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* (*PCSK9^{DY}*) 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 *PCSK9^{DY}* gene to liver. After injection with 3.5×10^{10} 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-*PCSK9^{DY}* 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-*PCSK9^{DY}* infection did not result in liver damage or signs of immunologic response. We further tested the use of AAV-*PCSK9^{DY}* to study potential genetic interaction with the *ApoE* gene. Histological analysis of *ApoE$^{-/-}$* AAV-*PCSK9^{DY}* mice showed a synergistic response to *ApoE* deficiency, with aortic lesions twice as extensive in *ApoE$^{-/-}$* AAV-*PCSK9^{DY}*-transexpressing mice as in *ApoE$^{-/-}$* AAV-*Luc* controls without altering serum cholesterol levels.

Conclusions—Single intravenous AAV-*PCSK9^{DY}* 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 *PCSK9^{DY}* 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 synthetized 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,

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Nonstandard Abbreviations and Acronyms

AAV	adeno-associated virus
ApoE	apolipoprotein E
CM	chylomicron
IDL	intermediate density lipoprotein
LDL	low-density lipoprotein
LDLR	LDL receptor
PCSK9	proprotein convertase subtilisin/kexin type 9
PCSK9^{DY}	D374Y PCSK9 mutant
tChol	total cholesterol
TG	triglyceride
VLDL	very low-density lipoprotein

who maintain excessive levels of cholesterol, particularly LDL.[9–11] Mice deficient for PCSK9 protein have low plasma LDL cholesterol levels and are protected against atherosclerosis development[12]; in contrast, gain-of-function *PCSK9* mutants[13] have hypercholesterolemia and accelerated atherosclerosis generation.[14–16] The most severe mutation described in *PCSK9*, identified in 2 populations,[10,17] 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.[18] 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.[19,20] These features make AAV vectors a successful gene therapy approach for reverting genetic dysfunctions in preclinical models,[21,22] 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-*PCSK9^{DY}* 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.[23,24] Dysregulation of this pathway by gain-of-function mutations in *PCSK9*, such as D374Y[25,26] (PCSK9^{DY}), is linked to hypercholesterolemia and atherosclerosis.[27–29] 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 PCSK9^{DY} (Figure 1A). *PCSK9^{DY}* gene expression was directed to hepatocytes by driving the open reading frame from the liver-specific promoter HCR-hAAH.[30] The AAV-*PCSK9^{DY}* 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 *PCSK9^{DY}* mRNA expression in

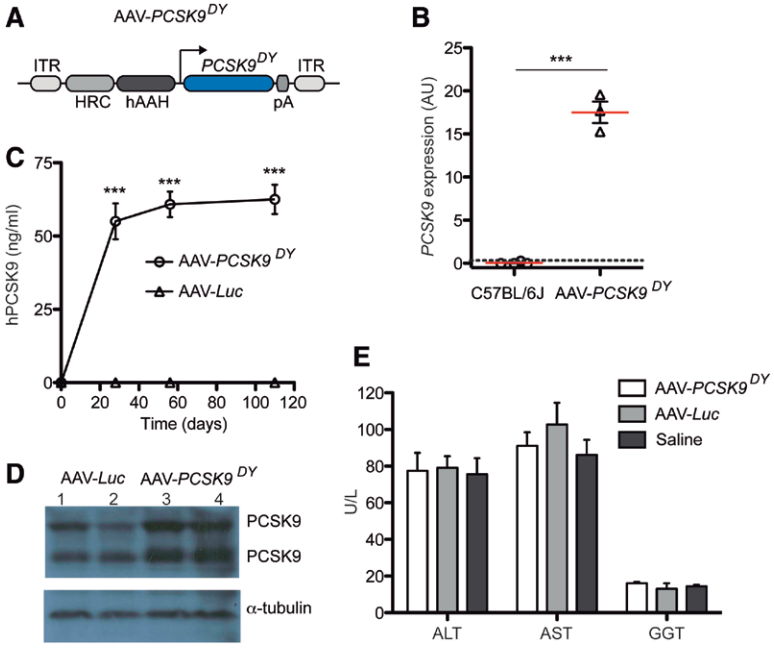

Figure 1. A, Structure of the adeno-associated virus (AAV) vector carrying the human D374Y proprotein convertase subtilisin/kexin type 9 mutant (*PCSK9^{DY}*) gene driven by the liver-specific *HRC-hAAT* promoter. **B**, Real-time PCR analysis of *PCSK9^{DY}* mRNA in mouse liver 110 days after injection. *PCSK9^{DY}* mRNA amounts are normalized to *Gapdh* mRNA and are presented relative to the level in wild-type (WT) animals (n=3–4; ***P<0.001, unpaired Student t test). Each data point denotes an individual mouse, horizontal red bars denote mean values, and black bars denote SEM. **C**, Serum levels of human PCSK9 protein in mice injected with *PCSK9^{DY}* virus. C57BL/6J, AAV *Luc*–injected control mice (n=5–8; ***P<0.001, unpaired Student t test). **D**, Liver PCSK9 protein levels analyzed by western blot and normalized to α-tubulin. Mice 1 and 2 were injected with AAV *Luc* control, mice 3 and 4 with AAV-*PCSK9^{DY}*. **E**, 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, poly-adenylation sequence.

the liver, measured after 110 days (Figure 1B). PCSK9^{DY} protein release to the bloodstream remained constant during the course of the experiment (Figure 1C). PCSK9^{DY} protein also accumulated in liver samples (Figure 1D), demonstrating that this approach is a robust and reproducible method for overexpressing human *PCSK9^{DY}*. 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^{DY}*-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^{DY} 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^{DY}* 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^{DY}* 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^{DY}*–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^{DY}*-expressing C57BL/6J mice were because of specific accumulation of LDL and intermediate density lipoprotein (IDL), suggesting that

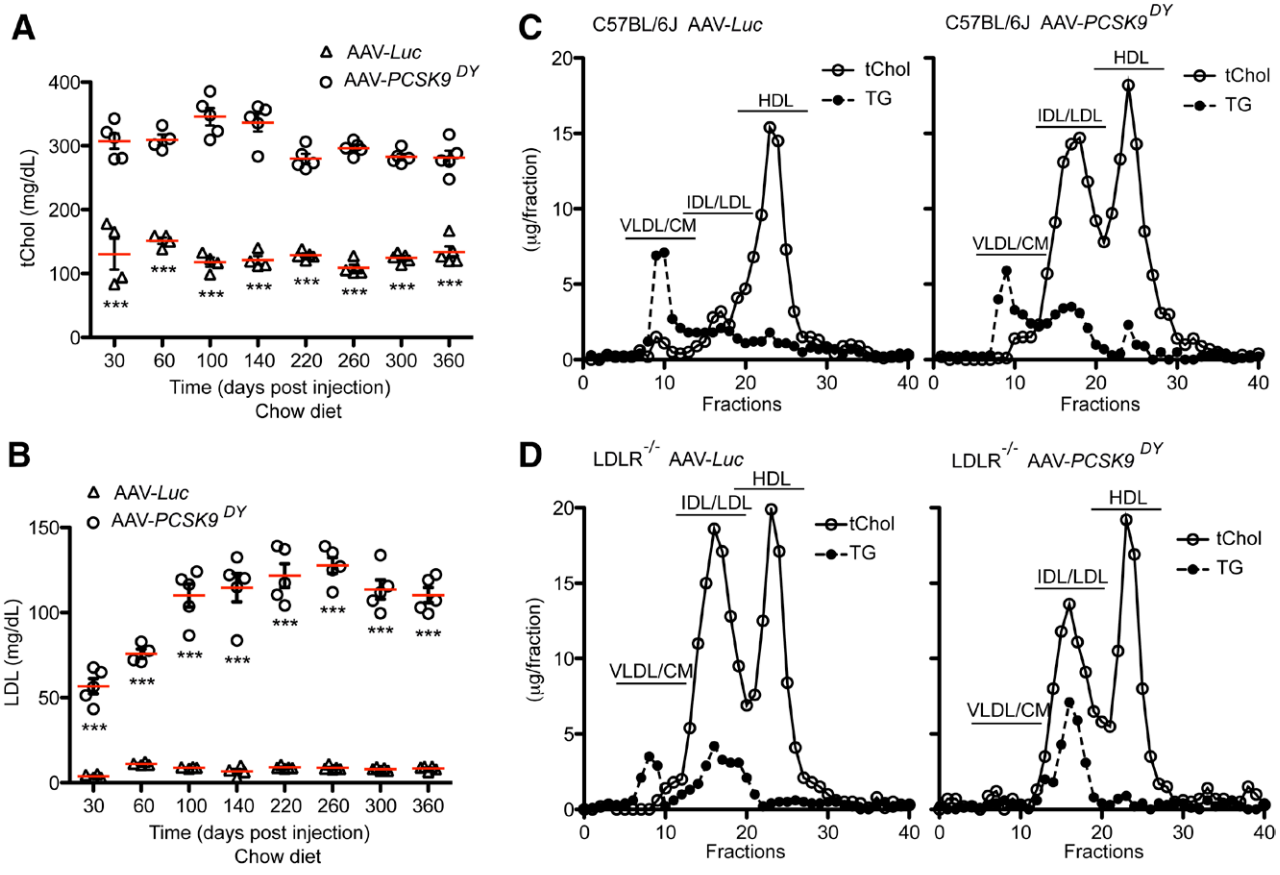

Figure 2. Serum levels of total cholesterol (tChol; **A**) and low-density lipoprotein (LDL; **B**) in control (AAV [adeno-associated virus] *Luc*–injected) C57BL/6J mice and AAV-*PCSK9^{DY}* (D374Y proprotein convertase subtilisin/kexin type 9 mutant) mice. Blood was obtained after overnight fasting. ***$P<0.001$ and ns, $P>0.05$ by 2-way ANOVA followed by Bonferroni post-test (n=4–5). Each data point denotes an individual mouse, horizontal red bars denote mean values, and black bars denote SEM. **C** and **D**, Fast protein liquid chromatography (FPLC) analysis of tChol and triglyceride (TG) in pooled serum samples from overnight-fasted C57BL/6J and *LDLR$^{-/-}$* mice transduced with AAV-*Luc* or AAV-*PCSK9^{DY}* (n=4–6). CM/VLDL indicates chylomicron and very low-density lipoprotein; HDL, high-density lipoprotein; IDL/LDL, intermediate density lipoprotein.

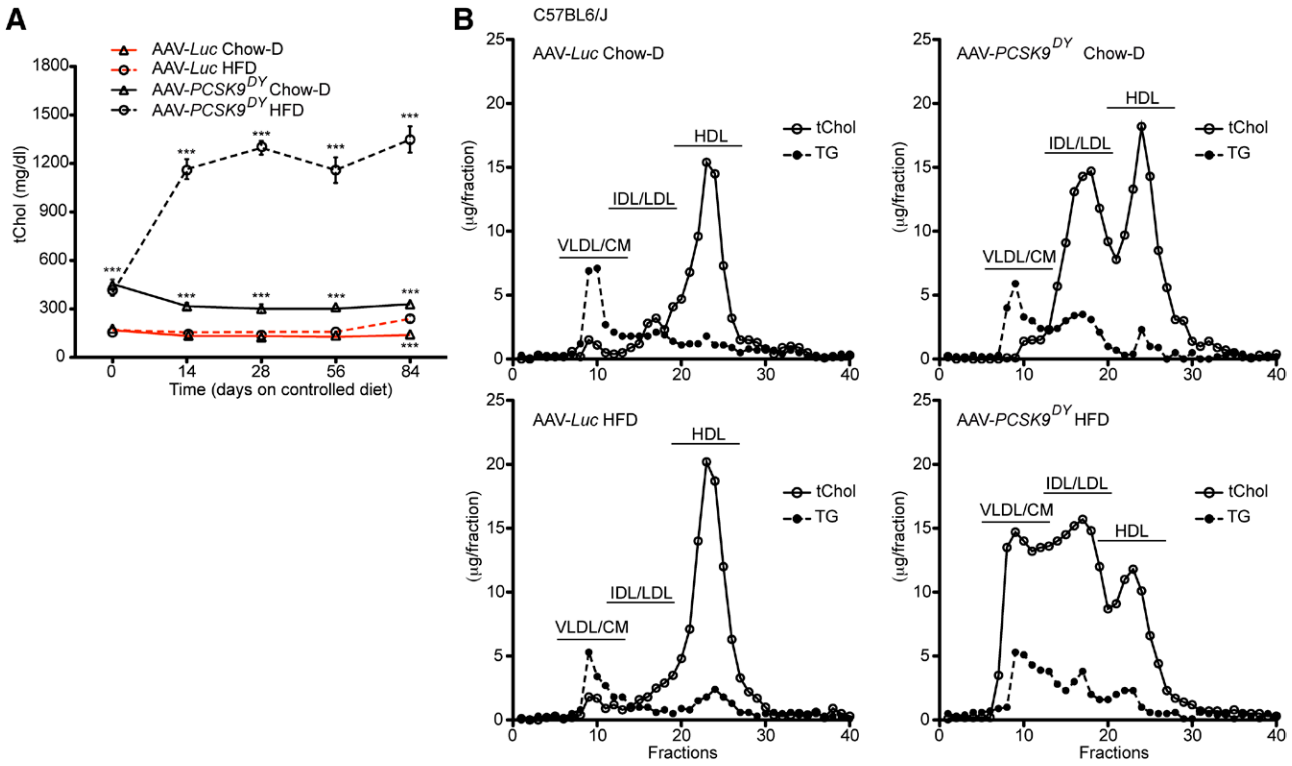

Figure 3. A, Serum levels (mg/dL) of total cholesterol (tChol) in C57BL/6J mice transduced with AAV-*Luc* or AAV-*PCSK9*^{DY} (D374Y proprotein convertase subtilisin/kexin type 9 mutant) 60 days before starting the analysis (time 0). Mice were maintained for the indicated periods on chow or high-fat-diet (HFD). Blood was obtained after overnight fasting. ***P<0.001; *P<0.05; ns, P>0.05 versus AAV-*Luc* or AAV-*PCSK9*^{DY} on chow diet (2-way ANOVA followed by Bonferroni post test; means±SEM, n=5–7). **B**, FPLC profile of tChol and triglyceride (TG) in pooled samples at the end of the experiment. AAV indicates adeno-associated virus; CM/VLDL, chylomicron and very low-density lipoprotein; HDL, high-density lipoprotein; IDL/LDL, intermediate density lipoprotein.

PCSK9^{DY}-mediated LDLR dysregulation induces a predominant IDL/LDL hyperlipidemia (Figure 2C). Consistent with this view, analysis of liver samples revealed below-normal LDLR protein levels in AAV-*PCSK9*^{DY}-transduced mice (Figure I in the online-only Data Supplement). If this abnormal LDLR degradation is the cause of the PCSK9^{DY}-induced hyperlipidemia, AAV-*PCSK9*^{DY} injection should not induce major changes in an LDLR genetic knockout; as predicted, LDLR$^{-/-}$ mice injected with AAV-*PCSK9*^{DY} 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 *PCSK9*^{DY}-mediated lipid dysregulation.

The in vitro binding affinity of mutant PCSK9^{DY} to the LDLR is >10× higher than that of wild-type PCSK9. We therefore evaluated whether AAV particles expressing wild-type PCSK9 were able to dysregulate LDL in vivo to a similar or lesser extent as *PCSK9*^{DY}. We observed that AAV-*PCSK9*-infected C57BL/6J mice had significantly lower levels of IDL/LDL lipoproteins in serum samples than AAV-*PCSK9*^{DY}-injected mice (Figure III in the online-only Data Supplement). This difference could explain why individuals expressing *PCSK9*^{DY} at similar levels to wild-type PCSK9 can develop severe dyslipidemia.

We also studied the effect of *PCSK9*^{DY} expression on the endogenous mRNA and protein levels of PCSK9. Although we did not detect significant differences at the transcriptional level, we observed a consistent accumulation of endogenous mouse PCSK9 in serum samples of mice infected with AAV-*PCSK9*^{DY} (Figure IV in the online-only Data Supplement). This result indicates that when the PCSK9^{DY} is present, the rate of endogenous mouse PCSK9 protein turnover is slower than normal.

High-Fat-Diet Exacerbates Hyperlipidemia in *PCSK9*^{DY}-Expressing Animals

To evaluate the response to fat intake and atherogenesis susceptibility in *PCSK9*^{DY}-expressing animals, we tested the effect of high-fat-diet (HFD) on cholesterol management. Mice were injected with AAV-*PCSK9*^{DY} 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 AAV-*PCSK9*^{DY}-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 AAV-*Luc*–infected mice, demonstrating that the diet-induced hyperlipidemia in AAV-*PCSK9*^{DY}–infected mice is a consequence of *PCSK9*^{DY} expression (Figure 3A and 3B). It is also

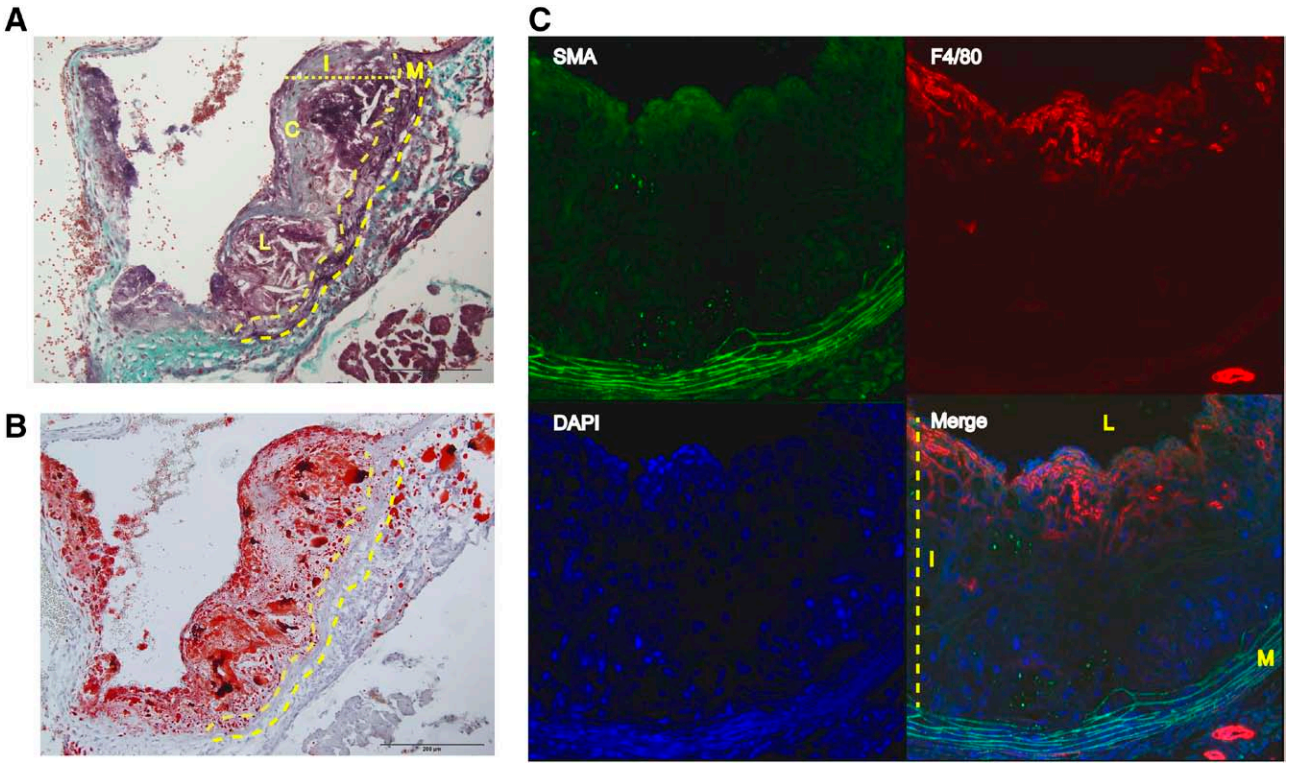

Figure 4. Atherosclerotic lesions in C57BL/6J mice transduced with AAV-*PCSK9*^{DY} (D374Y proprotein convertase subtilisin/kexin type 9 mutant) and fed a high-fat-diet (HFD). **A** and **B**, Representative staining of the aortic sinus with Masson's trichrome and elastin (connective tissue; **A**) and 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 AAV-*PCSK9*^{DY}-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.

notable that AAV-*Luc* and AAV-*PCSK9*^{DY} 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 *PCSK9*^{DY} 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 *PCSK9*^{DY} transgenic pig model.[28] These data demonstrate that AAV-mediated *PCSK9*^{DY} 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 AAV-*PCSK9*^{DY}-transduced mice but not in the vessels of similarly fed AAV-*Luc* mice (Figure VI in the online-only Data Supplement). These results confirm that hyperlipidemia induced by AAV-*PCSK9*^{DY} transduction is a useful tool for studies of atherosclerosis. Histological analysis at the aortic sinus also revealed that lesions in fat-fed AAV-*PCSK9*^{DY} mice were complex, progressing well beyond the fatty streaks seen in chow-diet-fed mice expressing the *PCSK9*^{DY} 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).

AAV-Mediated *PCSK9*^{DY} Expression Induces Hyperlipidemia and Atherosclerosis in Different Genetic Backgrounds

To test the potential of AAV-*PCSK9*^{DY} 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 3.5×10^{10} AAV-*PCSK9*^{DY} virus altered cholesterol homeostasis in all 3 lineages as measured at 30 days post-AAV injection. Liver *PCSK9*^{DY} 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 PCSK9^{DY} 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 mm^2) and FVB/NCrl mice, where lesions and foam cells were almost absent (Figure 5C and Ref. 31). Chow-fed AAV-*PCSK9^{DY}* 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^{DY}*-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[34] or mutant *PCSK9*[26] therefore resembles that of *LDLR$^{-/-}$* mice. We therefore hypothesized that AAV-*PCSK9^{DY}* transduction in an *ApoE$^{-/-}$* background would partially recapitulate features of the *ApoE$^{-/-}$ LDLR$^{-/-}$* double knockout[5] and *ApoE$^{-/-}$ PCSK9* transgenic[12] mice. We first compared serum lipoprotein levels in fasted *ApoE$^{-/-}$* mice transduced with AAV-*Luc* or AAV-*PCSK9^{DY}* 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^{DY}*-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^{DY}* 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^{DY}* 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^{DY}* 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^{DY}* 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^{DY}*-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^{DY}* (0.35±0.03 and 0.71±0.15 mm^2) and in *ApoE$^{-/-}$* transduced with AAV-*Luc* (0.36±0.02 mm^2). *En face* Oil Red O staining of aortas revealed lesions in the aortas and aortic branches (brachiocephalic, left common carotid, and left subclavian artery) of HFD-fed AAV-*PCSK9^{DY}* mice and AAV-*Luc*–transduced *ApoE$^{-/-}$* mice (Figure 7C). As predicted,[12] combined PCSK9^{DY} expression and ApoE deficiency had a synergistic effect, with lesions in

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

	FVB/NCrl		129/SvPasCrif		C57BL/6J	
Time	AAV-*Luc*	AAV-*PCSK9^{DY}*	AAV-*Luc*	AAV-*PCSK9^{DY}*	AAV-*Luc*	AAV-*PCSK9^{DY}*
tChol						
Chow diet						
4 weeks	204.12±6.38	337.1±7.12*	135.07±17.06	206.4±16.68	128±8.60	258.5±19.80
8 weeks	232.87±7.21	326.18±13.34*	203.66±11.45	281.72±16.02	163.38±32.90	274.02±7.87
HFD diet						
12 weeks	260.1±37.73	381.2±32.44*	226.08±19.86	317.62±25.22	225.76±2.59	321.58±17.02
24 weeks	359.9±23.40	524.28±47.30*	156.08±16.15	370.28±43.94	175.98±8.70	388.22±69.53
LDL						
Chow diet						
4 weeks	19.26±1.66	53.07±8.20*	9.41±1.17	20.02±5.00	10.88±1.76	51.38±3.62
8 weeks	24.41±6.65	50.49±4.70*	24.89±1.00	42.5±7.03	23.58±1.25	58.53±3.94
HFD diet						
12 weeks	35.89±4.54	77.15±4.97*	28.69±0.81	56.38±14.57	29.52±1.55	78.1±6.85
24 weeks	44.95±6.00	142.65±38.41*	13.49±1.87	108.05±32.01	22.21±2.97	145.97±47.84

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^{DY}* by 2-way ANOVA followed by Bonferroni post test; means±SEM; n=4–6.

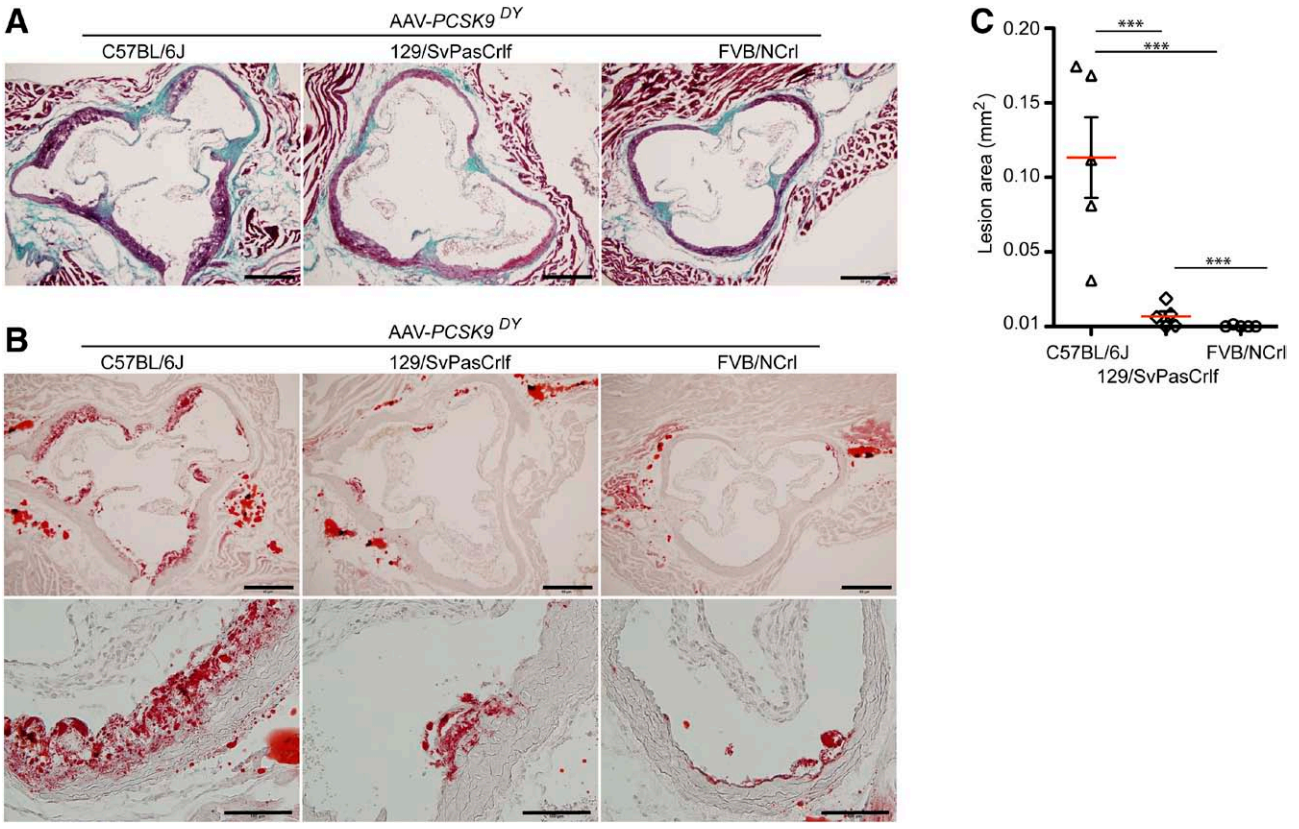

Figure 5. Representative Masson's trichrome (**A**) and Oil Red O (**B**) staining in aortic root sections from high-fat-diet (HFD)–fed AAV-*PCSK9*^{DY} (D374Y proprotein convertase subtilisin/kexin type 9 mutant)-transduced C57BL/6J, 129/SvPasCrlf, and FVB/NCrl mice. Scale bars, 100 μm (**A** and top row in **B**) and 50 μm (bottom row of magnified views in **B**). **C**, Quantitative analysis of atherosclerotic lesion size in Oil Red O–stained aortic sections from **B**. AAV indicates adeno-associated virus.

AAV-*PCSK9*^{DY}–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[22,36,37] and humans.[20] 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-*PCSK9*^{DY} 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-*PCSK9*^{DY}-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 *PCSK9*^{DY}-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 PCSK9^{DY} 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 PCSK9^{DY}, 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-*PCSK9*^{DY} in wild-type C57BL/6J mice fed standard chow or HFD (Figure 3C). Nonetheless, AAV-*PCSK9*^{DY}–injected mice do not develop plaques spontaneously on the chow diet

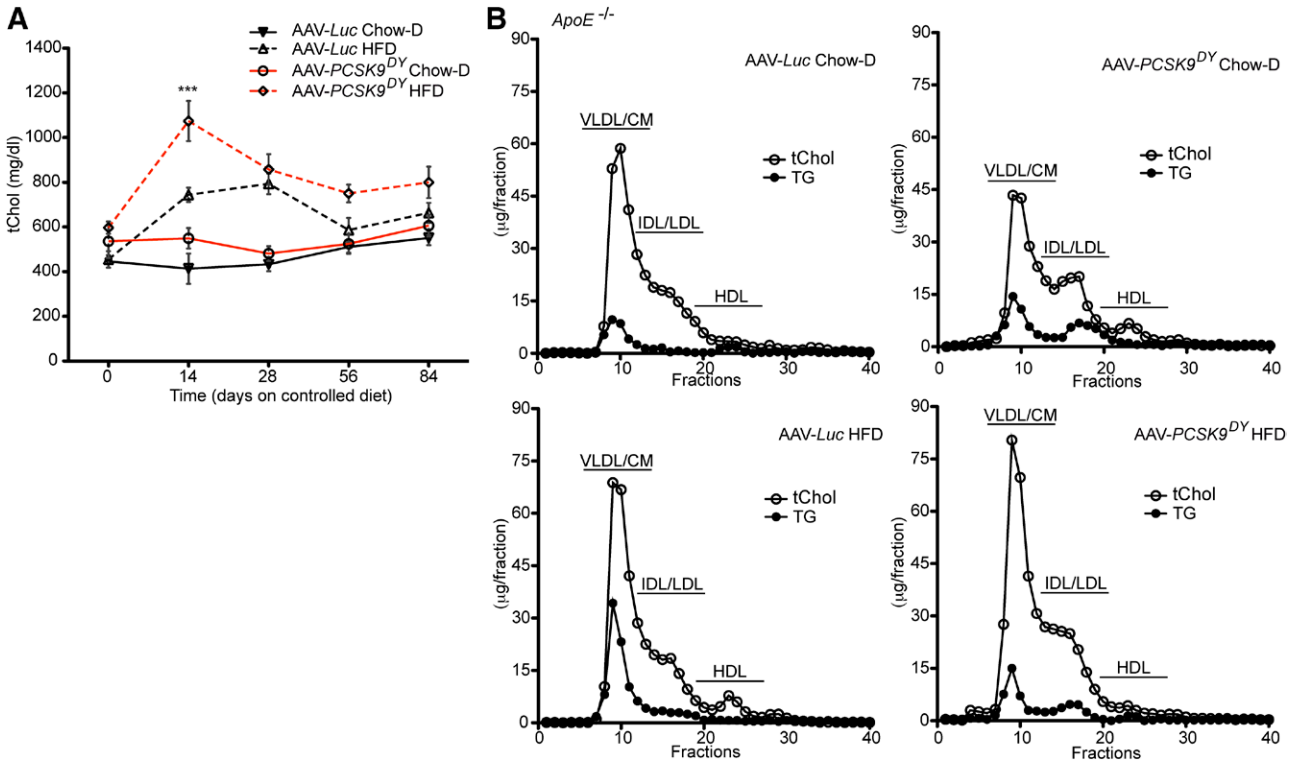

Figure 6. A, Serum levels (mg/dL) of total cholesterol (tChol) in *ApoE*$^{-/-}$ mice transduced with AAV-*Luc* or AAV-*PCSK9*^{DY} (D374Y proprotein convertase subtilisin/kexin type 9 mutant) 30 days before starting the analysis (time 0). Mice were maintained for the indicated periods on chow or high-fat-diet (HFD). Blood was obtained after overnight fasting. ***P<0.001; ns, P>0.05 versus AAV-*Luc* or AAV-*PCSK9*^{DY} on chow diet (2-way ANOVA followed by Bonferroni post test; means±SEM, n=5–7). **B**, Fast protein liquid chromatography (FPLC) profile of tChol and triglyceride (TG) in pooled samples at the end of the experiment. AAV indicates adeno-associated virus; CM/VLDL, chylomicron and very low-density lipoprotein; HDL, high-density lipoprotein; IDL/LDL, intermediate density lipoprotein.

and presented extensive lesions only when fed the HFD. In *ApoE*$^{-/-}$ mice fed a regular chow diet, AAV-*PCSK9*^{DY} 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 AAV-*PCSK9*^{DY}–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 *PCSK9*^{DY} 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.[20,44] These data suggest that the AAV-*PCSK9*^{DY} 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.[45] These strategies involve either reducing PCSK9 production or blocking circulating PCSK9 with neutralizing antibodies.[46–49] 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 AAV-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.

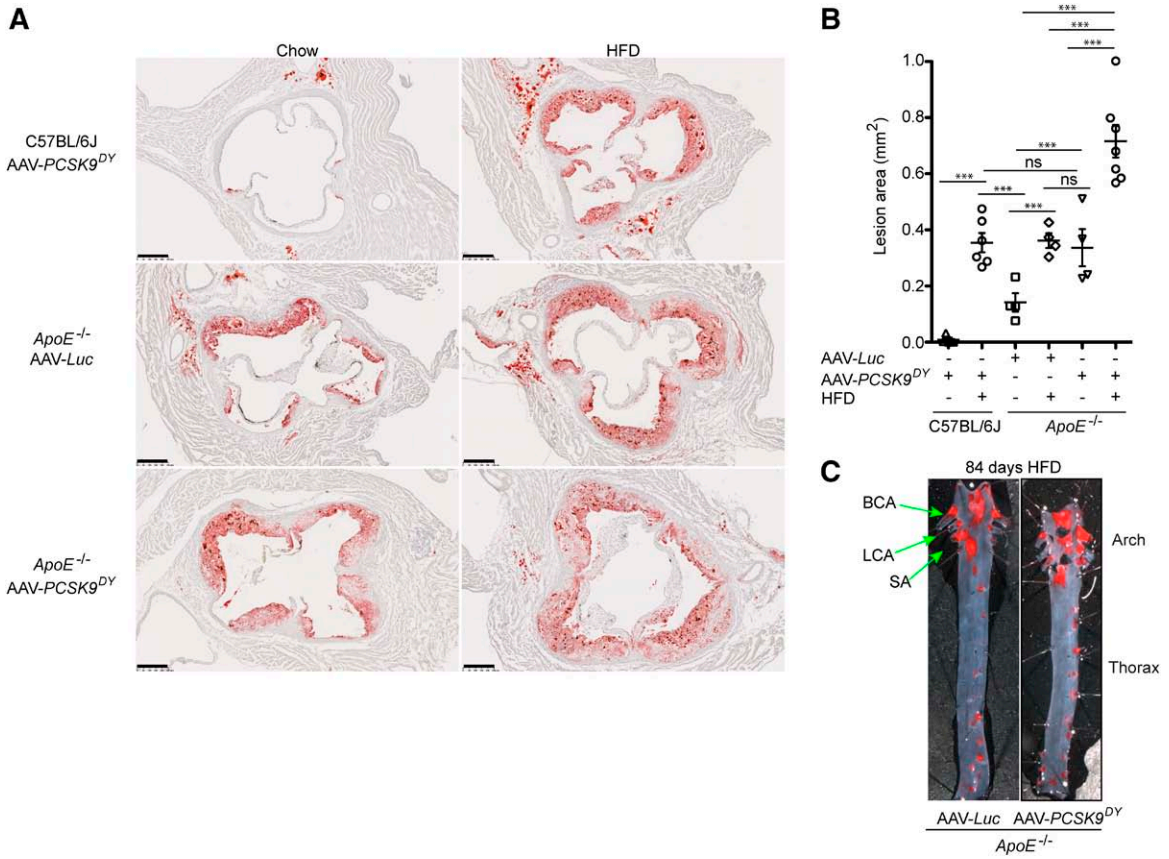

Figure 7. A, Representative Oil Red O–stained images from AAV-transduced C57BL/6J and *ApoE*$^{-/-}$ mice maintained on high-fat-diet (HFD) or chow diet for 84 days. Scale bars, 250 μm. **B**, Quantitative analysis of atherosclerotic lesion size in Oil Red O–stained aortic sections from **A**. ***P<0.001; ns, P>0.05 by 1-way ANOVA with Tukey multiple comparison test (n=4–7). Each data point denotes an aortic sinus section from an individual mouse, horizontal red bars denote mean values, and black bars denote SEM. **C**, Representative *en face* Oil Red O–staining of aortas from *ApoE*$^{-/-}$ mice injected with AAV-*PCSK9*^{DY} (D374Y proprotein convertase subtilisin/kexin type 9 mutant) or AAV-*Luc* and maintained on an HFD for 84 days. BCA indicates brachicephalic artery; LCA, left carotid common artery; and SA, subclavian artery.

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Disclosures

None.

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Significance

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 adeno-associated virus vectors, widely used for gene therapy approaches, to express the disease-causing dominant-negative PCSK9^{DY} mutant to generate a model of hyperlipidemia and atherosclerosis in wild-type mice. Single systemic injection of AAV-*PCSK9*^{DY} virus is more versatile, cost-effective, simpler, and time-efficient than transgenic approaches for generating hypercholesterolemic animals. These data suggest AAV-*PCSK9*^{DY}-transformed mice could become an advantageous platform for testing specific PCSK9-targeted therapies and demonstrate that adeno-associated virus–transfer methodology has the potential to make valuable contributions to the specific understanding of hyperlipidemia and atherosclerosis and to disease modeling in general.