# CRISPR/Cas9-Mediated Knockout of APOC3 Stabilizes Plasma Lipids and Inhibits Atherosclerosis in Rabbits

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**Research**

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# Abstract

**Background:** High levels of apolipoprotein C3 (APOC3) can lead to hypertriglyceridemia, which increases the risk of cardiovascular disease. We aim to create APOC3 knockout (KO) rabbits and explore the effects of APOC3 deletion on the occurrence and development of atherosclerosis.

**Methods:** A sgRNA anchored to exon 2 of APOC3 was designed to edit embryos using the CRISPR/Cas9 system. The founder rabbits were sequenced, and their lipid profile, inflammatory cytokines and atherosclerotic plaques were analyzed.

**Results:** When given a normal chow (NC) diet, all APOC3 KO rabbits had low plasma TG level, which was two times lower than that of the matched age control group. Additionally, their plasma lipoprotein lipase (LPL) increased. When fed a high-fat diet (HFD), it was observed that APOC3 deficiency was more conducive to the maintenance of plasma TG, total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in rabbits, the inhibition of inflammatory response and the protection of atherosclerosis in rabbits.

**Conclusion:** APOC3 KO rabbit models can be obtained with the CRISPR/Cas9 system to combat the formation of HFD-induced atherosclerosis, indicating a novel therapeutic target to treat atherosclerosis.


# Introduction

APOC3 is a key regulator of plasma triglycerides (TG) and shows significant correlation with plasma very low-density lipoprotein (VLDL) levels[1, 2]. Hypertriglyceridemia represents an independent risk factor for cardio- and cerebrovascular diseases[3, 4]. Although the in vivo studies on APOC3 are mainly based on mouse models, great differences in lipids metabolism have existed between mice and humans. Carriers with APOC3 mutations had a 40 percent lower risk of coronary heart disease[5], but no protective effect of APOC3 deficiency on atherogenesis was observed in KO mice[6]. Mice secrete VLDL containing either apolipoprotein B100 (Apob100) or apolipoprotein B48 (Apob48), while human and rabbit VLDL only contains APOB100. As opposed to mice, in which high-density lipoprotein (HDL) is the main plasma lipoprotein, rabbits and humans have LDL-rich lipoprotein profiles[7]. All animal models of human disease have strengths and limitations, and domestic rabbits (Oryctolagus cuniculus) continue to play significant roles in the study of lipid metabolism as well as histopathology[8].

CRISPR/Cas9 is a naturally occurring genome editing tool that forms part of the bacterial defense mechanism, and has since been developed as a tool for genetic manipulation in mice[9], rabbits[10, 11], dogs[12], hamsters[13] etc. Compared to other genome-editing technologies like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)[14], CRISPR/Cas9 has higher targeting efficiency, is less expensive, and is easier to design and implement[15]. In addition, CRIRP-mediated genome editing is emerging as a therapeutic strategy against cardiovascular diseases[16]. In this study, we aimed to generate APOC3 KO rabbits with biallelic mutations via the CRISPR/Cas9 system

anchored to exon 2 to study the relationship between APOC3 deficiency, abnormal lipid metabolism, and the formation and development of atherosclerosis.

## Material And Methods

# Animals

New Zealand White (NZW) rabbits were purchased from the Animal Genetic Engineering Laboratory at Yangzhou University. Rabbits were housed in a barrier facility with a 12-hour light/dark cycle at 24°C and 55% humidity. Rabbits were allowed access to water ad libitum and fed twice a day with normal chow (NC) diet or HFD (TP 2R301, Trophic, Ltd, Nantong, China). All animal studies were conducted according to the approval of the Animal Care Committee of the Yangzhou University.

# Cas9 mRNA and sgRNA preparation

To design mutant loci of the rabbit APOC3 gene, we obtained sequences from the Library of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and designed the CRISPR/Cas9 single-guide (sg)RNAs using the tool on the website: http:// crispr.mit.edu. Then we chose one sgRNA targeting loci of rabbit APOC3 by anchoring to exon 2, as shown in Fig. 1.

# Zygote injection and embryo transplantation

Female rabbits older than 6 months and fertile male rabbits were used to obtain embryos. Donor rabbits were superovulated with 60 IU pregnant mare serum gonadotropin, and 72 hours later, the donors and recipients were simultaneously injected with 10 IU human chorionic gonadotropin, and the donors were mated with male rabbits. Embryos were flushed out 18–20 hours later using PBS and collected for microinjection. A mixed solution containing Cas9 mRNA (33 ng/ul) and sgRNA (40 ng/ul) was microinjected into the cytoplasm of embryos under a Leica inverted light microscope. The injected embryos were transferred into M2 cushion fluid and incubated at 38°C, 5% CO2 for 30 min. Then, 10–15 injected zygotes were transferred into the oviducts of pseudo-pregnant females.

# Sequencing analysis of founder rabbits

Genomic DNA was extracted from tail biopsy specimens using the phenol-chloroform extraction method. The sgRNA target sites were amplified by PCR using the primers, F: 5'-GCTCACCCAGCTGAGATCCAT-3', R: 5'-CAAAGTGCTTACGGGCAGAGG-3'. PCR products were purified after agarose gel electrophoresis and cloned into a pMDTM 19-T vector (Takara Bio, Inc., Japan). The positive clones were sequenced and analyzed using Lasergene software (DNASTAR, Inc., U.S).

# Extraction and analysis of mRNA in liver and small intestine

Total RNA was extracted from liver or small intestine using TRIzol reagent (Gibco; Thermo Fisher Scientific, Inc.) and reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). cDNA was amplified by RT-qPCR using Mini Opticon Real-Time PCR detection system

(Bio-Rad) with GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The relative quantity of mRNA was calculated using the 2 − ΔΔCq method and normalized to GAPDH. Triplicate RT-qPCR reactions were preformed using three separate samples.

The sequences of the primers are as follows: APOC3: For 5’-CCTCCCTTCTCAGCGTCATG-3’, Rev 5’-GTCCCAGAACCCAGAGAACT-3’ and GADPH: For 5’-CATGTTTGTGATGGGCGTGAACAA-3’, Rev 3’-TAAGCAGTTGGTGGTGCAGGAT-3'.

# Off-target assay

The CRISPR/Cas9 system enables the efficient modification of genes in living cells and organisms for studying the phenotypic effects of genetic perturbations. However, certain off-target effects of this technology should be considered. To test whether off-target mutations occurred in the APOC3 KO rabbits, potential off-target sites (POTS) of the sgRNAs were predicted with the online CRISPR Design Tool (http://crispr.mit.edu/), and the top five POTS for each sgRNA were selected and PCR amplified with the primers listed in Table 1. The PCR products were subjected to Sanger sequencing.

Table 1
Primers for detection of possible off-target sites

| Primers | off-targets of GCAGGGCCAGAGCCCAGGTG AGG |
|---|---|
| off-targets-1 | 5’ TCGTCGGCAGCGTCACAAAGTGCTTACGGGCAGA 3’ |
| | 5’ GTCTCGTGGGCTCGGCTCCACTTTCCTCCCTGCAG 3’ |
| off-targets-2 | 5’ TCGTCGGCAGCGTCGAGAAGAAGCACCACCCCTC 3’ |
| | 5’ GTCTCGTGGGCTCGGACAGGGATCAAGGAAGGACTG 3’ |
| off-targets-3 | 5’ TCGTCGGCAGCGTCGGAAATGGTGAGTGAGCCCA 3’ |
| | 5’ GTCTCGTGGGCTCGGCAGTCACACGGCTTAGTCGT 3’ |
| off-targets-4 | 5’ TCGTCGGCAGCGTCTCGTGAAGGACTCTCCACCA 3’ |
| | 5’ GTCTCGTGGGCTCGGAATCTCACCTTCGACAGCCG 3’ |
| off-targets-5 | 5’ TCGTCGGCAGCGTCCGACAGGGGTGGGGAAAC 3’ |
| | 5’ GTCTCGTGGGCTCGGTTTCCCAGAAACCTGCCCTC 3’ |

# Phenotypic examinations

# Analysis of lipids and apolipoproteins

EDTA plasma was collected after 12 hours of fasting. The TC, TG, LDL-C and, HDL-cholesterol (HDL-C) levels in plasma were measured enzymatically using commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Western blotting was used to detect plasma APOC3, APOA1, APOE and PCSK9. The following primary antibodies were used: goat anti-APOC3 (genecreate, Wuhan, China), sheep anti-

apoA1 (Bio-Rad AbD Serotec, Kidlington, UK), goat anti-APOE (Rockland Inc., Limerick, PA, US), and mouse anti-PCSK9 (Abcam, Cambridge, MA, USA) polyclonal antibodies. The secondary antibodies were horseradish-peroxidase-conjugated donkey anti-goat IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA), donkey anti-sheep IgG (Chemicon, Temecula, CA, US) and goat anti-mouse IgG (Sangon Biotech, Shanghai, China).

# Analysis of plasma lipoprotein profiles

Plasma lipoprotein fractions were determined by agarose gel electrophoresis. Plasma (2 μl) was electrophoresed on 1% agarose gel (Helena Laboratories, Saitama, Japan) and stained for neutral lipids with Fat Red 7B.

# Analysis of plasma HL, LPL

The activities of hepaticlipase (HL) and LPL were assayed using commercial kits (Solarbio, Beijing, China).

# Analysis of plasma inflammatory mediators and white blood cell counts

Plasma levels of interleukin (IL) -1β and tumor necrosis factor alpha (TNF-α) were measured using rabbit ELISA kits (IL-1β, Elabscience Biotechnology Co., Ltd., Wuhan, China; TNF-α, Cusabio, Wuhan, China, https://www.cusabio.com.). Complete blood counts were measured using a BC-2800Vet auto hematology analyzer (Mindray Medical International Limited, Shenzhen, China).

# Analysis of fat tolerance

The rabbits were fasted for 12h, weighed, and orally gavaged with olive oil (Aladdin) at 10 ml

per kg body weight using a 20 ml syringe. Blood was collected before and after 1, 3, 5 and 7h of

gavage, and the plasma TG levels at each time point were measured as already described. Oral fat tolerance tests (OFTT) was evaluated in terms of the accumulation and clearance of TG.

# Analysis of atherosclerosis and liver pathological changes

We analyzed the atherosclerotic lesions of the aorta using previously described methods[17]. In brief, the rabbits were sacrificed by lethal injection with sodium pentobarbital solution. The aortic trees were isolated and opened out longitudinally and, after fixing in formalin for 24 hours, they were stained with Sudan IV (Solarbio Life Science, China). The Sudan IV-positive area was measured using Image-Pro Plus (Media Cybernetics, Inc. US) and expressed as a percentage of the total surface area.

For histological analysis, the aortic root samples were paraffin sectioned and stained with hematoxylin-eosin (HE) and Masson's trichrome (MT) and immunohistochemically stained with monoclonal antibodies against either macrophages (Mφ) (clone: RAM11, Dako, Carpinteria, CA, US) or α-smooth muscle actin for smooth muscle cells (clone: HHF35, Dako, Carpinteria, CA, US).

According to a method reported elsewhere, the formalin immersion-fixed hearts were cut into six blocks and embedded in paraffin[18]. The left coronary artery was cross-sectioned (5 μ/slice) and stained with HE. Fresh liver tissues were fixed in formalin for 48 hours, routinely embedded in paraffin, cut into 5-μm serial sections, and processed for HE staining.

## Results

# Production of APOC3 KO rabbits and genotype assay

We designed one sgRNA targeting the rabbit APOC3 anchored to exon 2 to generate mutant rabbits. The sgRNA were in vitro transcribed into mRNAs, which were co-injected with Cas9 mRNAs into rabbit zygotes. As shown in Table 2, 144 embryos were injected with Cas9 mRNA and 101 of which were transferred to five pseudo-pregnant recipient rabbits (16–20 embryos per recipient), three recipient rabbits became pregnant, and a total of five pups were born (numbered C1-C5), two of which died on the day of birth (C4 and C5).

A pair of primers were designed to amplify the target fragment of APOC3 (F:5'-GCTCACCCAGCTGAGATCCAT-3', R:5'-CAAAGTGCTTACGGGCAGAGG-3'), which was sequenced to determine whether the gene was mutated; then the PCR products were sequenced by TA-clone to further determine the mutation type and efficiency. The PCR results in Fig. 2A show that all 5 rabbits had overlapping peaks, suggesting that there may have been mutations, such as deletions, insertions, or frameshifts. The PCR products were cloned into the pMDTM 19-T vector, and the genomic DNA sequences are shown in Fig. 2B. The theoretical amino acid sequences of the APOC3 mutant alleles are shown in Fig. 2C. C1□, C2□and C3□both had frameshift mutations, which ultimately led to the termination of translation. The amino acids of C1(1) and C3(1) were the same, the amino acids of C1(2) and C2(1) were the same.

Western blotting analysis showed that the APOC3 was undetectable in the serum of the APOC3 KO rabbits (Fig. 2D). Furthermore, we measured RNA levels by RT-qPCR and shows that the content of APOC3 was still detectable among the WT rabbit liver and small intestine, but the expression was not detected in these founder rabbits (Fig. 2E). Finally, a total of five POTS were successfully amplified using PCR and subjected to Sanger sequencing, no overlapping peaks were detected around the POTS. Taken together, genetic KO of APOC3 was achieved in a rabbit model by CRISPR/Cas9 gene editing technology.

Table 2
Summary of Apoc3-KO rabbits generation and gene targeting efficiency.

| Number of microinjection | NO.1 | NO.2 | NO.3 |
|---|---|---|---|
| No. of injected embryos | 51 | 56 | 37 |
| No. of transferred embryos | 43 | 38 | 20 |
| No. of recipients | 2 | 2 | 1 |
| No. of pregnancies | 2 | 1 | 0 |
| No. of births in total | 3 | 2 | 0 |
| No. of live pups | 2 | 1 | 0 |
| No. of mutants | 4 | 1 | 0 |
| Rate of mutations (%) | 100 | | |

# Analysis of lipids, apolipoproteins, and lipoproteins

APOC3 can effectively increase the level of circulating TG[19–21], We collected fasting blood samples for the determination of blood lipid concentration. As predicted, APOC3 KO rabbits showed significantly lower plasma TG levels and higher plasma HDL-C levels than control rabbits of the same age under a normal chow (NC) diet (**Table 3**). However, there was no significant difference in plasma TC and LDL-C levels between the two groups.

To understand the effects of APOC3 on lipid metabolism in more detail, APOC3 KO rabbits and control rabbits were put on an HFD supplemented with 3% cholesterol and 10% soybean oil for 12 weeks, and their fasting plasma lipid profiles were measured on the 6th and 12th weeks while the plasma of WT rabbits appeared cloudy due to the high lipid content following high-fat feeding, no obvious chylomicron (CM) aggregation was observed in the sera of the APOC3 KO rabbits (Fig. 3A). According to the results, the levels of plasma TG, TC, and LDL-C increased significantly, while the levels of plasma HDL-C decreased significantly in all rabbits on an HFD, but changes in the blood lipid profile in the control group were more obvious than those in the KO group (**Table 3**). After calculation, the levels of plasma TG and LDL-C in the control group increased 13–18 times and 17 times, respectively, while those in the KO group increased 6–10 times and 12 times.

We further analyzed rabbit plasma lipoprotein profiles by agarose gel electrophoresis after 6 weeks of high-fat feeding, which showed that fewer lipoproteins in the plasma of C1-C3 migrated from the original position to the pre-β-area, and more lipoproteins migrated to the α-area (Fig. 3A ). Consistent with this, western blotting analysis showed that the plasma lipoprotein profiles of the WT and APOC3 KO rabbits showed significant differences, while APOB and APOE were significantly reduced in the APOC3 KO rabbits, APOA1 was enriched and led to the high HDL levels (Fig. 4B).

According to the results of OFTT test, the plasma TG level of APOC3 KO rabbits after olive oil gavage was lower than that of the control group (Fig. 3C). Furthermore, after 12 weeks of HFD, plasma LPL activity was significantly increased in KO rabbits compared with control rabbits (Fig. 3D).

Table.3 Plasma levels of TC, TG, LDL-C, and HDL-C of the rabbits.

| Diet | Group | TC (mg/dl) | TG (mg/dl) | LDL-C (mg/dl) | HDL-C (mg/dl) |
|---|---|---|---|---|---|
| NC | WT (n = 5) | 48.0 ± 8.1 | 59.9 ± 13.2 | 23.7 ± 2.9 | 28.7 ± 4.4 |
| | AC1 | 44.6 ± 7.5 | 23.8 ± 11.8 | 18.4 ± 2.7 | 31.1 ± 5.1 |
| | AC2 | 30.8 ± 6.9 | 36.3 ± 8.7 | 18.6 ± 3.1 | 21.5 ± 6.3 |
| | AC3 | 54.3 ± 8.8 | 30.5 ± 6.5 | 20.3 ± 2.6 | 33.7 ± 3.8 |
| HF (6W) | WT (n = 5) | 786.6 ± 48.5 | 877.4 ± 69.8 | 329.9 ± 23.3 | 26.4 ± 9.6 |
| | AC1 | 288.5 | 256.0 | 125.3 | 43.8 |
| | AC2 | 318.8 | 285.7 | 159.3 | 45.1 |
| | AC3 | 355.3 | 298.5 | 201.2 | 39.2 |
| HF (12W) | WT (n = 5) | 805.3 ± 39.7 | 890.7 ± 46.9 | 403.1 ± 49.3 | 25.1 ± 3.8 |
| | AC1 | 316.6 | 224.5 | 221.4 | 37.6 |
| | AC2 | 436.1 | 297.1 | 226.0 | 40.3 |
| | AC3 | 399.0 | 308.3 | 239.5 | 38.5 |

# Analysis of atherosclerosis and liver pathological changes

To directly observe the effects of APOC3 on the occurrence and development of atherosclerosis, we assessed the degree of atherosclerotic lesions in the aorta and coronary arteries of two groups of rabbits after an HFD regime. As shown in Fig. 4A, the WT rabbits had extensive plaques (stained bright red by the Sudan IV dye) in the aortic arch and smaller lesions scattered throughout the aorta. In contrast, only mild lesions were seen in the aorta of APOC3 KO rabbits. The lesioned area comprised 21% and 3% of the aortic region respectively in WT and APOC3 KO rabbits.

After 12 weeks of high-fat diet, HE, MT, anti-RAM-11, and α-SMA staining of the aortic root showed hyperplastic intima and increased collagen contents in WT rabbits, which were accompanied by the accumulation of macrophages and proliferation of smooth muscle cells (Fig. 4B). In comparison, aortic root lesions of APOC3 KO rabbits were much milder. However, no significant changes were observed in the coronary arteries of both groups (Fig. 4C), likely due to the short duration of HFD feeding. According to the results of the HE staining, WT rabbit had a slight accumulation of cytoplasmic lipid droplets in their liver while almost no damage was observed in APOC3 KO rabbits (Fig. 4D).

# Analysis of plasma inflammatory mediators and full blood count

Atherosclerosis is a chronic inflammatory disease, and the inflammatory process is important in both the initiation and progression of lesion development. Therefore, we also analyzed the levels of inflammatory cytokines (IL-1β and TNF-α) and immune cell counts in the rabbits after 12 weeks of HFD feeding. The APOC3 KO rabbits had fewer Leukocytes and monocytes in their peripheral blood compared to the WT rabbits, along with significantly lower serum levels of inflammatory cytokines (Fig. 5A and 5B). Taken together, APOC3 deficiency not only effectively reduced the number of macrophages in atherosclerotic plaques, but also had an effect on systemic inflammation.

## Discussion

Hypertriglyceridemia is a sign of abnormal lipid metabolism, and an independent risk factor in atherosclerotic development[4]. APOC3 is a key regulator of TG metabolism, is a water-soluble low molecular weight lipoprotein that is present in the plasma along with HDLs, VLDLs, CM and LDLs[22]. Studies show that elevated levels of APOC3 inhibits the activity of LPL and HL, which delays triglyceride-rich lipoprotein clearance and increases its levels in the plasma, eventually leading to impaired TG metabolism[23]. Although the in vivo studies on APOC3 are mainly based on mouse models, the rabbit model has several advantages such as easier maintenance, suitable size of the aorta, high fecundity and short gestation periods[24], and similar lipid metabolism and cardiovascular pathophysiology as humans[25]. For instance, the hepatic LDL receptor is normally inactive in the rabbits as in humans, which makes it a highly suitable model for studying the mechanistic basis of AS, as well as the effects of lipid-lowering drugs[26]. Furthermore, rabbits have abundant plasma cholesteryl ester transfer protein, which can help develop strategies to raise plasma HDL-c levels[27]. Finally, both humans and rabbits are more sensitive to an HFD compared to mice[25, 28].

We designed one sgRNA targeting exon 2 of rabbit APOC3 and finally obtained three KO rabbits by CRISPR/Cas9 gene-editing. None of the three founder rabbits contained APOC3 in their livers and small intestine, and no APOC3 protein was detected in plasma. Under an NC diet, the plasma TG levels of the KO rabbits decreased, but the plasma TC and LDL-C levels had no significant difference compared with the control group. It is worth noting that after an HFD, in addition to plasma TG levels, plasma TC and LDL-C levels also began to differ from the control group, indicating that the lack of APOC3 affects cholesterol transport, and this change is aggravated by a high-fat intake. Interestingly, all lipid indices were higher in AC1 compared to AC2 and AC3, which could be due to sex differences.

The plasma lipoprotein profiles of the WT and APOC3 KO rabbits showed significant differences; while APOB and APOE were significantly reduced in the APOC3 KO rabbits, APOA1 was enriched and led to the high HDL levels. This also confirmed that APOC3 may increase plasma TG levels by hindering APOE-mediated uptake of CM and VLDL in the liver by competitively inhibiting APOE binding to its receptor. We then detected a significant increase in plasma LPL activity in three KO rabbits and HL activity in two of

them (perhaps due to the insufficient sample size). LPL is present on the surface of extrahepatic capillary endothelial cells, and hydrolyzes CM and VLDLs[29, 30]. Its activity can be evaluated in terms of the amount of free fatty acids. Due to the role of HL in assisting the conversion of VLDL[31, 32], a large amount of VLDL accumulated in the plasma of the WT rabbits after 6 weeks of high-fat feeding, but few lipoproteins accumulated in the plasma of the KO rabbits. As the main regulator of HDL-C[33], the increase of HL activity also leads to an increase in HDL-C. Taken together, the absence of APOC3 significantly altered the lipoprotein profile and lipcatabolic related enzymes of the high-fat feeding rabbits, inhibited hypertriglyceridemia and improved their fat tolerance by enabling rapid clearance of the TGs.

Atherosclerosis is a chronic inflammatory disease, and the inflammatory process is important in both the initiation and progression of lesion development[34–36]. After detection, plasma IL-1β and TNF-a decreased significantly and the whole-blood monocyte, neutrophil, and platelet counts were significantly lower in KO rabbits than the WT rabbits. IL-1β and TNF-α can increase endothelial permeability and lipoprotein permeability[37, 38]. Extensive monocyte recruitment plays an important role in the development of early atherosclerotic lesions[34, 39, 40]. Neutrophils aggravate endothelial dysfunction, attract monocytes, enter atherosclerotic lesions, and accelerate the formation of foam cells[41]. Studies have confirmed that platelets contribute to the atherosclerotic process at both the early (endothelial disruption) and final stages (rupture of the vulnerable plaque), participating in the process by releasing chemokines, inflammatory mediators, and microparticles[42, 43]. Therefore, it can be boldly speculated that APOC3 is closely related to the formation and development of atherosclerosis, in which a series of inflammatory responses play an important role.

After 12 weeks of high-fat diet, the aortic tree lesion coverage rate of the WT rabbit was as high as 21%, while that in KO rabbits was much lower (only 3%). HE, MT, and immunohistochemical staining showed the WT rabbit had obvious atherosclerotic lesions and increased intima thickening and collagen content, accompanied by the accumulation of macrophages and the proliferation of smooth muscle cells, while KO rabbits only had slight early atherosclerotic lesions. However, there were no obvious pathological changes in the coronary arteries of the two groups. For the formation of coronary artery plaques in rabbits, higher blood lipids (such as seen in Watanabe heritable hyperlipidemic rabbits or LDLR KO rabbits) or other additional conditional interventions, such as hybridization or surgical ligation are usually required. Taken together, lack of APOC3 restrains atherogenesis, although the exact mechanistic basis needs to be elucidated.

Statins are often used to treat dyslipidemia, especially to control the elevation of LDL-C; although clinicians generally believe that the benefits of statins are exaggerated, while the potential side effects are underestimated[44]. In addition, other risk factors for atherosclerosis still exist, such as TG and TG-rich lipoproteins[45]. However, the efficacy of existing triglyceride-lowering drugs is still controversial. Therefore, using experimental animal models that are more similar to the characteristics of human lipid metabolism to study the association between genes and cardiovascular disease may be conducive to the development and application of new drugs.

# Conclusion

Using CRISPR/Cas9 gene editing technology to construct APOC3 deficient rabbit models is convenient, accurate and efficient, which lays a foundation for further research on APOC3 in the future. APOC3 deficiency alleviated cholesterol-induced hyperlipidemia and reduced atherosclerotic plaque formation, indicating that APOC3 is a highly promising therapeutic target for the treatment of AS.

# Study Strengths And Limitations

Only three APOC3 KO primary rabbits were obtained, and their genotypes were not completely consistent, even though in the end APOC3 was not detectable in plasma, liver and small intestine. Small sample size and different genotypes are not conducive to the statistics of experimental data.

# Abbreviations

APOC3
Apolipoprotein C3; TG:Triglycerides; TC:Total cholesterol; LDL-C:Low-density lipoprotein cholesterol; HDL-C:High-density lipoprotein cholesterol; VLDL-C:Very Low-density lipoprotein cholesterol; CM:Chylomicron; APOB:Apolipoprotein B; APOA1:Apolipoprotein A1; APOE:Apolipoprotein E; PCSK9:Proprotein convertase subtilisin/kexin type 9; LPL:lipoprotein lipase; HL:Hepaticlipase; IL-1β:Interleukin 1 beta:TNF-α:Tumor Necrosis Factor-alpha; OFTT:Oral fat tolerance tests; HFD:high-fat diet; NC:Normal chow

# Declarations


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### Authors' contributions

Jingyan Liang, Yong Cheng and Yingge Wang designed the experiments and provided the resources. Yiwen Zha and Yaoyao Lu performed the experiments, acquired and analyzed the data, and wrote the manuscript. Ting Zhang, Kunning Yan and Wenwen Zhuang performed the experiments and acquired data. All authors have read and approved the final paper.

### Competing interests

The authors declare no conflicts of interest.

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# Figures

### Figure 1

Schematic illustration of the CRISPR/Cas9-targeting sites of rabbit APOC3. One sgRNAs are designed for APOC3 anchored exon 2. The sgRNA-targeting sequence is indicated in red, and the protospacer adjacent motif (PAM) sequence is indicated in green.

### Figure 2

Identification of APOC3 knockouts in rabbits. (a): Sanger sequencing peak map of the rabbits that were visualized by Chromas software. (b): Genomic sequence of five mutant rabbits. Deletions are indicated by dotted lines, insertions are indicated in red, and substitutions are indicated in blue. The sizes of the deletions (-) , insertions (+) or substitutions (>) are shown in the right column. (c): Theoretical amino acid sequences of five mutant rabbits. Deletions are indicated by dotted lines, insertions are indicated in red,

substitutions are indicated in blue, and the frameshift mutations are indicated in orange. The sizes of the deletions (-), insertions (+) or substitutions(>) are shown in the right column. (d): Immunoblot showing presence of the 8.8 kDa APOC3 protein in the plasma. (e) The content of APOC3 in liver and in small intestine among WT and mutant rabbits.

## Figure 3

Analysis of plasma lipids, apolipoproteins and lipoproteins in APOC3 KO rabbits. (a) Agarose gel electrophoresis of plasma lipoproteins. 2μl of plasma was loaded in each well, fractionated on 1% agarose gel, and stained with fat red 7B for neutral lipids. The corresponding position of lipoprotein migration positions are marked on the left. (b) Analysis of plasma apolipoproteins by Western blotting. Rabbit plasma samples (8microl) were electrophoresed on SDS-PAGE using 10% gels, transferred to a cellulose membrane, and probed with appropriate antibodies. (c) Postprandial plasma TG levels at 0h, 1h, 2h, 3h, 4h and 5h in rabbits following olive oil gavage, as measured by OFTT. (d) Plasma LPL and HL activity after 12 weeks high-fat feeding. Values are expressed as means ± SD., *P<0.05, **P<0.01.

## Figure 4

Pathological analysis of atherosclerosis and liver in rabbits. (a) Gross lesions of aortic atherosclerosis of rabbits stained with Sudan IV (visualized as red area) and quantification of the lesion areas. (b) Micrographs of aortic atherosclerosis. Serial paraffin sections were stained with HE, MT and immunohistochemically stained with Abs against either RAM-11 for macrophages or α-SMA for SMC (Scale bar = 100μm). (c) Micrographs of coronary artery. Serial paraffin sections were stained with HE (Scale bar = 100μm or 20μm). (d) Micrographs of HE staining of liver (Scale bar = 100μm or 20μm).

## Figure 5

Analysis of plasma inflammatory mediators and full blood count. Values are expressed as means ± SD., *P<0.05, **P<0.01, ***P<0.001.