Permanent Alteration of PCSK9 With In Vivo CRISPR-Cas9 Genome Editing

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Rationale: **Individuals with naturally occurring loss-of-function proprotein convertase subtilisin/kexin type 9 (*PCSK9*) mutations experience reduced low-density lipoprotein cholesterol 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 system can efficiently introduce loss-of-function mutations into the endogenous *PCSK9* gene in vivo.**

Methods and Results: **We used adenovirus to express CRISPR-associated 9 and a CRISPR guide RNA targeting *Pcsk9* in mouse liver, where the gene is specifically expressed. We found that <3 to 4 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 low-density lipoprotein receptor levels, and decreased plasma cholesterol levels (by 35–40%). No off-target mutagenesis was detected in 10 selected sites.**

Conclusions: **Genome editing with the CRISPR–CRISPR-associated 9 system disrupts the *Pcsk9* gene in vivo with high efficiency and reduces blood cholesterol levels in mice. This approach may have therapeutic potential for the prevention of cardiovascular disease in humans. (*Circ Res*. 2014;115:488-492.)**

Key Words: coronary disease ■ genetic therapy ■ lipoproteins ■ molecular biology ■ prevention and control

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 statin drugs, has proven to be 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.

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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 2 loss-of-function mutations in *PCSK9*—resulting in ≈80% reduction in LDL-C levels—seem 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 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

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Nonstandard Abbreviations and Acronyms	
Cas	CRISPR-associated
CHD	coronary heart disease
CRISPR	clustered regularly interspaced short palindromic repeats
GFP	green fluorescent protein
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
PAM	protospacer-adjacent motif
PCSK9	proprotein convertase subtilisin/kexin type 9

(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 (protospacer-adjacent motif) recognized by Cas9.[7,8] CRISPR-Cas9 generates a double-strand break that is usually repaired by nonhomologous end-joining, 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

We hypothesized that CRISPR-Cas9 would disrupt the mouse *Pcsk9* gene in hepatocytes in vivo to a sufficient degree that plasma PCSK9 levels and cholesterol levels would be reduced. We further hypothesized that these would be specific effects, such that the use of CRISPR-Cas9 would affect neither plasma triglyceride levels nor alanine transaminase (ALT) levels.

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 a pilot experiment, a total of four 11-week-old male C57BL/6 mice were used, 2 each for the GFP and CRISPR-*Pcsk9* adenoviruses. In a second, more comprehensive experiment, a total of fifteen 5-week-old female C57BL/6 mice were used, 5 each for the GFP and CRISPR-*Pcsk9* adenoviruses and 5 with no virus. After 3 days (in the first experiment) or 4 days (in the second experiment) after virus administration, the mice were euthanized after overnight fasting, and the livers were harvested and terminal blood samples collected.

For the second experiment, we tested the null hypotheses that on CRISPR-Cas9 genome editing, each of 4 plasma analytes—PCSK9, triglyceride, cholesterol, and ALT—did not differ among the groups of mice. The levels of the analytes were each compared among 3 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 4 analytes, with a statistical significance threshold of $P<0.0125$ to account for multiple testing (Bonferroni correction for 4 tests). Two analytes (PCSK9 and total cholesterol) were found to reach statistical significance. For each of these 2 analytes, the Mann–Whitney U test was performed for each possible pairwise comparison among the 3 groups of mice, with a statistical significance threshold of $P<0.00833$ to account for multiple testing (Bonferroni correction for 2 sets of 3 pairwise comparisons performed). All statistical analyses were performed using GraphPad Prism 6 for Mac OS X.

An expanded Methods section is available in Online Data Supplement.

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; Online Figure I). 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.

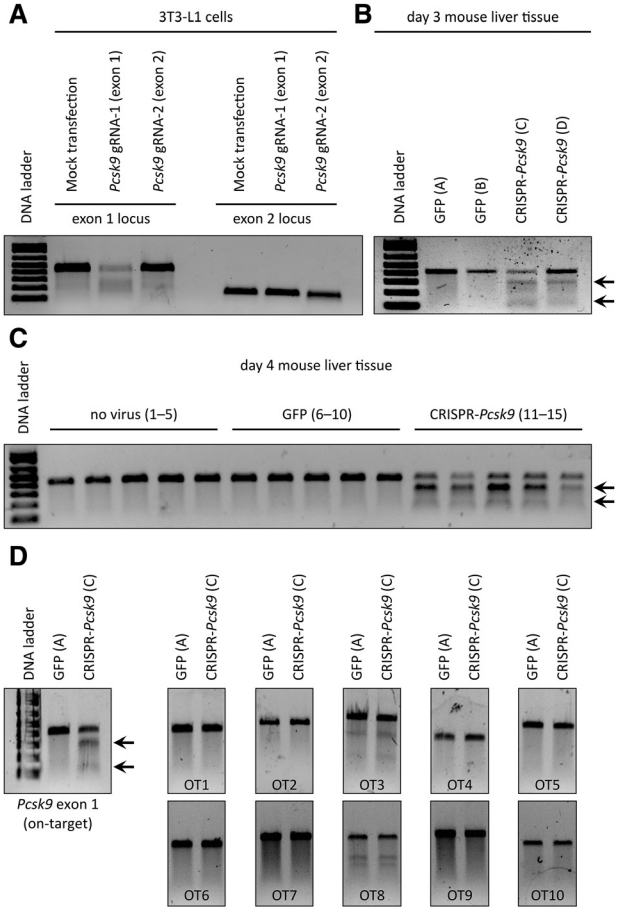

Figure 1. On-target and off-target effects in mouse cells and livers receiving clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9). **A**, Surveyor assays performed with genomic DNA from 3T3-L1 cells transfected with Cas9 and a guide RNA targeting *Pcsk9* exon 1 (gRNA-1) or a guide RNA targeting *Pcsk9* exon 2 (gRNA-2). **B**, Surveyor assays performed with genomic DNA from liver samples taken from mice 3 d after receiving a control adenovirus expressing green fluorescent protein (GFP; A and B) or an adenovirus expressing Cas9 and gRNA-1 (CRISPR-*Pcsk9*; C and D). **C**, Surveyor assays performed with genomic DNA from liver samples taken from mice 4 d after receiving no virus (1–5), the GFP virus (6–10), or the CRISPR-*Pcsk9* virus (11–15). **D**, Surveyor assays performed with liver genomic DNA from the A and C mice. The *Pcsk9* exon 1 on-target site and 10 genomic sites deemed to be the most likely off-target sites for CRISPR-Cas9 activity (OT1–OT10; see the 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.

After 3 days, we euthanized the mice 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 1 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; Online Figure I). Analyzing liver DNA from that mouse, we found that a wide variety of indels were produced in *Pcsk9*, ranging from 1 to 228 bp, with the possibility of larger indels that were not detected by polymerase chain reaction analysis (Online Figure I). 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 (6 sites with 3 mismatches to the on-target site and the 4 highest-scoring sites with 4 mismatches to the on-target site; see the Online Data Supplement for site sequences). We found no evidence of significant off-target mutagenesis, within the limit of detection by the Surveyor assay (Figure 1D).

To test the hypotheses that genome editing would result in reduced plasma PCSK9 and cholesterol and no differences in triglycerides and ALT, 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 5 mice receiving no virus. After 4 days, the CRISPR-*Pcsk9* mice all displayed substantial levels of mutagenesis, in some cases >50%, with no mutagenesis observed in any of the mice in the 2 control groups (Figure 1C). We compared plasma PCSK9 levels at 4 days by ELISA; the CRISPR-*Pcsk9* mice displayed substantially lower PCSK9 levels compared with each of the control groups of mice (2597 pg/mL with CRISPR-*Pcsk9* virus versus 26 461 pg/mL with GFP virus [*P*=0.0079] versus 21 734 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 3 groups at 4 days, CRISPR-*Pcsk9* mice had significantly lower levels of total plasma cholesterol, with 35% to 40% reduction compared with the control groups (101 mg/dL with CRISPR-*Pcsk9* virus versus 157 mg/dL with GFP virus [*P*=0.0079] versus 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 high-density lipoprotein and LDL fractions in CRISPR-*Pcsk9* mice (Figure 2B), consistent with prior observations in *Pcsk9*-knockout mice.[9] (Of note, reduction of PCSK9 in humans is not expected to reduce plasma high-density lipoprotein cholesterol levels, as observed in these experiments in mice, because of differences between human and mouse high-density lipoprotein metabolism.) No significant difference in blood ALT levels at 4 days among the 3 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% to 40% lower cholesterol levels in the CRISPR-*Pcsk9* mice compared with control mice is consistent with the 36% to 52% lower levels previously observed in *Pcsk9*-knockout mice compared with 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 because of 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 similar to the levels observed in the control group that received the GFP virus. There was no apparent inflammation in the liver within the timeframe of the experiments. Thus, we did not see any evidence of confounding because of the use of adenovirus.

Although 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 (eg, AAV2/8). Furthermore, the rapidity with which robust alteration of the *Pcsk9* gene occurred in our experiments—up to >50% mutagenesis in just 3 to 4 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 (eg, 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 several 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 long-term 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

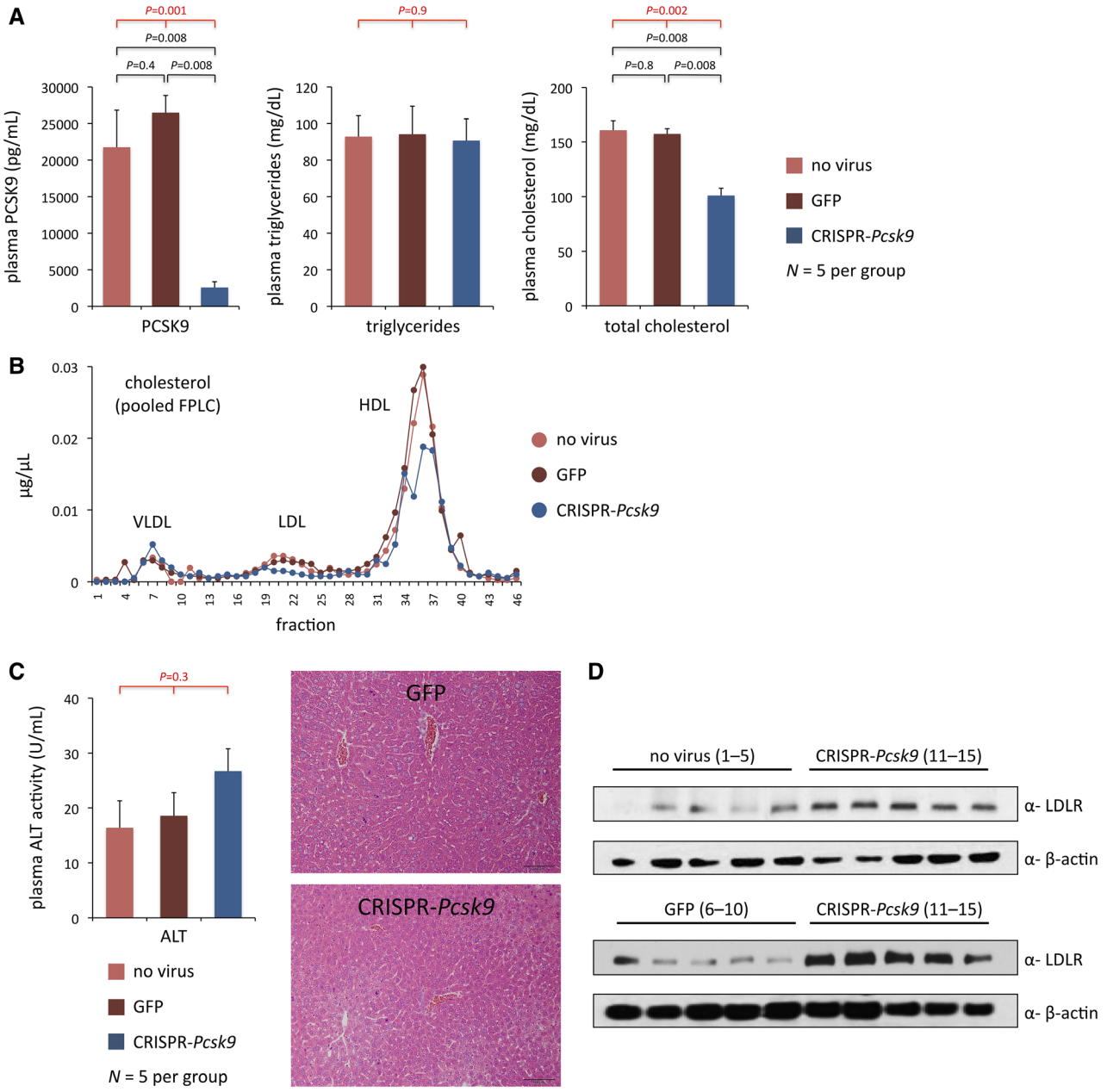

Figure 2. Effects of clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated 9 (Cas9) genome editing on mice. A, Results of ELISA for proprotein convertase subtilisin/kexin type 9 (PCSK9) protein, measurements of triglyceride levels, and measurements of total cholesterol levels in plasma samples from mice 4 d after receiving no adenovirus, green fluorescent protein (GFP) adenovirus, or CRISPR-*Pcsk9* adenovirus (n=5 mice for each group). **B**, Full plasma lipoprotein cholesterol (FPLC) profiles of pooled plasma samples from each group of mice. **C**, Plasma alanine transaminase (ALT) levels in mice 4 d after receiving virus (n=5 mice for each group) and hematoxylin/eosin stains of liver sections from representative mice. **D**, Results of Western blot analysis of liver samples taken from mice 4 d after receiving virus. LDLR indicates low-density lipoprotein receptor. For **A** and **C**, *P* values were determined by the Kruskal–Wallis test among all 3 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 SEM.

one might be able to target not just *PCSK9* but several 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.

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Disclosures

None.

References

1. Abifadel M, Varret M, Rabès JP, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat Genet*. 2003;34:154–156.
2. Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK, Hobbs HH. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat Genet*. 2005;37:161–165.
3. Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N Engl J Med*. 2006;354:1264–1272.
4. Zhao Z, Tuakli-Wosornu Y, Lagace TA, Kinch L, Grishin NV, Horton JD, Cohen JC, Hobbs HH. Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. *Am J Hum Genet*. 2006;79:514–523.
5. Hooper AJ, Marais AD, Tanyanyiwa DM, Burnett JR. The C679X mutation in PCSK9 is present and lowers blood cholesterol in a Southern African population. *Atherosclerosis*. 2007;193:445–448.
6. Stein EA, Swergold GD. Potential of proprotein convertase subtilisin/kexin type 9 based therapeutics. *Curr Atheroscler Rep*. 2013;15:310.
7. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339:819–823.
8. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823–826.
9. Rashid S, Curtis DE, Garuti R, Anderson NN, Bashmakov Y, Ho YK, Hammer RE, Moon YA, Horton JD. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proc Natl Acad Sci U S A*. 2005;102:5374–5379.
10. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T, Anderson DG. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype [published online ahead of print]. *Nat Biotechnol*. doi:10.1038/nbt.2884.

Novelty and Significance

What Is Known?

- Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems allow for high-efficiency genome editing, particularly gene knockout, in vitro.
- Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) loss-of-function mutations confer reduced blood cholesterol levels and protection against coronary heart disease in humans.

What New Information Does This Article Contribute?

- When delivered by adenovirus, CRISPR–CRISPR-associated 9 produces efficient knockout of *Pcsk9* in mouse hepatocytes in vivo.
- Knockout of *Pcsk9* by CRISPR–CRISPR-associated 9 in mouse liver results in reduced plasma PCSK9 protein and cholesterol levels.

Individuals with naturally occurring loss-of-function *PCSK9* mutations experience reduced blood cholesterol levels and protection against coronary heart disease without any known adverse consequences. Genome editing allows for permanent alteration of genes in mammalian cells. We report that a single administration of the CRISPR–CRISPR-associated 9 genome editing system to mice produced a high proportion of loss-of-function alleles of the *Pcsk9* gene in the liver in vivo, resulting in substantially reduced cholesterol levels (35–40%). This approach may be therapeutically useful in humans, potentially taking the form of a 1-time vaccination that would permanently reduce cholesterol levels and protect against coronary heart disease.