10.1161/CIRCULATIONAHA.119.042476

In Vivo AAV-CRISPR/Cas9-Mediated Gene Editing Ameliorates Atherosclerosis in Familial Hypercholesterolemia

Running Title: *Zhao et al.; Gene Editing of Familial Hypercholesterolemia*

Huan Zhao, PhD[1]; Yan Li, PhD[1]; Lingjuan He, PhD[1]; Wenjuan Pu, PhD[1]; Wei Yu, PhD[1]; Yi Li, PhD[1], Yan-Ting Wu, MD[2,3]; Chenming Xu, MD[2,3]; Yuda Wei, PhD[4]; Qiurong Ding, PhD[4]; Bao-Liang Song, PhD[5]; Hefeng Huang, MD[2,3]; Bin Zhou, MD, PhD[1,4,6-9]

[1]The State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Science, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Shanghai, 200031, China; [2]The International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China; [3]Shanghai Key Laboratory of Embryo Original Diseases, Shanghai, China; [4]CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Chinese Academy of Sciences, 200031, P. R. China; [5]Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, Institute for Advanced Studies, Wuhan University, Wuhan 430072, China; [6]School of Life Science and Technology, ShanghaiTech University, Shanghai, 201210, China; [7]Key Laboratory of Regenerative Medicine of Ministry of Education, Jinan University, Guangzhou, 510632, China; [8]The Collaborative Innovation Center for Cardiovascular Disease Translational Medicine, Nanjing Medical University, Nanjing 211100, China; [9]Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing, China

Addresses for Correspondence:
Hefeng Huang, MD
910 Hengshan Road
Shanghai, China, 200031
Tel: +86-21-64070434
Fax: +86-21-64070434
Email: huanghefg@hotmail.com
or huanghefg@sjtu.edu.cn

Bin Zhou, MD, PhD
320 Yueyang Road, A2112
Shanghai, China, 200031
Tel: +86-21-54920974
Fax: +86-21-54920974
Email: zhoubin@sibs.ac.cn

Abstract

Background: Mutations in low-density lipoprotein receptor (*LDLR*) are one of the main causes of familial hypercholesterolemia (FH), which induces atherosclerosis and has a high lifetime risk of cardiovascular disease. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is an effective tool for gene editing to correct gene mutations and thus ameliorate disease.

Methods: To determine whether *in vivo* somatic cell gene editing through the CRISPR/Cas9 system delivered by adeno-associated virus (AAV) could treat FH caused by the *Ldlr* mutant in a mouse model. We generated a nonsense point mutation mouse line, $Ldlr^{E208X}$, based on a relevant FH-related gene mutation. The AAV-CRISPR/Cas9 was designed to correct the point mutation in the *Ldlr* gene in hepatocytes and was delivered subcutaneously into $Ldlr^{E208X}$ mice.

Results: We found that homogeneous $Ldlr^{E208X}$ mice (n=6) exhibited severe atherosclerotic phenotypes after a High-fat diet regimen and the *Ldlr* mutation was corrected in a subset of hepatocytes after AAV-CRISPR/Cas9 treatment, with LDLR protein expression partially restored (n=6). Compared with the control groups (n=6 each group), the AAV-CRISPR/Cas9 with targeted sgRNA group (n=6) had significant reductions in total cholesterol, total triglyceride, and LDL-cholesterol in the serum, while the aorta had smaller atherosclerotic plaques and a lower degree of macrophage infiltration.

Conclusions: Our work shows that *in vivo* AAV-CRISPR/Cas9-mediated *Ldlr* gene correction can partially rescue LDLR expression and effectively ameliorate atherosclerosis phenotypes in *Ldlr* mutants, providing a potential therapeutic approach for the treatment of FH patients.

Key Words: Familial hypercholesterolemia; atherosclerosis; LDLR; CRISPR/Cas9; AAV; gene editing

Non-standard Abbreviations and Acronyms

LDLR	low-density lipoprotein receptor
FH	familial hypercholesterolemia
AAV	adeno-associated virus
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats /Cas9
LDL-C	low-density lipoprotein cholesterol
APOB	Apolipoprotein B
PCSK9	proprotein convertase subtilisin kexin9
HeFH	heterozygous individuals with FH
HoFH	homozygous individuals with FH
TBG	thyroxin-binding globulin
sgRNA	single guide RNA
PAM	prospacer adjacent motif
DSBs	double strand breaks
NHEJ	nonhomologous end-joining
HDR	homology directed repair
indels	insertions and deletions
α-SMA	α-smooth muscle actin
SM22α	smooth muscle protein 22α
SM-MHC	smooth muscle myosin heavy chains
TC	total cholesterol
TG	total triglyceride
T7E1	The T7 Endonuclease I
RFLP	restriction-fragment length polymorphism
ALT	alanine aminotransferase
AST	aspartate aminotransferase

Clinical Perspective

What is new?

- A new *Ldlr*^{E208X} knock-in mouse containing the E208X nonsense point mutation was generated for modeling of human familial hypercholesterolemia pathogenesis.
- *In vivo* gene editing of *Ldlr*^{E208X} by a single injection of AAV-CRISPR/Cas9 could reduce lipid accumulation and ameliorate atherosclerosis phenotypes.

What are the clinical implications?

- The new mutant knock-in mouse model serves as a valuable tool to study human familial hypercholesterolemia pathogenesis and to explore its new therapeutic approaches.
- *In vivo* somatic cell gene editing via an AAV-CRISPR/Cas9 delivery system may represent a potential therapeutic approach for the treatment of human familial hypercholesterolemia.

Introduction

Hypercholesterolemia is characterized by very high levels of low-density lipoprotein cholesterol (LDL-C) in the blood, and the patients have a high lifetime risk of premature cardiovascular disorders, such as atherosclerosis and heart attack. For some patients, hypercholesterolemia is inherited due to a genetic mutation, and the most common form of inherited hypercholesterolemia is called familial hypercholesterolemia (FH)[1, 2]. FH is an autosomal dominant genetic disease in humans with a gene dosage effect, and is due to mutations in the genes encoding either the low-density lipoprotein receptor (*LDLR*), Apolipoprotein B (*APOB*), or proprotein convertase subtilisin kexin9 (*PCSK9*), with mutations in each such gene accounting for approximately 90%, 5% and 1% of FH cases, respectively[2-4]. LDLR, which is a cell surface protein, is highly expressed in hepatocytes and is the primary mechanism to remove excess low-density lipoprotein from the circulation[3]. Mutations in *LDLR* that result in low protein expression level of functional LDLR leads to excess LDL in the circulation, which results in the development and progression of atherosclerotic plaques[5, 6]. The severity of atherosclerosis is closely related to the level and activity of LDLR in the liver[5-7].

In the population, 1 in 200 to 500 people have heterozygous mutations in *LDLR*, which may lead to cardiovascular disease in middle age[2, 6, 8]. Since heterozygote individuals have some residual function of LDLR (at a level of 2-25% compared to normal people), current drug treatments for FH, e.g., MG-CoA reductase inhibitors (statins), are usually effective in heterozygous individuals rather than those who are homozygous for a pathological mutation[6]. With early detection and proper drug treatment, plasma LDL levels in heterozygous individuals with FH (HeFH) harboring mutant *LDLR* can remain in a relatively acceptable range[6]. However, if underdiagnosed or undertreated, homozygous individuals with FH (HoFH), which occurs at a

frequency of 1 in a million in most countries, have very rapid development of severe atherosclerosis and cardiovascular disease, starting in infancy or childhood and leading to early premature death[2, 6, 7, 9-11]. As HoFH is marked by barely any functional LDLR protein, high-intensity statins and PCSK9 inhibitors have very limited therapeutic effect[6, 7]. To ensure survival of individuals with HoFH, lifelong therapeutic apheresis to remove excess LDL or liver transplantation must be implemented[12-15], which is very expensive and difficult to implement. The difference in disease outcomes and therapies between HeFH and HoFH strongly indicates that even low-level expression and activity of residual LDLR can significantly influence the speed of progression and the severity of clinical manifestation of FH.

Gene therapy delivered by Adeno-associated virus (AAV) is currently approved for more than 80 diseases and has been used in clinical trials[16-18]. AAV is a small replication-defective virus that can infect cells from humans or other species[16]. Many different serotypes of AAV have been developed to more precisely deliver the genes of interest to the targeted organ or specific cell types[16, 19]. For HoFH treatment, hepatic AAV-LDLR to deliver wild-type LDLR expression under the control of liver specific thyroxin-binding globulin (TBG) promoter directed gene therapy is currently in phase II clinical trials (ClinicalTrials.gov NCT02651675)[18]. Despite the success of liver AAV-directed gene therapy for FH, long-term therapeutic effects have yet to be seen. Because of the fast hepatocyte turnover rate in childhood, the non-integrating AAV vector could be lost overtime. Instead, correction of the pathological mutation at the DNA level *in vivo* may present a better alternative treatment for each individual because of its permanence in effect and standardized procedures for different *Ldlr* mutant FH patients.

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is a powerful genome-editing system that consists of the Cas9 nuclease and modified single guide

RNA (sgRNA)[20-23]. The Cas9-mediated double strand breaks (DSBs) can be repaired by mainly two mechanisms – the nonhomologous end-joining (NHEJ) and homology directed repair (HDR). The NHEJ pathway, however, can introduce insertions and deletions (indels) in an unpredictable way. The alternative HDR pathway enables precise genome editing accompanied by a DNA donor template, but is largely limited to dividing cells[24]. Both DNA repair mechanisms utilized by the CRISPR/Cas9 system have already been successfully implemented in a few disease settings, such as Duchenne muscular dystrophy (DMD) and liver metabolic disease, to modify disease-causing gene mutations *in vivo*, representing a promising new treatment for human disorders caused by genetic defects[25-33].

In this study, we generated an *Ldlr*^{E208X} mutant knock-in mouse model based on an E207X nonsense point mutation in *LDLR* that is observed in an individual with FH[8]. The *Ldlr*^{E208X} mutant mice, which harbors a nonsense mutation in the fourth exon of the endogenous *Ldlr* gene, showed no LDLR protein expression in the liver and developed severe atherosclerosis after feeding on a High-fat diet. When the mutant *Ldlr*^{E208X} strain was treated with AAV-CRISPR/Cas9, LDLR expression was partially restored, and the atherosclerosis was ameliorated. These results suggest that gene editing *in vivo* via an AAV-CRISPR/Cas9 delivery system may represent a promising therapeutic approach to the treatment of HoFH while potentially helping to improve the efficacy of current lipid-lowering drugs.

Methods

The data, mouse model, and materials that support the findings of this study are available from the corresponding author upon reasonable request. All animal procedures were approved by the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Institute for

Nutritional Sciences, and the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The *Ldlr*^{E208X} mouse line was generated by Cyagen Inc.

Statistical Analysis

All data are presented as the mean value ± standard error of the mean (S.E.M.). Unpaired two-tailed Student's *t*-test was used to compare two groups, one-way ANOVA was performed for multiple groups comparison, and the mixed-effects ANOVA was performed for the serial measurement.

Detailed Methods are included in the Supplemental Material.

Results

Generation of *Ldlr*^{E208X} Mutant Mice

Of the 18 exons in *LDLR*, most mutations occur in the fourth exon[34]. Previous studies have identified an E207X (a G to T point mutation, GAG-TAG) nonsense point mutation form of *LDLR* in an individual with FH (Figure 1A)[8]. We therefore delivered the CRISPR/Cas9 system into fertilized eggs to generate a knock-in mouse line expressing an E208X mutation (GAG>TAG) in the *Ldlr* gene by homology-directed repair, which was equivalent to the E207X mutation in the human *LDLR* gene (Figure 1B and C). A silent mutation (ATC>ATA) was also introduced to prevent the binding and re-cutting of the sequence by sgRNA (Figure 1C). Homozygous *Ldlr* knockout mouse (*Ldlr*$^{-/-}$) is a widely used mouse model for atherosclerosis disease research, while the heterozygous strain (*Ldlr*$^{+/-}$) has a very mild and variable atherosclerotic phenotype after High-fat diet feeding[5, 7, 35]. We therefore used homozygous *Ldlr-E208X* mutant mice in this study and refer to this model as *Ldlr*^{E208X} hereafter. As an internal

control, we used wild-type littermate mice for comparisons in experimental studies. Sanger sequencing results of the genomic DNA from the indicated mice validated the successful generation of *Ldlr*^{E208X} mutant mouse (Figure 1D). By semi-reverse transcription PCR (*RT-PCR*) and Western blotting assays, we did not detect *Ldlr* mRNA (Figure 1E and F) or LDLR protein (Figure 1G and H) in the livers of *Ldlr*^{E208X} mice. Immunostaining for LDLR on liver sections showed that hepatocytes in *Ldlr*^{E208X} mice did not express LDLR (Figure 1I and J). These data demonstrated the successful generation of a mouse genetic model for the human *LDLR* mutant[8].

Severe atherosclerosis is induced in *Ldlr*^{E208X} after High-fat diet feeding

The LDLR protein has a critical role in taking up LDL from the serum and thus regulating cholesterol levels in the circulation system[36]. Since the *Ldlr*^{E208X} mice have no functional LDLR, they have a higher incidence of atherosclerosis and cardiovascular disease. On a regular chow diet, we could detect a mild degree of atherosclerotic plaque development, macrophage infiltration and lipid accumulation in the 24 week-old *Ldlr*^{E208X} mice (Supplemental Figure 1). We therefore used a High-fat diet (containing 21% fat together with 0.21% cholesterol) to accelerate the atherosclerotic plaque formation in mutant mice and to determine whether the nonsense mutation in *Ldlr*^{E208X} mice could lead to an atherosclerotic phenotype. We fed *Ldlr*^{E208X} male mice and wild-type male mice with a 12-week-long High-fat diet starting at the age of 6 weeks (Figure 2A). The body weight of both groups increased after 12 weeks of High-fat diet feeding, but the weight of *Ldlr*^{E208X} mice was significantly higher than that of the wild-type control after 6 weeks of High-fat diet feeding and thereafter (Figure 2B). After High-fat diet feeding, atherosclerotic lesions were noticeable in the aorta in the *Ldlr*^{E208X} mice upon staining with Oil Red O, while atherosclerotic lesions were hardly detected in the wild-type control mice (plaque area: 7.72% ± 0.50% mutant vs. 0.0% ± 0.0% wild-type; Figure 2C and E). In addition,

Oil Red O staining on aortic root sections from *Ldlr*^{E208X} mice revealed excessive lipid accumulation in the aortic tunica media, and more than 80% of the perimeter was affected by atherosclerosis in *Ldlr*^{E208X} mice but not wild-type mice (87.17% ± 5.06% mutant vs. 0.0% ± 0.0% wild-type; Figure 2D and F). Immunofluorescent staining showed that the elastic tissue and smooth muscle layer in the aortic sections of *Ldlr*^{E208X} mice were discontinuous and disrupted (Figure 2G). Notably, several markers of smooth muscle cells, including α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), smooth muscle myosin heavy chains (SM-MHC) and calponin for immunofluorescent staining on aortic sections from two groups showed that these SMC cytoskeletal proteins were profoundly lower or even lost in the atherosclerotic plaque, representing a change from contractile to synthetic phenotypes, known as smooth muscle cell phenotypic modulation[37] (Figure 2G and Supplemental Figure 2). A substantial number of macrophages were seen to infiltrate and accumulate in the lesion area of *Ldlr*^{E208X} mice compared with that of wild-type mice (Figure 2G). Sirus Red staining of aortic tissue sections showed a markedly greater degree of fibrosis in the atherosclerotic plaques of the *Ldlr*^{E208X} mice compared with the littermate controls (Figure 2H). Additionally, Oil Red O staining of liver sections showed that large lipid droplets accumulated in the hepatocytes of the *Ldlr*^{E208X} mice compared with wild-type controls (26.67 ± 1.82% mutant vs. 1.25 ± 0.48% wild-type; Figure 2I and J). Compared with the wild-type control, the *Ldlr*^{E208X} group also displayed significantly higher plasma total cholesterol (TC; 38.28 ± 2.04 mmol/L mutant vs. 5.85 ± 0.77 mmol/L wild-type; Figure 2K), total triglyceride (TG; 9.71 ± 0.65 mmol/L mutant vs. 1.03 ± 0.12 mmol/L control; Figure 2K) and LDL-cholesterol (LDL-C; 28.59 ± 1.69 mmol/L mutant vs. 2.17 ± 0.08 mmol/L control; Figure 2K). These data indicated that this mouse model has many of the

pathological and clinical features of the human FH disease, such as a strong atherosclerotic phenotype[8].

AAV-CRISPR/Cas9 treatment partially restores LDLR expression in *Ldlr*^{E208X} mice

As AAV serotype 8 transduces hepatocytes at a high specificity and efficiency[17], an AAV8-CRISPR/Cas9 system was generated to target the point mutation, aiming to partially restore the expression of LDLR in the liver of *Ldlr*^{E208X} mice. We first tested 4 sgRNA sequences based on the prospacer adjacent motif (PAM) sequences NGG near the *Ldlr*^{E208X} point mutation (Supplemental Figure 3A). sgRNAs and Cas9 nuclease were both cloned into puromycin-resistant vectors and transfected into mouse Hepa1-6 cells to test their targeting efficiency. The T7 Endonuclease I (T7E1) assay, which could detect double-strand breaks and indels, was used to evaluate the targeting efficiency of sgRNAs. We found that among all designed sgRNA generated indels, sgRNA3 had the highest targeting efficiency (Supplemental Figure 3B). We therefore used sgRNA3 in the AAV-CRISPR/Cas9 system for *in vivo* somatic cell gene editing of mutant *Ldlr*.

The neonatal hepatocyte has a high proliferative ability and thus could be an ideal model for HDR-mediated gene correction[25, 29, 38]. Therefore, we also modified the 1.9kb donor template with the mutated PAM sequence to avoid re-cleavage of Cas9 and inserted a SpeI restriction enzyme site in the fourth intron (Supplemental Figure 6C) to evaluate the efficiency of HDR using the restriction-fragment length polymorphism (RFLP) assay. As AAV can be used to efficiently transduce neonatal hepatocytes[25, 38], and as HoFH should be gene corrected as early as possible, we injected the AAV8-GFP vectors subcutaneously using an insulin syringe into neonates at P1-P2 (Supplemental Figure 4A)[39]. We should mention that while intravenous injection of AAV typically leads to a high degree of transduction of hepatocytes, that approach is

more technically challenging than subcutaneous injection in neonatal mice, which is why we used the latter means of injection in our study here. After subcutaneous injection, the fluorescent staining of GFP in P6 liver sections showed high efficiency of viral transduction, as 87.08% ± 1.76% hepatocytes were GFP^+ (Supplemental Figure IVB). We also analyzed the livers of P6, P12, 4 week-old and 8 week-old mice for GFP fluorescence and found that the non-integrated AAV was gradually lost from the hepatocytes (Supplemental Figure 4C-D).

Due to the limited size capacity of the AAV vector for gene delivery, we used a dual AAV system, with one carrying the Cas9 with the liver-specific TBG promoter (named AAV-Cas9) and the other carrying the sgRNA and donor sequence driven by the U6 promoter (named AAV-sgRNA-Donor hereafter, Figure 3A). After successful generation of AAVs, we injected the AAV-Cas9 into neonatal mice at P1-P2, and found a high-targeting efficiency of Cas9 in 1 week-old mouse liver sections (Supplemental Figure 5A-B). Similar to the result of AAV-GFP, we could hardly detect Cas9 expression in the liver sections collected from adult mice (Supplemental Figure 5C-D).

Mixtures of the two AAVs (AAV-Cas9 and AAV-sgRNA-Donor) were injected subcutaneously into P1-P2 neonatal mice and analyzed at the adult stage (Figure 3B). We set up 4 different groups: Group 1, consisting of $Ldlr^{E208X}$ mice receiving 5×10^{10} genome copies of AAV-Cas9; Group 2, comprised of $Ldlr^{E208X}$ mice receiving 5×10^{11} genome copies of AAV-sgRNA-Donor; Group 3, which was $Ldlr^{E208X}$ mice receiving 5×10^{10} genome copies of AAV-Cas9 and 5×10^{11} genome copies of AAV-sgRNA-Donor, with all AAVs diluted in the same volume of PBS for injection; and Group 4, which was wild-type mice receiving an equal volume of PBS (Figure 3B). If gene editing was successful, a T-G mixture would be detected in the targeted group rather than other groups, as judged by Sanger sequencing of liver DNA nested

PCR from the indicated groups (Supplemental Figure 6A). Meanwhile the restoration would be detected only in liver DNA but not in genomic DNA from other tissues of the same mice (Supplemental Figure 6B), indicating the tissue specificity of the AAV8-TBG delivery system.

To assess the precision of AAV-CRISPR-mediated gene editing, we used deep sequencing to analyze on-target and off-target sites. Indels were detected in ~25% (24.84 ± 1.02%) of the *Ldlr* alleles, and the HDR-mediated correction of the T-G mutation was observed in ~6.7% (6.67 ± 0.64%) of the *Ldlr* alleles from the treated group (Figure 3C, Supplemental Figure 6E). By the RFLP assay, we confirmed the efficient HDR-mediated gene editing (Supplemental Figure 6D). The potential off-target sites were also amplified by PCR for deep sequencing in the Group 3 mice. We found that most of the off-target sites were located in the introns of different genes (Supplemental Table 1), and the indels frequency of these sites was negligible (Supplemental Figure 6F). To further strengthen the high HDR-mediated result, we added a mouse group receiving only AAV-Cas9 and AAV-sgRNA without donor at P1 and analyzed at 4 weeks old (Supplemental Figure 7A). The T7E1 assay, Sanger sequence, and RFLP assay showed similar high indels frequency and there was no detectable HDR in this control group (Supplemental Figure 7B-E).

Next, we examined the *Ldlr* mRNA level by semi-quantitative RT-PCR and detected *Ldlr* mRNA in Group 3 (Figure 3D). The real-time quantitative PCR found that the expressed *Ldlr* mRNA after DNA correction in the Group 3 was partial ~11% (11.09 ± 2.03%) of the wild-type, while no *Ldlr* mRNA was detected in Group 1, Group 2 and AAV-sgRNA treated group (Figure 3E, Supplemental Figure 7F). Consistently, the LDLR protein level in the Group 3 was restored to ~18% (18.01 ± 2.82%) of wild-type levels, while there was no detectable LDLR protein in the Groups 1 and 2 (Figure 3F-3G). Additional evidence of partial recovery of LDLR expression was

supported by immunostaining of liver sections of the different groups. While there was no noticeable signal in the Group 1 or 2, ~20% of hepatocytes expressed LDLR in the Group 3 (20.50 ± 2.29%, Figure 3H and I), demonstrating the partial restoration of LDLR expression in the liver of those mice.

We next examined liver toxicity after AAV-mediated treatment in *Ldlr*^{E208X} mice. Transaminase levels, including aspartate and alanine aminotransferase (AST and ALT, respectively), were similar between AAV- and PBS-treated groups at adult stage (Supplemental Figure 8A-B). We also conducted histological staining of the livers by H&E from virus-treated or PBS-treated mice in the adult stage. We did not detect any noticeable histological abnormalities in livers from AAV-treated mice (Supplemental Figure 8C), compared with PBS-treated controls. Meanwhile, immunohistochemistry for immune cell markers F4/80, CD11b; and T cell markers CD4, CD8 of *Ldlr*^{E208X} liver sections showed no significant difference in their number between AAV- and PBS-treated mice (Supplemental Figure 8D).

Together, these results indicated that AAV/CRISPR-mediated *Ldlr in vivo* gene editing could partially restore expression of LDLR protein in *Ldlr* mutant mice.

AAV-CRISPR/Cas9 significantly ameliorates atherosclerotic phenotypes in *Ldlr*^{E208X} mice

To assess further the potential translation of AAV-CRISPR/Cas9-mediated gene correction therapy for the treatment of HoFH, we next analyzed the atherosclerotic phenotype and plasma cholesterol levels after High-fat diet feeding in the 4 groups of mice (Figure 4A). On average, the body weight of all groups increased progressively over 12 weeks, but the weight of AAV-CRISPR/Cas9-targeted *Ldlr*^{E208X} mice (Group 3) was significantly lower than that of Groups 1 and 2 after 8 weeks of a High-fat diet regimen (Figure 4B). Importantly, there was no significant difference in body weight between AAV-CRISPR-targeted *Ldlr*^{E208X} mice (Group 3) and wild-

type control mice (Group 4, Figure 4B). Oil Red O staining of aortas showed that the atherosclerotic plaque burden was significantly less in the targeted *Ldlr*^{E208X} group (Group 3) compared with the Groups 1 and 2 (Figure 4C). Detailed histological analyses of aortic sections by Oil Red O staining and immunostaining for smooth muscle cells marker SMA and endothelial cells marker Ve-cad showed that the lipid accumulation in aortic tunica media and the disruption of the smooth muscle layer were substantially alleviated in Group 3 compared with those in Groups 1 and 2 (Figure 4D and E). Immunostaining for macrophages marker F4/80 and Sirius Red staining on tissue sections showed a lower degree of macrophage infiltration and collagen deposition (fibrosis) in the atherosclerotic plaques (Supplemental Figure 9). Quantification of the stained aortic tissue sections showed a significantly lower degree of both plaque area (7.25 ± 0.78% Group 1, 7.38 ± 0.59% Group 2 vs. 2.78 ± 0.39% Group 3) and lower percentage of perimeters affected by atherosclerosis in Group 3 mice compared to those in Groups 1 and 2 (87.83 ± 2.44% Group 1, 86.33 ± 2.95% Group 2 vs. 25.00 ± 4.08% Group 3, Figure 4F and G).

We also detected much smaller lipid droplets in liver sections from AAV-CRISPR-targeted *Ldlr*^{E208X} mice compared with those of Groups 1 and 2 (31.67 ± 2.17% Group 1, 31.50% ± 2.67% Group 2 vs. 7.83 ± 1.74% Group 3, Figure 4H and I). In the plasma, the total cholesterol (40.74 ± 2.17 mmol/L Group 1, 38.92 ± 2.98 mmol/L Group 2 vs. 12.84 ± 1.91 mmol/L Group 3), total triglyceride (9.12 ± 0.58 mmol/L Group 1, 8.59 ± 0.53 mmol/L Group 2 vs. 4.05 ± 0.55 mmol/L Group 3) and LDL cholesterol (30.08 ± 1.26 mmol/L Group 1, 28.61 ± 1.63 mmol/L Group 2 vs. 9.98 ± 0.86 mmol/L Group 3) were significantly lower in Group 3 mice than those in Groups 1 and 2 (Figure 4J). These data provide convincing evidence for the efficiency of the AAV-CRISPR/Cas9 system in *Ldlr* mutant gene correction and its efficacy in treatment of atherosclerotic phenotypes in the HoFH model (Figure 5).

Discussion

In this work, we generated a novel gene mutant mouse model (*Ldlr*^{E208X}) based on an individual with familial hypercholesterolemia who harbors a nonsense point mutation in the *LDLR* genome[8]. This *Ldlr*^{E208X} mice could be a new gene mutation mouse model and serve as a valuable tool for exploring different strategies for treating human HoFH pathogenesis. By *in vivo* genome editing using the AAV-CRISPR/Cas9 system, we managed to treat a familial hypercholesterolemia model in *Ldlr* mutant mice. We found that delivery of AAV-CRISPR/Cas9 to liver significantly lowered plasma cholesterol, triglyceride and LDL-cholesterol, protected the mutant from severe atherosclerosis and effectively alleviated hepatic fat accumulation in *Ldlr*^{E208X} mice.

In our work, although the genome editing efficiency was not high, the partial restoration of LDLR protein in the liver could significantly ameliorate atherosclerotic phenotypes in mutant mice. It appears interesting that the partial recovery of the wild type sequence (~7%) led to a higher percentage of repaired hepatocytes (~20%). There are several reasons that might explain this apparent discrepancy. First, about half of liver cells are nonparenchymal cells (e.g., Kupffer cells and endothelial cells)[40], which cannot be transfected with AAV8. This may underrepresent the actual genomic editing efficiency in hepatocytes. Second, repaired cells may have a greater propensity for proliferation[29-31, 33, 38]. It is possible that repaired hepatocytes have a higher proliferation rate than unrepaired hepatocytes[33]. Third, most hepatocytes have two or more nuclei[41]. Efficient gene editing in one nucleus might result in sufficient protein expression in that hepatocyte, leading to the discrepancy between the gene correction ratio and the repaired cell ratio. Fourth, murine hepatocytes are commonly polyploid[42], one corrected allele may sufficient to produce corrected proteins in the cell[25, 31, 33]. Fifth, LDLR is a cell membrane protein and thus

some negative neighboring hepatocytes might be counted falsely as positive, over-estimating the percentage of repaired cells by immunofluorescence approach. While the current AAV-CRISPR/Cas9-mediated *Ldlr* gene editing system was not effective enough to completely rescue the atherosclerotic- or hypercholesterolemia-related phenotypes, the partial amelioration by gene therapy could be combined with conventional interventions, such as medication for LDL cholesterol reduction and diet modification, to provide better therapeutic effects for individuals with HoFH. Importantly, the AAV-CRISPR/Cas9-mediated gene editing of *Ldlr* is a genetic modification, which may exert long-term therapeutic effects. Thus, this work provides a new approach for improving the therapeutic effect of current drug treatment in individuals with HoFH.

The CRISPR/Cas9 system has two major ways to repair gene defects: homology-directed repair (HDR) and nonhomologous end joining (NHEJ). Precise correction of a specific mutation through HDR is limited to proliferating cells. Individuals with homozygous *LDLR* mutant could have a very rapid development of severe cardiovascular disease in infancy or childhood. Thus, gene correction therapy should be employed at an early stage for such individuals to achieve better therapeutic outcomes. In our mouse model study, we injected AAV-CRISPR/Cas9 at the neonatal stage when most hepatocytes still have a proliferative capacity. Since the correction was made at the DNA level, the HDR-mediated restoration of the LDLR protein is permanent and durable as long as the gene corrected hepatocytes exist and self-renew, which would have a lifelong therapeutic effect. The neonatal hepatocytes have a high proliferative ability, thus enabling high HDR-mediated gene correction. Previously, Yang et al. used a dual AAV system to correct an *OTC* gene defect in livers of newborn mice. Their deep sequencing at the adult stage showed that the indels rate was 31% (26.5%-35.5%) and HDR rate was about 10% (6.7%-

20.1%)[25]. The frequency for HDR in our study was similar to the previous work, as we also treated mice with AAV at the neonatal stage. When Yang et al. injected high vector doses of AAVs in the adult stage, they observed that, while the indels frequency was 42.0%, HDR frequency was low (1.7%), which could be due to the reduced hepatocyte division in the adult stage compared to that of neonates. Recently, Wang et al. used the AAV-CRISPR/Cas9-mediated gene targeting to correct human factor IX in a mouse model of hemophilia B. A single injection of dual AAV vectors to livers of neonates led to about 35% indels and the HDR frequency was about 10%[38]. Therefore, HDR efficiency is much higher in the neonatal liver than the adult liver, which is due to active hepatocyte proliferation in neonates.

Recently, base editing has been shown to be an alternative gene editing tool for correction of one nucleotide base pair[43-46]. However, recent works have shown the genome-wide off-target effects of base editing[47, 48]. Nevertheless, with the continuous rapid development of this cutting-edge technology, we believe it will become an important tool for gene therapy in the future, especially for diseases involving genetic point mutations.

Here, we showed that an AAV-CRISPR/Cas9 system could be an effective therapeutic approach for correcting gene mutations in somatic cells for HoFH and thus has clinical implications. Future studies should address the ability to correct HoFH gene defects in large animal models, as much more careful consideration needs to be given before the approach here can be applied in the clinical. Along with the decrease in whole-genome sequencing costs, screening among family members and fetuses will become more convenient and accessible for individual patients, thus identifying FH at an earlier stage in disease progression, which would allow for earlier treatment via gene correction. In future, the AAV-delivered CRISPR/Cas9

system could be employed to correct some somatic gene mutations in heritable cardiovascular diseases, ameliorating or even rescuing the clinical manifestation of diseases.

Acknowledgments

We thank Shanghai Biomodel Organism Co., Ltd. and Cyagen Biosciences Incorporation. We also thank Baojin Wu, Guoyuan Chen, Zhonghui Weng and Aimin Huang for the animal husbandry; Wei Bian for technical help and the National Center for Protein Science Shanghai for helping in confocal imaging.

Sources of Funding

National Key Research & Development Program of China (2018YFA0108100, 2019YFA0110400, 2018YFA0107900, 2016YFC1300600, 2017YFC1001303), This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (CAS, XDA16010507, XDB19000000), National Science Foundation of China (31730112, 91639302, 31625019, 91849202, 81761138040, 81872241, 31701292, 31922032), Youth Innovation Promotion Association of CAS, Key Project of Frontier Sciences of CAS (QYZDB-SSW-SMC003), Shanghai Science and Technology Commission (17ZR1449600, 17ZR1449800, 19ZR1479800), the Program for Guangdong Introduction Innovative and Entrepreneurial Teams (2017ZT07S347), Innovative research team of high-level local universities in Shanghai, Major Program of Development Fund for Shanghai Zhangjiang National Innovtaion Demonstration Zone (Stem Cell Strategic Biobank and Stem Cell Clinical Technology Transformation Platform, ZJ2018-ZD-004), Young Elite Scientists Sponsorship Program Grant, Youth Innovation Promotion Association Grant, Shanghai Yangfan Project, China Postdoctoral Science

Foundation, China Postdoctoral Innovative Talent Support Program, Boehringer Ingelheim, Sanofi-SIBS Fellowship, Astrazeneca, Royal Society-Newton Advanced Fellowship.

Disclosures

None.

References

1. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL and Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J Clin Invest*. 1973;52:1544-68.
2. Defesche JC, Gidding SS, Harada-Shiba M, Hegele RA, Santos RD and Wierzbicki AS. Familial hypercholesterolaemia. *Nat Rev Dis Primers*. 2017;3:17093.
3. Hobbs HH, Brown MS and Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat*. 1992;1:445-66.
4. Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, Wiklund O, Hegele RA, Raal FJ, Defesche JC, Wiegman A, Santos RD, Watts GF, Parhofer KG, Hovingh GK, Kovanen PT, Boileau C, Averna M, Boren J, Bruckert E, Catapano AL, Kuivenhoven JA, Pajukanta P, Ray K, Stalenhoef AFH, Stroes E, Taskinen MR, Tybjaerg-Hansen A and Conse EAS. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease. *Eur Heart J*. 2013;34:3478-90a.
5. Ishibashi S, Brown MS, Goldstein JL, Gerard RD, Hammer RE and Herz J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J Clin Invest*. 1993;92:883-93.
6. Rader DJ, Cohen J and Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *J Clin Invest*. 2003;111:1795-1803.
7. Oka K, Pastore L, Kim IH, Merched A, Nomura S, Lee HJ, Merched-Sauvage M, Arden-Riley C, Lee B, Finegold M, Beaudet A and Chan L. Long-term stable correction of low-density lipoprotein receptor-deficient mice with a helper-dependent adenoviral vector expressing the very low-density lipoprotein receptor. *Circulation*. 2001;103:1274-81.
8. Sun XM, Patel DD, Webb JC, Knight BL, Fan LM, Cai HJ and Soutar AK. Familial hypercholesterolemia in China. Identification of mutations in the LDL-receptor gene that result in a receptor-negative phenotype. *Arterioscler Thromb*. 1994;14:85-94.
9. Raal F, Panz V, Immelman A and Pilcher G. Elevated PCSK9 levels in untreated patients with heterozygous or homozygous familial hypercholesterolemia and the response to high-dose statin therapy. *J Am Heart Assoc*. 2013;2:e000028.
10. Soutar AK and Naoumova RP. Mechanisms of disease: genetic causes of familial hypercholesterolemia. *Nat Clin Pract Cardiovasc Med*. 2007;4:214-25.

11. Vallejo-Vaz AJ, Kondapally Seshasai SR, Cole D, Hovingh GK, Kastelein JJ, Mata P, Raal FJ, Santos RD, Soran H, Watts GF, Abifadel M, Aguilar-Salinas CA, Akram A, Alnouri F, Alonso R, Al-Rasadi K, Banach M, Bogsrud MP, Bourbon M, Bruckert E, Car J, Corral P, Descamps O, Dieplinger H, Durst R, Freiberger T, Gaspar IM, Genest J, Harada-Shiba M, Jiang L, Kayikcioglu M, Lam CS, Latkovskis G, Laufs U, Liberopoulos E, Nilsson L, Nordestgaard BG, O'Donoghue JM, Sahebkar A, Schunkert H, Shehab A, Stoll M, Su TC, Susekov A, Widen E, Catapano AL and Ray KK. Familial hypercholesterolaemia: A global call to arms. *Atherosclerosis*. 2015;243:257-9.
12. Bilheimer DW, Goldstein JL, Grundy SM, Starzl TE and Brown MS. Liver transplantation to provide low-density-lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. *N Engl J Med*. 1984;311:1658-64.
13. Revell SP, Noble-Jamieson G, Johnston P, Rasmussen A, Jamieson N and Barnes ND. Liver transplantation for homozygous familial hypercholesterolaemia. *Arch Dis Child*. 1995;73:456-8.
14. Rader DJ, Cohen J and Hobbs HH. Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. *J Clin Invest*. 2003;111:1795-803.
15. Al-Ashwal A, Alnouri F, Sabbour H, Al-Mahfouz A, Al-Sayed N, Razzaghy-Azar M, Al-Allaf F, Al-Waili K, Banerjee Y, Genest J, Santos RD and Al-Rasadi K. Identification and Treatment of Patients with Homozygous Familial Hypercholesterolaemia: Information and Recommendations from a Middle East Advisory Panel. *Curr Vasc Pharmacol*. 2015;13:759-70.
16. Daya S and Berns KI. Gene Therapy Using Adeno-Associated Virus Vectors. *Clin Microbiol Rev*. 2008;21:583-593.
17. Mueller C and Flotte TR. Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Ther*. 2008;15:858-863.
18. Tontonoz P, Wu XH, Jones M, Zhang ZY, Salisbury D and Sallam T. Long Noncoding RNA Facilitated Gene Therapy Reduces Atherosclerosis in a Murine Model of Familial Hypercholesterolemia. *Circulation*. 2017;136:776-778.
19. Muramatsu S. Gene therapy using adeno-associated virus vectors. *Cancer Sci*. 2018;109:1200-1200.
20. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L and Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;154:1370-9.
21. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA and Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339:819-23.
22. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F and Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153:910-8.
23. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE and Church GM. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823-6.
24. Panier S and Durocher D. Push back to respond better: regulatory inhibition of the DNA double-strand break response. *Nat Rev Mol Cell Bio*. 2013;14:661-672.
25. Yang Y, Wang L, Bell P, McMenamin D, He Z, White J, Yu H, Xu C, Morizono H, Musunuru K, Batshaw ML and Wilson JM. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. *Nat Biotechnol*. 2016;34:334-8.

26. Tabebordbar M, Zhu K, Cheng JKW, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang F, Vandenberghe LH, Church GM and Wagers AJ. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science*. 2016;351:407-411.
27. Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R and Olson EN. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016;351:400-3.
28. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX, Asokan A, Zhang F, Duan D and Gersbach CA. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. 2016;351:403-7.
29. Traxler EA, Yao Y, Wang Y-D, Woodard KJ, Kurita R, Nakamura Y, Hughes JR, Hardison RC, Blobel GA, Li C and Weiss MJ. A genome-editing strategy to treat β-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition. *Nat Med*. 2016;22:987-990.
30. Amoasii L, Long C, Li H, Mireault AA, Shelton JM, Sanchez-Ortiz E, McAnally JR, Bhattacharyya S, Schmidt F, Grimm D, Hauschka SD, Bassel-Duby R and Olson EN. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Sci Transl Med*. 2017;9:eaan8081.
31. Amoasii L, Hildyard JCW, Li H, Sanchez-Ortiz E, Mireault A, Caballero D, Harron R, Stathopoulou TR, Massey C, Shelton JM, Bassel-Duby R, Piercy RJ and Olson EN. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Science*. 2018;362:86-91.
32. Long C, Li H, Tiburcy M, Rodriguez-Caycedo C, Kyrychenko V, Zhou H, Zhang Y, Min YL, Shelton JM, Mammen PPA, Liaw NY, Zimmermann WH, Bassel-Duby R, Schneider JW and Olson EN. Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. *Sci Adv*. 2018;4:eaap9004.
33. Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, Xue W and Anderson DG. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol*. 2016;34:328-33.
34. Austin MA, Hutter CM, Zimmern RL and Humphries SE. Genetic causes of monogenic heterozygous familial hypercholesterolemia: a HuGE prevalence review. *Am J Epidemiol*. 2004;160:407-20.
35. Kapourchali FR, Surendiran G, Chen L, Uitz E, Bahadori B and Moghadasian MH. Animal models of atherosclerosis. *World J Clin Cases*. 2014;2:126-32.
36. Nykjaer A and Willnow TE. The low-density lipoprotein receptor gene family: a cellular Swiss army knife? *Trends Cell Biol*. 2002;12:273-80.
37. Allahverdian S, Chaabane C, Boukais K, Francis GA and Bochaton-Piallat ML. Smooth muscle cell fate and plasticity in atherosclerosis. *Cardiovasc Res*. 2018;114:540-550.
38. Wang LL, Yang Y, Breton CA, White J, Zhang J, Che Y, Saveliev A, McMenamin D, He ZN, Latshaw C, Li MY and Wilson JM. CRISPR/Cas9-mediated in vivo gene targeting corrects hemostasis in newborn and adult factor IX-knockout mice. *Blood*. 2019;133:2745-2752.

39. Yu W, Huang XZ, Tian XY, Zhang H, He LJ, Wang Y, Nie Y, Hu SS, Lin ZQ, Zhou B, Pu W and Lui KO. GATA4 regulates Fgf16 to promote heart repair after injury. *Development*. 2016;143:936-949.
40. Friedman SL. Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. 2008;88:125-172.
41. Ganem NJ and Pellman D. Limiting the proliferation of polyploid cells. *Cell*. 2007;131:437-440.
42. Guidotti JE, Bregerie O, Robert A, Debey P, Brechot C and Desdouets C. Liver cell polyploidization: A pivotal role for binuclear hepatocytes. *J Biol Chem* 2003;278:19095-19101.
43. Kim K, Ryu SM, Kim ST, Baek G, Kim D, Lim K, Chung E, Kim S and Kim JS. Highly efficient RNA-guided base editing in mouse embryos. *Nat Biotechnol*. 2017;35:435-437.
44. Kim YB, Komor AC, Levy JM, Packer MS, Zhao KT and Liu DR. Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. *Nat Biotechnol*. 2017;35:371-376.
45. Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T and Kondo A. Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nat Biotechnol*. 2017;35:441-443.
46. Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D and Gao C. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat Biotechnol*. 2017;35:438-440.
47. Jin S, Zong Y, Gao Q, Zhu Z, Wang Y, Qin P, Liang C, Wang D, Qiu JL, Zhang F and Gao C. Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science*. 2019;364:292-295.
48. Zuo E, Sun Y, Wei W, Yuan T, Ying W, Sun H, Yuan L, Steinmetz LM, Li Y and Yang H. Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science*. 2019;364:289-292.

Figure Legends

Figure 1. Generation and characterization of *Ldlr*^{E208X} knock-in point mutation mouse line. **A,** Genomic sequence of the Familial hypercholesterolemia individual with a *LDLR* E207X mutation. The mutant and the wild-type codon is marked in red. **B,** The alignment result of human LDLR protein sequence and murine LDLR protein. **C,** Strategy for generation of the *Ldlr*^{E208X} knock-in point mutation mice line. The point mutation site is marked in red, the single guideRNA (sgRNA) sequence is marked as green, and the 5'-TGG protospacer adjacent motif (PAM) is marked as blue. **D,** The Sanger sequence result of the genomic DNA_extracted from *Ldlr*^{E208X} (homozygote) and the wild-type mice. **E,** Semi-quantitative RT-PCR result of *Ldlr* cDNA in the liver tissues from *Ldlr*^{E208X} and wild-type littermates using primers designed to distinguish the point mutation. **F**, Quantification of the *Ldlr* mRNA levels in the liver tissues from two groups. **G,** Western blot of the LDLR protein in the liver tissues from *Ldlr*^{E208X} and wild-type littermates. **H,** Quantification of the LDLR protein levels in the liver tissues from two groups. **I,** Immunostaining for LDLR on liver sections from *Ldlr*^{E208X} and wild-type littermates. **J,** Quantification of the percentage of hepatocytes expressing LDLR. Data are the mean ± s.e.m.; n = 6. Significance was accepted when ****$P < 0.0001$. Scale bars, 100 μm.

Figure 2. Atherosclerosis in *Ldlr*^{E208X} mice after a High-fat diet regimen. A, Schematic figure showing experimental strategy for High-fat diet feeding and analysis. **B,** Body weight of the indicated groups at different times. **C,** Whole-mount Oil Red O staining image of *en face* atherosclerotic lesions in aorta. **D,** Representative staining of aorta root sections with Oil Red O. **E,** Quantification of atherosclerotic lesion areas in the whole aorta from indicated groups. **F,**

Quantification of aortic perimeter affected by atherosclerosis (AS) in the aorta root from each groups. **G,** Immunostaining for smooth muscle actin (SMA), Ve-cad (left panel) and SMA, F4/80 (right panel) on aortic root slides collected from indicated mice. **H,** Sirus Red staining on aortic root sections collected from indicated mice. **I,** Oil Red O staining of liver sections collected from indicated mice. **J,** Quantification of Oil Red O positive areas in livers sections from indicated groups. **K,** Plasma levels of total cholesterol, total triglyceride, LDL-cholesterol in indicated mice. Significance was accepted when $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$. Data are the mean ± s.e.m.; n = 6. Scale bars, yellow, 1 mm; black and white,100 μm.

Figure 3. Partial recovery of LDLR expression in the livers of treated *Ldlr*^{E208X} mice. A, Strategy for generation of adeno-associated virus (AAV) -Cas9 and AAV-sgRNA-Donor. **B,** Schematic figure showing experimental strategy for AAV injection and analysis. **C,** Deep sequence result showing insertions and deletions (indels) frequency and homology directed repair (HDR) frequency in liver tissues of targeted Group3. **D,** Semi-quantitative RT-PCR result of the *Ldlr* cDNA in liver tissues from indicated groups. **E,** Quantification of qRT-PCR result of *Ldlr* expression levels of the liver tissues from indicated groups. **F-G,** Western blot result and quantification of the LDLR protein levels in livers of indicated groups. **H,** Immunostaining for LDLR on liver sections from indicated groups. **I,** Quantification of the percentage of hepatocytes expressing LDLR. Data are the mean ± s.e.m.; n = 5 to 6. Significance was accepted when $**P < 0.01$; $****P < 0.0001$. Scale bars, 100 μm.

Figure 4. Amelioration of atherosclerosis in *Ldlr*^{E208X} after AAV-CRISPR/Cas9 treatment. **A,** Experimental strategy for adeno-associated virus (AAV) injection, High-fat Diet induction

and analysis. **B,** Body weight of indicated groups at different times. **C,** Oil Red O staining of *en face* atherosclerotic lesions in aortas from indicated groups. **D,** Oil Red O staining on aortic root sections from indicated mice. **E,** Immunostaining for smooth muscle actin (SMA) and Ve-cad on aorta root sections collected from indicated groups. **F,** Quantification of atherosclerosis lesion areas in each group. **G,** Quantification of aortic perimeter affected by atherosclerosis (AS) in each group. **H,** Oil Red O staining on liver sections from indicated groups. **I,** Quantification of Oil Red O positive areas on liver sections in indicated groups. **J,** Plasma levels of total cholesterol, total triglyceride, LDL-cholesterol in indicated group. Significance was accepted when $*P < 0.05$; $***P < 0.001$; $****P < 0.0001$; n.s., non-significant; Data are the mean ± s.e.m.; n = 6. Scale bars, 100 μm.

Figure 5. Partial recovery of LDLR protein and amelioration of atherosclerosis phenotypes in *Ldlr*^{E208X} after AAV-CRISPR/Cas9 treatment. Cartoon image showing that the *Ldlr*^{E208X} mouse has severe atherosclerosis phenotype when treated with High-fat diet. After AAV-CRISPR/Cas9 treatment, the LDLR protein was partially recovered and atherosclerosis phenotypes were significantly ameliorated in *Ldlr*^{E208X} mice compared with controls.