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microRNA-483 ameliorates hypercholesterolemia by inhibiting PCSK9 production

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Introduction

Hypercholesterolemia is a major risk factor for cardiovascular disease. Circulatory levels of low-density lipoprotein cholesterol (LDL-C) are predominantly determined by hepatic clearance of LDL-C via the LDL receptor (LDLR). Proprotein convertase subtilisin/kexin type 9 (PCSK9) affects cholesterol homeostasis by targeting hepatic LDL receptor (LDLR) for lysosomal degradation. Clinically, PCSK9 inhibitors effectively reduce LDL-cholesterol (LDL-C) levels and the incidence of cardiovascular events. Because microRNAs (miRs) are integral regulators of cholesterol homeostasis, we investigated the involvement of miR-483 in regulating LDL-C metabolism. Using in silico analysis, we predicted that miR-483-5p targets the 3′-UTR of PCSK9 mRNA. In HepG2 cells, miR-483-5p targeted the PCSK9 3′-UTR, leading to decreased PCSK9 protein and mRNA expression, increased LDLR expression, and enhanced LDL-C uptake. In hyperlipidemic mice and humans, serum levels of total cholesterol and LDL-C were inversely correlated with miR-483-5p levels. In mice, hepatic miR-483 overexpression increased LDLR levels by targeting Pcsk9, with a significant reduction in plasma total cholesterol and LDL-C levels. Mechanistically, the cholesterol-lowering effect of miR-483-5p was significant in mice receiving AAV8 PCSK9-3′-UTR but not Ldlr-knockout mice or mice receiving AAV8 PCSK9-3′-UTR (ΔBS) with the miR-483-5p targeting site deleted. Thus, exogenously administered miR-483 or similarly optimized compounds have potential to ameliorate hypercholesterolemia.
Insight into the role of miR-483 in regulating PCSK9 expression and its therapeutic potential.

**Results**

**miRs targeting Pcsk9 alleviated hyperlipidemia and atherosclerosis.** The exogenously administered adeno-associated virus 8 (AAV8)-PCSK9 encoding a gain-of-function Pcsk9 mutant (D377Y) lacking the 3′-UTR region is commonly used to induce hyperlipidemia and experimental atherosclerosis in mouse models (12). To explore whether the 3′-UTR region of the PCSK9 transcript participates in PCSK9 regulation at the posttranscriptional level, we constructed an AAV8-based recombinant virus encoding mouse Pcsk9 mRNA encompassing its native 3′-UTR (hereafter called AAV8-PCSK9-3′-UTR). Mice were administered AAV8-PCSK9 or AAV8-PCSK9-3′-UTR, then fed a high-fat diet (HFD) for 12 weeks. The HFD-induced hyperlipidemia was significantly alleviated in mice receiving AAV8-PCSK9-3′-UTR compared with AAV8-PCSK9 (Figure 1A). Consistently, serum levels of total cholesterol, triglycerides, very LDL-C (VLDL-C), and LDL-C and atherosclerosis were reduced in mice with AAV8-PCSK9-3′-UTR (Figure 1B–E). Also, hepatic Pcsk9 mRNA levels and serum levels of PCSK9 were decreased in mice with AAV8-PCSK9-3′-UTR (Figure 2A). These results suggest that the 3′-UTR region of Pcsk9 mRNA exerts an endogenous mechanism against HFD-induced hyperlipidemia and atherosclerosis. To explore whether miR-dependent 3′-UTR targeting accounts for this atheroprotective mechanism, we used bioinformatics to profile miRs that likely target the PCSK9 mRNA 3′-UTR (Figure 2B and Supplemental Figure 2). A luciferase reporter fused with the PCSK9-3′-UTR was constructed to test the efficacy of miRs targeting the PCSK9-3′-UTR. Overexpression of miR-222, -191, -224, and -483 but not -1912 or -1295b greatly decreased the luciferase activity (Figure 2C). Among these miRs, only miR-483 increased significantly in the liver with AAV8-PCSK9-3′-UTR compared with AAV8-PCSK9 (Figure 2D). Such increased miR-483 levels, but not those of others, in the liver suggest that miR-483 would contribute to this posttranscriptional regulation of PCSK9 in vivo. Indeed, when validated by Western blot, miR-483 overexpression had the greatest effect on decreasing PCSK9 levels, with attendant increase in LDLR expression (Figure 2E).

miR-483-5p targeted PCSK9 in hepatocytes. Given that miR-483 might directly target the PCSK9 3′-UTR, we used bioinformatics to locate the binding site of miR-483-5p at the 3′-UTR of both human and mouse
PCSK9 mRNA (Figure 3A). A gain-of-function approach involving pre–miR-483 overexpression reduced the mRNA and protein levels of PCSK9 (Figure 3, B and C). In the complementary loss-of-function experiment, HepG2 cells were transfected with an anti–miR-483 construct. The inhibition of miR-483 increased PCSK9 mRNA and protein levels (Figure 3, B and C). Consistently, LDLR expression was increased in HepG2 cells transfected with pre–miR-483 and decreased in those transfected with anti–miR-483. As expected, these changes in LDLR expression were only at the protein level and not the mRNA level (Figure 3, B and C).

To investigate whether miR-483-5p directly targets the predicted binding site at the PCSK9 3′-UTR mRNA, we used a luciferase reporter fused to the WT PCSK9 3′-UTR [Luc-PCSK9 (WT)] or mutant PCSK9 3′-UTR [Luc-PCSK9 (MT)] with mutation of the miR-483-5p targeting site. Pre–miR-483 significantly decreased the luciferase activity of the cotransfected Luc-PCSK9 (WT) in HepG2 cells but only moderately decreased it in cells cotransfected with Luc-PCSK9 (MT) (Figure 3D). However, anti–miR-483 treatment significantly increased the reporter activity of Luc-PCSK9 (WT) but not Luc-PCSK9 (MT). Additionally, in HepG2 cells overexpressing pre–miR-483, both miR-483-5p and PCSK9 mRNA were enriched in the miRNA-induced silencing complexes (miRISCs) that contained Argonaute-1 or Argonaute-2 (Figure 3E). As a positive control, the mRNA level of CTGF, an established miR-483 target (18), was also increased in the miRISCs. The sequestration of PCSK9 and CTGF mRNA in miRISCs was decreased in HepG2 cells transfected with anti–miR-483.

To further confirm miR-483 targeting of PCSK9, we used CRISPR/Cas9 gene editing to create a HepG2 mutant cell line (mPCSK9 HepG2) with deletion of the miR-483 targeting site in the PCSK9 3′-UTR (Supplemental Figure 3). Transfection of pre–miR-483 or anti–miR-483 did not significantly change the mRNA or protein levels of PCSK9 or LDLR in mPCSK9 HepG2 cells (Figure 3, F and G).

miR-483-5p increased LDL uptake in hepatocytes. The functional consequence of miR-483 targeting PCSK9 was evaluated by testing its impact on LDLR-mediated LDL clearance. Overexpression of pre–miR-483 in HepG2 cells significantly increased the binding of fluorescence-labeled LDL, as assessed by flow cytometry and immunostaining (Figure 4, A and C). The miR-483–mediated increase in LDL binding was absent in mPCSK9 HepG2 cells.
HepG2 cells (Figure 4, B and D). Transfection of anti–miR-483 had the opposite effect and decreased LDL binding in HepG2 cells but had no impact on LDL binding in mPCSK9 HepG2 cells (Figure 4, A–D). Mature PCSK9 is secreted from cultured hepatocytes to the conditioned media. As expected, overexpression of pre–miR-483 decreased and anti–miR-483 transfection increased PCSK9 levels in conditioned media, as revealed by ELISA (Figure 4E, top) and Western blot analysis (Figure 4F, top). However, neither pre–miR-483 nor anti–miR-483 altered PCSK9 secretion in medium from mPCSK9 HepG2 cells (Figure 4E, bottom, and Figure 4F).

Part of a counterregulatory effect of statins is the SREBP2-mediated induction of both LDLR and PCSK9 in hepatocytes, which in turn decreases LDLR expression (3, 11). We tested whether miR-483-5p could reverse this unwanted effect of statins. Pre–miR-483 transfection in HepG2 cells decreased the atorvastatin-conferred increase in PCSK9 mRNA and protein levels (Figure 4, G and H). Importantly, this reduction in PCSK9 levels with combined atorvastatin and pre–miR-483 further increased LDLR expression compared with atorvastatin alone; this effect of miR-483-5p on rectifying LDLR expression was not observed in mPCSK9 HepG2 cells (Figure 4E, bottom, and Figure 4F).

Data in Figure 3 and Figure 4 suggest that miR-483-5p directly targets the 3′-UTR of PCSK9 mRNA in cultured hepatocytes, which results in augmented LDLR expression and increased LDL uptake.

Decreased circulating levels of miR-483 in hyperlipidemic mice and humans. Because miR-483, a secretory miR, and circulatory levels of PCSK9 are increased in hyperlipidemic rodent models and human subjects (18, 20, 22), we investigated whether hyperlipidemia is associated with reduced levels of miR-483 in circulation. Initially, we compared the circulatory levels of miR-483-5p and total cholesterol among 3 mouse groups: C57BL/6 mice fed an HFD or chow diet and Ldlr-knockout mice fed an HFD. Total cholesterol levels were greatly increased and serum levels of miR-483-5p were significantly reduced in...
**Ldlr**-knockout mice fed an HFD compared with WT mice fed an HFD or chow diet (Figure 5, A and B). Pearson’s correlation analysis showed an inverse correlation between serum levels of miR-483 and total cholesterol in these mice (\( P < 0.01, R^2 = 0.90, n = 24 \)) (Figure 5C). With these results from mouse models, we next explored whether this inverse correlation existed in a cohort of 179 humans without a diagnosis of cardiovascular disease. The serum levels of miR-483-5p and total cholesterol were indeed inversely correlated among these individuals (\( P < 0.01, R^2 = 0.20 \)) (Figure 5D). To this end, we separated these individuals into 4 groups based on their LDL-C levels (i.e., <100 mg/dL [optimal]; 100–129 mg/dL [near/above optimal]; 130–159 mg/dL [borderline high]; ≥160 mg/dL [high]; ref. 23).

Compared with individuals with LDL-C less than 100 mg/dL, for the other 3 groups, the serum levels of miR-483-5p were significantly lower (\( P = 0.699 \) vs. near/above optimal, 0.196 vs. borderline high, and < 0.0001 vs. high) (Supplemental Figure 4). Consistent with the correlation shown in Figure 5D, the serum levels of LDL-C and miR-483-5p were inversely correlated (Figure 5E). When comparing the group with LDL-C less than 100 mg/dL (optimal) with the other 3 groups (near/above optimal; borderline high; high), the larger difference in LDL-C levels, the inverse correlation of miR-483-5p levels was more significant (Figure 5F). Together, these data from mouse models and humans suggest that the serum levels of miR-483 were inversely correlated with hyperlipidemia.

miR-483 reduced LDL-C levels by targeting hepatic Pcsk9 in mouse models. Results from Figures 1–5 led us to investigate whether exogenously administered miR-483 can alleviate HDF-induced hyperlipidemia in
Figure 4. miR-483 overexpression in HepG2 cells increases LDL-C uptake.

(A–H) HepG2 and mPCSK9 HepG2 cells were transfected with pre-483 or anti-483 as indicated. Fluorescent-labeled LDL was incubated with HepG2 and mPCSK9 HepG2 cells. LDL uptake was detected by flow cytometry (A and B) or confocal microscopy (C and D) (original magnification, ×20; scale bars: 10 μm). (E and F) Levels of PCSK9 in conditioned media were measured by ELISA and Western blot analysis. (G and H) HepG2 and mPCSK9 HepG2 cells were incubated with 1 μM atorvastatin for 24 hours. mRNA and protein levels of PCSK9 and LDLR were determined by qPCR and Western blot analysis. In MT HepG2 cells, *LDLR in the same samples were detected in parallel in a separate gel (H). Data are mean ± SEM from at least 4 independent experiments. In A, B, and F, non-normally distributed data were analyzed using Mann-Whitney U test between 2 groups. In C–E, normally distributed data were analyzed by 2-tailed Student’s t test with Welch correction between 2 groups. In G and H, non-normally distributed data were analyzed using Kruskal-Wallis test with Dunn’s multiple comparisons between indicated groups. *P < 0.05 vs. Ctrl or between 2 indicated groups. miR, microRNA; HepG2, human hepatocellular carcinoma; LDL-C, LDL-cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9.
mouse models. We used AAV8-infected pri–miR-483 (hereafter called AAV-483) to achieve hepatic over-
expression of miR-483. Male and female C57BL/6 mice administered AAV-483 or parental empty AAV8 vector (AAV-null) were fed an HFD for 6 weeks. A group of mice administered AAV-null and fed a chow diet was a baseline control (Figure 6A). Hepatic miR-483-5p levels were significantly elevated in mice receiving AAV-483 versus AAV-null (Figure 6B). Moreover, the efficacy of hepatic delivery of miR-483 by AAV-483 was evidenced by much higher miR-483 levels in the liver than other nontarget tissues such as kidney, lung, and heart (Supplemental Figure 5). As anticipated, PCSK9 protein levels were decreased and LDLR protein levels were increased in the livers of animals receiving AAV-483 (Figure 6C). As a positive control, CTGF protein levels were decreased in the livers of these animals (Figure 6C). In line with the results that miR-483 targeted PCSK9 and CTGF, mRNA levels of Pcsk9 and Ctgf were enriched in miRISCs...
isolated from the livers of mice receiving AAV-483 versus AAV-null (Figure 6D). The HFD-induced hypercholesterolemia was evident from the lipoprotein profiles in male and female mice administered AAV-null, as measured by colorimetric assay and fast protein liquid chromatography (FPLC) (Figure 6, E and F, and Supplemental Table 1). AAV-483 administration reduced the HFD-induced hypercholesterolemia by reducing the levels of circulating IDL and LDL without affecting VLDL or HDL-associated cholesterol (Figure 6, E and F). Of note, the serum levels of PCSK9 and total cholesterol were inversely correlated with liver miR-483-5p expression (Figure 6G). We reasoned that the mechanism by which miR-483 decreases LDL-C levels depends on its suppression of the PCSK9–LDLR axis. Thus, as negative controls, male and female Ldlr-knockout mice were fed an HFD and administered AAV-483 or AAV-null. Although AAV-483 greatly increased miR-483-5p levels and blunted the PCSK9 and CTGF levels in the livers of Ldlr-knockout mice (Supplemental Figure 6, A and B), the levels of total cholesterol and LDL-C were comparable between mice administered AAV-483 or AAV-null (Supplemental Figure 6, C and D, and Supplemental Table 1).

**Discussion**

This study demonstrates that the exogenously administered miR-483 can substantially reduce total cholesterol and LDL-C levels in hypercholesterolemic mouse models. The underlying mechanism relies on miR-483 inhibiting PCSK9 expression, thereby increasing the hepatocyte expression of LDLR. Mechanistically, miR-483 targeting the 3′-UTR of PCSK9 mRNA was validated by luc-PCSK9 reporter assay (Figure 3D) and Ago-IP experiments (Figure 3E and Figure 6D). miR-483 and PCSK9 mRNA in the miRISCs were also enriched in the photoactivatable-ribonucleoside–enhanced cross-linking and immunoprecipitation data set and Ago-IP experiments (Figure 3E and Figure 6D). miR-483 and PCSK9 mRNA were also enriched in the photoactivatable-ribonucleoside–enhanced cross-linking and immunoprecipitation data set and Ago-IP experiments (Figure 3E and Figure 6D).

In the mouse experiments, the amount of miR-483 administered would be above physiological levels. Such supraphysiological doses of miR-483, when given to mice, robustly increase the hepatic and circulating levels of LDL-C in mice receiving AAV-3′-UTR WT or AAV-3′-UTR in vivo was validated by comparing reported by Hafner et al. (24). miR-483 targeting of the 3′-UTR of PCSK9 mRNA was validated by Luc-PCSK9 reporter assay (Figure 3D) and Ago-IP experiments (Figure 3E and Figure 6D). miR-483 and PCSK9 mRNA in the miRISCs were also enriched in the photoactivatable-ribonucleoside–enhanced cross-linking and immunoprecipitation data set and Ago-IP experiments (Figure 3E and Figure 6D).

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In the mouse experiments, the amount of miR-483 administered would be above physiological levels. Such supraphysiological doses of miR-483, when given to mice, robustly increase the hepatic and circulating levels of LDL-C. We also conducted experiments with doses as low as 4 × 10⁹ vector genomes of AAV-483, which still showed efficacy in modulating the expression of PCSK9 and LDLR (Supplemental Figure 7). The Further Cardiovascular Outcomes Research with PCSK9 inhibition in Subjects with Elevated Risk study and the ODYSSEY OUTCOME trial showed a greater reduction of LDL-C levels and cardiovascular events by combination therapy with PCSK9 mAb and statins than with statin monotherapy (10, 25, 26).

Data in Figure 4, G and H, show that miR-483 directly targeting the 3′-UTR of PCSK9 mRNA could mitigate the unwanted effect of statins in increasing plasma PCSK9 protein levels. These results demonstrate the potential use of miR-483 together with statins for treating hypercholesterolemia.

PCSK9 mAbs bind to the catalytic site of PCSK9, thereby inhibiting the extracellular interaction of PCSK9 with the EGF-A domain of the LDLR (6). Presumably, miR-483 inhibits PCSK9 via targeting PCSK9 mRNA intracellularly in hepatocytes. In humans, the plasma level of PCSK9 is positively correlated with the LDL-C level (22). PCSK9 levels were reduced in both the livers and the serum of our mice receiving AAV-483. These results suggest that miR-483 targeting PCSK9 mRNA in the liver decreased the amount of PCSK9 in circulation, which is consistent with decreased PCSK9 levels in patients receiving evolocumab or alirocumab (27, 28). Although miR-483 may decrease intracellular PCSK9 mRNA levels in hepatocytes, the PCSK9 mAbs could antagonize PCSK9 in circulation. The synergism of miR-483 and
PCSK9 mAbs may have greater cholesterol-lowering effects. Intriguingly, the circulatory levels of miR-483-5p contrasted with LDL-C levels in hyperlipidemic mouse models and humans, and miR-483-5p levels seemed to decrease more with high LDL-C levels (Figure 5). Despite unclear homeostatic ranges and regulatory mechanisms of the circulatory level of miR-483, our results support the use of exogenously administered miR-483 to ameliorate hyperlipidemia.

Besides miR-483, several other miRNAs, including miR-222, -191, and -224, have been predicted to target PCSK9 mRNA (17). However, the efficacy of these miRNAs in lowering LDL-C levels and attenuating atherosclerosis have not been tested in vitro and in vivo. Besides, results in Figure 2 suggest that miR-483 has a greater potency than other miRs in PCSK9 targeting. Besides targeting PCSK9 mRNA, miR-483 likely targets several genes and pathways involved in nonalcoholic fatty liver disease (NAFLD), including IL1B, IL6, TGFBI, and monocyte chemoattractant protein 1 (20). Thus, miR-483 may exert a pleiotropic effect to mitigate hyperlipidemia-associated NAFLD. In support of this thesis, we found that mice fed an HFD had...
less severe NAFLD when miR-483 was coadministered. This additional beneficial effect of miR-483 was evidenced by the alleviated lipid deposition in the liver, reduced levels of hepatic transaminases in circulation, and attenuated expression of \( \text{Il1b}, \text{Tnfa}, \text{Tgfb1}, \text{and Fbn1} \) (Supplemental Figure 8).

Both miR-483-3p and miR-483-5p are encoded by the \( \text{IGF2-miR-483} \) gene. From bioinformatics prediction, miR-483-5p may target the cognate sequence in the \( \text{PCSK9} \) mRNA better than miR-483-3p. Experimentally, luciferase reporter and LDLR expression assays validated the superiority of miR-483-5p in targeting \( \text{PCSK9} \) transcript (Figure 3, C and D, and Supplemental Figure 9). Thus, for therapeutic efficacy, miR-483-5p delivery should be considered. For miR-based therapeutics, the stability and specificity of miR delivery are 2 major challenges. Compared with the delivery systems used in current miR-related clinical trials (e.g., locked nucleic acids-, N-acetyl-D-galactosamine-, or cholesterol-conjugated miRs), the AAV8-based system features high efficacy for hepatic delivery, but this approach is still limited to experimental animals (29). Recent advancement in nanoparticles for tissue-specific drug delivery may be considered for therapeutic use of miR-483-5p (30, 31). Additionally, optimization of natural miR-483 function with medicinal chemistry approaches may allow for more translational therapeutic approaches in humans (32).

In summary, we found a mechanism by which miR-483-5p increases the hepatic expression of LDLR via targeting \( \text{PCSK9} \) 3′-UTR. The pharmaceutical efficacy of this mechanism relies on miR-483 administration greatly reducing total cholesterol and LDL-C levels in experimental hyperlipidemia.

**Methods**

**Cell culture.** Human hepatocellular carcinoma cells (HepG2) were obtained from ATCC (catalog HB-8065). Cells were maintained in DMEM (Gibco) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate at 37°C and 5% CO2.
Bioinformatics prediction of miRNA targeting sites. Putative miR-483 targeting sites in the 3′-UTR of the PCSK9 gene were predicted by using miRanda v3.3a with the parameters “-sc 120, -scale 4, -en -10” (33). The predicted binding sites with the highest score were selected for validation.

miR mimics and anti-miRs transfection. The miR mimics (pre-miR) and anti-miRs were purchased from Ambion. HepG2 cells at 50%–70% confluence were transfected with 60 nM pre-miR or anti-miR using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium for 6 hours and then changed to complete growth medium. At 24 hours after transfection, cells were lysed. Equal amounts of MirVana miRNA Mimic Negative Control #1 (i.e., Pre-Ctrl) or anti-miR miRNA Inhibitor Negative Control #1 (i.e., Anti-Ctrl) were used as controls for pre-miR or anti-miR experiments, respectively.

Luciferase reporter plasmids, transfection, and luciferase assay. The 908-bp human PCSK9 3′-UTR containing the 483 putative binding site was subcloned (forward primer, CGGACTAGTACTGGTGCGG-CATTTCACCAC, reverse primer, CGACGCCGTGCAACAGAGGACAGACCAC); restriction enzyme cutting on both ends with SpeI and MluI, respectively) into the pMIR-REPORT vector (Ambion) to generate the pMIR-Luc-PCS9K-3′-UTR WT reporter [Luc-PCS9K (WT)]. Then the CGG to GCC mutations in the miR-483 binding seed sequence of the PCSK9 3′-UTR were introduced into the Luc-PCS9K (WT) plasmid by using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies).

Renilla luciferase plasmid (pRL-TK) was used as the transfection control. Luc-PCS9K (WT), Luc-PCS9K (MT), and control pRL-TK plasmids were cotransfected into HepG2 cells by using Lipofectamine 2000 (Invitrogen). At 24 hours after transfection, cells were lysed for luciferase activity measurement with the Dual-Glo Luciferase Reporter Assay Kit (Promega).

Ago1 and Ago2 immunoprecipitation. HepG2 cells were cross-linked under 400 mJ/cm² UV light, then lysates were incubated with protein G Dynalbeads conjugated with anti-Ago1 or anti-Ago2 antibody (Wako Chemicals) at 4°C overnight. The same amount of mouse IgG was used as an isotype control. The immunoprecipitated RNAs and input RNAs were extracted by using Trizol for qPCR detection.

AAV8 hepatic-specific overexpression vectors. pAAV8/D377Y-mPCSK9 plasmid was obtained from Addgene. Mouse Pcsk9 3′-UTR was subcloned into the FseI site (WT, AAV8-Pcsk9-3′-UTR WT), then the 5′-CTGTCT-3′ miR-483 binding site was deleted (ABS, AAV8-Pcsk9-3′-UTR ABS) as shown in Figure 1A. AAV8-miR-483 was constructed with the Pcsk9 coding sequence replaced by mouse pri-miR-483 between Agel and FseI sites. AAVs and empty control viruses were enveloped by the UCSD Vector Development Core. Various AAV8 viruses (1 × 1012 vector genomes) were administered to mice by tail vein injection.

CRISPR/Cas9-mediated miR-483 binding-site disruption. Single-guide RNAs were designed by using an online tool (34) (Supplemental Figure 3). Oligonucleotide pairs with BbsI-compatible overhangs were annealed and cloned into the vector pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene). The XhoI restriction enzyme site was then introduced into the 5′ end of the inserted gRNA and subcloned with SnaBI in the 3′ end. The product including the gRNA and gRNA scaffold was inserted into the Adeno Cas9 plasmid (Addgene) for virus production. Adenovirus and empty control viruses were enveloped by the UCSD Vector Development Core. HepG2 cells were infected with the produced adenovirus or empty control virus at 3 × 10⁷ plaque-forming units to each well of a 6-well plate for 2 days. The mixed population of infected HepG2 cells was selected for genotyping.

RT-qPCR and Western blot analysis. RNA was isolated from cultured cells, liver tissues, or serum by using TRIzol or TRIzol LS (Invitrogen). Total RNA was reverse-transcribed with use of the PrimeScript RT-PCR Kit (Takara), followed by qPCR with SYBR Green (Bio-Rad) in a Bio-Rad CFX-96 real-time system. Primers used in this study are in Supplemental Table 2. The relative mRNA level was calculated by the 2^(-ΔΔCq) method with β-actin as an internal control. For miR quantification, the TaqMan MicroRNA Assay followed the manufacturer’s protocol (Life Technologies). U6 was detected as the internal control in cultured cells and tissues from animals. To quantify the miR-483 level in serum, a C. elegans miR (i.e., Cel-miR-39) was spiked in at 2 nM before RNA extraction, with the level used as the internal control.

Protein extracts from cultured cells or tissues were resolved by SDS-PAGE and transferred to 0.45 μm NC membranes. Western blot analysis was performed with the following antibodies: anti-PCSK9 (Abcam, catalog ab28770), anti-LDLR (Abcam, catalog ab30532), anti-CTGF (Abcam, catalog ab6992), anti-α-tubulin (Cell Signaling, catalog 2144), and anti-β-actin antibody (Santa Cruz Biotechnology, catalog sc-47778). Horseradish peroxidase-conjugated anti-rabbit (Cell Signaling, catalog 7074), anti-mouse (Cell Signaling, catalog 7076), or anti-goat (Dako, catalog P0449) were used as secondary antibodies.

LDL uptake assay. Monolayers of HepG2 cells were incubated with 50 μg/mL fresh, isolated, nonacetylated human LDL labeled with Dil (Invitrogen) for 1 hour in the dark with serum-free medium. Cells were
collected by venipuncture from median cubital vein after overnight fasting. After centrifugation at 1500 g of human subjects are summarized in Supplemental Tables 3 and 4. An amount of 3 mL whole blood was from the First Affiliated Hospital of Xi'an Jiaotong University during 2018–2019. The baseline characteristics fraction were determined by using cholesterol and triglycerides assay kits (SEKISUI Diagnostics). (Biovision) was used for hepatic lipid extraction. The levels of total cholesterol and triglycerides in each and 0.02% sodium azide, pH 7.4. Fractions (0.5 mL) were collected (0.5 mL/min). A lipid extraction kit ples were loaded on a GE Superose 6 10/30 GL column in 0.15 M sodium chloride containing 1 mM EDTA lipoprotein levels were analyzed by running 100 μL pooled serum onto a gel-filtration FPLC system. Sam-

ELISA. PCSK9 levels in cell culture medium and serum were measured with a human or mouse PCSK9 ELISA Kit (R&D Systems) according to the manufacturer’s protocols. Briefly, cell culture supernatants or serum samples were diluted and incubated in precoated plates for 2–4 hours. After 2 hours of incubation with horseradish peroxidase–conjugated secondary antibody, TMB substrate solution was added and sam-

Human serum samples and measurement of circulating miR-483. A group of 179 individuals were enrolled from the First Affiliated Hospital of Xi'an Jiaotong University during 2018–2019. The baseline characteristics of human subjects are summarized in Supplemental Tables 3 and 4. An amount of 3 mL whole blood was collected by venipuncture from median cubital vein after overnight fasting. After centrifugation at 1500g for 10 minutes, the serum was aliquoted into separator tubes, quickly frozen in liquid nitrogen, and stored at −80°C until use. The lipid profiles were detected by automatic chemical analysis (Hitachi LABOSPECT 008AS).

Statistics. All results are presented as mean ± SEM. Initially, data were tested for normality and equal vari-

Student’s t test for parametric data or Mann-Whitney U test for nonparametric data. Experiments with more than 2 groups were compared by 1-way ANOVA with a Bonferroni’s post hoc test for parametric data or Krus-
kal-Wallis test with Dunn’s multiple comparisons for nonparametric data. All statistical analyses were performed with GraphPad Prism version 5.01; 2-tailed P values of less than 0.05 were considered statistically significant.

Study approval. The UCSD animal care personnel maintained all animals in accordance with NIH guidelines, and the IACUC of UCSD approved all experimental procedures (approval no. S12263). UCSD has an Animal Welfare Assurance document (A3033-01) on file with the Office of Laboratory Animal Welfare and is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The study protocol for humans was approved by the ethics committees of Xi’an Jiaotong University. Written informed consent was obtained from all subjects.

Author contributions

JYJS, MH, and JD conceived the original idea and designed the overall experimental plan. JD, MH, J. Li, AP, CW, JZ, YS, WTW, YZ, J. Liu, SCW, and PHH performed experiments. JD and MH interpreted the data and performed statistical analysis. ST, ZYY, and PLSMG provided essential input to the overall research plan. MH, JD, JYJS, ST, and PLSMG wrote the manuscript.
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