New Methods in Cardiovascular Biology

Induction of Atherosclerosis in Mice and Hamsters Without Germline Genetic Engineering

Martin Mæng Bjørklund, Anne Kruse Hollensen, Mette Kallestrup Hagensen, Frederik Dagnæs-Hansen, Christina Christoffersen, Jacob Giehm Mikkelsen, Jacob Fog Bentzon

Rationale: Atherosclerosis can be achieved in animals by germline genetic engineering, leading to hypercholesterolemia, but such models are constrained to few species and strains, and they are difficult to combine with other powerful techniques involving genetic manipulation or variation.

Objective: To develop a method for induction of atherosclerosis without germline genetic engineering.

Methods and Results: Recombinant adeno-associated viral vectors were engineered to encode gain-of-function proprotein convertase subtilisin/kexin type 9 mutants, and mice were given a single intravenous vector injection followed by high-fat diet feeding. Plasma proprotein convertase subtilisin/kexin type 9 and total cholesterol increased rapidly and were maintained at high levels, and after 12 weeks, mice had atherosclerotic lesions in the aorta. Histology of the aortic root showed progression of lesions to the fibroatheromatous stage. To demonstrate the applicability of this method for rapid analysis of the atherosclerosis susceptibility of a mouse strain and for providing temporal control over disease induction, we demonstrated the accelerated atherosclerosis of mature diabetic Akita mice. Furthermore, the versatility of this approach for creating atherosclerosis models also in nonmurine species was demonstrated by inducing hypercholesterolemia and early atherosclerosis in Golden Syrian hamsters.

Conclusions: Single injections of proprotein convertase subtilisin/kexin type 9–encoding recombinant adeno-associated viral vectors are a rapid and versatile method to induce atherosclerosis in animals. This method should prove useful for experiments that are high-throughput or involve genetic techniques, strains, or species that do not combine well with current genetically engineered models. (Circ Res. 2014;114:1684-1689.)

Key Words: atherosclerosis • models, animal

Germline genetically engineered animals, mainly Apoe<sup>−/−</sup> or Ldlr<sup>−/−</sup> mice, have become the preferred models for studying atherosclerosis. However, several powerful genetic techniques are impractical to combine with these models because of the extensive amount of breeding required. These include conditional and inducible knockouts of candidate disease genes, high-throughput gene targeting, mapping of susceptibility genes in complex mouse crosses, and analysis of the atherosclerosis-modulating effect of polygenic traits, such as accelerated aging or type 2 diabetes mellitus. Furthermore, some aspects of human physiology may be better modeled in other small animals than mice.

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D374Y-hPCSK9; Online Figure 1). For both vectors, expression of PCSK9 was driven by an efficient liver-specific promoter. Viral vectors in serotype 8 capsids (rAAV8-D377Y-mPCSK9 and rAAV8-D374Y-hPCSK9) were produced by the UNC Vector Core (Chapel Hill, NC). Female C57BL/6NTac mice were from Taconic, female Ldlr−/− mice (B6.129S7-Ldlrtm1Her/J) from Jackson Laboratories, and male Golden Syrian Hamsters from Harlan Laboratories. Breeding pairs of C57BL/6-Jlnr<sup>−/−</sup> and C57BL/6J mice were from Jackson Laboratories, and male offspring were used for experiments. The Danish Animal Experiments Inspectorate approved all experiments.

**Experimental Design**

Viral vectors were delivered via a single tail vein injection in mice and a single intraperitoneal injection in hamsters, and animals were

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**Figure 1. Hypercholesterolemia induced by recombinant adeno-associated virus (rAAV) 8-D377Y-murine proprotein convertase subtilisin/kexin type 9 (mPCSK9).** A, Mouse injected with rAAV8-CMV-Luc (left) or saline (right) and analyzed for bioluminescence. B, Plasma mPCSK9 in mice injected with the rAAV8-D377Y-mPCSK9 vector and fed Western-type diet (n=4 for all groups). ***P<0.0001 for dose–response relationship among vector-injected groups (ANOVA for area under the curve with post-test for linear trend). C, Hepatic low-density lipoprotein receptor (LDLR) levels in different groups normalized to β-actin. Plasma total cholesterol on Paigen diet– (D) and Western-type diet–fed (E) mice (n=4 for all groups, except for Ldlr<sup>−/−</sup> n=5). **P<0.01 and ***P<0.001 for dose–response relationship among vector-injected groups.
Figure 2. Atherosclerosis induced by recombinant adeno-associated virus (rAAV) 8-D377Y-murine proprotein convertase subtilisin/kexin type 9 (mPCSK9). A, Atherosclerosis in the aortic arch (n=4 for all groups, except for Ldr−− n=5). *P<0.05 and **P<0.01 for dose–response relationship (ANOVA with post-test for linear trend). B, Aortic arches from Ldr−− (top left), high-dose rAAV8-D377Y-mPCSK9–injected (top right), and control mice (bottom left) on Western-type diet. Atherosclerosis in a high-dose rAAV8-D377Y-mPCSK9–injected mouse on Paigen diet is also shown (bottom right; scale bar, 1 mm). C, Aortic root lesion area in Western-type diet–fed mice. D, Plaque morphology in Western-type diet–fed Ldr−− (left) and high-dose rAAV8-D377Y-mPCSK9–injected (right) mice (scale bars, 500 μm). E, Necrotic core area in Western-type diet–fed mice. F, Analysis with Sirius Red staining (left, collagen red) and immunofluorescence for smooth muscle α-actin (right, SMCs red) showing necrotic core formation and SMC accumulation (arrows; scale bars, 100 μm).
challenged with either Western-type diet containing 21% fat and 0.21% cholesterol or cholate-supplemented Paigen diet containing 16% fat, 1.25% cholesterol, and 0.5% sodium cholate.

Aortic arch atherosclerotic lesion area (down to the supreme intercostal artery) was quantified en face after Oil Red O staining. Plaque morphology was examined in aortic root cross sections.

**Results**

**Hypercholesterolemia Induced by PCSK9 Gene Transfer**

Hydrodynamic injections of naked D377Y-mPCSK9– and D374Y-hPCSK9–encoding plasmids, a nonviral gene transfer approach, caused transient increase of plasma mPCSK9 and hPCSK9, respectively, in C57BL/6NTac mice (n=4 in each group; Online Figure IIA and IIB). This resulted in a temporally shifted phase of hypercholesterolemia (Online Figure IIC and IID), which, however, was not maintained long enough to cause atherosclerosis.

Tissue tropism for AAV serotype 8 was visualized in C57BL/6NTac mice by bioluminescence using the luciferase reporter gene (rAAV8-CMV-Luc, 5.0×10^{11} vector genomes). High levels of expression were seen in the upper abdomen consistent with efficient liver transduction (Figure 1A). rAAV8-CMV-Luc transduction did not increase plasma cholesterol levels, alanine aminotransferase activity, or serum amyloid A compared with saline-injected control mice (n=5 in each group; Online Figure IIIA–IIIC).

To achieve long-term hypercholesterolemia, C57BL/6NTac mice, 8 weeks of age, were given rAAV8-D377Y-mPCSK9 or rAAV8-D374Y-hPCSK9 at 3 different dosages (2.0×10^{10}, 1.0×10^{11}, or 5.0×10^{11} vector genomes) and fed either Western-type or Paigen diet. Ldlr^−/− and saline-injected control mice were treated similarly. All mice thrived well (Online Table I).

D377Y-mPCSK9 increased rapidly, peaking at day 56, and was stably maintained at high levels (Figure 1B). This resulted in strong reduction of hepatic LDL receptor levels (Figure 1C) and significant dose-dependent hypercholesterolemia on both diets (Figure 1D and 1E). As expected, cholesterol levels were higher on Paigen diet. Results with the rAAV8-D374Y-hPCSK9 vector were similar (Online Figure IV).

Size-exclusion chromatography showed that low-dose rAAV8-D374Y-PCSK9 vector injection caused an isolated increase in LDL-sized lipoproteins on Western-type diet, whereas...
more very-low-density lipoprotein (VLDL)-sized lipoproteins were seen at higher D374Y-hPCSK9 levels and in Ldlr−/− mice (Online Figure V). On Paigen diet, most plasma cholesterol was carried in VLDL-sized lipoproteins in all groups.

Plasma alanine aminotransferase activity and serum amyloid A were increased similarly in vector-injected and Ldlr−/− groups compared with controls (Online Figure VI), indicating no hepatocellular injury or systemic inflammation caused by rAAV8 transduction alone.

**Atherosclerosis Induced by PCSK9 Gene Transfer**

Mice were euthanized 12 weeks after rAAV8 vector injection to quantify atherosclerosis (Figure 2A). Controls had no or minimal lesions on either diet, whereas all rAAV8-injected mice developed atherosclerosis in a dose-dependent manner (Figure 2B). Similar results were seen with the rAAV8-D374Y-hPCSK9 vector (Online Figure VII).

Histological analysis of aortic root lesions showed advanced plaque development with foam cells, smooth muscle cells, and fibrous tissue (Figure 2C–2F). All, except 1 rAAV8-D377Y-mPCSK9–injected (high dose) and 1 Ldlr−/− mouse, exhibited necrotic core formation.

**Applications of the Method**

To show how the method can be used to examine the atherosclerosis susceptibility of a mouse strain without intercrossing with Apoe−/− or Ldlr−/− mice, we injected rAAV8-D377Y-mPCSK9 (1.0×10^{11} vector genomes) in 26-week-old diabetic Akita mice with the Ins2^Akita mutation and analyzed atherosclerosis after 10 weeks on Western-type diet. Consistent with previous studies in the Ldlr−/− background,10 we found augmented hypercholesterolemia and atherosclerosis compared with wild-type littermates (Figure 3). In this particular example, the effect of diabetes mellitus may have been underestimated because the level of plasma mPCSK9 was found to be lower in diabetic mice compared with controls (21 300 versus 56 000 ng/mL; n=4 both groups; P<0.03; Mann–Whitney). This will probably rarely be the case but serves to illustrate that analyzing for potential differences in the PCSK9 level obtained in groups is important when applying the atherosclerosis-inducing vector.

To provide an example of how the method can be used to create atherosclerosis models in nonmurine species, we injected Golden Syrian hamsters with a single intraperitoneal dose of the rAAV8-D374Y-hPCSK9 vector at 10 weeks of age (1.2×10^{12} vector genomes). After 10 days, D374Y-hPCSK9 was efficiently expressed in plasma and the level stayed elevated (>2000 ng/mL) throughout the study (Online Figure VIII). Marked hypercholesterolemia developed on Western-type diet (Figure 4A) with most cholesterol carried in VLDL- and LDL-sized lipoproteins (Figure 4B). After 12 weeks, all rAAV8-injected hamsters had scattered lesions in the aortic arch covering 3% to 6% of the surface area, whereas saline-injected controls showed no or minimal lesion development (Figure 4C and 4D). Histology of the aortic arch revealed foam cell lesions (Figure 4E).

**Figure 4. Hypercholesterolemia and atherosclerosis in hamsters.** A, Plasma total cholesterol in recombinant adeno-associated virus (rAAV) 8-D374Y-human proprotein convertase subtilisin/kexin type 9 (hPCSK9)–injected and control hamsters (n=4 in each group). *P<0.05 (Mann–Whitney test for area under the curve). B, Nonfasting cholesterol fast protein liquid chromatography profiles of pooled plasma samples from the 2 groups. C, Aortic arch atherosclerosis. *P<0.05 (Mann–Whitney test). D, Aortic arches from control (left) and vector-injected (right) hamsters (scale bar, 2 mm). E, Section marked in D showing foam cell lesion. Arrows mark the internal elastic lamina (scale bar, 50 μm). HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.
Discussion
Our experiments show that a single injection of PCSK9-encoding rAAV8 vectors is sufficient to induce atherosclerosis in mice and hamsters when combined with a cholate-free Western-type diet. This method should prove useful for high-throughput experiments or applications that involve genetic techniques, strains, or species that do not combine well with germine genetically engineered models. By circumventing the need for extensive breeding programs, it may also contribute to reducing the number of mice used for atherosclerosis research.

The efficiency of the technique stems from the noncell autonomous mechanism of action of PCSK9. PCSK9 is secreted into plasma and exerts its main function in the extracellular space. As a result, ubiquitous reduction of hepatic LDL receptor levels can be achieved even if only a subgroup of hepatocytes is transduced with the rAAV8 vector.

The extent of atherosclerosis induced by the viral vectors was less than that of Ldlr−/− mice on Western-type diet, but more important for most research applications, lesions progressed to the fibroatheromatous stage and the coefficient of variation did not seem to be increased. If more extensive arteriosclerosis is required, previous studies have shown that hepatic expression of rAAV8-encoded genes can be maintained far beyond the 12-week study period of the present study.

Because AAV vectors have broad species tropism and the PCSK9 and LDLR genes are conserved across mammals, we hypothesized that the technique would not be constrained to the mouse, and we were able to induce hypercholesterolemia and early aortic arteriosclerosis in Golden Syrian hamsters.

In conclusion, gain-of-function PCSK9-encoding rAAV8 vectors provide a versatile tool for experimental atherosclerosis research that overcomes some of the limitations of germine genetically engineered models and can be applied in mice and hamsters and potentially in other species.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
- Gene-modified models, such as Apoe- or Ldlr-deficient mice, are widely used for studying hypercholesterolemia and atherosclerosis.
- Hyperactivity of proprotein convertase subtilisin/kexin type 9, a key regulator of low-density lipoprotein receptor protein levels, causes hypercholesterolemia in mice and humans.
- Recombinant adeno-associated virus vectors are efficient tools for liver-directed gene delivery and provide long-term gene expression.

What New Information Does This Article Contribute?
- A single injection of recombinant adeno-associated virus encoding a proprotein convertase subtilisin/kexin type 9 gain-of-function mutant induces persistent hypercholesterolemia and atherosclerosis in mice and hamsters.
- This technique provides an alternative method for the induction of atherosclerosis that circumvents the need for breeding with Apoe- or Ldlr-deficient mice.

Several genetic techniques are available for studying the pathophysiology of atherosclerosis in mice, including complex strain crosses and conditional knockout techniques. However, ready application of these methods with current atherosclerosis models is limited by the extensive breeding required to generate the mice. Here, we report that injection of recombinant adeno-associated viruses encoding a gain-of-function proprotein convertase subtilisin/kexin type 9 mutant induces persistent hypercholesterolemia and atherosclerosis in mice and hamsters. This method could be useful for high-throughput experiments or for studies of atherosclerosis in complex mouse strains. It could also be used to generate nonmurine models.
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**Online Methods**

*Plasmid construction*

For construction of the rAAV plasmid encoding the human D374Y-hPCSK9 gene (Online Figure I), a PCR-amplified fragment containing Bovine Growth Hormone Polyadenylation Signal (BGHpA; amplified with primers 5’-aaaaacggtttaattaaaagctagcaagccgccgtgtgccttagttgcagc-3’ and 5’-aaagccgcatagccacccagcctacc-3’) was inserted into the MluI and Ascl restriction sites of pAAV/mU6.siRNA.shuttle (Applied Viromics, Fremont, CA, USA). In the resulting vector, designated pAAV/MCS-BGHpA, the BGHpA sequence flanked by an upstream linker (MCS) was inserted between the left and the right inverted terminal repeats (ITRs) and, hence, replaced the mU6.siRNA cassette. The ApoEHCR-hAAT promoter (1) was PCR-amplified from pPBT/D374Y-hPCSK9-forw (a piggybac transposon vector carrying the D374Y-hPCSK9 gene driven by the ApoEHCR-hAAT promoter) using the primers 5’-aaaaacggttgcagagctagccgacacc-3’ and 5’-aaagccgcatagccacccagcctacc-3’ and subsequently inserted into the MluI and FseI sites of the MCS in pAAV/MCS-BGHpA, leading to pAAV/ApoEHCR-hAAT-MCS-BGHpA. The D374Y-hPCSK9 sequence was PCR-amplified from pPBT/D374Y-hPCSK9-forw using the primers 5’-aaaaacggttgcagagctagccgacccagcctacc-3’ and 5’-aaagccgcatagccacccagcctacc-3’ and cloned into the AgeI and FseI sites between the promoter and the BGHpA sequence in pAAV/ApoEHCR-hAAT-MCS-BGHpA, resulting in pAAV/ApoEHCR-hAAT-D374Y-hPCSK9-BGHpA.

For generation of the murine variant of the human D374Y mutation of PCSK9 (Online Figure I), RNA was purified from liver cells from a female C57BL/6J mouse using TRI Reagent (Sigma) and cDNA was produced using Maxima First Strand cDNA synthesis Kit for RT-qPCR (Fermentas). A D377Y (G>T) mutation of the murine PCSK9 gene, which corresponds to the human D374Y mutation, was introduced by an overlap extension PCR on the cDNA generated from murine liver cells using for the upstream PCR fragment primers 5’-aaaaacggttgcagagctagccgacaccagcctacc-3’ and 5’-atgtgctgcagtaactggacgctcc-3’, and for the downstream fragment 5’-ggagcgtgcagtagctgctacc-3’ and 5’-aaagccgcatagccacccagcctacc-3’. The resulting PCR fragment D377Y-mPCSK9 was
AgeI/FseI-cloned into pAAV/ApoEHCR-hAAT-MCS-BGHpA, resulting in pAAV/ApoEHCR-hAAT-D377Y-mPCSK9-BGHpA.

Both plasmids were analyzed by restriction analysis and sequencing. Prior to rAAV production, the presence of both ITR’s in the rAAV vector plasmids was confirmed by digesting the plasmids with SmaI.

Animals, diets, and tissue sampling

Female C57BL/6NTac mice were from Taconic Europe (Ry, Denmark), female Ldlr-deficient (Ldlr<sup>−/−</sup>) mice (B6.129S7-Ldlr<sup>tm1Her</sup>/J, stock number 002207) from Jackson Laboratories (Bar Harbor, Maine, USA), and male Golden Syrian hamsters (Mesocricetus auratus) from Harlan Laboratories (Indianapolis, USA). Breeding pairs of C57BL/6-Ins<sub>2</sub><sup>Akita</sup>/J (stock number 003548) and C57BL/6J (stock number 00664) mice were from Jackson Laboratories, and male offspring (heterozygous for the Ins<sub>2</sub><sup>Akita</sup> mutation and wildtype littermates) were used for experiments. Genotyping for the Ins<sub>2</sub><sup>Akita</sup> mutation was performed by PCR and restriction fragment analysis as recommended by the Jackson Laboratories website. Animals were housed in a temperature-controlled facility with a 12-hour dark and light cycle and had ad libitum access to fresh water and feed. The Danish Animal Experiments Inspectorate approved all animal procedures.

Paigen diet (D12336, Research diets Inc., New Brunswick, NJ) contained 16% fat, 1.25% cholesterol, and 0.5% sodium cholate. Western-type diet (D12079B, Research diets Inc.) contained 21% fat and 0.21% cholesterol without added sodium cholate.

All murine blood samples (non-fasting) were obtained from the mandibular vein. Blood was drawn into an EDTA-coated tube and centrifuged at 3000 rpm for 10 minutes at 4°C. Plasma was stored at -80°C. At the end of the study, non-fasting mice were anesthetized (5 mg of pentobarbital with 2% lidocain i.p.), and exsanguinated by withdrawing blood from the right ventricle into EDTA-coated tubes. Mice were then flushed through the left ventricle with a cardioplegic solution, perfusion-fixed at ≈100 mm Hg for 5 minutes with 4% phosphate-buffered formaldehyde (pH 7.2), and then immersed in the fixative for 6 hours before storage in cold (4°C) phosphate buffer. Euthanization and sample collection from Golden Syrian hamsters was performed similarly with the exception that blood samples were drawn from the lateral saphenous vein under sevoflurane (4.0%) anaesthesia.
Tail vein hydrodynamic injections

Hydrodynamic injections of pAAV/ApoEHCR-hAAT-D377Y-mPCSK9-BGHpA and pAAV/ApoEHCR-hAAT-D374Y-hPCSK9-BGHpA plasmids were performed in female C57BL/6NTac mice (n=4 in each group) using a previously described procedure (2). Hydrodynamic injection of pUC19 plasmid was used as negative control (n=2). In brief, animals were kept at a high ambient temperature to dilate the tail veins and shortly anesthetized in a chamber containing isoflurane (3.75%) until the pain withdrawal reflex was absent. Plasmids were dissolved in sterile Ringer solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl2) at 50-55 µg/ml and a volume corresponding to 8% of the bodyweight was injected into the tail vein within 5-7 seconds. On day 1 after injection, the animals were put on Paigen diet. Blood samples were drawn after 1, 3, 7, 14, 21, and 42 days following injection.

Recombinant AAV vector production and delivery

Viral vectors in serotype 8 capsids (rAAV8-D377Y-mPCSK9, rAAV8-D374Y-hPCSK9, and rAAV8-CMV-Luc) were produced by the UNC Vector Core (Chapel Hill, North Carolina, USA) under a fee-for-service agreement. Vectors were prepared with a helper-virus-free method (3) and purified using a sequential process of nuclei isolation, density gradient centrifugation and heparin sulfate affinity column chromatography. Preparations were titrated by dot blot to determine the concentration of the virus particles. In the C57BL/6NTac mouse study, vectors were delivered via a single tail vein injection in three different doses: 2.0 × 10^{10} vector genomes (VG)/mouse, 1.0 × 10^{11} VG/mouse or 5.0 × 10^{11} VG/mouse. Injection of saline was used as control. The next day, mice were put on either Paigen or Western-type diet for 12 weeks. All groups consisted of four animals with the exception of the two Ldlr⁻/⁻ groups that consisted of 5 mice each. Blood was sampled 4 days before vector injection (baseline) and at day 1, 3, 7, 14, 21, 28, 42, 56, 70, and 84 (12 weeks) after injection.

For the bioluminescence study female C57BL/6NTac mice were injected intravenously through the tail vein with 5.0 × 10^{11} VG/mouse, and saline was used as control (n=5 in both groups). Mice were afterwards fed the Western-type diet. Blood samples were collected at baseline and at day 7, 14, 28 and 56 after vector injection.

Akita mice and wildtype littermates (n=4 in each group) were injected intravenously with rAAV8-D377Y-mPCSK9 (1.0 × 10^{11} VG) at 26 weeks of age and challenged with the Western-type diet. Blood samples were collected at day 28, 56, and 70 (10 weeks) after vector injection.
Golden Syrian hamsters were injected intraperitoneally with $1.2 \times 10^{12}$ VG of the rAAV8-D374Y-hPCSK9 vector (n=4) or saline (n=4). Blood was drawn at day 0 (baseline), 10, 28, 49, 70, and 84.

**In vivo analysis of bioluminescence**

The rAAV8-CMV-Luc injected mice were analyzed for bioluminescence 11 days after injection of the vector using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA, USA) and the included software Living Image 4.0. Mice were anesthetized with 3.75% isoflurane and then injected intraperitoneally with 200 µL luciferin (15 mg/mL) (Synchem OHG, Felsberg/Altenburg, Germany). Isoflurane-anesthesia was maintained at 2.0% while bioluminescence was analyzed. Images were acquired 10 minutes after luciferin injection.

**Western blotting**

For analysis of hepatic LDL receptor levels, liver tissue lysates were run in 8% Precise Protein Gels (Thermo Fisher Scientific) under non-reduced, denaturing conditions and blots were stained with rabbit anti-LDL receptor (1:200, Cat. # 3839-100, BioVision) and anti-β-actin antibodies (1:400, MA1-91394, Thermo Fisher Scientific) followed by peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies, respectively (Dako). Band intensities were measured using image analysis software (ImageJ64).

**Plasma PCSK9, ALAT activity, serum amyloid A, lipids and FPLC analysis**

A human PCSK9-specific ELISA was used to measure the level of D374Y-hPCSK9 and a murine-specific ELISA was used to measure the sum of D377Y-mPCSK9 and endogenous mPCSK9 (both R&D Systems). Alanine aminotransferase (ALAT) activity was measured in plasma using an ALAT activity assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Serum amyloid A (SAA) was quantified in plasma using a mouse-specific SAA ELISA kit (ab157723, Abcam, Cambridge, UK). Plasma total cholesterol was measured in duplicate with an enzymatic cholesterol reagent (CHOD-PAP, Roche Diagnostics). Size-exclusion chromatography of mouse and hamster plasma (120 µl) was performed at room temperature using PBS-EDTA and Superose 6 fast protein liquid chromatography (FPLC) columns (GE Healthcare). The flow rate was 0.4 ml/min. Fractions of 250 µl were collected and used for immediate cholesterol and triglyceride measurements.
Quantification and morphology of atherosclerotic lesions

Lesion development in the aortic arch was determined using the en face technique. For the mouse studies, the examined area was defined as the segment starting 0.8 mm before the branch point of the innominate artery and ending at the branch point of the supreme intercostal artery, including 0.5 mm of the major branching vessels. For the hamster study, the examined area was defined as the segment starting 1.6 mm before the branch point of the innominate artery and ending at the branch point of the supreme intercostal artery, including 1.0 mm into the three major branching vessels. Briefly, the entire aorta was removed and cleaned for periadventitial fat, cut open longitudinally, and stained with Oil Red O (60% solution for 10 minutes at 37°C). The opened aorta was then attached to a microscope slide and cover-slipped using Aquatex. Slides were scanned (Epson Perfection V600 Photo) and the percentage of aortic arch area stained by Oil red O was determined using image analysis software (ImageJ64). Lesion morphology and aortic root atherosclerosis was studied in cross-sections of the aortic root. Briefly, the base of the heart including the most proximal part of the ascending aorta was excised and embedded in paraffin. The part containing the aortic root was sectioned consecutively (3 µm sections) starting from the commissures of the aortic cusps upwards. Sections were stained with orcein. Slides were evaluated microscopically at three different levels (0, 80, and 160 µm) and the mean percentage of aortic root lesion area was determined using image analysis software (ImageJ64). For visualization of necrotic cores with loss of collagen-rich connective tissue, sections of the aortic root was stained with Sirius Red and evaluated microscopically. For the detection of migrated vascular smooth muscle cells, smooth muscle α-actin (SMαA) was visualized using biotinylated mouse monoclonal anti-SMαA (1:50, Neomarkers MS-113-BO) after blocking with normal donkey serum (DAKO, Denmark) incorporated in the blocking endogenous avidin/biotin kit (Biotin blocking kit, DAKO, Denmark) (15 min. avidin and serum followed by 15 min. biotin and 10 % serum). Primary antibodies were followed by Alexa Fluor 594–conjugated streptavidin (1:400, Jackson Immunoresearch). Nuclei were visualized by 4’′,6-diamidino-2-phenylindole (DAPI)-staining.

Statistics

Data are expressed as mean±SEM. Statistical analyses were performed in Prism (GraphPad Software Inc.). Dose-response relationships among vector-injected groups were analyzed by one-way ANOVA followed by post-test for linear trends. For multiple group comparisons data was
analyzed by one-way ANOVA followed by Tukey’s multiple comparisons post-test. Two-group comparisons were performed with un-paired t-test for normally distributed data or Mann-Whitney test. In two cases (Figure 3B and 3D), data were log-transformed before statistical analysis. Significance level was set at p<0.05.

Online References


## Online Table I

Overview with baseline and final weights in the different experimental groups.

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<td><strong>Mouse</strong></td>
<td>Paigen</td>
<td>rAAV8-D374Y-hPCSK9</td>
<td>High-dose</td>
<td>20.9±0.5</td>
<td>22.1±1.0</td>
<td>n=4</td>
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Mean±SEM
**Online Figure I. Overview of plasmids.** Plasmids pAAV/D377Y-mPCSK9 (A) and pAAV/D374Y-hPCSK9 (B) encoding gain-of-function forms of murine and human PCSK9, respectively. Plasmids have left and right inverted terminal repeats (ITR), a liver-specific promoter (ApoEHCR-hAAT) consisting of the hepatocyte control region (HCR) from the *APOE* gene and the human α1-antitrypsin promoter (hAAT) promoter, and a bovine growth hormone polyadenylation signal (BGHpA).
Online Figure II. Non-viral gene transfer of PCSK9-encoding plasmids. Hydrodynamic injections of pAAV/D377Y-mPCSK9 (A) and pAAV/D374Y-hPCSK9 (B) in C57BL6/NTac mice fed Paigen diet gave rise to transient high-level expression levels as measured by murine- and human-specific ELISAs, respectively. Note that the pre-injection level of plasma mPCSK9 is not zero because of endogenous expression. Total cholesterol levels increased with a slight delay in both pAAV/D377Y-mPCSK9 (C) and pAAV/D374Y-hPCSK9 (D)-injected groups.
Online Figure III. Injection of control luciferase-encoding rAAV8 vector does not induce hypercholesterolemia, hepatic injury or systemic inflammation. A, Total cholesterol levels on Western-type diet were similar between rAAV8-CMV-Luc- and saline-injected mice (n=5 in each group). Non-significant (ns), t-test for area-under-the-curve (AUC). B, Plasma ALAT activity was similar in groups at day 56 after rAAV8-CMV-Luc or saline injection. Non-significant (ns), t-test. C, Serum amyloid A was not increased by rAAV8 vector injection over the study period. In fact, the AUC was found to be slightly, but significantly (**p<0.01, t-test), lower in the rAAV8-CMV-Luc- compared to the saline-injected group (n=5 in each group).
Online Figure IV

**Online Figure IV.** Induction of hypercholesterolemia by rAAV8-D374Y-hPCSK9 vector injection. **A,** Plasma levels of human PCSK9 in mice injected with the rAAV8-D374Y-hPCSK9 vector and fed Western-type diet (n=4 for all groups). ***P<0.0001 for dose-response relationship among vector-injected groups (ANOVA for AUC with post-test for linear trend). **B,** Hepatic LDLR levels, normalized to β-actin levels, in mice injected with saline (control), low-dose rAAV8, high-dose rAAV8, and in Ldlr<sup>−/−</sup> mice. **C,** Plasma total cholesterol on Paigen diet (left) and Western-type diet-fed (right) mice (n=4 for all groups, except for Ldlr<sup>−/−</sup> n=5). **P<0.01 and ***p<0.001 for dose-response relationship among vector-injected groups (ANOVA for AUC with post-test for linear trend).
Online Figure V. FPLC profiles. Non-fasting cholesterol FPLC profiles of pooled plasma samples from the different mouse groups fed Paigen (upper panel) or Western-type diet (lower panel).
Online Figure VI

**A**, For both types of diets, the control group had significantly lower plasma ALAT activity compared to any of the other groups. N=4 for all groups, *P<0.05, ANOVA with Tukey’s post-test. ALAT activity levels were similar or lower in rAAV8-D377Y-mPCSK9-injected mice compared to Ldlr<sup>-/-</sup> mice indicating no hepatotoxicity of the rAAV8 vector alone. **B**, Measurements of serum amyloid A (SAA) in mice on Western-type diet showed that the control group had significantly lower SAA levels compared to any of the other groups. SAA levels were similar in rAAV8-D377Y-mPCSK9-injected mice compared to Ldlr<sup>-/-</sup> mice indicating no systemic inflammation caused by rAAV8 vector injection alone. N=4 for all groups, except for Ldlr<sup>-/-</sup> mice where n=5, *P<0.05, ANOVA with Tukey’s post-test.
Online Figure VII. Atherosclerosis induced by rAAV8-D374Y-hPCSK9 injection. Area of the aortic arch covered with atherosclerotic lesions measured in Oil Red O-stained en face preparations (n=4 for all groups, except for Ldlr⁻/⁻ n=5). *P<0.05 and **p<0.01 for dose-response relationship (ANOVA for AUC with post-test for linear trend).
Online Figure VIII

Online Figure VIII. Sustained expression of D374Y-hPCSK9 in Golden Syrian Hamsters. Plasma levels of human D374Y-hPCSK9 in hamsters injected with the rAAV8-D374Y-hPCSK9 vector and fed Western-type diet. *P<0.05 (Mann-Whitney test for AUC).