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# Increasing cholesterol synthesis in 7-dehydrosterol reductase (DHCR7) deficient mouse models through gene transfer

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ABSTRACT

Smith–Lemli–Opitz syndrome (SLOS) is caused by deficiency in the terminal step of cholesterol biosynthesis: the conversion of 7-dehydrocholesterol (7DHC) to cholesterol (C), catalyzed by 7-dehydrocholesterol reductase (DHCR7). This disorder exhibits several phenotypic traits including dysmorphia and mental retardation with a broad range of severity. There are few proven treatment options. That most commonly used is a high cholesterol diet that seems to enhance the quality of life and improve behavioral characteristics of patients, although these positive effects are controversial. The goal of our study was to investigate the possibility of restoring DHCR7 activity by gene transfer. We constructed an adeno-associated virus (AAV) vector containing the *DHCR7* gene. After we infused this vector into affected mice, the introduced *DHCR7* gene could be identified in liver, mRNA was expressed and a functional enzyme was produced. Evidence of functionality came from the ability to partially normalize the serum ratio of 7DHC/C in treated animals, apparently by increasing cholesterol production with concomitant decrease in 7DHC precursor. By 5 weeks after treatment the mean ratio (for 7 animals) had fallen to 0.05 while the ratio for untreated littermate controls had risen to 0.14. This provides proof of principle that gene transfer can ameliorate the genetic defect causing SLOS and provides a new experimental tool for studying the pathogenesis of this disease. If effective in humans, it might also offer a possible alternative to exogenous cholesterol therapy. However, it would not offer a complete cure for the disorder as many of the negative implications of defective synthesis are already established during prenatal development.


## 1. Introduction

Smith–Lemli–Opitz syndrome (SLOS) is an inherited disorder resulting in dysmorphias and mental retardation. It is caused by deficient 3β-hydroxysterol-$\Delta^7$-reductase (7-dehydrocholesterol reductase, DHCR7. EC 1.3.1.21), which catalyzes the last step in cholesterol synthesis. Thus, SLOS patients have elevated levels of dehydrocholesterol (DHC, 7-dehydrocholesterol [7DHC] and its isomer 8-dehydrocholesterol [8DHC]) and reduced ability to make cholesterol. The molecular and genetic basis of SLOS has been well described [1–3]. Due to the multiple and essential roles of cholesterol, the pathogenesis of SLOS is complex and not yet well understood. Among recessive inborn errors of metabolism, SLOS has a relatively high incidence, varying from 1 in 10,000 to 1 in 60,000 in different regions of Europe and North America [4–11]. The carrier frequency for mutant alleles is estimated as high as 1 in 30 for Caucasian populations and lower for African and Asian populations [2,4–6,12–14]. It is likely that the condition is underdiagnosed because patients having a mild disorder without distinctive phenotype may be missed. In addition early fetal demise may be common in the most severely affected cases [15]. Biochemical diagnosis is based on plasma serum levels of DHC [16], and prenatal diagnosis is available by measuring DHC in amniotic fluid [17,18] or chorionic villus cells [15]. Alternatively, a noninvasive technique involving measurement of dehydrosteroid metabolites in maternal urine has been shown to be equally effective [11,19,20].

Current treatment for SLOS is dietary cholesterol supplementation. Anecdotal reports show positive albeit limited effects of exogenous cholesterol on somatic growth and behavior, but development outcome does not appear to be altered [21–24]. However, an effect of dietary cholesterol on behavior would be somewhat

*Abbreviations:* DHC, dehydrocholesterol; C, cholesterol; DHCR7, 7-dehydrocholesterol reductase; AAV, adeno-associated virus; BSTFA, N,O-bis[trimethylsilyl] trifluoroacetamide; TMS, trimethylsilyl.

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surprising as the brain is believed to be impervious to external cholesterol [25], and a recent clinical trial showed no behavioral differences between short-term cholesterol supplementation and placebo [26]. Because both cholesterol deficiency and excess DHC likely contribute to the pathogenesis of SLOS, the therapeutic goal of treatment has been to enhance cholesterol accretion while decreasing accumulation of potentially toxic cholesterol precursors such as 7DHC. Some studies report a positive effect of combining a high cholesterol diet with administration of a statin inhibitor of sterol biosynthesis [27,28], however Haas et al. [29] could not confirm this positive effect in studies with a greater number of patients.

The single enzyme basis of SLOS makes it a candidate for gene therapy. Introduction of a functional *DHCR7* gene could increase the amount of cholesterol while simultaneously decreasing the amount of accumulated 7DHC. Using recombinant adeno-associated virus (AAV), the *DHCR7* gene could potentially be introduced and expressed in the liver, where most cholesterol is synthesized. Since DHCR7 is a membrane-bound protein [30], enzyme activity would be confined to the subset of hepatic cells successfully transduced by the viral vector; however, DHC could be shuttled between non-transduced and transduced cells via lipoprotein particles and their receptors. The present study reports the ability of AAV to introduce and express a functional *DHCR7* gene in mutant mice. This results in an improved serum 7DHC/C ratio, an important marker of DHCR7 deficiency in mutant mice and SLOS patients.

## 2. Experimental

### 2.1. Origin and breeding of mice

Animal work conformed to NIH guidelines and was approved by the Institutional Animal Care and Use Committee (OLAW Assurance number A3631-01). All animals were maintained in an AALAC certified facility and were fed a normal, cholesterol-free chow (Teklad irradiated rodent diet 2918; Harlan, Madison, WI). The two strains of mice carrying the *DHCR7* deletion (Δ) and the point mutation (T93M) were developed at NIH [31,32] and were then established at CHORI. A null mutation contained a partial deletion of *Dhcr7* (Δ) [31] and a hypomorphic mutant contained a point mutation in which methionine is substituted for threonine at amino acid 93 (T93M) [32]. We backcrossed both mutant strains to wild-type C57BL/6J (JAX) for multiple generations ($n > 12$ for Δ, $n = 9$ for T93M) to provide the mutations in a defined genetic background. Congenic T93M mutants were then maintained by inbreeding, but Δ/+ mutants were maintained by continued backcrossing, since homozygous (Δ/Δ) mutants die within 24 h of birth [31]. In the C57BL/6J background both mutant strains bred infrequently and many pups did not survive beyond 2 days of age. Therefore, it was very difficult to generate enough age-matched, mutant mice for experiments. To increase mutant animal viability, the FVB/N background was introduced; the FVB/N strain is particularly robust and prolific [33]. Homozygous C57BL/6J T93M/T93M males were crossed with wild-type FVB/N (JAX) females to produce T93M/+ progeny on a mixed background. These progeny were then intercrossed to generate T93M/T93M homozygotes. Heterozygous Δ/+ C57BL/6J males were also crossed with wild-type FVB/N females. Female Δ/+ progeny were then crossed with male T93M/T93M mixed background homozygotes to generate compound heterozygotes (Δ/T93M). In the mixed background Δ/T93M mice still had abnormal 7DHC/C ratios, but now had high survival rates. Δ/T93M mice were used for all experiments because they are phenotypically more severe than T93M/T93M mice. Genotypes of animals carrying Δ and T93M alleles were determined by PCR as described previously [31,32], and the mutant phenotypes of T93M/T93M and Δ/T93M animals were confirmed by measuring the serum 7DHC/C ratio, a biochemical marker indicative of SLOS.


| | Name | Sequence |
|---|---|---|
| → | Forward primer | 5'-AGGTGTGCGCAGGACTTTAG-3' |
| hDHCR7 | *Homo sapiens* 7-dehydrocholesterol reductase | See GenBank accession number: NM_001360 |
| ← | Reverse primer | 5'-GCTGGGCTCTCTCCAGTTTA-3' |

**Fig. 1.** DNA construct for AAV vector. The coding sequence for hDHCR7 (GenBank accession number NM_001360) was amplified from cDNA using the primers indicated in the table and cloned into pCR4® TOPO (Invitrogen). It was then excised using the plasmid's flanking EcoRI sites and subcloned into the EcoRI site of pV4.1c-WPRE [34]. Correct orientation in the pV4.1c plasmid was confirmed by sequencing. The complete vector construct contained: inverted terminal repeats (ITRs) from AAV2, CMV promoter/enhancer, intron from β-globin, human *DHCR7* coding sequence, woodchuck hepatitis virus post-translational regulatory element (WPRE) and human growth hormone polyadenylation signal (PA).

### 2.2. Preparation of AAV vector

Vector construction, production and purification were analogous to those in previous reports [34–36]. Human *DHCR7* cDNA was cloned in the EcoRI site of the pV4.1c plasmid, which contained the CMV promoter/enhancer and AAV inverted terminal repeats (ITR). A woodchuck hepatitis virus post-translational regulatory element (WPRE) was included at the 3′ untranslated end of *DHCR7* to increase translational utility of the transcript. The resulting construct (see Fig. 1), from ITR to ITR, was packaged in viral capsid. AAV2-DHCR7 particles were produced in an adenovirus-free system by cotransfecting HEK293 cells with plasmid pV4.1c-DHCR7, with a plasmid containing the AAV rep and cap genes (type 2 capsid was used), and with a plasmid containing adenovirus helper genes [37]. DNA packaged in capsid was purified by Optiprep Gradient (Iodixanol) and cesium chloride gradient centrifugation [38]. Finally, the viral vector was dialyzed and the titer in vector genomes (vg) per ml established by quantitative PCR.

### 2.3. Serum collection and administration of vector

Blood from Δ/T93M mice was collected via the retro-orbital sinus when the animals were 25 days old, and the ratio 7DHC/C in serum was measured by GC/MS. Prior to bleeding, mice were anesthetized with 2.5% (v/v) Isoflurane using a VetEquip Funnel-Fill Vaporizer (www.vetequip.com). Mutant mice were divided into two groups such that both groups had similar average ratios of 7DHC/C, as this ratio indicates disease severity. 200 μl of either AAV2-DHCR7 ($1.7 \times 10^{11}$ or $4.9 \times 10^{11}$ vg/ml) or saline was injected intravenously (IV) via the tail vein when the animals were 27 days old. Beginning at the age of 34 days, the ratio 7DHC/C was measured each week from blood collected via the retro-orbital sinus to monitor the effects of therapy.

### 2.4. Nucleic acid extraction and analysis

Mice were euthanized at 9 or 22 weeks of age by exsanguination via cardiac puncture. Livers were excised, immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Both DNA and RNA were extracted from a small sample of liver using the Qiagen Allprep DNA/RNA Mini Kit (www.qiagen.com). The presence or absence of vector DNA was determined by PCR using a commercial primer pair specific for human *DHCR7* cDNA (www.origene.com). *DHCR7* mRNA transcribed from the vector DNA was detected by

reverse transcription (oligo dT primer) followed by PCR using the human specific primer pair. The 123 base pair PCR product was detected in a 2% agarose electrophoretic gel. For quantitative PCR, real-time accumulation of product was detected using SYBR Green Master Mix (Fermentas) in an ABI 7900 instrument.

### 2.5. Serum sample preparation for sterol GC/MS

Blood was kept at 4 °C for at least 4 h, usually overnight, to optimize coagulation of red blood cells. Serum was separated by centrifugation for 3 min at 13,000 rpm on a bench centrifuge. 0.8 μg of internal standard (stigmasterol) was added to 8 μl of serum. The samples were then saponified by adding 500 μl of ethanol together with 300 μl of a 33% KOH solution, and heating at 55 °C for 45 min. After cooling to room temperature, 2 ml of water were added, and the samples were extracted twice with 2.5 ml of hexane by vortexing for 1 min. The organic layers were transferred to fresh tubes, evaporated and reacted with 50 μl of BSTFA (55 °C for 45 min) to form the trimethylsilyl (TMS) derivatives. The derivatives were finally diluted with 70 μl of cyclohexane and transferred to autosampler vials for analysis by GC/MS. To prevent conversion of 7DHC to previtamin $D_3$, all tubes were protected from light by covering with foil. The whole procedure was conducted under minimum lighting conditions.

### 2.6. Analysis of cholesterol and dehydrocholesterol by GC/MS

The analysis was essentially as previously described [39] with variation necessitated by changing to an Agilent 5975 instrument (www.agilent.com). Sterols were separated on a DB-1, 15 m × 0.25 mm i.d., film thickness 0.25 μm (J&W Scientific, Folsom, CA, USA). Helium was the carrier gas at a flow-rate of 1.2 ml/min. A 2 μl aliquot (in cyclohexane) of the final derivatized extract was injected in splitless mode (valve opened 2 min). The GC temperature was ramped as follows: initial 100 °C, held for 3 min, increased to 210 at 25 °C/min and finally, increased to 285 °C at 2.5 °C/min. The injector, transfer line, and ions source were kept at 260, 280 and 230 °C, respectively. The mass spectrometer was operated in the electron impact mode in a SIM (select ion monitoring) mode with ions 368 and 458 for cholesterol, 325 and 456 for 7DHC and 394 and 484 for stigmasterol, where the first ion is used for quantification, and the second one is used as a qualifier. Although DHC exists as both 7DHC and 8DHC due to an isomerase capable of their interconversion, serum and liver samples had almost exclusively the 7DHC isomer. Since 8DHC was a minor component often indistinguishable from background noise, only 7DHC was quantified.

#### 2.6.1. Preparation of calibration samples

Calibration curves were prepared daily for each analytical batch by combining increasing amounts of the analytes with fixed amount of stigmasterol, followed by derivatization. The final calibration curve for cholesterol contained six concentration levels (amount injected) at 6.67, 10, 15, 20, 25 and 50 ng with 13.33 ng stigmasterol. For 7DHC, the calibration samples had injected amounts of 0.5, 0.83, 1.67, 3.33, 6.67 and 10 ng.

### 2.7. Statistical analysis

Data comparing serum 7DHC/C ratios from treated and control groups did not meet the criteria for a normal distribution by Q–Q plot and the Shapiro–Wilk test. Therefore, we used the Mann–Whitney test to compare the medians of the vector treated group against the non-treated group at each week. We also compared these two groups longitudinally over the entire time course. This tested the overall difference based on all time points as opposed to cross-sectional differences at discreet time points. The mixed, random effects model was used for this longitudinal analysis where the outcome variable was the ratio of 7DHC/C, and independent variables were treatment (Y/N), sex and age in weeks of the mice at the times of sterol measurement. *P* values of 0.05 or less (two-tailed) were used as the test of significance. Statistical analysis employed Stata Statistical Software: Release 9 (Stata Corp. 2005 College Station, TX).

## 3. Results

### 3.1. Mouse model

T93M/T93M and T93M/Δ mice, which initially had a mixed genetic background, were originally shown to have near normal viability [32]. After extensive backcrossing, however, we observed a dramatic reduction in viability when these mutations were expressed in a uniform C57BL/6 genetic background. Normal viability was restored when this genetic background was mixed with alleles from FVB/N mice. This suggests that there are one or more genetic differences between C57BL/6 and FVB/N mice that contribute to the severity of the SLOS phenotype. For the work presented here, the mixed genetic background may have contributed to mouse to mouse variation independent of the DHCR7 mutations, but it was a practical necessity in order to have sufficient numbers of age-matched SLOS mice.

We previously showed that the 7DHC/C ratio in livers of T93M/Δ mice fluctuates considerably throughout the life span [39]. In general the ratio is relatively high at birth, drops to almost normal while suckling, rises again after weaning, and then gradually approaches (but does not reach) normal with advancing age. Presumably, serum ratios of 7DHC/C should follow the same general pattern as liver. To establish a baseline for serum 7DHC/C in T93M/Δ mice, we measured ratios weekly over an extended time course starting shortly after weaning (Fig. 2). The results showed both weekly fluctuations and animal to animal variations, but the overall trend was a modest rise (about 2-fold) followed by a return to original values. Although the details of weekly fluctuations varied, this general pattern was repeated in subsequent experimental controls. These results emphasized the need for age-matched controls, so we were careful to have comparable numbers of littermates in treated and non-treated experimental groups. Because, unlike humans, SLOS mice tend to normalize cholesterol metabolism as they age, we targeted treatment to young post-weaning mice, in which 7DHC/C ratios were still rising. All experimental and control groups included both males and females. Initially, males were compared to males only and females to females. Since we saw no correlation between sex and 7DHC/C, male and female data were then pooled for statistical analysis.

### 3.2. Delivery of DHCR7 gene to liver

A human *DHCR7* cDNA was cloned into a packaging plasmid (Fig. 1) to generate viral vector, which was then administered to mutant mice by intravenous (IV) injection. To study the effectiveness of the AAV vector in transferring the active gene, DNA was measured in liver samples. As seen in Fig. 3, only animals treated with vector particles present a DNA band after PCR with primers for the human *DHCR7* cDNA, showing that the gene was successfully introduced into the tissue and persisted for at least 5 weeks. In a longer-term experiment, however, delivered *DHCR7* DNA remaining in liver was reduced at 18 weeks after administration (Fig. 3B). This was in spite of the fact that the longer-term experiment used a higher dose of vector ($9.8 \times 10^{10}$ vector genomes (vg) vs. $3.4 \times 10^{10}$ vg).

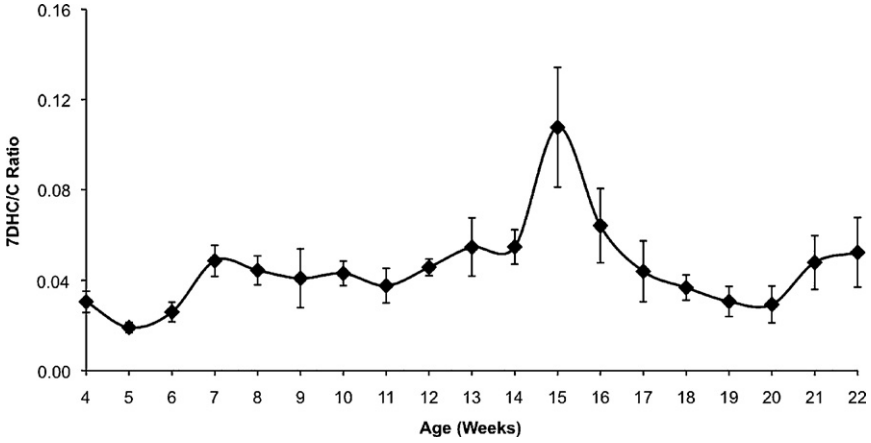


**Fig. 2.** Time course of ratio 7DHC/C in serum of untreated mutant mice. Each time-point represents the mean ± S.E.M. ($n = 5$).

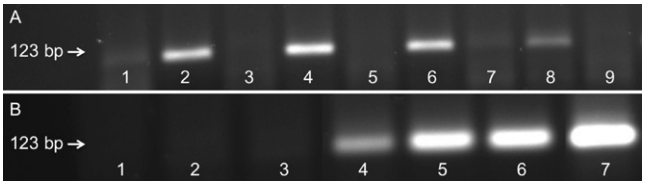


**Fig. 3.** Detection of human *DHCR7* DNA in liver after gene transfer. DNA was extracted from liver and subjected to PCR using primers specific for the human DHCR7 coding sequence and yielding a 123 bp product. (A) 5 weeks after AAV-DHCR7 administration. Treated mice (lanes 2, 4, 6, 8) received $3.4 \times 10^{10}$ vg via tail vein and non-treated mice (lanes 1, 3, 5, 7, 9) received saline. (B) 18 weeks after AAV-DHCR7 administration. Treated mice (lanes 4–6) received $9.8 \times 10^{10}$ vg via tail vein and non-treated mice (lanes 1–3) received saline. Lane 7 is DNA from the liver of a treated mouse 5 weeks after treatment (from same sample as lane 6 in A). As illustrated by comparison to lane 7, there was consistently less vector DNA after 18 weeks than after 5 weeks even though the longer-term experiment used a higher dose of vector.

### 3.3. Expression of DHCR7 mRNA

RNA extracted from the livers of treated and control mice was analyzed by RT-PCR. Human *DHCR7* mRNA was not detected in mice 5 weeks after administration of $3.4 \times 10^{10}$ vg (data not shown) even though vector DNA was clearly present (Fig. 3A). However, 18 weeks after administration of a higher dose of vector ($9.8 \times 10^{10}$ vg) h*DHCR7* mRNA was detected (Fig. 4). Expression was low and variable among 7 treated mice, and 2 of the treated mice appeared to be negative (like all of the saline controls). Quantitative RT-PCR confirmed expression indicating that hDHCR7 mRNA was actually present in all 7 treated mice. However, expression levels were quite low compared to mouse GAPDH mRNA, which was measured as an internal standard. Apparent levels of h*DHCR7* mRNA were only 0.57 ± 0.13% (mean ± S.E.M. for 5 males and 2 females) of GAPDH mRNA. Relative expression levels ranged from 0.2% (not visible in Fig. 4, lane 4) to 1.1%, while background levels in saline controls were 0.05%.

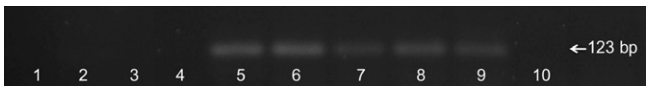


**Fig. 4.** Expression of human *DHCR7* mRNA. RT-PCR was performed on RNA extracted from individual livers 18 weeks after treatment with saline (lanes 1–3) or $9.8 \times 10^{10}$ vg of AAV-DHCR7 (lanes 4–10). Lanes 1, 3, 6 and 10 were females and the rest were males. The 123 bp PCR product is specific for human *DHCR7* cDNA and was seen only for treated mice. Although it was not readily visible in lanes 4 and 10, quantitative RT-PCR indicated that these two mice also expressed low levels of h*DHCR7* mRNA while saline controls did not (see text). RNA samples treated identically with the omission of reverse transcriptase gave no product (not shown) indicating that RNA was not contaminated with vector DNA.

### 3.4. 7DHC/C ratio in serum of treated and untreated mutant mice

In a preliminary experiment SLOS mice were injected IV with $3.4 \times 10^{10}$ vg at 4 weeks of age and serum sterols were followed weekly for 5 weeks, at which time mice were killed and tissues collected. At 5 weeks both serum and liver ratios of 7DHC/C were slightly lower than untreated littermate controls, but the difference was not statistically significant (data not shown). Even though vector DNA was clearly present in liver (see Fig. 3), the effect on cholesterol metabolism was minimal at best.

Using a higher titer vector preparation to give a three times higher dose ($9.8 \times 10^{10}$ vg), an effect on cholesterol metabolism became apparent (Fig. 5). As we had seen before, serum 7DHC/C ratios in untreated SLOS mice changed with time and varied considerably from animal to animal, but the general trend was first an increase followed by a decrease in ratio value. In treated littermates, however, the ratio decreased over 2 weeks and then remained relatively constant for an extended time. The extensive fluctuations seen in untreated mice were much attenuated in the treated mice. At 9 weeks of age (5 weeks after saline or vector administration) the ratio in untreated mice had increased about 2-fold to a mean ratio of 0.14 ± 0.04 while the ratio in treated mice had decreased to 0.05 ± 0.01. This difference was statistically significant as were the differences at several other time points indicated in Fig. 5. There were, however, several time points at which the differences did not meet the criteria for statistical significance. To test whether the treated and untreated groups had a significantly different response over the entire time course of the experiment, we did a longitudinal analysis using a mixed random effects model in which sex was included as a potential variable. This confirmed that the observed effect of treatment on cholesterol metabolism was significant ($P = 0.042$) and that sex was not a significant factor ($P = 0.64$). Although the 7DHC/C ratios were decreased in treated animals, the total sterols (i.e., 7DHC plus C) were indistinguishable between treated and untreated groups of mice (Fig. 6). These results imply that the vector treated animals have enhanced conversion of 7DHC to cholesterol rather than an effect on some other step in cholesterol metabolism.

Presumably, the partially normalized ratio in serum is the result of the introduction of an active hepatic enzyme by the gene transfer. At 22 weeks of age, 18 weeks after administration of vector, we looked at the ratios of 7DHC/C in liver, and the treated mice were not significantly lower than the untreated mice. Neither were serum ratios different by this time point. This was in spite of our observations that there was still vector DNA (Fig. 3B) and h*DHCR7* mRNA (Fig. 4) in liver at this time. However, it also appeared that the SLOS mice were starting to self-correct by this time.

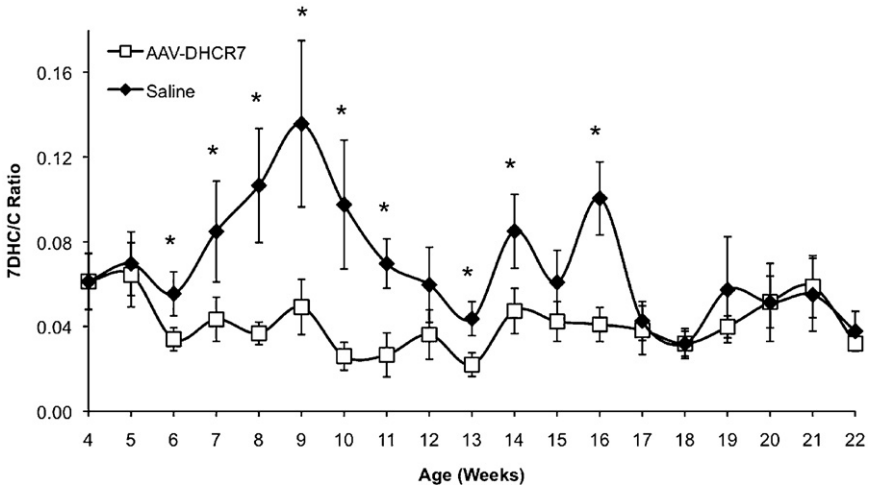


**Fig. 5.** Reduction of serum 7DHC/C in mutant mice treated with AAV-DHCR7. Mice were injected with vector ($9.8 \times 10^{10}$ vg) or saline at 4 weeks of age. Each time-point represents the mean ± S.E.M. ($n = 7$, 5 males and 2 females for mice treated with vector; $n = 6$, 3 males and 3 females for mice treated with saline). Time points at which treated and control are significantly different (*$P < 0.05$; Mann–Whitney test). By longitudinal analysis the two curves are significantly different ($P = 0.042$).

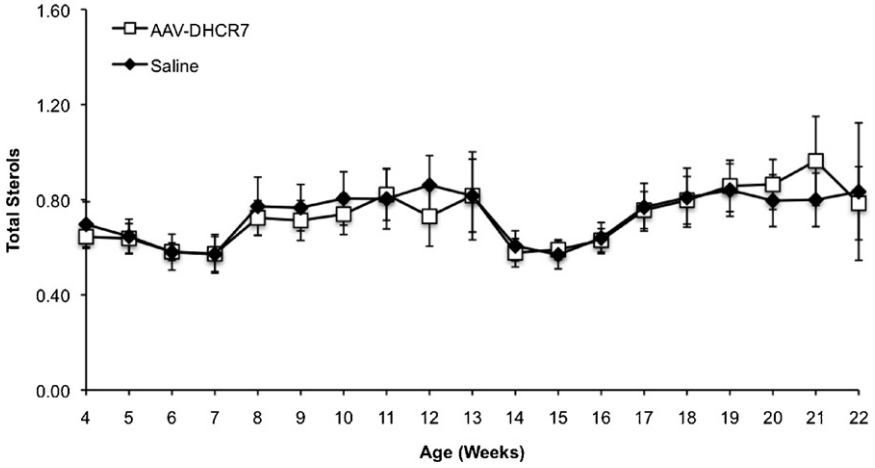


**Fig. 6.** No effect of gene transfer on total sterols. Treated and non-treated mice were as in Fig. 5. Total serum sterols were the sum of 7DHC and C measurements. Means ± S.E.M. are shown in μg/μl.

## 4. Discussion

The goal of the present study was to remove the block in cholesterol synthesis that causes SLOS. Our approach was to introduce an active *DHCR7* gene into a mouse model of SLOS using a recombinant AAV vector and then test for an improvement in the ratio of DHC to C. Results showed that several weeks after IV infusion of the vector, copies of the foreign gene were detectable in liver. Furthermore, treatment (at the higher dose) was able to reduce the serum DHC/C ratio in SLOS mice as compared to sham-treated, littermate controls. Because the total sterol levels (DHC plus C) were the same between treated and control mice, it appears that some, but not all, of the abnormally high DHC was reduced to C. That is, the block in cholesterol synthesis was indeed partially removed.

Normal mice (and humans) have very low levels of DHC in most tissues, so the DHC/C ratio is essentially zero using the measurement techniques employed here. Although significantly improved, the treated SLOS mice still had serum DHC/C ratios above normal. There are several factors that may have contributed to the less than 100% normalization of cholesterol metabolism. Most obvious is the dose of the AAV vector. At a dose of $3.4 \times 10^{10}$ vg there were clearly copies of human *DHCR7* DNA in liver cells 5 weeks after administration, but this was not sufficient to have a significant effect on serum DHC/C. However, at about three times the dose there was a definite effect on DHC/C. It remains to be seen if still higher doses of vector can further improve or even completely normalize DHC/C ratios.

Another factor that may impede complete normalization of DHC/C is that there appears to be an upper limit to the number of liver cells that can be stably transduced by AAV vectors with type 2 capsid. At best, we can expect only 5–10% of hepatocytes to express the delivered gene [40]. Since DHCR7 is a membrane-bound enzyme [30], we cannot expect it to move from transduced cells to non-transduced cells. Thus, a limited number of "cured" liver cells must take on the systemic burden of DHC, and DHC from non-transduced cells must be shuttled into transduced cells, reduced and exported again as cholesterol. Clearly, this happens at least to some extent as we see a reduction of DHC/C in serum, but we do not yet know what fraction of liver cells must be transduced to achieve a virtually complete normalization of DHC/C. We chose AAV2 as the vector because it has a long history of success in our hands and others [34–36]. More recently, it has been shown that AAV vector packaged with type 8 capsid can transduce more liver cells than AAV with type 2 capsid [40], so an alternative vector may be necessary.

Another possible limitation to complete normalization is that DHC is distributed in different pools or compartments, such as in membranes, lipoprotein particles or free molecules. Any DHC

sequestered in a low turnover pool might be expected to have lower exposure to the introduced DHCR7, and would, therefore, be more resistant to conversion to cholesterol. Of note in Fig. 5 is the large variability in DHC/C for untreated animals and small variability in the measurements for treated animals. These differences in variability are with respect to time (week to week changes) and also among individual animals at a given time point (note larger error bars in non-treated). This suggests that a particularly variable pool of DHC (perhaps more newly synthesized) is preferentially reduced to cholesterol in treated mice.

Our results show that the number of human *DHCR7* gene copies in liver was higher at 5 weeks after vector administration than at 18 weeks. This was in spite of the fact that the dose for the 5-week set of animals was only one third that for the 18-week animals. This is consistent with AAV2 vector results for both liver [40] and muscle [34] that showed a loss of vector copies over several weeks. Surprisingly, however, vector expression continued to increase in spite of vector copy losses. These results suggest that only a small fraction of vector copies are active and that activation is a prolonged process [34,40]. Low levels of active AAV vector can persist long-term, as expression has been shown to continue in mice for more than a year [41].

By 18 weeks, 14 weeks after vector administration, the difference between treated and non-treated SLOS littermates had disappeared. This may be due more to the eventual "self-correction" previously observed in SLOS mice [32,39] than to a loss of the introduced DHCR7 activity. Indeed, h*DHCR7* mRNA was detectable in liver at 22 weeks indicating at least some continued expression of the vector.

We have shown before that an AAV vector similar to the one used here preferentially targets liver when administered by IV infusion [34]. Thus, the observed reduction in serum DHC/C is most likely due to DHCR7 activity introduced into the liver. At the times measured, however, we did not see a significant reduction in liver DHC/C. In one case (5 weeks after administration) the vector dose was apparently too low, and in the other (18 weeks after administration) any effect was probably obscured by the tendency of SLOS mice to self-correct. This self-correction, which does not appear to happen in human SLOS patients, creates a limited window of opportunity for gene transfer experiments in our mouse model; possible effects of gene transfer in older SLOS mice become increasingly smaller.

The results presented provide proof of principle that it is possible to remove at least partially the block in cholesterol synthesis that is the root cause of SLOS. Thus, we are now able to manipulate cholesterol biosynthesis in our mouse model and test the phenotypic consequences. Furthermore, if success in the mouse model can be translated to humans, gene therapy might be able to provide clinical benefit to SLOS patients beyond that achieved by high dietary cholesterol. But there are limitations. Like dietary cholesterol, IV administration of viral vector is unlikely to benefit the central nervous system due to the blood brain barrier. Alternative methods of vector administration might circumvent this problem [35]. A more severe limitation is that many of the problems associated with SLOS are developmental and are already manifest in the fetus. Both very early diagnosis and *in utero* therapy would have to be developed. Thus, a "complete cure" for SLOS is not on the horizon. However, a gene therapy mediated correction of cholesterol synthesis in a fetus after mid-pregnancy and post-natally might attenuate some of the most negative affects of the disorder.

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