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Citation

Ding, Q., A. Strong, K. M. Patel, S.-L. Ng, B. S. Gosis, S. N. Regan, C. A. Cowan, D. J. Rader, and K. Musunuru. 2014. "Permanent Alteration of PCSK9 With In Vivo CRISPR-Cas9 Genome Editing." Circulation Research 115 (5) (June 10): 488–492. doi:10.1161/circresaha.115.304351.

Published Version

doi:10.1161/circresaha.115.304351

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Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing

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Word Count: 2,499

Subject Codes: [88] Gene therapy; [90] Lipids and lipoprotein metabolism

ABSTRACT

Rationale: Individuals with naturally occurring loss-of-function *PCSK9* mutations experience reduced blood low-density lipoprotein cholesterol (LDL-C) levels and protection against cardiovascular disease.

Objective: The goal of this study was to assess whether genome editing using a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system can efficiently introduce loss-of-function mutations into the endogenous *PCSK9* gene in vivo.

Methods and Results: We used adenovirus to express Cas9 and a CRISPR guide RNA targeting *Pcsk9* in mouse liver, where the gene is specifically expressed. We found that within three to four days of administration of the virus, the mutagenesis rate of *Pcsk9* in the liver was as high as >50%. This resulted in decreased plasma PCSK9 levels, increased hepatic LDL receptor levels, and decreased plasma cholesterol levels (by 35%–40%) in the blood. There was no evidence of significant off-target effects.

Conclusions: Genome editing with the CRISPR-Cas9 system can be used to disrupt the *Pcsk9* gene in vivo with high efficiency and reduce blood cholesterol levels. This approach may have therapeutic potential for the prevention of cardiovascular disease in humans.

KEYWORDS

Coronary disease; gene therapy; lipoprotein; molecular biology; prevention

ABBREVIATIONS

LDL, low-density lipoprotein cholesterol; CHD, coronary heart disease; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLR, low-density lipoprotein receptor; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; PAM, protospacer-adjacent motif; NHEJ, non-homologous end joining; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay.

INTRODUCTION

Among the best-established causal risk factors for cardiovascular disease, the leading cause of death worldwide, is the blood concentration of low-density lipoprotein cholesterol (LDL-C), and pharmacological therapy that reduces LDL-C levels—namely the statin drugs—has proven the most effective means of reducing the risk of coronary heart disease (CHD). Yet even with the use of statin therapy, there remains a large residual risk of CHD, and a substantial proportion of patients are intolerant of statin therapy. Thus, there is a critical need to develop new strategies for the reduction of LDL-C.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a promising therapeutic target for the prevention of CHD. A gene specifically expressed in and secreted from the liver, and believed to function primarily as an antagonist to the LDL receptor (LDLR), *PCSK9* was originally identified as the cause of autosomal dominant hypercholesterolemia in some families, with gain-of-function mutations in the gene driving highly elevated LDL-C levels and premature CHD.[1] In subsequent studies, individuals with single loss-of-function mutations in *PCSK9* were found to experience a significant reduction of both LDL-C levels (~30%–40%) as well as CHD risk (88%).[2,3] Notably, even individuals with two loss-of-function mutations in *PCSK9*—resulting in ~80% reduction in LDL-C levels—appear to suffer no adverse clinical consequences.[4,5] This observation suggests that therapies directed against PCSK9 would offer cardiovascular benefit without any accompanying undesirable effects. Just 10 years after the

discovery of *PCSK9*, PCSK9-targeting monoclonal antibodies are being evaluated in clinical trials.[6] Yet even if these antibody-based drugs prove effective, their effects on LDL-C are short-lived, and patients will have to receive injections of the drugs every few weeks, which will limit their use as preventative therapy.

The ability to permanently alter the human genome has been made possible by the technology now commonly known as "genome editing." Recently published clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems use *Streptococcus pyogenes* Cas9 nuclease that is targeted to a genomic site by complexing with a synthetic guide RNA that hybridizes a 20-nucleotide DNA sequence (protospacer) immediately preceding an NGG motif (PAM, or protospacer-adjacent motif) recognized by Cas9.[7,8] CRISPR-Cas9 generates a double-strand break that is usually repaired by non-homologous end-joining (NHEJ), which is error-prone and conducive to frameshift mutations resulting in knock-out alleles of genes.

In light of the observed high efficiencies of CRISPR-Cas9 in mammalian cells in vitro,[7,8] we assessed whether CRISPR-Cas9 can be used to disrupt the mouse *Pcsk9* gene in vivo with high efficiency. A proof of principle that the gene can be targeted in mammalian hepatocytes in vivo would suggest that the approach might be viable in humans.

METHODS

An expanded Methods section is available in the Online Supplement. Candidate guide RNAs were designed to target exon 1 or exon 2 of the *Pcsk9* gene, transfected into 3T3-L1 cells, and assessed for efficacy with Surveyor assays. Adenoviruses either expressing green fluorescent protein (GFP) or coexpressing Cas9 plus a guide RNA targeting *Pcsk9* exon 1 (CRISPR-*Pcsk9*) were generated. In one experiment, a total of four 11-week-old male C57BL/6 mice were used, two each for the GFP and CRISPR-*Pcsk9* adenoviruses. In a second, more comprehensive experiment, a total of 15 5-week-old female C57BL/6 mice were used, five each for the GFP and CRISPR-*Pcsk9* adenoviruses and five with no virus. Following the virus administration, after three days (in the first experiment) or four days (in the second experiment) the mice were sacrificed after overnight fasting, and the livers were harvested and terminal blood samples collected. For the second experiment, to test the null hypotheses that each of four plasma analytes—PCSK9, triglyceride, cholesterol, and ALT—did not differ among the groups of mice ($N = 5$ for each group), P values were determined by the Kruskal-Wallis test performed on the set of the three groups. For each analyte for which the Kruskal-Wallis P was statistically significant, the Mann-Whitney U test was performed between each pair of groups within the set of three groups.

RESULTS

We initially screened candidate CRISPR guide RNAs targeting sequences in exon 1 and exon 2 of the mouse *Pcsk9* gene in 3T3-L1 cells. We found that the guide RNA targeting exon 1 displayed ~50% mutagenesis at the on-target site in *Pcsk9*, as judged by the Surveyor assay (Figure 1A, Supplemental Figure 1). We made an adenovirus coexpressing Cas9 and this guide RNA (CRISPR-*Pcsk9*), using an adenovirus expressing GFP as a control.

In a pilot experiment, the CRISPR-*Pcsk9* virus and the GFP virus were administered to two 11-week-old male mice each. After three days, we sacrificed the mice in order to harvest liver tissue. Whereas there was no evidence of mutagenesis in the control mice, the CRISPR-*Pcsk9* mice displayed substantial levels of mutagenesis, with one of the mice showing ~50% mutagenesis in the Surveyor assay, consistent with alteration of at least half of the *Pcsk9* alleles in the liver (Figure 1B, Supplemental Figure 1). Analyzing liver DNA from that mouse, we found that a wide variety of indels were produced in *Pcsk9*, ranging from 1 bp to 228 bp, with the possibility of larger indels that were not detected by PCR analysis (Supplemental

Figure 1). The most frequent indels were a 1-bp insertion and a 2-bp deletion. We assessed for off-target mutagenesis at the 10 sites deemed most closely matched to the on-target site and most likely to harbor off-target effects (six sites with three mismatches to the on-target site and the four highest-scoring sites with four mismatches to the on-target site; see Online Supplement for site sequences). We found no evidence of significant off-target mutagenesis, within the limit of detection by the Surveyor assay (Figure 1D).

We next performed a more comprehensive experiment in which the CRISPR-*Pcsk9* virus and the GFP virus were administered to five 5-week-old female mice each, with an additional group of five mice receiving no virus. After four days, the CRISPR-*Pcsk9* mice all displayed substantial levels of mutagenesis, in some cases exceeding 50%, with no mutagenesis observed in any of the mice in the two control groups (Figure 1C). We compared plasma PCSK9 levels at four days by enzyme-linked immunosorbent assay (ELISA); the CRISPR-*Pcsk9* mice displayed substantially lower PCSK9 levels compared to each of the control groups of mice [2597 pg/mL with CRISPR-*Pcsk9* virus vs. 26461 pg/mL with GFP virus ($P = 0.0079$) and vs. 21734 pg/mL with no virus ($P = 0.0079$); $N = 5$ per group; Figure 2A].

Whereas there was no significant difference in plasma triglyceride levels among the three groups at four days, CRISPR-*Pcsk9* mice had significantly lower levels of total plasma cholesterol, with 35%–40% reduction compared to the control groups [101 mg/dL with CRISPR-*Pcsk9* virus vs. 157 mg/dL with GFP virus ($P = 0.0079$) and vs. 161 mg/dL with no virus ($P = 0.0079$); $N = 5$ per group; Figure 2A]. We performed complete lipoprotein profiling of pooled plasma samples from each group, observing reduced HDL and LDL fractions in CRISPR-*Pcsk9* mice (Figure 2B), consistent with prior observations in *Pcsk9* knockout mice.[9] No significant difference in blood ALT levels at four days among the three groups was observed, and hematoxylin/eosin staining of liver sections from representative mice that received either the GFP virus or the CRISPR-*Pcsk9* virus showed no inflammation (Figure 2C). Finally, we assessed LDLR levels in liver by Western blot analysis. PCSK9 functions to downregulate LDLR;[9] consistent with this relationship, the CRISPR-*Pcsk9* mice had higher levels of LDLR protein than the control groups of mice (Figure 2D).

DISCUSSION

In this proof-of-principle study, we found that CRISPR-Cas9 could disrupt the mouse *Pcsk9* gene in vivo with high efficiency and result in decreased circulating PCSK9 levels, increased hepatic LDLR levels, and decreased plasma cholesterol levels. The 35%–40% lower cholesterol levels in the CRISPR-*Pcsk9* mice compared to control mice is consistent with the 36%–52% lower levels previously observed in *Pcsk9* knockout mice compared to wild-type mice.[9] Thus, this approach may have therapeutic potential for the prevention of cardiovascular disease in humans.

Although the use of adenovirus allows for efficient delivery to the liver and sustained expression of the CRISPR-Cas9 system, it is not the optimal therapeutic vehicle due to the immune response to the virus. Indeed, inflammation and acute phase responses could potentially have affected the plasma cholesterol levels in the mice receiving adenovirus. However, we included a control group that did not receive any adenovirus, and the plasma PCSK9, triglyceride, cholesterol, and ALT levels were very similar to the levels observed in the control group that received the GFP virus. There was no apparent inflammation in the liver within the time frame of the experiments. Thus, we did not see any evidence of confounding due to the use of adenovirus.

While the use of adeno-associated virus would be preferable, the gene encoding *Streptococcus pyogenes* Cas9 (~4.2 kb) in combination with a CRISPR guide RNA-expressing cassette (~500 bp) is too large to fit into standard liver-targeting adeno-associated virus vectors (e.g., AAV2/8). Furthermore, the rapidity with

which robust alteration of the *Pcsk9* gene occurred in our experiments—up to >50% mutagenesis in just three to four days—suggests that a single brief pulse of CRISPR-Cas9 expression would be sufficient to achieve a therapeutic effect. Thus, a virus-free delivery method that transiently expresses CRISPR-Cas9 (e.g., RNAs in lipid nanoparticles) might be optimal. A recent study showed that hydrodynamic tail vein injection of DNA vectors encoding CRISPR-Cas9 successfully targeted a liver gene (*Fah*) with no apparent long-term adverse effects.[10]

A possible barrier to therapeutic CRISPR-Cas9 applications is the issue of off-target mutagenesis. In our study, we did not observe significant off-target mutagenesis at a number of potential off-target sites, but we cannot rule out low-frequency events in vivo. Strategies to greatly reduce off-target mutagenesis without impairing on-target mutagenesis are being developed and can be adapted for use in therapeutic applications.

With cardiovascular disease being the number one killer worldwide, a safe and effective *PCSK9*-genome-editing therapy could have a significant impact on human health. A single administration could confer the benefits of naturally occurring *PCSK9* loss-of-function mutations—a permanent reduction in LDL-C levels and CHD risk, equivalent to taking statins every day for the rest of one's life but without the need for chronic therapy. It could represent a paradigm shift in thinking about cardiovascular therapeutics: a one-shot, long-term solution—not unlike a vaccination—rather than a pill to be taken every day or an injection to be received every few weeks. It could also open the door to a whole new class of therapies, where one might be able to target not just *PCSK9* but a number of other potential therapeutic genes; indeed, given the multiplexing capacity of CRISPR-Cas9,[7,8] it might be feasible to efficiently target multiple genes simultaneously with a single therapy.

ACKNOWLEDGMENTS

We would like to thank the Vector Core Laboratory of the University of Pennsylvania for producing the adenoviruses and the Histology Core of the Harvard University Department of Stem Cell and Regenerative Biology for hematoxylin/eosin staining.

SOURCES OF FUNDING

This work was supported by the Harvard Stem Cell Institute (K.M.), Harvard University (Q.D., K.M.), and grants R01-DK097768 (K.M.) and R01-HL109489 (D.J.R.) from the United States National Institutes of Health (NIH).

DISCLOSURES

None.

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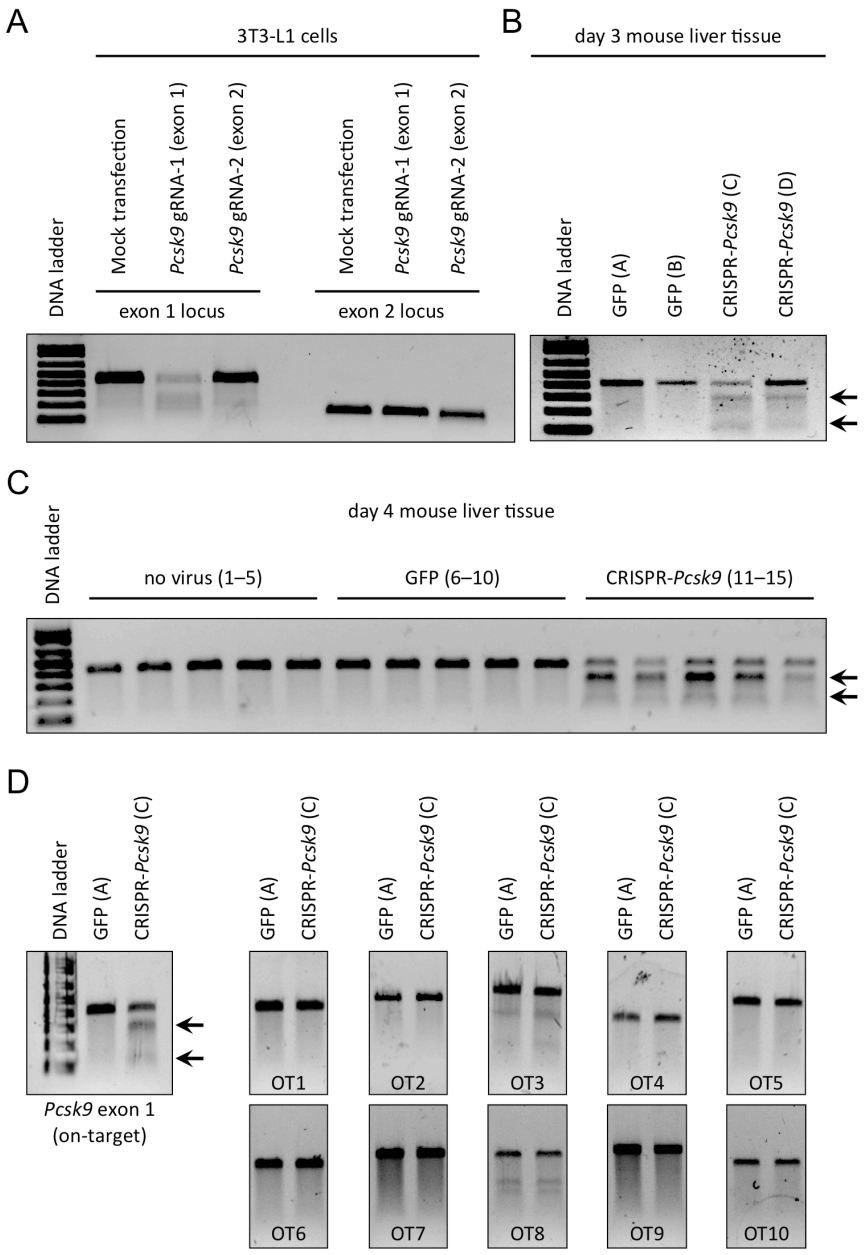


Figure 1. On-target and off-target effects in mouse cells and livers receiving CRISPR-Cas9. A shows Surveyor assays performed with genomic DNA from 3T3-L1 cells transfected with Cas9 and a guide RNA targeting *Pcsk9* exon 1 (gRNA-1) or a guide RNA targeting *Pcsk9* exon 2 (gRNA-2). **B** shows Surveyor assays performed with genomic DNA from liver samples taken from mice three days after receiving a control adenovirus expressing GFP (A, B) or an adenovirus expressing Cas9 and gRNA-1 (CRISPR-*Pcsk9*) (C, D). **C** shows Surveyor assays performed with genomic DNA from liver samples taken from mice four days after receiving no virus (1–5), the GFP virus (6–10), or the CRISPR-*Pcsk9* virus (11–15). **D** shows Surveyor assays performed with liver genomic DNA from "A" and "C" mice. The *Pcsk9* exon 1 on-target site and ten genomic sites deemed to be the most likely off-target sites for CRISPR-Cas9 activity (OT1–OT10; see Online Supplement for site sequences) were assessed. Arrows show the cleavage products resulting from the Surveyor assays; the intensity of the cleavage product bands relative to the uncleaved product band corresponds to the mutagenesis rate.

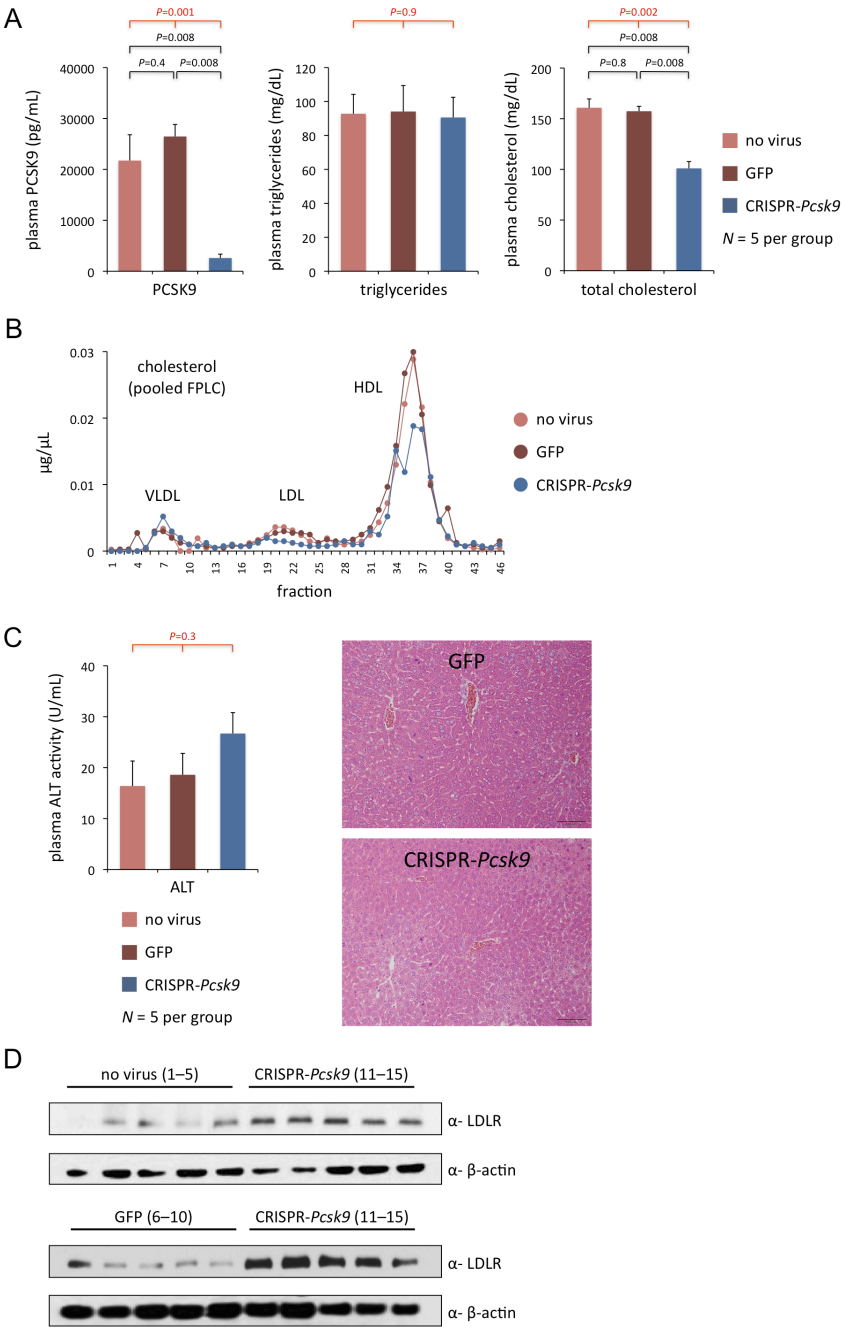


Figure 2. Effects of CRISPR-Cas9 genome editing on mice. A shows the results of ELISAs for PCSK9 protein, measurements of triglyceride levels, and measurements of total cholesterol levels in plasma samples from mice four days after receiving no adenovirus, GFP adenovirus, or CRISPR-*Pcsk9* adenovirus ($N = 5$ mice for each group). **B** shows the full plasma lipoprotein cholesterol profiles of pooled plasma samples from each group of mice. **C** shows the plasma ALT levels in mice four days after receiving virus ($N = 5$ mice for each group) and hematoxylin/eosin stains of liver sections from representative mice. **D** shows the results of Western blot analysis of liver samples taken from mice four days after receiving virus. For **A** and **C**, *P* values were determined by the Kruskal-Wallis test among all three groups (in red); if statistically significant, the Mann-Whitney U test between each pair of groups was performed (*P* values in black). Error bars show s.e.m.

ONLINE SUPPLEMENT

DETAILED METHODS

Screening of guide RNAs

Candidate guide RNAs were designed by visual inspection of the sequences of exons 1 and 2 of the *Pcsk9* gene. Protospacers corresponding to the guide RNAs were inserted into gRNA_Cloning Vector (Dr. George Church, http://www.addgene.org/41824/) as previously described.[11] Each guide RNA plasmid was co-transfected with pCas9_GFP (http://www.addgene.org/44719/) into mouse 3T3-L1 cells with TransIT-2020 Reagent (Mirus Bio) according to the manufacturer's instructions. After 2 days, the cells were subjected to fluorescent activated cell sorting (FACSAria II; BD Biosciences) to isolate green fluorescent protein (GFP)-positive cells. Genomic DNA was extracted from the isolated cells using the DNeasy Blood & Tissue Kit (QIAGEN), and *Pcsk9* exon 1 and exon 2 were PCR amplified. The PCR products were purified, analyzed using the Surveyor Mutation Detection Kit (Transgenomic) according to the manufacturer's instructions, and run on ethidium bromide-stained 1.5% agarose gels. The guide RNA showing the higher mutagenesis rate (exon 1) was chosen for further studies.

Generation of adenoviruses

The protospacer corresponding to the selected guide RNA was inserted into the pX330 plasmid (Dr. Feng Zhang, http://www.addgene.org/42230/) that coexpresses the guide RNA and Cas9, as previously described.[16] The coexpression cassette was cut out of the pX330 plasmid with AflIII and NotI and subcloned into the pShuttle vector (Clontech) cut with NcoI and NotI. A corresponding pShuttle vector with GFP was used as a control. In either case, the expression cassettes were then cut out of pShuttle with I-CeuI and PI-SceI and subcloned into the Adeno-X vector (Clontech) cut with the same enzymes. The Penn Vector Core at the University of Pennsylvania used these vectors to generate recombinant adenoviral particles (designated GFP and CRISPR-*Pcsk9*).

Animal studies

All procedures used in animal studies were approved by the pertinent Institutional Animal Care and Use Committees at the University of Pennsylvania and Harvard University and were consistent with local, state, and federal regulations as applicable. A total of four 11-week-old male C57BL/6 mice were simultaneously used for the first experiment, two each for the GFP and CRISPR-*Pcsk9* adenoviruses. A total of 15 5-week-old female C57BL/6 mice were simultaneously used for the second experiment, five each for the GFP and CRISPR-*Pcsk9* adenoviruses and five with no virus. Mice were administered 2.5×10^{10} particles each via retro-orbital injection. After three or four days, the mice were sacrificed by carbon dioxide asphyxiation after overnight fasting. Whole liver samples were harvested and split for DNA analysis, hematoxylin/eosin staining, and/or Western blot analysis, and terminal blood samples were collected.

Enzyme-linked immunosorbent assays (ELISAs), measurement of plasma analytes, and Western blot analysis

ELISAs were performed on mouse plasma samples using the Mouse Proprotein Convertase 9/PCSK9 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions. Triglyceride and total cholesterol levels were measured using Infinity Triglycerides Reagent and Infinity Cholesterol Reagent (Thermo Fisher) independently according to the manufacturer's instructions. Pooled plasma from each experimental group (150 μL for each group) was separated by FPLC gel filtration. Cholesterol plate assays were performed on FPLC fractions using the Infinity Cholesterol Reagent. ALT levels were

measured using the Alanine Transaminase Activity Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Western blot analysis was performed with liver lysates using antibodies against LDLR and against β-actin (Sigma).

On-target and off-target mutagenesis analyses

Liver genomic DNA samples were isolated and subjected to Surveyor assays as described above. To further analyze the *Pcsk9* exon 1 on-target site, PCR amplicons for the on-target site were subcloned using the TOPO TA Cloning Kit with the pCRII-TOPO vector (Thermo Fisher) according to the manufacturer's instructions. Plasmid DNAs obtained from individual bacterial clones were subjected to Sanger sequencing; usable sequence data was obtained from ~135 clones.

Off-target sites were predicted using the CRISPR Design server (http://crispr.mit.edu/).[16] Ten sites were chosen based on either sequence similarity (three-base mismatches) or high scoring (the top-scoring four-base mismatches), as shown below. There were no sites that were zero-base, one-base, or two-base mismatches.

Guide RNA sequence:

GGCTGATGAGGCCGCACATG**TGG**

top genome-wide off-target sites

sequence	score	mismatches	label	locus
AGATGAAGGGGCCGCACATGGAG	0.6	4MMs [1:3:7:9]	OT1	chr3:-53797274
CTGTGATGAGGCAGCACATGTGG	0.6	4MMs [1:2:3:13]	OT2	chr2:-35727358
GGGTCATTAGGCAGCACATGAGG	0.6	4MMs [3:5:8:13]	OT3	chr15:+27353354
GAATGATGAAGCCGCACAGGCGG	0.5	4MMs [2:3:10:19]	OT4	chr2:-129514031
GGGTGATGAGGCAGCACAGGCAG	0.4	3MMs [3:13:19]	OT5	chr7:-53619772
GGGTGCTGAGGCCGCACCTGTGG	0.4	3MMs [3:6:18]	OT6	chr10:-90187208
GGCTTATCAAGCCGTACATGAAG	0.4	4MMs [5:8:10:15]		chr10:-43915219
GGCTCATTTGGCAGCACATGAGG	0.3	4MMs [5:8:9:13]		chr7:-143656909
GGTTGAAGAGGCCACACATGAAG	0.3	3MMs [3:7:14]	OT7	chr2:-154450561
AGCTGACGTGGCTGCACATGGGG	0.2	4MMs [1:7:9:13]		chr10:+125595116
GGGTGTGGAGGCAGCACATGTGG	0.2	4MMs [3:6:7:13]		chr17:+51584115
GACTGATGAGGCCCCACATCTGG	0.2	3MMs [2:14:20]	OT8*	chr14:+26837439
GACTGATGAGGCCCCACATCTGG	0.2	3MMs [2:14:20]	OT8*	chr14:+26697669
GACTGATGAGGCCCCACATCTGG	0.2	3MMs [2:14:20]	OT8*	chr14:+26977053
GGAGCATGAGGCCCCACATGGAG	0.2	4MMs [3:4:5:14]		chr11:+120134262
GACTGATTATGCCTCACATGCAG	0.2	4MMs [2:8:10:14]		chr15:+43680258
GGCTGAGGGAGCAGCACATGTGG	0.2	4MMs [7:9:10:13]		chr7:-56244753
GGATGCTGAGGTCGCACATCCAG	0.2	4MMs [3:6:12:20]		chr1:-170137588
GGTTGATGAGGCGGCTCATGAGG	0.2	3MMs [3:13:16]	OT9	chr2:+165087015
GTCTGCTGAGTCTGCACATGAGG	0.2	4MMs [2:6:11:13]		chr13:-118529657
GGCTGATGAAGCTGCACCTGTGG	0.2	3MMs [10:13:18]	OT10	chr2:-127603103

*duplicated region in the annotated mouse genome

Primer sequences

Genomic amplification of on-target sites. *Pcsk9* exon 1: 5', GGAGGACACGTTTTCTGCAT; 3', CTGCTGCTGTTGCTGCTAC. *Pcsk9* exon 2: 5', TGGGAAAATCTGTGATACGC; 3', TGTAGCCTCTGGGTCTCCTC.

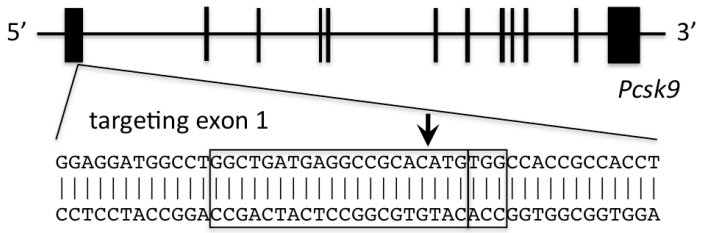
Genomic amplification of off-target sites. OT1: 5', TGTAGCCTCTGGGTCTCCTC; 3', GAATTCTCCCTCGTGAGCTG. OT2: 5', CAGTTGCAGGGAGAGGAGAC; 3',

GGAACTGACCCTACGATCCA. OT3: 5', GGATTCAATGGCCAGAGCTA; 3', CTCACCTCTAGGGCCGAACT. OT4: 5', ACCATAGCGCTTCTGGTTGT; 3', TCCTGAAGGAGCTGGAGAAA. OT5: 5', CGTGCACACACATTCATTCA; 3', CGTCTCATGGTGCATAGTGG. OT6: 5', TGTTCCTTCTGCCTCTGCTT; 3', CAGCAAAACGATGCTGATGT. OT7: 5', ATGAGGAAGTGGCATTGGAG; 3', AGGCCAAGGAAGGAATGACT. OT8: 5', CTTTCAGGCAAAAGCTGACC; 3', TCTCAGACAGGCATGCAGAC. OT9: 5', GTCAGACCCTGTCTGGAGGA; 3', CAAACTCGGTTCATGTGGTG. OT10: 5', CTTCCAAGGCAGCATGTGTA; 3', TGTGTCTTCCACTGGAGCAG.

Statistical analyses

Statistical analyses were performed solely for the second experiment. The levels of four plasma analytes—PCSK9, triglycerides, total cholesterol, and ALT—were each compared among three groups—mice that received no virus ($N = 5$), mice that received GFP virus ($N = 5$), and mice that received CRISPR-*Pcsk9* virus ($N = 5$). Initially, the Kruskal-Wallis test was performed for each of the four analytes, with a statistical significance threshold of $P < 0.0125$ to account for multiple testing (Bonferroni correction for four tests). Two analytes (PCSK9 and total cholesterol) were found to reach statistical significance. For each of the these two analytes, the Mann-Whitney U test was performed for each possible pairwise comparison among the three groups of mice, with a statistical significance threshold of $P < 0.00833$ to account for multiple testing (Bonferroni correction for two sets of three pairwise comparisons performed). All statistical analyses were performed using GraphPad Prism 6 for Mac OS X.

SUPPLEMENTAL FIGURES

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GGAGGATGGCCTGGCTGATGAGGCCGCAC-TGTGGCCACCGCCACCT    del_1 x 2
GGAGGATGGCCTGGCTGATGAGGCCGCA-ATGTGGCCACCGCCACCT    del_1
GGAGGATGGCCTGGCTGATGAGGCCGCAccATGTGGCCACCGCCACCT   ins_1 x 9
GGAGGATGGCCTGGCTGATGAGGCCGCA--TGTGGCCACCGCCACCT    del_2 x 6
GGAGGATGGCCTGGCTGATGAGGCCGCAC--GTGGCCACCGCCACCT    del_2 x 2
GGAGGATGGCCTGGCTGATGAGGCCGCgcCATGTGGCCACCGCCACCT   ins_2/del_1
GGAGGATGGCCTGGCTGATGAGGCCGCACAccaTGTGGCCACCGCCACCT ins_3
GGAGGATGGCCTGGCTGATGAGGCCGCAC-----GCCACCGCCACCT    del_5
GGAGGATGGCCTGGCTGATGAG------CATGTGGCCACCGCCACCT    del_6
GGAGGATGGCCTGGCTGATGAGGCCGCACcg-------CCGCCACCT    ins_2/del_9
GGAGGATGGCCTGGCTGATG--------CATGTGGCCACCGCCACCT    del_8
GGAGGATGGCCTGGCTGA-----------ATGTGGCCACCGCCACCT    del_11
GGAGGATGGCCTGGCTGATGAGGCC------------ACCGCCACCT    del_12
GGAGGATGGCCTGGCTG------------ATGTGGCCACCGCCACCT    del_12
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------CCACCT    del_12
GGAGGATGGCCTGG---------------TGTGGCCACCGCCACCT    del_16
GGAGGATGGCCTGGCTGATGAGGCCccgttgcctgg-----------    ins_11/del_27
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_21 x 2
GGAGGATG---------------------ATGTGGCCACCGCCACCT    del_21
GG---------------------------CATGTGGCCACCGCCACCT    del_26
GG---------------------------ATGTGGCCACCGCCACCT    del_27
G----------------------------ATGTGGCCACCGCCACCT    del_28
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_28
GGAGGATGGC-------------------------------CACCT    del_32
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_35 x 2
----------------------------ATGTGGCCACCGCCACCT    del_36
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_43
--------------------------------TGGCCACCGCCACCT    del_49
GGAGGATGGCCTGGCTGATGAGGCC----------------------    del_49 x 2
GGAGGATGGC-------------------------------------    del_50
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_50
GGAGGATGGCCTGGCTGATGAGGCCGCA-------------------    del_51
-----------------------------------------CACCT    del_52
----------------------------ATGTGGCCACCGCCACCT    del_54
GGAGGATGGCCTGGCTGATGAGGCCGCAC------------------    del_55
GGAGGATGGCCTGGCTGATGAGGCCGCACc-----------------    ins_1/del_58
-------------------------tctATGTGGCCACCGCCACCT    ins_3/del_76
GGAGGATGGCCTGGCTGATGAGGCCGCt-------------------    ins_1/del_88
----------------------------ATGTGGCCACCGCCACCT    del_82
GGAGGATG---------------------------------------    del_110
-----------------------------------------------
del_59, del_82, del_98, del_122, del_125 x 2,
del_137, del_143, del_174, del_185, del_228
```

Supplemental Figure 1. Mutagenesis of *Pcsk9* in vivo with CRISPR-Cas9. The CRISPR-Cas9 target site in *Pcsk9* exon 1 is shown. The boxes indicate the 20-bp sequence matching the protospacer and the 3-bp PAM. The predicted site of Cas9-induced double-strand breaks is indicated with an arrow. Deletions and insertions detected by PCR amplification and Sanger sequencing of the on-target site in liver genomic DNA from a representative mouse ("C" in Figure 1) that received an adenovirus expressing Cas9 and the guide RNA for the target site (CRISPR-*Pcsk9*) are shown. At the bottom are deletions that spanned the entire site.