



Human apolipoprotein A-II reduces atherosclerosis in knock-in rabbits

Tomonari Koike^{a,b}, Yui Koike^a, Dongshan Yang^a, Yanhong Guo^a, Oren Rom^a, Jun Song^a, Jie Xu^a, Yajie Chen^b, Yanli Wang^c, Tianqing Zhu^a, Minerva T. Garcia-Barrio^a, Jianglin Fan^{b, **}, Y. Eugene Chen^{a, ***}, Jifeng Zhang^{a, *}

^a Center for Advanced Models for Translational Sciences and Therapeutics, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI, USA

^b Department of Molecular Pathology, Faculty of Medicine, Graduate School of Medical Sciences, University of Yamanashi, Yamanashi, Japan

^c Department of Pathology, Xi'an Medical University, Xi'an, China

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ABSTRACT

Background and aims: Apolipoprotein A-II (apoAII) is the second major apolipoprotein of the high-density lipoprotein (HDL) particle, after apoA1. Unlike apoA1, the biological and physiological functions of apoAII are unclear. We aimed to gain insight into the specific roles of apoAII in lipoprotein metabolism and atherosclerosis using a novel rabbit model.

Methods: Wild-type (WT) rabbits are naturally deficient in apoAII, thus their HDL contains only apoA1. Using TALEN technology, we replaced the endogenous apoA1 in rabbits through knock-in (KI) of human apoAII. The newly generated apoAII KI rabbits were used to study the specific function of apoAII, independent of apoA1.

Results: ApoAII KI rabbits expressed exclusively apoAII without apoA1, as confirmed by RT-PCR and Western blotting. On a standard diet, the KI rabbits exhibited lower plasma triglycerides (TG, 52%, $p < 0.01$) due to accelerated clearance of TG-rich particles and higher lipoprotein lipase activity than the WT littermates. ApoAII KI rabbits also had higher plasma HDL-C (28%, $p < 0.05$) and their HDL was rich in apoE, apoAIV, and apoAV. When fed a cholesterol-rich diet for 16 weeks, apoAII KI rabbits were resistant to diet-induced hypertriglyceridemia and developed significantly less aortic atherosclerosis compared to WT rabbits. HDL isolated from rabbits with apoAII KI had similar cholesterol efflux capacity and anti-inflammatory effects as HDL isolated from the WT rabbits.

Conclusions: ApoAII KI rabbits developed less atherosclerosis than WT rabbits, possibly through increased plasma HDL-C, reduced TG and atherogenic lipoproteins. These results suggest that apoAII may serve as a potential target for the treatment of atherosclerosis.

1. Introduction

High-density lipoprotein (HDL) and apolipoprotein AI (apoAI) [1] are well-known plasma markers that are negatively correlated with atherosclerotic disease [2]. HDL is known for its anti-atherogenic properties through enhancement of reverse cholesterol transport, anti-inflammatory and anti-oxidative effects as well as improved vascular function [2]. The anti-atherogenic properties of HDL are attributed to the biological function of its major apolipoprotein, apoAI. Nevertheless, it remains controversial whether therapeutic elevation of plasma HDL or apoAI can alleviate atherosclerotic disease [3–5]. A

potential explanation for this controversy is that the protein components of the HDL particles are highly heterogeneous as indicated by proteomics studies [6–12]. Furthermore, the biological functions of the major apolipoproteins in the HDL particles are not fully understood.

Apolipoprotein A-II (apoAII) is the second major constitutive protein in the human HDL particle, accounting for 15–20% of the total proteins [13–15]. Unlike apoAI, the biological and physiological functions of apoAII are largely unknown. Epidemiological and genetic studies reported inconsistent results regarding the relationship between apoAII and atherosclerotic disease [16–18]. Studies using transgenic mice and knockout (KO) mice also resulted in conflicting findings indicating that

* Corresponding author.

** Corresponding author.

*** Corresponding author.

E-mail addresses: jianglin@yamanashi.ac.jp (J. Fan), echenum@umich.edu (Y.E. Chen), jifengz@umich.edu (J. Zhang).

apoAII may exert either anti-atherogenic [19,20] or pro-atherogenic effects [21–25]. These contradicting findings may be explained by species differences with regards to the apoAII protein structure, as well as differences in HDL metabolism in humans and mice. Human apoAII forms a homodimer while mouse apoAII exists as a monomer. Furthermore, the kinetics of HDL and LDL in mice is different from humans due to the lack of cholesteryl ester transfer protein (CETP) in mice [26].

To define the functional roles of apoAII, we previously generated transgenic rabbits expressing the human *apoAII* gene and found that apoAII expression substantially affected lipoprotein metabolism and atherosclerosis [27,28]. Wild-type (WT) rabbits are naturally deficient in the *apoAII* gene and their HDL contain only apoAI [27]. This feature allows a unique opportunity to study the specific biological and physiological functions of apoAII using genetically modified rabbits. Indeed, we previously observed an increased cholesterol efflux capacity of HDL isolated from transgenic rabbits with both apoAI and apoAII in comparison to HDL from WT rabbits with only apoAI [27,28]. These findings are consistent with human studies demonstrating that the presence of apoAII in HDL enhances ATP binding cassette subfamily A member 1 (ABCA1)-mediated cholesterol efflux compared to HDL containing only apoAI [29].

In the current study, we aimed to gain insight into the specific roles of apoAII, independent of apoAI. We applied transcription activator-like effector nucleases (TALEN) technology to knock-in (KI) the human *apoAII* coding sequence in rabbits, replacing the rabbit *apoAI*. The newly generated *apoAII* KI rabbits are a unique animal model in which their HDL contains apoAII without apoAI. Comparison of KI rabbits to WT rabbits, in which their HDL contains only apoAI, allowed us to study the specific roles of apoAII in lipoprotein metabolism and atherosclerosis. Using this novel model, we found that replacement of apoAI with apoAII resulted in profound effects on both apoB-containing lipoprotein and HDL metabolism and significantly attenuated cholesterol diet-induced atherosclerosis in rabbits.

2. Materials and methods

Extended methods are described in the [Supplemental Data](#).

2.1. Animals

New Zealand White rabbits (Covance Inc. Princeton, NJ) were used for this study. All rabbits were housed individually in cages under constant 21 °C temperature and 12-h light/dark cycles. Rabbits were fed a standard diet (Teklad global rabbit diet 2030, Envigo, Indianapolis, IN), unless indicated otherwise. All animal studies were performed in accordance with animal protocols (PRO00008268) approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan and complied with the National Institutes of Health (NIH) Guidelines for the care and use of laboratory animals.

2.2. Induction of atherosclerosis with a cholesterol-rich diet

To induce atherosclerosis, we fed the *apoAII* KI and WT rabbits a standard diet supplemented with different amounts of cholesterol for 16 weeks. Since the *apoAII* KI rabbits exhibited an attenuated response to a cholesterol-rich diet (see below), WT rabbits were fed a diet containing 0.3% cholesterol, whereas the *apoAII* KI rabbits were fed a diet containing ~1% cholesterol. This allowed us to maintain comparable hypercholesterolemia in the two groups. The plasma total cholesterol (TC) levels were monitored weekly and each KI rabbit was fed an adjusted amount of cholesterol in the diet to keep their plasma TC similar to that of WT rabbits (Fig. 4A and [Supplemental Figure IVA](#)) [28,30,31].

2.3. Statistical analysis

All data are expressed as mean \pm SEM. Statistical tests performed are

detailed in the figure legends and all analyses were conducted using software GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). Each dataset was first assessed for normality using the Shapiro-Wilk test. If data were found not to follow a normal distribution, a nonparametric test was performed as indicated. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Generation of human *apoAII* knock-in rabbits with TALEN-mediated homologous recombination

The human *apoAII* coding sequence was designed to be knocked in the rabbit *apoAI* locus by TALEN-mediated homologous recombination, as illustrated in Fig. 1A. The TALEN mRNAs to target rabbit *apoAI* and human *apoAII* donor DNA vector were injected into 90 rabbit embryos. The embryos were subsequently implanted into 7 surrogate female rabbits, and generated 20 kits. Among them, two founder rabbits carried the human *apoAII* coding sequences, as determined by PCR and sequencing ([Supplemental Figure IB](#)). After breeding with WT rabbits, we established a human *apoAII* KI rabbit line for the current study as verified by genomic PCR (Fig. 1B). Western blot analysis of HDL isolated from rabbit plasma showed that HDL from homozygous *apoAII* KI rabbits contained only human apoAII without the rabbit apoAI, HDL from the heterozygous *apoAII* KI rabbits contained both rabbit apoAI and human apoAII and that from WT rabbits contained only rabbit apoAI (Fig. 1C). *ApoAII* KI rabbits showed no apparent differences in body weight, and necropsy examination revealed no abnormalities in the heart, kidneys, liver, lungs and other organs (data not shown).

3.2. Plasma lipid and lipoprotein profiles of *apoAII* knock-in rabbits fed a standard diet

On a standard diet, homozygous (but not heterozygous) 4–6 month-old *apoAII* KI rabbits showed higher plasma TC (1.3-fold increase over WT, $p < 0.05$), but significantly lower TG (52% reduction, $p < 0.01$) compared with the WT littermates, with no sex differences observed. Plasma phospholipids were comparable between genotypes (Fig. 1D). Next, we compared the plasma lipoprotein profile of homozygous *apoAII* KI rabbits with that of WT rabbits, including both male and female rabbits. Agarose gel electrophoresis of lipoprotein fractions revealed that pre- β migrating lipoproteins (VLDL) were prominently decreased whereas α -migrating lipoproteins (HDL₁₋₂) were increased in *apoAII* KI rabbits compared to WT rabbits (Fig. 2A). These findings were confirmed by quantifying the TC content in each density fraction, which indicated a significant increase in HDL₁ and HDL₂ ($d = 1.06$ – 1.08 and 1.08 – 1.10 g/mL) but a reduction in the apoB-containing particles in the *apoAII* KI rabbits. The reduced apoB protein contents in the plasma of *apoAII* KI rabbits was also identified by proteomics assay ([Supplemental Figure II](#)). The TC content in the VLDL and IDL fractions was reduced by 81% and 71%, respectively. The TC content in the HDL₁, HDL₂ (1) and HDL₂ (2) fractions was increased by 3.2-fold, 3.2-fold and 2.1-fold, respectively (Fig. 2B, upper panel). Quantification of the TG content in each fraction revealed a significant reduction in the majority of lipoproteins isolated from *apoAII* KI rabbits compared to WT rabbits, particularly in the TG-rich lipoproteins (VLDL and IDL fractions in $d < 1.006$ and $d = 1.006$ – 1.02 g/mL, respectively) and the HDL fractions. The TG content in all fractions except for sLDL/HDL₁ was significantly reduced by 76%, 59%, 58%, 32%, 42% and 75% in the VLDL, IDL, LDL, HDL₂ (1), HDL₂ (2) and HDL₃ fractions, respectively (Fig. 2B, lower panel). We further analyzed the apolipoprotein composition in each fraction using SDS-PAGE and Western blotting. We confirmed that rabbit apoAI was replaced by human apoAII in the HDL fractions isolated from *apoAII* KI rabbits, which was accompanied by several remarkable changes in the HDL and apoB-containing particles. Unlike the WT rabbits, the HDL₂₋₃ ($d = 1.06$ – 1.21 g/mL) isolated from *apoAII* KI

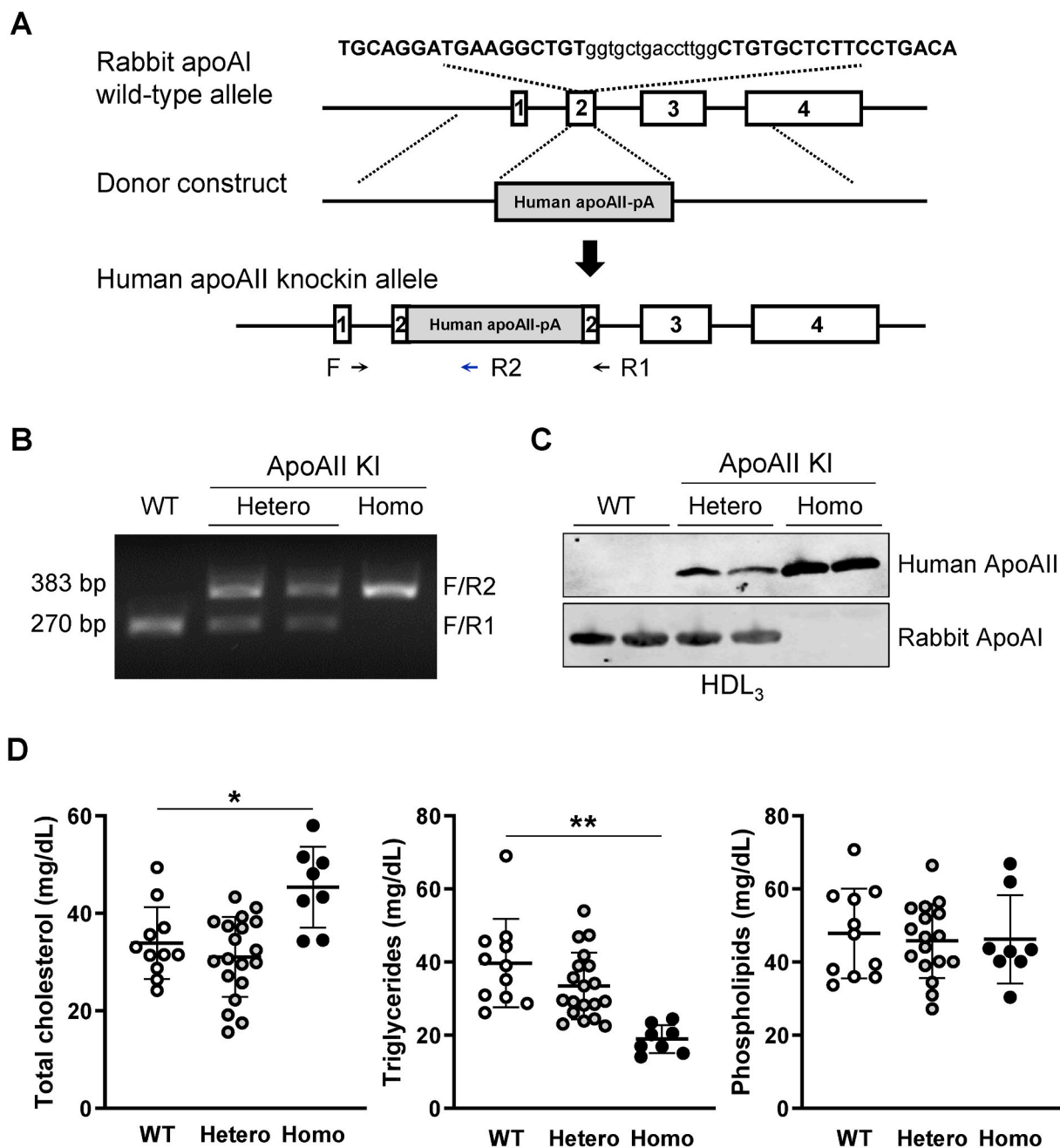


Fig. 1. Generation of human apolipoprotein *apoAII* knock-in rabbits by TALEN genome editing.

(A) Strategy for knocking-in the human *apoAII* coding sequence into the rabbit *apoAI* locus. The rabbit *apoAI* gene structure, donor vector and *apoAI* locus after knock-in are shown in the scheme. The sequence shown at the very top is the TALEN targeting sequence. (B) Electrophoresis result showing the PCR products of rabbit genotyping. The location of the genotyping primers is shown at the bottom of panel A, which results in a 270bp and 383bp amplicon from WT and homozygous *apoAII* knock-in allele, respectively. (C) Rabbit apoAI and human apoAII proteins detected by Western blot of the HDL₃ fraction using antibodies against rabbit apoAI and human apoAII. 4 μ l of the HDL₃ fraction was loaded into each lane. (D) Total cholesterol (TC, n = 8–19 in each group, 5–12 male and 3–7 female rabbits), triglycerides (TG, n = 8–19 in each group, 5–12 male and 3–7 female rabbits) and phospholipids (n = 8–17 in each group, 5–10 male and 3–7 female rabbits) in the plasma from 4 month-old WT and *apoAII* KI rabbits. Plasma samples were collected from both male and female rabbits.

rabbits was rich in apoE. Also, the apoB-100 content in the small LDL (d = 1.04–1.08 g/mL) was increased in *apoAII* KI rabbits (Fig. 2C). Furthermore, Western blot analysis of the HDL₃ fraction revealed a larger size apoE in *apoAII* KI rabbits. ApoAIV and apoAV were detected only in HDL₃ isolated from the *apoAII* KI rabbits (Fig. 2D).

3.3. Catabolism of triglyceride-rich lipoproteins in *apoAII* knock-in rabbits

We next sought to explore potential mechanisms underlying the reduction in TG-rich lipoproteins observed in the *apoAII* KI rabbits. First, we performed a lipid clearance test by intravenous lipid loading. As shown in Fig. 3A, the rate of TG clearance from the circulation was significantly accelerated in the *apoAII* KI rabbits compared to the WT rabbits. In addition, post-heparin lipoprotein lipase (LPL) activity was

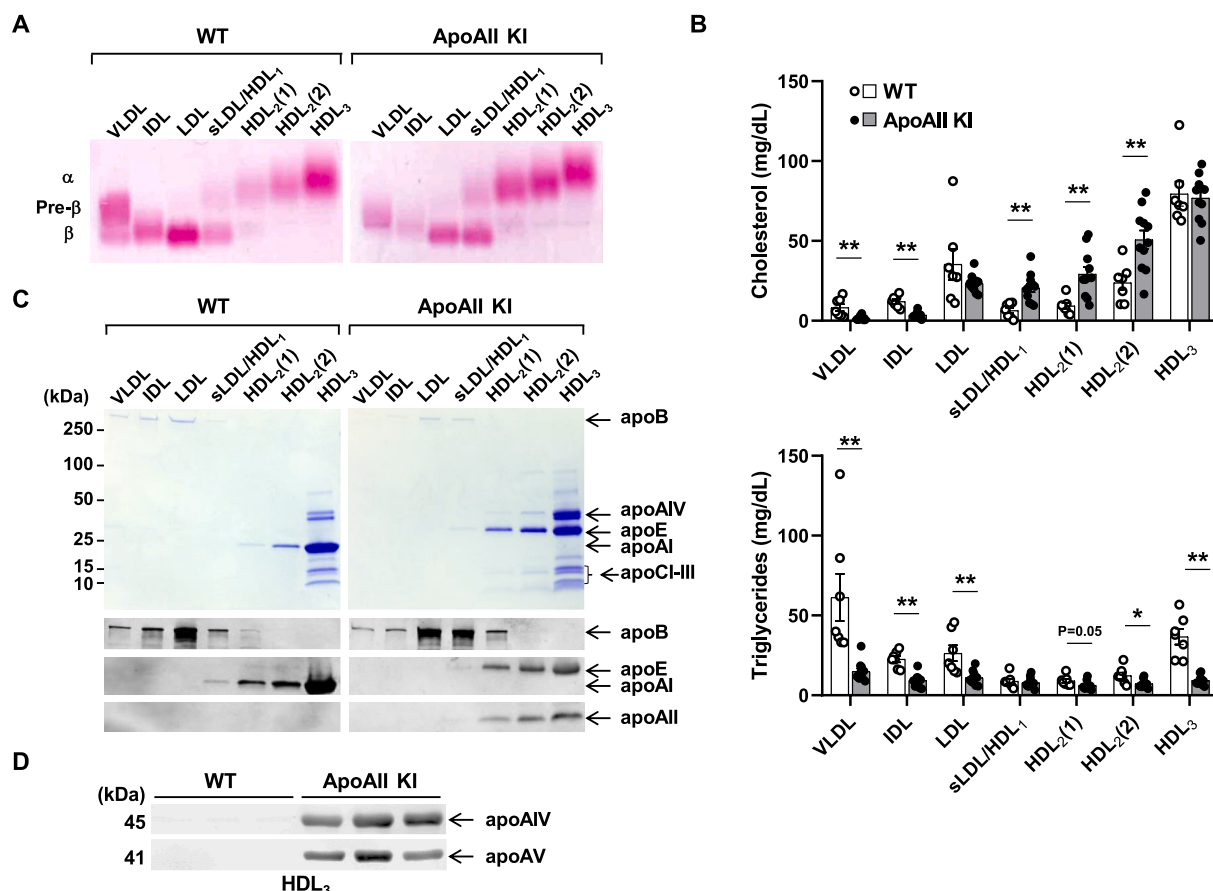


Fig. 2. Plasma lipid and lipoprotein profiles of *apoAII* knock-in and WT rabbits on a standard diet.

(A) Representative agarose gel electrophoresis of lipoproteins stained with Fat red 7B. An equal volume of each fraction was resolved by electrophoresis in a 1% agarose gel. α and β indicate electrophoretic mobility. (B) Quantification of cholesterol and triglyceride content in lipoproteins. $n = 7$ –11 per group (wt: 3 males and 4 females; *apoAII* KI: 6 males and 5 females), 4–5 month-old rabbits. Each fraction corresponds to the densities of <1.006 (VLDL), 1.006–1.02 (IDL), 1.02–1.04 (LDL), 1.04–1.06 (small LDL and HDL₁), 1.06–1.08 [HDL₂(1)], 1.08–1.10 [HDL₂(2)], 1.10–1.21 (HDL₃) g/ml. (C) SDS-PAGE gel (upper panel) and immunoblot using apoB, apoE, apoAI and apoAII antibodies (bottom panel). Lipoproteins were isolated from WT and *apoAII* KI rabbits by sequential density ultracentrifugation according to the density ranges shown above the gels. (D) ApoAIV and apoAV were detected in the HDL₃ fraction from WT rabbits and *apoAII* KI rabbits by Western blot. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with WT group. Statistical analyses were performed using ANOVA for comparison among 3 groups and 2-tailed, unpaired Student's *t*-test between 2 groups.

about 2-fold higher in KI rabbits than WT rabbits (Fig. 3B), suggesting that apoAII may increase LPL activity, which in turn enhances the catabolism of TG-rich particles. To examine the effects of *apoAII* KI on VLDL metabolism, we fed the KI rabbits a diet containing 3% soybean oil and 1% cholesterol. We found that the TG-rich lipoproteins in the *apoAII* KI rabbits remained low even after feeding the rabbits a cholesterol-rich diet for 6 h (Supplemental Figure IIIA and B). Both the TG and TC contents in the VLDL fractions ($d < 1.006$ g/mL) isolated from the KI rabbits were significantly lower compared with the WT rabbits (Fig. 3C). In addition to plasma lipids, the abundance of both apoB100 and apoB48 in the VLDL fractions at the postprandial state was lower in *apoAII* KI rabbits compared with WT rabbits, suggesting that *apoAII* KI rabbits are less responsive to a cholesterol-rich diet (Fig. 3D). This notion was further confirmed by feeding the cholesterol-rich diet for a longer period. After one week on cholesterol-rich diet, plasma TC was consistently lower in *apoAII* KI rabbits compared to WT rabbits (Fig. 3E). The hepatic expression of microsomal triglyceride transfer protein (MTTP) was examined by quantitative real time PCR (data not shown) and Western blotting (Supplemental Figure IIIC) and was not significantly different between the two groups, excluding the possibility that lower VLDL levels in *apoAII* KI rabbits were caused by reduced hepatic VLDL synthesis.

3.4. Lipid metabolism in *apoAII* knock-in rabbits after prolonged feeding with a cholesterol-rich diet

To investigate the role of apoAII in the development of atherosclerosis, we fed the rabbits a cholesterol-rich diet for 16 weeks. Since hypercholesterolemia is the major determinant for the lesion size, we kept the *apoAII* KI rabbits with similar hypercholesterolemia as the WT rabbits by adjusting the dietary cholesterol content as described in the Materials and methods section. This approach allowed us to examine the effects of *apoAII* KI on atherosclerosis development without the confounding effect of different TC levels. At similar hypercholesterolemia (Fig. 4A and Supplemental Figure IVB, left panel), we found considerable differences in the levels of plasma TG and cholesterol distribution to lipoproteins between the two genotypes. Although the plasma TC was maintained at similar levels, the TG levels were consistently lower in the KI rabbits (Fig. 4B and Supplemental Figure IVB, right panel). In WT rabbits, cholesterol was mainly carried by the more atherogenic apoB-containing particles (β -VLDL and LDL, $d < 1.006$ –1.04 g/mL), whereas in *apoAII* KI rabbits, the cholesterol content in all HDL fractions was increased (Fig. 4C and Supplemental Figure II). Trends towards reduced TG content in all the apoB-containing particles were found, without reaching statistical significance due to the limited sample size and large variation. The relative large variation in lipid contents in each lipoprotein, especially for VLDL and IDL, may be partially caused by the

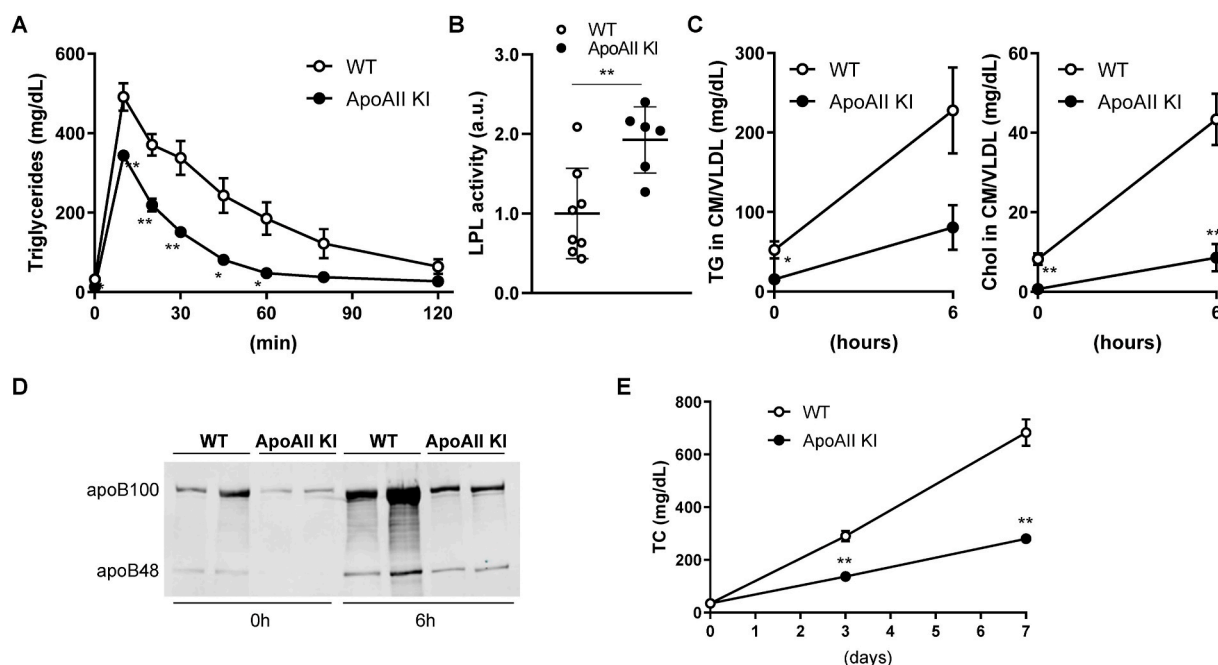


Fig. 3. Increased lipid clearance in *apoAII* knock-in rabbits.

(A) Rabbits were fasted for 16 h followed by a lipid clearance test performed by intravenous injection of intralipid emulsion (2 mL/kg). The blood was collected sequentially after the injection at the indicated times, and TG levels in each blood sample were quantified. $n = 4$ for each group (2 male and 2 female rabbits). (B) Plasma post-heparin lipoprotein lipase (LPL) activity was measured with an LPL activity assay kit. $n = 8$ (5 male and 3 female) in WT and $n = 6$ (4 male and 2 female) in KI rabbits group. (C) TC and TG levels in the TG-rich lipoprotein fraction (density; < 1.006 g/mL) isolated by ultracentrifugation before and 6 h after feeding a 1% cholesterol diet. $n = 7$ in WT (4 male and 3 female) and $n = 5$ (3 male and 2 female) in KI rabbits group. (D) Western blot analysis of apoB100 and apoB48 protein in the TG-rich lipoprotein fraction before and after 6 h of feeding a 1% cholesterol diet. (E) Plasma TC monitored for one week after feeding a cholesterol rich diet (1% cholesterol). $n = 8$ (5 male and 3 female) in WT and $n = 5$ (3 male and 2 female) in KI rabbits group. Data are expressed as mean \pm SEM. $*p < 0.05$, $**p < 0.01$ compared with WT. Statistical analyses were performed using 2-tailed, unpaired Student's *t*-test.

recovery rate of samples after ultracentrifugation. In addition, the rabbit is not an inbred animal, which also contributes to the variation in plasma lipid contents among rabbits. No significant differences in TG content in HDLs were found between the genotypes (Fig. 4D). Although the plasma TC levels were maintained at similar levels between WT and *apoAII* KI rabbits, the HDL-C levels were higher in *apoAII* KI rabbits (Fig. 4C right pane). We also measured the CETP activity in rabbits fed the cholesterol-rich diet. In line with increased HDL-C, plasma CETP activity in *apoAII* KI rabbits was significantly lower than in WT rabbits (Fig. 4E).

3.5. *In vitro* functional analyses of HDL from *apoAII* knock-in rabbits

To explore functional differences in HDL from WT rabbits and *apoAII* KI rabbits, we compared their cholesterol efflux capacity and anti-inflammatory effects *in vitro*. As shown in Supplemental Figure VA, no significant differences in HDL-mediated cholesterol efflux were found between the two genotypes using either apoB-depleted serum as HDL or using HDL₃. Furthermore, HDL isolated from WT and *apoAII* KI rabbits exhibited a similar anti-inflammatory response, and suppressed tumor necrosis factor alpha (TNF α)-induced overexpression of pro-inflammatory genes (Supplemental Figure VB). These results indicate that apoAII in HDL isolated from *apoAII* KI rabbits has a similar function as apoAI in HDL in WT rabbits.

3.6. Atherosclerosis in *apoAII* KI rabbits after feeding a cholesterol-rich diet

We next analyzed the aortic atherosclerotic lesions in WT and *apoAII* KI rabbits fed a cholesterol-rich diet for after 16 weeks. *En face* analysis of aortic lesions visualized by Sudan IV staining revealed that the aortic lesions of *apoAII* KI rabbits were significantly less in the thoracic (60% decrease), abdominal (52% decrease) and total (47% decrease) aortic

area than those of WT littermates (Fig. 5A and B). Pathologic analysis of aortic atherosclerosis showed that the proportions of lesion-associated macrophages and smooth muscle cells were not significantly different between WT and *apoAII* KI rabbits (Fig. 5C and Supplemental Figure VI).

4. Discussion

In the current study, we generated *apoAII* KI rabbits in which their HDL contains the human apoAII instead of the rabbit apoAI. This novel rabbit model allowed us to investigate the specific roles of apoAII in lipoprotein metabolism and atherosclerosis, independent of apoAI. The *apoAII* KI rabbits presented remarkable differences in plasma lipid profile compared to WT rabbits including increased HDL, reduced TG and a marked reduction in pro-atherogenic lipoproteins. Furthermore, a significant increase in LPL activity and a decrease in CETP activity were found in *apoAII* KI rabbits. In line, the *apoAII* KI rabbits showed substantially lower atherosclerotic burden than the WT rabbits, albeit with no significant differences in the cellular compositions of the plaques.

Replacement of apoAI with apoAII in the KI rabbits resulted in several prominent changes in HDLs. First, compared with apoAI-HDLs, apoAII-HDL particles were rich in apoE along with apoAIV and apoAV, which are known to play important roles in the metabolism of TG-rich lipoproteins. However, replacement of apoAI with apoAII did not alter the HDL function as cholesterol efflux capacity and anti-inflammatory effects were similar in HDL isolated from *apoAII* KI rabbits and WT rabbits. These findings support the notion that apoAII is equally important as apoAI with regards to HDL function. It is noteworthy that the plasma levels of HDL-C were higher in *apoAII* KI rabbits than WT rabbits, which may explain the observed anti-atherogenic effects.

Another important feature of the *apoAII* KI rabbits is a significant reduction in plasma TG both on a standard diet and on a cholesterol-rich

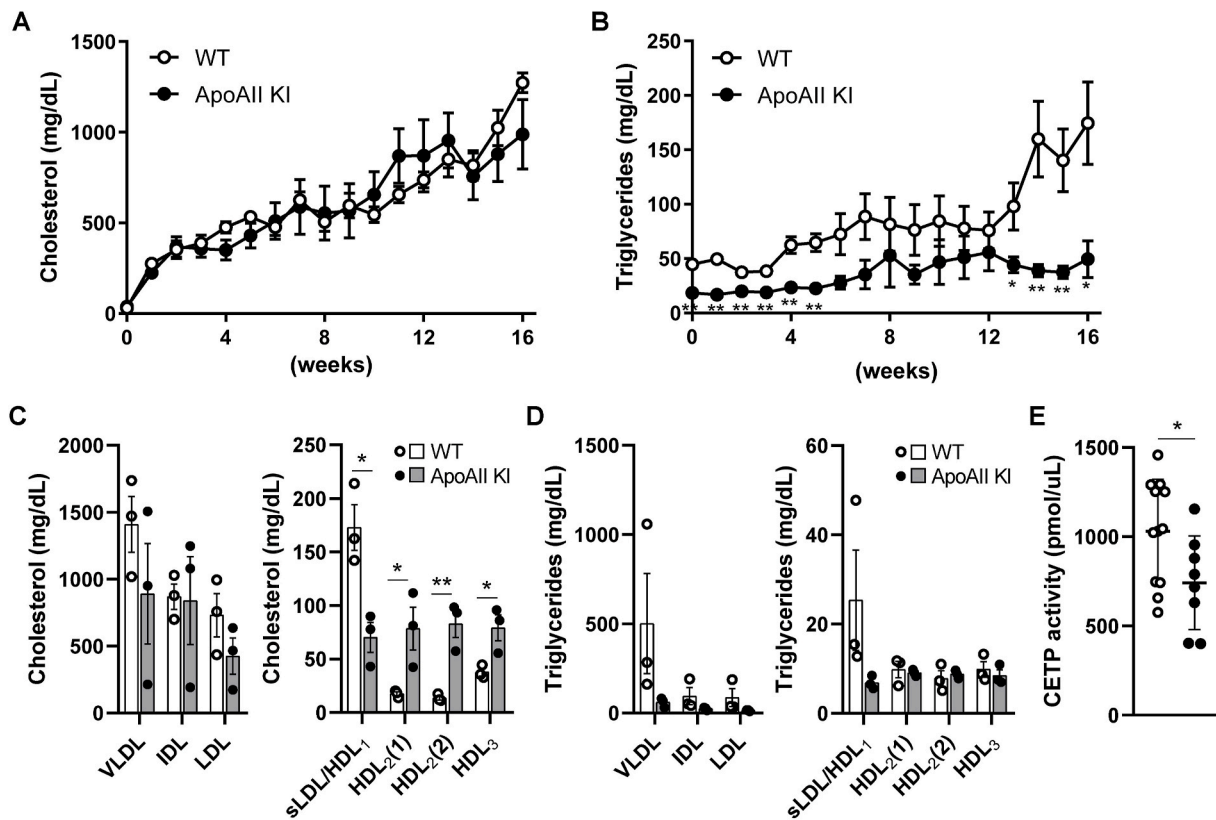


Fig. 4. Plasma lipid profiles of WT and knock-in rabbits fed a cholesterol-rich diet.

Plasma TC (A) and TG (B) levels monitored during the 16 weeks of cholesterol-rich diet treatment. TC in WT and *apoAII* KI rabbits was maintained at similar levels by adjusting the cholesterol content of the diet. $n = 11$ (8 male and 3 female) in WT and $n = 8$ (5 male and 3 female) in KI rabbits group. Quantification of TC (C) and TG (D) content in each lipoprotein fraction. Lipoproteins were isolated from rabbits at 12 weeks on the cholesterol-rich diet. $n = 3$ (male) for each group. (E) Plasma CETP activity was measured after feeding the cholesterol-rich diet for 16 weeks. $n = 12$ (7 male and 5 female) in WT and $n = 8$ (5 male and 3 female) in KI rabbits group. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ compared with WT rabbits. Statistical analyses were performed using 2-tailed, unpaired Student's *t*-test.

diet. These findings indicate that apoAII has profound effects on the metabolism of apoB-containing particles. The lower TG levels found in the KI rabbits suggest that apoAII, and/or apoAIV, apoAV, and apoE, which were increased in their HDL particles, accelerate the catabolism of VLDL in the circulation. This notion was supported by our findings in which *apoAII* KI rabbits showed accelerated clearance of TG and were resistant to hypertriglyceridemia induced by a cholesterol-rich diet. Importantly, these effects were independent of altered VLDL production in the liver since the hepatic expression of MMTP was comparable between *apoAII* KI and WT rabbits.

In plasma from *apoAII* KI rabbits, CETP activity was significantly lower than that of WT rabbits. This finding is in agreement with previous reports indicating that CETP can bind to apoAI, which extends the half-life of CETP in the plasma as compared to unbound CETP [32,33]. The TG-lowering effects of apoAII can also be explained by enhanced LPL activity that was observed in *apoAII* KI rabbits (2-fold increase over the control). In our previous studies using transgenic rabbits expressing human apoAII together with apoAI, LPL activity was found to be lower [26,27]. In the current study, replacing apoAI with apoAII in the KI rabbits enhanced LPL activity. Our findings, together with previous reports demonstrating that apoAI itself can inhibit LPL activity [34], suggest that apoAII may act as an activator of LPL, although this remains to be verified. The enhanced LPL activity and reduction in plasma TG found in the *apoAII* KI rabbits can also be explained by increased abundance of apoAIV and apoAV in their HDL fractions. ApoAIV is known to enhance the activity of LPL [35], and apoAV lowers plasma TG by enhancing TG clearance through binding to LPL and/or heparin sulfate proteoglycans and interaction with LDL receptors [36–38].

Although we did not find an increase in abundance of apoAIV, apoAV, and apoE in TG-rich lipoproteins, apolipoproteins in HDL particles can interact with apoB in LDL through bridging molecules like CETP.

With regards to lipoprotein metabolism and atherogenicity, our findings from studying the *apoAII* KI rabbit model indicate an overall beneficial effect of apoAII. To examine the role of apoAII on atherogenesis, we fed KI and WT rabbits a cholesterol-rich diet. To investigate the effects on atherosclerosis without the confounding effects of differences in hypercholesterolemia, we fed the rabbits diets with different amount of cholesterol and maintained similar levels of plasma TC throughout the study. Importantly, we found that the whole aortic lesion area was significantly lower in *apoAII* KI rabbit than WT littermates, even when maintaining similar hypercholesterolemia. The findings of the current work, together with our previous studies in apoAII transgenic rabbits [28], clearly indicate the potent anti-atherogenic effects of apoAII in the presence or absence of apoAI.

The anti-atherogenic effects of apoAII may be mediated by multiple mechanisms that are independent of plasma cholesterol levels. First, as we found in *apoAII* KI rabbits, apoAII expression reduced plasma TG and pro-atherogenic TG-rich lipoproteins including β -VLDLs, IDLs and LDLs through enhancing LPL activity, the latter is also known to reduce atherosclerosis in rabbits [39]. Second, although the cholesterol efflux capacity and anti-inflammatory effects of HDL isolated from *apoAII* KI and WT rabbits were comparable, a net increase in HDL particles was found in *apoAII* KI rabbits. HDL is known to display anti-oxidant activities [40], maintain endothelial integrity [41], regulate immunity and T cell functions [42,43], that may further contribute to the atheroprotective effects observed in *apoAII* KI rabbits. Although *apoAII* KI

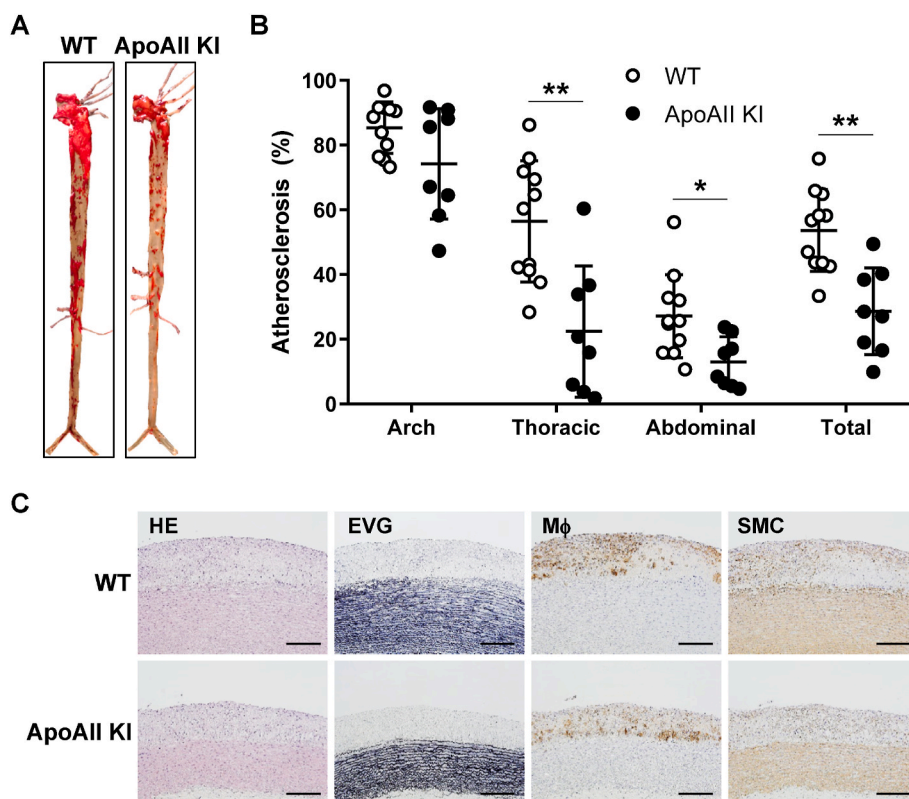


Fig. 5. Aortic atherosclerotic lesions after 16 weeks of cholesterol-rich diet feeding.

(A) Representative images of aortic atherosclerosis from WT and *apoAII* KI rabbits stained with Sudan IV. (B) The lesion area (defined by sudanophilic staining as red) was quantified using an image analysis system. Each dot represents the lesion area of an individual rabbit. The horizontal bar represents the mean value for each group. $n = 11$ (8 male and 3 female) in WT and $n = 8$ (5 male and 3 female) in KI rabbits group. $**p < 0.01$ or $*p < 0.05$ compared with WT. Statistical analyses were performed using 2-tailed, unpaired Student's *t*-test. (C) Representative micrographs of the aortic arch lesions from WT and *apoAII* KI rabbits. Serial sections of paraffin embedded specimens were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically using monoclonal antibodies against macrophages (M ϕ) or α -smooth muscle actin for smooth muscle cells (SMCs). The lesions were characterized by intimal accumulation of macrophage-derived foam cells intermingled with smooth muscle cells. Scale bars represent 200 μ m.

rabbits exhibited lower atherosclerosis, it should be noted that rabbits have low activity of hepatic lipase [44,45], which may affect the function of apoAII observed in KI rabbits. The interaction between apoAII and hepatic lipase could be addressed in future studies by crossbreeding *apoAII* KI rabbits with hepatic lipase transgenic rabbits.

Whereas the whole aortic lesion area was significantly lower in *apoAII* KI rabbits, the presence of lesion-associated macrophages and smooth muscle cells was similar to comparable to WT rabbits. These findings are consistent with our previous findings using human apoAII transgenic rabbits and suggest that apoAII does not directly influence the lesion morphological and cellular characteristics rather the overall atherosclerotic burden [28]. Our findings suggest a therapeutic potential for HDL-associated apoAII against atherosclerotic disease; however, the beneficial roles of HDL in clinical settings remain controversial. A recent report shows that extremely high HDL-C levels in humans were found to increase all-cause mortality [46]. Furthermore, to date, there is no pharmaceutical treatment for cardiovascular diseases that is based on HDL, apoAI or apoAII [3–5]. Moreover, genetic variants associated with increased [47] or decreased [48] plasma HDL-cholesterol did not affect the risk of cardiovascular diseases. These observations highlight the considerable discrepancies between humans and animal models and emphasize the need for further studies to define the role of HDL and that of apoAII in physiological and pathophysiological conditions.

In conclusion, using newly generated *apoAII* KI rabbits, we uncovered the specific roles of human apoAII in lipoprotein metabolism and atherosclerosis. ApoAII plays multiple roles in modulating apoB-containing and HDL particles and reduces atherosclerosis in rabbits. Our findings suggest that apoAII may serve as a novel therapeutic target for the treatment of atherosclerosis.

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CRediT authorship contribution statement

Tomonari Koike: Formal analysis, Data curation, Writing - original draft, performed experiments, collected, analyzed and interpreted the data, wrote the manuscript. **Yui Koike:** Formal analysis, Data curation, Writing - original draft, performed experiments, collected, analyzed and interpreted the data. **Dongshan Yang:** generated the apoA2 KI rabbits. **Yanhong Guo:** Formal analysis, Data curation, Writing - original draft, performed experiments, collected, analyzed and interpreted the data. **Oren Rom:** Writing - review & editing, did critical editing and discussed results. **Jun Song:** generated the apoA2 KI rabbits. **Jie Xu:** generated the apoA2 KI rabbits. **Yajie Chen:** Formal analysis, Data curation, Writing - original draft, performed experiments, collected, analyzed and interpreted the data. **Tianqing Zhu:** Formal analysis, Data curation, Writing - original draft, performed experiments, collected, analyzed and interpreted the data. **Minerva T. Garcia-Barrio:** Writing - review & editing, did critical editing and discussed results. **Jianglin Fan:** Writing - review & editing, designed the study, did critical editing and discussed results. **Y. Eugene Chen:** Writing - review & editing, designed the study, did critical editing and discussed results. **Jifeng Zhang:** Writing - original draft, designed the study, generated the apoA2 KI rabbits, wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2020.11.028>.

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