Establishment of a lipid metabolism disorder model in \textit{ApoEb} mutant zebrafish

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Abstract

Background and aims: ApoEb is a zebrafish homologous to mammalian ApoE, whose deficiency would lead to lipid metabolism disorders (LMDs) like atherosclerosis. We attempted to knock out the zebrafish ApoEb, then establish a zebrafish model with LMD.

Methods: ApoEb was knocked out using CRISPR/Cas9 system, and the accumulation of lipids were confirmed by Oil Red O staining, confocal imaging, and lipid measurements. The lipid-lowering effects of simvastatin (SIM), ezetimibe (EZE) and Xuezhikang (XZK), an extract derived from red yeast rice, were evaluated through in vivo imaging in zebrafish larvae.

Results: In ApoEb mutant, significant vascular lipid deposition occurred, and lipid measurement performed in whole-body homogenate of larvae and adult plasma showed significantly increased lipid levels. SIM, EZE and XZK apparently relieved the hyperlipidemia in ApoEb mutants, and XZK had a significant inhibitory effect on the recruitment of neutrophils and macrophages.

Conclusions: In this study, a LMD model has been established in ApoEb mutant zebrafish. We suggest that this versatile model could be applied in studying hypercholesterolemia and related vascular pathology in the context of early atherosclerosis as well as the physiological function of ApoE.

1. Introduction

Apolipoprotein E (ApoE) is a kind of apolipoprotein mainly expressed in mammal livers [1]. It is crucial in lipid metabolism, especially in the metabolism of very low-density lipoproteins (VLDLs), cholesterol and high-density lipoproteins (HDLs) [2]. The relationship between ApoE and risk of cardiovascular disease (CVD) such as hyperlipidemia and ischemic heart disease has been discovered through numerous epidemiological observational and genetics studies as well as interventional clinical trials [3]. Approximately half of plasma ApoE is associated with triglyceride-rich lipoproteins, where ApoE serves as the main ligand for the low-density lipoprotein receptor (LDLR) and the LDLR related protein (LRP) [3]. Previous studies found that the sterol-regulatory element binding protein (SREBP) regulates the expression of LDLR and other proteins involved in biosynthesis of cholesterol like hydroxy-3-methyl glutaryl coenzyme A reductase (HMGCR) [4], fatty acid synthase (FASN) [5] and proprotein convertase subtilisin/kexin type 9 (PCSK9)[6].

Lipid-lowering drugs targeting various pathways have successfully helped human manage hyperlipidemia. Simvastatin (SIM), one of the principal drugs targeting Ldlr expression, is able to efficiently lower LDL-C and reduce the incidence of CVDs in human. Xuezhikang (XZK) is a commonly used traditional Chinese medicine extracted from red yeast rice [7]. It has shown powerful lipid-lowering effect in clinic both in China and abroad [7,8]. Despite its common use, the pharmacological and toxicological effects of XZK are still not totally clarified.
Although various lipid-lowering drugs were invented, familial hypercholesterolemia (FH) patients are not sensitive to them, hence new lipid-lowering drugs remain to be developed, and efficient animal models are essential. But the procedures of the drug screening and hyperlipidemia-related research using ApoE⁻/⁻ mice might be expensive, time-consuming, and labor-intensive. As a novel model animal, zebrafish have the advantages like body transparency, large progeny numbers and low maintenance costs. What’s more, genes between human and zebrafish have a relative high conservation, including the genes encoding apolipoprotein family members like the ApoA family, ApoB, ApoC2 and Ldlr [9-11]. In order to study lipid metabolism disorders (LMDs) like FH in depth, zebrafish lines with corresponding gene mutations have been established in recent years. The known main causes of FH are pathogenic variants in Ldlr, ApoB (the gene encodes the apolipoprotein B) and Pcsk9, as well as ApoE [12]. Among them, Ldlr [11], ApoB [13] and Pcsk9 [14] mutant zebrafish lines have been established recently, yet the pathophysiology of FH remains elusive. To provide a different perspective for the study of the occurrence and development of FH and related CVDs, in this study, we used the CRISPR/Cas9 system to interrupt the expression of the zebrafish ApoEb gene, following with a short high-fat diet (HFD) feeding, established a novel zebrafish LMD model. And using this disease model, we have made a preliminary study on the mechanism of XZK in preventing LMDs. Our study paved another way for future pathophysiology studies of LMDs like hyperlipidemia and FH, as well as pharmacological and toxicological studies of lipid-lowering drugs.

2. Materials And Methods

2.1 Zebrafish maintenance and feeding

Wild-type (Tuebingen), Tg (fli1: EGFP), Tg (cmcl2:mCherry) and Tg (flk1:dsRed; lyz: EGFP) zebrafish embryos were obtained by in vitro fertilization and natural spawning of adults maintained at 28°C on a 14/10-hour light/dark cycle and staged as described [15]. In the Tg (fli1: EGFP) fish line, green fluorescent protein labels vascular endothelial cells. In Tg (flk1:dsRed; lyz: EGFP) larvae, red fluorescent protein labels vascular endothelial cells, while green fluorescent protein labels neutrophils. In the Tg (cmcl2:mCherry) line, cardiomyocytes were labeled by red fluorescent protein. To prevent pigmentation during embryonic development, 0.003% 2-phenylthiourea (PTU, Sigma-Aldrich, CA, USA) was added to the culture water. Zebrafish larvae were fed twice a day, starting at the 5th day post fertilization (dpf), with either a normal diet (ND) (paramecium, from China Zebrafish Resource Center) as described [16] or a high-fat diet (HFD) (Yuanye, Shanghai, China), which contains 4% weight per weight (w/w) cholesterol and 20% w/w triglycerides. For drug treatment experiments, simvastatin (Sigma-Aldrich, Munich, Germany), ezetimibe (Selleckchem, Shanghai, China) and XZK (Beijing Peking University WBL Biotech Co., Ltd., Beijing, China) were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Munich, Germany) and thoroughly mixed with HFD. According to the experiment, drugs were dissolved into different concentrations when used. The final DMSO concentration was ≤0.5% volume/volume (v/v) in all treatment groups. Adult zebrafish of the Tuebingen strain were maintained in the same environment and fed brine shrimp (Bohai, Binzhou, China) twice a day as described [16]. The HFD feed (containing 5% w/w cholesterol and 24% w/w
triglycerides) for adult fish were bought from Trophic Corporation (Nantong, China). All animal studies were approved by the Experimental Animal Ethics Committee of Shanghai Changzheng Hospital.

2.2 ApoEb knockout using CRIPR/Cas9 system

The gRNA plasmid and Cas9 mRNA plasmid used in this study were kind gifts from Dr. Jing-Wei Xiong from Peking University. In vitro transcription of gRNAs and Cas9 mRNA were performed using T7 RNA polymerase (Ambion, TX, USA) according to the manufacturer’s instructions and purified by phenol chloroform extraction. Two ApoEb genomic target sequences were selected: 5'-gcccatgggaggagatggTGG-3' and 5'-gatgacgtgaagaaccgtgtCGG-3' in which the last 3 nt were the protospacer adjacent motif (PAM) required for CRISPR/Cas9 function. One hundred pg of gRNAs and 200 pg of Cas9 mRNA were co-injected into 1 to 2-cell stage embryos. For genotyping, the caudal fins of adult fish were partly cut down, and whole embryos were homogenized. The genomic DNA was extracted using the TransDirect Animal Tissue PCR kit (Transgen, Beijing, China). The genomic DNA fragment containing the target site was amplified using Ex Taq DNA polymerase (Takara, Tokyo, Japan). The primers used for PCR amplification of ApoEb cDNA flanking exon 3 to exon 4 were listed in Supplementary Table 1. All primers and the Sanger sequencing of the PCR amplification products to confirm the genotypes in detail were provided by Biosune Ltd, Shanghai, China.

2.3 Quantitative RT-PCR (rt-qPCR)

Total RNA was isolated from 15 larvae using Trizol reagent (Thermo Fisher, MA, USA), and reverse transcribed using FastQuant RT Kit (Tiangen, Beijing, China). Quantitative RT-PCR assays were performed using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). The primers used for mRNA detection were all listed in Supplementary Table 1.

2.4 Plasma lipase activity assay

Adult zebrafish were anesthetized in an ice-water mixture, and then tail amputation was performed. Blood collected from 5 adults were pooled and diluted 1:200 in natural saline and plasma was obtained by centrifugation at 3,000 g for 10 minutes. Two hundred μl of the diluted plasma was used to measure lipase activity with a Lipase activity assay kit (Solarbio, Beijing, China), following the manufacturer’s manual. The assay buffer was used as a negative control. The reaction was conducted at 37 °C.

2.5 Oil O Red staining

For Oil Red O (ORO) staining, the lipid in zebrafish body were stained with Oil Red O dye (Sigma-Aldrich, Munich, Germany). The zebrafish larvae were fixed in 4% paraformaldehyde (Sangon, Shanghai, China) for 12 h, washed twice with phosphate buffer saline (PBS) (Sangon, Shanghai, China), and then incubated in 0.3% ORO solution for 3 h. Stained larvae were rinsed with PBS before imaging.

2.6 Whole-mount Immunofluorescence (IF-Wm)
Three dpf *Tg (flk1:dsRed; lyz:EGFP)* larvae were fixed in 4% paraformaldehyde, and used for *IF-Wm*, which were performed as described previously [17]. The following antibodies were used: anti-Mpo (1:200; rabbit; Abcam), anti-MFAP4 (1:200; rabbit; Abcam), and Donkey anti-Rabbit IgG H&L (Alexa Fluor 647) antibody (1:200; Abcam).

2.7 **Imaging and counting**

For *in vivo* visualization of vascular lipid deposits, the HFD was supplemented with 1 μg/g of a fluorescent cholesteryl ester analog CHOLESTERYL BODIPY 576/589 C11 (Invitrogen, Carlsbad, USA), as previously described [18]. After fed with this fluorescently labeled diet for a specific period according to the procedures of different experiments, *Tg (fli1:EGFP)* larvae were mounted in low melting point agarose (Sigma-Aldrich) in Danieu’s solution with 0.04% Tricaine to keep them immobilized. Then, the larvae were scanned on a Leica TCS SP5 confocal microscope with 1.5 μm step size, 1,024 × 1,024 pixel at 400 Hz. The *in vivo* imaging of angiogenesis of *Tg (fli1: EGFP)* fish line was performed using either the confocal microscope with the same parameters, or a Leica M205 fluorescent microscope, which was also used to image the *Tg (flk1:dsRed; lyz: EGFP)* fish line. The amount of the neutrophils in 10 consecutive segments of the trunk was counted using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA). The heart beating images and movies of the *Tg (cmcl2: mCherry)* fish line were also taken using the Leica M205 fluorescent microscope. The consequent measurements on the images were performed using Image-Pro Plus 6.0 software. Bright field imaging for ORO staining and neutral red staining was taken using a stereomicroscope (Zeiss, Oberkochen, Germany). The integrated optical density (IOD) value of 5 consecutive segments of the trunk in all ORO staining or neutral red staining images were measured using Image-Pro Plus 6.0 software to relatively quantify the vascular lipid accumulation or recruitment of the macrophages [19]. All the images are lateral views, anterior to the left, and dorsal is up unless specifically noted.

2.8 **Lipid level measurement**

Fifteen larvae in the same group were euthanized in icy PBS and homogenized by ultrasonic fragmentation, supernatants were collected and used as 1 homogenate sample. Lipid levels in diluted pooled adult plasma or larvae homogenate were measured using total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein (VLDL) and high-density lipoprotein cholesterol (HDL-C) quantification kits (Jiancheng, Nanjing, China) according to the manufacturer’s protocols, respectively.

2.9 **Neutral red staining**

To evaluate recruitment of the macrophages, 15 live larvae from each group were respectively cultured in a 12-well plate containing 2.5 mg/L of neutral red dye solution (Solarbio, Beijing, China), which can aggregate in the macrophages, forming red dots due to the recruitment of macrophages. After incubation for 8 h, the larvae were anesthetized and imaged under a microscope.
2.10 Statistical analysis

Normality was evaluated by Shapiro-Wilk test. The data fit normal distribution were presented as the mean ± standard error of mean (SEM), while boxplots were used to present skew distribution data. GraphPad Prism software was used for data analysis. For the normal distribution data, Levene's test of homogeneity of variance was further performed. When the data fitted the homogeneity of variance, two-tailed Student's t test or one-way ANOVA was applied. For the data that did not fit the homogeneity of variance, Mann-Whitney test, Brown-Forsythe ANOVA test, or Kolmogorov-Smirnov test was performed. Mann-Whitney test or Kruskal-Wallis test was performed for skew distribution data. All P-values were two-sided and P < 0.05 was considered statistically significant.

3. Results

3.1 Generation of the ApoEb mutant zebrafish

In zebrafish, the ApoE gene has two paralogs - ApoEa (NCBI gene ID: 553587) and ApoEb (NCBI gene ID: 30314) [20-22]. Despite of its less than 30% identity with mouse or human ApoE (Fig. S1A), ApoEb, but not ApoEa, contains a conserved sequences encoding amino acid sequence similar to the lipoprotein receptor-binding region (LRR) of human ApoE [23], and its protein sequences satisfy the common structural features depicted for potential amphipathic helices characteristic of plasma apolipoprotein [24]. Previous study also has found that ApoEa expression gradually reduced during fish development, while ApoEb increased [25]. All these indicated that with conserved functional motifs like mammalian ApoE (Fig. S1B), ApoEb may play an important role in lipid metabolism of zebrafish. In order to effectively interrupt the expression of ApoEb gene using CRISPR/Cas9, we designed 2 gRNAs targeting its exon 3 and 4 respectively (Fig. 1A). These could cause a 378 nt deletion in ApoEb mRNA, and consequently lead to a 126 amino acid (aa) loss and a 1 aa mistranslation in the corresponding ApoEb protein (Fig. 1B). The truncated protein translated might lost parts of the apolipoprotein domain and the LRR required for ApoEb function (Fig. 1C). Hence, the mutant might only have loss-of-function ApoEb protein. The gRNAs and Cas9 mRNAs were synthesized and injected immediately into the cytoplasm of the fertilized egg cells as soon as possible, to obtain mutant founders (F0). Sanger sequencing of the F0 genomic DNA showed there were 542 nucleotide deletions (Δ542) occurred in several mutants, as shown in Fig. 1D. In order to carry this mutation to other zebrafish lines with various transgenic backgrounds, successfully mutagenized F0 animals were raised to adulthood and crossed to other fish lines to obtain the F1 generations. After the sexual maturity, the F1 male were crossed with F1 female to obtain F2 embryos carrying the same mutation. Through 2 generations, homozygous ApoEb mutated Tg (fl1:EGFP), Tg (flk1:dsRed; lyz: EGFP) and Tg (cmcl2:mCherry) lines were then screened out. Rt-qPCR detection for the normal ApoEb mRNA with the integrated sequence showed ApoEb mRNA level was almost undetectable in 5 dpf ApoEb⁻/⁻ mutants, as well as in the livers of 2-month-old adults (Fig. 1E). This part of results indicated that there existed not normally functionable ApoEb protein in homozygous.

3.2 Hyperlipidemia in adult ApoEb mutants
No apparent physical differences were found between 3-month-old male wild-type siblings and *ApoEb* mutant zebrafish (Fig. 2A-B). To test whether *ApoEb* deficiency resulted in hypertriglyceridemia, we drew blood from adult male zebrafish fed with an 8-week normal diet (ND) or high-fat diet (HFD) to measure the lipid profile in plasma. The results showed that ND fed *ApoEb* mutants had higher plasma TC, LDL-C and VLDL level. Although there was no statistical difference, plasma TG level in *ApoEb* mutant tended to increase. After HFD feeding, TC, TG, LDL-C and VLDL levels in *ApoEb* mutant’s plasma significantly increased, while HDL-C remained unchanged compared with siblings (Fig. 2C). Meanwhile, plasma lipase activity was significantly decreased in normally fed *ApoEb* mutants compared with siblings (Fig. 2D). Due to ApoEb probably was a ligand for zebrafish LDLR, which is required for extracellular cholesterol uptake and regulation of the Srebp pathways in the liver to regulate plasma cholesterol [25], we next tested whether *ApoEb* knockout had influence on the Srebp pathways, which was also a target of many lipid-lowering drugs, in the livers of normally fed 2-month-old *ApoEb* mutants (Fig. 2E). The detection of the expression level of *Hmgcr* and *Ldlr*, two target genes of *Srebp*-2, showed an interesting upregulation without that of *Srebp*-2 in *ApoEb* mutant. An increase in mRNA expression of *Srebp*-1 and *Fasn*, one of its target genes, could be observed in *ApoEb* mutant.

### 3.3 Early angiogenesis defects in *ApoEb* mutant larvae

Three dpf *ApoEb* mutant larvae showed no significant defects in development and yolk utilization and growth (Fig. 3A). The body length, yolk area and yolk extension did not show differences between 3 dpf siblings and *ApoEb* mutants (Fig. 3B). However, in about 40% of *ApoEb* mutants at 3 dpf, cerebral hemorrhage could be observed, mainly in midbrain and hindbrain (Fig. 3C-D, indicated by red triangles). Confocal imaging of 3 dpf *ApoEb* mutant Tg (*fli1*: EGFP) larvae showed the integrity of the cerebral vascular network was significantly interfered. For instance, in Fig. 3E, the red triangles indicated the narrowed posterior mesencephalic central arteries (PMctAs), while the white triangles pointed out the absence of the metencephalic arteries (MtAs) in *ApoEb* mutants. We then observed the development of the mutant’s intersegmental vessels (ISVs). Fig. 3F showed the development of wild-type and *ApoEb* mutated larvae ISVs within 48 hpf, with significant narrowed ISVs at 30, 36 and 48 hpf in the mutants. However, within 6 dpf, the time and length of ISV spreading were not statistically different (Fig. 3F), though the diameter of ISVs shortened in 48 hpf *ApoEb* mutants. These results demonstrated that angiogenesis defects occurred at early developmental stage of *ApoEb* mutants.

### 3.4 LMD occurred in *ApoEb* mutant fed with HFD

We performed ORO staining, confocal imaging, and lipid measurements to test whether the *ApoEb* mutant developed LMD. We found that both the yolk sac and blood vessels of *ApoEb* mutants had lipid content (Supplementary Fig. S2A). Lipid deposition gradually decreased with development, and existed until 6 dpf, indicating that the lipids derived from the yolk sac had been consumed completely at this stage (Supplementary Fig. S2A). Compared with ND feeding for 1 day, 7 dpf *ApoEb* mutant larvae fed with ND for 2 days showed slightly ORO staining in the blood vessels (Supplementary Fig. S2B), indicating moderately elevated neutral lipid levels in the circulation. The IOD value, which could be used
to relatively quantify the intensity of intravascular ORO staining confirmed these findings (Supplementary Fig. S2C). When fed for 5 days, ORO staining of 10 dpf larvae fed with ND showed significant intravascular lipid deposition in ApoEb mutants (Supplementary Fig. S2D). However, fluorescently labeled cholesterol esters (576/589-CE, the numbers indicate excitation/emission wavelengths) accumulated in vessels did not show significant change in 5-day ND fed 10 dpf ApoEb mutants (Supplementary Fig. S2D-E). Interestingly, the angiogenesis defects in ISVs at early stage seemed to be corrected at this stage. In other words, the vasculature of 7 dpf mutants was normal (Supplementary Fig. S2D). We then tested whether a 2-day HFD feeding was able to induce LMD in ApoEb mutant larvae, as previously reported in wild-type [26]. The results showed that the phenotype in ApoEb mutants fed with a 2-day HFD was more robust (Supplementary Fig. S2F-G). In 10 dpf ApoEb mutants fed with a 5-day HFD, much more intravascular lipid accumulation also could be observed through ORO staining (Fig. 4A-B). A HFD feeding, even for just 2 days, might lead to significantly increased 576/589-CE deposition in 7 dpf ApoEb mutant larvae (Fig. 4A). The calculation of average lipid deposits per segment also told this (Fig. 4C).

To figure out the primary effects of ApoEb knockout on the Srebp pathways, we performed detections in 5 dpf fasted sibling and ApoEb mutant larvae (Supplementary Fig. S2H), which showed an interesting upregulation of Ldlr. Though a 2-day ND did not alter the expressions of the Srebp pathways much, a 2-day HFD feeding significantly activated both Srebp pathways in ApoEb mutant (Supplementary Fig. S2I). This was consistent with the expression of Srebp-1 and -2 pathways in 10 dpf ApoEb mutant fed with a 5-day diet, which showed that ND might induce the upregulation of Hmgcr, while HFD upregulated the expressions of all these genes (Fig. 4D). So as the results from the livers of 8-week HFD feeding ApoEb mutants (Supplementary Fig. S2J). Furthermore, lipid measurement of whole-body homogenates of 7 dpf larvae showed significant higher TC, TG, LDL-C and HDL-C levels in ApoEb mutants fed with HFD for 2 days, while a 2-day ND also increased the cholesterol levels in ApoEb mutants (Supplementary Fig. S2K). However, a continuous 5-day ND did not elevate the lipid levels (Supplementary Fig. S2L), while a 5-day HFD feeding increased the levels of TC, TG, VLDL and LDL-C in whole-body homogenate of 10 dpf ApoEb mutants, with unchanged HDL-C level (Fig. 4E).

We also investigated the effect of ApoEb deficiency on systemic inflammation. We found that the expression of IL-1β and TNF-α were significantly upregulated in 5-day HFD fed 10 dpf ApoEb mutants, while IL-6 expression remained unchanged (Supplementary Fig. S2M). Besides, we discovered that acute lipid burden like a 2-day HFD feeding might result in impaired cardiac function and arrhythmia in ApoEb mutant Tg (cmcl2: mCherry) larvae. Supplementary Fig. S2N showed that after 2-day HFD feeding, ApoEb mutants manifested higher heart rates, lower ventricular fractional shortening (FS), and smaller heart area as well as shorter venous sinus (SV) – artery ball (BA) distance which indicated restricted heart beats. Supplementary Movie 1&2 demonstrated significant diastolic prolongation and premature beats in 2-day HFD fed 7 dpf ApoEb mutant, compared to the sibling. The results above suggested that HFD feeding might lead to serious LMD in ApoEb mutant zebrafish.

3.5 Simvastatin, ezetimibe and Xuezhikang significantly relieved LMD in ApoEb mutant
To evaluate whether ApoEb mutant zebrafish subjected to a short-term (2 days) HFD feeding could be useful for drug screening experiments, we need to find proper positive controls. We tested the effect of simvastatin (SIM), an Hmgcr inhibitor as well as a widely applied lipid-lowering drug, on intravascular lipid accumulation. Firstly, 5 dpf larvae were fed with a 2-day HFD, supplemented with various concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 μg/mL) of SIM at the last 24 hours. The survival test of 24-hour SIM treatment showed that when treated with 0.4 μg/mL SIM for 12 hours, the survival rate maintained at a high level (Supplementary Fig. S3A). However, a 24-hour SIM treatment has relatively higher fatality rate (Supplementary Fig. S3A), which was consistent to a previous report [27], as well as significant teratogenic effect to zebrafish larvae. When treated for 24 hours, the larvae demonstrated doubled rate of slow touch-evoked escape (a parameter used to measure the body movement ability and the ability to respond to external stimuli of zebrafish larvae, Supplementary Fig. S3B) as well as elevated rate of abnormal morphology (Supplementary Fig. S3C-D, red triangles indicate the bent tails). For longer drug treatment experiments, according to former studies [27], we tested ezetimibe (EZE), which was much safer than SIM for larvae when treated for more than 2 days. As a result, larvae treated with 5 or 10 mM EZE within 5 days showed higher survival rates (Supplementary Fig. S3E-F). We also found that larvae had an acceptable 12-hour survival rate when treated with Xuezhikang (XZK) ≤120 μg/mL (Supplementary Fig. S3E-F), and the optimal concentration (30 μg/mL) of XZK for 5-day treatment (Supplementary Fig. S3E).

Having this confirmed, the lipid lowering effect of SIM, EZE, and XZK had been tested. ORO staining demonstrated that a 12-hour treatment with 30 μg/mL XZK was effective to reduce intravascular lipid accumulation in 7 dpf ApoEb mutants fed with a 2-day HFD, while 10 mM ezetimibe or 0.4 μg/mL SIM treatment was more potent (Fig. 5A-B). We also examined the intravascular accumulation of lipid deposits directly through confocal imaging (Fig. 5C). Consistently, the vascular deposition of 576/589-CE in the XZK treatment group was significantly reduced, and further reduced in the EZE and SIM treatment groups (Fig. 5C-D). In 5-day treatment experiments, the lipid measurements of whole-body homogenates of 10 dpf ApoEb mutant larvae fed with 5-day HFD suggested that compared to the DMSO group (treated with 0.5% DMSO), both 10 mM EZE and 30 μg/mL XZK significantly reduced lipid levels in tissue, with an elevated HDL-C level (Fig. 5E). We also detected the effects of EZE or XZK treatments on the expressions of Srebp-1 and -2 pathways in 10 dpf ApoEb mutant larvae fed with 5-day HFD (Fig. 5F). The results implied that EZE might activates both pathways while XZK mainly upregulated the Srebp-2 pathway, with increased Hmgcr and Ldlr expression (Fig. 5F).

The above results proved that the ApoEb mutant LMD model could be utilized in drug screening procedures. SIM and EZE could be applied as positive controls in 12-hour and 5-day treatments, respectively. Through a rapid relatively quantification method, we also found that XZK was able to relief LMD by lowering general TC, TG, VLDL and LDL-C levels while increasing HDL-C level in zebrafish.

3.6 The effect of XZK on LMDs
ORO staining for XZK treated *Tg (flk1:dsRed; lyz:EGFP)* larvae carrying the *ApoEb* mutation showed that along with the increase of XZK concentrations, the intravascular lipid deposition gradually decreased ([Supplementary Fig. S4A-B, Fig. 6A](#) showed the representative images), which could be confirmed by the quantification of intravascular staining intensity ([Fig. 6B](#)). This lipid-lowering effect might play an important role in the preventive effect of XZK on hyperlipidemia. In order to prove that the cells with green fluorescence in this fish line were neutrophils rather than macrophages, we conducted immunofluorescence experiments to verify ([Fig. 6C](#)). As showed in [Fig. 6C](#), EGFP (green) driven by the *Lyz* promoter had obvious colocalization (yellow) with neutrophil specific marker *Mpo* (red), but no colocalization with macrophage specific marker *MFAP4*. This indicated that the cells expressing EGFP in the fish line were indeed neutrophils. On the other hand, fluorescence photography revealed that after HFD feeding for 2 days, EGFP-expressing neutrophils gathered in large numbers around the trunk large blood vessels ([Fig. 6D](#)). While in XZK and SIM treatment groups, neutrophils were mostly concentrated in the intestine rather than blood vessels ([Supplementary Fig. S4C-D, Fig. 6D](#) showed the representative images), and as the drug concentration increased, neutrophils accumulated in blood vessels decreased ([Fig. 6E](#)). These results indicated that XZK has an inhibitory effect on the aggregation of neutrophils, which might be another mechanism of XZK’s LMD-preventing effect. To evaluate whether XZK treatment affected macrophage recruitment, Neutral Red staining was performed in 10 dpf *ApoEb* mutants fed with 5-day HFD supplemented with EZE (10 μM) and XZK (30 μg/mL). It was observed that both of them effectively reduced the recruitment of macrophages ([Fig. 6F-G](#)). Besides, the mRNA measurements of IL-1β, IL-6 and TNF-α in the pooled whole-body homogenates of 10 dpf larvae fed a 5-day HFD supplemented with 0.5% DMSO (DMSO), XZK or EZE showed that EZE significantly induced the downregulation of IL-1β and TNF-α. XZK treatment reduced mRNA expression of IL-1β either but did not affect that of TNF-α. IL-6 was not affected by both of XZK and EZE ([Fig. 6H](#)).

4. Discussion

At present, both *ApoE* and *Ldlr* knockout mice supplemented with HFD have become routine animal models of LMDs such as atherosclerosis and hyperlipidemia. *ApoE* knockout mouse demonstrate a marked increase in blood lipids, a large amount of lipid deposits in the aortic wall – as a result, developed to atherosclerosis in a short period of time [28]. Even though the phenotype of *ApoE* and *Ldlr* mutant mice are similar to each other, they differ in their dietary needs for developing atherosclerosis, prominent lipoproteins and effect of hepatic lipase deficiency [29]. The advantage of the *ApoE*−/− mice model is that complex vascular lesions readily develop in animals fed the normal low-fat rodent chow, and atherogenesis can be notably accelerated by the feeding of a high-fat, high-cholesterol Western type diet [30], which is a necessary condition for establishing an atherosclerosis model in *Ldlr* knockout mice. In addition, apolipoprotein ApoE was reported to have several other roles in the pathological process of atherosclerosis besides directly participating in lipid metabolism, such as the regulation of monocyte activation [31] and smooth muscle cell (SMC) migration and proliferation [32], nitric oxide synthase (NOS) mediated platelet aggregation [33], and so on. In brief, different factors may show
different functions in ApoE and Ldlr knockout mice, respectively. In other words, ApoE and LDLR mutants cannot be substituted for each other.

In a recent review, Vedder et al wrote, “The development of ApoE−/− zebrafish would create a new tool for atherosclerosis research that could be compared with mouse models” [34]. In this study, we aimed to establish a model of LMD in zebrafish with loss-of-function of ApoEb, which is encoded by one of the 2 subfunctionalized paralogs of ApoE during the evolution of zebrafish [22]. Zebrafish ApoEb gene expressed in a species-specific manner [22,35,20], and its specific function and mechanism research is still lacking. Several studies had chartered zebrafish ApoEb protein has similar functional domains to mammalian ApoE protein and their high homology [24,23]. Hereinafter in this study, we supposed zebrafish ApoEb functioned as the ApoE gene in mammals, and the ApoEb mutant fish line adds to the set of zebrafish models of lipid abnormalities including ApoC2 mutant zebrafish and others [18]. Although these mutated zebrafish lines have been established, there are various physiological and pathophysiological differences between ApoEb mutants and others. In the present study, we discovered that ApoEb deficiency in zebrafish led to cerebral hemorrhage, defected cerebral vascular network, and ISV stenosis. Under lipid burden, ApoEb mutants manifested early cardiac dysfunction and significant vascular lipid deposits. Previous studies reported a WT larvae zebrafish hypercholesterolema model with accumulation of vascular lipid deposits whose establishment required a 2-week HCD feeding [36], and Ldlr loss-of-function mutant zebrafish model with a similar phenotype which needed HCD feeding for 5 days [11]. However, the time consumption to induce robust vascular lipid accumulation by HFD feeding in our new ApoEb mutant could be reduced to 48 hours, according to specific research purpose, for instance, the rapid reaction of neutrophils in acute lipid burden. What's more, the establishment of our ApoEb mutant zebrafish model is not only valuable for research on LMDs, but also can be used for nervous system development and disease research like Alzheimer's disease [37] as well as research on the innate immune system like bacterial antigen immunity [38], etc.

In our results, fasting 5 dpf ApoEb mutants showed increased Ldlr expression which indicated the activation of Srebp-2 pathway and decreased expression of Fasn which indicated the suppression of the Srebp-1 pathway. Through the measurements at various time points of feeding, we confirmed the activation of both Srebp-1 and -2 pathways in HFD feeding ApoEb mutants, and consistently, the upregulation of Ldlr. It is well-known that Ldlr is the mediator of hepatic cholesterol uptake [39], and predominantly regulated by SREBP-2 [40]. When hepatocytes lack cholesterol relative to that required for their physiological needs, the upregulation of Ldlr would be mediated via a negative feedback mechanism that is tightly controlled by SREBP-2 [41]. On the other hand, FASN is a multifunctional protein directly correlated with the fatty acids synthesis, playing a crucial role in de novo lipogenesis in mammals [42]. Remarkably, the downregulation of Fasn in ApoEb mutant we found in the current study was in contrast to its upregulation in Ldlr mutant [11]. However, other Srebp genes, including Hmgcr and Pcsk9, were not significantly changed in fasting larvae. It seems that different target genes are regulated by Srebp-1 or Srebp-2 in different manners, and the underlying mechanisms, which might provide new
insights and suggest new approaches for differential targeting of genes regulating cholesterol homeostasis, are worth further exploring.

In the 12-hour drug treatment experiments, we used SIM to test if the established model could be applied in lipid-lowering drug screening. SIM prevented HFD-induced accumulation of vascular lipid deposits effectively, elevated the plasma HDL-C level, indicating a promoted reverse transportation of cholesterol back to the liver. These results were in contrast to a previous report that SIM did not show explicit lipid-lowering effect in WT zebrafish [27]. This discrepancy may be caused by the underlying coupling effect of Srebp pathway activation induced by ApoEb dysfunction and HMGCR inhibition caused by SIM treatment and partly by the different absorption of SIM due to different methods of administration. Notably, previous work reported the skeletal muscle toxicity of SIM to zebrafish larvae [43], as well as human patients [44]. Consistently, our results confirmed in vivo the myotoxicity of SIM characterized by decreased body movement, slow response to external stimuli and increased abnormal morphology (bent tails and scoliosis). In the 5-day drug treatment experiments, EZE was employed as a safe and efficient positive control to be compared to XZK. Our results showed EZE significantly activated the Srebp-2 pathway, upregulated the expression of Srebp-2 and its downstream genes, Hmgcr, Pcsk9 and Ldlr, consistent to previous studies in mice [45], and XZK demonstrated similar effects on the Srebp-2 pathway. Which was inconsistent to mice studies [46], we found EZE also upregulated Srebp-1 expression, without affecting its downstream, Fasn. In the present study, it was hard to confirm whether and where the fatty acid synthesis was activated, which is worthy of further exploration. Therefore, we supposed that the ApoEb mutant larvae can be used for mechanistic studies, as well as for initial genetic or drug screening to identify new therapeutic targets, as well as their potential hazards.

In the early stages of atherosclerosis, neutrophils are recruited in large quantities under the vascular endothelium, releasing a variety of cytokines and inflammatory mediators, causing local inflammatory reactions. Neutrophils then induce monocytes and macrophages to enter and engulf oxidized LDL (oxLDL), forming foam cells, promoting the formation of lipid streaks, further activating macrophages, and aggravating atherosclerosis. A number of clinical studies have found that in advanced atherosclerotic plaques, the number and activity of neutrophils are positively correlated with the incidence of acute coronary syndrome (ACS), unstable plaque rupture, thrombosis and other cardiovascular events [47,48]. Previous study reported that traditional Chinese medicines such as mustard seeds and their extracts can inhibit the recruitment of neutrophils and exert their anti-atherosclerotic effects [49]. In our experiments, we have observed significant suppression in intravascular neutrophil recruitment after XZK treatment, which indicated that XZK may also have a similar inhibitory effect on neutrophils. In 5-day EZE treatment, reduced intravascular macrophage recruitment could be observed, which was consist to mice models [50]. Like EZE, XZK also demonstrated an inhibitory effect on macrophage recruitment. What’s more, both EZE and XZK significantly downregulated IL-1β expression, while EZE alone reduced TNF-α expression, either.

There were several limitations in our study. Firstly, adult zebrafish has a long experimental period and is not suitable for large-scale experiments like drug screening. The larvae fish have a short cycle and low
cost, but the volume is tiny, and the development of organs are still in the early stage, making it difficult to conduct blood tests and slices. In fact, even for adult zebrafish, due to the small size and disease progression limitations, research on zebrafish atherosclerosis is heavily leaning on the lipid metabolism [34]. Therefore, only the adult fish blood lipids were measured in this study, and it was not observed whether atherosclerotic plaque formation occurred early after feeding HFD. This could be a drawback, but the easy genetic manipulation and opacity of larvae offers an opportunity to study early atherosclerosis pathomechanisms. Maybe, with the progress of detection technology, the additional effect of ApoEb deletion on leukocyte accumulation and atherosclerotic plaque formation in adult zebrafish will be clarified in the future. Secondly, it is impossible to accurately quantify the feeding, consumption, and excretion of each larva. In the experiment, we observed the development and feeding of larvae under the microscope regularly and abandoned the unhealthy or non-feeding larvae in time, but still could not completely eliminate the influence of individual differences on the experimental results. Thirdly, whether ApoEb knockout will lead to other pathological consequence related to lipid metabolism is still unclear, which requires further observation.

5. Conclusion

In summary, we introduced a new genetic model of LMD. ApoEb mutant zebrafish larvae subjected to a short HFD feeding resulted in robust and consistent accumulation of intravascular lipid deposits. We propose that this new model may pave another way for the pathophysiological and therapeutic target screening research on LMDs like hyperlipidemia and FH, as well as the pharmacological and toxicological studies of novel lipid-lowering drugs.

Declarations

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CRediT authorship contribution statement

Yang-Xi Hu: Conceptualization, Methodology, Software, Formal analysis, Data curation, Visualization, Writing - Original draft preparation. Hong-Min You: Validation. Rong-Fang Zhu: Conceptualization, Methodology. Yu-Lai Liang and Fang-Fang Li: Methodology. Yong-Wen Qin and Xian-Xian Zhao: Supervision, Resources. Chun Liang and Qing Jing: Writing - Review & Editing, Project administration, Funding acquisition.
Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

References


**Abbreviations**

ApoE, Apolipoprotein E; CVD, cardiovascular disease; DMSO, dimethyl sulphoxide; dpf, days post fertilization; EZE, ezetimibe; FASN, fatty acid synthase; FH, familial hypercholesterolemia; Hetero, heterozygous; HDL, high-density lipoprotein; HDL-C, high-density lipoprotein cholesterol; HFD, high-fat diet; HMGCR, hydroxy-3-methyl glutaryl coenzyme A reductase; Homo, homozygous; hpf, hours post fertilization; IOD, integrated optical density; ISV, intersegmental vessels; LDL, low-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LMD, lipid metabolism disorder; LRP, LDL receptor related protein; LRR, lipoprotein receptor-binding region; MtA, metencephalic artery; ND, normal diet; ORO, Oil Red O; PAM, protospacer adjacent motif; PBS, phosphate buffer saline; PCSK9, proprotein convertase subtilisin/kexin type 9; PMcTa, posterior mesencephalic central artery; PTU, 2-phenylthiourea; rt-qPCR, quantitative polymerase chain reaction; SEM, error of mean; SIM, simvastatin; SREBP, sterol-regulatory element binding protein; TC, total cholesterol; TG, triglycerides; VLDL, very low-density lipoprotein; WT, wild type, v/v, volume /volume; w/w, weight per weight; XZK, Xuezhikang.
Establishment of *ApoEb* knockout zebrafish mutant. (A) Schematic diagram of the gRNA target sites at the *ApoEb* loci. Two gRNA target sites were designed to induce a large fragment deletion. The fragments in white in Exon 3 and 4 indicate those to be deleted. Red arrows indicate to the position of the primers.
used for genotyping. Green arrows represent the position of the primers used to detect the existence of normal ApoEb mRNA. +1, the translation initiation site; +748 and +1290, the target sites of the gRNAs; +2242, the stop codon site of ApoEb. (B) Sequences of the ApoEb mutant showing a 375 nt deletion in the open reading frame (ORF), which means a loss of 126 amino acids (aa) and a mis-interpreted amino acid (R → C). (C) Zebrafish ApoEb protein contains conserved signal peptide (SP) and apolipoprotein domain (Pfam ID: PF01442), in which exists a lipoprotein receptor-binding region (LRR). Parts of the apolipoprotein domain and the LRR required for ApoEb function are lost in the predicted truncated ApoEb mutant protein. (D) Consistent with the result of sequencing, genotyping shown a genomic Δ542 deletion in F2 heterozygote (+/-) and homozygote (-/-). (E) Quantitative analysis of ApoEb mRNA levels in the pooled whole-body homogenate of 5 dpf F3 larvae showed a significant decrease of ApoEb expression in homozygote (-/-), as well as in the livers of 2-month-old adults. In the left graph, 15 larvae pooled per sample, n = 12 in each group. In the right graph, n = 10 in each group.
Adult ApoEb mutant zebrafish develop hyperlipidemia. (A) No apparent differences between 3-month-old male wild-type siblings and ApoEb mutant zebrafish. (B) No significant change occurred in body length and weight after ApoEb deletion in 3-month-old adults. N = 14 in each group. (C) Plasma lipid profile of 8-week normal diet (ND) or high-fat diet (HFD) fed 3-month-old sibling or ApoEb mutants. Five animals pooled per sample, n = 6 in each group. (D) Plasma lipase activity assayed as described in the Materials

Figure 2
Development and angiogenesis in ApoEb mutant larvae. (A) Morphology of siblings and ApoEb mutant embryos at 3 dpf. The red and blue lengths highlighted in (A) were used to delineate the size of the body length and yolk extension, respectively. The area circled by the green dotted line was used to calculate the yolk area. Scale bar, 200 μm. (B) Body lengths, yolk areas or yolk extensions between siblings and ApoEb mutants were not significantly different. N = 12 in each group. (C-D) In about 40% of ApoEb mutants at 3 dpf, cerebral hemorrhage could be observed in midbrain and hindbrain (indicated by red triangles). N = 9 in each group; scale bar, 200 μm. (E) The integrity of the cerebral vascular network was significantly interfered. Red triangles indicated the narrowed posterior mesencephalic central arteries (PMCTAs), while white triangles pointed out the absence of the metencephalic arteries (MtAs) in ApoEb mutants. Scale
bars, 200 μm (upper) or 150 μm (lower). (F) The development of intersomitic vessels (ISVs) of fltl:EGFP background siblings and ApoEb mutant larvae within 48 hpf were shown. Note the narrowed ISVs at 30, 36 and 48 hpf in ApoEb mutants. The timing of sprouting and the length of spreading of ISVs between siblings and ApoEb mutant showed no significant difference ($P = 0.168$) at various time points within 6 dpf ($n = 3$ in each group). However, the diameter of ISV shortened ($P = 0.001$) in ApoEb mutants within 48 hpf ($n = 15$ in each group). Scale bars, 200 μm (24 hpf) or 50 μm (28, 30, 36 and 48 hpf).
Lipid metabolism disorders occurred in *ApoEb* mutant larvae. (A) Oil Red O (ORO) staining of 10 dpf larvae fed with 5-day high-fat diet (HFD) showed significant intravascular lipid deposition in *ApoEb* mutants. Fluorescently labeled cholesterol esters (576/589-CE, the numbers indicate excitation/emission wavelengths) were added to the HFD, so that vascular lipid deposits in 7 dpf sibling and *ApoEb* mutant larvae fed with 2-day HFD could also be visualized. DA, dorsal aorta; PCV, posterior cardinal vein; scale bars, 200 μm. (B) Intravascular ORO staining intensity in (A) was quantified through integral optical density (IOD). N = 15 in each group. (C) Average lipid deposits per segment were calculated (n = 15 in each group). (D) The expression of Srebp-1 and -2 pathways in 10 dpf sibling and *ApoEb* mutant larvae fed with 5-day normal diet or high-fat diet were measured by rt-qPCR assays (15 larvae pooled per sample, n=6 in each group). (E) Five-day high-fat diet feeding increased the total cholesterol level (TC), triglyceride level (TG), very-low-density lipoprotein level (VLDL) and low-density lipoprotein cholesterol level (LDL-C) in whole-body homogenate of 10 dpf *ApoEb* mutants, with unchanged high-density lipoprotein cholesterol level (HDL-C). Fifteen embryos pooled per sample, n = 6 in each group.
Simvastatin, ezetimibe and Xuezhikang significantly relieved lipid metabolism disorder in ApoEb mutant larvae. (A) ORO staining of intravascular lipid deposition in 7 dpf ApoEb mutant larvae fed a 2-day HFD feeding, followed by a 12-hour treatment with 0.5% (v/v) DMSO (DMSO), 30 μg/mL Xuezhikang, 10 mM ezetimibe or 0.4 μg/mL simvastatin. Scale bar, 100 μm. (B) The quantitative data showed lipid accumulation in vessels were significantly reduced in the Xuezhikang treatment group, and further
reduced in the ezetimibe and simvastatin treatment group. N = 15 in each group. (C-D) Representative images and quantitative data for vascular lipid deposits. N = 14 in each group; scale bar, 100 mm. (E) Compared to the DMSO group (treated with 0.5% DMSO), the lipid measurement of whole-body homogenate of 10 dpf ApoEb mutant larvae fed 5-day HFD supplemented with 10 mM ezetimibe or 30 μg/mL Xuezhikang showed significant decreased total cholesterol level (TC), triglyceride level (TG), very-low-density lipoprotein level (VLDL) and low-density lipoprotein cholesterol level (LDL-C), and an increase in high-density lipoprotein cholesterol level (HDL-C). Fifteen embryos pooled per sample, n = 6 in each group. (F) The effects of ezetimibe or Xuezhikang treatment on the expression of Srebp-1 and -2 pathways in 10 dpf ApoEb mutant larvae fed with 5-day HFD were measured by rt-qPCR assays (15 larvae pooled per sample, n=6 in each group).
The effect of Xuezhikang on preventing lipid metabolism disorder may be caused by various mechanisms. (A) After a 12-hour drug treatment, compared with the DMSO group (0.5% DMSO), the intravascular lipid deposition in both low-concentration Xuezhikang (XZK) treatment group (36 μg/mL, Lo-XZK) and high-concentration XZK treatment group (500 μg/mL, Hi-XZK) significantly decreased, comparable to 0.4 μg/mL simvastatin (SIM). Scale bar, 100 μm. (B) Quantification of intravascular ORO.
staining intensity confirmed the findings in (A). N = 15 in each group. (C) Lyz-promoter-driven EGFP (green) had obvious colocalization (yellow) with neutrophil specific marker Mpo (red in the upper row), but no colocalization with macrophage specific marker MFAP4 (red in the lower row). Scale bar, 200 μm. (D) In the DMSO group (7 dpf ApoEb mutant larvae fed a 2-day HFD supplemented with 0.5% DMSO), neutrophils gathered in large numbers around the trunk large blood vessels (green arrows). Among the high-concentration Xuezhikang treatment group (500 μg/mL, Hi-XZK), the low-concentration Xuezhikang treatment group (18 μg/mL, Lo-XZK) and simvastatin treatment group (SIM), neutrophils were mostly concentrated in the intestine (white arrows) rather than trunk blood vessels. Scale bar, 200 μm. (E) Counts of neutrophils in trunk vessels showed that ≥18 μg/mL Xuezhikang treatment significantly reduced the number of neutrophils recruited, indicating that specific concentrations of XZK have an inhibitory effect on the recruitment of neutrophils. DMSO, 0.5% DMSO; Lo-XZK, 18 μg/mL Xuezhikang; Hi-XZK, 500 μg/mL Xuezhikang; SIM, 0.4 μg/mL simvastatin; n = 12 in each group. (F-G) Both ezetimibe (10 μM) and Xuezhikang (30 μg/mL) treatment effectively reduced the recruitment of macrophages in 10 dpf ApoEb mutants fed with 5-day HFD. N=9 in each group; scale bar, 200 μm. (H) The mRNA expression of IL-1β, IL-6 and TNF-α were measured by rt-qPCR assays in the pooled whole-body homogenates of 10 dpf larvae fed a 5-day HFD supplemented with 0.5% DMSO (DMSO), 30 μg/mL Xuezhikang or 10 μM ezetimibe, respectively. Fifteen larvae pooled per sample, n=6 in each group.

**Supplementary Files**

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