

# Vaccine Against PCSK9 Improved Renal Fibrosis by Regulating Fatty Acid $\beta$ -Oxidation

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**Background**—Defects in the renal fatty acid  $\beta$ -oxidation pathway have been implicated in the development of renal fibrosis. Our group has developed a therapeutic vaccine targeting PCSK9 (proprotein convertase subtilisin/kexin type 9), named PCSK9Q $\beta$ -003. In this study, we investigated the potential effectiveness of the PCSK9Q $\beta$ -003 vaccine on hypercholesterolemia with renal fibrosis.

**Methods and Results**—The low-density lipoprotein receptor<sup>+/-</sup> male mice fed with a high-cholesterol (1%) Western diet were randomly assigned into 4 groups: the sham group (or the control group), the phosphate-buffered saline group, the Q $\beta$  virus-like particles group and the PCSK9Q $\beta$ -003 vaccine group. Mice of the PCSK9Q $\beta$ -003 group were injected with the PCSK9Q $\beta$ -003 vaccine (100  $\mu$ g/time) every 2 or 4 weeks. The mice were administered with either unilateral ureteral obstruction for 2 weeks or N-nitro-L-arginine methyl ester (50 mg/kg per day) for 6 weeks to establish a renal fibrosis model. Compared with the other 3 groups, the PCSK9Q $\beta$ -003 vaccine obviously decreased total cholesterol and low-density lipoprotein cholesterol in low-density lipoprotein receptor<sup>+/-</sup> mice with hypercholesterolemia. Compared with the phosphate-buffered saline and Q $\beta$  virus-like particles groups, the PCSK9Q $\beta$ -003 vaccine improved hepatic steatosis and renal function. Histology analysis showed that the PCSK9Q $\beta$ -003 vaccine significantly ameliorated renal lipid accumulation and renal fibrosis. Moreover, the PCSK9Q $\beta$ -003 vaccine obviously upregulated the expression of low-density lipoprotein receptor, very-low-density lipoprotein receptor, sterol-regulatory element binding protein 2, and fatty acid  $\beta$ -oxidation-related factors, and ameliorated renal fibrosis-related molecules both in the unilateral ureteral obstruction and N-nitro-L-arginine methyl ester models.

**Conclusions**—This study suggested that the PCSK9Q $\beta$ -003 vaccine improved renal lipid accumulation and renal fibrosis by regulating fatty acid  $\beta$ -oxidation, which may provide a promising method for treating hypercholesterolemia with renal fibrosis. (*J Am Heart Assoc.* 2020;9:e014358. DOI: 10.1161/JAHA.119.014358.)

**Key Words:** fatty acid  $\beta$ -oxidation • hyperlipidemia • proprotein convertase subtilisin/kexin type 9 • renal fibrosis • vaccine

Renal fibrosis is a common pathological process during the progression of chronic kidney disease (CKD) to end-stage renal disease.<sup>1</sup> CKD has exerted a great burden on public health worldwide. According to the World Health Organization estimate, CKD accounted for 1.5% of deaths worldwide in 2012.<sup>2</sup> Patients with CKD have an increased prevalence of hyperlipidemia and an increased risk of cardiovascular disease.<sup>3</sup> Hyperlipidemia is known to escalate

the progression of glomerular injury. Unfortunately, little evidence exists to guide the optimal treatment of dyslipidemia with CKD. Statins are the most common pharmacological intervention to treat dyslipidemia with CKD. However, statins are associated with skeletal muscle complaints, ranging from mild serum creatine kinase elevations and myalgia to severe muscle weakness, muscle cramps, myositis, and rhabdomyolysis.<sup>4,5</sup> Moreover, previous studies indicated that statins

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Accompanying Table S1 and Figures S1 and S2 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.014358>

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## Clinical Perspective

### What Is New?

- This is the first report showing that the PCSK9 (proprotein convertase subtilisin/kexin type 9) Q $\beta$ -003 vaccine significantly improved renal lipid accumulation and attenuated the development of renal fibrosis induced in low-density lipoprotein receptor <sup>+/−</sup> mice with hypercholesterolemia administered with unilateral ureteral obstruction and N-nitro-L-arginine methyl ester by regulating the fatty acid  $\beta$ -oxidation pathway.

### What Are the Clinical Implications?

- It is remarkable that immunization of the PCSK9 Q $\beta$ -003 vaccine significantly improved renal lipid accumulation and attenuated the development of renal fibrosis, and has some potentially superior advantages, including the ability to stably decrease lipid levels, improvement of patient compliance, and low cost.
- Accordingly, the PCSK9Q $\beta$ -003 vaccine may provide a promising avenue for treating hypercholesterolemia with renal fibrosis.

showed limited effectiveness in reducing dyslipidemia in nephrotic syndrome.<sup>6–9</sup> Therefore, current therapies are insufficient, which necessitates the search for new therapeutic strategies in hyperlipidemia with CKD.

The relationship between lipid disturbances and renal diseases has been studied for several decades. It is well recognized that when the balance of renal lipid uptake, synthesis, oxidation, and outflow is disrupted, lipids will undergo excessive oxidation and be sequestered as lipid droplets, generating toxic metabolites that could cause nephrotoxicity in diverse renal diseases.<sup>10,11</sup> Fatty acid  $\beta$ -oxidation (FAO) is one of the major pathways for ATP production in the normal kidney.<sup>12</sup> Defects in the FAO pathway have been implicated in the development of renal fibrosis. Serial studies indicated that improving FAO may offer a new approach for the treatment and prevention of kidney fibrosis.<sup>12,13</sup>

PCSK9 (proprotein convertase subtilisin/kexin type 9) is a hepatic enzymatic protein that negatively regulates low density lipoprotein receptor (LDLR). Plasma PCSK9 binds to the extracellular domain of LDLR, and then mediates internalization and degradation of LDLR, which results in the increase of low-density lipoprotein cholesterol (LDL-C) level. To date, the most advanced approach for PCSK9 inhibition is monoclonal antibody (mAb), which has become one of the most promising therapeutic targets for hypercholesterolemia and could reduce incidences of cardiovascular events in clinical trials.<sup>14</sup> Also, PCSK9 inhibitor was well tolerated in

dyslipidemia with CKD. Of note, in rats with experimental CKD, serum PCSK9 level was significantly increased, and liver LDLR was decreased, accompanied by higher cholesterol.<sup>10</sup> In patients with CKD, there was a significantly direct correlation between increased PCSK9 and high total cholesterol (TC) and LDL-C in plasma.<sup>15</sup> Additionally, PCSK9 level was elevated in patients with CKD and negatively correlated with estimated glomerular filtration rate.<sup>16</sup> An increased level of PCSK9 in patients with nephrotic syndrome was directly correlated with proteinuria.<sup>15–17</sup> Genetic ablation of podocytes and nephrotoxic serum administration resulted in hypercholesterolemia and increased level of PCSK9 in mice.<sup>16,17</sup> Recently, successful treatment of resistant nephrotic syndrome with PCSK9 mAb has been described.<sup>18</sup> Moreover, studies showed that selective endothelin-A antagonism decreased proteinuria and improved lipid profiles in optimally managed patients with CKD through a reduction in PCSK9 level.<sup>19,20</sup> These findings suggested that increased PCSK9 may play a significant role in CKD, and aggravate renal dysfunction by worsening lipid disturbances. Therefore, possible benefits of PCSK9 inhibitor in hyperlipidemia with CKD need to be investigated.

Peroxisome proliferator-activated receptors (PPARs) and PPAR gamma coactivator-1 $\alpha$  (PGC1) are the key transcription factors that regulate the expression of proteins involved in fatty acid uptake and oxidation in various types of tissues and organs.<sup>21–23</sup> Apart from SREBP2 (Sterol-regulatory element binding protein 2), PPAR is another transcription factor that regulate PCSK9 transcription.<sup>24</sup> Considering the significant role of PCSK9 in lipid metabolism, we hypothesized that PCSK9 may participate in the regulation of FAO pathway. PCSK9 mAb is expensive and faces important shortcomings including frequent administration, anti-mAb antibodies production, and high doses. In contrast, active vaccination could overcome these shortcomings.<sup>25,26</sup> Previously, our group has developed a therapeutic vaccine targeting PCSK9 named PCSK9Q $\beta$ -003.<sup>27</sup> In this study, we investigated the potential effectiveness of the PCSK9Q $\beta$ -003 vaccine on renal fibrosis by regulating FAO.

## Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Peptide Synthesis and Vaccine Preparation

The PCSK9-003 epitope peptide (F-A-Q-S-I-P-W-N) derived from human PCSK9 was synthesized by GL Ltd. (Shanghai, China). The purity was above 98% and was determined using high-performance liquid chromatography and mass

spectrometry. The peptide was conjugated to the Q $\beta$  virus-like particles using Sulfo-SMCC crosslinker (Thermo Fisher Scientific, Waltham, MA, USA) to produce the PCSK9Q $\beta$ -003 vaccine. The conjugation rate of PCSK9Q $\beta$ -003 vaccine was analyzed on an SDS-PAGE gel. The vaccine concentration was determined using Bradford protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

## Animals

LDLR<sup>-/-</sup> mice (B6/JNju-Ldlrem1Cd82/Nju) were purchased from the Nanjing BioMedical Research Institute of Nanjing University (Nanjing, China). Wild-type C57BL/6 mice were from Beijing HFK Bio-Technology Co., LTD. Mice were maintained in the Animal Center of Tongji Medical College of Huazhong University of Science and Technology under a specific pathogen-free condition (Permit Number, No. 00261727). LDLR<sup>+/-</sup> mice were bred using F1 from wild-type and LDLR<sup>-/-</sup> mice. All interventions and animal caring procedures were approved by the Animal Care and Use Committee of Union Hospital of Huazhong University of Science and Technology. LDLR<sup>+/-</sup> mice were fed a high-cholesterol (1%) Western diet containing 21% fat from 4 weeks old to the end of the experiment.

## Animals Experiment

### Unilateral ureteral obstruction model

The male LDLR<sup>+/-</sup> mice aged 10 weeks were randomly divided into the following 4 groups (n=6 per group), namely the sham group, the phosphate-buffered saline (PBS) group, the Q $\beta$  virus-like particles (VLP) group, and the PCSK9Q $\beta$ -003 vaccine group. Mice of the PCSK9Q $\beta$ -003 group were subcutaneously immunized with 100  $\mu$ g of PCSK9Q $\beta$ -003 vaccine at weeks 10, 12, 14, and 18. Mice of other groups were administered with an equal volume of PBS or VLP, respectively. The specific peptide antibody titers were detected by ELISA. At week 20, the mice weighing 27 to 29 g were used to establish a unilateral ureteral obstruction (UUO) model. After general anesthesia (1% sodium pentobarbital, 0.5  $\mu$ g/g, intraperitoneal injection), complete UUO was carried out by double ligating the left ureter through 4-0 silk following the left abdominal incision. The ureters of the sham group were exposed but not ligated. Mice were weighed each week. Systolic blood pressure was determined using the tail-cuff method (BP-2010A, Softron, Tokyo, Japan) every 2 weeks. Mice were euthanized at week 22. The ligated kidney was immediately frozen and saved for histology analysis. The detailed proceeding is shown in Figure 1A.

### N-nitro-L-arginine methyl ester-induced renal injury

Male LDLR<sup>+/-</sup> mice aged 10 weeks were randomly divided into the following 4 groups (n=6 per group), namely, the control group, the PBS group, the VLP group and the

PCSK9Q $\beta$ -003 vaccine group. Mice of the PCSK9Q $\beta$ -003 group were subcutaneously immunized with 100  $\mu$ g of PCSK9Q $\beta$ -003 vaccine at weeks 10, 12, 14, 18, and 22. Mice of other groups were administered with an equal volume of PBS or VLP, respectively. The specific peptide antibody titers were detected by ELISA. The PBS, VLP, and PCSK9Q $\beta$ -003 groups were fed with N-nitro-L-arginine methyl ester (L-NAME; 50 mg/kg per day) in drinking water from weeks 22 to 28. Mice were weighed each week. Systolic blood pressure was determined using the tail-cuff method every 2 weeks. The detailed proceeding is shown in Figure S1A.

### Biochemical measurement

Before the measurement, all mice were fasted for 12 hours. The sera were separated by centrifugation at a rate of 1000g for 10 minutes at room temperature. The sera lipids including TC, triglyceride (TG) and LDL-C were measured using biochemical kits (Najing Jianchen Bioengineering Institute, Najing, China). The serum creatinine and blood urea nitrogen were also measured using biochemical kits (Najing Jianchen Bioengineering Institute, Najing, China). Urine samples were collected by using metabolic cages for 24 hours, and the supernatant was used for examination of the urinary protein.

### Serum total PCSK9 quantification

Serum total PCSK9 level was tested by a mouse PCSK9 ELISA kit (R&D Systems, Minneapolis, MN, USA) comparing experimental sera samples to an internal standard curve according to manufacturer's protocol.

### Histology and immunostaining

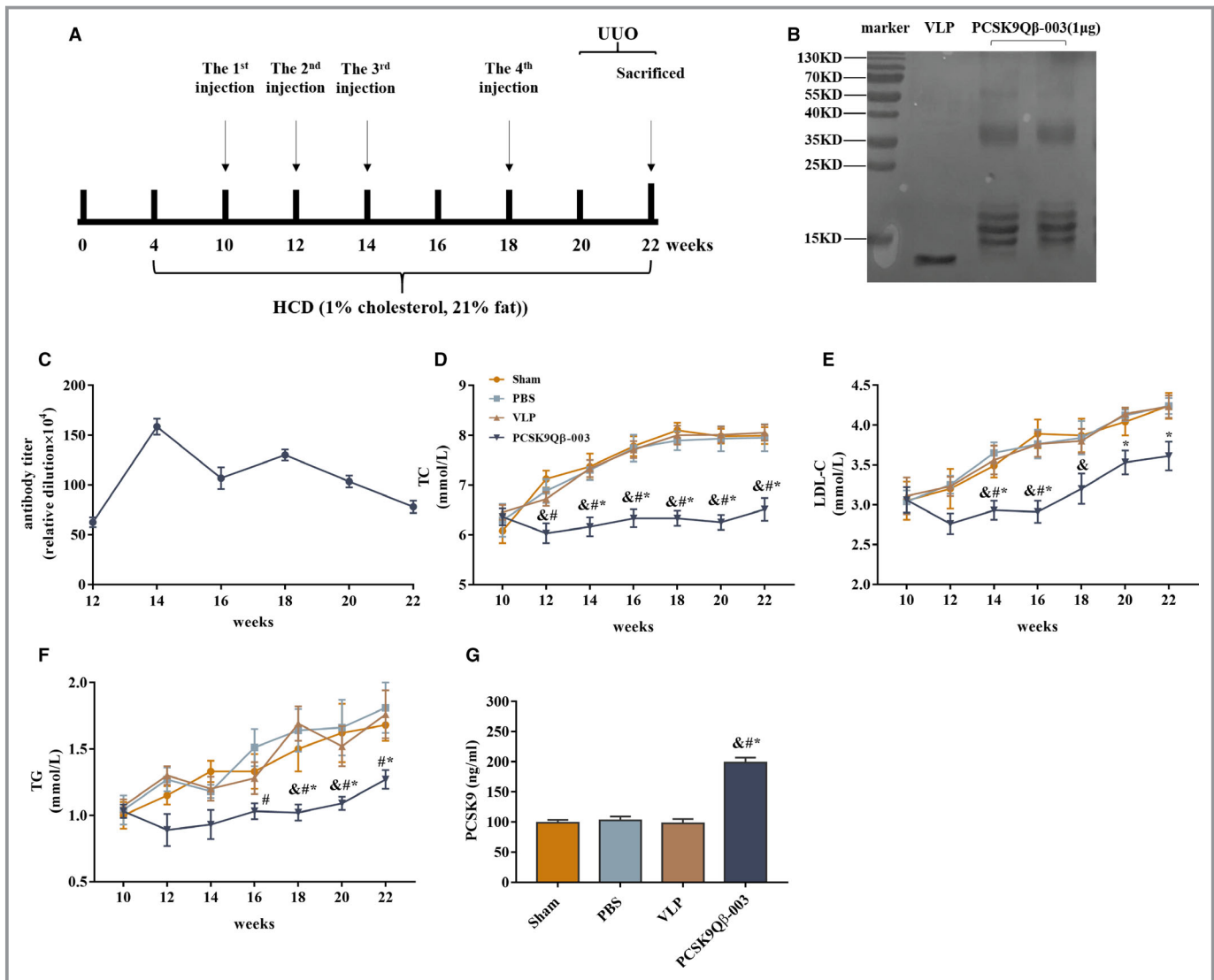
The fresh liver and kidney tissues were immediately fixed in 4% paraformaldehyde overnight and then cut into optimal sections for Oil Red O staining. The other parts were embedded in paraffin for hematoxylin-eosin, Masson's trichrome, and periodic acid-Schiff (PAS) staining.

### Real-time quantitative polymerase chain reaction analysis

Total RNA from liver and kidney was extracted using RNAiso Plus (Takara, Shiga, Japan) following the manufacturer's protocol. The expression of associated genes was assessed by real-time quantitative polymerase chain reaction with a Step One Real-Time PCR machine (Applied Biosystems, Foster City, CA, USA) using TB Green Premix Ex Taq (Takara, Shiga, Japan). Primers are shown in Table S1.

### Western blotting

Liver or kidney samples (20-mg tissues of equivalent location) were homogenized by electric homogenizer (Thundersci, Shanghai, China) in ice-cold protein extraction buffer (Pierce, Dallas, TX, USA) containing a protease inhibitor



**Figure 1.** PCSK9Qβ-003 vaccine decreased serum TC and LDL-C in the UUO model. **A**, Process of animal experiments in the LDLR<sup>+/-</sup> mice+UUO. **B**, The vaccine was analyzed on a SDS-PAGE gel. The figure showed the PCSK9 peptide conjugated to the Qβ virus-like particles. **C**, The LDLR<sup>+/-</sup> mice were immunized subcutaneously, and the anti-PCSK9Qβ-003 titers were measured by ELISA. **D** through **F**, TC, LDLC and TG was decreased in the PCSK9Qβ-003 vaccine group compared with the other groups. **G**, The total PCSK9 level was significantly elevated in the PCSK9Qβ-003 vaccine group compared with the other groups. n=6 per group. Data are expressed as means±SEM. &P<0.05 vs the Sham group; #P<0.05 vs the PBS group; \*P<0.05 vs the Qβ virus-like particles group. LDL-C indicates low-density lipoprotein cholesterol; PBS, the phosphate-buffered saline group; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9Qβ-003, the PCSK9Qβ-003 vaccine group; Sham, the sham group; TC, total cholesterol; TG, triglyceride; UUO, unilateral ureteral obstruction; VLP, the Qβ virus-like particles group.

cocktail (Roche, Basel, Switzerland). Then the homogenates were centrifuged at 15 294g for 15 minutes at 4°C. Protein concentrations were confirmed via the BCA assay kit (Pierce, Dallas, TX, USA). Equivalent amounts of the extracted protein were electrophoresed on 10% SDS polyacrylamide gels and then electro-transferred onto polyvinylidene fluoride membranes (Roche Applied Science, Penzberg, Germany). After being blocked with 5% skim milk, the membranes were incubated with appropriate primary antibodies at 4°C overnight, followed by incubation with an horseradish peroxidase-conjugated secondary antibody. The antigens

were bound by the primary antibodies as follows: anti-SREBP2 (Abcam, ab30682), anti-LDLR (Abcam, ab52818), anti-PPARα (Arigo, ARG55240), anti-peroxisomal acyl-coenzyme A oxidase 1 (ACOX1; Abcam, ab184032), anti-pSmad3 (CST, 9520S), anti-Smad3 (CST, 9523S), and anti-transforming growth factor (TGF)-β (Abcam, ab6179965). The specific bands were detected using Super ECL reagent (Thermo Fisher Scientific, Waltham, MA, USA). The protein levels were determined by densitometry analysis using the Image Lab 3.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

**Table.** Systemic Parameters of Mice at the End of Study

Parameters	Sham/Con	PBS	VLP	PCSK9Q $\beta$ -003
Mice intervened by UUO				
Body weight, g	29.35 $\pm$ 0.80	28.88 $\pm$ 0.73	28.98 $\pm$ 0.93	29.06 $\pm$ 0.71
Kidney weight, mg	309 $\pm$ 6	365 $\pm$ 13	365 $\pm$ 8	335 $\pm$ 13
KW/BW, mg/g	10.53 $\pm$ 0.13	12.65 $\pm$ 0.16*	12.66 $\pm$ 0.44*	11.53 $\pm$ 0.31
Heart rate, bpm	653 $\pm$ 14	657 $\pm$ 11	622 $\pm$ 19	642 $\pm$ 27
SBP, mm Hg	132 $\pm$ 3	131 $\pm$ 2	134 $\pm$ 3	133 $\pm$ 3
Mice intervened by L-NAME				
Body weight, g	29.08 $\pm$ 0.73	28.63 $\pm$ 0.82	28.88 $\pm$ 1.03	28.79 $\pm$ 0.81
Kidney weight, mg	318 $\pm$ 12	308 $\pm$ 5	304 $\pm$ 17	337 $\pm$ 9
KW/BW, mg/g	10.96 $\pm$ 0.52	10.78 $\pm$ 0.37	10.86 $\pm$ 0.47	10.95 $\pm$ 1.42
Heart rate, bpm	587 $\pm$ 18	572 $\pm$ 20	569 $\pm$ 22	582 $\pm$ 20
SBP, mm Hg	130 $\pm$ 3	138 $\pm$ 2	135 $\pm$ 3	137 $\pm$ 4

Data are expressed as means $\pm$ SEM. Con indicates the control group; KW/BW, kidney weight-to-body weight ratio; L-NAME, N-nitro-L-arginine methyl ester; PBS, the phosphate-buffered saline group; PCSK9Q $\beta$ -003, the PCSK9Q $\beta$ -003 vaccine group; SBP, systolic blood pressure; Sham, the sham group; UUO, unilateral ureteral obstruction; VLP, the Q $\beta$  virus-like particles group.

\* $P$ <0.05 vs the control group or the sham group.

## Statistical Analysis

Data are expressed as mean $\pm$ SEM. One-way analysis of variance using Bonferroni's method (for comparison of >2 groups) was used for the statistical analyses. The calculations were performed using Prism 7.0 (GraphPad Software, La Jolla, CA, USA).  $P$ <0.05 was considered as significant.

## Results

### Animal Characteristics

In comparison with the control animals, the 2 batches of mice in the PCSK9Q $\beta$ -003 group had no difference in body weight and kidney weight (Table). Compared with the sham group, the ratio of kidney weight/body weight in the PBS and VLP groups was increased, but no difference was observed between the PCSK9Q $\beta$ -003 and sham groups in LDLR $^{+/-}$  mice intervened by UUO. In mice intervened by L-NAME, the ratio of kidney weight/body weight had no obvious difference among all groups. Moreover, all groups had similar systolic blood pressure and heart rate. No evidence of skin damages and mortality was noted in the vaccine-treated animals.

### PCSK9Q $\beta$ -003 Vaccine Decreased TC and LDL-C

Hypercholesterolemia was induced in male LDLR $^{+/-}$  mice fed with high-cholesterol Western diet, which presented changes with steadily increased blood lipid level (Figure S1B through S1D). The conjugated PCSK9Q $\beta$ -003 vaccine manifested that 1

monomer of VLP coupled with 1 to 4 PCSK9 epitopes (Figure 1B). To confirm the effect of the PCSK9Q $\beta$ -003 vaccine on hypercholesterolemia, the vaccine was used to vaccinate male LDLR $^{+/-}$  mice with hypercholesterolemia. After the second injection of the vaccine, the PCSK9Q $\beta$ -003 specific antibody titer was 1:400 000 to 1:900 000 and rose after the third injection, then peaked at week 14 (1:1 500 000) and gradually decreased thereafter (Figure 1C, Figure S1E). Except the PCSK9Q $\beta$ -003 group, the other 3 groups had similar serum lipids level in the UUO model. Compared with the sham group, obvious decline of TC (6.33 $\pm$ 0.14 versus 8.10 $\pm$ 0.15 mmol/L, 21.8% decline;  $P$ <0.001), LDL-C (2.91 $\pm$ 0.14 versus 3.89 $\pm$ 0.18 mmol/L, 25.2% decline;  $P$ =0.002) and TG (1.02 $\pm$ 0.06 versus 1.50 $\pm$ 0.17 mmol/L, 32.0% decline;  $P$ =0.033) following PCSK9Q $\beta$ -003 vaccination was observed in LDLR $^{+/-}$  mice after the third injection (Figure 1D through 1F). These results are in agreement with that of the L-NAME model (Figure S1F through S1H). To evaluate serum PCSK9 level, total PCSK9 level was measured at the end of the experiment. Total PCSK9 level was significantly elevated in mice vaccinated with the PCSK9Q $\beta$ -003 vaccine compared with the other groups (Figure 1G, Figure S1I).

### PCSK9Q $\beta$ -003 Vaccine Reduced Hepatic Steatosis

To investigate the liver lipid composition, we performed lipid analyses on livers from high-cholesterol Western diet -fed male mice. As shown, the PCSK9Q $\beta$ -003 group had almost no steatosis, whereas other groups had severe steatosis and

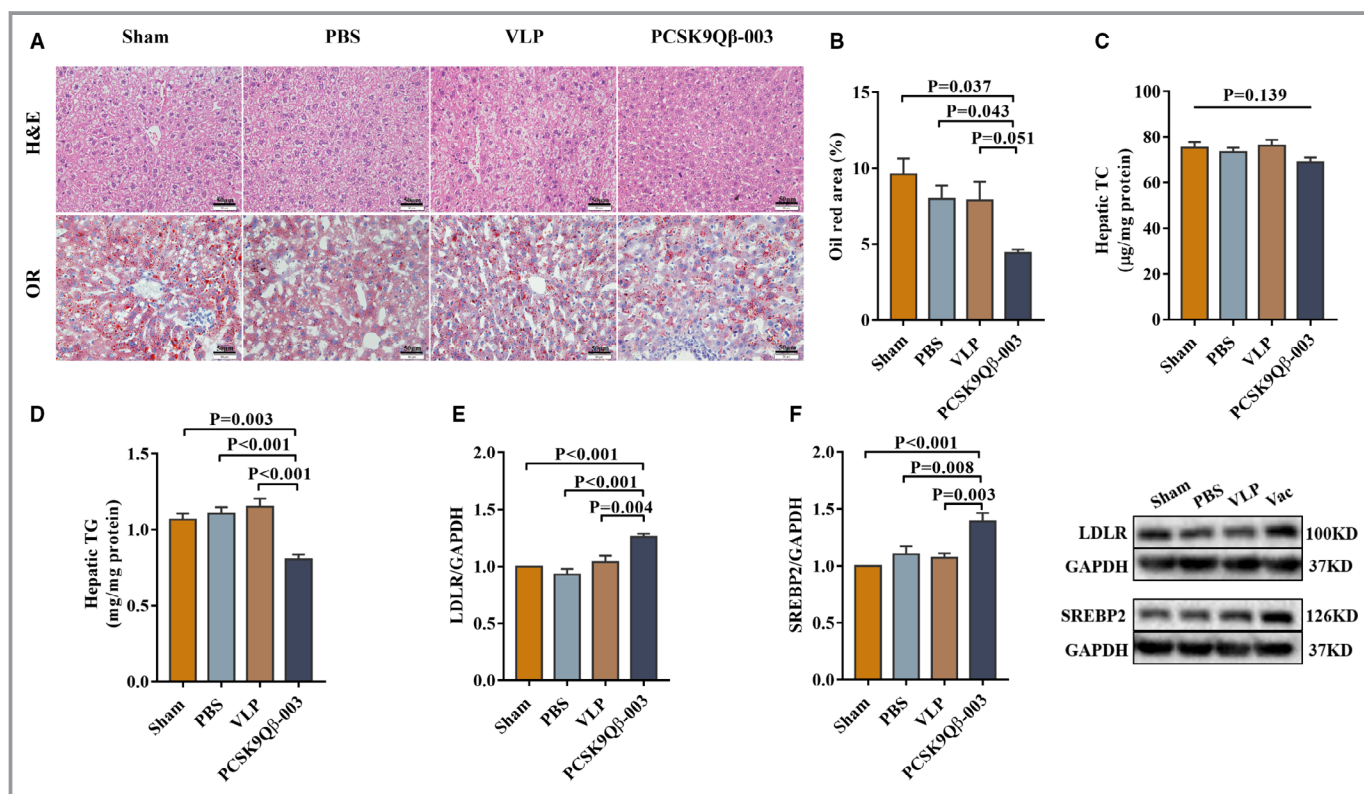
large lipid droplet (Figure 2A). Oil Red O staining showed that PCSK9Q $\beta$ -003 markedly reduced the formation of fatty liver (Figure 2B). The hepatic TG content in the PCSK9Q $\beta$ -003 group was significantly decreased compared with that in other groups; however, the hepatic TC only had a descending trend (Figure 2C and 2D). These results were basically consistent with the mice induced by L-NAME (Figure S2A through S2D).

Next, transcription factors and target genes associated with the development of hepatic steatosis were investigated. The PCSK9Q $\beta$ -003 vaccine increased the expression level of LDLR, very-low-density lipoprotein receptor (VLDLR), and SREBP2 in the liver in both UUO and L-NAME models (Figures 2E, 2F, and 3A through 3C). In addition, the PCSK9Q $\beta$ -003 vaccine remarkably upregulated the mRNA levels of FAO-related factors, including PPAR $\alpha$ , PGC1, ACOX1, carnitine palmitoyl transferase alpha, and lipoprotein lipase (LPL) (Figure 3D and 3E). Moreover, the PCSK9Q $\beta$ -003 vaccine upregulated the level of transporting factors ATP binding cassette transporter A1 (ABCA1) and ATP binding cassette subfamily G member 1 (ABCG1), while downregulated acetyl-CoA acetyltransferase 1 (ACAT1) (Figure 3F). In addition, PCSK9Q $\beta$ -003 administration

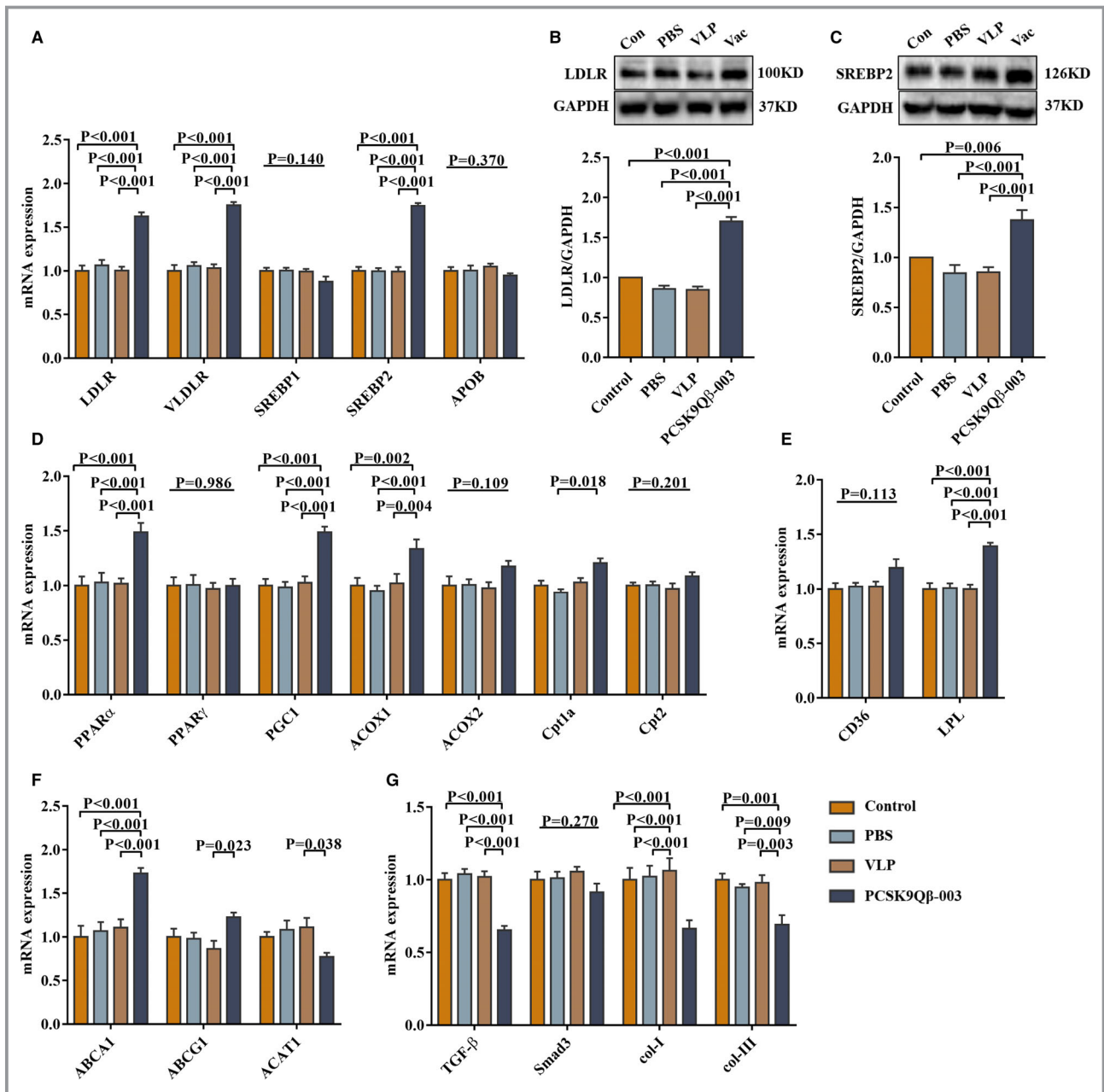
obviously reduced the level of hepatic fibrosis including TGF- $\beta$ , collagen alpha-1 and collagen alpha-3 (Figure 3G).

### PCSK9Q $\beta$ -003 Vaccine Ameliorated Renal Lipid Deposition and Renal Fibrosis Induced by UUO

We further investigated the effect of the PCSK9Q $\beta$ -003 vaccine on renal pathology induced by UUO. Compared with the PBS and VLP groups, hematoxylin and eosin staining showed that the PCSK9Q $\beta$ -003 vaccine significantly ameliorated tubular dilatation, edema, inflammatory cell infiltration, and interstitial fibrosis of kidney (Figure 4A). Oil Red O staining showed that Oil Red O-positive regions mostly located in the renal tubule was significantly decreased in the PCSK9Q $\beta$ -003 group (Figure 4A and 4B). Masson's staining showed all UUO mice exhibited large areas of renal interstitial fibrosis; however, the degree of interstitial fibrosis was remarkably reduced by PCSK9Q $\beta$ -003 treatment in contrast to the PBS and VLP groups (Figure 4A and 4C). PAS analysis showed that the PAS-positive area of the glomerulus was obviously reduced in the PCSK9Q $\beta$ -003 group compared with other UUO groups (Figure 4A and 4D).



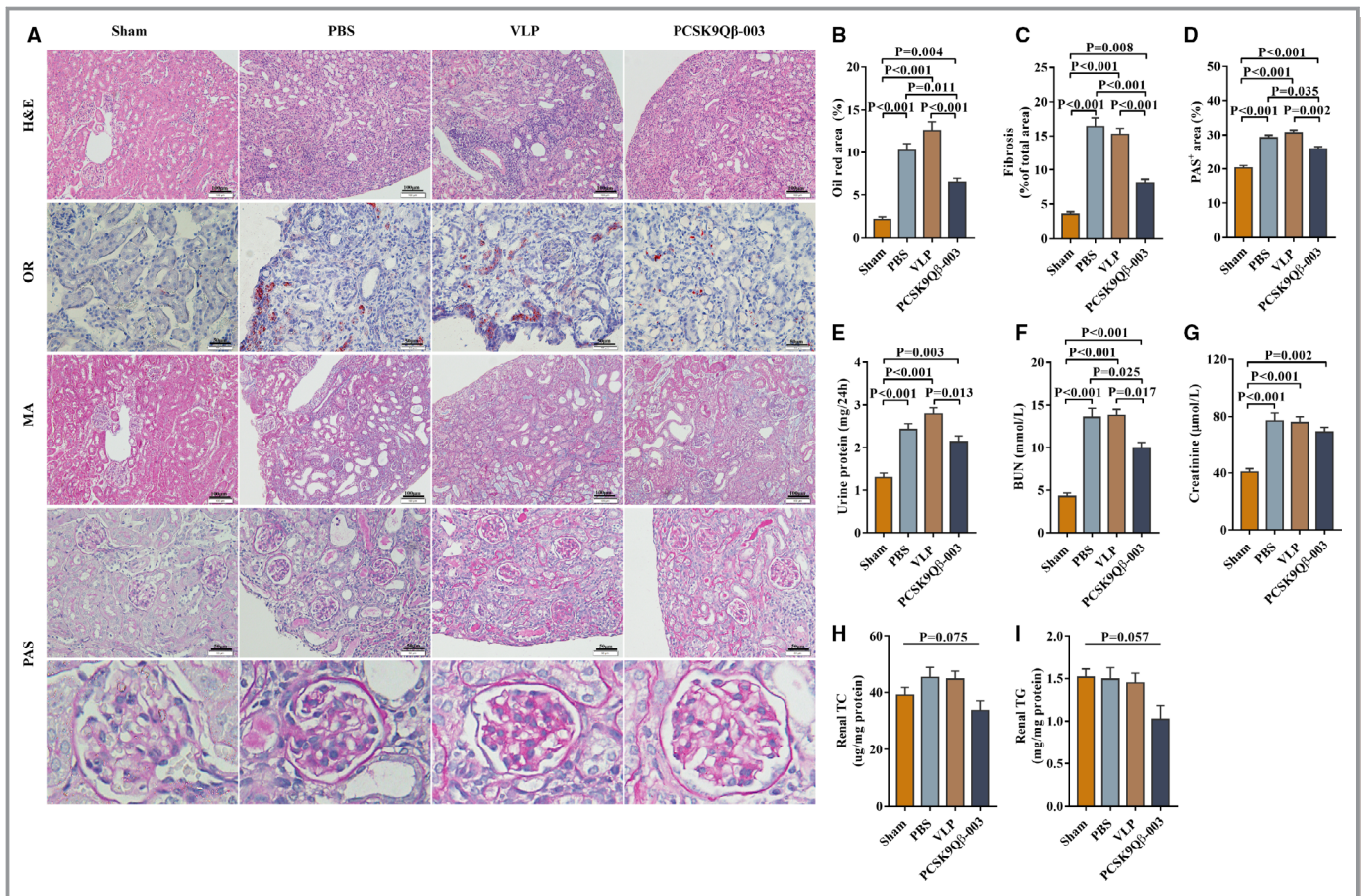
**Figure 2.** PCSK9Q $\beta$ -003 vaccine reduced hepatic steatosis in the UUO model. **A**, Histology of the livers stained with hematoxylin-eosin (H&E) and Oil Red O (bar=50  $\mu$ m). **B**, Representative Oil Red O-stained liver sections show that PCSK9Q $\beta$ -003 markedly reduced the formation of fatty liver. **C** and **D**, Hepatic TC and TG contents. **E** and **F**, Compared with the other groups, the protein expression level of LDLR and SREBP2 in liver was increased obviously in the PCSK9Q $\beta$ -003 vaccine group. n=6 per group. Data are expressed as means $\pm$ SEM. H&E indicates hematoxylin-eosin; LDLR, low-density lipoprotein receptor; OR, Oil Red O; PBS, the phosphate-buffered saline group; Sham, the sham group; SREBP2, sterol regulatory element-binding protein 2; TC, total cholesterol; TG, triglyceride; Vac, the PCSK9Q $\beta$ -003 vaccine group; VLP, the Q $\beta$  virus-like particles group.



**Figure 3.** PCSK9Qβ-003 vaccine remarkably improved the levels of transcription factors and target genes associated with the development of hepatic steatosis. **A** through **C**, PCSK9Qβ-003 increased the expression level of LDLR, VLDLR, and SREBP2. **D** and **E**, PCSK9Qβ-003 remarkably upregulated the mRNA levels of fatty acid oxidation related factors. **F**, PCSK9Qβ-003 up-regulated the level of transporting factors ABCA1 and ABCG1. **G**, PCSK9Qβ-003 administration obviously reduced the level of hepatic fibrosis-related factors. n=6 per group. Data are expressed as means±SEM. ABCA1 indicates ATP binding cassette transporter A1; ABCG1, ATP binding cassette subfamily G member 1; ACAT1, acetyl-CoA acetyltransferase 1; ACOX1, acyl-CoA oxidase 1; ACOX2, acyl-CoA oxidase 2; APOB, apolipoprotein B; CD36, cluster of differentiation 36; col-I, collagen alpha-1; col-III, collagen alpha-3; Con, the Control group; Cpt1a, carnitine palmitoyl transferase 1α; Cpt2, carnitine palmitoyl transferase 2; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; PBS, the phosphate-buffered saline group; PGC-1, PPARγ coactivator-1a; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP1, sterol regulatory element-binding protein 1; SREBP2, sterol regulatory element-binding protein 2; TGF-β, transforming growth factor-β; Vac, the PCSK9Qβ-003 vaccine group; VLDLR, very-low-density lipoprotein receptor; VLP, the Qβ virus-like particles group.

Renal function was determined in the UUO model. Results showed, compared with the VLP group, the PCSK9Qβ-003 vaccine significantly reduced the level of

24-hour urine protein (Figure 4E). All of the mice intervened by UUO had a higher level of blood urea nitrogen and creatinine. Compared with other UUO groups, the blood



**Figure 4.** PCSK9Qβ-003 vaccine ameliorated renal lipid deposition and renal fibrosis induced by UUO. **A**, Histology of the livers stained with H&E, Oil Red O, Masson (bar=100 μm) and PAS (bar=50 μm). **B**, Representative Oil Red O–stained renal sections, showed that PCSK9Qβ-003 markedly reduced the renal lipid accumulation. **C**, Interstitial fibrosis was significantly ameliorated in the PCSK9Qβ-003 group compared with the other 3 groups. **D**, PAS analysis showed that GME was reduced by in PCSK9Qβ-003 group. **E** through **G**, The PCSK9Qβ-003 vaccine significantly reduced the 24-hour urine protein and blood urea nitrogen of mice compared with the PBS and VLP groups. The creatinine level had a tendency to decrease in the PCSK9Qβ-003 group (**H** and **I**). Renal TC and TG contents. n=6 per group. Data are expressed as means±SEM. BUN indicates blood urea nitrogen; H&E, hematoxylin and eosin; MA, Masson's trichrome; OR, Oil Red O; PAS, periodic acid-Schiff; PBS, the phosphate-buffered saline group; PCSK9Qβ-003, the PCSK9Qβ-003 vaccine group; Sham, the sham group; TC, total cholesterol; TG, triglyceride; VLP, the Qβ virus-like particles group.

urea nitrogen level was notably decreased after PCSK9Qβ-003 treatment; however, the creatinine level only had a tendency to decrease (Figure 4F and 4G). In addition, lipid analysis showed that the renal TC and TG contents in the PCSK9Qβ-003 group tended to be decreased than that in other 3 groups (Figure 4H and 4I).

### PCSK9Qβ-003 Vaccine Upregulated FAO-Related Factors and Ameliorated Renal Fibrosis-Related Molecules in the UUO Model

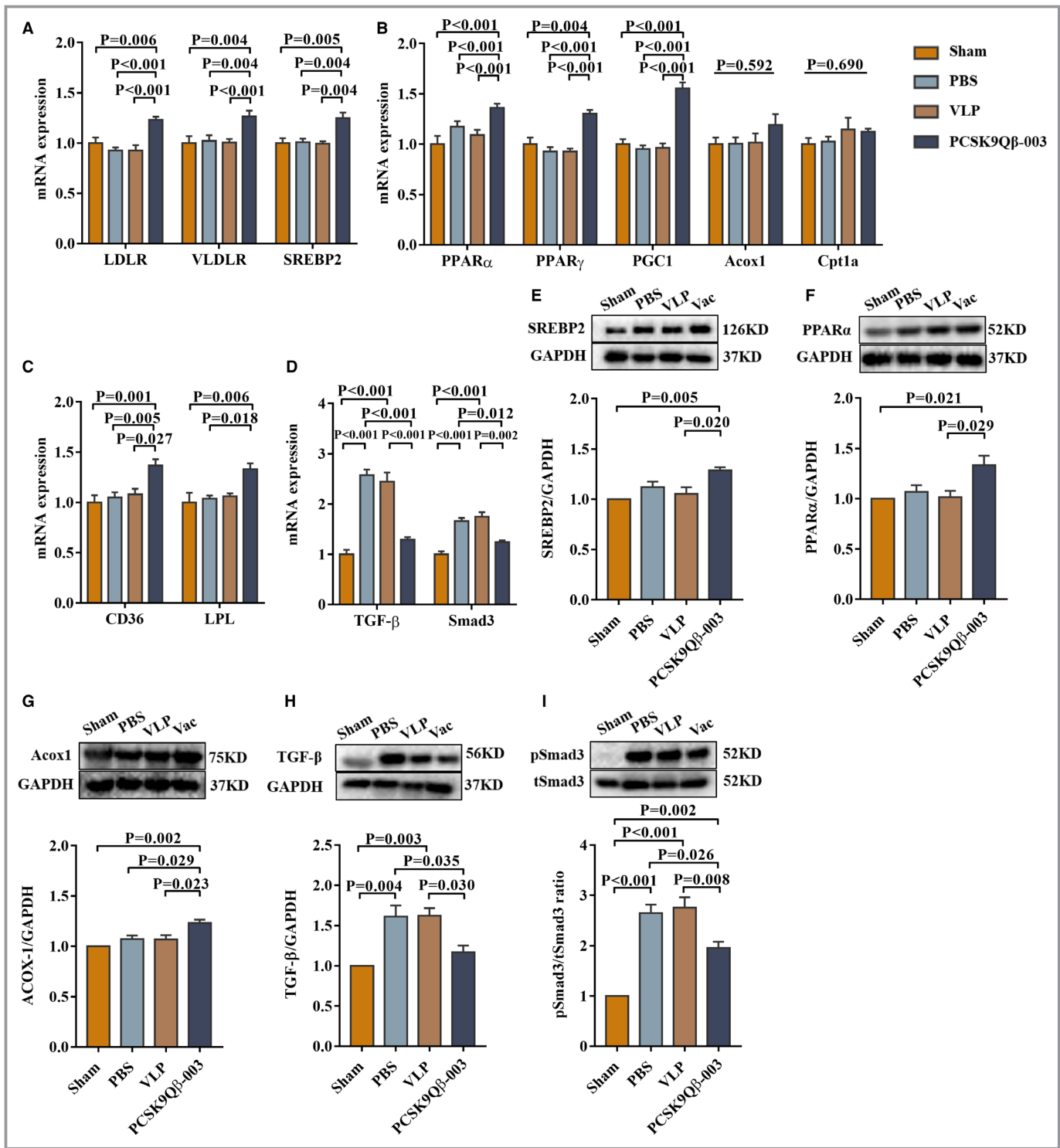
FAO-related transcription factors and target molecules associated with the development of renal fibrosis were investigated. Compared with the other 3 groups, the PCSK9Qβ-003 vaccine increased the expression of LDLR, VLDLR, and SREBP2 (Figure 5A and 5E). And, the PCSK9Qβ-003 vaccine remarkably upregulated the levels of fatty acid metabolism–related factors,

including PPARα, PPARγ, PGC1, ACOX1, CD36, and LPL, in contrast with the other 3 groups (Figure 5B, 5C, 5F, and 5G). In addition, compared with the PBS and VLP groups, the PCSK9Qβ-003 vaccine administration obviously reduced the level of TGF-β and pSmad3, which indicated the antifibrosis effect of the PCSK9Qβ-003 vaccine (Figure 5D, 5H, and 5I).

### PCSK9Qβ-003 Vaccine Improved Renal Lipid Accumulation and Renal Fibrosis Induced by L-NAME

Renal pathology was also investigated in the renal fibrosis model induced by L-NAME. Compared with the PBS and VLP groups, Oil Red O staining showed that the Oil Red O–positive region mostly located in the renal tubule, and was significantly decreased in the PCSK9Qβ-003 group (Figure 6A and 6B). Masson's staining showed that the degree of interstitial





**Figure 5.** PCSK9Qβ-003 vaccine upregulated FAO-related factors and ameliorated renal fibrosis-related molecules in the UUO model. **A**, PCSK9Qβ-003 increased the expression level of LDLR, VLDLR, and SREBP2. **B** and **C**, PCSK9Qβ-003 remarkably upregulated the levels of fatty acid metabolism-related factors. **D**, The PCSK9Qβ-003 vaccine obviously reduced the level of TGF-β and Smad3. **E** through **I**, PCSK9Qβ-003 treatment improved the protein expression of SREBP2, PPARα, ACOX1, TGF-β, and pSmad3. n=6 per group. Data are expressed as means±SEM. ACOX1 indicates acyl-CoA oxidase 1; CD36, cluster of differentiation 36; Cpt1a, carnitine palmitoyl transferase 1α; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; PBS, the phosphate-buffered saline group; PGC-1, PPARγ coactivator-1α; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; Sham, the sham group; SREBP2, sterol regulatory element-binding protein 2; TGF-β, transforming growth factor-β; Vac, the PCSK9Qβ-003 vaccine group; VLDLR, very-low-density lipoprotein receptor; VLP, the Qβ virus-like particles group.

fibrosis was remarkably reduced by PCSK9Q $\beta$ -003 treatment in contrast with the PBS and VLP groups (Figure 6A and 6C). PAS analysis showed that the PAS-positive area of the glomerulus was obviously reduced in the PCSK9Q $\beta$ -003 group compared with other L-NAME groups (Figure 6A and 6D). Renal function detection showed that, compared with the PBS and VLP groups, the PCSK9Q $\beta$ -003 vaccine not only significantly reduced the level of 24-hour urine protein at weeks 22 and 24 (Figure 6E) but also notably decreased the level of blood urea nitrogen and creatinine (Figure 6F and 6G). All of these results indicated the positive effect of the PCSK9Q $\beta$ -003 vaccine on renal protection.

### PCSK9Q $\beta$ -003 Vaccine Upregulated FAO-Related Factors and Ameliorated Renal Fibrosis-Related Molecules in the L-NAME Model

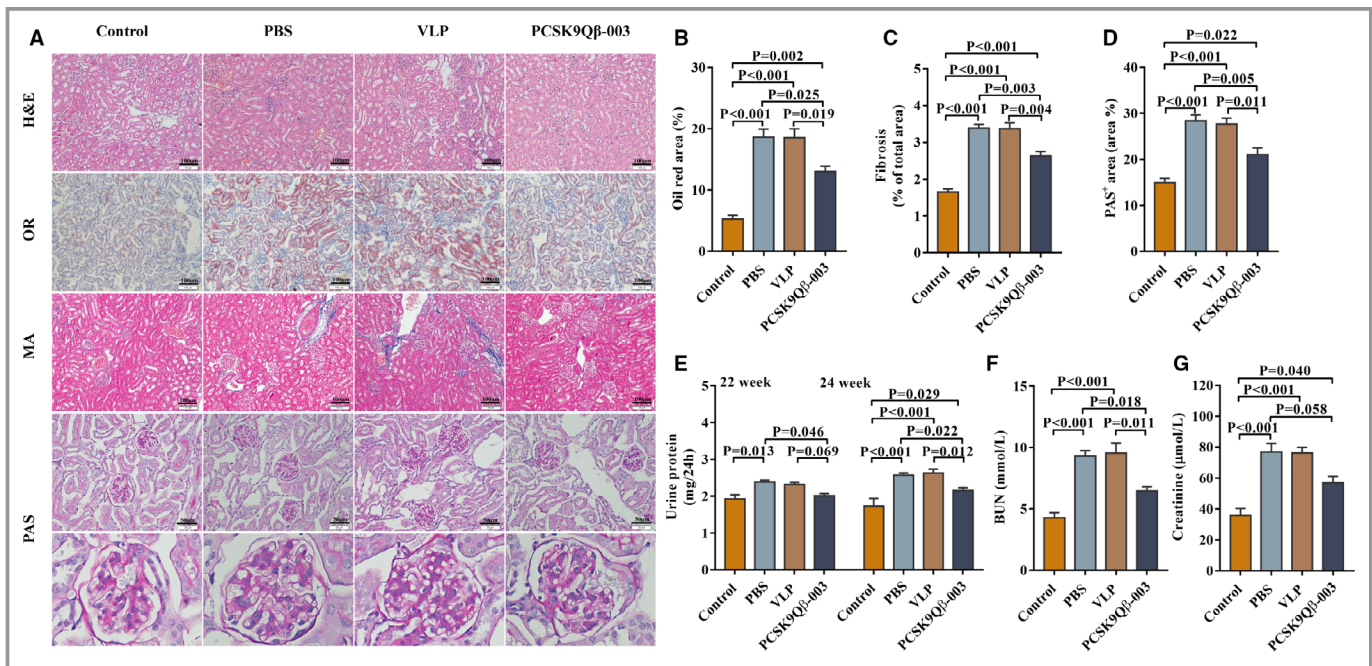
FAO-related transcription factors and target molecules associated with the development of renal fibrosis were also investigated in the L-NAME renal fibrosis model. Compared with the other 3 groups, the PCSK9Q $\beta$ -003 vaccine increased the expression level of LDLR, VLDLR, and SREBP2 (Figure 7A). In addition, the PCSK9Q $\beta$ -003 vaccine remarkably upregulated the levels of fatty acid metabolism-related factors,

including PPAR $\alpha$ , PPAR $\gamma$ , PGC1, ACOX1, CD36, and LPL (Figure 7B, 7C, 7E, and 7F). Moreover, compared with the PBS and VLP groups, the PCSK9Q $\beta$ -003 vaccine administration obviously reduced the level of TGF- $\beta$  but only tended to decrease the level of pSmad3 (Figure 7D, 7G, and 7H).

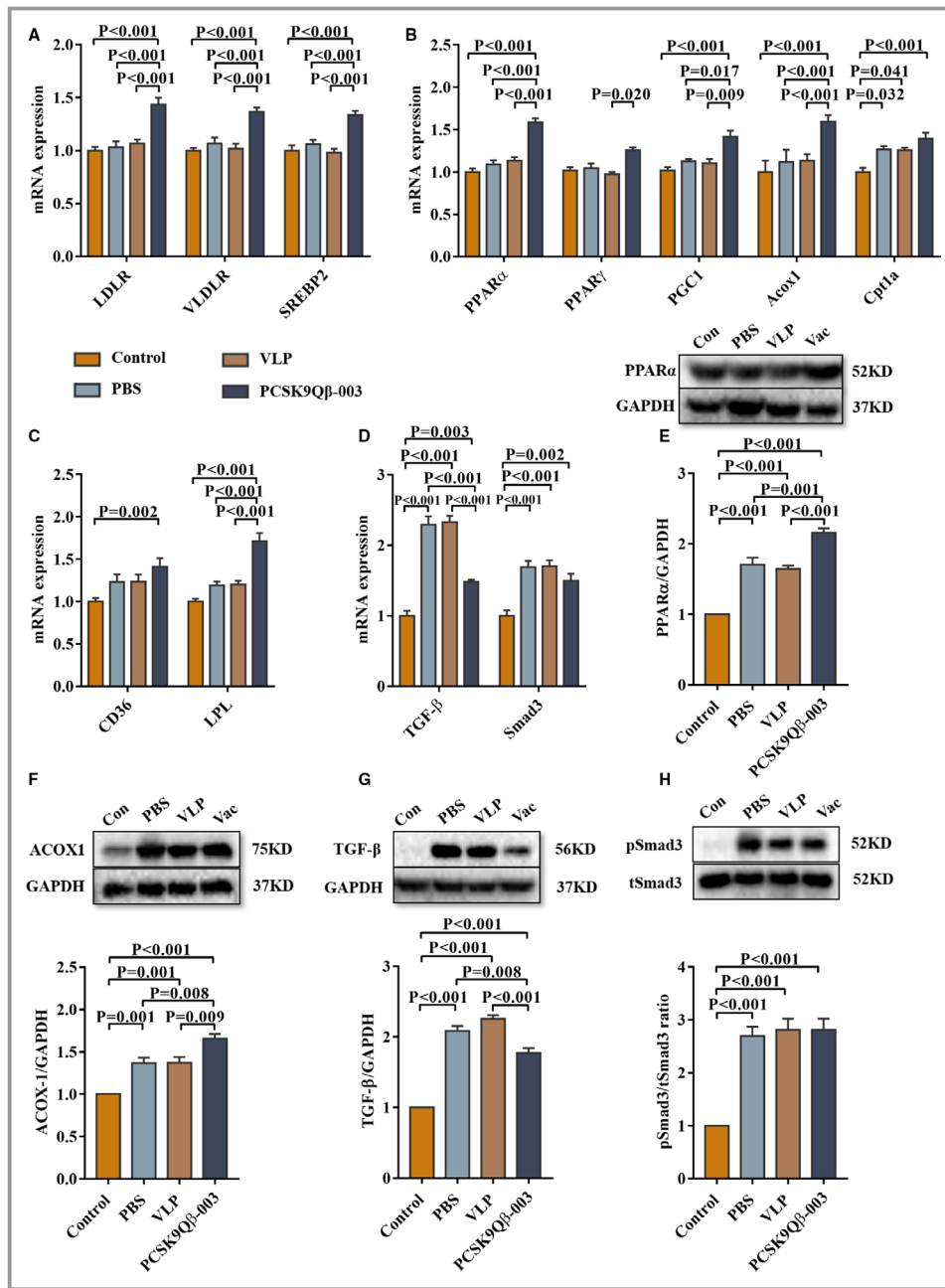
### Discussion

In the present study, we demonstrated that the PCSK9Q $\beta$ -003 vaccine significantly decreased serum TC and LDL-C, improved renal lipid accumulation, and attenuated the development of renal fibrosis induced by UUO and L-NAME in LDLR<sup>+/-</sup> mice with hypercholesterolemia for the first time. Moreover, the PCSK9Q $\beta$ -003 vaccine obviously upregulated FAO-related factors and ameliorated renal fibrosis-related genes both in the UUO and L-NAME models.

Our results showed that total PCSK9 level was significantly elevated in mice vaccinated with the PCSK9Q $\beta$ -003 vaccine compared with the other 3 groups. The results were consistent with previous studies. Clinical trials demonstrated that anti-PCSK9 antibodies engaged PCSK9 in vivo and formed an immune complex, which could actually raise plasma total PCSK9 level.<sup>28</sup> However, the level of free PCSK9 after treatment of PCSK9 inhibitors is decreased. This result



**Figure 6.** PCSK9Q $\beta$ -003 vaccine improved renal lipid accumulation and renal fibrosis induced by L-NAME. **A**, H&E, Oil Red O, Masson (bar=100  $\mu$ m) and PAS (bar=50  $\mu$ m). **B**, Representative Oil Red O-stained renal sections, showed that PCSK9Q $\beta$ -003 markedly reduced the tubule epithelial lipid accumulation. **C**, Interstitial fibrosis was significantly ameliorated in the PCSK9Q $\beta$ -003 group compared with the other 3 groups. **D**, PAS analysis showed that GME was reduced in PCSK9Q $\beta$ -003 group. **E** through **G**, The PCSK9Q $\beta$ -003 vaccine significantly reduced the 24-hour urine protein, BUN and creatinine compared with PBS and VLP groups. n=6 per group. Data are expressed as means $\pm$ SEM. BUN indicates blood urea nitrogen; Con, the control group; H&E, hematoxylin and eosin; MA, Masson's trichrome; OR, Oil Red O; PAS, periodic acid-Schiff; PBS, the phosphate-buffered saline group; PCSK9Q $\beta$ -003, the PCSK9Q $\beta$ -003 vaccine group; VLP, the Q $\beta$  virus-like particles group.



**Figure 7.** PCSK9Qβ-003 vaccine upregulated FAO-related factors and ameliorated renal fibrosis-related molecules in the L-NAME model. **A**, PCSK9Qβ-003 increased the expression level of LDLR, VLDLR, and SREBP2. **B** and **C**, PCSK9Qβ-003 remarkably upregulated the levels of fatty acid metabolism-related factors. **D**, The PCSK9Qβ-003 vaccine obviously reduced the level of TGF-β and Smad3. **E** through **H**, PCSK9Qβ-003 treatment improved the protein expression of PPARα, ACOX1, TGF-β and pSmad3. n=6 per group. Data are expressed as means±SEM. ACOX1 indicates acyl-CoA oxidase 1; CD36, cluster of differentiation 36; Con, the control group; Cpt1a, carnitine palmitoyl transferase 1α; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; PBS, the phosphate-buffered saline group; PGC-1, PPARγ coactivator-1a; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; SREBP2, sterol regulatory element-binding protein 2; TGF-β, transforming growth factor-β; Vac, the PCSK9Qβ-003 vaccine group; VLDLR, very-low-density lipoprotein receptor; VLP, the Qβ virus-like particles group.

also has been reported in our previous study, which showed that free PCSK9 level was substantially decreased in the PCSK9Qβ-003 vaccine group, similar to anti-PCSK9 mAbs.<sup>27</sup>

That is, the increase of total PCSK9 level after immunization with the PCSK9Qβ-003 vaccine suggested the effectiveness of the vaccine.

Clinical observations indicated a potential association between lipid levels and CKD development.<sup>29</sup> Higher intracellular lipid accumulation has been proposed to have a key role in the development of fibrosis by inducing lipotoxicity.<sup>12</sup> PCSK9 might aggravate dyslipidemia and exacerbate renal dysfunction in patients with CKD. Conversely, the effect of PCSK9 inhibitors on renal function and fibrosis is worth exploring.<sup>30,31</sup> Significantly, in addition to the decrease of LDL-C level, the PCSK9Q $\beta$ -003 vaccine further ameliorated renal lipid accumulation and renal fibrosis, and then improved renal function in the LDLR<sup>+/-</sup> UUO and L-NAME mice. This was a novel and important finding for PCSK9 inhibitors.

Defects in the FAO pathway have received substantial attention in the context of acute and CKD. Defective FAO in tubule epithelial cells has been associated with ATP depletion, cell death, dedifferentiation, and lipid accumulation, which were the phenotypes of renal fibrosis.<sup>12</sup> It has been shown that improvement of FAO could protect mice from tubulointerstitial fibrosis. The PPARs and PGC1 are the key transcription factors that regulate the expression of proteins involved in fatty acid uptake and oxidation.<sup>22,32,33</sup> Metabolism of fatty acids requires that they be transported into the mitochondria, which is mediated by carnitine palmitoyl transferase alpha, an enzyme could conjugate fatty acids with carnitine.<sup>34</sup> In our study, the results showed that the PCSK9Q $\beta$ -003 vaccine remarkably improved FAO-associated molecules expression, such as PPAR $\alpha$ , PGC1, ACOX1, and CD36 but significantly inhibited TGF- $\beta$  expression and Smad3 phosphorylation. These data suggested that the PCSK9Q $\beta$ -003 may ameliorate renal fibrosis by regulating the FAO pathway.

PCSK9 was reported to affect the activity of other receptors beyond LDLR, such as CD36, VLDLR, apolipoprotein E receptors, and LDLR-related protein 1.<sup>35,36</sup> Our results showed that the PCSK9Q $\beta$ -003 vaccine significantly increased the expression of LDLR and VLDLR. Thus, in addition to kidney protection, PCSK9 inhibitors may have other pleiotropic effects that need to be clarified in the future studies. It is noteworthy that PCSK9 is also produced by the kidneys, and PCSK9 seems to reduce the expression of the epithelial sodium channel in renal tubules known to markedly affect sodium reabsorption.<sup>36</sup> Theoretically, PCSK9 inhibitors might be associated with increased sodium reabsorption and hypertension, but this is not the case in experimental models, in individuals with PCSK9 loss-of-function mutations or after the administration of monoclonal antibodies.<sup>34,37</sup> In our study, coincident results showed that, compared with other groups, the systolic blood pressure had no significant difference in PCSK9Q $\beta$ -003 treatment.

Given the efficacy of PCSK9 mAbs in treating dyslipidemia and the potential link between acquired LDLR deficiency and the pathogenesis of CKD,<sup>38</sup> future trials exploring the use of PCSK9 inhibitors in treating dyslipidemia with CKD are

warranted. Compared with traditional chemical drugs, the PCSK9Q $\beta$ -003 vaccine has some potentially superior advantages, including the ability to stably decrease lipid levels, improvement of patient compliance, and the low cost, which may provide a promising method for treating hypercholesterolemia with renal fibrosis.

## Sources of Funding

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## Disclosures

None.

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# **SUPPLEMENTAL MATERIAL**

**Table S1. Detail information of primers.**

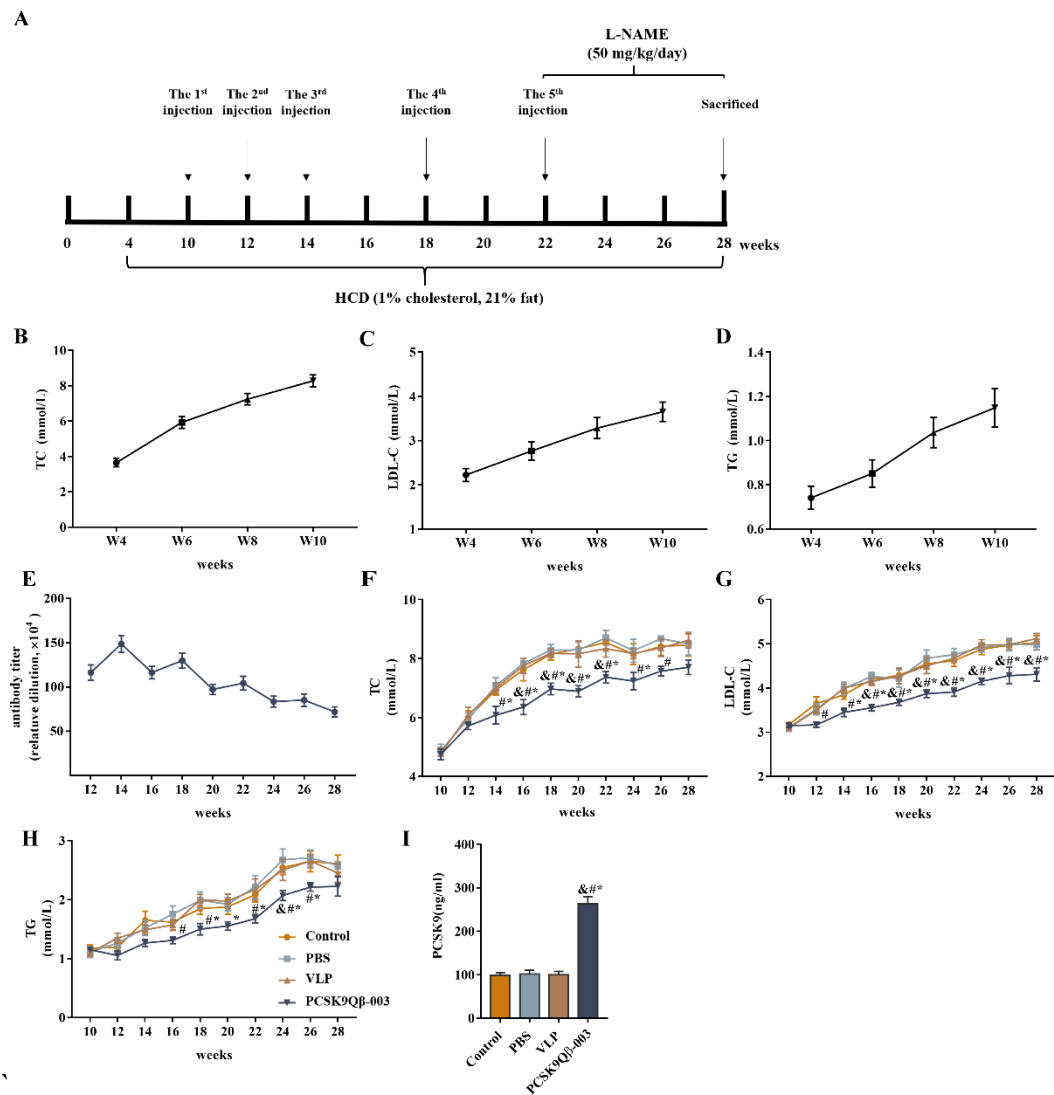
<b>Gene</b>		<b>Sequence</b>
<b>LDLR</b>	Forward	5'-CCTCAAGTACCTTGGTATGACGC-3'
	Reverse	5'-GAGGCTGTCCGGTCAGGATG-3'
<b>VLDLR</b>	Forward	5'-GAGTCTGACTTCGTGTGCAAA-3'
	Reverse	5'-GAACCGTCTTCGCAATCAGGA-3'
<b>SREBP1</b>	Forward	5'-CAAGGCCATCGACTACATCCG-3'
	Reverse	5'-CACCACTTCGGGTTTCATGC-3'
<b>SREBP2</b>	Forward	5'-GCAGCAACGGGACCATTCT-3'
	Reverse	5'-CCCCATGACTAAGTCCTTCAACT-3'
<b>APOB</b>	Forward	5'-TTGGCAAACCTGCATAGCATCC-3'
	Reverse	5'-TCAAATTGGGACTCTCCTTTAGC-3'
<b>PPAR<math>\alpha</math></b>	Forward	5'-AGAGCCCCATCTGTCCTCTC-3'
	Reverse	5'-ACTGGTAGTCTGCAAAACCAAA-3'
<b>PPAR<math>\gamma</math></b>	Forward	5'-GGAAGACCACTCGCATTTCCTT-3'
	Reverse	5'-GTAATCAGCAACCATTGGGTCA-3'
<b>PGC1</b>	Forward	5'-TATGGAGTGACATAGAGTGTGCT-3'
	Reverse	5'-CCACTTCAATCCACCCAGAAAG-3'
<b>Acox1</b>	Forward	5'-TAACTTCCTCACTCGAAGCCA-3'
	Reverse	5'-AGTTCCATGACCCATCTCTGTC-3'
<b>Acox2</b>	Forward	5'-CATCCAACGTGACCCAGTGT-3'
	Reverse	5'-AAATGCGTTCAGGACCGTCTT-3'

<b>Cpt1a</b>	Forward	5'-TGGCATCATCACTGGTGTGTT-3'
	Reverse	5'-GTCTAGGGTCCGATTGATCTTTG-3'
<b>Cpt2</b>	Forward	5'-CCTGCTCGCTCAGGATAAACA-3'
	Reverse	5'-GTGTCTTCAGAAACCGCACTG-3'
<b>CD36</b>	Forward	5'-ATGGGCTGTGATCGGAACTG-3'
	Reverse	5'-GTCTTCCCAATAAGCATGTCTCC-3'
<b>LPL</b>	Forward	5'-GGGAGTTTGGCTCCAGAGTTT-3'
	Reverse	5'-TGTGTCTTCAGGGGTCCTTAG-3'
<b>ABCA1</b>	Forward	5'-CAGATGAAGCAGTTTTTCAGTCCT-3'
	Reverse	5'-CACATTCCGGCTCTTTAGAAGG-3'
<b>ABCG1</b>	Forward	5'-CTTTCCTACTCTGTACCCGAGG-3'
	Reverse	5'-CGGGGCATTCCATTGATAAGG-3'
<b>ACAT1</b>	Forward	5'-CAGGAAGTAAGATGCCTGGAAC-3'
	Reverse	5'-TTCACCCCCTTGGATGACATT-3'
<b>TGF-β</b>	Forward	5'-CCACCTGCAAGACCATCGAC-3'
	Reverse	5'-CTGGCGAGCCTTAGTTTGGAC-3'
<b>Smad3</b>	Forward	5'-CACGCAGAACGTGAACACC-3'
	Reverse	5'-GGCAGTAGATAACGTGAGGGA-3'
<b>Col-I</b>	Forward	5'-TAAGGGTCCCAATGGTGAGA-3'
	Reverse	5'-GGGTCCCTCGACTCCTACAT-3'
<b>Col-III</b>	Forward	5'-CTGTAACATGGAAACTGGGGAAA-3'
	Reverse	5'-CCATAGCTGAACTGAAAACCACC-3'

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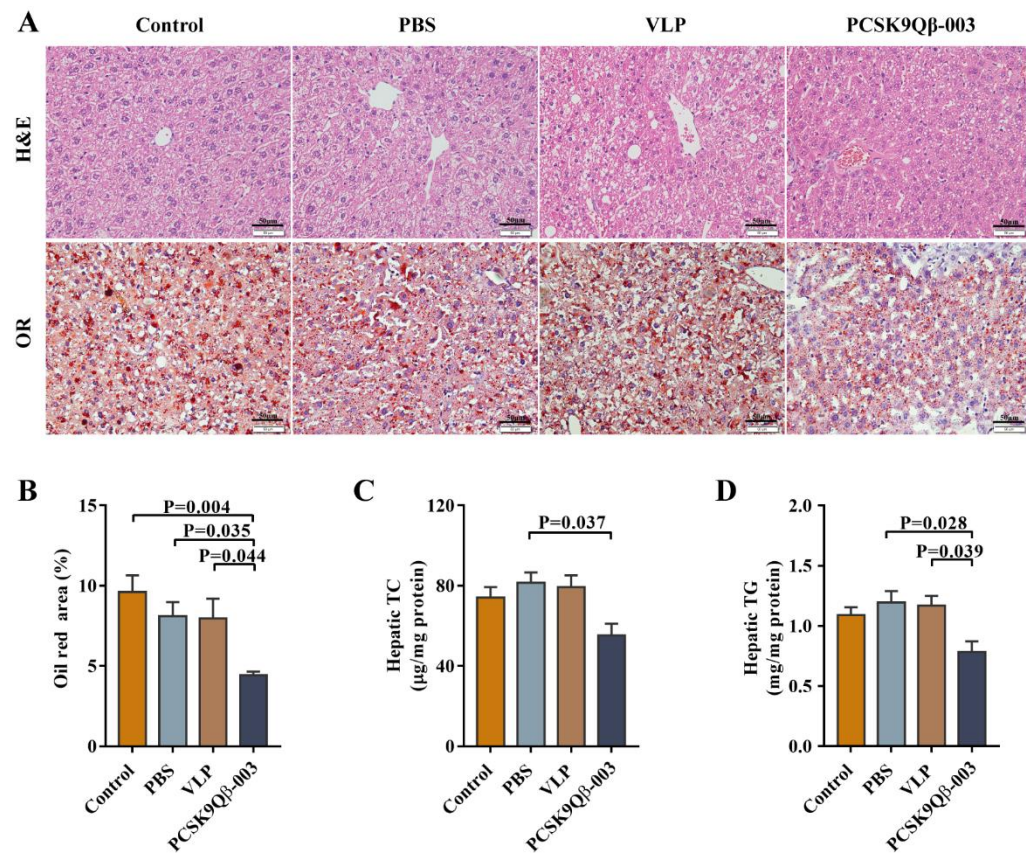


**Figure S1. PCSK9Q $\beta$ -003 vaccine decreased plasma TC in mice intervened by L-NAME.**



(A) Process of animal experiments in the LDLR<sup>+/-</sup> mice + L-NAME. (B-D) LDLR<sup>+/-</sup> mice fed with HCD presented changes of steadily increased blood lipid level. (E) The anti- PCSK9Q $\beta$ -003 titers were measured by ELISA. (F-H) The TC, LDL-C and TG was decreased in the PCSK9Q $\beta$ -003 vaccine group compared with the other groups. (I) The total PCSK9 level was significantly elevated in the PCSK9Q $\beta$ -003 vaccine group compared to the other groups. n = 6 per group. Data were expressed as means  $\pm$  SEM. &P<0.05 versus the Control group; #P<0.05 versus the PBS group; \*P<0.05 versus the Q $\beta$  virus-like particles group.

**Figure S2. PCSK9Q $\beta$ -003 vaccine decreased hepatic steatosis in mice induced by L-NAME.**



(A) Histology of the livers stained with H&E and Oil-red O (Bar 50= $\mu$ m). (B) Representative Oil Red O stained liver sections showed that the PCSK9Q $\beta$ -003 vaccine markedly prevented the formation of fatty liver. (C, D) Hepatic TC and TG contents. n = 6 per group. Data were expressed as means  $\pm$  SEM.