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

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A B S T R A C T

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.

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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 homoeostasis. 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...
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/kinin 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. 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. 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. In the past five years, the CRISPR/Cas9 system has been successfully used to introduce zygotes to generate gene-edited rabbits. 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 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 cysteine which form three disulfide bonds [21,22]. Proprotein convertase subtilisin/kinin 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.
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.

gRNA oligos were respectively sub-cloned into the linearized PYSY-T7-Cas9-T7-Casein-gRNA cloning vector by Nanjing YSI 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]. Transiently transfected cell lines 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-α-apoA-I (0650–0180, Bio-Rad AbD Serotec, Kidlington, UK), goat anti-α-apoB (600–101–111, Rockland Inc., Limerick, PA, US), and goat anti-α-apoE (600–101–197, Rockland Inc., Limerick, PA, US) polyclonal antibodies at 4 °C overnight, followed by three washes with TBST. 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

| Primers | Primer sequences |
|---------|----------------|
| Off-target-1 | 5′-CTGTTAGTGTTGTTCCAGATGC-3′ |
| Off-target-2 | 5′-CTGTTAGTGTTGTTCCAGATGC-3′ |
| Off-target-3 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-4 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-5 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-6 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-7 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-8 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-9 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |
| Off-target-10 | 5′-CAGGTCTGTCTTCTGTGTC-3′ |

Table 1

Primer sequences for detection of possible off-target sites.
Labs, 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 (M0) (clone: RAM11, Dako, Carpenteria, CA, US) or α-smooth muscle actin for smooth muscle cells (SMC) (clone: HHF35, Dako, Carpenteria, 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. Among the 91 zygotes that were in vitro transcribed into mRNAs, which were respectively co-injected with Cas9 mRNA into rabbit zygotes, 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 frame-shift mutations in exon 7 of the LDLR gene that did not cause frameshift mutation. The structure of each protein differs from the wild-type, L15♂ and L17♀ were homozygous and both of them lost a distal splice 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, 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♂ (192, 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♀, 588.36 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♀, 440.60 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 site that migrated from the original position to the

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)
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.
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).

### Table 3

| 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 |
| L10♀        | 1013.15    | 290.51     | 15.87         | 730.00         |
| L12♀        | 812.84     | 223.20     | 18.19         | 625.60         |
| L13♂        | 272.24     | 73.51      | 29.55         | 124.00         |
| L15♂        | 435.04     | 146.14     | 18.51         | 351.60         |
| L16♀        | 442.77     | 149.68     | 23.03         | 233.20         |
| L17♀        | 588.56     | 255.97     | 19.35         | 440.00         |

* The data does not contain vLDL.

### Table 4

| 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       |
| L12♀      | 86.04         | 87.00        | 683.64       |
| L13♂      | 79.28         | 76.83        | 624.00       |
| L15♂      | 87.20         | 78.70        | 496.00       |
| L16♀      | 588.56        | 255.97       | 19.35        |
| L17♀      | 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 (×10^9/L)   | Lymph (×10^9/L) | Mon (×10^9/L)    |
| WT (n = 4)      | 11.1 ± 1.5      | 5.9 ± 1.3       | 0.4 ± 0.1        |
| L10♀           | 8.0 ± 0.3       | 3.9 ± 0.3       | 0.3 ± 0.1        |
| L12♀           | 11.8 ± 0.3      | 6.1 ± 0.3       | 0.3 ± 0.1        |
| L13♂           | 9.1 ± 0.3       | 8.2 ± 0.4       | 0.2 ± 0.1        |
| L15♂           | 12.4 ± 0.4      | 9.0 ± 0.4       | 0.4 ± 0.1        |
| L16♀           | 15.2 ± 0.4      | 6.6 ± 0.4       | 0.6 ± 0.1        |
| L17♀           | 15.2 ± 0.4      | 7.0 ± 0.4       | 0.7 ± 0.1        |

### References

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).
The founder rabbit L10Q 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, L9Q and L10Q 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; L13Q showed a milder hypercholesterolemia, although the deficiency in both alleles were frame-shift mutation and resulted in a premature termination. The reason for this need to be investigated in the further experiments; L15Q and L17Q also showed severe hypercholesterolemia although it was milder than in L9Q and L10Q. 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 fail 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 pro tease of the proprotein convertase family that regulates circulating LDL 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]; L12Q 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, L12Q could be counted as a hemizygote; L16Q 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 (L15Q and L17Q) 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 in 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 tri glyceride 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], L9Q, L10Q, L16Q, and L17Q LDLR-KO rabbits showed severe hypercholesterolemia on a NC diet by this standard. L9Q 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 L9Q, we believe that our severe hypercholesterolemia KO rabbits will spontaneously develop myocardial infarction within a reasonable period.

Like humans but unlike mice, rabbits have abundant plasma cholesteryl ester transfer protein (CETP) activity, intestine-only ApoB mRNA editing, considerable hepatic cholesterol synthesis, and LDL as predominant 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 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 LDLR receptor KO mice on a chow diet, TC levels have been found to be around 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 human are effective in this rabbit model. Although the current LDLR-KO rabbits...
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.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors’ contributions

Yong Cheng, Jingyin Liang and Jiqiang 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. Yinghe Wang, Ting Zhang, Yuguoyuan Dajin Wu, Minya Zhou, Zhengyi He, Yaoyao Lu and Yajie Chen performed the experiments and acquired data. Rui Lu, Tingting Yuan and Yinghe Wang contributed equally. All authors approved the final paper.

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