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Hyperlipidemia induces typical atherosclerosis development in Ldlr and Apoe deficient rats

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Abstract

Background and aims: Low-density lipoprotein receptor (*Ldlr*) and apolipoprotein E (*Apoe*) knockout (KO) mice have been widely used as animal models of atherosclerosis. However, data suggested that it is difficult to develop typical atherosclerosis in rats. To this end, *Ldlr* and *Apoe* KO rats were generated and the potential to develop novel atherosclerosis models was evaluated.

Methods: We established *Apoe*/*Ldlr* single and double KO (DKO) rats via the CRISPR/Cas9 system in the same background. Phenotypes of dyslipidemia and atherosclerosis in these *KO* rats were systematically characterized.

Results: Knockout of either gene led to severe dyslipidemia and liver steatosis. Significant atherosclerotic plaques were observed in the abdominal aorta of all mutant rats fed a normal diet for 48 weeks. Western diet greatly aggravated atherosclerosis and fatty liver. In addition, we found mononuclear cell infiltration in early lesions. Increased expression of inflammatory cytokines, as well as macrophage accumulation in lesions of mutants, was observed, indicating that mononuclear cell trafficking and endothelial inflammation affected atherogenesis. Moreover, mutant rats displayed a sex difference profile more similar to

humans in which males had heavier plaque burdens than females.

Conclusions: Deficiency of either *Ldlr* or *Apoe* genes induced hyperlipidemia, which promoted endothelial inflammation and led to typical atherosclerosis in rats on normal or Western diets. These models display certain advantages, which will benefit future investigations of atherosclerotic pathology and antiatherosclerotic therapeutics.

Key Words: Apoe; Ldl receptor; atherosclerosis; rat; gene knockout

Introduction

Atherosclerosis is the pathological and physiological basis, as well as a pre-symptom, of many cardiovascular diseases. Low-density lipoprotein receptor (LDLR) and apolipoprotein E (ApoE) participate in the transport of cholesterol-rich lipoproteins. *Ldlr* or *Apoe* deficiency in humans is related to elevated plasma total cholesterol and consequently higher risk of hypercholesterolemia, atherosclerosis and coronary artery disease [1, 2]. Both *Apoe* and *Ldlr* knockout (KO) mice have become the most used animal models of atherosclerosis, and helped a great deal understand the biology of atherosclerosis [3-5]. However, the translation of discovery from mice to humans has been slow and unconvincing, largely due to differences between mice and humans in the pathogenesis and phenotype of atherosclerosis. For example, typical atherosclerosis in mice is usually induced by severe hypercholesterolemia. The most important clinical consequences of atherosclerosis in humans arise from lesions in the coronary, carotid, and cerebral arteries, whereas the focus in mice is on the aorta and proximal great vessels [6, 7]. Intimal-thickening occurs in early lesions in human disease but not in mice [8]. Furthermore, data exclusively obtained from mouse models may be compromised by species-dependent effects and have limited value in identifying features unique to human disease. Therefore, it is necessary to develop novel animal models, to obtain a more comprehensive understanding of the initiation, progression and consequences of atherosclerosis, as well as to find effective therapies.

As certain characteristics of lipid metabolism in rats are in-between those of humans and mice [9], we speculated that rat atherosclerosis models might have advantages compared to mouse models. In fact, it is accepted that rat as a model animal has advantages over other animal models, especially for studying cardiovascular diseases such as hypertension and stroke [10]. So far, only two papers related to atherosclerosis in *Apoe* gene knockout rats have been published. One study described that occlusal disharmony accelerated atherosclerosis in *Apoe* KO rats, but only showed very early signs of atherosclerosis and lipid deposition [11]. Another report about *Apoe* KO rats generated through TALEN technology stated that *Apoe* KO rats were totally different from *Apoe* KO mice as the rats were resistant to hyperlipidemia-induced endothelial inflammation and did not develop atherosclerosis [12]. As for *Ldlr*, a paper in 2016 reported the generation of an *Ldlr* KO rat via Zinc-finger nuclease (ZFN) technology and stated that it did not develop plaques but could be a new model of hypercholesterolemia [13]. During the preparation of our paper, another *Ldlr* KO rat generated by ZFN was reported, which developed typical plaque formation when fed a Western diet, whereas no aortic lesions were found in the normal diet-fed group [14].

Here, using the CRISPR/Cas9 technique, we generated *Apoe*/*Ldlr* single and double KO rat models in the same genetic background. Phenotypes of the KO rats were systematically characterized. The three mutant rats developed major phenotypic and biochemical characteristics present in human atherosclerosis, including marked hyperlipidemia, different

stages of atherosclerosis and liver steatosis. More importantly, heavier plaques were found in males, a sex difference more similar to humans than to mouse models, thus creating several promising alternative animal models for the study of human hyperlipidemia and atherosclerosis.

Materials and methods

Animals and diet

All techniques and procedures were performed according to the NIH guidelines, and were approved by the Animal Ethics Committee of East China Normal University (Permit number: M20150505). Heritable total *Ldlr* or *Apoe* gene knockout (KO) rats were generated by CRISPR-Cas9 system in our lab according to the method described previously in Nature Protocol [15]. More detailed methods are provided in Supplementary material.

Biochemical analysis

Rats were fasted overnight (12-14 h) and blood samples from the retro-orbital plexus were collected. Serum was obtained by centrifugation at 3000 rpm for 15 min at 4 °C, then kept frozen at −80 °C until analysis. Lipids and lipoproteins including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), ApoB and lipoprotein (a) were analyzed using AU680 Automatic Biochemistry Analyzer (Beckman Coulter, USA). FPLC lipoprotein profiles were assessed by size-exclusion chromatography on Superose 6 10/300 GL column and AKTA purifier (GE Healthcare) [16]. We also measured liver and kidney indexes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid (UA) and creatinine levels. Furthermore, the atherosclerosis index and LDL/HDL ratio were calculated. Atherosclerosis

index was calculated as [TC- HDL-C]/HDL-C [17]. Serum insulin, leptin and free fatty acid (FFA) levels were measured using ELISA kits (Hengyuan Biological Technology Co. Ltd., Shanghai, China).

Hematoxylin-eosin (H&E) staining

After perfusion with cold PBS buffer (pH7.2) and fixation in 4% paraformaldehyde, aortas were excised from the carotid artery and heart to the iliac artery. Kidney, visceral adipose tissue and liver were collected and analyzed histologically following H&E staining.

Oil Red O staining

Liver and aorta sections were fixed overnight, followed by immersion in 15% then 30% sucrose for dehydration, and stained with Oil Red O solution. The *en face* aortas were excised from adhesion tissue and adipose tissue, opened lengthwise and edges and corners were flatted on black boards. Staining was then performed with Oil Red O solution.

Analysis of aortic atherosclerosis

Histologic analysis of aortic lesions was performed as stated above. H&E staining or Oil Red O staining images were captured on a Leica DM4000 B LED microscope with Leica DFC310FX Camera and software kit. Oil Red O staining was quantified by Image-Pro® Plus

version 6.0 software, and the percentage of plaque coverage was calculated.

Statistical analyses

Data are presented as mean ± SEM, and analyzed by Graphpad prism 6 software. One-way ANOVA followed by Dunnett's multiple comparisons test was applied to analyze differences among groups. For body weight, the comparisons were analyzed using repeated measures analysis followed by Tukey's multiple comparisons test. Differences were considered statistically significant at $p < 0.05$.

Details of other methods were provided in Supplementary material.

Results

Generation of heritable total *Ldlr* and *Apoe* KO rats using CRISPR/Cas9 system

Two sgRNAs targeting *Ldlr* exon 4 were transcribed. Zygotes of SD rats were microinjected with a mixture of Cas9 mRNA (50 ng/μl) and sgRNA (25 ng/μl each). A total of 24 pups were born from 2 pseudopregnant female SD rats transferred with 100 injected zygotes. PCR analysis showed that nine rats (founder 2, 3, 7, 10, 16, 21-24) had an *Ldlr* deletion (Supplementary Fig. 1A). Further sequence analysis revealed that 23 rats (founder 1-4, 6-24) had a frameshift mutation (Supplementary Fig. 1B). In the end, founder 1 was chosen to establish a colony (*Ldlr* KO), which carried a 118 bp deletion from No.22759599bp to 22759716bp in the *Ldlr* gene (NC_005107.4), resulting in a termination codon TAG, and deletion of 768 amino acids of LDLR.

Similarly, two sgRNAs targeting *Apoe* exon 4 were transcribed. Following Cas9 and sgRNA microinjection, 100 injected zygotes were transferred to 2 pseudopregnant female rats. 7 pups were born. PCR analysis showed that all seven rats (founder 1-7) had *Apoe* deletions (Supplementary Fig. 2A). Sequencing data confirmed that six rats (founder 1, 2, 4-7) had a frame shift mutation (Supplementary Fig. 2B). Among them, founder 1 was chosen to establish a colony (*Apoe* KO), which carried a 130 bp deletion from No.80613571bp to 80613700bp in *Apoe* gene (NC_005100.4), resulting in a termination codon TAA, and deletion of 231 amino acids of ApoE.

The *Apoe/Ldlr* double KO (DKO) was derived from cross-breeding the two KO rats above. Typical genotyping results are shown in Supplementary Fig. 1C and Fig. 2C. The genotypes of successive rat generations were verified as correct (over a period of two and a half years). In addition, homozygous mutants showed a markedly decreased *Apoe* or *Ldlr* mRNA level (Supplementary Fig. 2D) as expected, with the decrease in mRNA stability induced by frameshift or nonsense mutations [18]. No obvious adverse phenotype was observed in the development, viability and fertility of mutant rats.

Characterization of body weight and serum lipid profile

We first monitored the body weight of male and female rats of four different genotypes. No significant change was observed in either *Ldlr* KO or DKO rats. However, the body weight of *Apoe* KO rats displayed a slight increase as compared to that of wild type (WT) littermates, in both male normal diet and female Western diet groups (Supplementary Fig. 3).

Next, we checked the serum lipid and lipoprotein profiles. All three mutant rats had markedly upregulated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) levels, both in normal diet and Western diet groups as compared to the levels in respective WT controls. Upregulation of triglycerides (TG) was also observed, but the small increase in 24 week-old normal diet-fed *Apoe* KO rats did not reach significance (Fig. 1A-C). The levels of ApoB and lipoprotein (a) were increased in all mutants, while no alteration was observed for

lipoprotein (a) in *Ldlr* KO rats (Fig. 1E). Single or double deletion of *Ldlr*/*Apoe* caused a 7-9 fold increase in plasma LDL-c in the normal diet groups (Fig. 1C). Western diet aggravated the elevation of TC and LDL-c in all mutants (Fig. 1A and 1C). Interestingly, Western diet-fed *Ldlr* KO rats showed a particularly dramatic elevation of TG (Fig. 1B). On the other hand, only *Ldlr* KO rats displayed a significant increase in high-density lipoprotein cholesterol (HDL-c), whereas *Apoe* KO showed a decreased HDL-c level (Fig. 1D). Not much alteration was observed in the level of leptin, except in the 14 week-old Western diet-fed group. Serum free fatty acid (FFA) was increased in 48 week-old Western diet-fed mutants, although the change in DKO did not reach significance (Fig. 1F). In addition, plasma lipoprotein profiles of *Ldlr* KO and *Apoe* KO maintained on normal chow for 24 weeks showed elevated levels of cholesterol in the VLDL fraction (Supplementary Materials, Fig. 4). Altogether, the *Apoe* KO, *Ldlr* KO and DKO rats displayed severe hyperlipidemia and redistribution of cholesterol in different lipoprotein fractions, resulting in selective enrichment of cholesterol associated with VLDL. Western diet dramatically exacerbated hyperlipidemia especially as measured by TC and LDL-c.

To examine whether there was any change in TG degradation, we analyzed mRNA levels of lipoprotein lipase (LPL) and adipose triglyceride lipase (ATGL) in 24 week-old Western diet-fed rats. Both LPL and ATGL increased in *Ldlr* KO and *Apoe* KO. On the other hand, acetyl-CoA carboxylase 1 (ACC) and fatty acid synthase (FAS) increased significantly in *Apoe*

KO, suggesting increased TG synthesis. The increase of LPL was confirmed by plasma activity. In addition, mRNA and protein levels of proprotein convertase subtilisin kexin 9 (PCSK9) in the liver and plasma of mutant rats showed no changes (Supplementary Fig. 4).

Atherosclerotic phenotypes in normal diet-fed rats

No obvious aortic plaques were identified in the *en face* aortas of all four genotypes of 24 week-old normal diet-fed rats (Data not shown). However, early vascular lesions were observed in mutant carotid arteries and abdominal aortas. Mononuclear cell infiltration typical of inflammation was also observed along the intima of the aortic arch in DKO rats and in the abdominal aorta of all three mutants. In addition, Oil Red O staining showed evidence of lipid deposition in the aortic sinus of *Ldlr* KO and DKO rats, as well as in the coronary artery orifices of DKO rats (Supplementary Fig. 5). By 48 weeks of age, all three mutant rats developed markedly increased lipid deposition. Plaques in transverse aortic root sections and in flat *en face* aortic preparations were also clearly observed. Plaques were more often found in carotid bifurcation and abdominal aorta, and frequently were distributed at the entrance of small vessels (Fig. 2A and B). The mean lesion area of KO rats was significantly increased as compared to that of control rats. A trend towards heavier lesions in DKO rats was also observed, though it did not reach significance due to considerable individual variation. The lesion areas accounted for 6.3-10.7% of total aortic intima in *Ldlr* KO, 5.0-10.7% in *Apoe* KO,

and 4.7-13.2% in DKO, as compared to 2.6-5.2% of total aortic intima in WT controls. No significant difference was found among the three mutants (Fig. 2B). However, although both LDL/HDL ratios and atherosclerosis indices increased significantly in all three mutants, greater increases were observed in *Apoe* KO and DKO rats. The LDL/HDL ratios and atherosclerosis indices in Western diet-fed mutants increased more dramatically as rats aged, when compared to normal diet-fed mutants (Fig. 2C). Therefore, our three KO rats developed endothelial inflammation and aortic lesions, simulating typical atherosclerosis even when kept on a normal diet.

We proceeded to measure atherosclerosis-related gene expression in liver and aortic arch, to gain more insight into different factors involved in the process. In livers of 24 week-old normal diet-fed groups, the mRNA expression of the adhesive molecule VCAM1 increased significantly in all three mutant rats, whereas Toll-like receptor 4 (TLR4), the primary receptor in innate immunity, was mainly increased in *Ldlr* KO and *Apoe* KO rats. In aortic arch, TLR4 expression showed a threefold increase in *Apoe* KO, together with a moderate increase of ICAM1 mRNA (Supplementary Fig. 6A and B). The lipid scavenger receptor CD36 and lectin-like oxidized LDL receptor-1 (LOX-1), involved in scavenging modified forms of LDL and foam cell formation, were also detected, and a significant increase of CD36 was observed in *Apoe* KO and DKO rats, together with a modest increase of LOX-1 in the mutants, suggesting increased foam cell formation. C-reactive protein (CRP), which may play a causal role in

atherogenesis, had little alteration in the liver, but a dramatic increase was observed in aortic arch of all three mutants, with an increase ranging from 133 to 891 fold (Supplementary Fig. 6C and D), indicating prominent endothelial inflammation and atherosclerotic changes at lesion sites. We further assessed VACM1 in the aortic arch of 24 week-old Western diet-fed rats. Result showed significant elevation in *Apoe* KO rats. When we examined VACM1 expression in lesions of 72 week-old Western diet-fed rats by immunohistochemical staining, all mutants displayed enhanced expression as compared to WT (Supplementary Fig. 6E and F). There were no compensatory increases in expression of remnant receptors such as low-density lipoprotein receptor-related proteins (LRPs) and ATP-binding cassette members (ABCs). On the contrary, decrease in LRP1, LRP 5, LRP 6, and ABCG1 in both *Apoe* KO and *Ldlr* KO rats was observed. ABCA1 was reduced in *Apoe* KO rats. Expression of scavenger receptors such as SRB1, LOX-1 and CD36 was increased in 24 week-old Western diet-fed *Ldlr* KO and *Apoe* KO rats (Supplementary Fig. 6G and H). The above data suggested that dyslipidemia induced by *Ldlr* and *Apoe* deficiency accelerated foam cell formation and atherosclerosis development by promoting atherosclerosis-related adhesive and inflammatory gene expression, as well as alteration of remnant receptors.

The Western diet accelerated atherosclerosis development

The above results showed that Western diet aggravated dyslipidemia and inflammation. We

258 further investigated if Western diet affected atherosclerosis development in aortas. Earlier
259 atherosclerosis development in *Ldlr* and *Apoe* deficient rats fed a Western diet was observed.
260 At 24 weeks of age (16 weeks on Western diet), both *Ldlr* KO and *Apoe* KO displayed certain
261 lesions in aortic lumen and arterial wall, including bulging in lumen and interrupted endothelial
262 lining (Fig. 3A). Lipid deposition was also observed at sections in different parts of the aortic
263 root (Fig. 3B and C). At 48 weeks of age (i.e., after 40 weeks on Western diet), the *en face*
264 aortas of all three mutants showed a much heavier plaque burden (Fig. 3D). When the
265 mutant rats reached 72 weeks of age, there were severe atherosclerotic plaques, widely
266 distributed throughout the arterial tree, including the carotid arteries. The percentage of aortic
267 intima covered by plaques reached 9.7-20.3% in *Ldlr* KO, 7.2-22.0% in *Apoe* KO and
268 9.4-39.1% in DKO rats *versus* 2.5-9.4% in WT rats on a Western diet. Similar to data from
269 normal diet groups, there was no significant difference in the average lesion area among the
270 three mutants (Fig. 4A and4B). Data in DKO strongly indicated that male rats had a higher
271 plaque burden compared to female rats. The two single KO mutants showed the same trend
272 although it did not reach a significant level (Fig. 4C). These plaques contained a significant
273 increase in macrophages (Fig. 4D). The aortas covered with plaques were clearly more
274 fragile, more easily broken than younger aortas when excised, suggesting a higher likelihood
275 of hemorrhage occurrence. Altogether, Western diet positively exacerbated atherosclerosis
276 development in mutant rats.

Increased hepatic steatosis and adiposity

Next, we tested whether there was any change in the liver of mutant rats. Significantly elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were shown in 24 week-old *Apoe* KO and *Ldlr* KO rats in Western diet-fed groups. ALT level was also increased in 24 week-old normal diet-fed rats (Fig. 5). In addition, H&E staining of liver sections from normal diet groups revealed large cavities in *Apoe* KO and *Ldlr* KO rats, with *Apoe* KO being more severe. Oil Red O staining confirmed that these cavities were related to adipose deposition (Fig. 5A). When rats were fed a Western diet, even WT rats displayed cavities and Oil Red O stained areas. *Apoe* KO and *Ldlr* KO rats presented with more severe steatosis, both by H&E and Oil Red O staining (Fig. 5B). In addition, mutant rats fed aWestern diet showed a moderately enlarged average adipocyte size compared to WT littermates, although the difference in *Ldlr* KO did not reach significance. There was a trend towards an increase in the ratio of visceral *versus* subcutaneous adipose tissue (VAT/SAT) in Western diet-fed mutants (Supplementary Fig. 7). Thus, the mutant rats showed marked hepatic steatosis, together with a moderate increase in adiposity.

In contrast, no significant change in oral glucose tolerance and fasting blood glucose was observed, except in 24 week-old Western diet-fed *Apoe* KO rats (Supplementary Fig. 8). Moreover, when the effect of *Apoe* and *Ldlr* deficiency on kidney was examined, no significant

296 change was observed in normal or Western diet-fed rats based on the H&E staining results.
297 Only some minor alterations were found in creatinine and uric acid levels in *Apoe* KO rats
298 (Supplementary Fig. 9).
299

300 Discussion

301 This is the first report of typical atherosclerotic rat models based on CRISPR/Cas9 generated
302 *Ldlr/Apoe* single or double knockouts. This work not only provides new insights into ApoE and
303 LDLR functions in lipid metabolism and atherosclerosis related diseases, but also presents
304 novel options for animal models of hyperlipidemia and atherosclerosis. The generated rat
305 models had certain advantages, including a sex difference more similar to humans and the
306 ability to develop lesions under normal diet.
307 Although there was little doubt that *Ldlr* and *Apoe* deficiency in rats could lead to
308 hyperlipidemia and proatherogenic conditions, whether knockout of these two genes could
309 lead to typical atherosclerotic plaques was still uncertain [12, 13]. In this study, we
310 demonstrated that *Ldlr* KO, *Apoe* KO and DKO rats fed a normal diet for 48 weeks developed
311 significant aortic plaques. When fed a Western diet, typical lipid deposition and plaques
312 generally developed earlier, with severe plaques in aortas appearing at 48 weeks of age. At
313 72 weeks of age, after 64 weeks on a Western diet, the lesion area could reach more than 30
314 percent. Our data strongly demonstrated that *Ldlr* or *Apoe* deficiency led to severe

dyslipidemia as well as typical atherosclerosis. These results supported the notion that atherosclerosis is developed mainly from hyperlipidemia [19]，and counter the previous view that the rat is resistant to hyperlipidemia and cannot develop typical atherosclerosis.

Several reports previously described the attempts to generate *Apoe* and *Ldlr* KO rats. The previous TALEN-generated *Apoe* KO rat did not develop typical plaques even when fed a high-cholesterol diet containing much higher cholesterol than the diet used in our study [12]. The *Ldlr* KO rat generated using the ZFN technology was reported to lack an obvious phenotype and arterial plaque formation when fed a normal diet, although no data was presented [13]. Since all the mutations were generated in SD rats, the differences might result from different deletion sites, analytical methods or observation time. For example, the TALEN-*Apoe* KO was only analyzed up to 18-20 weeks of age, which might be too short. Quite recently, another ZFN-*Ldlr* KO rat model was reported to developed exuberant atherosclerotic lesions when induced by Western diet [14]. The hyperlipidemia and atherosclerosis phenotypes were more similar to our *Ldlr* KO rats. However, there are still some differences. For example, although there were higher levels of vLDL, LDL and TG in both models, HDL in our *Ldlr* KO rats increased as compared to that in WT rats while in ZF-*Ldlr* KO the level decreased. In addition, the ZFN-*Ldlr* KO rat had no lesions after 64 weeks on a normal diet, but we found significant early lesions in our 48 week-old normal diet fed *Ldlr* KO, probably due to the capability of our rat model to develop higher inflammatory

responses, including early mononuclear cell infiltration and increased expression of inflammatory molecules. The higher inflammation may be associated with deficiency in LRPs, which was not observed in the ZFN model. Another significant difference was that ZFN-*Ldlr* KO rat gained more body weight and developed glucose intolerance. Our *Ldlr* KO rats displayed no change in body weight and glucose tolerance, which was consistent with data from *Ldlr* KO mice [20, 21]. Both of these *Ldlr* mutants were generated in SD rats, and used similar normal or Western diets. However, there were modest differences in gene targeting (the deletion in the ZFN-*Ldlr* KO rat was 337 bp at the junction of intron 3 and exon 4, while ours was 118 bp in exon 4), raising the possibility that some regulatory elements in intron 3 may cause the observed difference. It is well known that CRISPR/Cas9 has advantages over the ZFN technology simplicity, higher target density, higher success rate, lower cytotoxicity and multiplex genome editing possibility. Thus, CRISPR/Cas9 could be a better approach in creating animal models [22, 23].

Our *Apoe* KO rats had relatively similar phenotypes to *Apoe* KO mice, except for the following few aspects. The advanced atherosclerosis appeared at an older age in thr *Apoe* KO rat model than in the *Apoe* KO mouse model, which may due to a difference in artery size [24]. *Apoe* KO mice were leaner and had reduced adiposity [25, 26], but our mutant rats, especially the *Apoe* KO rat, showed a tendency towards increased adiposity and unchanged or even increased body weight. On the contrary, the *Ldlr* KO rat was quite different from the

353 *Ldlr* KO mouse. The *Ldlr* KO mouse showed a moderate increase of plasma LDL-c and
354 needed a Western diet or to be crossed with other models to induce atherosclerosis [27, 28].
355 *Ldlr* deficiency in our rat displayed a significant increase of LDL-c, similar to the two
356 ZFN-generated *Ldlr* KO rats [13, 14]. Moreover, our *Ldlr* KO rats developed atherosclerosis,
357 including typical plaques, even when fed a normal diet. Actually, there was no significant
358 variation in the atherosclerotic phenotype among our three mutant rats. This was consistent
359 with data from a mouse model which showed that DKO did not increase hypercholesterol
360 beyond *Apoe* KO alone [29]. Overall, the phenotype of our *Ldlr* rat was more in line with
361 previous reports that pathogenic variants in human *Ldlr* accounted for a high percentage of
362 familial hypercholesterolemia cases [1]. As for plasma TG levels in this study, no alteration in
363 normal diet-fed ApoE deficient rats was shown until 24 week of age. However, a significant
364 increase was observed in 8 week-old LDLR deficient rats. After a 40-week induction with
365 Western diet, both *Apoe* and *Ldlr* KO showed markedly increased TG levels. It seemed that
366 the increase of TG levels occurred earlier in *Ldlr* KO, while in *Apoe* KO. TG levels showed an
367 increase with age and Western diet induction.

368 Another interesting point is that our rat models, especially the DKO rats, displayed a sex
369 difference profile more similar to human diseases in which men have earlier onset of clinical
370 atherosclerotic plaque burdens [30, 31]. In mouse *Apoe* or *Ldlr* KO models, atherosclerosis
371 phenotypes were more prominent in females [32-34]. In addition, the mutant rats developed

372 atherosclerosis with a normal diet, which had a less extreme lipid disturbance. As a result, our
373 rat atherosclerosis models provided more suitable alternatives to be used in evaluating the
374 atherosclerosis regulatory effect of some specific genes, which have no impact on lipids [35].
375 H&E staining of the aortic intima indicated that there were inflammatory responses involved
376 in the initiation of atherosclerosis in these mutants, which was confirmed by results of
377 atherosclerosis-related gene expression. It was reported that inflammatory disorders in
378 humans, such as rheumatoid arthritis and psoriasis, are strongly associated with
379 atherosclerotic cardiovascular diseases, while development and progression of
380 atherosclerosis is enhanced by chronic inflammation, among which macrophages play a
381 critical role [36, 37]. In our preliminary study, we indeed observed enhancement of monocyte
382 trafficking and macrophage phagocytosis in the mutants. We also detected significant
383 macrophages infiltrations in lesions. These indicated that, in our models, immune responses
384 were directly linked to the metabolic disturbance and pathogenesis of the disease, mimicking
385 the situation in humans.
386 In conclusion, we successfully established rat models of atherosclerosis using
387 CRISPR/Cas9-generated *Ldlr* and *Apoe* deficient rats. A systematic comparison of typical
388 phenotypes in atherosclerosis development was performed for the first time in a single
389 background. In addition to the larger size making them more suitable for intravascular
390 angioplasty and stenting, our mutant rats have several extra advantages, including a sex

391 difference profile more similar to humans, and the formation of preliminary plaques under a
392 normal diet. These will greatly benefit future investigations of atherosclerosis-related genetic
393 and environmental factors, as well as facilitate the development of new therapeutics for
394 cardiovascular diseases.

395

396

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

YZ, HC, DL, WP and ML conceived and designed the experiments. YZ, YY, RX, XC, YX, LX, PY, TW and LZ conducted the experiments. HC, DL, YZ, YY, XC, and WP analyzed the results. HC, YZ, DL and ML wrote the manuscript. All authors critically revised the manuscript, read and approved the manuscript.

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Fig. Legends

Fig. 1 Lipid and lipoprotein profiles in serum.

(A) Total cholesterol (TC), (B) triglyceride (TG), (C) LDL-c, (D) HDL-c, (E) ApoB and lipoprotein (a) of *Ldlr* KO, *Apoe* KO, DKO rats and WT littermates. (F) Leptin and free fatty acid (FFA) levels of each genotype on a normal diet or Western diet. Data are shown as mean ± SEM. Data from male and female rats were merged (n = 10-16) as they showed a similar pattern. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. WT littermates (one-way ANOVA with Dunnett's multiple comparisons test).

Fig. 2 Atherosclerotic characteristics of normal diet-fed mutants.

(A) Oil Red O staining of aortic root sections. Aortas of different genotypes of 48 week-old rats were used. Aorta sections were cut and stained. Scale bar = 200 μm. (B) Representative images (left) and lesion area (right) of Oil Red O stained *en face* aortas of normal diet-fed mutants (n = 5-6). Lesion area was quantified by Image-Pro® Plus version 6.0. Data are shown as mean ± SEM. *$p < 0.05$ *vs.* WT littermates. (C) LDL-c/HDL-c ratio and Atherosclerosis index of different genotypes under normal or Western diet (n = 6-9). Data are shown as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs*. WT littermates (one-way

511 ANOVA with Dunnett's multiple comparisons test).

512

513 **Fig. 3** Representative atherosclerosis results of Western diet-fed rats.

514 (A) H&E staining of vessel wall of different arteries of 24 week-old rats. Arrows indicate

515 lesions. (B) Oil Red O staining of aortic root sections of 24 week-old rats, and (C)

516 quantification of the lesion area (% aortic root). AA, ascending aorta; OCA, coronary artery;

517 AS, aortic sinus. (D) Oil Red O staining of the *en face* aortas of indicated genotypes at the age

518 of 48 weeks.

519

520 **Fig. 4** Severe atherosclerosis in aortas and macrophage accumulation in lesions of Western

521 diet-fed 72 week-old rats.

522 (A) Percentage of total aorta occupied by plaques (n = 6-9) and (B) representative Oil Red O

523 images of aortas. (C) Different plaque burdens in male and female rats of the four genotypes.

524 (D) Macrophages in aortic root of four different genotypes. Aortic roots were perfused with

525 PBS followed by 4% paraformaldehyde, then embedded in paraffin for sectioning.

526 Macrophages were identified by immunohistochemical staining with anti-CD68 antibody.

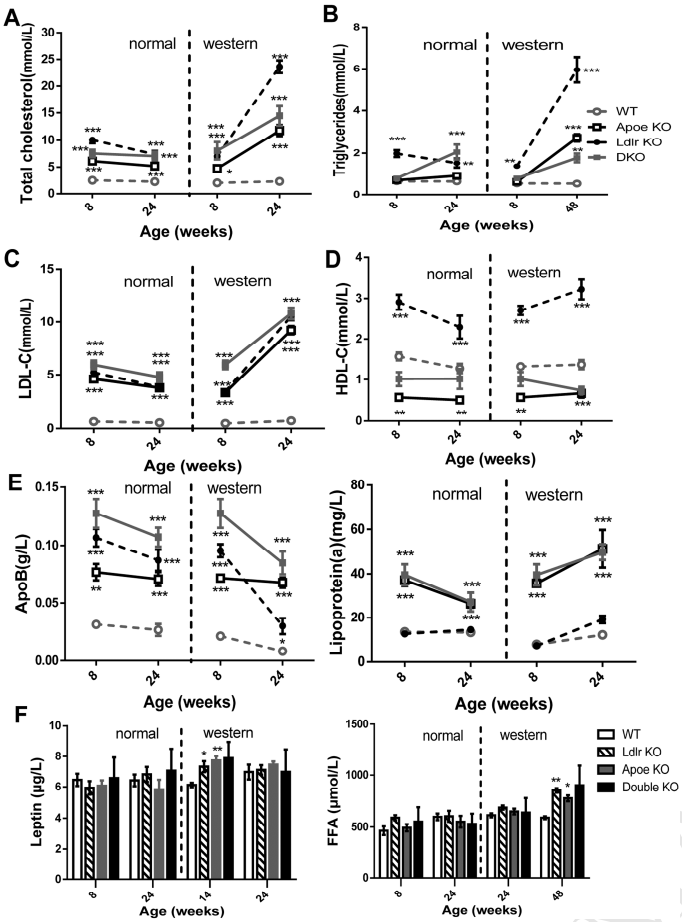
527 Images were captured using Leica DM4000 B LED. Data are shown as mean ± SEM. * $p <$

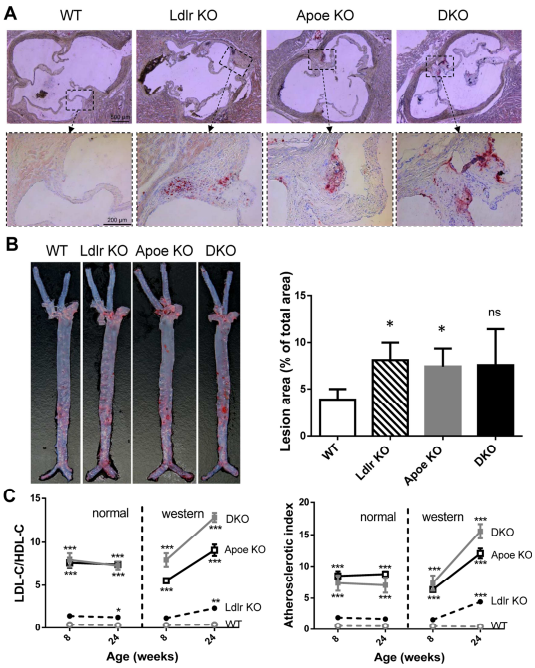
528 0.05, ** $p < 0.01$ and *** $p < 0.001$ *vs.* WT control (one-way ANOVA with Dunnett's multiple

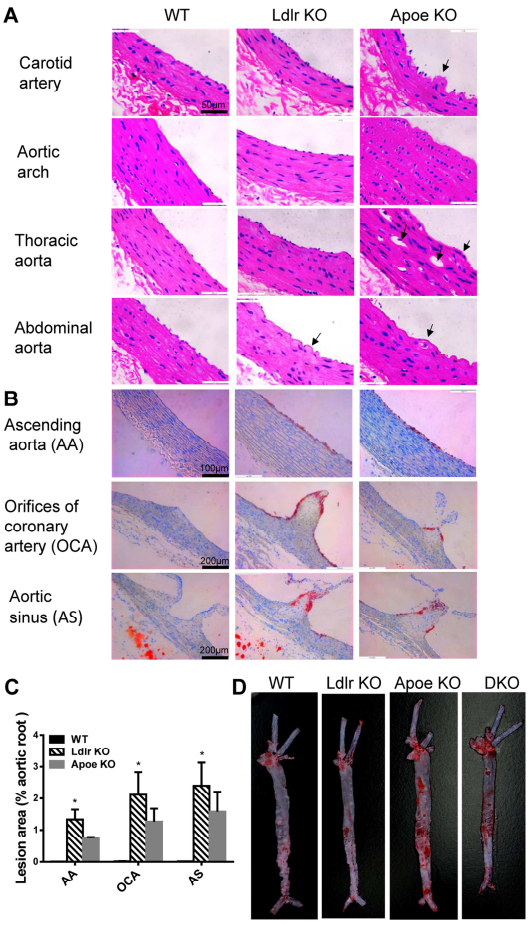
529 comparisons test).

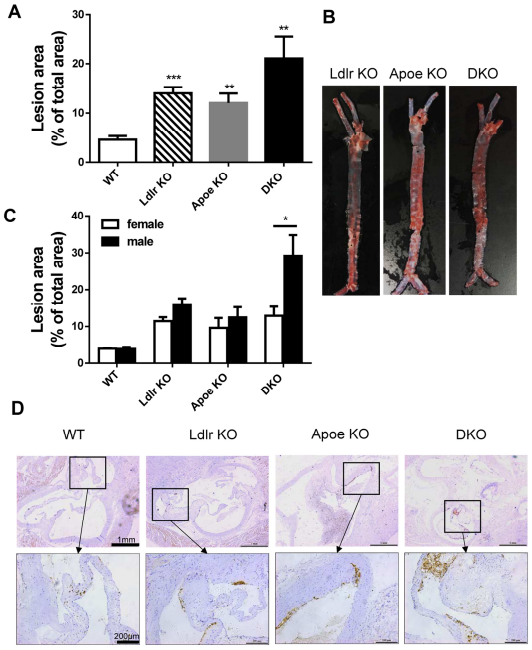
Fig. 5 Alteration of the liver in *Ldlr* and *Apoe* deficient rats.

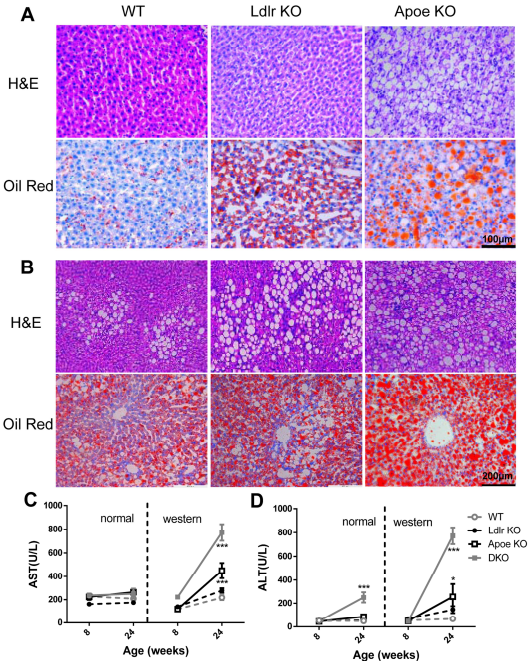
(A) H&E and Oil Red-O staining of livers from 24 week-old rats fed a normal diet (magnification 200X). (B) H&E and Oil Red-O staining of livers from 24 week-old rats fed a Western diet (magnification 100X). (C) AST and ALT of *Ldlr* KO, *Apoe* KO, DKO and WT littermates (n = 6-8). Data are shown as mean ± SEM. * $p < 0.05$, *** $p < 0.001$ *vs*. WT littermates (one-way ANOVA with Dunnett's multiple comparisons test).











Highlights

- Rat models of atherosclerosis established using CRISPR/Cas9.
- Systematic comparison of *Apoe*/*Ldlr* single and double knockout rats.
- First demonstration for *Apoe* deficiency in rat led to typical atherosclerosis.
- Sex difference profile similar to human disease in which males had heavier plaques.