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Original Article

Establishment of a rat model with diet-induced coronary atherosclerosis

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Abstract

Coronary atherosclerotic disease is a serious disease in humans, but no suitable animal model is available currently for further studies. We used apolipoprotein E gene knockout (*ApoE* KO) rats to induce hypercholesterolemia through a special high cholesterol/bile salt diet (Paigen diet), then analyzed aortic and coronary atherosclerosis lesions and the myocardial injury in order to establish a novel small animal model of coronary atherosclerosis. Plasma cholesterol of *ApoE* KO rats increased 7.6-fold compared with wild-type rats after 8 weeks on the Paigen diet. After 10 to 12 weeks of subsisting on the Paigen diet, *ApoE* KO rats developed mild aortic atherosclerosis with severe coronary atherosclerosis. Hematoxilyn and eosin staining showed that 11 out of 12 *ApoE* KO male rats had right coronary artery atherosclerosis, 7 of them were>70% occluded. Oil Red O (Lipid Stain), Mac2 immuno-staining and Masson's trichrome staining demonstrated substantial amounts of lipid, macrophages and collagen fibers in coronary atherosclerosis plaques. In addition, *ApoE* KO male rats had severe myocardial focal lesions with cholesterol ester as the main component in the lesions. In conclusion, *ApoE* KO rats developed severe hypercholesterolemia, coronary atherosclerosis and myocardial cholesterol ester deposition after subsisting on the Paigen diet and can be used as a novel animal model for studies on cholesterol metabolism and coronary atherosclerotic disease.

Keywords: ApoE knockout rats, hypercholesterolemia, coronary atherosclerosis

Introduction

Coronary atherosclerotic disease (CAD) is a human disease with a high morbidity and mortality. It is caused by coronary artery atherosclerosis which leads to stenosis or occlusion of the vessels and progresses to myocardial ischemia. Choosing a suitable animal model for coronary heart disease is a necessary approach to study the occurrence, development and outcome of the human disease. Although human atherosclerosis mainly occurs at cardiovascular and cerebrovascular arteries, causing such clinical events as myocardial infarction and stroke, the majority of atherosclerosis lesions mainly occur in the aorta, and rarely, in the coronary arteries in most atherosclerotic animal models[1]. Myocardial ischemia can be artificially induced in mice by coronary artery ligation or injection of isopropyl epinephrine without the pathological lesions of coronary arteries. Therefore, it cannot mimic the natural progression of human CAD. Application of genetic modification techniques including gene deletion of B1 type scavenger receptor (*SRB1*)[2] and nitric oxide

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synthase (*NOS*)[3] has now been developed to generate a number of mouse models of CAD, but extensive genetic manipulations and inefficient long-term breeding are required.

ApoE is one of the most important apolipoprotein in human plasma, and distributed mainly in the low density protein (VLDL), intermediate density lipoprotein (IDL), chylomicrons (CM) and their residues (CMR). ApoE plays its function mainly through the interaction with low density lipoprotein receptors (LDLR), very low density lipoprotein receptors (VLDLR), and LDL receptor related protein (LRP). It can regulate the lipolysis and clearance of plasma lipoprotein, and the synthesis of VLDL. In human plasma, there are 3 ApoE isomers, E2, E3 and E4, with E3 being the wild type isomer. E2 and E4 polymorphism were associated with a higher risk of cardiovascular disease[4]. It has been reported that *ApoE* null mutation patients suffered from early onset of severe cardiovascular disease, xanthoma and type III hyperlipidemia. The plasma total cholesterol (TC) level can reach as high as 500-700 mg/dL[5–6].

In 1992, two groups separately generated *ApoE* knockout mice[7–9]. The plasma TC in these mice can reach about 400 mg/dL on a diet comprised of chow. These mice can even develop spontaneous atherosclerosis at 3 months of age. High cholesterol diet can induce severe hypercholesterolemia and aortic atherosclerosis in *ApoE* KO mice. Currently, *ApoE* knockout mice have become the most commonly used animal model for the research of hypercholesterolemia and aortic atherosclerosis. Even though simultaneous modification of certain genes at the same time could induce severe coronary atherosclerosis in *ApoE* KO mice[10–11], the genetic modification and breeding are difficult.

Compared with mice, rats are large enough to allow surgical operation and imaging examination, thus enabling an easier evaluation of their cardiovascular function. In effect, it is possible to obtain larger biological samples from rats than from mice. Therefore, rats are widely used in the study of cardiovascular physiology and pathology. Most importantly, under some special conditions, certain degrees of coronary atherosclerosis can be induced in wild-type rats, but not mice. If we can promote the development of atherosclerosis through *ApoE* gene knockout, it is likely to induce more severe coronary atherosclerosis in rats. This type of rat may become a more suitable small animal model for human CAD research.

In this study, *ApoE* knockout rats (*ApoE* KO), generated by transcriptional activator-like effector nucleases (TALEN) mediated gene editing method, were used to analyze their plasma lipid contents, aortic and coronary atherosclerotic lesions and myocardial injury produced by chow diet or Paigen diet feeding.

Material and methods

Animals were maintained on a 12-hour light/12-hour dark cycle at 24°C, given water *ad libitum* and fed a standard laboratory chow diet. The Principles of Laboratory Animal Care (NIH publication No.85Y23, revised 1996) were followed, and the experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center (LA2010-059).

Generation of *ApoE* knockout rats

TALEN construction

A pair of TALENs targeting exon 3 of the *ApoE* gene was created. Each TALEN binds to 20 bp of DNA and the binding sites are separated by a 14-bp spacer region as illustrated in ***Fig. 1A***. TALEN plasmids were linearized and transcribed in vitro using a mMessage mMachine T7 Ultra Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Capped, polyA-tailed mRNAs were cleaned up with a MEGAclear Kit (Ambion). In addition, mRNAs were precipitated, washed, and resuspended at 1 μg/μL in RNase free water, dispensed into aliquots, and stored at 80°C. TALEN mRNAs were subsequently diluted at a final concentration of 10 ng/μL for embryo injection.

Embryo manipulation

Sprague-Dawley (SD) rats were used for generating *ApoE* knockout rats. Female embryo donors were superovulated and subsequently individually caged with a male SD rat. The following morning, donors were sacrificed and embryos collected from the oviducts. TALEN mRNAs were injected into the cytoplasm using glass injection pipettes. Embryos that survived the injection procedure were surgically transferred to the oviduct of day 0.5 post coitum psuedopregnant recipient SD females that had successfully mated with vasectomized males.

DNA sequencing of genome mutated sites

Offspring from injected embryos were screened for mutations in the *ApoE* locus using DNA sequencing. The target sites were amplified by PCR with specific primers (ApoE-F, 5′-GTTGGTCCCATTGCTGACAG-3′, and ApoE-R, 5′-CAGATAGGAGGAACCCCTG-

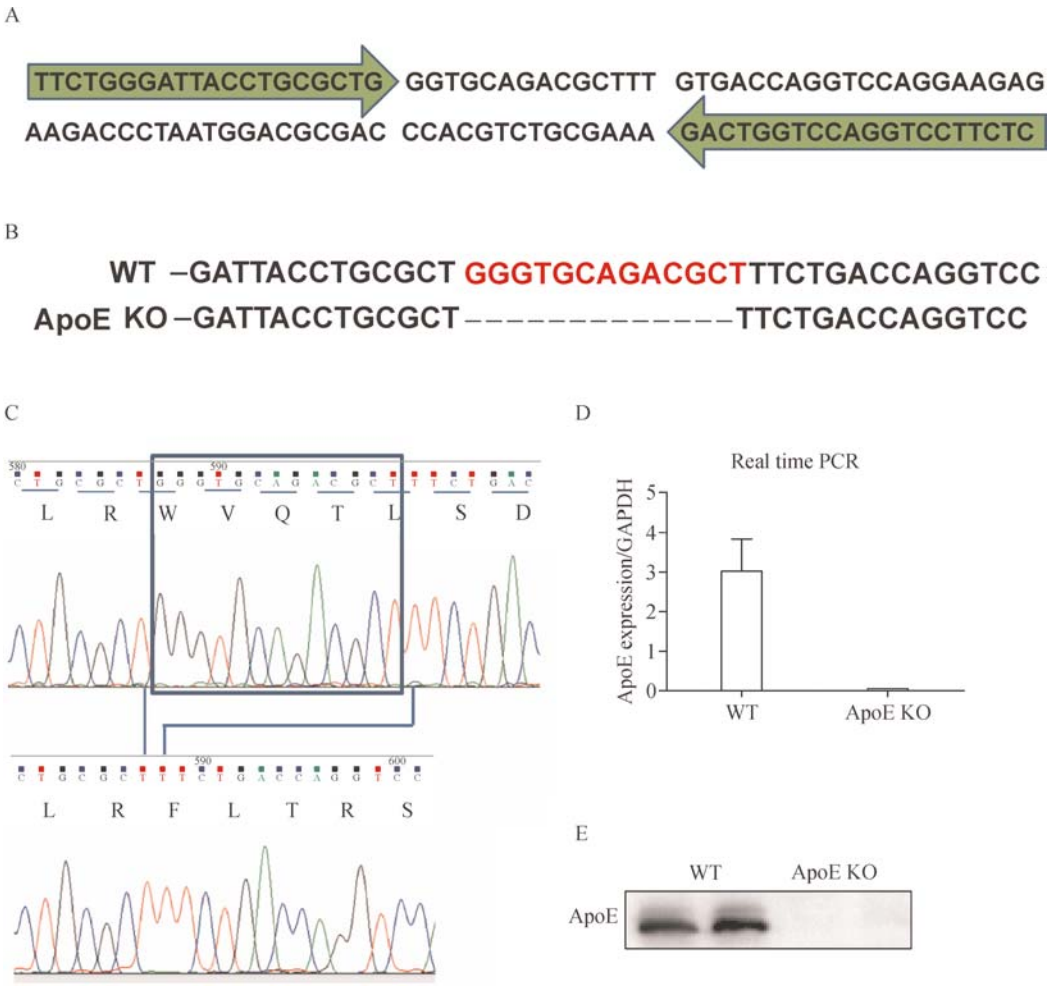

Fig. 1 **Generation of *ApoE* knockout rats.** A: Double-stranded DNA sequence of the *ApoE* locus that was targeted with TALENs. The TALEN binding sites are represented by the large green arrows. B: Mutation site and sequence of *ApoE* KO rats which possess 13bp deletion mutation. C: Sequencing peak figure and amino acid changes in *ApoE* KO rats. The top one is from WT rat and the bottom one from *ApoE* KO rat. D: ApoE mRNA expression levels in the liver, $n = 6$. E: Western blot of plasma ApoE.

GAT-3′) from genomic DNA. PCR was performed with 35 cycles of a reaction consisting of 45 s of denaturation at 95°C, 45 seconds of annealing at 62°C and 45 seconds of elongation at 72°C. The PCR products were 748 bp. DNA sequencing was performed by SinoGenoMax Co., Ltd (Beijing, China).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the liver using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was generated by using an RT kit (Invitrogen). Quantitative real-time PCR was performed using specific primers (r-ApoE-F, 5′-CTGCTGTTGGTCCCATTGCT-3′, and r-ApoE-R, 5′-CCGAGTCGGTTGCGTAGATC-3′). Amplifications were performed in 35 cycles using an opticon continuous fluorescence detection system (MJ Research, Foster City, CA, USA) with SYBR green fluorescence (Molecular Probes, Eugene, USA). Each cycle consisted of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 56°C, and extension for 30 seconds at 72°C. All samples were quantitated by using the comparative CT method for relative quantitation of gene expression, normalized to GAPDH[12].

Animal groups

Homozygous *ApoE* KO rats were used for phenotype analysis. Ten to 12 weeks old WT (male $n = 8$; female $n = 5$) and *ApoE* KO (male $n = 12$; female $n = 10$) rats were fed a Paigen diet (15% [w/w] lard fat; 1.25% [w/w] cholesterol; 0.5% [w/w] sodium cholate) for 10-12 weeks. Plasma lipids and atherosclerotic lesions were analyzed.

Blood lipid analysis

Blood was obtained by retro-orbital bleeding. Plasma TC and triglyceride (TG) were determined by using enzymatic methods (Bio Sino, Beijing, China). High

density lipoprotein cholesterol (HDL-C) was measured with the TC kit after ApoB-lipoprotein had been precipitated with 20% polyethylene glycol solution. Free cholesterol (FC) was measured using enzymatic methods (Applygen Technologies Inc, Beijing, China). For lipoprotein distribution analysis, pooled plasma samples from 6 rats per group were separated by fast protein liquid chromatography (FPLC) and cholesterol were determined in each fraction. For Western blot analysis, 1 μL of plasma from each sample were loaded and separated by SDS-polyacrylamide gel electrophoresis. The proteins transferred on nitrocellulose membranes were recognized with the required primary antibodies and secondary antibodies conjugated to horseradish peroxidase. The blots were developed by use of the enhanced chemiluminescence detection reagents.

Histological studies

Atherosclerotic lesion area in the aortic root and coronary artery were quantified on cross sections of the aorta as previously described[13]. In brief, rats were sacrificed and flushed with 80 mL 0.01 mol/L phosphate-buffered saline (PBS) through the left ventricle. Tissues were harvested and stored in –80°C or fixed in 4% paraformaldehyde (PFA) for 4 hours, and then transferred to 20% sucrose. The heart was embedded in OCT, snap-frozen in liquid nitrogen and stored at –30°C prior to sectioning. Serial 7 μm sections were obtained working from the apex of the heart toward the origin of the aorta, and sections were mounted from the point where all three aortic valve cusps became clearly visible. Every 20th section was used for oil red O staining, counterstained with hematoxylin. Atherosclerotic lesion areas were measured using Image J graphic Analysis System and were reported as the average oil red O staining area per section in the first such section for each rat.

Sections were also stained with hematoxylin and eosin (H&E) or Masson's trichrome method for fibrosis analysis. Paraffin-embedded hearts were sectioned at a thickness of 5 μm and stained with H&E or Sirius red for fibrosis analysis. Immuno-detection was performed with Mac2 antibody (Santa Cruz Biotechnology, Dallas, TX) to examine macrophage infiltration.

Heart lipid analysis

Hearts (~50 mg wet weight) were weighed and homogenized in 1 mL PBS. Lipids were extracted as described by Folch *et al.*[14] and dissolved in 200 μL 3% Triton X-100 for TC and TG analysis using enzymatic methods as described earlier or dissolved in 50 μL choloroform for thin layer chromatography (TLC) to determine cholesterol ester (CE) contains. TLC was performed on silica G-24 plates. The chromatographic developing solution was heptane/diethylether/acetic acid (74:21:4, vol/vol/vol)[15] using lipid from white adipose tissue as TAG control.

Statistical analysis

Quantitative data were given as mean±SEM. Statistical significance was tested using two-tailed Student's *t* test and one way ANOVA (Turkey posttest) by the computer program Prism (GraphPad Software). A value of $P < 0.05$ was considered statistically significant.

Results

Generation of *ApoE* KO rats

Traditional methods of gene targeting are complex with low efficiency and need culture of rat ES cells. In this study, the currently widely used genome editing methods TALEN was used to generate *ApoE* KO rats. A pair of TALEN plasmids targeting exon 3 of the *ApoE* gene was created and the target sequence is shown in ***Fig. 1A***. We chose 13 bp deletion mutation founders to generate stability passaged strain. The mutation site and sequences are shown in ***Fig. 1B***. The sequencing peak graph and amino acid changes are shown in ***Fig. 1C***. To verify *ApoE* knockout efficiency, we examined *ApoE* mRNA levels in the liver, which possessed high level ApoE expression in wildtype rats. As shown in ***Fig. 1D***, *ApoE* mRNA expression was undetectable in *ApoE* KO rats. In addition, we measured ApoE protein levels in plasma and there was no detectable ApoE protein in *ApoE* KO rats (***Fig. 1E***). We successfully established *ApoE* KO rats using TALEN mediated gene editing technique.

Hypercholesterolemia in *ApoE* KO rats

It is well known that *ApoE* KO mice displayed hypercholesterolemia upon chow diet (~400 mg/dL), and become more aggravated after high-fat diet feeding. In this study, 10 to 12 weeks old *ApoE* KO homozygous rats and wild type (WT) control rats were fed with Paigen diet (containing 15% lard, 1.25% cholesterol, and 15% sodium cholate). The plasma TC levels increased 2-fold in *ApoE* KO rats compared with WT rats (170.7±16.4 vs 82.7±4.4 mg/dL) on chow diet, and 4-fold after 2 weeks of Paigen diet (1339.0±88.8 vs. 326.5±35.6 mg/dL). After Paigen diet for 8 weeks, *ApoE* KO rats had 7.6-fold TC levels than WT rats (2462.4±238.6 vs 323.6±37.6 mg/dL) (***Fig. 2A***). *ApoE* KO rats also had moderate hypertriglyceridemia (378.8±55.0 vs. 85.4±12.0 mg/dL) after 2 weeks of

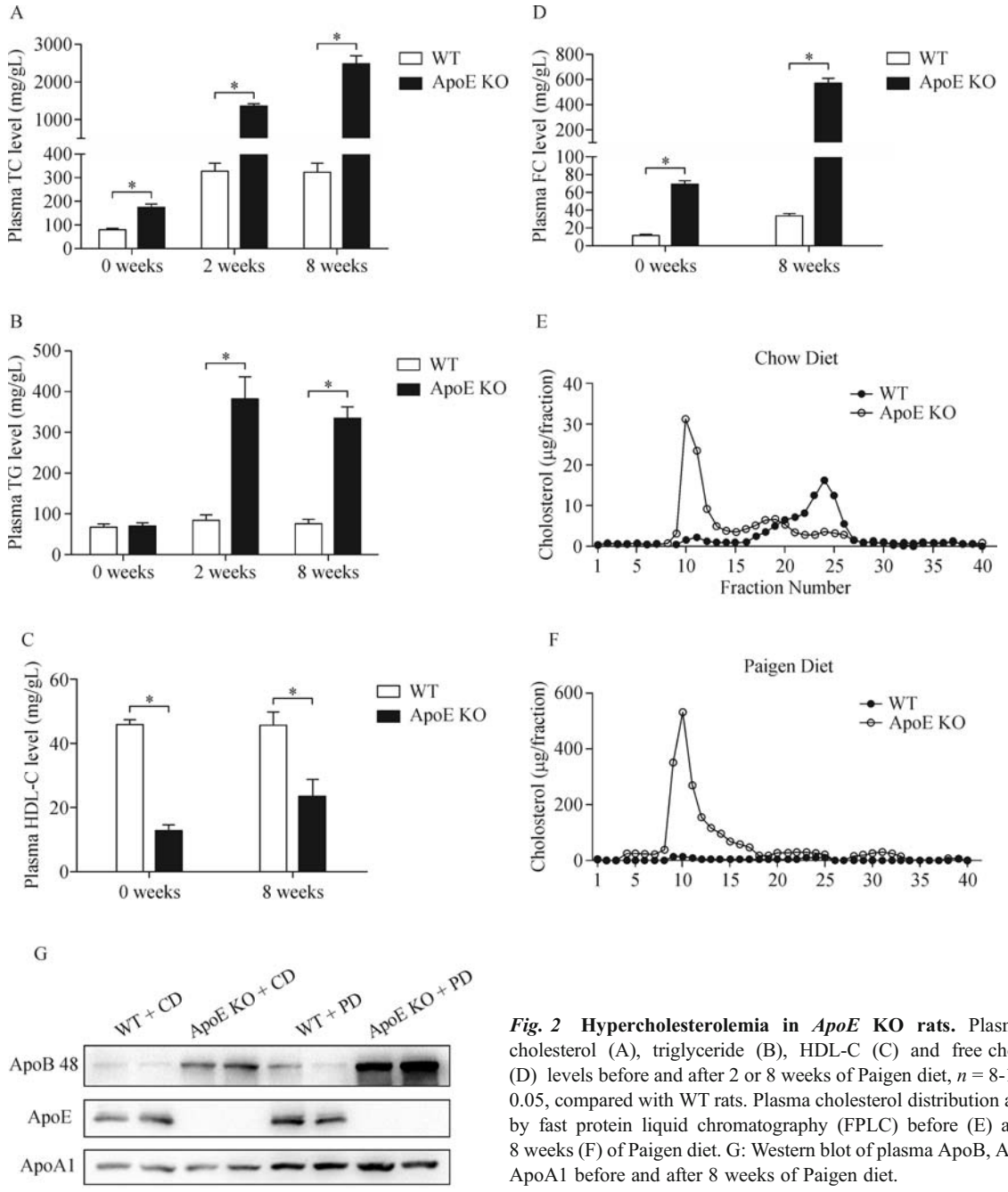

Fig. 2 **Hypercholesterolemia in *ApoE* KO rats.** Plasma total cholesterol (A), triglyceride (B), HDL-C (C) and free cholesterol (D) levels before and after 2 or 8 weeks of Paigen diet, n = 8-10, *P < 0.05, compared with WT rats. Plasma cholesterol distribution analyzed by fast protein liquid chromatography (FPLC) before (E) and after 8 weeks (F) of Paigen diet. G: Western blot of plasma ApoB, ApoE and ApoA1 before and after 8 weeks of Paigen diet.

Paigen diet feeding (***Fig. 2B***). Plasma HDL-C levels decreased 50% in *ApoE* KO rats upon both chow diet and Paigen diet (***Fig. 2C***). *ApoE* KO rats also showed markedly increased plasma FC levels with a 5.8-fold increase (34.6±1.9 vs. 11.8±0.6 mg/dL) on chow diet, and 16.4-fold increase after 8 weeks of Paigen diet (566.3±42.7 vs. 35.6±1.9 mg/dL) compared with WT rats (***Fig. 2D***). Plasma FPLC showed that cholesterol in chylomicron remnants (CMR) and VLDL fractions were significantly increased in *ApoE* KO rats compared with WT rats on chow diet (***Fig. 2E***). The differences were more striking after 8 weeks of Paigen diet (***Fig. 2F***). Cholesterol in HDL fraction was significantly decreased in *ApoE* KO rats, consistent with plasma HDL-C levels (***Fig. 2E***). We also detected several apolipoprotein (ApoB, ApoE and ApoA1) levels in plasma. ApoB48 was significantly increased in *ApoE* KO rats regardless of diet (***Fig. 2G***). Therefore, Paigen diet feeding could induce severe hypercholesterolemia in *ApoE* KO rats.

Mild aortic atherosclerosis in *ApoE* KO rats

ApoE KO mice displayed spontaneous aortic atherosclerosis at 3 months of age, and had severe aortic

atherosclerosis after high-fat diet feeding [9]. In this study, we can hardly detect atherosclerotic lesions even in 16-month old *ApoE* KO rats upon chow diet (Data not shown). After 10 to 12 weeks of Paigen diet feeding, oil red O staining in frozen section of aortic roots and the full length of the aorta showed that *ApoE* KO rats displayed mild aortic atherosclerosis with aortic root lesions (WT: $1.50\pm0.47\times10^3$ μm^2; *ApoE* KO:$254.2\pm33.4\times10^3$ μm^2) (***Fig. 3A*** and ***3B***) and full length of aorta lesion (WT: 0.59±0.15%; *ApoE* KO:2.58±0.42%) (***Fig. 3C*** and ***3D***).

Severe coronary atherosclerosis in *ApoE* KO rats

Though aortic atherosclerosis was mild in *ApoE* KO rats, severe coronary atherosclerosis was observed after 10 to 12 weeks of Paigen diet feeding. H&E staining showed 11 of 12 male *ApoE* KO rats having coronary artery atherosclerosis in the ostium of the right coronary artery, 7 of them with>70% occlusion. Among the females, there were also 4 of 10 *ApoE* KO rats displaying mild right coronary artery atherosclerosis with<25% occlusion. We did not detect any coronary atherosclerosis in WT rats (***Fig. 4***). Oil red O, Mac2 immuno-staining and Masson's trichrome staining showed coronary atherosclerosis plaque in *ApoE* KO rats containing substantial amounts of lipid, macrophages and collagen fibers (***Fig. 4***).

Myocardial cholesterol ester deposition in *ApoE* KO rats

ApoE KO male rats also exhibited severe myocardial focal lesions after Paigen diet feeding. They could be observed by naked eyes on the transverse section of the myocardium (***Fig. 5A***). Oil red O, Mac2 immuno-staining and Sirius red staining showed that the main components of myocardial lesions were neutral lipids, together with macrophages and collagen fibers (***Fig. 5B***). To detect the lipid composition in the myocardial lesions, we conducted myocardial lipid extraction, and then measured cholesterol and triglyceride contents. It was found that cholesterol levels were dramatically increased (***Fig. 5C***). We also conducted TLC using myocardial lipid extraction samples. It was shown that the major lipid component in myocardial lesions was CE (***Fig. 5D***).

Discussion

It was hard to generate gene knockout rats based on the traditional method using ES cell homologous recombination. Nowadays, with the application of novel gene editing techniques (TALEN and CRISPR/Cas system) knockout rats can be produced quickly and efficiently. There were already reports about *ApoE*

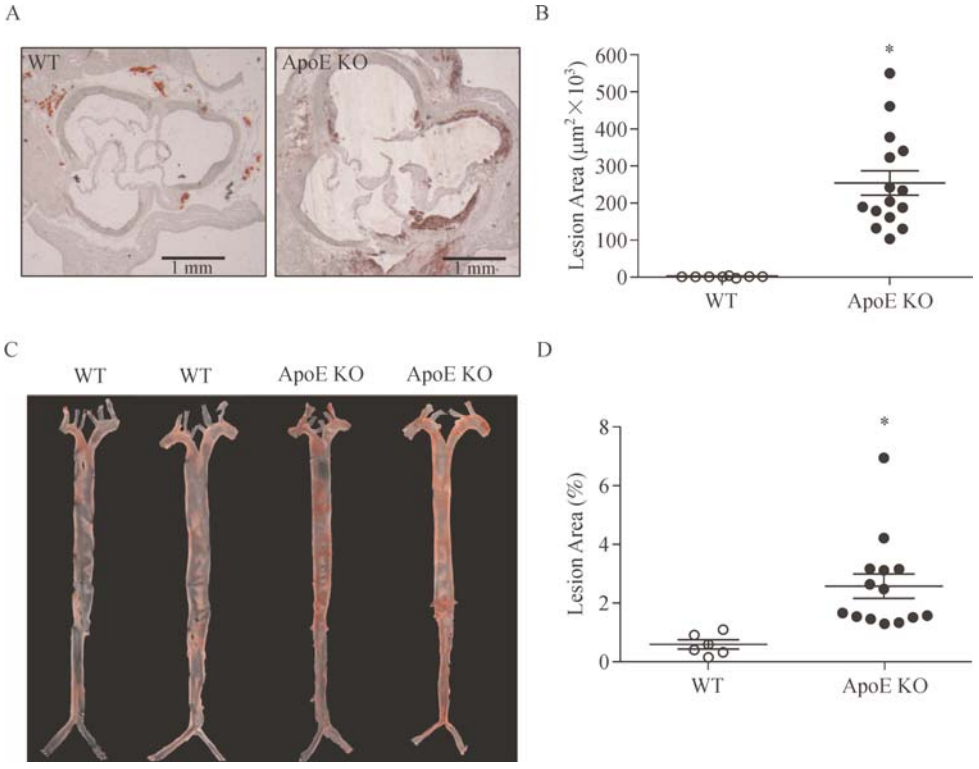

Fig. 3 **Mild aortic atherosclerosis in *ApoE* KO rats.** A: Histological analysis of aortic roots from WT and ApoE KO rats stained with oil red O. B: Quantification of atherosclerosis lesions area of aortic roots. C: Representative photographs of the aorta en face from WT and ApoE KO rats stained with oil red O. D: Quantification of lesions area of full length aorta. n = 6-15, *$P<0.05$, compared with WT rats.

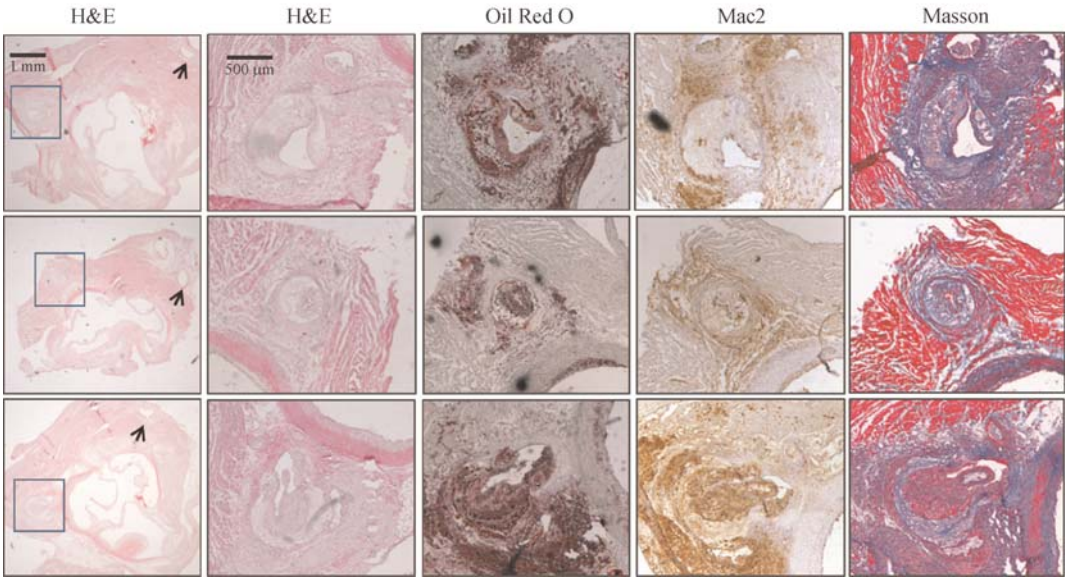

Fig. 4 **Severe coronary atherosclerosis in *ApoE* KO rats.** Histological analysis of coronary artery sinus with H&E, oil red O, Mac2 and Masson staining from 3 male *ApoE* KO rats after Paigen diet.

knockout rats[16–17]. The plasma cholesterol levels of *ApoE* KO rats were 1.5-fold higher than WT controls, but significantly lower than *ApoE* KO mice, which are consistent with the present data. However, there was no report on the phenotypes upon high-fat diet feeding in these *ApoE* gene deleted rats. In this study, we analyzed plasma lipid and atherosclerotic features in *ApoE* KO rats generated by TALEN method. Plasma cholesterol levels were slightly increased in *ApoE* KO rats upon chow diet. After Paigen diet feeding, *ApoE* KO rats had severe hypercholesterolemia, coronary atherosclerosis and myocardial cholesterol ester deposition.

There were many differences in phenotypes between *ApoE* KO rats and mice. 1) Plasma cholesterol levels upon chow diet are mildly elevated in *ApoE* KO rats but highly elevated in *ApoE* KO mice (about 170 mg/dL vs. 400 mg/dL). 2) There were more aortic atherosclerotic lesions in *ApoE* KO rats than in mice either as spontaneous occurrence or by high fat diet induction. 3) There was obvious coronary atherosclerosis in *ApoE* KO rats, which, however, was nearly completely absent in mice during 3-month Paigen diet induction[18]. 4) *ApoE* KO rats also displayed severe myocardial CE accumulation after Paigen diet feeding, while there was no report on this phenomenon in *ApoE* KO mice.

The apparent difference between *ApoE* KO mice and rats in aortic and coronary atherosclerosis may be related to the difference of vascular diameter and wall structure which would lead to hemodynamic changes. *ApoE* KO rats readily develop coronary atherosclerosis but fewer lesions in the aorta. Hence, *ApoE* KO rats can certainly serve as a better model for coronary atherosclerosis, which is the major pathological process in humans. Because *ApoE* KO rats do not develop spontaneous atherosclerosis in the aorta and the atherosclerotic lesions must be induced by Paigen diet feeding *ApoE* KO rats are therefore not suitable to serve as a model for the study of spontaneous atherosclerosis. In this regard, *ApoE* KO mice are superior to the rats.

ApoE KO rats displayed severe myocardial CE accumulation, which probably was a type of cardiac xanthoma, resulting from severe hypercholesterolemia. The reason for massive accumulation of myocardial CE in *ApoE* KO rats, but not in *ApoE* KO mice, may be related to undefined difference in metabolic features between the two genera. *ApoE* KO rats also exhibited skin xanthoma (Supplementary Data), which is similar to certain types of *ApoE* mutated patients[5–6]. We found that the mRNA expression of oxidized low density lipoprotein (lectin-like) receptor 1 (Lox1) was increased in the heart of *ApoE* KO rats. Lox1 mediates the uptake of oxidized lipoproteins[19], and it was reported that lipoproteins from *ApoE* KO mice were highly oxidized[20]. This may play a certain role in the accumulation of myocardial CE in *ApoE* KO rats.

According to the results of echocardiography, *ApoE* KO rats did not display myocardial systolic functional changes after Paigen diet feeding (Supplementary Data). Therefore, although *ApoE* KO rats had severe coronary atherosclerosis and myocardial CE accumulation, the heart function was still in the compensation phase. Male *ApoE* KO rats displayed more severe phenotypes than female rats. This may be related to the cardiac protective role of estrogen.

Severe coronary atherosclerosis can be induced in *ApoE* KO rats by 10 to 12 weeks of Paigen diet. This rat

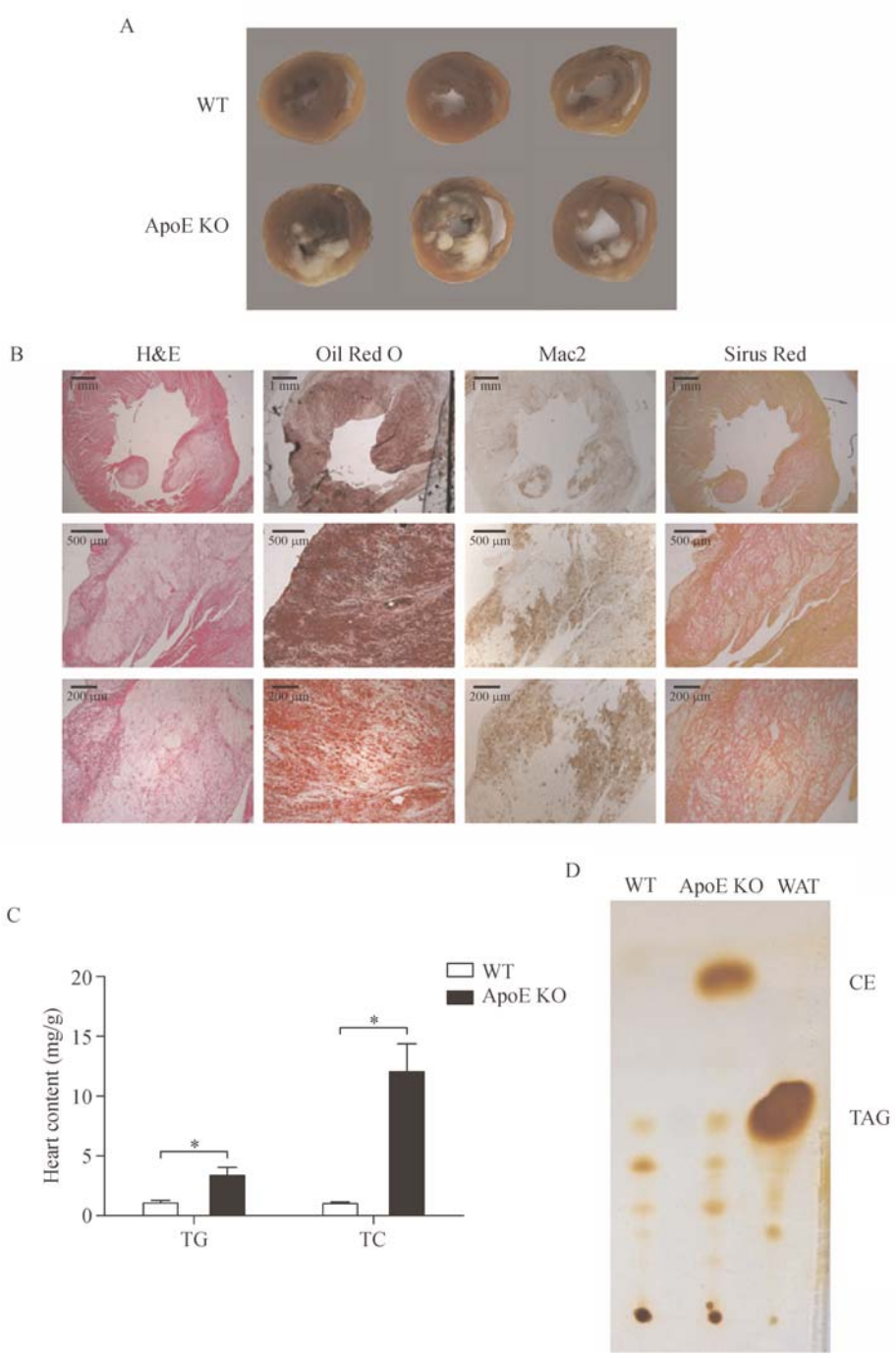

Fig. 5 **Myocardial cholesterol ester deposition in ApoE KO rats.** A: Representative photographs of heart transverse section from 3 WT and 3 *ApoE* KO male rats. B: Histological analysis of myocardial with H&E, oil red O, Mac2 and Sirius red staining from 3 male *ApoE* KO rats. C: The total cholesterol and triglyceride contents of heart form WT and *ApoE* KO rats by lipid extraction. $n = 7$-8, $^*P < 0.05$, compared with the WT rats. D: Myocardial lipid thin-layer chromatography. WAT lane: extracted from rat white adipose tissue, representative the location of TAG.

model is easy to operate and handle. Furthermore, the process of diet induced atherosclerosis is similar to the pathological progression of human diseases. *ApoE* KO rats could serve as a good animal model of coronary atherosclerosis that could be applied to the pathogenic studies and drug screening, and so on.

In the future, we will analyze the impact of different diet compositions and feeding time on the progression and regression of coronary atherosclerosis, cardiac function and the occurrence of acute myocardial infarction in *ApoE* KO rats. We will also study the pharmacological intervention with lipid-lowering and anti-atherosclerotic agents in these *ApoE* KO rats. To evaluate the advantages and disadvantages of *ApoE* KO rats as a novel animal model for coronary atherosclerosis and hypercholesterolemia.

In conclusion, *ApoE* KO rats, generated by the TALEN mediated gene editing method, developed server hypercholesterolemia, coronary atherosclerosis and myocardial CE deposition after subsisting on a Paigen diet. *ApoE* KO rats can be used as a novel coronary atherosclerosis and hypercholesterolemia animal model.

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