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AAV8-mediated overexpression of mPCSK9 in liver differs between male and female mice

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Abstract

Background and aims: The recombinant adeno-associated viral vector serotype 8 expressing the gain-of-function mutation of mouse proprotein convertase subtilisin/kexin type 9 (AAV8-PCSK9) is a new model for the induction of hypercholesterolemia. AAV8 preferentially infects hepatocytes and the incorporated liver-specific promoter should ensure expression of PCSK9 in the liver. Since tissue distribution of AAVs can differ between male and female mice, we investigated the differences in PCSK9 expression and hypercholesterolemia development between male and female mice using the AAV8-PCSK9 model.

Methods: Male and female C57BL/6 mice were injected with either a low-dose or high-dose of AAV8-PCSK9 and fed a high-fat diet. Plasma lipid levels were evaluated as a measure of the induction of hypercholesterolemia.

Results: Injection of mice with low dose AAV8-PCSK9 dramatically elevated both serum PCSK9 and cholesterol levels in male but not female mice. Increasing the dose of AAV8-PCSK9 threefold in female mice rescued the hypercholesterolemia phenotype but did not result in full restoration of AAV8-PCSK9 transduction of livers in female mice compared to the low-dose male mice. Our data demonstrate female mice respond differently to AAV8-PCSK9 injection compared to male mice.

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CONFLICT OF INTEREST

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Conclusions: These differences do not hinder the use of female mice when AAV8-PCSK9 doses are taken into consideration. However, localization to and production of AAV8-PCSK9 in organs besides the liver in mice may introduce confounding factors into studies and should be considered during experimental design.

Keywords

PCSK9; AAV8; Hypercholesterolemia; Atherosclerosis

INTRODUCTION

The two most commonly used mouse models of atherosclerosis are the *ApoE*^{-/-} (apolipoprotein E-deficient) and *Ldlr*^{-/-} (low-density lipoprotein receptor-deficient) mice which develop “spontaneous” or diet-induced plaque development^{1–3}. Investigating the contribution of specific genes of interest on atherosclerosis typically requires backcrossing of these mouse strains, which is time intensive and expensive. As an alternative method for inducing atherosclerosis while circumventing the necessity for germline-encoded mice, a recombinant adeno-associated viral vector 8 expressing a gain-of-function mutation (D377Y) of mouse proprotein convertase subtilisin/kexin type 9 (referred to as AAV8-PCSK9) was developed^{4–6}. Injection of AAV8-PCSK9 in mice results in transduction of hepatocytes and subsequent over- production of the gain-of-function mPCSK9 that binds the low-density lipoprotein (LDL) receptor resulting in its degradation and a corresponding increase in serum cholesterol levels that eventually lead to atherosclerosis⁴.

AAV serotypes, as a tool for gene therapy, have been extensively studied as serotype influences tissue tropism^{7, 8}. Therefore, choosing the correct AAV serotype for gene delivery is critical. The AAV8 serotype has a high affinity for hepatocytes and depending on the dose can achieve up to 95% transduction of hepatocytes, making it very effective for liver-targeted gene therapy⁷. However, AAV8 targeting of liver is more efficacious in male mice as AAV8 tissue tropism is more widely distributed in female mice^{9, 10}. Gene therapy studies using AAV8-*GFPLuc* constructs have demonstrated AAV8 distribution is higher in the extremities, including the brain in female mice, while in male mice distribution is largely seen in the liver⁹. Our laboratory previously observed that female mice did not develop hypercholesterolemia when injected with the same AAV8-PCSK9 dose (3×10^{10} vector genomes) as male mice¹¹. Our observations, along with the observations from other gene therapy studies, led us to question if there are differences in the ability to elicit hypercholesterolemia in male and female mice using the AAV8-PCSK9 model

MATERIALS AND METHODS

AAV8-PCSK9 viral vector preparation –

DNA for pAAV/D377Y-mPCSK9 (Addgene plasmid #58376), a gift from Jacob Bentzon⁴ was packaged into adeno-associated virus serotype 8 (AAV8) using helper and capsid plasmids from the University of Pennsylvania by previously described methods^{12–14}. Viral stocks were sterilized via Millipore Millex-GV syringe filter (Billerica, MA), titered by dot

blot assay, aliquoted, and stored frozen until use¹⁵. Final product will be referred to as AAV8-PCSK9.

Animals and tissue harvest –

Animal protocols were approved by the LSU Health Sciences Center-Shreveport institutional animal care and use committee, and all animals were cared for according to the National Institute of Health guidelines for the care and use of laboratory animals. 6- to 8-week old, C57BL/6J and *Ldlr*^{-/-} male and female mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained on normal diet (5053; PicoLab® Rodent Diet 20) prior to AAV8-PCSK9 injection and continued for mice that were not placed on high-fat diet. The same viral preparation was aliquoted prior to storage and used for all studies. Mice were either non-transduced (no injection) or given retro-orbital injections of 3×10^{10} or 9×10^{10} vector genomes of AAV8-PCSK9. Retro-orbital injections were chosen as they have been shown to have similar delivery and efficacy as tail-vein injections while minimizing distress to the animal^{16, 17}. Immediately following the AAV8-PCSK9 injection, the mice were switched to a high-fat, Research Diet (D12451) that contained 24% fat by weight (0.02% cholesterol) or a high-fat/high-cholesterol diet, Western diet (TD 88137; Harlan-Teklad, Madison, WI) that contained 21% fat by weight (0.15% cholesterol and 19.5% casein without sodium cholate) for 8 weeks. Mice were weighed once a week after starting a high-fat diet and blood was collected by cheek puncture into heparinized blood collection tubes centrifuged at 5,000 rpm for 5 minutes, and plasma was isolated and frozen at -80°C until analysis. After 8 weeks on a high-fat diet, mice were euthanized by exsanguination and pneumothorax under isoflurane anesthesia. Blood was collected by vena cava puncture into heparinized blood collection tubes as described above. Following blood collection, mice were perfused with 1X phosphate-buffered saline (PBS) and tissue (liver, brain, lung and heart) was collected for RNA, DNA and protein extraction.

Blood analysis –

Total and HDL cholesterol (Wako) and triglyceride levels (Pointe Scientific) were measured using commercially available kits. LDL was calculated using the Friedewald equation.

PCSK9 ELISA –

PCSK9 levels were measured from the collected blood serum samples using commercially available kits (BioLegend cat# 443208).

Tissue processing –

Liver, brain, heart, and lung tissue were flash frozen in liquid nitrogen and stored at -80°C until use. For RNA and DNA isolation, tissues were thawed, and 30 mg of each tissue was homogenized using the Biojector® 2000 (iHealthNet). Then the Qiagen AllPrep DNA/RNA Mini Kit was used to collect DNA and STAT60 was used to collect RNA. For protein isolation, tissues were thawed, and 50 mg of each tissue was lysed in 1 mL RIPA buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1X protease inhibitor cocktail (Thermo Scientific), 1X phosphatase inhibitor cocktail 2 (Sigma Aldrich), and 1X phosphatase inhibitor cocktail 3 (Sigma Aldrich) using

the Biojector® 2000 (iHealthNet). Homogenized tissues were incubated on ice for 30 minutes. Samples were then centrifuged at 16,000xg for 35 minutes at 4°C. Supernatant below the lipid layer was collected as the lysate.

Quantitative PCR analysis –

Quantitative PCR was performed using DNA isolated from tissue on a BioRad iCycler with SsoAdvanced Universal SYBER Green SuperMix (BioRad). Primers were designed using online Beacon Designer software. PCR products were verified by the presence of a single peak in melt curve analysis. Results were normalized to the housekeeping gene, *GAPDH*, and expressed as fold change using the 2^{-C_t} method. Primers include:

HCRapoE/hAAT promoter Forward: 5' – TTCACCATGTTGGTCAGG – 3', HCRapoE/hAAT promoter Reverse: 5' – GCAGAATCCTTAGTGGCT – 3', *PCSK9* Exon2/3 Forward: 5' – TATGTCATCAAGGTTCTAC – 3', *PCSK9* Exon2/3 Reverse: 5' – GTCTTCCTCAATGTACTC – 3', *GAPDH* Forward: 5' – AGTGGCAAAGTGGAGATT – 3', *GAPDH* Reverse: 5' – GTGGAGTCATACTGGAACA – 3'.

Quantitative RT-PCR analysis –

qRT-PCR was performed using RNA isolated from tissue on a BioRad iCycler with PerfeCTa® SYBR® Green SuperMix, ROX (QuantaBio). DNase treatment was performed using the TURBO DNA-free™ Kit (Thermo Scientific). RNA was converted to cDNA using qScript cDNA SuperMix (Quantabio). Primers were purchased from Harvard Bank. PCR products were verified by the presence of a single peak in melt curve analysis and appropriate reverse transcriptase negative controls were included to rule out single-stranded DNA contamination. Results were normalized to the housekeeping gene, *GAPDH*, and expressed as fold change using the 2^{-C_t} method. Primers include: *PCSK9* Forward: 5' – GAGACCCAGAGGCTACAGATT – 3', *PCSK9* Reverse: 5' – AATGTACTCCACATGGGGCAA – 3', *GAPDH* Forward: 5' – GCCTCCCGTGTTCTTACC – 3', *GAPDH* Reverse: 5' – CTTCAACACCTTCTTGATGTC – 3'.

Western blot analysis –

Tissue lysates were sonicated, and heat denatured at 95° C. Protein concentration was determined by Pierce® 660 nm Protein Assay (Thermo Scientific). 40–50 µg total protein of each sample was diluted in 4X NuPAGE buffer (Thermo Scientific). Samples were placed in 95°C water bath for 7 minutes and then cooled to room temperature. Samples were centrifuged at 16,000xg for 1 minute before being loaded into a 4 to 12% polyacrylamide NuPAGE Novex gel (Invitrogen). 1X MES (20X MES: 50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA) running buffer was used. Proteins were separated at 200 volts for 50 minutes. Semi-dry transfer was performed at 20 volts for 45 minutes onto an Immobilon-FL membrane (EMD Millipore). Li-Cor Odyssey® blocking buffer (Li-Cor) was used to block membranes for 1 hour at room temperature. Primary antibodies were diluted 1:5,000 in 1% BSA in PBS with 0.01% sodium azide and incubated on membranes at 4°C overnight. The membranes were washed in 1X TBST (1X Tris-buffered saline and 0.1% Tween 20). Goat anti- Rabbit and Rabbit anti-Goat IgG (H+L) secondary antibodies were diluted in 5% milk with 1X TBST with 0.01% SDS at 1:2,000 and 1:1,000 respectively and incubated for

2 hours at room temperature. Membranes were washed in 1X TBST. ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz) was mixed and added to blots for 1 minute. Exposures were taken using the Amersham Typhoon Imager 680. Densitometry was performed using Image Quant TL Toolbox v8.2.0. Bands of interest were normalized to GAPDH for statistical analysis. Primary antibodies were as follows: anti-m/rPC9 (R&D Systems; AF3985), LDLR¹⁸, GAPDH (Cell Signaling; 2118S). Secondary antibodies were as follows: Rabbit Anti-Goat IgG (H+L) (Jackson ImmunoResearch; 305–035-003) and Goat anti-Rabbit (Jackson ImmunoResearch; 111–035-003)

Statistical analysis –

GraphPad Prism 5.0 (La Jolla, CA) was used for statistical analyses. Data was tested for Gaussian distribution, followed by either a two-tailed student T test or one-way ANOVA with a Bonferroni's post-test.

RESULTS

Female mice have lower total cholesterol and PCSK9 levels as compared to male mice when infected with the same dose of AAV8-PCSK9.

In previous experiments, we were able to recapitulate hypercholesterolemia and plaque development in male mice injected with AAV8-PCSK9 similar to that seen in *Ldlr*^{-/-} mice, however we failed to induce hypercholesterolemia and subsequent plaque development in female mice injected with AAV8-PCSK9 (Supplemental Figure 1). These differences suggested that AAV8 tissue tropism between male and female mice, which has been well studied in the gene therapy field, may be responsible for differences in production of PCSK9 that ultimately influences hypercholesterolemia induction^{7, 8}. To determine if differences in AAV8 tissue tropism between male and female mice may be responsible for observed differences in induction of hypercholesterolemia using the AAV8-PCSK9 vector, male and female mice were retro-orbitally injected with 3×10^{10} vector genomes of AAV8-PCSK9 and placed on high-fat diet (Research Diet) for 8 weeks. The AAV8-PCSK9 dose was based on previous publications using this model of atherosclerosis^{4, 11}. Male and female mice gained weight at equivalent rates on the high-fat diet (Figure 1A). Blood was collected at the time of injection (week 0), 3 weeks after injection, and at 8 weeks (at euthanasia). Serum lipid analysis of total cholesterol (TC) content was performed on the serum samples. Male mice had significantly higher serum TC than the female mice at both 3 and 8 weeks on high-fat diet (Figure 1B). Correspondingly, male mice also had higher serum PCSK9 levels than female mice at both 3 and 8 weeks post-injection (Figure 1C). A full serum lipid analysis was performed on the blood samples collected at the time of euthanasia (8 weeks), which included analysis of TC, high-density lipoprotein (HDL), triglycerides (TG), and LDL cholesterol levels. Male mice had significantly higher serum levels of TC, HDL, TG, and LDL, compared to female mice (Figure 1D).

Surprisingly, the TC levels of the male mice in this study were much lower compared to male mice from our previous study (Supplemental Figure 1 & Figure 1). The difference between the two experiments was the high-fat diet, as the mice in Supplemental Figure 1 were fed a high-fat/high-cholesterol “Western” diet (Harlan-Teklad) and the mice in Figure 1

were fed a high-fat diet (Research Diet). To determine if diet was influencing the results, we fed the high-fat (Research Diet) diet to *Ldlr*^{-/-} mice for 8 weeks and measured TC levels. *Ldlr*^{-/-} mice on high-fat (Research Diet) diet had significantly more TC compared to their AAV8-PCSK9 injected counterparts (Supplemental Figure 2A). Regardless, diet does not appear to inhibit transduction of AAV8-PCSK9, as female mice have observed reductions in TC regardless of diet.

We next wanted to examine liver LDL receptor (LDLR) protein levels to determine how effective LDLR depletion was in the mice. There was almost no detectable LDLR protein in the livers of low-dose male mice and although protein levels were low in the low-dose female mice, the fact that it was still detectable suggests that LDLR expression is not reduced enough to result in increased TC levels (Supplemental Figure 2B). This led us to hypothesize that increasing the AAV8-PCSK9 vector dose in female mice will increase serum PCSK9 to a level that would lower liver LDLR expression enough to induce sufficient hypercholesterolemia.

Increasing viral titers induces hypercholesterolemia and PCSK9 levels in female mice.

We chose the AAV8-PCSK9 dose (3×10^{10} vector genomes) based on previous studies in this model^{4, 11}. However, a recent study reported equivalent induction of PCSK9 serum levels and TC in male and female mice when injected with 1×10^{11} vector genomes of AAV8-PCSK9, suggesting that altering the injection dose may be sufficient to induce hypercholesterolemia in female mice⁶. To test this, female mice were retro-orbitally injected with 9×10^{10} vector genomes of AAV8-PCSK9 (referred to as high-dose) which was three times more than the initial 3×10^{10} vector genome dose (referred to as low-dose). The mice were then placed on high-fat diet (Research Diet) for 8 weeks. Serum analysis demonstrated that the high-dose AAV8-PCSK9 significantly increased TC levels in the female mice to levels comparable to the low-dose males (Figure 2A). Additionally, PCSK9 levels were increased to levels comparable to the low-dose male mice (Figure 2B).

Increasing viral titers does not increase viral delivery or PCSK9 expression in the liver of female mice.

Since increasing the AAV8-PCSK9 dose rescued the serum TC and PCSK9 levels in female mice (Figure 2), we wanted to know if this was due to better viral transduction in the liver of female mice. For this experiment, both male and female mice were non-transduced or injected with either low-dose (3×10^{10} vector genomes) or high-dose (9×10^{10} vector genomes) AAV8-PCSK9. To evaluate transduction of the AAV8-PCSK9 viral vector without confounding factors, these mice were kept on a normal diet (PicoLab® Rodent Diet 20). Three weeks after injection, blood and tissues were collected for analysis. On a normal diet at 3 weeks, male low-dose mice had higher TC levels as compared to female low- and high-dose mice (Supplemental Figure 3). Similar to data shown in Figure 2, male low-dose mice had significantly more serum PCSK9 compared to female low-dose mice, but female high-dose mice rebounded to low-dose male serum PCSK9 levels (Figure 3A).

To determine if the increase in serum PCSK9 observed in female high-dose mice was due to an increase in viral transduction of the liver, we examined liver tissue for the presence of

AAV8-PCSK9 DNA. AAV8-PCSK9 vector was detected using quantitative PCR primers that target the unique liver-specific hepatic control region-apolipoprotein enhancer/alpha1-antitrypsin promoter found on the viral vector (referred to as Promoter) and primers to span exons 2 and 3 (referred to as Exon 2/3) of PCSK9. No viral DNA was detected in either male or female non-transduced control mice, demonstrating specificity of our primers to detect the virus. Male low-dose mice had significantly more detectable DNA than female low-dose mice in the liver. However, the liver DNA in female high-dose mice was not statistically different from the male low-dose mice (Figure 3B). Additionally, to determine if mRNA expression of PCSK9 was increased in the female high-dose mice, we used a primer designed for qRT-PCR for PCSK9 and evaluated PCSK9 mRNA in the liver tissue. PCSK9 mRNA levels in the liver demonstrated equivalent PCSK9 mRNA expression in the female livers regardless of dose (Figure 3C).

The observation of increased PCSK9 in the serum of high-dose female mice without a significant increase in PCSK9 mRNA led us to question the tissue tropism of AAV8-PCSK9. The gene therapy field has demonstrated that although AAV8 is strongly targeted to the abdominal cavity in male mice, AAV8 tends to localize in the extremities of female mice as well^{9, 10}. As such, we also looked in the brain, lung, and heart tissues to determine if the AAV8-PCSK9 vector was transducing alternative tissues. AAV8-PCSK9 DNA was not detectable in the brain, lung, or heart of any of the treatment groups. However, PCSK9 mRNA was detectable in the brains of all groups except the male high-dose. The levels of mRNA detected in the brain were very low, one thousand-fold lower than the male low-dose liver samples (Figure 3D). Additionally, PCSK9 mRNA was detectable in the heart tissue of the female high-dose mice at one hundred thousand-fold lower than the male low-dose liver samples (Figure 3E). PCSK9 mRNA was undetectable in the heart tissue of the remaining treatment groups, as well as the lung in all treatment groups. This highlights the possibility that cells other than the liver may be producing PCSK9.

PCSK9 protein is similarly expressed in male livers but not female livers.

Previous studies have demonstrated that in wild-type mice, the liver is the major source of circulating PCSK9 and is primarily expressed in the hepatocytes¹⁹. The AAV8-PCSK9 viral vector results in overexpression of PCSK9 which may result in ectopic expression of PCSK9 in other tissues. Since PCSK9 mRNA was detected in the brain and heart tissues of AAV8-PCSK9 injected mice, we wanted to determine if the mRNA expression was leading to protein production. Therefore, protein was collected from the liver, brain, lung, and heart tissue and analyzed by Western blot. PCSK9 protein in the liver was significantly lower in the female low-dose mice compared to the male low-dose mice, but the female high-dose levels of PCSK9 were increased in comparison to the female low-dose (Figure 4A and B). There was no statistical difference in PCSK9 protein in the lungs of any of the treatment groups (Figure A and C).

Taken together, these data suggest that although high-dose AAV8-PCSK9 in female mice restores the hypercholesterolemia phenotype, the additional PCSK9 in the serum might be produced by sources other than the liver, which is possible in both sexes as our data demonstrates.

DISCUSSION

We demonstrated that dosage of AAV-PCSK9 affects induction of hypercholesterolemia between male and female mice. A dose of 3×10^{10} vector genomes of AAV8-PCSK9 induced robust PCSK9 production and increased TC and LDL serum levels in male mice but failed to in female mice. Increasing the dose of AAV8-PCSK9 was sufficient to elicit hypercholesterolemia in female mice to levels seen in male mice. However, we were able to detect mRNA and protein in tissues besides the liver. Therefore, differences in tissue tropism of AAV8 between male and female mice may lead to production of PCSK9 in tissues besides the liver. Thus, increasing AAV8-PCSK9 dose can rescue the hypercholesterolemia phenotype in female mice similar to that observed in male mice. Increasing the AAV8-PCSK9 dose in female mice increases circulating PCSK9, but it is unclear where that additional PCSK9 protein is coming from, which may introduce confounding factors into studies that should be considered.

AAV8 was likely chosen as the vector for PCSK9 model due to studies showing AAV8 efficacious for transduction of hepatocytes^{7, 20}. However, both sex and dosage of AAV8 has been shown to alter tissue distribution and tropism^{10, 20, 21}. Low-dose AAV8 targets primarily to the liver of male mice in contrast to female mice where AAV8 is more widely distributed²⁰. To better control the localization of PCSK9 gene expression in AAV8 infected mice a liver-specific promoter (hepatic control region-apolipoprotein enhancer/alpha1-antitrypsin) was incorporated into the vector⁴. Our observation of detectable expression of PCSK9 mRNA in brains and hearts of female high-dose mice suggests the promoter is more promiscuous than previously thought. Unintended overexpression of PCSK9 in tissue other than the liver may introduce confounding factors that influence other aspects of mouse physiology. For instance, ectopic expression of PCSK9 is observed in smooth muscle cells found in atherosclerotic plaques which could contribute to disease phenotype²². PCSK9 is also known to be involved in neuronal apoptosis, which would be important to consider when using AAV8-PCSK9²³. These data highlight the delicate balance between sex, viral titer, and viral tropism. Therefore, the AAV8-PCSK9 model is a cost- and time-efficient alternative to the traditional *Apoe*^{-/-} and *Ldlr*^{-/-} models, but it is imperative to appropriately titer the virus, so the minimal amount can be used to achieve the desired phenotype and to consider the limitations of the AAV8-PCSK9 model when designing experiments.

The National Institute of Health and the Arteriosclerosis, Thrombosis and Vascular Biology Council both emphasize the importance of including both sexes in *in vitro* and *in vivo* studies^{24, 25}. The ability to utilize both male and female animals during experiments can help bridge the gap between animal and human studies, as some disease states are strongly correlated with genetic factors²⁶. The AAV8-PCSK9 model of hypercholesterolemia allows for the study of mutations without the requirement of complex and time-consuming backcrossing of mice. Our data clearly demonstrate differences between male and female mice when using the AAV8-PCSK9 model of hypercholesterolemia, which highlights the importance of titrating the appropriate dosage of virus in order to induce the correct phenotype with minimal off-target effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Hypercholesterolemia differs between male and female mice in response to AAV8-PCSK9
- AAV8-PCSK9 transduction of female mice is less effective
- High-dose AAV8-PCSK9 injection results in PCSK9 mRNA in tissues besides the liver

Figure 1.

Female mice have lower total cholesterol and serum PCSK9 levels as compared to male mice. Male and female mice were retro-orbitally injected with 3×10^{10} vector genomes of AAV8-PCSK9 and placed on high-fat diet (Research Diet) for 8 weeks. Blood was collected at the time of injection (week 0), 3 weeks, and 8 weeks after injection. Statistical analysis was performed by two-tailed student T test (** $p < 0.01$, *** $p < 0.001$). (A) Percent weight gain over 8 weeks ($n = 9-10$). (B) Plasma lipid analysis of total cholesterol over 8 weeks on high-fat diet ($n = 4-10$). (C) Analysis of PCSK9 levels in blood serum by ELISA ($n = 4-10$). (D) Plasma lipid analysis of total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), and low-density lipoprotein (LDL) after 8 weeks on high-fat diet ($n = 9-10$).

Figure 2.

Increasing the dose of AAV8-PCSK9 in female mice restores the total cholesterol levels and serum PCSK9 levels to those similar to low-dose male mice. Male and female mice were retro-orbitally injected with 3×10^{10} (low-dose) or 9×10^{10} (high-dose) vector genomes of AAV8-PCSK9 and placed on high-fat diet (Research Diet) for 8 weeks. Blood was collected at the time of injection (week 0), 3 weeks, and 8 weeks after injection. Statistical analysis was performed by one-way ANOVA with a Bonferroni post-test (** $p < 0.01$, *** $p < 0.001$). Data for male and female low-dose is the same as Figure 1. (A) Plasma lipid analysis of total cholesterol over 8 weeks on high-fat diet ($n = 3-10$). (B) Analysis of PCSK9 levels in blood serum by ELISA ($n = 3-10$).

Figure 3.

Increasing the dose of AAV8-PCSK9 in female mice restores serum PCSK9 levels but did not fully restore PCSK9 mRNA in the liver. Male and female mice were retro-orbitally injected with 3×10^{10} (low-dose) or 9×10^{10} (high-dose) vector genomes of AAV8-PCSK9 for 3 weeks on normal (PicoLab® Rodent Diet 20) diet. Blood and tissues were collected at the time of euthanasia. Liver, brain, lung, and heart tissue was collected from non-transduced, low-dose, and high-dose male and female mice. RNA and DNA were isolated and quantitative PCR was performed. DNA Primers were designed to span exons 2 and 3 of *PCSK9* (Exon 2/3) and to detect the liver-specific hepatic control region apolipoprotein enhancer/alpha 1-antitrypsin promoter (Promoter). Statistical analysis was performed by one-way ANOVA with a Bonferroni post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (A) Analysis of PCSK9 levels in blood serum by ELISA ($n = 5-17$). (B) Quantitative PCR

analysis of Promoter DNA and *PCSK9* DNA in the livers of low- and high-dose male and female mice (n = 3–8). Fold change was calculated relative to the low-dose male liver samples. (C) Quantitative RT-PCR analysis of *PCSK9* mRNA in the livers of low- and high-dose male and female mice (n = 5–11). Fold change was calculated relative to the non-transduced male liver samples. (D) Quantitative RT-PCR analysis of *PCSK9* mRNA in the brains of low- and highdose male and female mice (n = 6–10). Fold change was calculated relative to non-transduced liver samples. Male low-dose liver mRNA from Figure 3C is shown for visual comparison. (E) Quantitative RT-PCR analysis of *PCSK9* mRNA in the hearts of high-dose female mice (n = 4). Fold change was calculated relative to non-transduced liver samples. Male low-dose liver mRNA from Figure 3C is shown for visual comparison.

Figure 4.

PCSK9 protein is detectable in the liver and lungs of mice injected with AAV8- PCSK9. Male and female mice were retro-orbitally injected with 3×10^{10} (low-dose) or 9×10^{10} (high-dose) vector genomes of AAV8-PCSK9 for 3 weeks on normal (PicoLab® Rodent Diet 20) diet. Tissues were collected at the time of euthanasia. Liver, brain, lung, and heart tissue was collected from non-transduced, low-dose, and high-dose male and female mice. Protein was isolated from the tissues for Western blot analysis. Quantification was performed using Image Quant TL Toolbox. Statistical analysis was performed using a two-tailed student T-test ($*p < 0.05$). (A) Representative Western blot image of PCSK9 protein detected in the liver and lung ($n = 3-5$). PCSK9 was not detectable in the brain or the heart. (B) Graphical representation of fold change analysis of PCSK9 in the liver over non-transduced samples. (C) Graphical representation of fold change analysis of PCSK9 in the lung over non-transduced samples.