Induction of Sustained Hypercholesterolemia by Single Adeno-Associated Virus–Mediated Gene Transfer of Mutant hPCSK9

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Objectives—Patients with mutations in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene have hypercholesterolemia and are at high risk of adverse cardiovascular events. We aimed to stably express the pathological human D374Y gain-of-function mutant form of PCSK9 (PCSK9DY) in adult wild-type mice to generate a hyperlipidemic and proatherogenic animal model, achieved with a single systemic injection with adeno-associated virus (AAV).

Approach and Results—We constructed an AAV-based vector to support targeted transfer of the PCSK9DY gene to liver. After injection with 3.5×1010 viral particles, mice in the C57BL/6J, 129/SvPasCrlf, or FVB/NCrl backgrounds developed long-term hyperlipidemia with a strong increase in serum low-density lipoprotein. Macroscopic and histological analysis showed atherosclerotic lesions in the aortas of AAV-PCSK9DY mice fed a high-fat diet. Advanced lesions in these high-fat-diet–fed mice also showed evidence of macrophage infiltration and fibrous cap formation. Hepatic AAV-PCSK9DY infection did not result in liver damage or signs of immunologic response. We further tested the use of AAV-PCSK9DY to study potential genetic interaction with the ApoE gene. Histological analysis of ApoE−/− AAV-PCSK9DY mice showed a synergistic response to ApoE deficiency, with aortic lesions twice as extensive in ApoE−/− AAV-PCSK9DY-transexpressing mice as in ApoE−/− AAV-Luc controls without altering serum cholesterol levels.

Conclusions—Single intravenous AAV-PCSK9DY injection is a fast, easy, and cost-effective approach, resulting in rapid and long-term sustained hyperlipidemia and atherosclerosis. We demonstrate as a proof of concept the synergy between PCSK9DY gain-of-function and ApoE deficiency. This methodology could allow testing of the genetic interaction of several mutations without the need for complex and time-consuming backcrosses. (Arterioscler Thromb Vasc Biol. 2015;35:50-59. DOI: 10.1161/ATVBAHA.114.303617.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ PCSK9

Cardiovascular complications derived from progressive degeneration of the vascular system are expected to remain leading causes of morbidity and mortality worldwide.1 Hypercholesterolemia and associated atherosclerosis develop through interaction of complex genetic networks with environmental cues. Animal models of atherosclerosis have greatly increased our understanding of the disease and have been instrumental in the development of treatment approaches, with the apolipoprotein-E-deficient (ApoE−/−) mouse being the most widely used mouse model of atherosclerosis to date.2 The ApoE protein is synthesized in liver and macrophages and plays an important role in lipid homeostasis.3,4 As a component of plasma lipoproteins, it serves as a ligand for cell–surface receptors, such as low-density lipoprotein (LDL) receptor (LDLR) and related proteins. This interaction promotes the cellular uptake of atherogenic particles from the circulation.5 Homozygous gene deletion of ApoE or Ldlr causes severe hypercholesterolemia and spontaneous atherosclerosis.6,7 Another key regulator of lipid homeostasis is proprotein convertase subtilisin/kexin type 9 (PCSK9). Recent animal studies show that PCSK9 reduces hepatic uptake of LDL by increasing the endosomal and lysosomal degradation of LDLR,8 suggesting a possible treatment target for the nonresponsiveness of a subset of patients treated with cholesterol-lowering statins.
who maintain excessive levels of cholesterol, particularly LDL. Mice deficient for PCSK9 protein have low plasma LDL cholesterol levels and are protected against atherosclerosis development; in contrast, gain-of-function PCSK9 mutants have hypercholesterolemia and accelerated atherosclerosis generation. The most severe mutation described mutants have hypercholesterolemia and accelerated atherosclerosis generation. The most severe mutation described in PCSK9, identified in 2 populations, results in cholesterol levels above 500 mg/dL. The mutation, an amino-acid substitution of Asp374 by Tyr (D374Y), increases the affinity of PCSK9 for the LDLR by 10-fold. Further animal research is needed to increase understanding of the biology of PCSK9 in different scenarios, genetic backgrounds, and in association with lipid-altering genetic modifications. More versatile models would help to characterize the effect of different therapies targeting PCSK9.

Adeno-associated virus (AAV) vectors efficiently transduce dividing and nondividing cells, escape immune surveillance, and achieve long-term gene transfer. These features make AAV vectors a successful gene therapy approach for reverting genetic dysfunctions in preclinical models, and to date, these vectors have been tested as a tool for reverting genetic disease. However, the same rationale could be used to cause a disease, generating a model for experimental analysis, but to our knowledge, this alternative application has not been tested to date. Here, we present a method for generating a mouse model of disease by AAV injection and subsequent stable expression of a disease-causing mutation in wild-type mice, demonstrating that AAV-mediated PCSK9 gene transfer induces hyperlipidemia and subsequent atherosclerosis. This method provides a convenient system for exploring potential genetic interactions of PCSK9 and its contribution to atherosclerosis development.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Generation and Long-Term Lipid Profile of AAV-PCSK9DY Expressing Mice
The liver is the main site of lipoprotein transit and metabolism. Hepatocytes control blood LDL levels through the expression of PCSK9, the major regulator of the LDLR. Dysregulation of this pathway by gain-of-function mutations in PCSK9, such as D374Y (PCSK9DY), is linked to hypercholesterolemia and atherosclerosis. To test the effect of stable liver transexpression of this mutant on plasma lipoprotein homeostasis and atherosclerosis development in adult animals, we generated an AAV vector encoding human PCSK9DY (Figure 1A). PCSK9DY gene expression was directed to hepatocytes by driving the open reading frame from the liver-specific promoter HRC-hAAH. The AAV-PCSK9DY vector was used to encapsidate viral particles in serotype 9. A single intravenous femoral injection of 30-day-old wild-type C57BL/6J mice with 3.5×10^10 viral particles resulted in stable PCSK9DY mRNA expression in

Figure 1. A. Structure of the adeno-associated virus (AAV) vector carrying the human D374Y proprotein convertase subtilisin/kexin type 9 mutant (PCSK9DY) gene driven by the liver-specific HRC-hAAH promoter. B. Real-time PCR analysis of PCSK9 mRNA in mouse liver 110 days after injection. PCSK9DY mRNA amounts are normalized to Gapdh mRNA and are presented relative to the level in wild-type (WT) animals. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyltransferase (GGT) were measured at the same time point and determined as described in Materials and Methods. ITR indicates AAV inverted terminal repeat; and pA, polyadenylation sequence.
the liver, measured after 110 days (Figure 1B). PCSK9<sup>DY</sup> protein release to the bloodstream remained constant during the course of the experiment (Figure 1C). PCSK9<sup>DY</sup> protein also accumulated in liver samples (Figure 1D), demonstrating that this approach is a robust and reproducible method for overexpressing human PCSK9<sup>DY</sup>. AAV viral infection does not elicit any reported adverse responses in animals, and postinjection levels of serum alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transferase were similar to those of uninfected mice and mice infected with AAV9 viral particles expressing Luciferase (AAV-Luc) from a nonspecific liver promoter (Figure 1E). AAV viral infection and ectopic specific-liver expression thus does not induce hepatotoxicity in PCSK9<sup>DY</sup>-expressing animals. Consistent with these results, we did not observe changes in white cell counts 2 weeks after infection (Table I in the online-only Data Supplement), indicating that the viral transduction and extracellular transgene expression did not elicit an immunologic response in these mice.

PCSK9<sup>DY</sup> transexpression in hepatocytes increased serum cholesterol levels in overnight-fasted mice maintained on a regular chow diet at all postinjection times analyzed (Figure 2A and 2B). At 30 days post injection, total serum cholesterol (tChol) in AAV-PCSK9<sup>DY</sup> transexpressing mice was double that in AAV-Luc–injected controls (307±12 versus 130±24 mg/dL). These differences were moreover maintained 1 year after injection, demonstrating the chronic effect of a single AAV injection on systemic lipid levels. Among cholesterol fractions, LDL levels increased significantly between days 30 and 100 after PCSK9<sup>DY</sup> gene transfer (56±4.5 versus 110±6.7 mg/dL; P<0.001) and then remained stable between days 140 and 260 (114.6±8.1 and 127.6±4.6 mg/dL; Figure 2B). After 1 year, serum LDL in AAV-PCSK9<sup>DY</sup>–injected animals fed a regular chow diet was 10-fold higher than in AAV-Luc–injected controls. The AAV PCSK9 gain-of-function approach thus induces a dyslipidemia with a strong LDL component that is sustained over long periods.

To confirm the increase in LDL, we analyzed cholesterol and triglyceride distribution in serum samples by fast protein liquid chromatography. The increases in tChol and triglyceride in PCSK9<sup>DY</sup>-expressing C57BL/6J mice were because of specific accumulation of LDL and intermediate density lipoprotein (IDL), suggesting that...
PCSK9DY-mediated LDLR dysregulation induces a pre-dominant IDL/LDL hyperlipidemia (Figure 2C). Consistent with this view, analysis of liver samples revealed below-normal LDLR protein levels in AA V-PCSK9DY-transduced mice (Figure I in the online-only Data Supplement). If this abnormal LDLR degradation is the cause of the PCSK9DY-mediated lipid dysregulation, AA V-PCSK9DY injection should not induce major changes in an LDLR genetic knockout; as predicted, LDLR−/− mice injected with AA V-PCSK9DY showed no increase in total triglyceride, tChol, or IDL/LDL (Figure 2D; Figure II in the online-only Data Supplement), confirming that LDLR degradation contributes to PCSK9DY-mediated lipid dysregulation.

The in vitro binding affinity of mutant PCSK9DY to the LDLR is >10× higher than that of wild-type PCSK9. We therefore evaluated whether AA particles expressing wild-type PCSK9 were able to dysregulate LDL in vivo to a similar or lesser extent as PCSK9DY. We observed a consistent accumulation of endogenous mouse PCSK9 in serum samples of mice infected with AAV-PCSK9DY (Figure IV in the online-only Data Supplement). This result indicates that when the PCSK9DY is present, the rate of endogenous mouse PCSK9 protein turnover is slower than normal.

**High-Fat-Diet Exacerbates Hyperlipidemia in PCSK9DY-Expressing Animals**

To evaluate the response to fat intake and atherogenesis susceptibility in PCSK9DY-expressing animals, we tested the effect of high-fat-diet (HFD) on cholesterol management. Mice were injected with AAV-PCSK9DY or AAV-Luc 60 days before starting the dietary regime. At that time, mice were randomized to the HFD or standard chow for an additional 84 days. Fourteen days after HFD initiation, serum tChol in HFD-fed AA V-PCSK9DY-transexpressing mice was almost 3× higher than in similarly injected mice fed the chow diet (1165±61 versus 316±21 mg/dL; Figure 3A). Fast protein liquid chromatography revealed that the hyperlipidemic response to HFD was stronger in the very-low-density lipoprotein (VLDL) and chylomicron (CM) fractions than in the IDL/LDL fraction (Figure 3B). In contrast, diet had little significant effect on the cholesterol profile in AA V-Luc–infected mice, demonstrating that the diet-induced hyperlipidemia in AAV-PCSK9DY–infected mice is a consequence of PCSK9DY expression (Figure 3A and 3B).
notable that AA V-Luc and AA V-PCSK9DY mice maintained a baseline difference in serum tChol and LDL 60 days after virus injection, corresponding to day 0 of the dietary regime (168±2 versus 454±28 mg/dL for tChol, 3A); this difference strengthens the results shown in Figure 2. We also confirmed that expression of the PCSK9DY transgene was not suppressed in response to 84 days of HFD (Figure V in the online-only Data Supplement), consistent with results obtained in the PCSK9DY transgenic pig model.28 These data demonstrate that AA V-mediated PCSK9DY transexpression is a robust and easy methodology for generating animals that develop hyperlipidemia in response to HFD.

Hyperlipidemia can provoke the development of lesions throughout the vasculature, and this is greatly exacerbated by HFD.5–7,26 En face staining of aortas with Oil Red O revealed lesions in the thoracic aortas, aortic arches, and secondary arterial branches of all HFD-fed AA V-PCSK9DY-transduced mice but not in the vessels of similarly fed AA V-Luc mice (Figure VI in the online-only Data Supplement). These results confirm that hyperlipidemia induced by AA V-PCSK9DY transduction is a useful tool for studies of atherosclerosis. Histological analysis at the aortic sinus also revealed that lesions in fat-fed AA V-PCSK9DY mice were complex, progressing well beyond the fatty streaks seen in chow-diet-fed mice expressing the PCSK9DY gene (Figure 4A and 4B). Immunofluorescence staining for markers of macrophages (F4/80) and smooth muscle (α-smooth-muscle-actin) confirmed macrophage infiltration of the plaque and the migration of smooth muscle cells from the intima to the aortic lumen to form a fibrous cap, features of a developed plaque (Figure 4C).

**Figure 4.** Atherosclerotic lesions in C57BL/6J mice transduced with AA V-PCSK9DY (D374Y proprotein convertase subtilisin/kexin type 9 mutant) and fed a high-fat-diet (HFD). A and B, Representative staining of the aortic sinus with Masson’s trichrome and elastin (connective tissue; A) and Oil-red O (B). The elastic lamina (yellow dashed lines) is stained black. Bars, 200 μm. C, Representative immunostaining of macrophages (right) and smooth muscle cells (SMC; left) in aortic sinus lesions of AA V-PCSK9DY-transduced C57BL/6J mice fed an HFD for 84 days. Lesions were stained for biomarkers of macrophages (F4/80; red) and SMCs (α-actinin; green); nuclei were stained with DAPI (blue). Merged images are also shown. AAV indicates adeno-associated virus; C, collagen in lesions; L, lipids; M, tunica media; and I, tunica intima.

AAV-Mediated PCSK9DY Expression Induces Hyperlipidemia and Atherosclerosis in Different Genetic Backgrounds

To test the potential of AA V-PCSK9DY for generating hyperlipidemic animals with different genetic backgrounds, we compared the responses of C57BL/6J, 129/SvPasCrlf, and FVB/NCrl mice. A single intravenous injection of 3.5×10¹⁰ AAV-PCSK9DY virus altered cholesterol homeostasis in all 3 lineages as measured at 30 days post-AAV injection. Liver PCSK9DY transexpression led to significant increases in tChol (68%, 36%, and 40%, respectively) with concomitant increases in LDL (152%, 70%, and 138%). Changes in cholesterol fractions remained stable for 60 days (Table), when the mice were randomized for feeding with HFD or standard chow. All 3 mouse strains showed an HFD-dependent increase in serum lipoprotein levels (Table).

After killing, mice were analyzed for fast protein liquid chromatography lipid profile (Figure VII in the online-only Data Supplement), liver PCSK9DY content, and serum protein accumulation (Figure VIII in the online-only Data Supplement). Aortic atherosclerotic lesions were analyzed...
by Masson’s tricrome and Oil Red O-staining (Figure 5A and 5B). Quantification of the cross-sectional area of plaques at the level of the aortic sinus confirmed larger plaques in the atherosclerosis-susceptible C57BL/6J strain than in 129/SvPasCrlf mice (0.112±0.027 versus 0.007±0.003 mm²) and FVB/NCrl mice, where lesions and foam cells were almost absent (Figure 5C and Ref. 31). Chow-fed AAV-PCSK9DY C57BL/6J mice did not show the notable basal lesion development seen in HFD-fed mice. Although the overall pattern of lipoprotein changes was seen across all 3 mouse strains, with a diet-induced increase in the IDL/LDL fraction, there were differences suggestive of differing susceptibility to atherosclerosis, consistent with the reported influence of genetic background on atherosclerosis in ApoE−/−.32,33

**Atherosclerotic Lesion Development in ApoE−/− and PCSK9−/−-Expressing Mice**

PCSK9 induces posttranslational downregulation of hepatic LDLR by diverting recycling LDLR into the endosomal–lysosomal pathway, leading to degradation.8,24 The hypercholesterolemic phenotype of transgenic mice overexpressing wild-type or mutant PCSK925,26 therefore resembles that of LDLR−/− mice. We therefore hypothesized that AAV-PCSK9−/− transduction in an ApoE−/− background would partially recapitulate features of the ApoE−/− LDLR−/− double knockout1 and ApoE−/− PCSK9 transgenic mice. We first compared serum lipoprotein levels in fasted ApoE−/− mice transduced with AAV-Luc or AAV-PCSK9−/− and maintained on a chow diet (Figure 6A and 6B). Consistent with previous findings in ApoE−/− PCSK9 transgenic mice,12 at 120 days postinjection, lipoprotein levels in AAV-PCSK9−/− transduced ApoE−/− mice did not differ significantly from the levels in the ApoE−/− AAV-Luc controls fed the same diet (Figure 6A). In both AAV groups, lipoprotein levels increased markedly in response to HFD. Over the 84-day dietary regime, ApoE−/− AAV-PCSK9−/− showed higher lipoprotein levels than ApoE−/− AAV-Luc mice, but the difference was statistically significant only at 14 days (743±32 versus 1074±89 mg/dL for tChol). Fast protein liquid chromatography lipoprotein analysis showed that AAV-Luc and AAV-PCSK9−/− mice on an ApoE−/− genetic background and maintained on a chow diet accumulated mainly VLDL/CM lipoproteins. This analysis also confirmed that mice transduced with AAV-PCSK9−/− accumulated more IDL/LDL lipoproteins and that the hyperlipidemic response to HFD was stronger in the VLDL/CM fractions than in the IDL/LDL fraction (Figure 6B).

We then compared plaque size in C57BL/6J and ApoE−/− mice injected with AAV-Luc or AAV-PCSK9−/− particles (Figure 7A). ApoE-deficient mice on a chow diet spontaneously develop atheroma plaques at a young age,6 whereas appearance of lesions in LDLR-deficient or PCSK9-transgenic mice requires a longer period or HFD.12,35 Quantification of plaque cross-sectional area in slices at the level of the aortic sinus (Figure 7B) confirmed that only ApoE−/− mice develop plaques on a chow diet, with plaques being significantly larger in AAV-PCSK9−/−-transduced ApoE−/− mice than AAV-Luc. After 84 days on the HFD, lesions were observed in C57BL/6J and ApoE−/− mice transduced with AAV-PCSK9−/− (0.35±0.03 and 0.71±0.15 mm²) and in ApoE−/− transduced with AAV-Luc (0.36±0.02 mm²). 

**Table. Overnight-Fasted Serum Levels of tChol and LDL (mg/dL) in C57BL/6J, 129/SvPasCrif, and FVB/NCrl Mice Injected With AAV-Luc (control) or AAV-PCSK9−/− Before the Analysis and Fed Standard Chow Diet or HFD for the Indicated Times**

<table>
<thead>
<tr>
<th>Time</th>
<th>FVB/NCrl</th>
<th>129/SvPasCrif</th>
<th>C57BL/6J</th>
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<tr>
<td></td>
<td>AAV-Luc</td>
<td>AAV-PCSK9−/−</td>
<td>AAV-Luc</td>
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<tr>
<td>tChol</td>
<td></td>
<td></td>
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<tr>
<td>Chow diet</td>
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<tr>
<td>4 weeks</td>
<td>204.12±6.38</td>
<td>337.1±7.12*</td>
<td>135.07±17.06</td>
</tr>
<tr>
<td>8 weeks</td>
<td>232.87±7.21</td>
<td>326.18±13.34*</td>
<td>203.66±11.45</td>
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<tr>
<td>HFD diet</td>
<td></td>
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<tr>
<td>12 weeks</td>
<td>260.1±37.73</td>
<td>381.2±32.44*</td>
<td>226.08±19.86</td>
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<tr>
<td>24 weeks</td>
<td>359.9±23.40</td>
<td>524.28±47.30*</td>
<td>156.08±16.15</td>
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<td>LDL</td>
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<tr>
<td>Chow diet</td>
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<tr>
<td>4 weeks</td>
<td>19.26±1.66</td>
<td>53.07±8.20*</td>
<td>9.41±1.17</td>
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<td>8 weeks</td>
<td>24.41±6.65</td>
<td>50.49±4.70*</td>
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<td>HFD diet</td>
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<tr>
<td>12 weeks</td>
<td>35.89±4.54</td>
<td>77.15±4.97*</td>
<td>28.69±0.81</td>
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<tr>
<td>24 weeks</td>
<td>44.95±6.00</td>
<td>142.65±38.41*</td>
<td>13.49±1.87</td>
</tr>
</tbody>
</table>

AAV indicates adeno-associated virus; ANOVA, analysis of variance; HFD, high-fat diet; LDL, low-density lipoprotein; and tChol, total cholesterol.

\*P<0.001 versus AAV-PCSK9−/− by 2-way ANOVA followed by Bonferroni post test; means±SEM; n=4–6.
AAV-PCSK9DY-transduced ApoE−/− mice at least double the size of those in single mutants on the same diet (Figure 7B), without significantly altering serum cholesterol levels (Figure 6).

Discussion

Our data demonstrate that AAV-mediated long-term gain-of-function of human PCSK9 in mice provides a versatile model of dyslipidemia and atherosclerosis, established with a single intravenous injection. Recombinant AAV vectors support long-term transgene expression in many animal models and humans.22,36,37 Highly attractive features of AAV vectors include their tropism for postmitotic as well as mitotic cells, their intracellular genetic stabilization as predominantly non-integrated DNA, and their low immunogenicity.38 One major advantage of AAV-mediated transexpression is its robust stability after a single administration. The clear association between hyperlipidemia and atherosclerotic lesion development in the AAV-PCSK9DY model could be easily used to test genetic interactions in combination with new transgenic or knockout models without the need for tedious, costly, and time-consuming backcrosses, as we have demonstrated here with the ApoE−/− mice.

PCSK9 binds to the low-density lipid receptor family members LDLR, VLDLR, and apolipoprotein receptor-2 (ApoER2)39 and targets them for degradation.40 It is possible that the increase in VLDL/CM fractions observed in AAV-PCSK9DY-infected mice when fed the HFD, together with the minor change in the IDL/LDL fractions, could be mediated by these last 2 receptors. Furthermore, VLDLR knockout mice have normal plasma lipoprotein levels when fed a chow diet.41 However, in HFD, these mice show a slight increase in circulating triglyceride,42,43 similar to what is observed in HFD-fed PCSK9DY-transduced mice.

The hypercholesterolemia model presented here is based on the expression of a pathological variant of human PCSK9 in wild-type animals. Expression of PCSK9DY induced the accumulation of endogenous PCSK9 protein without any evident change in mRNA levels. We propose that this alteration might be induced by the sequestration of LDLR by mutant PCSK9DY, rendering it unable to interact with mouse PCSK9. The endogenous PCSK9 would therefore not be recycled and degraded, increasing its total serum levels (Figure IV in the online-only Data Supplement).

AAV-PCSK9 expression induces only a modest increase in IDL/LDL levels (Figure III in the online-only Data Supplement), contrasting with the strong effect of AAV-PCSK9DY in wild-type C57BL/6J mice fed standard chow or HFD (Figure 3C). Nonetheless, AAV-PCSK9DY-injected mice do not develop plaques spontaneously on the chow diet.
and presented extensive lesions only when fed the HFD. In ApoE−/− mice fed a regular chow diet, AA V-PCSK9DY transduction has a marginal effect on serum lipoprotein levels, despite the doubling of plaque size compared with single ApoE mutants. A similar doubling of plaque size is seen in AA V-PCSK9DY–injected ApoE−/− mice fed the HFD. Our data suggest that ApoE is a key factor in atheroma plaque development and clearly uncouples lipid cholesterol levels from plaque size measured at the aortic root.

Our long-term results demonstrate induction of persistent hyperlipidemia over 1 year follow-up. AAV-mediated liver-specific PCSK9D74Y transfer induced hyperlipidemia in 100% of injected animals, with no hepatotoxicity or signs of inflammatory response activation. These data are consistent with several ongoing or completed phase I/II clinical trials that show an absence of adverse hepatic events. These data suggest that the AA V–PCSK9D74Y transfer strategy is a robust approach for inducing stable liver-specific expression.

Because the link between gain-of-function mutations in PCSK9 and autosomal dominant hypercholesterolemia was made 10 years ago, drug-development strategies for hypercholesterolemia have targeted PCSK9. These strategies involve either reducing PCSK9 production or blocking circulating PCSK9 with neutralizing antibodies. A major advantage of our AAV-injection model is that it is easily applicable in any genetic background in a cost-effective manner. This feature makes this system an ideal inhibitor test platform for PCSK9 in multiple genetic contexts.

In summary, we think that AAV transfer methodology has the potential to make valuable contributions to the specific understanding of hyperlipidemia and atherosclerosis and to disease modeling in general. The ability to transexpress human disease–causing mutated genes in a tissue-specific manner in wild-type mice obviates the need for complex backcrosses, nonphysiological gene mutations, and the maintenance of large colonies of genetically modified animals. Moreover, the requirement for small numbers of readily available wild-type animals fits with public concerns and the minimal-use concept expressed in the 3 Rs (3Rs) principle for the rational use of animals in research: Replacement, referring to the use of nonanimal methods; Reduction, referring to the use of fewer animals to obtain comparable information; and Refinement, referring to methods that alleviate or minimize potential suffering or distress. We further envision that the AA V-based approach for gene transfer described here is suitable for general use in studies for which expression of any given gain-of-function transgene induces a disease phenotype and is especially applicable to the generation of disease models in larger animals.
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Disclosures

None.

References

Hypercholesterolemia and associated atherosclerosis are leading causes of morbidity and mortality worldwide. Animal models of atherosclerosis are essential investigative tools for expanding our understanding of the disease; however, the generation and maintenance of genetically modified mouse colonies for research is costly. We have developed an alternative method that uses adenovirus vectors, widely used for gene therapy approaches, to express the disease-causing dominant-negative PCSK9 mutant to generate a model of hyperlipidemia and atherosclerosis in wild-type mice. Single systemic injection of AAV-PCSK9DY virus is more versatile, cost-effective, simpler, and time-efficient than transgenic approaches for generating hypercholesterolemic animals. These data suggest that AAV-PCSK9DY can be used as an alternative platform for testing specific PCSK9-targeted therapies and demonstrate that adenovirus-based vector methodologies have the potential to make valuable contributions to the specific understanding of hyperlipidemia and atherosclerosis and to disease modeling in general.
Induction of Sustained Hypercholesterolemia by Single Adeno-Associated Virus–Mediated Gene Transfer of Mutant hPCSK9
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Supplement Material

Supplementary Figure I. Liver LDLR protein levels analyzed by western blot and normalized to β-actin in C57BL/6J mice transduced with AAV-Luc or AAV-PCSK9DY.

Supplementary Figure II. (A) Serum levels of total cholesterol (tChol) and (B) triglycerides (TG) in LDLR−/− mice transduced with AAV-Luc or AAV-PCSK9DY. Blood was obtained after overnight fasting. ns, p>0.05 by unpaired Student’s t test (n = 4-5). Each data point denotes an individual mouse, horizontal bars denote mean values and SEM.

Supplementary Figure III. (A) Serum levels of human PCSK9 protein in mice injected with PCSK9 virus. C57BL/6J, AAV Luc-injected control mice. (n=5-8; ***p< 0.001, unpaired Student’s t test). (B) Fast protein liquid chromatography (FPLC) analysis of total cholesterol (tChol) in pooled serum samples from overnight-fasted C57BL/6J transduced with AAV-Luc, AAV-PCSK9DY or AAV-PCSK9 (n=4-6). IDL/LDL: intermediate density lipoprotein; CM/VLDL: chylomicron and very low-density lipoprotein; HDL: high-density lipoprotein.

Supplementary Figure IV. Serum levels of endogenous mouse PCSK9 protein in mice injected with PCSK9DY virus. C57BL/6J, AAV Luc-injected control mice. (n=5-8; ***p< 0.001, unpaired Student’s t test). Each data point denotes an individual mouse, horizontal red bars denote mean values, and black bars denote SEM.

Supplementary Figure V. Real-time PCR analysis of PCSK9DY mRNA in livers of HFD-fed AAV-transduced mice. PCSK9DY mRNA amounts are normalized to Gapdh mRNA and are expressed in arbitrary units (AU) relative to the level in control animals and expressed (n=5-6; ***p< 0.001, unpaired Student’s t test). Each data point denotes an individual mouse, horizontal red bars denote mean values, and black bars denote SEM.

Supplementary Figure VI. (A) The diagram shows the sinus section plane (red line) and the aortic anatomy examined in histological studies. (B) Representative en face Oil Red O staining of aortas from AAV-PCSK9DY and AAV-Luc mice maintained on HFD for 84 days.

Supplementary Figure VII. FPLC cholesterol profile in pooled samples from overnight-fasted C57BL6/J, 129/SvPasCrlf and FVB/NCrl mice transduced with AAV-Luc or AAV-PCSK9DY.

Supplementary Figure VIII. (A,B) Real-time PCR analysis in liver samples of mRNA expression of (A) PCSK9DY mRNA (hPCSK9) and (B) endogenous mouse PCSK9 mRNA. PCSK9 mRNA amounts are normalized to Gapdh mRNA and are expressed in arbitrary units (AU) relative to the level in non-transduced animals. (C) Serum levels of PCSK9DY protein measured by ELISA in C57BL6/J, 129/SvPasCrlf and FVB/NCrl mice 120 days after transduction with AAV-PCSK9DY.

Supplementary Table I. Effect of AAV-PCSK9DY injection on white blood cell counts in C57Bl6/J mice (n=5, mean±SEM).
**Supplementary Figure I.** Liver LDLR protein levels analyzed by western blot and normalized to β-actin in C57BL/6J mice transduced with AAV-Luc or AAV-PCSK9DY.

**Supplementary Figure II.** (A) Serum levels of total cholesterol (tChol) and (B) triglycerides (TG) in LDLR-l- mice transduced with AAV-Luc or AAV-PCSK9DY. Blood was obtained after overnight fasting. ns, \( p > 0.05 \) by unpaired Student’s t test (n = 4-5). Each data point denotes an individual mouse, horizontal bars denote mean values and SEM.
Supplementary Figure III. (A) Serum levels of human PCSK9 protein in mice injected with PCSK9 virus. C57BL/6J, AAV Luc-injected control mice. (n=5-8; **p<0.001, unpaired Student’s t test). (B) Fast protein liquid chromatography (FPLC) analysis of total cholesterol (TC) in pooled serum samples from overnight-fasted C57BL/6J transduced with AAV-Luc, AAV-PCSK9 or AAV-PCSK9 (n=4-6). IDL/LDL: intermediate density lipoprotein; CM/VLDL: chylomicron and very low-density lipoprotein; HDL: high-density lipoprotein.
Supplementary Figure IV. Serum levels of endogenous mouse PCSK9 protein in mice injected with PCSK9DY virus. C57BL/6J, AAV Luc-injected control mice. (n=5-8; ***p<0.001, unpaired Student’s t test).
**Supplementary Figure V.** Real-time PCR analysis of PCSK9DY mRNA in livers from HFD fed mice. PCSK9DY mRNA amounts are normalized to Gapdh mRNA and are presented relative to the level in control animals expressed as arbitrary units (AU). (n=5-6 ***p< 0.001, unpaired Student's t test). Each data point denotes an individual mouse, horizontal red bars denote mean values, and black bars denote SEM.
Supplementary Figure VI. (A) The diagram shows the sinus section (red line) and the aorta taken for histological studies. (B) Representative Oil Red O-stained en face aortas from AAV-PCSK9DY and AAV-Luc mice maintained on HFD for 84 days.
**Supplementary Figure VII.** Cholesterol FPLC profile of pooled samples from overnight fasted C57BL6/J, 129/SvPasCrlf and FVB/NCrI mice transduced with AAV-Luc or AAV-PCSK9DY.
Supplementary Figure VIII. (A) Real-time PCR analysis of PCSK9DY and (B) endogenous mouse PCSK9 mRNA in liver samples. PCSK9DY mRNA amounts are normalized to Gapdh mRNA and are presented relative to the level in WT animals and expressed as arbitrary units (AU). (C) Serum levels of PCSK9DY protein by ELISA in C57BL/6J, 129/SvPasCrlf and FVB/NCrI mice transduced with AAV-Luc or AAV-PCS9DY at the end of the experiment.
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**Supplementary Table1**

Effect after 4 weeks of AAV-PCSK9\textsuperscript{DY} injection on white blood cells in C57Bl6/J (n=5, mean±SEM). On the left are shown the average values for wild-type animals (n=100) and normal range.
Materials and Methods

AAV vector production and purification. AAV vectors were all produced by the triple transfection method using HEK 293A cells. AAV plasmids were cloned and propagated in the Stbl3 E. coli strain (Life Technologies). Shuttle plasmid pAAV-PCSK9<sup>DY</sup> was derived from pAcTnT (a gift from Dr B.A. French, University of Virginia, USA) and packaged into capsids from AAV-9, using helper plasmids ρAdDF6 (providing the three adenoviral helper genes) and plasmid pAAV2/9 (providing rep and cap viral genes), obtained from PennVector. Shuttle vector pAAV-PCSK9<sup>DY</sup> was generated by direct cloning of the Psil-BgIII fragment from pSB-D374Y-PCSK9 (a gift from Dr JA Bentzon, Aarhus University, Denmark) into pAcTnT cut with BamHI and Eco47III. This fragment contains the PCSK9<sup>DY</sup> ORF under the liver-specific promoter HCR-hAAH<sup>2</sup>.

The AAV shuttle and helper plasmids were transfected into HEK 293A cells by calcium-phosphate co-precipitation. A total of 840µg plasmid DNA (mixed in an equimolar ratio) was used per Hyperflask (Corning) seeded with 1.2x10<sup>8</sup> cells the day before. Seventytwo hours after transfection, the cells were collected by centrifugation and the cell pellet was resuspended in TMS (50 mmol/L Tris HCl, 150 mmol/L NaCl, 2 mmol/L Na<sub>2</sub>Mg) on ice before digestion with DNase I and RNaseA (0.1 mg/mL each; Roche) at 37 °C for 60 minutes. Clarified supernatant containing the viral particles was obtained by iodixanol gradient centrifugation<sup>3</sup>. Gradient fractions containing virus were concentrated using Amicon UltraCel columns (Millipore) and stored at -70°C.

Determination of AAV vector titer. Titers for the AAV vectors (viral genomes per ml) were determined by quantitative real-time PCR as described<sup>4</sup>.

The following primers were used: PCSK9F, 5′ AGCTGGTGCTAGCCTTGCGTTC 3′; PCSK9R, 5′ AGACATGCAGGATCTTGAG 3′; HCRpromF, 5′ CATCCTCAGCAGCTGTGGTG 3′; HCRpromR 5′ CTCCTCCTCCAGGAAATTCCAG3′.

Known copy numbers (10<sup>5</sup>–10<sup>6</sup>) of the respective plasmid (pAAV-HRC-hAAT-PCSK9<sup>DY</sup>) carrying the appropriate complementary DNA were used to construct standard curves.

Animals and diets. Wild-type mice and homozygous ApoE-deficient mice (ApoE<sup>-/-</sup>), both on the C57BL/6J genetic background, originated from Jackson Laboratories (Charles River Laboratories). FVB/NCrI and 129/SvPasCrIff were obtained from Charles River Laboratories. Adult males at different time points were used for metabolic analysis. Mice were individually housed in wire-bottomed cages in a temperature-controlled room (22±0.8°C) with a 12 h light–dark cycle and a relative humidity of 55±10%. The mice had free access to food and water. Mice were fed a low-fat standard rodent diet (reference 2014, Teklad global rat/mouse chow, Harlan Interfauna). When indicated, 60-day-old mice were switched to a high-fat diet containing 0.75% cholesterol (reference S8492-E010, Ssniff), and analyzed for atheroma plaque formation after 84 days. All animals were maintained and handled according to the recommendations of the CNIC Institutional Ethics Committee.

AAV injection. Thirty-day-old mice were anesthetized with 100µl of ketamine (60 mg/kg), xylazine (20 mg/kg) and atropine (9mg/kg) via the intraperitoneal route. Once asleep, animals were located on a heated pad at 37±0.5°C to prevent hypothermia. A small incision (4mm) was made in the skin to reveal the right femoral vein. To increase vessel diameter and facilitate infusion, blood flow was interrupted with a cotton bud for a couple of seconds. Once the vein was dilated, and insulin syringe vessel was introduced into the vein and 3.5x10<sup>10</sup> virus particles were inoculated in a final volume of
50µL, taking care to prevent introduction of air bubbles. Animals were then analgesized with buprenorphine (S.C., 0.1 mg/kg) and maintained on the heating pad until recovery. Paracetamol was administered orally for 1 week.

**Serum analysis.** Overnight-fasted serum levels of triglyceride (TG), total cholesterol (tChol), low-density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate transaminases (AST) and gamma-glutamyl transferase (GGT) were assayed with a Dimension RxL Max HM clinical chemistry system from SIEMENS. Lipid amounts are shown in milligram per deciliter (mg/dl) and enzyme activities in units per liter (U/L).

**Serum lipoprotein fractionation and analysis.** For lipoprotein fractionation analysis, equal volumes of plasma samples from each group were pooled. Lipoproteins were fractionated using a Superose 6 10/300 GL fast-performance liquid chromatography (FPLC) column in an AKTApurifier P900 apparatus. Fractions were collected and used for lipid measurement.

**Determination of hematological parameters.** The Horiba ABX Pentra 80 Diagnostics system (ABX pentra Montpellier, France) was used to determine hematological parameters, including white blood cells (WBC), neutrophils, monocytes, lymphocytes, eosinophils, and basophils.

**Histological analysis and immunostaining.** Mouse hearts and aortas were perfused with PBS, removed, fixed in 4% paraformaldehyde for 24 h, incubated 24h in PBS supplemented with 30% sucrose and embedded in OCT and cryopreserved at -70°C. Cryocut cross-sections (5 µm) were then prepared. Samples were blocked for 30 min with 10% horse serum plus 2% BSA (for immunofluorescence) in PBS. Cryocut cross-sections were stained with Oil Red O (0.5% in isopropanol) or Masson’s trichrome stain. Images were acquired using an Olympus BX51 microscope fitted with 10x or 20x UPlanSApo objectives and Cell Sens Entry.Ink acquisition software. Immunofluorescence samples were stained with rat anti-mouse F4/80 (MCA497R; AbD Serptec) and Cy3-conjugated mouse anti-smooth muscle actin (SMA) (C6198; Sigma-Aldrich). Images were acquired using an Olympus BX51 microscope fitted with 10x or 20x UPlanSApo objectives and Cell Sens Entry.Ink acquisition software. The secondary antibody for immunofluorescence was Alexa Fluor® 633 goat anti-rat IgG (A-21094; Invitrogen). Nuclei were stained with DAPI (excitation 405 nm, emission 420-475 nm). Immunofluorescence images were acquired using an inverted confocal microscope (Carl Zeiss Axio Imager Z2, Apotome.2) fitted with 20x or 40x HCX PL Apo oil immersion objectives and ZEN acquisition software. Images were analyzed using ImageJ (http://rsbweb.nih.gov/ij/index.html) and were processed for presentation with Adobe Photoshop.

**Quantitative real-time PCR.** Total RNA was isolated from mouse livers using the Direct-zol RNA Miniprep Kit (Zymo) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The complementary DNAs were then used for real time PCR using the Power SYBR® Green PCR Master Mix (Applied Biosystems). Amplification, detection and data analysis were done with an ABI PRISM® 7900HT Sequence Detection System. The crossing threshold values for individual mRNAs were normalized to Gapdh. Changes in the expression of mRNA were expressed as the fold change relative to the control. We used the following primers in this study. Gapdh: GapdhF, 5’ TTGATGGCAACAATCTCCAC 3’; GapdhR, 5’ GTCCCGTAGACAAAAATGGT 3’. Human PCSK9F and PCSK9R were as shown above. Endogenous mouse PCSK9: mPCSK9F, 5’ TGGAAGACCTTAGTGTCCGGAGGCAG 3’; mPCSK9R, 5’ GTCCACGCTGTAGGCTCCAGAGTGTA 3’.
Statistics. The number of mice chosen for each experiment was the minimum needed to provide sufficient statistical power. No animals were excluded from the analysis. Data were analyzed by one-way ANOVA with Tukey’s multiple comparison test, Student’s t test or two-way ANOVA followed by Bonferroni post-test. Error bars represent SEM. In all corresponding figures, * represents p< 0.05, ** represents p< 0.01, *** represents p< 0.001, and ns represents p> 0.05.

References: