral (HDAd) vectors are attractive for gene therapy of inherited diseases because they can provide long term transgene expression without chronic toxicity, as shown by several studies in small and large animal models.[21, 22] However, the dose-dependent acute inflammatory response elicited against the viral capsid remains a major obstacle for clinical applications of these vectors for systemic delivery. To achieve efficient hepatocyte transduction by intravenous injections, high doses of adenoviral vector are required in both rodents and nonhuman primates. Several studies have shown a nonlinear dose response, with low doses yielding very low to undetectable levels of transgene expression, but higher doses resulting in disproportionately high levels of transgene expression in both mice[23, 24] and nonhuman primates.[25-27] In mice, this steep threshold effect can be transiently saturated[23, 24] and cells of the reticulo-endothelial system, including Kupffer cells in the liver, play a significant role in the nonlinear dose

response.[23, 24, 28] Therefore, efficient hepatic transduction is achieved only after intravenous injection of high doses of adenoviral vectors. Such systemic high vector dose results in activation of an acute inflammatory response with potentially severe and lethal consequences[29, 30]. The mechanism(s) responsible for this Ad-mediated activation of the acute inflammatory response is not completely understood, however, it is clearly dose-dependent.[30, 31] To overcome the obstacle of the acute toxicity, a balloon occlusion catheter method for preferential hepatic delivery of HDAd has been previously developed. This method resulted in higher levels and long-term transgene expression from a single injection, and minimal toxicity in nonhuman primates (baboon and rhesus) with clinically relevant doses.[32, 33]

In PH1, combined liver and kidney transplantation has sufficient risk to make the attempt of hepatocyte gene therapy justifiable from the perspective of a risk:benefit ratio. This is particularly the case with HDAd vectors because they result in long-term transgene expression after a single injection.[22] The main goal of this study was to evaluate the efficacy of HDAd vectors for liver-directed gene therapy in a mouse model of PH1.

RESULTS

The available mouse model of PH1 is homozygous for the deletion of exons 4-8 of the *Agxt* gene (*Agxt*$^{-/-}$) and has been previously shown to have hyperoxaluria independent of the dietary oxalate content but normal growth and lifespan.[18, 19] *Agxt−/−* mice were not found to develop nephrocalcinosis spontaneously[18] but only after enhancement of oxalate production by oral administration of 0.5-0.7% ethylene glycol (EG), a precursor of glycolate and glyoxalate.[18, 19] In contrast to these studies, we did not observe development of calcium oxalate stones in *Agxt−/−* mice that received 0.6% of EG for 4 weeks in their drinking water (n=5; Supplementary Table 1). Therefore, we investigated the higher dose of 1.25% EG in the drinking water for 4 weeks in *Agxt−/−* (n=21) and wild-type mice (n=10) as controls. Survival was 95% in *Agxt−/−* and 100% in wild-type mice and mild-to-moderate accumulation calcium stones were observed in kidneys of 6/21 (28.5%) *Agxt−/−* mice whereas none of the wild-type controls showed nephrocalcinosis (Supplementary Table 1 and Supplementary Fig. 1). Therefore, calcium oxalate stone formation under 1.25% EG challenge does not appear to be a sensitive marker of the disease, at least in our colony of *Agxt−/−* mice. Nevertheless, *Agxt−/−* mice show oxalate excretions of 2.48 μmoles/24 hours that were significantly different from oxalate excretion in age- and gender-matched wild-type SV129 controls (n=7; 0.5-0.8 μmoles/24 hours, 95% confidence interval) (Fig. 1).

We constructed an HDAd vector expressing the human AGT under the control of a liver-specific expression cassette (HDAd-AGT)[34] that was injected intravenously into *Agxt−/−* mice at the doses of 1×10^{13}, 5×10^{12}, or 1×10^{12} viral particles (VP) per kg body weight (n=5 mice per group). As control, an additional group of *Agxt−/−* mice (n=5) were injected with saline. Following vector administration, 24-hour urines were collected at multiple time points for 24 consecutive weeks (Fig. 1). Significant reduction of hyperoxaluria was observed in *Agxt−/−* mice injected with all three vector doses compared to saline-injected controls. *Agxt−/−* mice injected with 1×10^{13} VP/kg or 5×10^{12} VP/kg of HDAd-AGT showed a statistically significant 3.6- and 2.7-fold reduction of urinary oxalate levels

compared to saline-injected mice, respectively (Gaussian Processes, Bayes Factor 35.98 and 26.08, respectively; $p<0.05$ at each time point by Wilcoxon-Mann-Whitney test) (Fig. 1). Urinary oxalate excretions in mice injected with the two higher doses were within the normal range detected in wild-type SV129 mice. Partial reduction of urinary oxalate levels that were above the normal range were detected with the lowest dose of 1×10^{12} VP/kg (Gaussian Processes, Bayes Factor 9.98) (Fig. 1). In all mice the reduction of urinary oxalate was sustained for the entire period of observation of 24 weeks (Fig. 1). Following 24 weeks from vector administration, vector- and saline-injected mice were challenged for 4 consecutive weeks with 1.25% EG added to the drinking water. As expected urinary oxalate levels were increased under EG challenge in *Agxt−/−* as well as in wild-type mice (Fig. 2). Under EG challenge, urinary oxalate excretions were significantly lower in mice injected with 1×10^{13} VP/kg and 5×10^{12} VP/kg compared to saline-injected mice ($p<0.05$) and were similar to wild-type mice. No significant reduction in oxalate levels was detected in mice that received 1×10^{12} VP/kg compared to saline controls under EG challenge (Fig. 2).

Real time PCR showed a dose-dependent increase of HDAd vector genome copies in the liver at the three tested doses (Supplementary Fig. 2). By immunofluorescence performed on livers harvested at 28 weeks post-vector injections, a larger number of hepatocytes positive for AGT expression at the doses of 1×10^{13} VP/kg and 5×10^{12} VP/kg was observed whereas a smaller percentage of AGT-expressing liver cells was detected at the dose 1×10^{12} VP/kg (Fig. 3). By Western blotting, livers of mice injected with 5×10^{12} VP/kg and 1×10^{13} VP/kg of HDAd-AGT revealed robust AGT expression while no protein was detected in saline-injected *Agxt−/−* mice (Fig. 4A). AGT enzyme activity on liver homogenates from *Agxt−/−* mice injected with vector was higher than saline-injected controls at all three tested doses (Fig. 4B; $p<0.05$). The dose of 1×10^{13} VP/kg resulted in supraphysiologic levels of enzyme activity ($p<0.05$; 1×10^{13} VP/kg vs. wild-type controls) and 5×10^{12} VP/kg resulted in enzyme activity that was similar to wild-type controls (Fig. 4B). *Agxt−/−* mice that received 1×10^{12} VP/kg showed 67% of normal enzyme activity (Fig. 4B). Vector-encoded human AGT protein was also found to be enriched in the peroxisomal fraction of liver lysates thus showing that vector-encoded AGT was predominantly localized in peroxisomes (Fig. 4C).

DISCUSSION

PH1 is an attractive candidate for gene therapy: its pathophysiology is well characterized, it has a favorable risk-benefit ratio, direct measures of clinical benefit are available, and a sufficient number of patients would be available for enrollment in a clinical trial. Moreover, in humans AGT is only expressed in the liver[35] and thus, liver-directed gene therapy offers the possibility of replacing the total body's requirement for AGT.

In this study, we showed that a single injection of an HDAd vector expressing the AGT results in long-term correction of hyperoxaluria in a mouse model of PH1. Phenotypic correction was achieved with the two highest doses of 1×10^{13} VP/kg and 5×10^{12} VP/kg of vector whereas partial reduction was detected with the lowest dose of 1×10^{12} VP/kg. AAV vectors also resulted in similar levels of correction of hyperoxaluria at relatively high doses.[19] The requirement of higher vector doses for phenotypic correction is not surprising and was predicted by disease pathophysiology. In PH1 a large portion of the liver, although

structurally normal, produces an excess of toxic oxalate and has to be replaced/corrected. For gene therapy to be effective, a large proportion of hepatocytes has to be transduced to minimize the deleterious effect of uncorrected hepatocytes which will continue to produce oxalate. Although hepatocytes suffer no damage from the enzymatic defect, PH1 behaves like cell-autonomous defects in which one corrected hepatocyte cannot compensate for overproduction of toxic metabolites in its neighboring cells.[36] This model explains the partial correction observed with the lower dose of 1×10^{12} VP/kg despite the increased levels of total AGT activity measured in livers. Higher percentages of hepatocyte transduction achieved with the higher doses of 5×10^{12} VP/kg and 1×10^{13} VP/kg, as shown by immunofluorescence, are needed for correction of hyperoxaluria in *Agxt−/−* mice.

The AGT is an example of a dual localized protein with targeting sequences directing the protein to two distinct cellular compartments. In mice and rats, AGT is present both in the mitochondria and peroxisomes, while in humans it is exclusively located in the peroxisomes.[37] Consistent with previous studies,[18, 19] we also showed that the vector-encoded human AGT protein is correctly targeted to peroxisomes in mouse hepatocytes.

In recent years, there has been clear success in the clinic using AAV vectors for hemophilia B that resulted in long-term expression of factor IX (FIX).[38, 39] Although no immune reaction was observed at lower vector doses, participants who received the higher dose of vector developed a transient, asymptomatic elevation of serum alanine transaminase (ALT) levels associated with detection of AAV-capsid-specific T cells in peripheral blood. A short course of glucocorticoid therapy was associated with rapid normalization of ALT levels and sustained FIX in the therapeutic range. This and the previous trial with AAV2 in hemophilia B patients[40] have highlighted the limitations of immunologic responses against transduced hepatocytes and while short-course corticosteroid therapy appeared to be sufficient to blunt the immune response, it still needs to be determined whether long-term expression can be achieved in diseases such as PH1 that require an higher percentage of hepatocyte transduction compared to hemophilia.

AAV vectors have shown in general excellent safety profile in human trials. However, the safety of AAV has been challenged by at least two studies that documented hepatocellular carcinoma (HCC) and vector genomic integration after AAV gene delivery in mice.[41, 42] Moreover, a recent study reports that natural infections in humans with AAV serotype 2 result in chromosomal insertions activating proto-oncogenes in the liver and it suggests that the AAV integrations cause the tumors, similarly to the hepatitis B.[43] While the risk of HCC development remains to be fully understood, it is important that other vector systems continue to be investigated, particularly for disorders such as PH1 that require higher percentage of liver transduction and therefore, higher vector doses. HDAd genomes appear to exist in the nucleus of transduced cells as replication-deficient linear monomers both in cell culture and in mouse livers.[44] Intracellular HDAd genome is assembled into chromatin through association with cellular histones which promotes efficient transgene expression.[45, 46] Several studies in cell culture have investigated the frequency of HDAd genome integration and found random integration frequencies to be 10^{-3} to 10^{-5} per cell[47-51] whereas an even lower integration frequency in hepatocytes has been detected *in*

vivo.[52] Therefore, HDAd vector appear to be predominantly episomal with very low frequency of genomic integration.

The acute toxicity elicited by high doses of HDAd vector is an obstacle preventing their clinical applications. This acute toxic response is dose-dependent: it has been consistently shown in nonhuman primates that low vector doses result in little, if any, acute toxicity and hepatic transduction, while high doses, required for efficient hepatocyte transduction, lead to severe acute toxicity that can be lethal.[26, 27, 30, 53] If this acute response can be avoided, then HDAd should be able to provide long-term transgene expression without further chronic toxicity.[22] Strategies allowing the use of lower vector doses are attractive for clinical applications because they can overcome the issue of acute toxicity. The method of balloon catheter-assisted delivery of HDAd in nonhuman primates resulted in high level of hepatic transduction and multi-year transgene expression with relatively low and clinically relevant doses, minimal evidence of acute toxicity and no chronic toxicity.[32, 33] The use of such delivery method has potential for severe disorders such as PH1 that is currently treated with invasive, high risk surgical transplant procedure and require high percentage of hepatocyte transduction to obtain clinical benefit.

MATERIALS AND METHODS

HDAd vector

The HDAd-AGT vector bears the PEPCK-WL-hAGT expression cassette including the liver-specific promoter of phosphoenolpyruvatecarboxykinase (PEPCK) and other regulatory elements including the woodchuck hepatitis virus post regulatory element (WPRE), the Locus Control Region (LCR) from the apoE locus, and the bovine growth hormone poly-adenylation signal as described elsewhere.[34] HDAd was produced in 116 cells with the helper virus AdNG163 as described previously.[54, 55] The cells were regularly tested and found to be negative for Mycoplasma by real time PCR. DNA analyses of HDAd genomic structure was confirmed as described elsewhere.[54]

Agxt−/− mice and injections

Agxt−/− mice[18] were obtained from Dr. Roy-Chowdhury, Albert Einstein College of Medicine, New York, USA and bred into a SV129 background. Wild-type SV129 mice were purchased from Charles River (Calco, Lecco, Italy). Animal procedures were approved by the Italian Ministry of Health. Purified, high titer preparations of HDAd vectors diluted in 0.2 ml of normal saline were administered via injections in the retro-orbital venous plexus to 12-20 week-old male *Agxt−/−* mice. The sample size was of at least n=5 per experimental group, no randomization was performed to allocate mouse to experimental groups, and investigators were not blinded to the experimental group. None of the animals or samples was excluded from the study. Urines were collected placing mice into metabolic cages for twenty-four hours with food and water *ad libitum*. At 24 weeks post-injection, 1.25% Ethylene Glycol (EG, Sigma-Aldrich, Milan, Italy) was added to drinking water of the injected mice for four consecutive weeks. The Jaffe alkaline picrate test was used to measure urine creatinine concentrations.[56] The 24-hour urine collections with low creatinine were excluded because considered incomplete and re-collected. Urinary oxalate was determined

using the oxalate kit (Trinity Biotech, Bray Co Wicklow, Ireland) according to manufacturer's protocol.

AGT western blot, enzyme activity, and stainings

Liver specimens were homogenized in radio-immunoprecipitation assay (RIPA) buffer and complete protease inhibitor cocktail (Sigma-Aldrich). Samples were incubated for 20 minutes at 4°C and centrifuged at 13,200 rpm for 10 minutes. Pellets were discarded and cell lysates were used for western blots. Subcellular fractionation was performed as described elsewhere.[57] Whole cell lysates were quantified and equivalent cellular fractions were loaded for each one of the three enriched fractions. Proteins were loaded on a 12% SDS-PAGE and after transfer to a polyvinylidene fluoride (PVDF) membrane; blots were blocked with Tris-buffered saline(TBS)-Tween-20 containing 5% non-fat milk for 1 hour at room temperature followed by incubation with primary antibody overnight at 4°C. The primary antibodies used were: rabbit anti-human AGT (#HPA035370, Sigma-Aldrich), rabbit anti-calnexin (#ADI-SPA-860, Enzo Life Science, Farmingdale, NY, USA), rabbit anti-PMP70 (#S34201, Invitrogen, Monza, Italy), and mouse anti-GAPDH (#SC-32233, Santa Cruz Biotechnology, Dallas, TX, USA). An anti-rabbit IgG (#NA934V, GE Healthcare, Milan, Italy) and an anti-mouse IgG (#NA931, GE Healthcare) were used as secondary antibodies. The AGT enzyme activity was measured using a previously developed assay with modifications.[58] Briefly, liver specimens were homogenized in a buffer containing 250 mM sucrose plus 2 mM Tris HCl pH 7.4 and sonicated at 4°C three times for 15 seconds at 45 seconds interval. Samples were then incubated in a medium containing 100 mM potassium phosphate, 10 mM glyoxylate, and 100 nM pyridoxal-5'-phosphate. Alanine at the concentration of 80 mM was used to start the reaction that was performed for 4 hours at 37°C and stopped by addition of 2M perchloric acid. Samples were centrifuged at 20,000 × g at 4°C for 10 minutes and supernatants were neutralized with 2M KOH and 0.2 M 3-(N-morpholino)propanesulfonic acid (MOPS). Samples were centrifuged again to remove potassium perchlorate. Pyruvate was determined by adding neutralized samples to a solution containing 200 mM Tris HCl, 200 μM NADH and 7.5 U/mL of lactate dehydrogenase. The decreased in absorbance at 340 nm was measured by spectrophotometer.

For immunofluorescence, livers were embedded in optimal cutting temperature (OCT) compound after dehydration in 30% sucrose. 5-μm cryosections were blocked in 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton- phosphate buffered saline (PBS) and blocked with donkey serum, normal goat serum, bovine serum albumin (BSA) and PBS; anti-AGT antibody[19] was used at 1:1,000 dilution in blocking solution for an overnight incubation. The AlexaFluor-488 anti-rabbit antibody made in donkey (#A21206, Invitrogen) was used as secondary antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Stained liver sections were mounted in mowiol, cover-slipped and examined under a Zeiss LSM 710 confocal laser-scanning microscope. The percentage of AGT positive cells was determined manually analyzing at least 6 randomly taken images from wild-type uninjected mice and *Agxt−/−* mice injected with saline or with each dose of HDAd-AGT. Each cell in the field of view was evaluated for the presence of AGT signals and was considered positive when at least one green signal was observed within the cell area. Percentage of transduction was reported as ratio between positive and

total number of cell. The total number of cells was determined using DAPI staining for nuclei and hepatocytes with two or more nuclei were counted as one. Oxalate staining on kidneys were performed as described elsewhere.[59]

Vector genome copy number

HDAd vector genome copies were determined in livers. Total DNA was extracted from mouse tissues using standard phenol-chloroform extraction and quantitated by absorbance at 260 nm. Three different specimens per liver per mouse were processed. Quantitative real-time PCR was performed in duplicate for each sample using the LightCycler FastStart DNA Master SYBER Green I (Roche) in a total volume of 20 μl using 1 mM of each HDAd-specific primers (5'-TCTGAATAATTTTGTGTTACTCATAGCGCG-3' and 5'-CCCATAAGCTCCTTTTAACTTGTTAAAGTC-3'). Cycling conditions consisted of 95° C for 10 minutes followed by 45 cycles at 95° C for 10 seconds, 60° C for 7 seconds and 72° C for 20 seconds. Serial dilutions of a plasmid bearing the PCR target sequence were used as a control to determine the amounts of HDAd. Results were analyzed with Light Cycler software version 3.5 (Roche).

Statistical analyses

Data are presented as mean± s.d. Statistical significance was computed using the Wilcoxon-Mann-Whitney test and p-values <0.05 were considered significant. Reduction of urinary oxalate over time was compared in saline- versus vector-injected mice also by means of Gaussian Processes which enables to quantify the true signal and noise embedded in data profiles over time.[60]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Piccolo P, Brunetti-Pierri N. Gene therapy for inherited diseases of liver metabolism. Hum Gene Ther. 2015; 26(4):186–92. [PubMed: 25830689]
2. Leumann E, Hoppe B. The primary hyperoxalurias. J Am Soc Nephrol. 2001; 12(9):1986–93. [PubMed: 11518794]
3. Hoppe B, Langman CB. A United States survey on diagnosis, treatment, and outcome of primary hyperoxaluria. Pediatr Nephrol. 2003; 18(10):986–91. [PubMed: 12920626]
4. Cochat P, Liutkus A, Fargue S, Basmaison O, Ranchin B, Rolland MO. Primary hyperoxaluria type 1: still challenging! Pediatr Nephrol. 2006; 21(8):1075–81. [PubMed: 16810517]
5. Cochat P, Koch Nogueira PC, Mahmoud MA, Jamieson NV, Scheinman JI, Rolland MO. Primary hyperoxaluria in infants: medical, ethical, and economic issues. J Pediatr. 1999; 135(6):746–50. [PubMed: 10586179]

6. Mandrile G, van Woerden CS, Berchialla P, Beck BB, Acquaviva Bourdain C, Hulton SA, et al. Data from a large European study indicate that the outcome of primary hyperoxaluria type 1 correlates with the AGXT mutation type. Kidney Int. 2014; 86(6):1197–204. [PubMed: 24988064]
7. Cochat P, Faure JL, Divry P, Danpure CJ, Descos B, Wright C, et al. Liver transplantation in primary hyperoxaluria type 1. Lancet. 1989; 1(8647):1142–3. [PubMed: 2566086]
8. Watts RW, Calne RY, Rolles K, Danpure CJ, Morgan SH, Mansell MA, et al. Successful treatment of primary hyperoxaluria type I by combined hepatic and renal transplantation. Lancet. 1987; 2(8557):474–5. [PubMed: 2887776]
9. Millan MT, Berquist WE, So SK, Sarwal MM, Wayman KI, Cox KL, et al. One hundred percent patient and kidney allograft survival with simultaneous liver and kidney transplantation in infants with primary hyperoxaluria: a single-center experience. Transplantation. 2003; 76(10):1458–63. [PubMed: 14657686]
10. Danpure CJ. Primary hyperoxaluria: from gene defects to designer drugs? Nephrol Dial Transplant. 2005; 20(8):1525–9. [PubMed: 15956068]
11. Danpure CJ. Molecular etiology of primary hyperoxaluria type 1: new directions for treatment. Am J Nephrol. 2005; 25(3):303–10. [PubMed: 15961951]
12. Sidhu H, Allison MJ, Chow JM, Clark A, Peck AB. Rapid reversal of hyperoxaluria in a rat model after probiotic administration of Oxalobacter formigenes. J Urol. 2001; 166(4):1487–91. [PubMed: 11547118]
13. Campieri C, Campieri M, Bertuzzi V, Swennen E, Matteuzzi D, Stefoni S, et al. Reduction of oxaluria after an oral course of lactic acid bacteria at high concentration. Kidney Int. 2001; 60(3): 1097–105. [PubMed: 11532105]
14. Lieske JC, Goldfarb DS, De Simone C, Regnier C. Use of a probiotic to decrease enteric hyperoxaluria. Kidney Int. 2005; 68(3):1244–9. [PubMed: 16105057]
15. Alonso MH, Ryckman FC. Current concepts in pediatric liver transplant. Semin Liver Dis. 1998; 18(3):295–307. [PubMed: 9773429]
16. Newstead CG. Assessment of risk of cancer after renal transplantation. Lancet. 1998; 351(9103): 610–1. [PubMed: 9500312]
17. Jiang J, Salido EC, Guha C, Wang X, Moitra R, Liu L, et al. Correction of hyperoxaluria by liver repopulation with hepatocytes in a mouse model of primary hyperoxaluria type-1. Transplantation. 2008; 85(9):1253–60. [PubMed: 18475180]
18. Salido EC, Li XM, Lu Y, Wang X, Santana A, Roy-Chowdhury N, et al. Alanine-glyoxylate aminotransferase-deficient mice, a model for primary hyperoxaluria that responds to adenoviral gene transfer. Proc Natl Acad Sci U S A. 2006; 103(48):18249–54. [PubMed: 17110443]
19. Salido E, Rodriguez-Pena M, Santana A, Beattie SG, Petry H, Torres A. Phenotypic correction of a mouse model for primary hyperoxaluria with adeno-associated virus gene transfer. Mol Ther. 2011; 19(5):870–5. [PubMed: 21119625]
20. Brunetti-Pierri N, Ng P. Progress towards the clinical application of helper-dependent adenoviral vectors for liver and lung gene therapy. Curr Opin Mol Ther. 2006; 8(5):446–54. [PubMed: 17078387]
21. Brunetti-Pierri N, Ng P. Helper-dependent adenoviral vectors for liver-directed gene therapy. Hum Mol Genet. 2011; 20(R1):R7–13. [PubMed: 21470977]
22. Brunetti-Pierri N, Ng T, Iannitti D, Cioffi W, Stapleton G, Law M, et al. Transgene Expression up to 7 Years in Nonhuman Primates Following Hepatic Transduction with Helper-Dependent Adenoviral Vectors. Hum Gene Ther. 2013; 24(8):761–5. [PubMed: 23902403]
23. Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, et al. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. Mol Ther. 2001; 3(1): 28–35. [PubMed: 11162308]
24. Schiedner G, Hertel S, Johnston M, Dries V, van Rooijen N, Kochanek S. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. Mol Ther. 2003; 7(1):35–43. [PubMed: 12573616]
25. Sullivan DE, Dash S, Du H, Hiramatsu N, Aydin F, Kolls J, et al. Liver-directed gene transfer in non-human primates. Hum Gene Ther. 1997; 8(10):1195–206. [PubMed: 9215737]

26. Nunes FA, Furth EE, Wilson JM, Raper SE. Gene transfer into the liver of nonhuman primates with E1-deleted recombinant adenoviral vectors: safety of readministration. Hum Gene Ther. 1999; 10(15):2515–26. [PubMed: 10543616]
27. Morral N, O'Neal WK, Rice K, Leland MM, Piedra PA, Aguilar-Cordova E, et al. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. Hum Gene Ther. 2002; 13(1):143–54. [PubMed: 11779418]
28. Piccolo P, Vetrini F, Mithbaokar P, Grove NC, Bertin T, Palmer D, et al. SR-A and SREC-I Are Kupffer and Endothelial Cell Receptors for Helper-dependent Adenoviral Vectors. Mol Ther. 2013; 21(4):767–74. [PubMed: 23358188]
29. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab. 2003; 80(1-2):148–58. [PubMed: 14567964]
30. Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. Hum Gene Ther. 2004; 15(1):35–46. [PubMed: 14965376]
31. Muruve DA, Cotter MJ, Zaiss AK, White LR, Liu Q, Chan T, et al. Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses in vivo. J Virol. 2004; 78(11):5966–72. [PubMed: 15140994]
32. Brunetti-Pierri N, Stapleton GE, Law M, Breinholt J, Palmer DJ, Zuo Y, et al. Efficient, long-term hepatic gene transfer using clinically relevant HDAd doses by balloon occlusion catheter delivery in nonhuman primates. Mol Ther. 2009; 17(2):327–33. [PubMed: 19050700]
33. Brunetti-Pierri N, Liou A, Patel P, Palmer D, Grove N, Finegold M, et al. Balloon catheter delivery of helper-dependent adenoviral vector results in sustained, therapeutic hFIX expression in rhesus macaques. Mol Ther. 2012; 20(10):1863–70. [PubMed: 22828499]
34. Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ, et al. Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates. Hum Gene Ther. 2006; 17(4):391–404. [PubMed: 16610927]
35. Kamoda N, Minatogawa Y, Nakamura M, Nakanishi J, Okuno E, Kido R. The organ distribution of human alanine-2-oxoglutarate aminotransferase and alanine-glyoxylate aminotransferase. Biochem Med. 1980; 23(1):25–34. [PubMed: 7387639]
36. Danpure, CJ. Primary Hyperoxaluria. McGraw Hill; New York, NY, USA: 2009.
37. Danpure CJ. Primary hyperoxaluria type 1: AGT mistargeting highlights the fundamental differences between the peroxisomal and mitochondrial protein import pathways. Biochim Biophys Acta. 2006; 1763(12):1776–84. [PubMed: 17027096]
38. Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med. 2011; 365(25): 2357–65. [PubMed: 22149959]
39. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med. 2014; 371(21):1994–2004. [PubMed: 25409372]
40. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med. 2006; 12(3):342–7. [PubMed: 16474400]
41. Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, et al. AAV vector integration sites in mouse hepatocellular carcinoma. Science. 2007; 317(5837):477. [PubMed: 17656716]
42. Chandler RJ, LaFave MC, Varshney GK, Trivedi NS, Carrillo-Carrasco N, Senac JS, et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. The Journal of clinical investigation. 2015; 125(2):870–80. [PubMed: 25607839]
43. Nault JC, Datta S, Imbeaud S, Franconi A, Mallet M, Couchy G, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. Nat Genet. 2015; 47(10):1187–93. [PubMed: 26301494]

44. Jager L, Ehrhardt A. Persistence of high-capacity adenoviral vectors as replication-defective monomeric genomes in vitro and in murine liver. Hum Gene Ther. 2009; 20(8):883–96. [PubMed: 19364285]
45. Ross PJ, Kennedy MA, Parks RJ. Host cell detection of noncoding stuffer DNA contained in helper-dependent adenovirus vectors leads to epigenetic repression of transgene expression. J Virol. 2009; 83(17):8409–17. [PubMed: 19515759]
46. Ross PJ, Kennedy MA, Christou C, Risco Quiroz M, Poulin KL, Parks RJ. Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. J Virol. 2011; 85(8):3950–8. [PubMed: 21307207]
47. Harui A, Suzuki S, Kochanek S, Mitani K. Frequency and stability of chromosomal integration of adenovirus vectors. J Virol. 1999; 73(7):6141–6. [PubMed: 10364373]
48. Stephen SL, Sivanandam VG, Kochanek S. Homologous and heterologous recombination between adenovirus vector DNA and chromosomal DNA. J Gene Med. 2008; 10(11):1176–89. [PubMed: 18773501]
49. Hillgenberg M, Tonnies H, Strauss M. Chromosomal integration pattern of a helper-dependent minimal adenovirus vector with a selectable marker inserted into a 27.4-kilobase genomic stuffer. J Virol. 2001; 75(20):9896–908. [PubMed: 11559822]
50. Ohbayashi F, Balamotis MA, Kishimoto A, Aizawa E, Diaz A, Hasty P, et al. Correction of chromosomal mutation and random integration in embryonic stem cells with helper-dependent adenoviral vectors. Proc Natl Acad Sci U S A. 2005; 102(38):13628–33. [PubMed: 16174752]
51. Suzuki K, Mitsui K, Aizawa E, Hasegawa K, Kawase E, Yamagishi T, et al. Highly efficient transient gene expression and gene targeting in primate embryonic stem cells with helper-dependent adenoviral vectors. Proc Natl Acad Sci U S A. 2008; 105(37):13781–6. [PubMed: 18768795]
52. Stephen SL, Montini E, Sivanandam VG, Al-Dhalimy M, Kestler HA, Finegold M, et al. Chromosomal integration of adenoviral vector DNA in vivo. J Virol. 2010; 84(19):9987–94. [PubMed: 20686029]
53. Schnell MA, Zhang Y, Tazelaar J, Gao GP, Yu QC, Qian R, et al. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. Mol Ther. 2001; 3(5 Pt 1):708–22. [PubMed: 11356076]
54. Palmer D, Ng P. Improved system for helper-dependent adenoviral vector production. Mol Ther. 2003; 8(5):846–52. [PubMed: 14599819]
55. Suzuki M, Cela R, Clarke C, Bertin TK, Mourino S, Lee B. Large-scale production of high-quality helper-dependent adenoviral vectors using adherent cells in cell factories. Hum Gene Ther. 2010; 21(1):120–6. [PubMed: 19719388]
56. Jaffe M. Ueber den Niederschlag, welchen Pikrinsäure im normalen Harn erzeugt, und über eine neue Reaction des Kreatinins. Zeitschrift für Physiologische Chemie. 1886; 10:391–400.
57. Celis, JE. Cell Biology. A laboratory handbook. Elsevier Academic Press; Burlington, MA, USA: 2006.
58. Wanders RJ, Ruiter J, van Roermund CW, Schutgens RB, Ofman R, Jurriaans S, et al. Human liver L-alanine-glyoxylate aminotransferase: characteristics and activity in controls and hyperoxaluria type I patients using a simple spectrophotometric method. Clin Chim Acta. 1990; 189(2):139–44. [PubMed: 2397596]
59. Yasue T. Histochemical identification of calcium oxalate. Acta Histochem. Cytochem. 1969; 2(3): 83–95.
60. Kalaitzis AA, Lawrence ND. A simple approach to ranking differentially expressed gene expression time courses through Gaussian process regression. BMC Bioinformatics. 2011; 12:180. [PubMed: 21599902]

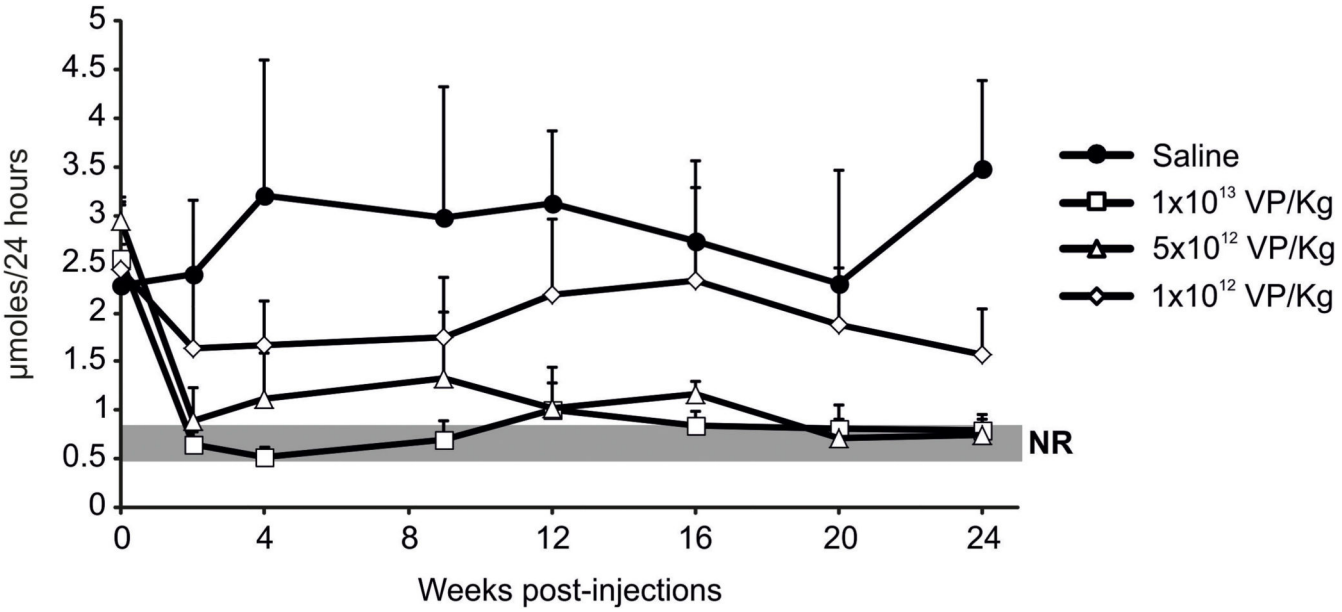

Fig. 1. Long-term correction of hyperoxaluria by a single intravenous injection of HDAd encoding AGT

Urinary oxalate measured on 24-h urine collections at multiple time points post-injection in *Agxt−/−* mice injected with various doses of HDAd-AGT vector or saline (at least n=5 per group; $p<0.05$ at each time-point for 1×10^{13} viral particles (VP) per kg and 5×10^{12} VP per kg and at 24 weeks post-injection for 1×10^{12} VP per kg compared to saline controls). Normal range is expressed as 95% confidence interval for the mean in wild-type SV129 mice (n=7). *NR= normal range*.

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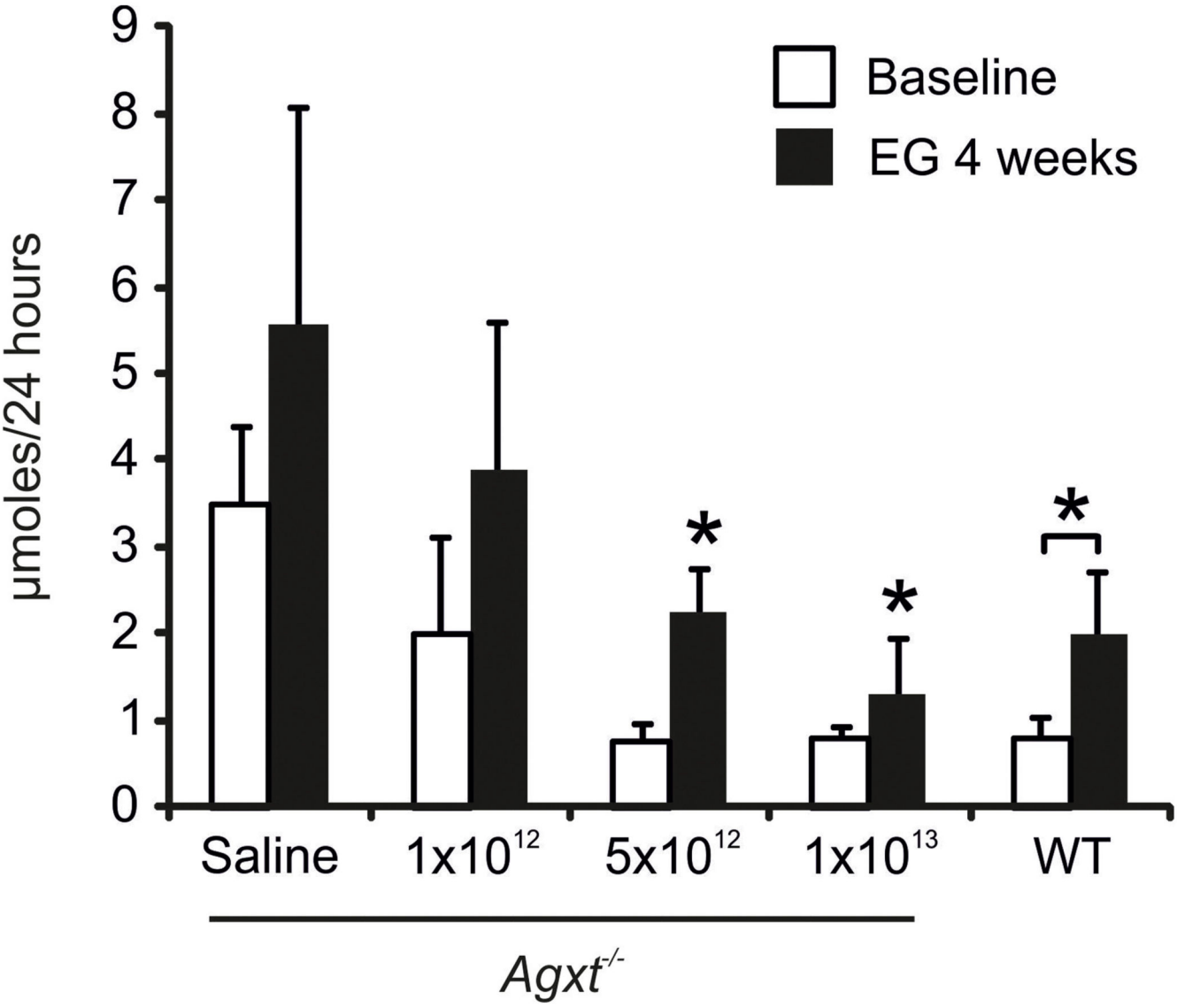

Fig. 2. Correction of hyperoxaluria in HDAd-AGT injected *Agxt*−/− mice challenged with ethylene glycol

Agxt−/− mice were challenged with 1.25% ethylene glycol (EG) 24 weeks after injections of saline or HDAd vectors (at least n=5 per group). Urinary oxalate excretions were lower in mice injected with 1×10^{13} VP/kg and 5×10^{12} VP/kg compared to saline-injected controls (*, $p<0.05$). Urinary oxalate increased significantly in WT mice administered with EG compared to baseline levels (*, $p<0.05$). Doses are expressed as VP/Kg. *EG= Ethylene glycol; WT= wild type*.

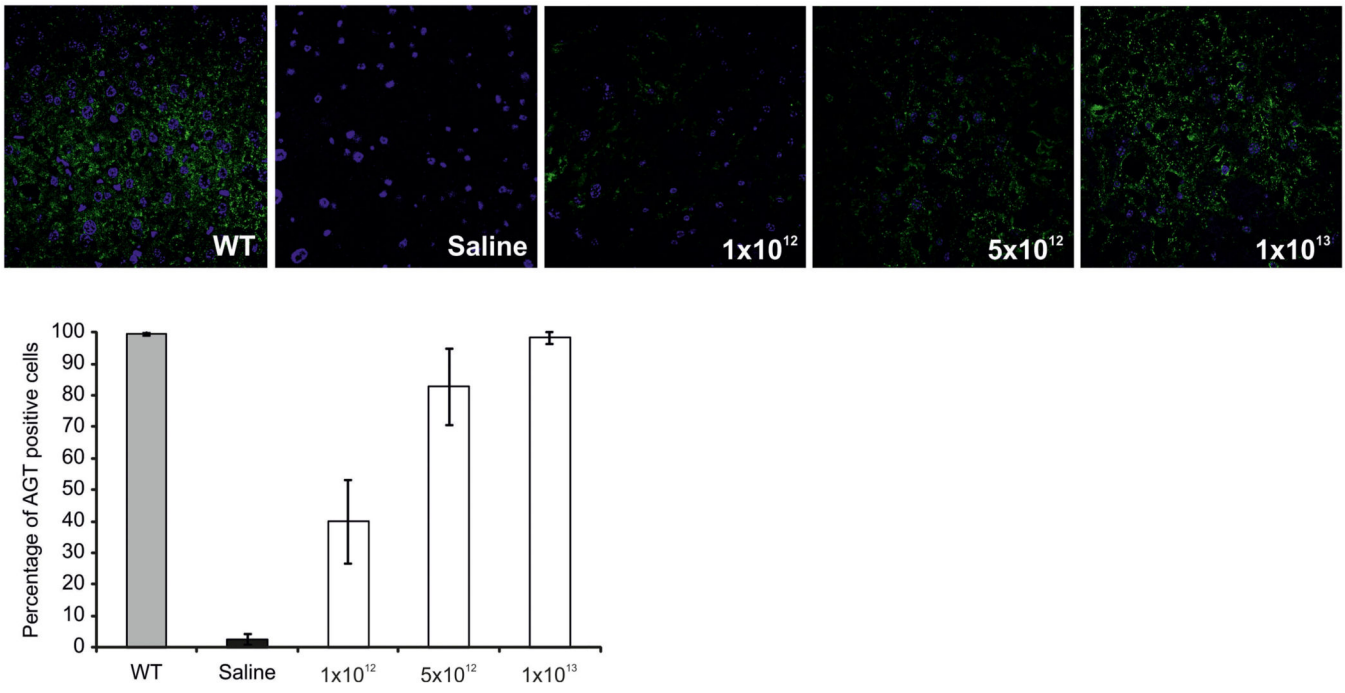

Fig. 3. Liver AGT immunofluorescence

Staining for AGT (green) of liver specimens harvested at 28 weeks post-injection showed a dose-dependent increase following injection of HDAd-AGT vector (magnification 63×). The graph shows the percentage of AGT positive hepatocytes per field of view. The vector doses are expressed as VP/kg. *WT= wild type*.

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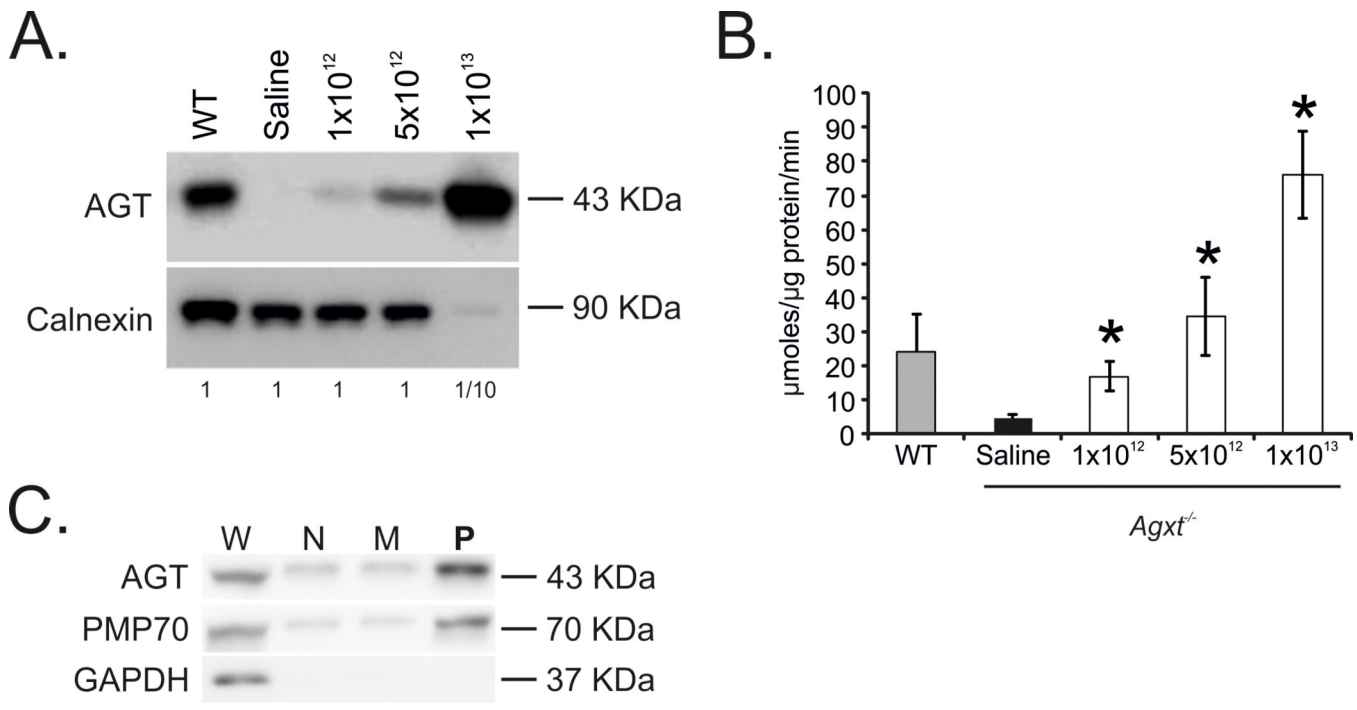

Fig. 4. AGT expression, activity, and subcellular localization

A. Western blot for AGT in liver lysates of *Agxt−/−* mice injected with three doses of HDAd-AGT expressed as VP/kg. The amount of protein extract loaded for the lysate of mice injected with 1×10^{13} VP/kg was 1 µg (1/10) whereas 10 µg (1) of proteins were loaded for all remaining lysates. Livers of saline-injected mice were used as negative control and WT mice as positive control. Calnexin was used as loading control. **B.** Hepatic AGT enzyme activity showed a dose-dependent increase following injection of HDAd-AGT vector (n=3 per group; *, $p<0.05$ vs. saline). **C.** Western blot analysis of subcellular organelle-enriched fractions of livers of mice injected with 1×10^{13} VP/kg of HDAd-AGT: the AGT protein band is more abundant in the whole cell lysates (W) and in the peroxisome-enriched fraction (P) whereas nuclear (N) and mitochondrial (M) enriched fractions show minimal amount of AGT. Peroxisomal protein PMP-70 was used as a peroxisomal marker and Glyceraldheyde-3-phosphate dehydrogenase (GAPDH) as a cytosolic marker. The vector doses are expressed as VP/kg. *WT= wild type*.