EBioMedicine 36 (2018) 29–38

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

Spontaneous severe hypercholesterolemia and atherosclerosis lesions in rabbits with deficiency of low-density lipoprotein receptor (*LDLR*) on exon 7

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ARTICLE INFO

Article history:
Received 12 April 2018
Received in revised form 2 September 2018
Accepted 12 September 2018
Available online 19 September 2018

Keywords:
LDLR
Hypercholesterolemia
Atherosclerosis
Rabbits
Cas9
Knockout

ABSTRACT

Rabbits (*Oryctolagus cuniculus*) have been the very frequently used as animal models in the study of human lipid metabolism and atherosclerosis, because they have similar lipoprotein metabolism to humans. Most of hyperlipidemia and atherosclerosis rabbit models are produced by feeding rabbits a high-cholesterol diet. Gene editing or knockout (KO) offered another means of producing rabbit models for study of the metabolism of lipids and lipoproteins. Even so, apolipoprotein (Apo)E KO rabbits must be fed a high-cholesterol diet to induce hyperlipidemia. In this study, we used the CRISPR/Cas9 system anchored exon 7 of low-density lipoprotein receptor (*LDLR*) in an attempt to generate KO rabbits. We designed two sgRNA sequences located in E7:g.7055–7074 and E7:g.7102–7124 of rabbit *LDLR* gene, respectively. Seven *LDLR*-KO founder rabbits were generated, and all of them contained biallelic modifications. Various mutational LDLR amino acid sequences of the 7 founder rabbits were subjected to tertiary structure modeling with SWISS-MODEL, and results showed that the structure of EGF-A domain of each protein differs from the wild-type. All the founder rabbits spontaneously developed hypercholesterolemia and atherosclerosis on a normal chow (NC) diet. Analysis of their plasma lipids and lipoproteins at the age of 12 weeks revealed that all these KO rabbits exhibited markedly increased levels of plasma TC (the highest of which was 1013.15 mg/dl, 20-fold higher than wild-type rabbits), LDL-C (the highest of which was 730.00 mg/dl, 35-fold higher than wild-type rabbits) and TG accompanied by reduced HDL-C levels. Pathological examinations of a founder rabbit showed prominent aortic atherosclerosis lesions and coronary artery atherosclerosis.In conclusion, we have reported the generation *LDLR*-KO rabbit model for the study of spontaneous hypercholesterolemia and atherosclerosis on a NC diet. The *LDLR*-KO rabbits should be a useful rabbit model of human familial hypercholesterolemia (FH) for the simulations of human primary hypercholesterolemia and such models would allow more exact research into cardio-cerebrovascular disease.

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by an elevation of cholesterol in plasma and severe atherosclerosis [1–3]. The low-density lipoprotein receptor (LDLR) plays a key role in the regulation of cholesterol homeostasis. When the LDLR is defective, low-density lipoprotein (LDL) cannot enter cells by receptor-mediated endocytosis and the lipoprotein accumulates in plasma, eventually producing atherosclerosis [4,5]. Although mutations

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https://doi.org/10.1016/j.ebiom.2018.09.020

Research in context section

Evidence before this study

When the LDLR is defective, low-density lipoprotein (LDL) cannot enter cells by receptor-mediated endocytosis and the lipoprotein accumulates in plasma, eventually producing atherosclerosis. Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the EGF-A domain (encoded by exon 7) of the LDLR in a pH- and calcium-dependent manner and decreases total LDLR levels in liver by directing the receptor to the lysosomes for degradation. Deficiency of this region results in a failure of internalized LDLRs to release bound ligand and prevents recycling to the cell surface. The rabbits have features of lipoprotein metabolism similar to those of humans and rapidly develop hypercholesterolemia and atherosclerosis. However, the generation of KO rabbits is often impractical because of the lack of embryonic stem (ES) cells and genomic information of rabbits in the past. In the past five years, the CRISPR/Cas9 system has been successfully used to introduce zygotes to generate gene-edited rabbits.

Added value of this study

Our *LDLR*-KO rabbits with biallelic mutations via the CRISPR/Cas9 system anchored exon 7 spontaneously developed severe hypercholesterolemia and atherosclerosis on a normal chow (NC) diet. During the past two decades, *LDLR*-KO mice have been widely used for studying many facets of lipid metabolism and atherosclerosis. However, homozygous LDL receptor KO mice on a chow diet, TC levels have been found to be only mildly elevated (200–300 mg/dl) and developed no or only mild atherosclerosis.

Implications of all the available evidence

There is currently only one strain of spontaneous endogenous hypercholesterolemia rabbit, the Watanabe heritable hyperlipidemic (WHHL) strain. However, >1700 human *LDLR* mutations have been reported worldwide. Limited hyperlipidemic rabbit models hamper the investigation of all these mutations, since the rabbits have features of lipoprotein metabolism more similar to those of humans than mouse. We can expect that the *LDLR*-KO rabbit model will provide new insights into the understanding of FH and atherosclerosis and expand the power of the rabbit model for translational research in cardiovascular disease.

in other genes encoding proteins involved in cholesterol metabolism or LDLR function and processing can also be causative less frequently [6].

The deficiency of different genes causes hypercholesterolemia in many models, including mice [7–9], rats [10,11], and pigs [12]. Analysis of these animal models shows that changes in systemic lipid metabolism and plasma lipoproteins, and the formation of atherosclerotic lesions are variably affected. *LDLR* and apolipoprotein (*Apo*)E KO mice are widely used in atherosclerosis research, and they show milder hypercholesterolemia but do not develop atherosclerotic lesions on a normal chow (NC) diet [13]. Atherosclerotic lesions in mice develop only under severe hypercholesterolemia when placed on a high-cholesterol diet, and the lesions are distinctly different from human atherosclerotic plaques [7]. The differences between mice and humans hamper their usage as animal models for the discovery of cholesterol-lowering drugs. The exclusive use of mice makes it difficult to rule out model- and species-dependent effects or to identify additional features that might be important in humans but not in mice [11]. Hence, new animal models are needed to study hypercholesterolemia and atherosclerosis.

Some animals, including humans, monkeys, rabbits, and hamsters, are susceptible to hypercholesterolemia and atherosclerosis. Others, such as mice, rats, dogs, and tree shrews, exhibit resistance to hypercholesterolemia and atherosclerosis [14–17]. The rabbits have features of lipoprotein metabolism similar to those of humans and rapidly develop hypercholesterolemia and atherosclerosis. Both human and rabbit LDLR genes comprise 18 exons and 17 introns, and deficiency in any of the 18 exons would result in the structural abnormalities and functional defects of the LDLR [18–20]. The mature rabbit LDLR consists of five discrete domains identical to human LDLR, among which epidermal growth factor (EGF) precursor-like domain is the most highly conserved domain [5]. It contains two EGF homology domains (EGF-A and EGF-B) separate from a third EGF-like domain (EGF-C) by a β-propeller domain. EGF-A encoded by the seventh exon of LDLR contains 40 amino acid residue, including 6 cysteines which form three disulfide bonds [21,22]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the EGF-A domain of the LDLR in a pH- and calcium-dependent manner and decreases total LDLR levels in liver by directing the receptor to the lysosomes for degradation [23–25]. Deficiency of this region results in a failure of internalized LDLRs to release bound ligand and prevents recycling to the cell surface [26,27]. According to LOVD, *LDLR* variants on exon 7 that encode EGF repeat A account for 6% of the total 1741 variants [20,28,29]. However there have been only a few reports about animal models with deficiency in the exon 7 of the *LDLR* gene [10].

There is currently only one strain of spontaneous endogenous hypercholesterolemia rabbit, the Watanabe heritable hyperlipidemic (WHHL) strain. However, >1700 human *LDLR* mutations have been reported worldwide (http://www.ucl.ac.uk/ldlr) [28,29]. Limited hyperlipidemic rabbit models hamper the investigation of all these mutations. The generation of KO rabbits is often impractical because of the lack of embryonic stem (ES) cells and genomic information of rabbits in the past [30]. CRISPR/Cas9 gene editing technique is a newly emerging versatile genome engineering tool, which is composed of a single guide RNA (sgRNA) and the Cas9 enzyme for genome cutting [31,32]. In the past five years, the CRISPR/Cas9 system has been successfully used to introduce zygotes to generate gene-edited rabbits [33,34]. This showed that it is feasible to generate *LDLR*-KO rabbits using the CRISPR/Cas9 gene editing technique.

In this study, we aimed to generate *LDLR*-KO rabbits with biallelic mutations via the CRISPR/Cas9 system anchored exon 7 to induce spontaneous hypercholesterolemia and atherosclerosis on a NC diet. Our study showed that LDLR-KO rabbits are suitable for use as a human FH model for studying hypercholesterolemia, atherosclerosis, and related translational research.

2. Materials and methods

2.1.1. Animals and ethics statement

The New Zealand rabbits used in this study were maintained in the Animal Genetic Engineering Laboratory of Yangzhou University. Rabbits were housed with a 12/12 h light/dark cycle, and had free access to food and water. All animal experiments were performed in accordance with the guide of the Animal Care Committee of the Yangzhou University and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.1.2. CRISPR/Cas9 construction

We obtained *LDLR* genomic sequence (Genbank: NW_003159540.1) from the NCBI Library of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and designed the CRISPR/Cas9 single guide RNAs (sgRNA) using the tool available at the website http://crispr.mit.edu. After screening, we obtained 2 sgRNAs targeting the rabbit *LDLR* gene anchored exon 7 (sgRNA1 sited E7:g.7055–7074 and sgRNA2 sited E7:g.7102–7124) as shown in Fig. 1. The annealed

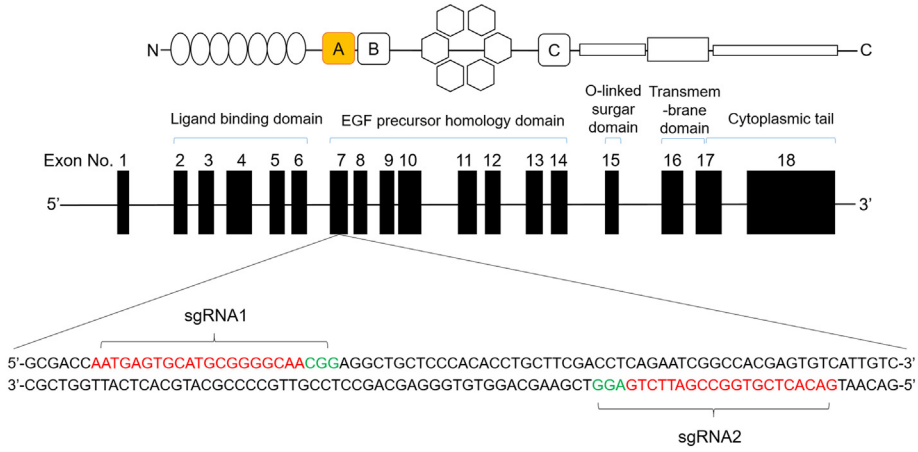


Fig. 1. Schematic illustration of the CRISPR/Cas9-targeting sites of the rabbit *LDLR* gene. Exons are shown in boxes. Two sgRNAs are designed for the LDL receptor gene anchored exon 7 encode EGF A domain (labeled in orange). The sgRNA-targeting sequences are labeled in red, and the protospacer-adjacent motif (PAM) sequences are labeled in green.

sgRNA oligos were respectively sub-cloned into the linearized PYSY-T7-Cas9-T7-Casein-gRNA cloning vector by Nanjing YSY Biotech Com., Ltd., Nanjing, China. PCR products for Cas9 mRNA and sgRNAs were transcribed in vitro using a Script MAX Thermo T7 Transcription Kit (TSK-101) (Toyobo, Com., Ltd., Tokyo, Japan) and purified using an Easy Pure PCR Purification Kit (EP101–1, TransGen, Com, Beijing) according to the manufacturers' instructions. Cas9 mRNA and sgRNAs were diluted into RNase-free water and stored at −80 °C for future use.

2.1.3. Micro-injection and embryo implantation

The *LDLR*-KO rabbits were generated by zygote micro-injection. The protocols for the superovulation, surgery, and embryo transfer were described elsewhere [35–37]. Mixed solution containing Cas9 mRNA (40 ng/μL) and sgRNA (13 ng/μL) were microinjected into the cytoplasm of the zygotes under a Leica inverted light microscope (Leica, Germany). After microinjection, rabbit zygotes were implanted into the uteruses of synchronous recipient rabbits. The average pregnancy lasted 31 days.

2.1.4. Genotype and off-target analysis of LDLR-KO rabbits

Genomic DNA was extracted from a small piece of ear biopsy using phenol chloroform extracting method. sgRNA target sites were amplified by PCR using the primers as follows: F1: 5′-AGGGACCCAGCCCAAACA-3′, R1: 5′-TCAGACGAGCCCATCAAAGAG-3′. PCR products were cloned into a pMDTM 19-T vector cloning kit (Takara Bio, Inc. Japan) and the positive clones were Sanger sequenced and analyzed using Lasergene (DNASTAR, Inc., U.S.). The tertiary structure of the theoretical amino acid sequences were modeled by SWISS-MODEL (https://www.swissmodel.expasy.org/) [38–42].

The top off-target sites were predicted using the online CRISPR design tool developed by Zhang's group at MIT (http://crispr.mit.edu/). The PCR products of these potential off-target sites using the primers listed in Table 1 were Sanger sequenced for further analysis.

2.1.5. Analysis of plasma lipids, apolipoproteins lipoproteins

Blood samples were obtained from rabbits that had fasted for 16 h, and EDTA-plasma was collected after centrifugation at 4 °C for 20 min.

Plasma total cholesterol (TC), triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL—C) were measured using commercial kits (A111–1, A110–1, A113–1 and A112–1. Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Plasma ApoA-I, ApoB, and ApoE contents in the plasma were determined by Western blotting. Briefly, plasma samples (2 μL) were boiled at 100 °C for 10 min in SDS-PAGE loading buffer and then separated on 4–15% polyacrylamide tris-glycine gels. Separated proteins were electrophoretically transferred to PVDF membranes (0.45 μm, Pall, US). The membranes were blocked with 5% non-fat milk/TBST overnight at 4 °C and then incubated with sheep anti-apoA-I (0650–0180, Bio-Rad AbD Serotec, Kidlington, UK), goat anti-apoB (600–101-111, Rockland Inc., Limerick, PA, US), and goat anti-apoE (600–101-197, Rockland Inc., Limerick, PA, US) polyclonal antibodies at 4 °C overnight, followed by three washes with TBST. Then the membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (Jackson Immuno Research Laboratories, West Grove, PA, US) and donkey anti-sheep IgG (Chemicon, Temecula, CA, US) polyclonal Abs, followed by three washes with TBST. Chemiluminescence signals were detected using a gel documentation system (Syngene, UK) after membranes were incubated with ECL substrates (Millipore Corporation, Billerica, MA, US). As a loading control for Western blot analysis of plasma samples, rabbit TF (transferrin) was detected by sequential incubation with a mouse monoclonal antibody against TF (ab769, Abcam, Cambridge, UK), peroxidase-conjugated goat anti-mouse IgG (Sangon Biotech, Shanghai, China) and ECL substrates (Millipore Corporation, Billerica, MA, US).

Plasma lipoprotein profiles were analyzed using agarose gel electrophoresis and fast-protein liquid chromatography (FPLC). In brief, plasma (2 μL) was electrophoresed on 1% agarose gel (Helena

Table 1
Primers for detection of possible off-target sites.

Primers	Primer sequences
Off-target-1	5′-GTGTAGTGTTGTTCCGAGTGC-3′
	5′-GCGGTTTCGAGATTGCTG-3′
Off-target-2	5′-GTGTCAGTCACCAGCCAGAG-3′
	5′-CTGTTCCCACCCAAGTCC-3′
Off-target-3	5′-CAGGGTGCTCTTCTGTGC-3′
	5′-CCTATCTGCCTCTTCTTGTCTA-3′
Off-target-4	5′-CAGACAGGCAGAGGCAGAG-3′
	5′-CGTTTGGGAAGTGAGTAGTAGG-3′
Off-target-5	5′-CACTTCCTACCACCCTCC-3′
	5′-CTTGCGGTTTGAACTTTG-3′
Off-target-6	5′-AAGGACAACCTATGACCCAC-3′
	5′-CCAGGACTGAAACCCACA-3′
Off-target-7	5′-CTGGAAGAGGAGCAACCG-3′
	5′-TCCCTCGTGAACTTGAAACA-3′
Off-target-8	5′-GTTTCCCACGCTGCTTCC-3′
	5′-CGCCCACTCATCTCCTCA-3′
Off-target-9	5′-TTTTATGCGGAAGACAGA-3′
	5′-GAAGTGAAGCAGCCAAGA-3′
Off-target-10	5′-TTCCAGCCTTGTCCATAA-3′
	5′-GAGGTCGTCACTGTTTGTTG-3′

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

Microinjected sgRNAs	SgRNA1	SgRNA2
No. of injected embryos	14	82
No. of transferred embryos	12	79
No. of recipients	1	4
No. of pregnancies	1	4
No. of births in total	3	10
No. of live pups	3	4
No. of mutants	3	10
Rate of mutations (%) =NO. of mutants /No. of born	100	100

Laboratories, Saitama, Japan) and stained for neutral lipids with Fat Red 7B; Plasma (4 μL) was analyzed by FPLC on a Superose 6 10/300 GL column (GE Healthcare Life Sciences, Sweden).

2.1.6. Analysis of plasma inflammatory mediators and white blood cell counts

Plasma levels of Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6) and C—C Motif Chemokine Ligand 2(CCL2) in plasma were measured using ELISA kits (IL-1β & IL-6, Elabscience Biotechnology Co., Ltd., Wuhan, China) and (CCL2, Cusabio, Wuhan, China, https://www.cusabio.com.)

White blood cell counts in the peripheral blood of the rabbits were measured using an auto hematology analyzer (BC-2800Vet, Mindray, Shenzhen, China).

2.1.7. Analysis of atherosclerosis

The aortic atherosclerosis lesions were analyzed using previously described methods [43,44]. In brief, rabbits were sacrificed by venous injection of an overdose of sodium pentobarbital solution. The aortic trees were stained with Sudan IV (Solarbio Life Science, China) after opened out longitudinally and fixing in formalin. The Sudan IV-positive area was calculated using Image-Pro Plus™ (Media Cybernetics, Inc. US) and was expressed as a percentage of the total surface area.

For histological analysis, serial paraffin sections of aortic atherosclerosis lesions were stained with hematoxylin-eosin (HE), Masson Trichrome (MT) and immunohistochemically stained with monoclonal antibodies against either macrophages (Mφ) (clone: RAM11, Dako, Carpinteria, CA, US) or α-smooth muscle actin for smooth muscle cells (SMC) (clone: HHF35, Dako, Carpinteria, CA, US).

According to the method reported elsewhere [45,46], the immersion-fixed hearts treated with formalin were cut into 6 blocks and embedded in paraffin. The left coronary artery was cross-sectioned (5 μm per slice), and sections were stained with H&E staining.

3. Results

3.1.1. Production of LDLR-KO rabbits and genotype assay

We designed two sgRNAs (sgRNA1 and sgRNA2) targeting the rabbit *LDLR* anchored exon 7 to generate knockout rabbits. The two sgRNAs were in vitro transcribed into mRNAs, which were respectively co-injected with Cas9 mRNA into rabbit zygotes. In total, 91 zygotes were injected (12 for sgRNA1, 79 for sgRNA2) and subsequently transferred to implanted into the uteruses of 5 (1 for sgRNA1, 4 for sgRNA2) synchronous recipient rabbits (Table 2). 13 (3 for sgRNA1, 10 for sgRNA2) pups were born and all of them harbored mutations in the *LDLR* gene analyzed by PCR and TA-clone. 6 pups treated with sgRNA2 died by accident.

The 7 live pups were numbered L9♀, L10♀, L12♀, L13♂, L15♂, L16♀, and L17♀ respectively. The genomic DNA sequences, theoretical amino acid sequences, and tertiary structure of the *LDLR* knockout alleles are shown in Fig. 2. Here, 3 rabbits (L15♂, L16♀, L17♀) harbored deletion/insertion mutations in exon 7 of the *LDLR* gene that did not cause frame-shift mutation. The structure of each protein differs from the wild-type, L15♂ and L17♀ were homozygous and both of them lost a disulfide in EGF-A domain. There even appeared a helix (marked by red arrow) in L17♀ (Fig. 2E). One allele of L16♀ caused exon 7 to be skipped, while the other alleles remained similar to their wild-type counterparts; 3 pups (L9♀, L10♀, L13♂) harbored mutations that caused a shift in the reading frame in exon 7, as shown in Fig. 2F, all the proteins resulted in a premature termination; 1 pup (L12♀) harbored both of the mutations named above, a shift in the reading frame in exon 7 in one allele and another similar to the wild-type alleles.

A total of 10 (5 for sgRNA1 and 5 for sgRNA2) potential off-target sites (OTs) were successfully amplified and subjected to Sanger sequencing via PCR. No overlapping peaks were detected near the OTs. No appearance defects or reproductive problems were observed in the founder rabbits.

3.1.2. Analysis of plasma lipids, apolipoproteins, lipoproteins

The LDLR is responsible for the clearance of LDL-C from the circulation. We therefore measured parameters of blood chemistry in the *LDLR*-KO rabbits at the age of 12 weeks. As expected, on a NC diet, all the *LDLR*-KO rabbits showed hyperlipidemia and their plasma TC and LDL-C levels were significantly higher than those of WT rabbits (Table 3). Striking differences were observed, TC levels of L9♀ and L10♀ (L9♀, 1013.15 mg/dl; L10♀, 812.84 mg/dl) were 16–20 fold higher than those of the WT rabbits while LDL-C levels (L9♀, 730.00 mg/dl; L10♀, 625.60 mg/dl) were 30–35 fold higher than those of the WT rabbits. Pronounced changes were observed in L15♂, L16♀, and L17♀, TC levels (L15♂, 442.77 mg/dl; L16♀, 588.56 mg/dl; L17♀, 798.54 mg/dl) were 9–16 fold higher than those of the WT rabbits while LDL-C levels (L15♂, 233.20 mg/dl; L16♀, 440.00 mg/dl; L17♀, 513.60 mg/dl) were 11–24 fold higher than those of the WT rabbits, because L9♀ and L10♀ were frame-shift mutations while L15♂, L16♀, and L17♀ were deletion/insertion mutations. Nevertheless, TC and LDL-C levels were lower in L12♀ and L13♂ than in the 5 rabbits mentioned above. Changes in TG were much less pronounced than those of TC and LDL-C. And HDL-C levels of all the founder rabbits were much lower than the WT ones. These results indicated that the increase in TC was mainly caused by the increase of LDL-C.

Western blot analysis showed that plasma ApoB and ApoE levels increased significantly and those of whereas ApoA-I reduced observably (Fig. 3A) at the age of 12 weeks. These results verified the analysis of the plasma lipids above.

All these 7 founder rabbits exhibited hyperlipidemia, we further analyzed plasma lipoprotein profiles at the age of 12 weeks. Agarose gel electrophoresis revealed that increased plasma lipids in *LDLR*-KO rabbits on a NC diet were essentially caused by increased lipoproteins that migrated from the original position to the β and pre-β area, assuming that these lipoproteins were those of chylomicron, (very low-density lipoprotein) VLDL, and especially LDL particles (Fig. 3B). Results of FPLC revealed that *LDLR*-KO rabbits (L12♀, especially L9♀and L17♀) on a NC diet exhibited markedly higher total cholesterol levels in plasma, accompanied by lower HDL-C levels, than wild-type rabbits (Fig. 3C), and the fractions of VLDL and especially LDL were the most common type of lipoprotein.

3.1.3. Analysis of plasma inflammatory mediators and white blood cell counts

Because high levels of cholesterol could stimulate inflammatory responses, plasma samples of the rabbits (except L9♀) were subjected to inflammatory mediators tests (including IL-1β, IL-6 and CCL2) at the age of 48 weeks. These results were shown in Table 4. Plasma IL-1β and IL-6 of the KO rabbits were similar to that of the wild type ones. Plasma CCL2 of the KO rabbits seems to be quite irregular. (See Table 5.)

White blood cell counts in the peripheral blood of the rabbits (except L9♀) were measured at the age of 48 weeks. Results didn't seem to be quite different from the wild type ones, and confirmed the plasma inflammatory mediators test. (See Table 5)

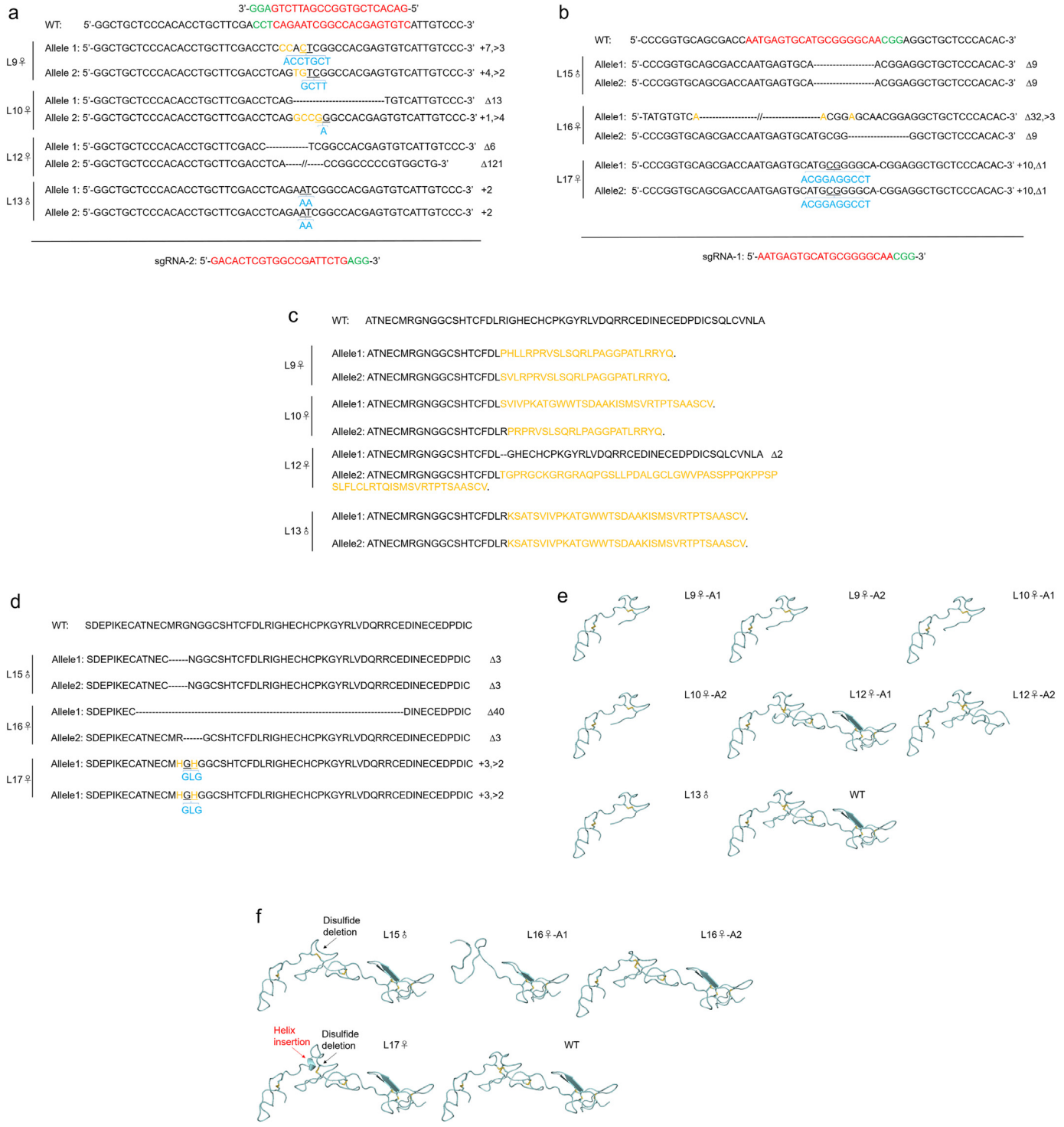


Fig. 2. Mutations of *LDLR* induced by CRISPR/Cas9. A: Details of the mutations of the modified alleles targeted by sgRNA2 detected in the founder rabbits (L9♀, L10♀, L12♀, L13♂). B: Details of the mutations of the modified alleles targeted by sgRNA1 detected in the founder rabbits (L15♂, L16♀, L17♀). C: Theoretical amino acid sequences of the founder rabbits mediated by sgRNA2 (L9♀, L10♀, L12♀, L13♂). D: Theoretical amino acid sequences of the founder rabbits mediated by sgRNA1 (L15♂, L16♀, L17♀). Insertions (+) and deletions (−) are shown to the right of each allele, substitutions (>) are labeled in orange and insertions are labeled in blue. E: The tertiary structures of the modified alleles targeted by sgRNA2. F: The tertiary structures of the modified alleles targeted by sgRNA1. The tertiary structures are partly shown in pictures above (from amino acid 280 to 380), EGF-A domain (encoded by exon 7) is marked by a blue square, disulfide deletions are marked by black arrows, and helix insertion is marked by red arrow.

These results show that plasma inflammatory mediators analysis and white blood cell counts of the LDLR KO rabbits cannot be correlated with their plasma cholesterol and TG concentration. The mechanisms need to be investigated in the further experiments.

3.1.4. KO rabbits developed aortic and coronary atherosclerosis and xanthoma

Because the progression of hyperlipidemia leads to build-up of atherosclerotic plaques that cause narrowing of the arterial lumen, we further assessed aortic and coronary atherosclerosis lesions.

Table 3
Plasma levels of TC, TG, HDL-C, and LDL-C of the *LDLR*-KO rabbits at the age of 12 weeks.

Cholesterol	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)*
WT ($n = 4$)	50.54 ± 1.45	48.80 ± 5.44	34.74 ± 2.37	21.10 ± 11.56
L9♀	1013.15	290.51	15.87	730.00
L10♀	812.84	223.20	18.19	625.60
L 12♀	272.24	73.51	29.55	124.00
L 13♂	435.04	146.14	18.51	351.60
L 15♂	442.77	149.68	23.03	233.20
L 16♀	588.56	255.97	19.35	440.00
L 17♀	798.54	330.37	10.45	513.60

* The data does not contain vLDL.

Table 4
Plasma levels of IL-1β, IL-6 and CCL2 of the *LDLR*-KO rabbits at the age of 48 weeks.

Cytokine	IL-1β (pg/ml)	IL-6 (pg/ml)	CCL2 (pg/ml)
WT (n = 4)	73.70 ± 0.94	92.19 ± 11.95	462.16 ± 21.39
L10♀	74.60	79.70	318.00
L 12♀	86.08	87.00	683.64
L 13♂	79.28	76.83	624.00
L 15♂	87.20	78.70	496.00
L 16♀	81.57	74.93	500.60
L 17♀	80.43	89.68	461.62

Table 5
White blood cell counts of the *LDLR*-KO rabbits at the age of 48 weeks.

Cells	WBC ($\times 10^9$/L)	Lymph ($\times 10^9$/L)	Mon ($\times 10^9$/L)	Gran ($\times 10^9$/L)	Lymph (%)	Mon (%)	Gran (%)
WT (n = 4)	11.1 ± 1.5	5.9 ± 1.3	0.4 ± 0.1	4.9 ± 2.0	53.9 ± 12.5	3.3 ± 0.7	42.8 ± 12.7
L10♀	11.8	6.1	0.3	5.5	51.1	2.4	46.6
L12♀	8.0	5.0	0.3	2.8	62	3.2	34.6
L13♂	9.1	3.9	0.3	5.0	42.2	2.7	55.2
L15♂	11.8	8.2	0.4	3.3	68.5	3.8	27.7
L16♀	15.2	6.6	0.6	9	43.2	3.5	53.4
L17♀	12.4	7.0	0.4	5.0	56.4	3.3	40.6

WBC, white blood cells; Lymph, lymphocytes; Mon, monocytes; Gran, granulocytes.

When maintained on a NC diet, the *LDLR*-KO founder rabbit L9♀ (20 weeks), L13♂ (48 weeks), L17♀ (48 weeks) and wild type♀ (48 weeks) showed different degrees of aortic lesions (red areas stained with Sudan IV) (Fig. 4A). The lesioned areas of different rabbits were positively related to their plasma cholesterol concentration. L9♀ showed appreciable atherosclerotic lesion formation from the aortic arch to the abdominal aorta while the others were much milder. The lesioned area covered 60%, 4%, 8% and 0% of the four rabbit's aorta respectively as measured by Image-Pro Plus™.

High levels of cholesterol could stimulate inflammatory responses. We therefore examined inflammation in the lesions. Staining of the aortic lesions with HE, MT, anti-RAM-11 and α-smooth muscle actin antibody showed the accumulation of macrophages in the intima (Fig. 4B). Typical fibrous plaques contained rich foam cells derived from macrophages, depositions of calcium and covered by thin fibrous caps (smooth muscle cells and extracellular matrix) were observed. This type of cap is very vulnerable [47].

Because homozygous FH patients without any treatment die from premature coronary artery disease (CAD), we further investigated whether the *LDLR*-KO rabbits had CAD. HE staining of the cross-section revealed that the founder rabbit L9♀had advanced coronary artery atherosclerosis (Fig. 4C).

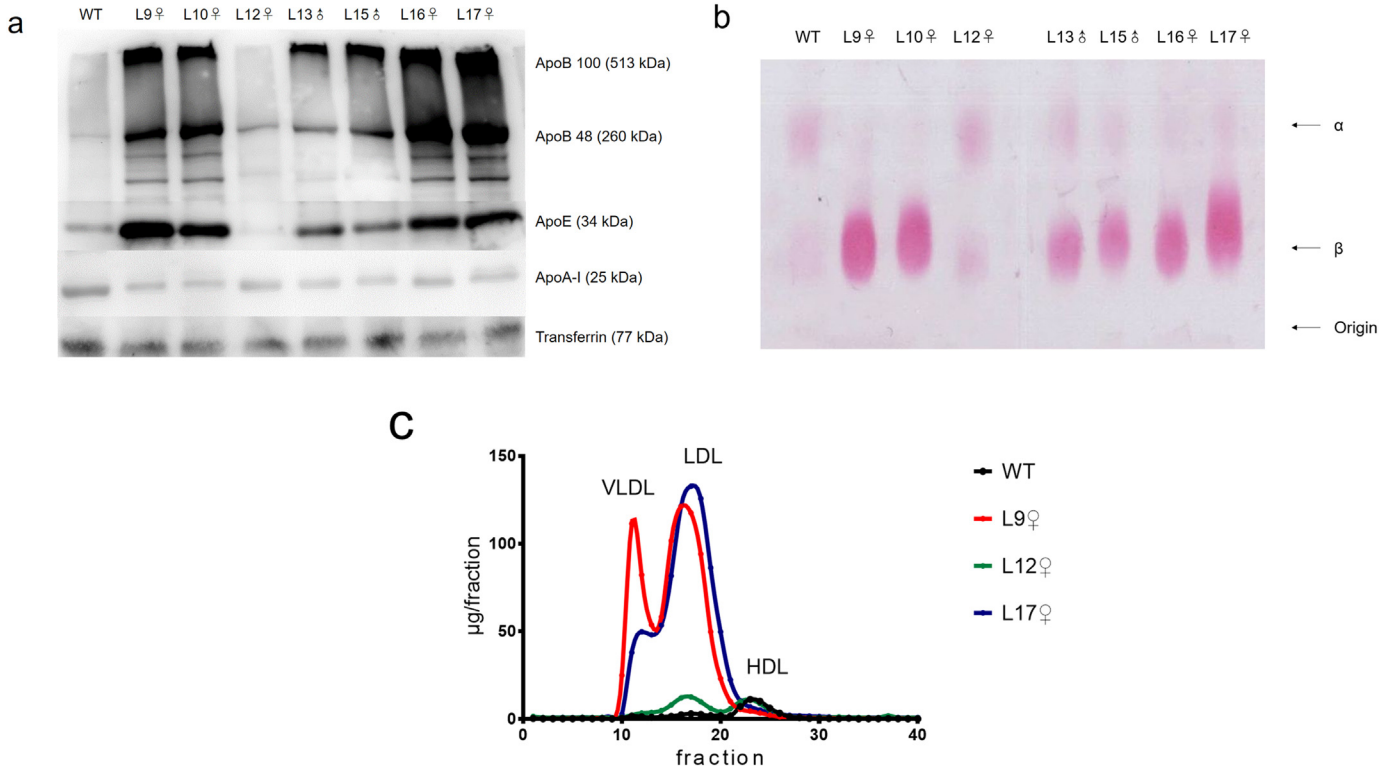


Fig. 3. Analysis of plasma TC, TG, LDL-C, and HDL-C in the *LDLR*-KO rabbits. A: Analysis of plasma apolipoproteins by Western blot. Plasma samples (0.5 μL) were fractionated on 4–15% SDS-PAGE and transferred to a PVDF membrane probed with Abs anti ApoA-I, ApoB, and ApoE as described in the Materials and Methods section. B: Agarose gel electrophoresis of plasma lipoproteins. 4 μL of plasma was loaded in each well, fractionated on 1% agarose gel, and stained with fat red 7 B for neutral lipids. Lipoprotein migration positions are indicated by arrows. C: Analysis of plasma lipoprotein profiles by fast-protein liquid chromatography (FPLC). Black, red, green, and blue lines showed the fractions of WT, L9♀, L12♀, and L17♀ respectively; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

The founder rabbit L10♀ also exhibited xanthoma in its forepaws (Fig. 4D), which was often observed in FH patients.

Collectively, these data demonstrate that the *LDLR*-KO rabbit is a human-like animal model of cardiovascular disease.

4. Discussion

In the current study, we characterized a spontaneous rabbit hyperlipidemia model generated by deletion of the *LDLR* gene using CRISPR/Cas9 editing system. LDLR is a cell-surface transmembrane protein that controls the level of blood plasma cholesterol and triglycerides using LDLR-mediated endocytosis in the liver [48]. Genetic deficiency of LDLR prevents low-density lipoprotein (LDL) from entering cells, which accounts for the reduced clearance of LDL molecules from the plasma via the receptor-mediated pathway [48]. Rabbits have lipid metabolism similar to that of humans [49] and the available WHHL rabbit line is far from sufficient to satisfy the study of so many mutation sites. Hence, we designed two sgRNAs anchored exon 7 of rabbit *LDLR* and generated *LDLR*-KO rabbits to establish a model for the study of human hyperlipidemia and atherosclerosis.

Among the 7 founder *LDLR*-KO rabbits, L9♀ and L10♀ showed extremely severe hypercholesterolemia. The deficiency in both alleles was a frame-shift mutation result in a premature termination of the LDLR synthesis. The deficient LDLR lost all the domains after EGF-A, ceased to produce a transmembrane cell surface protein, and lost the ability to eliminate LDL-C; L13♂ showed a milder hypercholesterolemia, although the deficiency in both alleles were frame-shift mutation and resulted in a premature termination. The reason for this needs to be investigated in the further experiments; L15♂ and L17♀ also showed severe hypercholesterolemia although it was milder than in L9♀ and L10♀. They harbored deletion/insertion of several amino acids in EGF-A domain. As modeled by SWISS-MODEL, both of them lost a disulfide in the EGF-A domain (although cysteine was not deleted) and had great changes in the tertiary structures. These mutations of LDLR might result in the increased affinity to PCSK9, and so disturbed the dissociation of LDLR/PCSK9 so the internalized LDLRs fails to release bound ligand and prevents recycling to the cell surface [50]. So, in theory, the content of LDLR on the cell surface is reduced, as well as the ability to eliminate LDL-C. PCSK9 is a serine protease of the proprotein convertase family that regulates circulating LDLR levels by controlling LDLR degradation [51,52]. The two proteins interact via a flat contact patch between the catalytic domain of PCSK9 and the EGF-A domain (encoded by exon 7) in the LDLR [26]; L12♀ harbored both of the mutations mentioned above, and showed mild hypercholesterolemia. This might be because the deletion of RI (319–320) did not result in loss of a disulfide, and did not accelerate the degradation of LDLR. As a result, L12♀ could be counted as a hemizygote; L16♀ showed severe hypercholesterolemia and harbored two kind of mutations: exon 7 (encodes EGF-A domain) skipping and deletion of three amino acids in EGF-A domain. The mechanism need to be investigated in the further experiments.

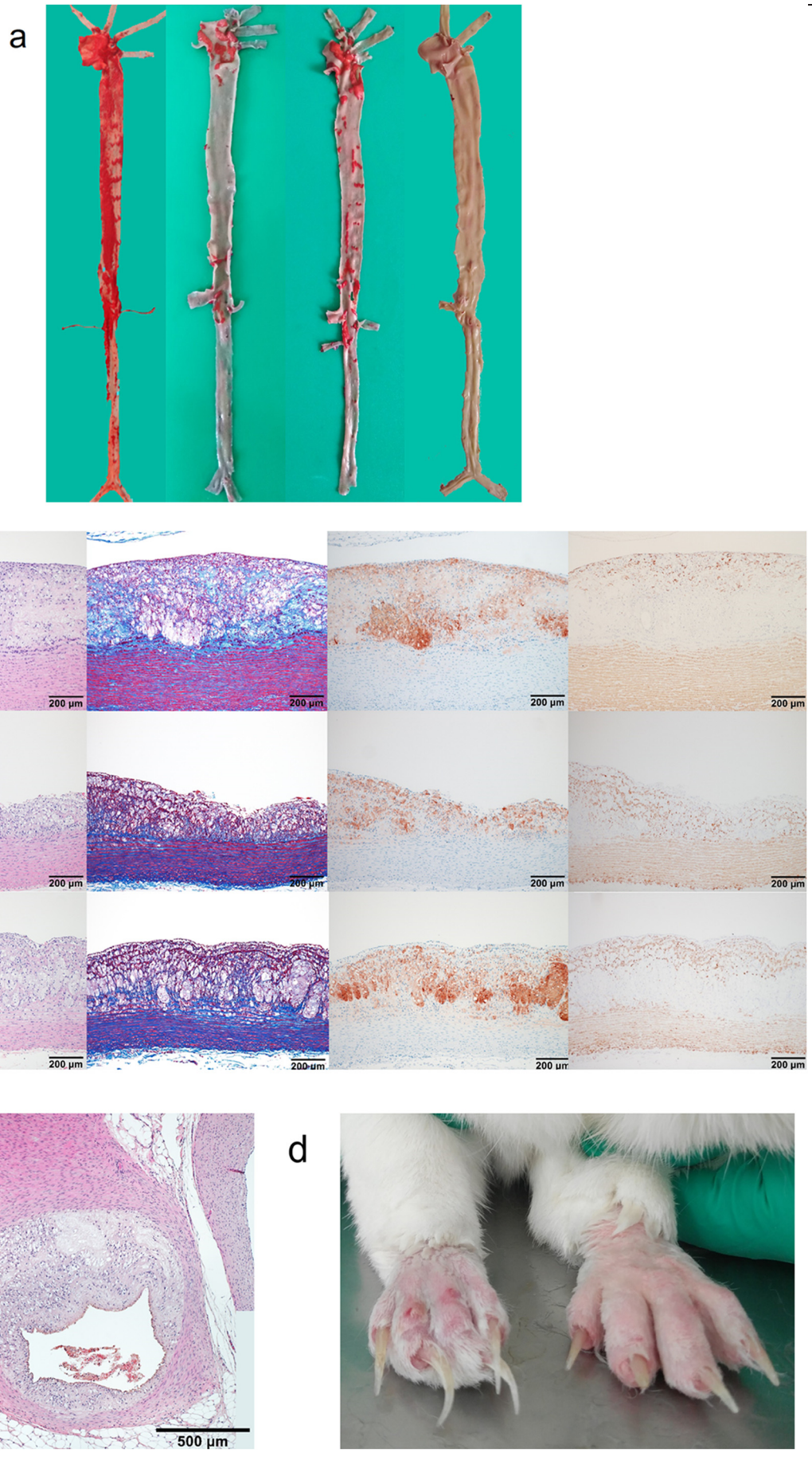
Our LDLR-KO rabbits with disruption of exon 7 were similar to human in various diseases. We generated 7 LDLR-KO rabbits with different base mutations and exhibited different levels of hypercholesterolemia and atherosclerosis. Two homozygous founder rabbits (L15♂ and L17♀) harbored deletion/insertion of several amino acids in EGF-A domain, and exhibited severe hypercholesterolemia and atherosclerosis. These results are consistent with human familial hyperlipidemia. Walus-Miarka, Malgorzata et al. [53] reported a mutation c986G > T (Cys308Phe) in the exon 7 (encodes EGF-A domain) of LDLR gene. Concentrations of serum LDL-C in probands before treatment were between 9.5 and 10.5 mmol/L. All patients had corneal arcus and tendon xanthoma.

The Western blots and agarose gel electrophoresis, shown in Fig. 3A & 3B indicate that ApoB 100, ApoB 48 and ApoE containing lipoprotein particles (LDL, VLDL and chylomicron) increased in the plasma of founder rabbits. Based on the existing theory and prior studies, LDLR knock out also be able to accumulate VLDL remnants and chylomicron remnants. Ishibashi et al. [54] studied retinyl palmitate clearance after fat feeding in mice lacking either LDL receptors, ApoE or both proteins. The plasma of LDLR-deficient mice accumulate 4-fold more retinyl palmitate than normal mice while ApoE-deficient mice and mice deficient in both ApoE and LDL receptors accumulate 12-fold more plasma retinyl palmitate. Most of the accumulation is in larger, triglyceride-rich particles such as chylomicrons and VLDL. They concluded that LDL receptors play a major role in the catabolism of remnant particles. Wang, H. Y. et al. [10] reported the generation of LDLR-KO rat with disruption of exon 7, the deletion of the LDLR elevated total cholesterol and total triglyceride in the plasma, and caused a tenfold increase of plasma LDL and a fourfold increase of plasma VLDL on high cholesterol diet. In this study, mutations of the founder rabbit LDLR gene can be divided into two kinds: frame-shift mutation and deletion/insertion of several amino acids. And we think that both of them resulted in reduced content of LDLR on the cell surface, as well as the reduced ability to eliminate ApoB 100 and ApoE containing lipoprotein particles. We think that is the reason of the accumulation plasma LDL-C, VLDL remnants and chylomicron remnants.

The IAS published recommendations to identify the severe FH criteria includes LDL cholesterol >400 mg/dl (corresponding to 10 mmol/L) [55]. L9♀, L10♀, L16♀, and L17♀ *LDLR*-KO rabbits showed severe hypercholesterolemia on a NC diet by this standard. L9♀ was sacrificed at the age of only 20 weeks, HE staining of the cross-section revealed it developed coronary artery atherosclerosis. Considering WHHL rabbits develop coronary artery atherosclerosis and spontaneous myocardial infarction [56] and the young age of L9♀, we believe that our severe hypercholesterolemia KO rabbits will spontaneously develop myocardial infarction within a reasonable period.

Like humans but unlike like mice, rabbits have abundant plasma cholesteryl ester transfer protein (CETP) activity, intestine-only ApoB mRNA editing, considerable hepatic cholesterol synthesis, and LDL as predominate plasma lipoprotein, and other traits [49,57,58]. It is generally better to use an animal model with lipid metabolism mechanisms similar to that of humans. However, during the past two decades, *LDLR*-KO mice have been widely used for studying many facets of lipid metabolism and atherosclerosis [59,60]. There are some differences in the phenotype among *LDLR*-negative humans, rabbits, and mice. LDLR deficiency leads to massive increases of plasma TC levels and to the development of severe atherosclerosis in both WHHL rabbits (700–1200 mg/dl) [61] and FH humans (650–1000 mg/dl) [62], and, in this study, the highest level was 1013 mg/dl at the age of 12 weeks. Nevertheless, in homozygous LDL receptor KO mice on a chow diet, TC levels have been found to be only mildly elevated (200–300 mg/dl) and developed no or only mild atherosclerosis [7,13,17]. In this study, the *LDLR*-KO rabbits, like *LDLR*-negative humans, displayed advanced coronary artery atherosclerosis while *LDLR*-KO mice typically did not develop coronary artery atherosclerosis [63]. It is likely that LDL receptors are more critical for maintenance of cholesterol homeostasis in rabbits and humans than in mice. Rabbits are larger than small rodents, which allows for more convenient physiological and surgical manipulations as well as imaging [64,65]. Our method to establish a cardiovascular disease rabbit model on a NC diet saves time and effort and may be appropriate in many circumstances.

The *LDLR*-KO rabbits could also be used to bridge the gap between smaller rodents and larger animals, such as dogs, pigs, and monkeys, and may play an important role in many translational research activities such as pre-clinical testing of drugs and diagnostic methods for patients [47,65]. An animal model with high predictive efficacy to humans is needed. We here seek to establish whether drugs that have been successful in humans are equally effective in rabbits and whether drugs that have been shown to be efficacious in mice but useless in humans are effective in this rabbit model. Although the current *LDLR*-KO rabbits


a
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represent a useful model for the study of human lipid metabolism and atherosclerosis, one should be aware that rabbits are more expensive than mice and they also have low hepatic lipase activity [65]. These are the major drawbacks to the use of this model.

In conclusion, we have reported the generation of *LDLR*-KO rabbits, which spontaneously develop severe hypercholesterolemia and atherosclerosis on a NC diet. We can expect that the *LDLR*-KO rabbit model will provide new insights into the understanding of FH and atherosclerosis and expand the power of the rabbit model for translational research in cardiovascular disease.

Acknowledgements

This work was supported by the National Key Research and Development Program of China, China (No. 2016YFE0126000), the National Natural Science Foundation of China, China (No. 81570392 and 81770457), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, China (PAPD), Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology, Japan (15H04718) and the Natural Science Foundation of Shaanxi Province, China (2017JZ028).

Conflicts of interest

The authors have no conflicts of interest to declare.

Authors' contributions

Yong Cheng, Jingyan Liang and Jianglin Fan designed the experiments and provided the resources. Rui Lu, Tingting Yuan and Yong Cheng performed the experiments, acquired and analyzed the data, and wrote the manuscript. Yingge Wang, Ting Zhang, Yuguo Yuan, Daijin Wu, Minya Zhou, Zhengyi He, Yaoyao Lu and Yajie Chen performed the experiments and acquired data. Rui Lu, Tingting Yuan and Yingge Wang contributed equally. All authors approved the final paper.

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Fig. 4. Pathological analysis of aortic lesions and coronary atherosclerosis in *LDLR*-KO rabbit. A: Gross lesions of aortic atherosclerosis in *LDLR*-KO rabbit L9♀ (20 weeks), L13♂ (48 weeks), L17♀ (48 weeks) and wild type ♀ (48 weeks) (from left to right) stained with Sudan IV (visualized as red area). The lesioned area covered 60%, 4%, 8% and 0% of the four rabbit's aorta respectively, positively related to their plasma cholesterol concentration. B: Micrographs of aortic atherosclerosis lesions of *LDLR*-KO rabbit L9♀ (20 weeks), L13♂ (48 weeks) and L17♀ (48 weeks) (from top to bottom). Serial paraffin sections were stained with hematoxylin-eosin (HE), Masson Trichrome (MT) and immunohistochemically stained with Abs against either RAM-11 for macrophages (Mφ) or α-smooth muscle actin for smooth muscle cells (SMC) (from left to right). C: Micrographs of coronary artery atherosclerosis lesions of *LDLR*-KO rabbit (L9♀). HE stained cross-section revealed that L9♀ displayed advanced coronary artery atherosclerosis. D: *LDLR*-KO rabbit L10♀ exhibited xanthoma in its paws. The forepaw of L10♀ is shown on the right and a wild-type rabbit's on the left.

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