Proprotein Convertase Subtilisin/Kexin Type 9 Interacts With Apolipoprotein B and Prevents Its Intracellular Degradation, Irrespective of the Low-Density Lipoprotein Receptor

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Objective—Proprotein convertase subtilisin/kexin type 9 (PCSK9) negatively regulates the low-density lipoprotein (LDL) receptor (LDLR) in hepatocytes and therefore plays an important role in controlling circulating levels of LDL-cholesterol. To date, the relationship between PCSK9 and metabolism of apolipoprotein B (apoB), the structural protein of LDL, has been controversial and remains to be clarified.

Methods and Results—We assessed the impact of PCSK9 overexpression (~400-fold above baseline) on apoB synthesis and secretion in 3 mouse models: wild-type C57BL/6 mice and LDLR-null mice (Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>Apoeb<sup>−/−</sup>). Irrespective of LDLR expression, mice transduced with the PCSK9 gene invariably exhibited increased levels of plasma cholesterol, triacylglycerol, and apoB. Consistent with these findings, the levels of very-low-density lipoprotein and LDL were also increased whereas high-density lipoprotein levels were unchanged. Importantly, we demonstrated that endogenous PCSK9 interacted with apoB in hepatocytes. The PCSK9/apoB interaction resulted in increased production of apoB, possibly through the inhibition of intracellular apoB degradation via the autophagosome/lysosome pathway.

Conclusion—We propose a new role for PCSK9 that involves shuttling between apoB and LDLR. The present study thus provides new insights into the action of PCSK9 in regulating apoB metabolism. Furthermore, our results indicate that targeting PCSK9 expression represents a new paradigm in therapeutic intervention against hyperlipidemia. (Arterioscler Thromb Vasc Biol. 2012;32:00–00.)

Key Words: apolipoprotein B □ autophagy □ hyperlipidemia □ low-density lipoprotein receptor □ proprotein convertase subtilisin/kexin type 9

In 2003, positional cloning identified dominant point mutations (S127R and F216L) of proprotein convertase subtilisin/kexin type 9 (PCSK9) in French families with familial hypercholesterolemia. This finding has led to intense investigation of the role played by PCSK9 in the regulation of lipoprotein metabolism. PCSK9 has emerged as a novel therapeutic target for the treatment of dyslipidemia.

PCSK9, a 692-amino-acid–long glycoprotein, is synthesized in the endoplasmic reticulum as a precursor that undergoes autocatalytic processing in which the propeptide (14 kDa) is cleaved at the N terminus. The propeptide remains attached noncovalently to the mature peptide after being secreted into circulation. Once in circulation, PCSK9 binds to the low-density lipoprotein (LDL) receptor (LDLR) on the cell surface and is further internalized via clathrin-coated pits. The LDLR/PCSK9 complex traffics through the endosome to the lysosome for degradation, resulting in reduced LDLR activity and increased levels of plasma LDL-cholesterol. A gain-of-function mutation of PCSK9 (D374Y) enhances binding of PCSK9 to the LDLR and thus strongly promotes LDLR degradation compared with wild-type PCSK9. The finding that PCSK9 negatively regulates LDLR levels has led to numerous attempts to interfere with the LDLR/PCSK9 interaction through pharmacological means.

Although it is well established that PCSK9 negatively regulates LDLR levels, the impact of PCSK9 on apolipoprotein B (apoB) production remains controversial and poorly understood. ApoB is the structural protein of LDL, which is removed from circulation via LDLR. Thus, the regulation of LDLR by PCSK9 might alter apoB levels. Clinically, increased apoB concentration is associated with coronary artery disease. Thus, the question of whether PCSK9 plays a role in apoB production is important.

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The production of apoB is regulated at the posttranscrip-
tional level. It has been shown that substantial amounts of
newly synthesized apoB are degraded intracellularly. The
newly synthesized apoB can be degraded either through
ubiquitin-mediated proteasomal degradation or by propro-
tosomal degradation, the latter of which involves autophag-
osomes. Autophagosomes are membrane structures that
cloace intracellular substrates and deliver them to lysosomes
for disposal, in a process termed autophagy. The apoB pro-
tein has been shown to accumulate in this structure, which is
in close proximity to cytosolic lipid droplets. It is suggested
that autophagy may represent an alternative pathway for the
disposal of apoB. The present study was designed to investig-
ate the effect of PCSK9 expression on apoB production and
whether PCSK9 regulates apoB levels via autophagy.

We used a second-generation adenovirus vector to achieve
long-term (30 days) expression of PCSK9 in mice with or with-
out the LDLR, including C57BL/6, LDLR-deficient (Ldlr−/−), and
LDb (Ldlr−/−Apobec1−/−) mice. Teng et al. as well as others,
developed the LDb mouse model by deleting the genes encoding
the LDLR and the apoB mRNA editing enzyme (Apobec1). In contrast to wild-type mice or
Ldlr−/− mice, the phenotype of the LDb mice closely mimics
humans with hyperlipidemia characterized by the secretion of
apoB100-containing lipoproteins only, with increased plasma
levels of LDL-cholesterol and decreased levels of high-den-
sity lipoprotein (HDL)-cholesterol. In addition, these LDb
mice spontaneously develop severe atherosclerotic lesions,
even when fed on a normal chow diet. Thus, LDb mice are
distinctly different from Ldlr−/− mice, despite both lacking the
LDLR. In the present study, we showed that PCSK9 regulates
the synthesis and secretion of apoB in an LDLR-independent
fashion. Moreover, our results have provided novel evidence
for a physical interaction between PCSK9 and apoB: The
PCSK9/ apoB interaction inhibits intracellular degradation of
apoB via the autophagosome/lysosome pathway, and this, in
turn, results in increased secretion of apoB-containing lip-
proteins and increased levels of cholesterol and triacylglycerol
(TAG).

Materials and Methods

Animal Studies

C57BL/6J, Pcsk9−/−, and Ldlr−/− mice were purchased from Jackson
Laboratory (Bar Harbor, ME). LDb mice were generated in Teng’s
laboratory, as described previously. Ldlr−/−Apobec1−/−Pcsk9−/−
triple knockout mice were generated by crossing Pcsk9−/− with LDb
mice and confirming the genotype by polymerase chain reaction.
Mice were kept in a barrier facility with a 12:12 light–dark cycle,
and maintained on a standard laboratory chow diet. All animal
experiments were conducted in accordance with the Guidelines of
the Animal Protocol Review Committee of the University of Texas
Health Science Center at Houston.

The E1-, E2b-, E3-deleted adenovirus type 5 vector pAd-del-pol,
adeno-viral-cytomegalovirus shuttle vector pD2007MCS, and E.C7
cell line were obtained from Euthetics Corporation (Seattle, WA) to
produce the adenovirus Pcsk9 (Ad-Pcsk9) vector. The plasmid
vector Pcsk9 cDNA containing a FLAG tag was obtained from the
laboratory of Dr Jay D. Horton (University of Texas Southwestern
Medical Center, Dallas, TX). The detailed construction and produc-
tion of Ad-Pcsk9 is described in Methods in the online-only Data
Supplement.

Male C57BL/6J, Ldlr−/−, and LDb mice (n=24 for each group) aged
2 months were transduced with Ad-PCSK9 or Ad-null virus (1×1010
viral particles per mouse) via tail vein injection. Fasting (~16 hours)
blood samples were collected pre- (day 0) and postadenovirus injec-
tion on days 7, 14, 21, and 30 via retro-orbital plexus by using a
heparin-coated capillary tube (Fisher Scientific, Pittsburgh, PA). The
liver and other tissues were collected, snap-frozen in liquid nitrogen,
and stored at −80°C. Detailed lipid analyses, Western blotting analy-
sis, real-time quantitative polymerase chain reaction, and ELISA
for mouse PCSK9 and human PCSK9, reagents, and antibodies are
described in Methods in the online-only Data Supplement.
GraphPad Prism software (version 5) unpaired t tests with 2-tailed P values. A P<0.05 was considered to be statistically significant.

Results

Overexpression of PCSK9 Increases Plasma Cholesterol and TAG Levels in an LDLR-Independent Fashion

To assess the effects of PCSK9 on apoB and lipoprotein metabolism, we expressed a FLAG-tagged human PCSK9 (Ad-PCSK9) by using an E2b-deleted second-generation adenovirus vector that exhibits long-term gene expression and low toxicity.24,25 We transduced control virus Ad-null and Ad-PCSK9 to wild-type C57BL/6J mice and Ldlr−/− mice and LDb. As shown in Figure 1A, the levels of overexpressed PCSK9 proteins were similar (~400-fold above the baseline) in all 3 mouse models studied, and the levels remained elevated 30 days after treatment. Furthermore, as expected, the expressed recombinant PCSK9 protein was biologically active; the expression of PCSK9 in C57BL/6 mice markedly decreased LDLR protein levels in the liver (Figure 1A).

Most of the endogenous Pcsk9 mRNA and protein were expressed in the liver, with some expressed in the small intestine also (Figure 1B). This is similar to the findings of previous studies.26-27 After Ad-PCSK9 transduction, >95% of the adenovirus-mediated PCSK9 gene was expressed in the liver, with minute amounts expressed in other organs such as the spleen, small intestine, heart, and kidney (Figure 1B).

PCSK9 expression resulted in highly significant increased levels of plasma cholesterol and TAG in all 3 mouse models (Figure 2A; Table I in the online-only Data Supplement). The effect of PCSK9 expression on plasma cholesterol and TAG was much greater in C57BL/6 mice than that in the LDLR-null mice (Ldlr−/− and LDb). In C57BL/6 mice, the levels of plasma cholesterol and TAG were increased to an average of 3.7-fold and 8.5-fold, respectively, on PCSK9 expression compared with Ad-null. The increase in the levels of plasma cholesterol and TAG in Ldb−/− mice were 1.7-fold and 3.4-fold, respectively, and 1.5-fold and 1.5-fold, respectively, in LDb mice on PCSK9 expression. The smaller increase in plasma cholesterol and TAG levels on PCSK9 expression in LDLR-null mice was attributable to elevated baseline levels of the lipids in these animals. It was also noted that the increase in plasma TAG was much greater than that observed in plasma cholesterol on PCSK9 expression, suggesting the increased very-low-density lipoprotein (VLDL) secretion. The present results showing an effect of PCSK9 expression on
plasma lipid levels in C57BL/6 mice are in accord with previous observations.\textsuperscript{20,28–31} The effect of PCSK9 expression on plasma lipids in the LDLR-null mouse models is in agreement with results reported by Benjannet et al\textsuperscript{28} showing increased plasma lipid levels in \textit{Ldlr} \textsuperscript{−/−} mice. Fractionation of plasma lipoproteins by fast protein liquid chromatography showed that the increased cholesterol and TAG levels on PCSK9 expression were mainly associated with VLDL and LDL (but not HDL) in all 3 mouse models examined (Figure IA and IB, respectively in the online-only Data Supplement). In agreement with the total plasma lipid data (Figure 2A), an increase in TAG levels was more pronounced than that for cholesterol, suggesting further that PCSK9 expression promotes VLDL production, irrespective of the LDLR.

To determine whether PCSK9 expression had an effect on lipid metabolism, we quantified the mRNA of the genes involved in lipid metabolism by using real-time quantitative polymerase chain reaction. No consistent differences were noted in the expression of cholesterol synthesis genes (Figure IIA in the online-only Data Supplement), fatty acid/TAG synthesis genes (Figure IIB in the online-only Data Supplement), or lipid gene transcription factors (Figure IIC in the online-only Data Supplement) between mice transduced with Ad-null or Ad-PCSK9. Moreover, no significant differences were noted in the protein levels of these gene products, as determined by Western blotting analysis (Figure IIIA and IIIB in the online-only Data Supplement). Our results, in agreement with previous studies,\textsuperscript{20,29} indicate that PCSK9 expression has no obvious effects on the genes governing cholesterol and TAG biosynthesis.

**Overexpression of PCSK9 Increases Plasma ApoB Levels in an LDLR-Independent Fashion**

Expression of PCSK9 markedly increased plasma apoB levels in all 3 mouse models studied (Figure 2B). On day 7 after PCSK9 expression, plasma apoB100 and apoB48 levels had markedly increased in both C57BL/6 and \textit{LDb} mice. The apoB levels remained elevated throughout the 30-day study. In C57BL/6 mice, PCSK9 expression resulted in
1.93-, 3.79-, 2.22-, and 1.68-fold increases in apoB100 levels on days 7, 14, 21, and 30, respectively. Likewise, apoB48 levels were increased 3.53-, 4.73-, 4.34-, and 2.0-fold on days 7, 14, 21, and 30 after transduction with adenovirus PCSK9 (Ad-PCSK9) were fractionated by FPLC. Each fraction was resolved by SDS-PAGE, followed by Western blotting analysis using an anti-FLAG antibody to detect human PCSK9. The position of PCSK9 is marked. The corresponding fractions from day 7 were probed with anti-mouse apolipoprotein B (apoB) and anti-mouse apoA-I antibodies, respectively. The positions of apoB100 and apoA-I are marked.

Figure 3. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is associated with very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), but mostly with LDL. A, Association of endogenous mouse PCSK9 with lipoproteins. Plasma samples of C57BL/6, LDL receptor–deficient (Ldlr−/−), and LDb mice were fractionated by fast protein liquid chromatography (FPLC). The levels of total cholesterol (mg/dL) and mouse PCSK9 (ng/mL) were determined. The total cholesterol levels are shown as open squares, and the mouse PCSK9 levels are shown as closed circles. B, Pooled plasma samples of LDb mice (n=3 for each time point) on days 7, 14, 21, and 30 after transduction with adenovirus PCSK9 (Ad-PCSK9) were fractionated by FPLC. Each fraction was resolved by SDS-PAGE, followed by Western blotting analysis using an anti-FLAG antibody to detect human PCSK9. The position of PCSK9 is marked. C, Pooled/concentrated FPLC-fractionated VLDL, LDL, and high-density lipoprotein (HDL) of C57BL/6, Ldlr−/−, and LDb mice on day 7 after Ad-PCSK9 transduction were resolved by SDS-PAGE, followed by Western blotting analysis using an anti-FLAG antibody to detect human PCSK9. The position of PCSK9 is marked.

PCSK9 Is Associated With VLDL and LDL, But Mostly With LDL

Next, we determined whether PCSK9 is associated with apoB-containing lipoprotein particles. We measured endogenous mouse plasma PCSK9 levels in nontreated C57BL/6, Ldlr−/−, and LDb mice by using ELISA (92±7.8, 2015±140, and 1646±380 ng/mL, respectively); mice lacking LDLR had ∼20-fold more PCSK9 than wild-type C57BL/6 mice. Fractionation of plasma lipoproteins revealed that the increased apoB100 and apoB48 levels were associated with VLDL and LDL fractions (Figure IVA in the online-only Data Supplement). As expected, the levels of apoE, another apolipoprotein component of VLDL, were also observed in LDL from day 7 to day 30 on PCSK9 expression. In Ldlr−/− mice, the increase in apoB100 and apoB48 levels was prominent on day 14 and maintained through day 30. Fractionation of plasma lipoproteins revealed that the increased apoB100 and apoB48 levels were associated with VLDL and LDL fractions (Figure IVA in the online-only Data Supplement). As expected, the levels of apoE, another apolipoprotein component of VLDL, were also increased (Figure IVB in the online-only Data Supplement). However, the levels of apoA-I, the apolipoprotein component of HDL, were unchanged with PCSK9 expression (Figure IVB in the online-only Data Supplement, bottom). Taken together, the results showing increased apoB secretion, together with the increased lipids associated with VLDL and LDL, suggest strongly that PCSK9 exerts an impact on apoB-containing lipoprotein production. This effect is independent of LDLR expression.
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(Figure 3C). These results indicate that PCSK9 is associated with both VLDL and LDL, but mostly with LDL particles.

**PCSK9 Interacts With apoB**

To gain an insight into the mechanisms by which PCSK9 expression affects apoB production, we examined the possibility of a protein-protein interaction between PCSK9 and apoB. We used 3 different approaches. First, a PCSK9/apoB interaction was demonstrated by coimmunoprecipitation using an anti-FLAG antibody on cell lysates and media of McA-RH7777 cells stably expressing human apoB18 or apoB48 (representing the N terminus, 18% and 48% of the full-length apoB100, respectively) transduced with Ad-PCSK9 (+) or Ad-null (−). As shown in Figure 4A, apoB18 and apoB48 coimmunoprecipitated with PCSK9 in both cell lysates and media. ApoB was not detected in the precipitates from the control cells transduced with Ad-null. These results suggested that PCSK9 could bind to amino acid sequences within the N-terminal region of apoB, namely, apoB18. In addition, the association of PCSK9 with apoB occurred intracellularly and in the media.

Second, the PCSK9/apoB18 interaction was determined with a mammalian matchmaker two-hybrid system using secreted alkaline phosphatase (SEAP) as the reporter. Protein–protein interaction between PCSK9 and apoB18 was observed with a mammalian matchmaker two-hybrid system. The 293 and COS-1 cells were transfected with the indicated plasmids. At 48 hours after transfection, the secreted alkaline phosphatase (SEAP) activities in the cell media were assayed. The results are expressed as light unit (mean±SD). Control assays included pM-apoB18/pG5SEAP, pVP16-PCSK9/pG5SEAP, pM/pVP16/pG5SEAP, pM-Apobec1/pVP16-PCSK9/pG5SEAP, and pG5SEAP. The pM-LDLR/pVP16-PCSK9 pair in 293 cells was used as a positive control. The assay was carried out 3× with duplicate samples.

Third, we used an in situ PLA, termed Duolink in situ PLA, to determine the intracellular interaction between endogenous PCSK9 and apoB. The images were captured using a Leica DM 6000 B confocal microscope with 488-nm, 594-nm, and 638-nm filters. The blue nuclear staining is DAPI. Each experiment was performed in triplicate.
PCSK9 and apoB100 in HepG2 cells. Duolink in situ PLA detects protein–protein interactions (within 40 nm) at physiological concentrations with high specificity.33 In HepG2 cells, apoB and PCSK9 were detected in the cytoplasm whereas the LDLR was localized to the membranes (Figure 4C, top). In HepG2 stably expressing Apobec1 cells, Apobec1 and PCSK9 were also detected in the cytoplasm (Figure 4C, bottom). Figure 4D shows that endogenous apoB100 interacted with PCSK9 in the cytoplasm of HepG2 cells, as determined by Duolink in situ PLA (Figure 4D, top marked αApoB/αPCSK9 in red dots). As expected, there was also a detectable interaction signal between PCSK9 and LDLR (top marked αLDLR/αPCSK9). The following control experiments are not shown here because no signals were detected in the HepG2 cells hybridized with only anti-apoB or anti-PCSK9 antibodies, no antibody control, between PCSK9 and GAPDH, or between apoB and GAPDH. Although Apobec1 did not interact with PCSK9 (αApobec1/αPCSK9) in HepG2 stably expressing Apobec1 cells, a strong interaction between apoB and PCSK9 was observed again in the cytoplasm of these cells (Figure 4D, bottom marked αApoB/αPCSK9). Collectively, these 3 different approaches confirmed a physical interaction between PCSK9 and apoB within the hepatocytes.

Increased Synthesis/Secretion and Decreased Autophagic Degradation of ApoB on PCSK9 Expression

We next performed pulse-chase experiments to determine the impact of the PCSK9/apoB interaction on apoB biosynthesis by using primary hepatocytes isolated from C57BL/6, Ldlr<sup>−/−</sup>, and Ldb mice. The hepatocytes were transfected with either Ad-PCSK9 or Ad-null vectors. The hepatocytes were then pulsed with 35S-methionine/cysteine for 15 minutes and chased for 30, 60, 120, 180, and 240 minutes. PCSK9 expression in the hepatocytes of C57BL/6 mice significantly increased the incorporation of 35S-methionine/cysteine into apoB100 and apoB48 in cell lysates compared with Ad-null transduced cells (Figure 5A). Similarly, primary hepatocytes

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of Ldlr−/− mice expressing PCSK9 had significantly increased incorporation of 35S-methionine/cysteine into apoB100 and apoB48 in both cell lysates and media (Figure 5B), and the incorporation of methionine into apoB100 in LDb mice was ∼3-fold higher than that in either C57BL/6 or Ldlr−/− mice. The effect of PCSK9 expression on apoB synthesis is specific, because no effect was observed for albumin synthesis. The effect of PCSK9 expression on apoB synthesis is specific, because no effect was observed for albumin synthesis. Thus, our results demonstrated that PCSK9 increased apoB because no effect was observed for albumin synthesis. The effect of PCSK9 expression on apoB synthesis is specific, because no effect was observed for albumin synthesis.

Autophagic degradation and the protection of apoB by PCSK9 expression in the mouse liver protects/inhibits apoB from degradation in Pcsk9-positive mice (Figure 6B). Thus, there was less apoB in the autophagosomes of C57BL/6 mice. These results together suggest that decreased apoB (apoB100 and apoB48) levels in autophagosomes.

To examine the effect of endogenous PCSK9 on apoB levels, we compared the apoB levels in autophagosomes isolated from the livers of Ldlr−/− mice with or without PCSK9 (LDb=Ldlr−/−/Apobecl1−/−/Pcsk9−/− mice and LDbPcsk9−/−=Ldlr−/−/Apobecl1−/−/Pcsk9−/− mice, respectively). Similar to what was observed in McA-RH7777 cells, the ratio of apoB100/LC3 was significantly lower in LDb mice (LDb−/−/Apobecl1−/−/Pcsk9−/−) compared with that in LDbPcsk9−/− triple knockout mice (Figure 6B). Thus, there was less apoB in the autophagosomes of Pcsk9-positive mice (LC3), which suggested that decreased amount of apoB was shunted to autophagosomes for degradation. This was reflected on LDbPcsk9−/− triple knockout mice, which had significantly decreased plasma levels of apoB (↓34%), cholesterol (↓29%), and TAG (↓33%) compared with LDb mice.

Finally, we determined whether the effect of PCSK9 on apoB degradation was dependent on the LDLR by comparing apoB100/LC3 and apoB48/LC3 ratios in autophagosomes isolated from C57BL/6 and Pcsk9−/− mice. As shown in Figure 6C, C57BL/6 mice (Pcsk9+/+) had significantly less apoB100/LC3 and apoB48/LC3 ratios in autophagosomes compared with Pcsk9−/− mice. The Pcsk9−/− mice have lower plasma cholesterol (↓53%) and apoB48 levels compared with C57BL/6 mice. These results together suggest that PCSK9 expression in the mouse liver protects/inhibits apoB from autophagic degradation and the protection of apoB by PCSK9 is independent of the LDLR.

Discussion
The present study demonstrates that long-term expression of PCSK9 in mice, irrespective of the presence of the LDLR, increases plasma cholesterol, TAG, and apoB levels. PCSK9 interacts with apoB to prevent/inhibit/decrease the mobilization of apoB toward autophagosomes for degradation via the autophagosome/lysosome pathway. This, in turn, results in increased production and secretion of apoB and apoB-containing lipoproteins. Most importantly, our study demonstrates a direct protein–protein interaction of PCSK9 and apoB in cells under physiological conditions. Taken together, these data establish that PCSK9 regulates apoB metabolism independent of the LDLR. The present study provides a new mode by which PCSK9 could regulate apoB degradation, and suggests the importance of lowering PCSK9 levels to reduce the production/secrection of apoB-containing lipoproteins.

Previous investigations involving cells, mice, and humans have suggested that PCSK9 might play a role in regulating apoB production, but other studies failed to observe an effect of PCSK9 on apoB production. Evidence from the present study led us to conclude that these discordances might have resulted from experimental designs; those studies were terminated too early (3–6 days). Most importantly, we used 3 different methods, coimmunoprecipitation, mammalian matchmaker two-hybrid system, and Duolink in situ PLA, to show that PCSK9 interacts with apoB. These results provided convincing evidence of a direct endogenous protein–protein interaction of PCSK9 with apoB. Previous studies have shown that PCSK9 mutants such as D374Y and S127R exert greater effect on increasing apoB production than wild-type PCSK9. Patients with D374Y mutation in PCSK9 have a particularly severe form of autosomal dominant hypercholesterolemia. The D374Y mutant form of PCSK9 binds to the LDLR and results in reduced LDLR activity 10-fold greater than that observed for wild-type PCSK9.42 These mutants might also have higher binding affinity toward apoB. Further study is warranted to elucidate the structure–function relationship between PCSK9 and apoB.

Zhang et al have demonstrated that PCSK9 selectively interacts with the epidermal growth factor-A repeat of the LDLR (but not with other members of the LDLR family) in a calcium-dependent manner. Moreover, they have shown that the PCSK9-mediated degradation of the LDLR requires the LDLR ligand-binding domain and the β-propeller domain. Further, the prodomain, catalytic domain, and C-terminal domain of PCSK9 are all essential for PCSK9-mediated degradation of the LDLR. ApoB is a large protein with a molecular weight of 550 kDa. Now that we know that apoB18 interacts with PCSK9 efficiently, the question will be whether the interaction of apoB18 with PCSK9 is sufficient to inhibit apoB from moving to the autophagosome for degradation. Furthermore, the PCSK9-binding site on the LDLR is distinct from the ligand-binding region that recognizes the LDL particle. Because we have shown that PCSK9 associates with LDL, it would be of interest to determine whether PCSK9 can mask the LDLR-binding domain of apoB. In that case, the PCSK9-associated LDL would not be taken up by the LDLR. These questions remain to be addressed experimentally.

ApoB is constitutively expressed in the liver and is regulated at the posttranscriptional and translational levels. Various mechanisms have been proposed for the intracellular
Figure 6. Proprotein convertase subtilisin/kexin type 9 (PCSK9) expression decreases the levels of apolipoprotein B100 (apoB100) and apoB48 in hepatic autophagosomes. 

A, Autophagosomes were isolated from McA-RH7777 cells transfected with either pcDNA3 vector or pcDNA3-PCSK9 vector. B, Hepatic autophagosomes were isolated from LDb and LDbPcsk9−/− mice. C, Hepatic autophagosomes were isolated from C57BL/6 and Pcsk9−/− mice. The lysed autophagosomes (30 μg) were subjected to Western blotting analysis to detect apoB100, apoB48, PCSK9, and light chain 3-II (LC3-II). Each protein was detected and quantified using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). The results are expressed as a ratio of the intensities of apoB100/LC3-II and apoB48/LC3-II and are shown as mean±SD. *P<0.05 compared with the corresponding samples. The assays were performed 3× with duplicate samples.

degradation of apoB. They include an endoplasmic reticulum-associated degradation mediated by the proteasome/lysosome pathway, a postendoplasmic reticulum presecretory proteolysis possibly mediated through the autophagosome/lysosome pathway, and a LDLR-mediated presecretory apoB degradation possibly mediated by the proteasome/lysosome pathway. Collectively, it is likely that 2 major degradation pathways for apoB exist in hepatocytes: the proteasome/lysosome pathway and the autophagosome/lysosome pathway. In the endoplasmic reticulum, some of the apoB might interact with LDLR and be degraded through the proteasome pathway. In this case, if LDLR were
dysfunctional, the cells would increase the secretion of apoB-containing lipoproteins. We propose an LDLR-independent apoB degradation process. ApoB is found in cytoplasmic lipid droplets as apoB-crescents,13 which fuse with autophagosomes/lysosomes for degradation. This process is likely to be the preferred or more important pathway for apoB degradation. In the present study, we demonstrated PCSK9-regulated apoB degradation in hepatocytes, independent of LDLR. The physical interaction of PCSK9 with apoB acts to shunt apoB away from autophagosomes and degradation. In turn, most of the apoB would be destined for assembly and secretion as VLDL from hepatocytes. This observation is consistent with increased apoB production on overexpression of PCSK9. This is the first report linking PCSK9 regulation of apoB to apoB degradation via the autophagosome/lysosome pathway.

Autophagy is an essential cellular regulatory process that mediates degradation in lysosomes, and it is involved in normal and pathological pathways. Oxidized LDL is removed through the autophagosome/lysosome pathway in human vascular endothelial cells.46 Moreover, LDL is broken down in activated macrophages via autophagy.47 It would be interesting to determine whether the association of PCSK9 with LDL via apoB would inhibit the uptake of LDL via macrophages, and in turn, hinder the removal of lipids through an autophagy-mediated efflux process. Thus, our study has raised many unexpected questions related to PCSK9/apoB/LDL that remain to be answered.

In summary, we have demonstrated the important discovery that PCSK9 interacts with apoB, and this interaction leads to decreased degradation of apoB via the autophagosome/lysosome pathway. The present study also provides evidence for how overexpression of PCSK9 increased cholesterol, TAG, and apoB levels, independent of LDLR. Taken together, our results suggest that decreasing intracellular PCSK9 levels may represent a new therapeutic approach for regulating the production of atherogenic apoB-containing lipoproteins.

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Disclosures

None.

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Proprotein Convertase Subtilisin/Kexin Type 9 Interacts With Apolipoprotein B and Prevents Its Intracellular Degradation, Irrespective of the Low-Density Lipoprotein Receptor

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Supplemental Figure legends

Supplemental Figure I. Distribution of cholesterol and triacylglycerol in plasma lipoproteins separated by FPLC.

C57BL/6 (red), Ldlr−/− (blue), and LDb (Ldlr−/−Apobec1−/−) (Purple) mice were transduced with either Ad-PCSK9 (closed circle) or Ad-Null (open circle). Pooled plasma samples from each group (n = 3) at indicated time were fractionated by FPLC. Total cholesterol (A) and triacylglycerol (B) from each fraction were measured and expressed as mg/dl. Positions of VLDL, LDL, and HDL are marked.

Supplemental Figure II. Quantitative RT-PCR hepatic gene expression analysis from C57BL/6, Ldlr−/−, and LDb (Ldlr−/−Apobec1−/−) mice after transduction with Ad-PCSK9 or Ad-Null.

C57BL/6, Ldlr−/−, and LDb mice (n = 3 for each time point) were transduced with either Ad-Null or Ad-PCSK9. The hepatic gene expression levels of cholesterol biosynthesis genes (Fig. S2A), TAG biosynthesis genes (Fig. S2B), and transcription factors (Fig. S2C) were determined using SYBR quantitative RT-PCR method. The results were normalized with 16S RNA and presented as Mean±SD. The black bars represent Ad-PCSK9 group and the open bars represent Ad-Null group. The * represents p<0.05, compared Ad-PCSK9 treated group with Ad-Null treated group.

Supplemental Figure III. Western blot analysis of hepatic protein expressions in C57BL/6, Ldlr−/−, and LDb (Ldlr−/−Apobec1−/−) mice after transduction with Ad-PCSK9 or Ad-Null.
C57BL/6, Ldlr-/-, and LDb mice (n = 3 for each time point) were transduced with either Ad-Null or Ad-PCSK9.

(A). The protein expression levels of HMG CoA Reductase, Srebp1, and Srebp2 were analyzed by Western blot.

(B). The protein expression levels of FAS, Scd1, and control Gapdh were analyzed by Western blot.

**Supplemental Figure IV. Western blot analysis of FPLC-fractioned plasma apolipoproteins B, E, and AI in LDb (Ldlr-/-Apobec1-/-) mice after transduction with Ad-PCSK9 or Ad-Null.**

(A). Pooled plasma samples from LDb mice at days 0, 7, 14, 21, and 30 were fractionated by FPLC. Fractions (10 µl) of VLDL, LDL, and HDL were analyzed by SDS/PAGE, followed by Western blotting with anti-mouse apoB. The fraction numbers of VLDL, LDL, and HDL were indicated. The intensity of each band was determined by using an Odyssey Infrared Imaging system (Li-COR, Lincoln, NE).

(B). Western blot analysis of FPLC fractions from LDb mice with anti-mouse apoE (Top panel) and anti-mouse apoAI (Bottom panel).

**Supplemental Figure V. Quantitative RT-PCR hepatic gene expression analysis of apoB mRNA from C57BL/6, Ldlr-/-, and LDb (Ldlr-/-Apobec1-/-) mice after transduction with Ad-PCSK9 or Ad-Null.**

C57BL/6, Ldlr-/-, and LDb mice (n = 3 for each time point) were transduced with either Ad-Null or Ad-PCSK9. The gene expression levels of apoB mRNA were determined by TaqMan
quantitative RT-PCR. The gene expression levels were normalized with 18S RNA. The results were expressed as Mean±SD.
**Supplemental Materials and Methods:**

**Animal studies**

*C57BL/6, Pcsk9-/- and Ldlr-/- mice were purchased from Jackson Laboratory (Bar Harbor, ME). LDb (Ldlr-/-Apobec1-/-) mice were generated in Teng’s laboratory as described previously 1-3. The Ldlr-/-Apobec1-/-Pcsk9-/- (LDbPcsk9-/-) triple knockout mice were generated by crossing Pcsk9-/- with LDb mice, and genotype of which was confirmed by PCR. Mice were kept in a barrier facility with a 12:12 h dark-light cycle and maintained on a standard laboratory chow diet. All animal experiments were conducted in accordance with the Guidelines of the Animal Protocol Review Committee of the University of Texas Health Science Center at Houston.*

**Production of E1-, E2b-, E3-deleted second-generation adenovirus type 5 vector expressing hPCSK9**

The E1-, E2b-, E3-deleted adenovirus type 5-vector pAd-del-pol, adenoviral-CMV shuttle vector pD2007MCS, and E.C7 cell line were obtained from Etubics Corporation (Seattle, WA). The full-length hPCSK9 cDNA with a FLAG tag was obtained from the laboratory of Dr. Jay D. Horton (University of Texas Southwestern Medical Center, Dallas, TX) 4 and inserted into the pAdEasy shuttle vector (at HindIII and EcoRV sites) that contains the CMV promoter and the Kan resistant gene (Invitrogen, Carlsbad, CA). The resulting pAdEasy-PCSK9-Kan was co-transformed with pAd-del-pol into BJ5183 competent cells (Invitrogen) to generate virus-ready
plasmid vector pAd-PCSK9-Kan. The pAd-PCSK9-Kan was co-transformed with the shuttle vector pD2007-CMV-PCSK9-Amp into BJ5183 cells to replace the pD2007 backbone through homologous recombination. The resulting plasmid vector pAd-PCSK9-Amp was linearized with FseI and transfected into E.C7 cells to produce recombinant adenovirus (Ad-PCSK9). The virus titer (10^{12}-10^{13} viral particles/ml) was determined by qPCR using a pair of primers (forward: 5’ATACGTCATTATTGACGTCAATG3’; reverse: 5’CGTTACATAACTTACGGTAAATG3’) and the PCR product was probed with 5’6-FAM/CCTGGCTGACCGCCAACGAC3’. A control adenovirus, referred as Ad-Null, was similarly constructed without cDNA insert. Expression of Ad-PCSK9 was confirmed in 293 cells. Western blot analysis using anti-FLAG monoclonal antibody demonstrated that both the expected pre-PCSK9 (74 kDa) and mature PCSK9 (63 kDa) were synthesized and secreted from cells.

Transduce mice with Ad-PCSK9 and Ad-Null

Male C57BL/6J, Ldlr-/-, and LDb mice (n=24 for each group) at 2-months of age were transduced with Ad-PCSK9 or Ad-Null virus (1×10^{10} viral particles/mouse) via tail vein injection. Fasting (~16 h) blood sample was collected prior to (day 0) and post adenovirus injection at days 7, 14, 21, and 30, respectively, via retro-orbital plexus using a heparin coated capillary tube (Fisher Scientific). The liver and other tissues were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

Analysis of plasma lipid and lipoprotein levels
Pooled plasma (225 µl) was separated by FPLC on two Superose 6 columns (Amersham Biosciences, Piscataway, NJ). Fractions (0.5 ml each) were eluted using 150 mM NaCl, 1 mM EDTA and 0.02% NaN3 pH 8.2 to separate VLDL, LDL, and HDL. 3,5 Cholesterol and TAG concentrations in both plasma and each FPLC fraction were determined using the Cholesterol E and the L-Type Triglyceride M kits (Wako Chemicals, Richmond, VA), respectively.

**Western blot analysis**

Mouse plasma samples (3 µl from C57BL/6J, 1 µl from Ldlr−/− or LDb) or FPLC fractions (10 µl/fraction) were resolved by gel electrophoresis. The ProSieve-50 gel (6%) (FMC BioProducts, Rockland, ME) was used for apoB, and SDS-PAGE was used for apoE, apoA-I (12.5% gel) and PCSK9 (7.5% gel). The respective proteins were detected by anti-mouse apoB (Abcam, Cambridge, MA), anti-mouse apoE, or anti-mouse apoA-I (generously given by B. Ishida, University of San Francisco), or anti-FLAG antibody (Sigma). The intensity of protein bands was semi-quantified using an Odyssey Infrared Imaging system (Li-COR, Lincoln, NE).

In some experiments, equal volumes of pooled VLDL, LDL, and HDL (FPLC fractions) of C57BL/6J, Ldlr−/−, and LDb mice (at day 7 post-PCSK9 expression) were concentrated and resolved by SDS-PAGE (7.5% gel) and subjected to Western blot analysis for PCSK9.

**ELISA Quantification of PCSK9**
We used a commercial ELISA kit to measure the concentration of mouse PCSK9 (CycLex Co., Ltd, Nagano, Japan) and the human PCSK9 levels were determined by using Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN).

**Real-time Quantitative PCR**

Total RNA were extracted from mouse livers using the Qiazol reagent (Qiagen, Valencia, CA), digested with DNase I (Ambion, Austin, TX), and transcribed into cDNA using Achieve Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was performed on ABI Prism 7900 Sequence Detection System (Applied Biosystems). RNA was quantified using the SYBR Green PCR Master Mix (Bio-Rad). Each primer set was optimized to eliminate primer-dimer formation. The mouse apoB RNA levels were quantified using the TaqMan primer/probe. The results are expressed as the ratio of specific RNA/house-keeping RNA. The mouse apoB RNA was normalized with 18S RNA. The other RNAs were normalized with 16S RNA. Sequences of primers and probes used in this study are listed in *Supplemental Table 2*.

**Pulse-chase analysis of apoB biosynthesis in mouse primary hepatocytes**

Mouse primary hepatocytes were isolated using a modified procedure established by David Moore’s laboratory at Baylor College of Medicine, Houston, TX. 6 Mice (*C57BL/6J, Ldlr−/−*, and *LDb*) were anesthetized, the livers were perfused at a rate of 3 ml/min via the portal vein with 50 mL HBSS (Invitrogen) containing 0.5 mM EGTA, followed by 50 mL EBSS
(Invitrogen) containing 0.3 mg/ml liberase blendzyme (Roche) and 0.04 mg/ml trypsin inhibitor (Invitrogen) at 37°C. After perfusion, the liver was removed, the hepatic capsule was peeled off, and hepatocytes were dispersed by shaking the digested liver in a pre-warmed William’s E Medium (Invitrogen) at 37°C, followed by filtration through 100 µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ). The cells were washed twice, re-suspended in the William’s E Medium, and loaded onto a 50 ml falcon tube containing discontinuous Percoll gradient solution (Invitrogen) (5 ml 1.06 g/ml, 7 ml 1.08 g/ml, 5 ml 1.12 g/ml). After centrifugation at 700 g for 20 min, the cells were collected between 1.08 and 1.12 g/ml, washed twice with 20 ml William’s E Medium, and re-suspended in hepatocyte culture medium (William’s E Medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 1×insulin-transferrin-selenium solution (Invitrogen). The viability of cells (90-95%) was determined using trypan blue dye exclusion.

The cells were plated (0.5 × 10⁶ cells/well) onto a 6-well plate coated with mouse type IV collagen, incubated for 6 h, and infected overnight with Ad-Null or Ad-PCSK9 (multiplicity of infection; MOI=50:1). On the next day, the media were changed to Met/Cys-free DMEM for 30 min prior to pulse labeling. The cells were labeled with [³⁵S]Methionine/Cysteine (Perkin Elmer, Waltham, MA) (100 µCi/ml Met/Cys-free DMEM) for 15 min. The cells were chased with serum-free DMEM containing 2 mM Met for 30, 60, 120, 180, and 240 min. At the indicated time point, media were collected, and cells were lysed with 100 µl RIPA buffer (Cell Signaling) containing a protease inhibitor mixture (Roche). The media (400 µl) and cell lysates (50 µl) were incubated with 10 µl anti-mouse apoB (Abcam) or anti-albumin (GenWay Biotech, San Diego, CA) antibodies in 500 µl 1× RIPA buffer containing protein-A-agarose beads (Roche) at 4 °C for
1 h with rotation. The immunoprecipitates were washed with 500 µl 1× RIPA buffer for 5 times and then eluted with SDS sample loading buffer and subjected to SDS-PAGE (6% Prosieve gel for apoB and 10% gel for albumin). The gels were fixed and amplified in 1 M sodium salicylate (Sigma-Aldrich) for 15 min, dried, and scanned by a PhosphoImage system (Bio-Rad). The bands were quantified using QuantityOne software (Bio-Rad). The results are expressed as total radioactivity of each band.

**Co-immunoprecipitation of PCSK9 and apoB**

McA-RH7777 cells stably expressing human apoB-18 or apoB-48 were generated as described previously. The cells were plated onto 6-well plates, and cultured for 18 h in DMEM containing 10% FBS and 200 µg/ml G418. The cells were infected with Ad-PCSK9-FLAG or Ad-Null (MOI=50:1). After 24 h, the cells were lysed with 1× RIPA buffer. Cell lysates were incubated with 50 µl anti-FLAG M2 agarose (Sigma-Aldrich) to precipitate FLAG-tagged PCSK9. The immunocomplexes were washed 3 times with 500 µl RIPA buffer, eluted in SDS sample-loading buffer by boiling for 5 min, and subjected to SDS-PAGE (6% Prosieve gel for apoB and 7.5% gel for PCSK9). After gel electrophoresis, the proteins were transferred to Immobilon P membrane; the membrane was hybridized with mAb 1D1 to detect human apoB-18 and apoB-48, and with anti-FLAG to detect PCSK9.

**Protein-Protein interaction: Mammalian matchmaker Two-hybrid System.**

We used mammalian matchmaker two-hybrid system (Clontech, Mountain View, CA) to examine protein-protein interaction. Briefly, full-length human PCSK9 cDNA was cloned into
pVP-16 vector (EcoRI and XbaI sites), which generated a fusion protein of the VP-16 activation domain with PCSK9 as pVP16-PCSK9. We designed primers flanking the apoB-18 cDNA (NM_000384, nucleotides 129-2555) to amplify apoB18 DNA from pCMV-apoB-18. The amplified fragment was sequenced and cloned into EcoRI and BamHI sites of pM vector. This vector produced a fusion GAL4-binding domain with apoB18 as pM-apoB-18. The control vector of pM-Apobec1 was obtained from our previous study. 8 We transfected three plasmid vectors using lipofectamine 2000 (Invitrogen) into either 293 cells or COS-1 cells; pM-apoB-18 (0.5 µg), pVP-16-PCSK9 (0.5 µg), and pG5-SEAP (0.5 µg). At the end of experiment (48 h after transfection), the interaction between two proteins was assayed by measuring SEAP activities in the cell media using Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech). The Light Unit was determined using Tecan infinite-200 chemiluminescent reader (TECAN, San Jose, CA). Control vector pM-Apobec1, vector without insert pM or pVP16, and pM-LDLR were performed simultaneously. The results are expressed as Light Units.

**Autophagosome isolation**

We isolated autophagosomes from cells or mouse liver using the method described by Stromhaug et al.. 9 Briefly, the pelleted cultured McA-RH7777 cells or mouse liver were homogenized gently using glass tissue grinder type B (Kimble Chase) in a homogenization buffer (HB, 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, 0.5 mM glycyl-l-phenylalanine 2-naphthylamide (GPN), 1% DMSO, pH 7.3, ρ=1.030g/ml). After 6 min incubation at 37°C, the homogenate was centrifuged at 2,000 g for 2 min to separate nuclear pellet from supernatant. The supernatants were layered on top of a discontinuous Nycodenz gradient solution (4 ml 1.072
g/ml and 4 ml 1.127 g/ml). The Nycodenz gradient was centrifuged at 4°C for 1 h at 141,000 g using NVT65 rotor (Bechman Coulter). The interface band (~1ml), which contained the autophagosomes, was collected using 21G needle. This band was diluted with 5 ml of HB and layered on top of the 2nd discontinuous gradient (4 ml 33% Percoll in HB and 5 ml 22.5% Nycodenz (1.127g/ml) in HB). The gradients were centrifuged for 30 min at 72,000 g using NVT65 rotor (Bechman Coulter). We collected the purified autophagosomes band at the interface between Percoll and Nycodenz using 21G needle. The autophagosomes were lysed in 1× RIPA buffer and 30 µg of it was subjected to Western-blot analysis to identify apoB, Pcsk9, and LC3. Proteins were resolved by SDS-PAGE (6% Prosieve gel for apoB; 12.5% gel for Pcsk9 and LC3), transferred to Immobilon-FL membrane, and probed with anti-mouse apoB (1:2,000 dilution), anti-Pcsk9 (Cayman Chemical, 1:2,000 dilution), and anti-LC3 (Cell Signaling, at 1:3,000 dilution) antibodies. The intensities of the protein bands were quantified using an Odyssey Infrared Imaging system (Li-COR).

**Duolink in situ proximity ligation assays (PLA) and Immunofluorescence analysis**

In situ PLA assay kit was obtained from OLINK Bioscience (Uppsala, Sweden). The primary antibodies used for this assay were mAb PCSK9 (mC33-Mab1), anti-ApoB (Abcam), anti-Apobec1 (gift from Dr. Lawrence Chan’s lab, Baylor College of Medicine), and anti-LDLR (Cayman).

In general, cells (HepG2 cells and HepG2 stably expressed Apobec1 cell line) were seeded in a chamber slide (NUNC, Naperville, IL) and cultured overnight at 37°C in the incubator. On the next day, the cells were washed with ice-cold PBS, fixed in ice-cold methanol for 10 min,
followed by permeabilization with PBS containing 0.25% Triton X-100 for 10 min. The cells were then blocked with blocking buffer (PBS containing 1% BSA) for 1 h, followed by incubation with primary antibody (1:100 dilution in blocking buffer) at 4 °C overnight.

For immunofluorescence analysis, the cells were washed with PBS and incubated with fluorescence dye conjugated-secondary antibodies at 1:300 for 1 h at room temperature. After washing off the secondary antibody, the slides were incubated with DRAQ5 nuclear Staining reagent (Abcam) at 1:1,000 in PBS for 2 h at room temperature. The slides were mounted and examined using a Leica DM 600B confocal microscope with 488nm, 594nm and 633nm filters.

For in situ PLA, we followed the commercial kit instruction. Briefly, after primary antibodies incubation the cells were washed with PBS, followed by incubation with PLA secondary probes (1:5 dilution in blocking solution; anti-rabbit/PLUS and anti-mouse/MINUS PLA probes) for 1 h at 37°C. The slide was washed 2 times with Wash Buffer-A, 5 min each time. The cells were then incubated with 1:40 diluted Ligase for 30 min at 37 °C in a pre-heated humidity chamber. After ligation, the slides were washed 2 times with Wash buffer-A, 2 min each. We then added 40 µl diluted polymerase (5 units, 1:80 dilution) to amplify the reaction for 100 min at 37 °C in a pre-heated humidity chamber. The slides were washed quickly with Wash Buffer-B, followed by 10 min wash with Wash Buffer-B again 2 times each and finally rinsed with 1:100 diluted Wash Buffer-B for 1 min. The slides were dried at room temperature in the dark, mounted with Duolink II mounting medium containing DAPI. The slides were examined using a Zeiss Axio Observer.D1m fluorescence microscopy with DAPI, FITC and Texas Red filters. Control slides without primary or secondary antibodies were performed at the same time.
Since LDLR was expressed on the cell membrane, we treated the cells differently to detect the interaction of LDLR and PCSK9. HepG2 cells were seeded onto a chamber slide and cultured overnight at 37°C in the incubator. On the next day, the cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 10 min and the cells were NOT permeabilized to avoid the destruction of cell membrane. After the fixation, we used the same procedure as described above for the assay.

**Statistical Analysis**

All the results were expressed as mean ± SD. We used STAT software to conduct one-way and two-way analysis of variance ANOVA (http://www.stata.com/capabilities/anova-manova/). For each fixed time, one-way ANOVA was used to test for significant difference between the means of phenotypes (Cholesterol and Triacylglycerol) of Ad-PCSK9 group and control virus Ad-Null group. Two-way ANOVA was used to analyze the main effects and interaction effects of treatment and time on the phenotype. The two factors are treatment (Ad-PCSK9 and control virus Ad-Null) and time. Two-way analysis of variance tests for significant difference between the means of phenotypes with Ad-PCSK9 and control virus Ad-Null, and for interaction between treatment and time. The results are shown in Supplemental Table 1. Other statistical comparisons were performed using GraphPad Prism software (ver 5) unpaired t-test with two-tailed p value. \( p < 0.05 \) were considered to be statistically significant


Supplemental Figure IA. Hua Sun et al.

C57BL/6 at Day 0

Day 7

Day 14

Day 21

Day 30

C57BL/6 at Day 0

Ldlr−/− at Day 0

Day 7

Day 14

Day 21

Day 30

Ldlr−/− at Day 0

Ldb at Day 0

Day 7

Day 14

Day 21

Day 30

LDb at Day 0
Supplemental Figure IB. Hua Sun et al.

**C57BL/6 at Day 0**

- Ad-Null
- Ad-PCSK9

**Ldlr−/− at Day 0**

- Ad-Null
- Ad-PCSK9

**Ldb at Day 0**

- Ad-Null
- Ad-PCSK9

**Triglycerides (mg/dl)**

**Days:** 0, 7, 14, 21, 30

**Fractions:**
Supplemental Figure IIA Hua Sun et al.

C57BL/6

Ldlr/-

LDb (Ldlr/-Apobec1/-)

HMGCoA Synthetase

HMGCoA Reductase

Ad-Null
Ad-PCSK9

*
Supplemental Figure IIB Hua Sun et al.

**C57BL/6**

- **Acetyl CoA Carboxylase**
  - Ad-Null
  - Ad-PCSK9

- **Fatty Acid Synthase**

- **Stearoyl CoA Desaturase**

- **Gpat**

**Ldlr-/-**

- **Acetyl CoA Carboxylase**

- **Fatty Acid Synthase**
  - * (*Significant difference)

- **Stearoyl CoA Desaturase**
  - * (*Significant difference)

- **Gpat**

**LDb (Ldlr-/-Apobec1-/-)**

- **Acetyl CoA Carboxylase**

- **Fatty Acid Synthase**
  - * (*Significant difference)

- **Stearoyl CoA Desaturase**
  - * (*Significant difference)

- **Gpat**
Supplemental Figure IIC Hua Sun et al.

*C57BL/6*  | *Ldlr/-*  | *LDb (Ldlr/-Apobec1/-)*

**Srebp-1a**
- Ad-Null
- Ad-PCSK9

**Srebp-1c**
- Ad-Null
- Ad-PCSK9

**Srebp-2**
- Ad-Null
- Ad-PCSK9
Supplemental Figure IIIA  Hua Sun et al.

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<td><img src="C57BL/6_Day14.png" alt="Image" /></td>
<td><img src="C57BL/6_Day21.png" alt="Image" /></td>
<td><img src="C57BL/6_Day30.png" alt="Image" /></td>
</tr>
<tr>
<td>Ldlr-/-</td>
<td><img src="Ldlr_Day7.png" alt="Image" /></td>
<td><img src="Ldlr_Day14.png" alt="Image" /></td>
<td><img src="Ldlr_Day21.png" alt="Image" /></td>
<td><img src="Ldlr_Day30.png" alt="Image" /></td>
</tr>
<tr>
<td>LDb</td>
<td><img src="LDb_Day7.png" alt="Image" /></td>
<td><img src="LDb_Day14.png" alt="Image" /></td>
<td><img src="LDb_Day21.png" alt="Image" /></td>
<td><img src="LDb_Day30.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**HMGCoA Reductase**
- Day-7: Ad-Null, Ad-PCSK9
- Day-14: Ad-Null, Ad-PCSK9
- Day-21: Ad-Null, Ad-PCSK9
- Day-30: Ad-Null, Ad-PCSK9

**Srebp1**
- Day-7: C57BL/6, Ldlr-/-, LDb
- Day-14: C57BL/6, Ldlr-/-, LDb
- Day-21: C57BL/6, Ldlr-/-, LDb
- Day-30: C57BL/6, Ldlr-/-, LDb

**Srebp2**
- Day-7: C57BL/6, Ldlr-/-, LDb
- Day-14: C57BL/6, Ldlr-/-, LDb
- Day-21: C57BL/6, Ldlr-/-, LDb
- Day-30: C57BL/6, Ldlr-/-, LDb