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Research Paper

LDL Receptor Gene-ablated Hamsters: A Rodent Model of Familial Hypercholesterolemia With Dominant Inheritance and Diet-induced Coronary Atherosclerosis

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant genetic disease caused mainly by LDL receptor (*Ldlr*) gene mutations. Unlike FH patients, heterozygous *Ldlr* knockout (KO) mice do not show a dominant FH trait. Hamsters, like humans, have the cholesteryl ester transfer protein, intestine-only ApoB editing and low hepatic cholesterol synthesis. Here, we generated *Ldlr*-ablated hamsters using CRISPR/Cas9 technology. Homozygous *Ldlr* KO hamsters on a chow diet developed hypercholesterolemia with LDL as the dominant lipoprotein and spontaneous atherosclerosis. On a high-cholesterol/high-fat (HCHF) diet, these animals exhibited severe hyperlipidemia and atherosclerotic lesions in the aorta and coronary arteries. Moreover, the heterozygous *Ldlr* KO hamsters on a short-term HCHF diet also had overt hypercholesterolemia, which could be effectively ameliorated with several lipid-lowering drugs. Importantly, heterozygotes on 3-month HCHF diets developed accelerated lesions in the aortas and coronary arteries.
Our findings demonstrate that the *Ldlr* KO hamster is an animal model of choice for human FH and has great potential in translational research of hyperlipidemia and coronary heart disease.

1. Introduction

Familial hypercholesterolemia (FH) is a major form of hypercholesterolemia characterized by elevated blood cholesterol levels due to delayed clearance of plasma low density lipoproteins (LDLs), leading to the early onset of cardiovascular and cerebrovascular diseases (Hobbs et al. 1992). FH pathogenesis hinges upon loss-of-function mutations in the *Ldlr* gene, which interacts with additional genetic and environmental factors. Significant resources have been allocated to FH research, including animal models based on various species in which human FH can be simulated by deleting the *Ldlr* gene with current genetic engineering techniques (Ishibashi et al. 1993; Li et al. 2016). Among these species, mice have been the most popular because of their small size, rapid breeding and low cost. However, lipid metabolism in mice differs from that in humans with respect to 1) the absence of cholesteryl ester transfer protein (CETP), 2) the presence of both intestinal and hepatic ApoB editing activity, and 3) the high hepatic expression of *Ldlr* mRNA. Thus, mice are "HDL predominant" because they lack CETP-mediated cholesterol transfer from HDL to VLDL and display a fast clearance of ApoB48-containing lipoproteins resulting from additional hepatic ApoB editing. Despite the current widespread use of *Apoe* KO and *Ldlr* KO mice to mimic human atherosclerosis, obvious differences in murine genetic and metabolic profiles limit their further applications

Abbreviations: ApoB, apolipoprotein B; ApoE, Apolipoprotein E; CETP, cholesteryl ester transfer protein; CAD, coronary artery disease; CD, chow diet; CHD, cardiovascular heart diseases; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HCHF, high cholesterol high fat; HeFH, heterozygous familial hypercholesterolemia; HoFH, homozygous familial hypercholesterolemia; KO, knockout; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; PCSK9, proprotein convertase subtilisin/kexin type 9; VLDL, very low density lipoprotein.

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(Getz and Reardon 2006). In contrast, golden Syrian hamsters are small rodents that exhibit many features that are similar to those of humans, including lipoprotein profile, high plasma CETP levels, and ApoB editing only in the intestine (Reaves et al. 2000). In particular, hamsters are susceptible to high-fat diet-induced combined hyperlipidemia (Haidari et al. 2002). In addition, one study (Bjorklund et al. 2014) recently used AAV-mediated hepatic gene delivery to introduce a PCSK9 gain-of-function mutant gene (rAAV8-D377Y-mPCSK9) to hamsters, and their results revealed hypercholesterolemia and atherosclerosis that were similar to those observed in mice when both species were fed a western-type diet. However, this non-germline approach is questionable because although >95% of hepatic LDLR is reduced in the rAAV8-D377Y-mPCSK9 model, the plasma cholesterol levels are not as high as those in the *Ldlr*−/− model, indicating that LDLR from extrahepatic or other tissues can contribute to this cholesterol-lowering effect. Additionally, a systemic review of the literature reveals that genes associated with atherosclerosis in mice are scarcely confirmed in human diseases with common genetic variants, suggesting that the use of gene-modified mouse models is insufficient to fully understand the potential pathogenesis underlying atherosclerosis (Pasterkamp et al. 2016). Furthermore, due to the technical limitations of hamster embryo manipulation, genetically modified hamster models have not been generated to study the human hereditary hyperlipidemia pathogenesis and experimental interventions.

Recently, we achieved a technological breakthrough in hamster embryo manipulation and successfully generated the genetically engineered hamster (Gao et al. 2014). Herein, we report the generation of *Ldlr* knockout hamsters using the CRISPR/CAS9 gene editing technique (Cong et al. 2013) and show that this model manifests pathological phenotypes similar to those of humans with respect to autosomal inherited hypercholesterolemia and coronary atherosclerotic lesions.

2. Materials and Methods

2.1. Hamster Model

Golden Syrian hamsters were purchased from Vital River Laboratories (Beijing, China) and maintained in an air-conditioned room with a 14/10-hour light/dark cycle. For HCHF diet intervention experiments, 8–10 weeks old animals were fed with HCHF for the indicated time points. All experiments were monitored according to the principles of laboratory animal care (NIH publication no.85Y23, revised 1996) and approved by the Animal Care and Use Committee of the Peking university health science center (LA2010-059).

2.2. sgRNA Design

The sgRNA targeting sites of interest were identified by searching for the GG(N)18NGG motifs in the DNA sense or antisense strands. The specificity of the sgRNA target sites was analyzed according to the basic local alignment search tool (BLAST) applied to the golden Syrian hamster genome. The targeting sequence of the *Ldlr* gRNAs was gaaatgcatcgccagcaag[*tgg*].

2.3. Production of sgRNA and Cas9 mRNA

The sgRNA template was amplified by PCR. In a 50-μL PCR volume, the reaction comprised a unique oligonucleotide (CRISPRF) harboring the T7 polymerase binding site; the sgRNA target sequence, GG(N)18; and a common oligonucleotide (sgRNAR) encoding the remainder. The reaction was performed on a thermal cycler (98 °C for 30 s and 35 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 10 min and 4 °C for ∞), and the PCR products were purified using a Gel DNA Extraction Kit (TAKARA) and by phenol chloroform extraction, with subsequent isopropanol precipitation. The sgRNA template was transcribed using Megascript T7 Kit (Ambion) in vitro, purified with the MEGAclear Kit (Ambion), diluted in RNase-free water to a final concentration at 200 ng/μL and stored at −80 °C for future use.

The PXT7 construct carrying a humanized cas9 cDNA was used as the DNA template for the amplification of the cas9 coding sequence. The PXT7 vector was linearized with *Xba*I and purified by ethanol precipitation. Cas9 mRNA was transcribed using linearized template DNA with the mMESSAGE mMACHINE T7 kit (Ambion) and purified by phenol chloroform extraction and isopropanol precipitation. Cas9 mRNA was diluted into RNase-free water to a final concentration at 500 ng/μL and stored at −80 °C for future use.

2.4. Microinjection

Microinjections were performed under a microscope with red filters. M2 medium covered by mineral oil was used as the injection medium. sgRNA (20 ng/μL) and cas9 mRNA (50 ng/μL) were co-injected into the cytoplasms of fertilized eggs with well-recognized pronuclei in M2 medium (Sigma-Aldrich, St. Louis, MO, USA). The injected zygotes were cultured in HECM-10 medium at 37.5 °C under 10% CO_2 for 30 min. Thereafter, the injected embryos with normal morphology were transferred into each oviduct (approximately 15 embryos per oviduct) through the fimbriae of the female recipients; the females were naturally mated with male litters for 24 h before the transfer, as described previously (Fan et al. 2014).

2.5. Genotyping

Genomic DNA was extracted from the toes into 200 μL of squishing buffer (10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, 500 mg/mL proteinase K, pH 8.2) and incubated at 55 °C for 12 h, followed by inactivation at 95 °C for 10 min. The targeted fragments were amplified from the extracted DNA, and mutations were identified by Sanger sequencing. The *Ldlr* KO hamsters were genotyped with primers *Ldlr*-F (5′-CGGCCC AGATGTCAATAT-3′) and *Ldlr*-R (5′-GTGAAACCCTCCAAACCC-3′).

Furthermore, the amplified products were cloned into the pEASY-T1 vector (Transgen Biotech). After transformation of the reactions, the targeted DNAs isolated from single clones were sequenced.

2.6. Off-target Analysis

Potential off-target analysis was based on the rules indicating that sequences matching the final 12 nt of the target sequence and PAM sequence may cause off-target effects (Cong et al. 2013). Potential off-target fragments were amplified from the extracted DNA and identified by Sanger sequencing.

2.7. Plasma Lipids, Oral Fat Load, VLDL Secretion and LPL Activity Assay

The total plasma cholesterol (TC) and triglyceride (TG) levels were determined enzymatically using commercially available kits (Sigma). After ApoB-containing lipoproteins were precipitated with 20% polyethylene glycol solution, HDL cholesterol (HDL-c) was measured with the aforementioned kit. Fast-Protein Liquid Chromatography (FPLC) of plasma lipoproteins was performed from pooled plasma aliquots (250 μL) and applied to Tricorn high-performance Superose S-6 10/300GL columns using a fast-protein liquid chromatography system (Amersham Biosciences), followed by elution with PBS at a constant flow rate of 0.25 mL/min. Eluted fractions (500 μL per fraction) were assessed for triglyceride and cholesterol concentrations using the same TG and cholesterol kits.

In the oral fat load assay, 8–10 weeks old male hamsters on chow diet were fasted for 12 h and then gavaged with olive oil (10 mL/kg body weight). Plasma was collected for TG measurement at the indicated time points (0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, and 8 h) after gavage.

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