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Recombinant adeno-associated virus-mediated gene delivery of long chain acyl coenzyme A dehydrogenase (LCAD) into LCAD-deficient mice

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Abstract

Background—Very long chain acyl coenzyme A (CoA) dehydrogenase (VLCAD) deficiency is a relatively common mitochondrial β -oxidation disorder. The most severe form of VLCAD deficiency presents with neonatal cardiomyopathy and hepatic failure and is generally fatal within the first year of life. Mice deficient for long chain acyl CoA dehydrogenase (LCAD) closely resemble the clinical syndrome observed in VLCAD-deficient humans. Recombinant adeno-associated viral (rAAV) vectors with pseudotype capsids were investigated for their potential towards correcting the phenotype observed in mice heterozygous (+/−) for LCAD (i.e. liver and muscle steatosis).

Methods—rAAV containing the mouse LCAD cDNA (mLCAD) under the transcriptional control of the CMV/chicken β -actin hybrid promoter were injected intramuscularly into the tibialis anterior (TA) muscle of LCAD^{+/-} mice or injected into the portal vein to transduce hepatocytes.

Results—Ten weeks post-injection of rAAV1-mLCAD into the TA muscle, significantly increased levels of mLCAD within mitochondria were demonstrated by immunostaining of TA sections, immunoblotting of mitochondrial isolates and by the electron transfer flavoprotein (ETF) fluorescence reduction enzyme activity assay. Magnetic resonance spectroscopy of vector-injected TA muscle demonstrated a reduction in the lipid content compared to phosphate-buffered saline-injected mice, whereas a systemic effect was observed as a reduction in liver macrosteatosis. Eight weeks after portal vein injection of rAAV8-mLCAD into LCAD^{+/-} mice, increased levels of mLCAD within hepatocyte mitochondria were demonstrated by immunostaining and also by the

ETF assay. Scoring of the hepatosteatosis observed in partially deficient LCAD mice indicated a reduction in the lipid content within livers of vector-treated mice.

Conclusions—These studies show that rAAV-mediated delivery of mLCAD was efficient and led to an amelioration of local and systemic pathologies observed in partially deficient LCAD mice.

Keywords

adeno-associated virus; fatty acid oxidation; gene therapy; LCAD; liver; mitochondria; vector; VLCAD

Introduction

Mitochondrial fatty acid oxidation is the primary source of energy for heart and slow-twitch skeletal muscle. Moreover, during prolonged fasting or physiological stress, β -oxidation of fatty acids in the liver generates ketone bodies that supply energy for the brain, muscles and other organs. Mitochondrial catabolism of fatty acids occurs by repeated cycling through a four-step β -oxidation spiral, with each round producing one molecule of FADH₂, one NADH, and one acetyl-CoA. Inborn errors of metabolism affecting this pathway are typically inherited in a recessive fashion. Affected individuals deficient for medium- and very long chain acyl coenzyme A (CoA) dehydrogenase (MCAD and VLCAD, respectively) may present with heart and skeletal muscle dysfunction, nonketotic hypoglycaemia or sudden infant death syndrome. Post-mortem histological findings almost always include organ lipidosis [1].

The first step in the β -oxidation spiral is rate-limiting and is catalysed by a family of acyl CoA dehydrogenases (ACADs) that differ in their substrate specificity based on the carbon chain length of the fatty acid. Long chain acyl CoA dehydrogenase (LCAD; OMIM 609 576) has a broad specificity, with most activity towards medium and long chain C10 to C18 species, although it is largely responsible for catalysing dehydrogenation of C12–C14 fatty acid thioesters [2]. As mono- and polyunsaturated fatty acids such as oleate (C18 : 1) and linoleate (C18 : 2) are β -oxidised and repeatedly chain shortened, the double bond reaches the 5,6 (or 4,5) position producing C14 : 1 and C14 : 2, respectively, at which point LCAD is the ACAD responsible for initiating the next round of β -oxidation in the mitochondria. Consistent with this role in the metabolism of unsaturated species, accumulation of acylcarnitines of C14 : 1 and C14 : 2 have been demonstrated in LCAD-deficient mice [3,4].

Until the discovery of very long chain acyl CoA dehydrogenase (VLCAD) deficiency in the early 1990s, patients diagnosed with deficiencies in VLCAD were considered to have a defect in LCAD. To date, no specific mutations have been identified in the gene encoding human LCAD (*Acadl*) and no definitively diagnosed cases of human LCAD deficiency are known. The failure to identify patients with LCAD deficiency is surprising given the prevalence of disease caused by deficiencies in all other members of this gene family. This has put into question both the role of LCAD in the metabolism of long chain fatty acids and the potential of LCAD deficiency to produce disease. Human LCAD deficiency may not

cause clinical disease or it could result in gestational lethality, either of which could account for the lack of identified human cases.

Kurtz *et al.* [3] produced a mouse model of LCAD deficiency with severely impaired fatty acid oxidation. Matings between heterozygous mice yielded an abnormally low number of LCAD heterozygous and homozygous deficient offspring, indicating frequent gestational loss. LCAD^{-/-} mice that reached birth appeared normal but had severely reduced fasting tolerance with hepatic and cardiac lipidosis, hypoglycaemia, elevated serum free fatty acids and nonketotic dicarboxylic aciduria. Gestational loss was observed in both homozygous deficient and heterozygous mice. Guerra *et al.* [5] found that LCAD-deficient mice are sensitive to the cold, similar to BALB/cByJ mice, which have mutations in the gene for short chain acyl CoA dehydrogenase (SCAD).

Replacement of the murine SCAD gene has previously been demonstrated in our laboratory [6]. rAAV vector with pseudotype 1 capsid, containing the murine SCAD cDNA was injected into the tibialis anterior (TA) muscle of 8-week old SCAD^{-/-} mice. Ten weeks after injection, SCAD enzyme activity was detected within the TA muscle and the levels of circulating C4 butyrylcarnitine were significantly reduced in vector treated mice. Magnetic resonance spectroscopy (MRS) demonstrated a reduction of the lipid peak in injected TA muscle. Biochemical correction of SCAD-deficiency after rAAV8-mediated delivery of mSCAD to the livers of SCAD-deficient mice has also been recently demonstrated [7]. A significant reduction of circulating butyrylcarnitine was found in AAV8-mSCAD injected SCAD^{-/-} mice 6 weeks after treatment. In addition, MRS demonstrated a reduction in the lipid content within the livers of rAAV8-mSCAD-treated mice. In the present study, we describe LCAD gene replacement to the TA muscle and livers of partially deficient LCAD mice, a surrogate model for human VLCAD deficiency.

Materials and methods

Producing and purifying recombinant AAV vectors

The University of Florida Powell Gene Therapy Center produced all rAAV with pseudotype capsids for these studies. The AAV recombinant genome, *pCB-mLCAD*, contains the coding sequence for murine LCAD under the transcriptional control of the cytomegalovirus enhancer/chicken β -actin promoter [8]. Virus production included use of the helper/ packaging plasmids *pXYZ1* and *pDG8* for AAV1 and AAV8, respectively. These helper plasmids supply all the necessary helper functions as well as *rep* and *cap* proteins *in trans*. Vector plasmid *pCB-mLCAD* was co-transfected by calcium phosphate precipitation with into a single cell factory (632 cm² surface area; Nunc, Rochester, NY, USA) of approximately 80–95% confluent 293 cells. rAAV were purified by iodixanol step gradient centrifugation and anion exchange chromatography as described by Zolotukhin *et al.* [9]. rAAV were purified to 7.5×10^{12} vector genomes (vg) per ml for rAAV1-mLCAD and 5.4×10^{12} vg/ml for rAAV8-mLCAD, as determined by dot blot assay.

LCAD-deficient mice

Mice heterozygous for a mutation in LCAD (strain B6.129S6-*Acadl*^{tm1Uab}/Hsd) were purchased from the Mutant Mouse Regional Resource Center (University of Missouri/Harlan, Columbia, MO, USA). Heterozygotes (+/-) were used for these studies because of difficulties in breeding a homozygous-deficient colony, due to gestational loss. Mice were maintained in plastic cages in pathogen-free facilities under a 12 : 12 h light/dark cycle. Mice were fed a standard chow and watered *ad libitum*. All animal surgeries and care were approved by the University of Florida Institutional Animal Care and Use Committee in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International specifications. Mice were fasted overnight prior to MRS scanning and prior to tissue collection upon sacrifice.

Tibialis anterior muscle injection

Twelve 10-week old mice were injected with AAV1-mLCAD (seven males, five females) and 10 mice were injected with phosphate-buffered saline (PBS; six males, four females). When performing intramuscular injections, all animals were anaesthetised with 3% isoflurane (Abbott Laboratories, Abbott Park, IL, USA). The tibialis anterior (TA) muscle was exposed by removing hair from the hind right leg by applying depilatory cream diluted 1 : 1 with PBS. Using a 31-gauge Ultra-Fine II Insulin syringe short needle (Becton Dickinson, Franklin Lakes, NJ, USA), 1×10^{11} vg rAAV1-mLCAD were injected intramuscularly in a total volume of 15 μ l. To determine the concentration of circulating butyrylcarnitines, serum samples were obtained via the tail vein at regular intervals (0, 2, 4, 6, 8 and 10 weeks post-injection). At 10 weeks post-rAAV administration, mice were sacrificed by anaesthetisation with 5% isoflurane followed by cervical dislocation, prior to tissue collection.

Portal vein injection

Eight 6-week old female LCAD^{+/-} mice were injected via the hepatic portal vein with 1×10^{11} vg AAV8-mLCAD vector. Seven mice received PBS, acting as the negative control group. Mice were randomly assigned to both treatment groups. To perform the portal vein injections, all animals were anaesthetised with 3% isoflurane. A ventral midline abdominal incision was made into the peritoneal cavity, and the portal vein was exposed. rAAV vector or PBS (30 μ l) were administered into the portal vein using a 31 gauge Ultra-Fine II Insulin syringe short needle. Haemostasis was achieved by application of a sterile cotton bud tip directly onto the site of injection. Surgeries were performed on a thermoregulated operating board designed to maintain a temperature of 37 °C throughout the procedure. Serum samples were obtained biweekly for up to 8 weeks when the mice were sacrificed and tissues harvested.

Immunohistochemistry

TA muscle (from both injected and contralateral legs) liver and hearts were completely removed. Representative sections were frozen in a mixture of isopentane and dry ice for subsequent mitochondrial isolation or fixed in 10% neutral-buffered formalin for histology and immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections (4 μ m) were

sequentially deparaffinised, blocked for endogenous peroxidase activity with 3% hydrogen peroxidase in methanol for 10 min, and rehydrated. Slides were subsequently rinsed in Tris buffered saline (TBS) and incubated with the universal protein blocker, Background Sniper (Biocare Medical, Concord, CA, USA) for 15 min. After rinsing in TBS, slides were incubated in the polyclonal primary antibody, rabbit anti-human LCAD (provided by Dr Jerry Vockley) diluted 1 : 1000, for 1 h at room temperature (RT). Slides were rinsed for 5 min followed by incubation in Mach2 Rabbit HRP-polymer (Biocare Medical) for 30 min at RT. Detection of LCAD was achieved by incubating slides in Vulcan Fast Red (Biocare Medical) for 5 min at RT. Slides were rinsed for 5 min in water then counterstained with haematoxylin (Vector Laboratories Inc., Burlingame, CA, USA) before mounting. Negative control slides included incubation of the PBS-injected muscles without primary antibodies. Representative digital images were captured using a Zeiss Axioskop equipped with an Axiocam camera (Carl Zeiss, Jena, Germany). Camera exposure settings were constant for all images.

Western blotting

Mitochondrial proteins were extracted from 30 mg of TA muscle and liver using a mitochondria isolation kit (MITOISO1; Sigma, St Louis, MO, USA) following the manufacturer's instructions for isolation from hard and soft tissues, respectively. Treating the mitochondrial isolate with a final concentration of 0.25% (w/v) lubrol allowed for efficient lysis of mitochondria. Fifty micrograms of mitochondrial protein from each mouse were heat-denatured and resolved by electrophoresis on a 10% Criterion sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Purified mouse LCAD served as a positive control. Separated proteins were transferred to 0.2 μ m Optitran nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) using a TE Series Transphore Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, USA). LCAD antigen was detected using the same rabbit anti-human LCAD antibody used for immunohistochemistry, diluted 1 : 1000. This was followed by incubation of the membrane in alkaline phosphatase conjugated goat anti-rabbit IgG, diluted 1 : 3000, and visualisation with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colour development solution according to the manufacturer's instructions.

LCAD enzyme activity determination

Flash-frozen tissue pieces were homogenised for 30 s in 600 μ l of 50 mM Tris-HCl, pH 8.0, supplemented with 2 mM ethylenediaminetetraacetic acid and Halt protease inhibitor cocktail (Pierce, Rockford, IL, USA). The homogenate was centrifuged at 4 °C for 20 min and the supernatant collected. The protein concentration was determined by the Bradford method. Enzyme activity was measured with the anaerobic electron transfer flavoprotein fluorescence reduction assay [10] using an LS50B fluorescence spectrophotometer from PerkinElmer Life Sciences (Wellesley, MA, USA) with a heated cuvette block set to 32 °C. The enzymatic reaction was started by addition of palmitoyl CoA (Sigma) or 2-methyl-C7-CoA to yield a final concentration of 5 μ M. Specific activity was calculated with one milliunit (mU) of activity defined as the amount of protein necessary to completely reduce 1 nmol of electron transfer flavoprotein (ETF) in 1 min.

Electron spray ionisation tandem mass spectrometry (ESI-MS/MS) of serum acylcarnitines

Acylcarnitines in serum were measured as their C14 esters as previously described [1].

MRS of LCAD^{+/-} TA muscle

Magnetic resonance imaging (MRI) and MRS of injected LCAD^{+/-} TA muscle were performed on an 11T Bruker Avance spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA). LCAD^{+/-} mice were anaesthetised using 1.5% isofluorane with an oxygen flow of approximately 1 l/min. The animals were placed prone in a home-built quadrature birdcage coil and respiration used for MRS gating was monitored using a small animal monitoring and gating system (SA Instruments, Inc., Stony Brook, NY, USA). A survey scan was conducted to visualise the TA muscle for voxel placement. Proton spectroscopy from a 6.75 μ l voxel was acquired using a volume selective PRESS sequence with a TR of 2000 ms and a TE of 18 ms. Data were acquired into a 6000-Hz spectral width, comprised of 4096 points. Five hundred and twelve averages were used for a total scan time of 17 min. Processing of the data was conducted using XwinNMR (Bruker BioSpin Corporation). In addition to lipid peak values, area integration of the peaks for both lipid and creatine were assigned. Because MRS lipid peak values are arbitrary, lipid to creatine ratios were used to determine the relative lipid content within rAAV1-mLCAD-injected TA muscle.

Statistical analysis

Student's *t*-test was used to compare groups. $p < 0.05$ was considered statistically significant.

Results

Tibialis anterior muscle expression of mLCAD delivered by rAAV1 persists up to at least 10 weeks post-injection.

Ten-week old LCAD heterozygous (LCAD^{+/-}) mice were injected into the TA muscle with either PBS ($n = 10$) or rAAV1-mLCAD ($n = 12$) at a dose of 1×10^{11} vg per animal. Ten weeks later, injected TA muscle sections were collected and immunostained with a rabbit anti-human LCAD antibody. No LCAD protein was detectable in the PBS-treated animals (Figure 1A), whereas, in mice injected with the rAAV1-mLCAD vector, mLCAD expression was observed in a punctate manner throughout the myofibres (Figures 1B and 1C), a pattern consistent with mitochondrial localisation. LCAD is encoded in the nuclear genome as a precursor containing a mitochondrial targeting peptide at the amino terminus. After translation in the cytoplasm, the precursor protein is transported to the mitochondria where it is then imported into the matrix, proteolytically processed to its 44.4 kDa mature form, and assembled into an active homotetrameric holoenzyme. The human ACADL protein shares 85.3% identity with the rat protein [11]. To confirm that the rAAV1-delivered murine LCAD was properly targeted to the mitochondrial matrix, western blotting was carried out on mitochondria isolated from TA muscle samples. Mitochondrial isolates were probed with a rabbit anti-human LCAD antibody, where 15 ng and 7.5 ng purified mouse LCAD protein were loaded into the first and last wells, respectively, as positive controls. Mitochondria from four representative LCAD^{+/-} mice from each group were analysed.

LCAD^{+/-} mice injected with rAAV1-mLCAD had detectable levels of murine LCAD present in TA muscle mitochondria 10 weeks post-injection, whereas there was no or low levels of LCAD detected from those that received PBS (Figure 2). The faint LCAD bands observed in the lanes for the PBS-injected TA muscle mitochondria are background levels of LCAD found in heterozygous mice. However, in the mice injected with rAAV1-mLCAD, significantly increased levels of LCAD were detected.

Having demonstrated that LCAD protein encoded by the rAAV vector properly targets to mitochondria and persists for 10 weeks post-injection, we next asked whether the vector-delivered enzyme was active. LCAD enzyme activity was measured in crude TA muscle extracts using the anaerobic ETF reduction assay. This assay is highly sensitive and specific for the ACAD enzyme family [10]. With C16-CoA as substrate, mean activity in TA muscle of rAAV1-mLCAD injected mice was 254.63 ± 45.22 mU/mg tissue. Background activity in PBS-treated LCAD^{+/-} mice was low but still detectable (20.22 ± 3.46 mU/mg) (Table 1).

TA muscle transduction diminishes the physiological effects of partial LCAD deficiency

Nonfasting levels of C14, C14 : 1 and C14 : 2 acylcarnitine were measured by ESI-MS/MS on sera collected at biweekly intervals post-administration of rAAV1-mLCAD vector or PBS. There was a nonsignificant reduction of C14, C14 : 1 and C14 : 2 acylcarnitine species in LCAD^{+/-} mice that received vector compared to those that received PBS (data not shown). However, serum concentrations of C14 acyl carnitines from all mice were determined to be below 0.1 $\mu\text{mol/l}$ for both groups of mice.

MRS was performed on LCAD^{+/-} mice that received either rAAV1-mLCAD ($n = 5$) or PBS ($n = 3$) 10 weeks post-injection. All mice were fasted overnight prior to scanning. MRI allowed for selection of homogenous areas within the TA muscle for MRS. Figure 3A illustrates a magnetic resonance spectrum from a LCAD^{+/-} mouse TA muscle 10 weeks post-injection of rAAV1-mLCAD. The three-pronged cluster of peaks towards the left of the spectrum corresponds to taurine, trimethyl-ammonium groups and creatine. An additional smaller creatine peak occurs to the left of the three-pronged cluster and with the other creatine peak, collectively, represent total creatine. The wider peak to the right represents lipid. Area integration of the peaks for both total creatine and lipid from water-suppressed spectra were determined. A significant reduction in total lipid content within the TA muscle was demonstrated in the partially deficient mice that received rAAV1-mLCAD ($p = 0.038$; Figure 3B). Because the creatine values do not remain constant within the LCAD^{+/-} mice, the ratio of lipid to total creatine was also used to indicate a reduction in the relative lipid content within the vector-treated muscle. The mean ratio of lipid to water peaks in LCAD^{+/-} mice that received rAAV1-mLCAD was eightfold lower than those that received PBS ($p = 0.087$; not shown).

Oil-red-O (ORO) staining of frozen livers harvested from LCAD^{+/-} fasted mice, 10 weeks post-injection of PBS, presented the hepatic fatty change reported in LCAD^{-/-} mice [3]. Light micrographs of ORO-stained liver sections from LCAD^{+/-} mice that received PBS contain relatively large lipid vesicles (Figures 4A and 4B). Such macrosteatosis within the LCAD^{+/-} liver was generally not observed in fasted mice 10 weeks after injection of rAAV1-mLCAD (representative sections are shown in Figures 4C and 4D). Liver sections

from all mice were scored for microsteatosis and macrosteatosis. Because approximately equal numbers of male and female mice were included in the two treatment groups, the extent of microsteatosis and macrosteatosis within male and female mice was also examined. There was no difference in microsteatosis between the two treatment groups (for both genders) where low scores were assigned. There was no difference in macrosteatosis observed in LCAD^{+/-} males. However, there was a significant reduction in macrosteatosis between groups of females, where those treated with rAAV1-mLCAD all scored 1.0 compared to a mean score of 2.75 in females that received PBS ($p < 0.005$; Figure 4E).

Hepatocyte expression of mLCAD delivered by rAAV8 persists up to at least 8 weeks post-injection

After demonstrating expression of mLCAD in rAAV1-injected TA muscle, the liver was studied as a target for exogenous rAAV vector-mediated LCAD expression. Eight weeks post-injection of 1×10^{11} vg rAAV8-mLCAD, LCAD^{+/-} livers were harvested and immunostained with a rabbit anti-human LCAD antibody. Only background levels of LCAD protein were detected in the PBS-treated animals (Figure 5A). However, in mice injected with the rAAV8-mLCAD vector, over 80% of hepatocytes stained positive for LCAD (Figure 5B). There was no histological evidence of liver toxicity from the vector or its effects as demonstrated by haematoxylin and eosin staining (not shown). LCAD enzyme activity was measured in crude liver extracts using the anaerobic ETF reduction assay. With 2,6-dimethyl-C7-CoA as the specific substrate, mean activity in livers of rAAV8-mLCAD injected mice was 60.37 ± 27.19 mU/mg tissue, respectively. Activity in PBS-treated LCAD^{+/-} mice was low but still detectable (13.18 ± 3.16 mU/mg; Table 2). Immunohistochemistry was performed on heart sections from LCAD^{+/-} mice injected with rAAV8-mLCAD. LCAD was detected throughout the heart muscle (Figure 5C). However, this was comparable to background levels of LCAD expression seen in the hearts of LCAD^{+/-} mice injected with PBS (Figure 5D).

Liver transduction reduces the degree of hepatosteatosis observed in mice with partial LCAD deficiency

Liver, diaphragm and heart sections were ORO stained and images blindly scored between 1 and 4 for tissue lipid infiltration (steatosis). Figure 6A illustrates an ORO-stained liver section taken from a LCAD^{+/-} mouse that received PBS via the portal vein. There is an equal distribution of fatty microvesicles and macrovesicles, resulting in a score of 2 for both microsteatosis and macrosteatosis. By contrast, Figure 6B shows an ORO-stained liver section from a mouse that received rAAV8-mLCAD, which scored zero for both micro- and macrosteatosis. There was a nonsignificant reduction in macrosteatosis, microsteatosis and total hepatosteatosis in rAAV8-mLCAD-treated mice compared to those that received PBS (Figures 6C to 6E). Diaphragm and heart sections stained with ORO did not show a difference between groups (not shown).

Discussion

The presented study demonstrates the feasibility of efficient LCAD gene transfer to the TA muscle and liver of mice with partial LCAD deficiency. Despite LCAD^{-/-} mice being the

intended model for such a gene replacement approach, partially deficient, heterozygote mice were used to demonstrate LCAD gene delivery in these ‘proof principle’ studies. Generating an LCAD^{-/-} mouse colony proved extremely difficult due to gestational loss and small litter size [3]. In our experiments with LCAD^{+/-} mice, vector delivery of mLCAD was sufficient that the desired effects were easily detected over the background of the remaining endogenous LCAD allele. Immunoblotting of mitochondrial isolates from TA muscle, immunostaining of tissue sections and tissue enzyme activity illustrated efficient delivery and sustained expression of rAAV1-mediated mLCAD in LCAD^{+/-} mice. MRS was applied as a non-invasive clinically relevant end-point to show a reduction in the lipid content within the TA muscle of LCAD^{+/-} mice that had received rAAV1-mLCAD. This reduction in lipid in LCAD heterozygotes is of interest since it indicates that carrier status for defects within β -oxidation of fatty acids can lead to ectopic fat storage within muscle and that treatment with a rAAV1-mLCAD vector was able to decrease the amount of fat within the TA muscle.

Further to reducing lipid content within LCAD^{+/-} TA muscle after injection of rAAV1-mLCAD, a systemic effect was indicated when the degree of hepatic macrosteatosis was also reduced in female LCAD^{+/-} mice (at 20 weeks of age) but not males. Kurtz *et al.* [3] reported that 10% of 14–16 week-old LCAD^{-/-} males display severe multifocal myocardial fibrosis. Such cardiomyocyte degeneration was not seen in age-matched LCAD^{-/-} females [3]. Further studies in LCAD^{+/-} and LCAD^{-/-} mice should investigate whether higher levels of rAAV1-mediated expression in muscle are required to ameliorate hepatic macrosteatosis in males, rather than females.

rAAV1-mediated expression of mLCAD in the TA muscle of nonfasting LCAD^{+/-} mice did not reduce serum circulating levels of C14 or C14 : 1 acylcarnitine. A simple explanation may lie with the use of sera rather than whole blood or dried blood spots as sample material. It is feasible that the long chain fatty acyl carnitine esters co-pelleted with erythrocytes when attempting to separate blood cells from sera by centrifugation. Dried blood spots have higher concentrations of acylcarnitines not only in patients or mice deficient for ACAD enzymes, but also in unaffected controls. In a study conducted in our laboratory, a significant reduction in the circulating levels of short chain (C4) butyrylcarnitine was observed in SCAD-deficient mice 10 weeks after injection of rAAV1-mSCAD into the TA muscle [6]. Short chain fatty acids are able to freely diffuse into the mitochondrial matrix, whereas long- and very long-chain fatty acids require carnitine as a carrier to transfer across the mitochondrial membranes (with the aid of carnitine palmitoyltransferases I and II and acylcarnitine translocase). Despite the reported reduction in circulating butyrylcarnitines in SCAD-deficient mice after rAAV1-mediated expression of SCAD within TA muscle, skeletal muscle may not act as an ideal metabolic sink for longer chain fatty acids. TA muscle, chosen due to its accessibility for injection in the mouse, is white fast-twitch muscle rather than red mitochondria-rich slow-twitch muscle, and normally oxidises very little fatty acids for energy. Long chain fatty acids and acylcarnitines are much more hydrophobic than butyrylcarnitine and may not as readily enter TA muscle for oxidation.

By contrast, the liver is a central processing center for long chain fatty acids, and delivering rAAV vectors expressing the acyl CoA dehydrogenase to the liver may represent a more viable approach towards observing substantial whole-body changes. rAAV8-mLCAD

vectors were administered to the livers of LCAD^{+/-} mice. Consistent with published reports after intravenous administration of rAAV8, wide-spread transduction of the liver was observed with rAAV8-mLCAD vector resulting in up to 80% hepatocytes expressing mLCAD 8 weeks after portal vein injection [12,13]. Circulating levels of long chain acylcarnitine species in LCAD^{+/-} mice that received rAAV8-mLCAD vector via the portal vein were unable to be determined and MRS analysis of livers was not amenable. However, rAAV8-mediated expression of exogenous LCAD in the liver was indicated to reduce the degree of hepatosteatosis observed in partially deficient fasted mice.

LCAD deficiency has not been found in human patients, perhaps unsurprisingly, considering the gestational loss observed in the LCAD^{-/-} mouse model. Berger and Wood [14] tested the hypothesis that LCAD deficiency disrupts normal embryonic development by culturing LCAD^{-/-}, ^{+/-} and wild-type embryos [14]. They found a significantly increased rate of death in LCAD^{-/-} embryos at the morula-to-blastocyst conversion indicating a deficient ability to complete the development of a blastocoele and formation of a blastocyst. As a result, LCAD^{-/-} mice have reduced litter size (approximately 4–5 pups). Additionally, LCAD^{-/-} mice demonstrate cardiomyopathy [3], cold intolerance [5] and fasting-induced acute metabolic crisis. Zhang *et al.* [15] recently used LCAD-deficient mice to show that primary defects in mitochondrial fatty acid oxidation capacity can lead to hepatic insulin resistance [15]. Sudden death has also been witnessed in LCAD^{-/-} mice during conditions of no apparent external stress [3]. The LCAD^{-/-} mouse develops a clinical syndrome that closely resembles human VLCAD deficiency.

LCAD gene delivery and replacement in the mouse acts as an informative model for the more common VLCAD and medium-chain acyl CoA dehydrogenase (MCAD) deficiencies. As judged by tandem mass spectrometry blood spot screening of 8.25 million screened newborns, the incidence of MCAD deficiency is 1 : 14 600 [16]. Following the introduction of neonatal screening programs for VLCAD deficiency [17,18] the apparent incidence has increased and is now estimated at 1 : 30 000–1 : 120 000 [19]. Disorders of mitochondrial fatty acid β -oxidation have serious clinical consequences. The disease phenotypes are complex and highly variable, ranging from mild to severe episodes of metabolic decompensation, often presenting as a recurrent Reye Syndrome-like disorder (hypoglycaemia, hyperammonaemia and hepatosteatosis) or resulting in sudden unexpected death [20,21]. There are also additional inherited defects in fatty acid catabolism in humans that are being studied with a view towards rAAV-based gene delivery, including fatty aldehyde dehydrogenase deficiency (i.e. Sjögren–Larsson syndrome) [22]. The data presented here provide further evidence for using rAAV vectors as part of a gene replacement strategy for patients deficient for enzymes responsible for mitochondrial β -oxidation of fatty acids.

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Figure 1.

rAAV1-mLCAD vector-mediated expression of murine LCAD in LCAD^{+/-} TA muscle. Sections from LCAD^{+/-} mouse TA muscle 10 weeks after injection of rAAV1-mLCAD were incubated with a rabbit anti-human LCAD antibody as the primary antibody. (A) LCAD^{+/-} mouse TA muscle that received PBS. Original image at $\times 20$ magnification. (B) Transverse section from LCAD^{+/-} mouse TA muscle that received rAAV1-mLCAD. Image at $\times 20$ magnification. (C) Longitudinal section from LCAD^{+/-} mouse TA muscle that received rAAV1-mLCAD. Image at $\times 40$ magnification. (D) LCAD^{+/-} mouse TA muscle that received rAAV1-mLCAD but stained without rabbit-anti-human LCAD primary antibody. Image at $\times 40$ magnification

Figure 2.

LCAD protein detection in TA muscle from mitochondrial isolates in PBS and rAAV1-mLCAD injected LCAD^{+/-} mice, 10 weeks post-injection. Mitochondrial isolates were probed with a rabbit anti-human LCAD antibody, where 15 ng and 7.5 ng purified human LCAD protein were loaded into the first and last wells, respectively, as a positive control

Figure 3.

Injection of rAAV1-mLCAD decreases the lipid content within the TA muscle ten weeks post-injection, as demonstrated by proton magnetic resonance spectroscopy. (A) Water-suppressed magnetic resonance spectrum from a LCAD^{+/-} mouse TA muscle that received rAAV1-mLCAD. LCAD^{+/-} mice were fasted for 18–20 h prior to analysis. An 11.T1-Advance spectrometer proton MRS was used to determine lipid infiltration of tissue. (B) Lipid peak values in TA muscle of LCAD^{+/-} mice 10 weeks post-injection of 1×10^{11} vg rAAV1-mLCAD or PBS. Error bars are the mean $\pm$ SE. PBS ($n = 3$); rAAV1-mLCAD ($n = 5$). * $p < 0.05$

Figure 4.

rAAV1-mLCAD-mediated over-expression of mLCAD in the TA muscle of $LCAD^{+/-}$ mice reduces the degree of hepatosteatosis. ORO-staining of $LCAD^{+/-}$ livers 10 weeks post-injection of rAAV1-mLCAD or PBS via the tibialis anterior. (A) $LCAD^{+/-}$ mouse that received PBS. Original image at $\times 40$ magnification. (B) $LCAD^{+/-}$ mouse that received PBS. Image taken using a $\times 100$ oil immersion objective. Arrows indicate ORO-stained lipid droplets. (C) $LCAD^{+/-}$ mouse that received rAAV1-mLCAD. Original image at $\times 40$ magnification. (D) $LCAD^{+/-}$ mouse that received rAAV1-mLCAD. Image taken using a 100

oil immersion objective. (E) Degree of macrosteatosis within the livers of female LCAD^{+/-} mice. * $p < 0.05$ ×

Figure 5. rAAV8-mLCAD vector-mediated expression of murine LCAD in LCAD^{+/-} liver and heart. Sections from LCAD^{+/-} mouse liver 8 weeks after injection of rAAV8-mLCAD were incubated with a rabbit anti-human LCAD antibody as the primary antibody. (A) LCAD^{+/-} mouse liver that received PBS; ×20 original magnification. (B) LCAD^{+/-} mouse liver that received rAAV8-mLCAD; ×10 magnification. (C) LCAD^{+/-} mouse heart after injection of PBS into the portal vein; Longitudinal section; ×10 magnification. (D) LCAD^{+/-} mouse heart after injection of rAAV8-mLCAD into the portal vein; Transverse section; ×20 magnification

Figure 6.

ORO-staining of LCAD^{+/-} livers 10 weeks post-injection of rAAV8-mLCAD vectors or PBS via the hepatic portal vein. (A) LCAD^{+/-} mouse liver that received PBS. Arrows indicate lipid droplets. (B) LCAD^{+/-} mouse liver that received rAAV8-mLCAD. Both images at $\times 20$ original magnification. (C) Degree of macrosteatosis within LCAD^{+/-} livers. (D) Degree of microsteatosis within LCAD^{+/-} livers. (E) Degree of total hepatosteatosis within LCAD^{+/-} livers

Table 1

LCAD enzyme activity in tibialis anterior muscle of LCAD-heterozygous deficient mice 10 weeks post-injection 1×10^{11} vg rAAV1-mLCAD

Group	Mean enzyme specific activity (mU/mg)	SE
PBS ($n = 10$)	20.22	3.46
rAAV1-mLCAD ($n = 10$)	254.63	45.22

Tibialis anterior muscles were extracted on sacrifice at 10 weeks and the EFT reduction enzyme assay was performed using palmitoyl CoA as the substrate. Each tissue sample was assayed at least twice. rAAV1-mLCAD and PBS groups were analysed by Student's *t*-test and found to be significant ($p < 0.001$).

Table 2

LCAD enzyme activity in livers of partially deficient LCAD mice 8 weeks after injection of rAAV8-mLCAD vector via the hepatic portal vein

Group	Mean enzyme specific activity (mU/mg)	SE
PBS (<i>n</i> = 7)	13.18	3.16
rAAV1-mLCAD (<i>n</i> = 8)	60.37	27.19

Liver samples were extracted on sacrifice at 8 weeks and the EFT reduction enzyme assay was performed using palmitoyl CoA as the standard substrate. Each tissue sample was assayed at least twice. rAAV8-mLCAD and PBS groups were analysed by Student's *t*-test ($p = 0.15$).