Novel Reversible Model of Atherosclerosis and Regression Using Oligonucleotide Regulation of the LDL Receptor

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Rationale: **Animal models have been used to explore factors that regulate atherosclerosis. More recently, they have been used to study the factors that promote loss of macrophages and reduction in lesion size after lowering of plasma cholesterol levels. However, current animal models of atherosclerosis regression require challenging surgeries, time-consuming breeding strategies, and methods that block liver lipoprotein secretion.**

Objective: **We sought to develop a more direct or time-effective method to create and then reverse hypercholesterolemia and atherosclerosis via transient knockdown of the hepatic LDLR (low-density lipoprotein receptor) followed by its rapid restoration.**

Methods and Results: **We used antisense oligonucleotides directed to LDLR mRNA to create hypercholesterolemia in wild-type C57BL/6 mice fed an atherogenic diet. This led to the development of lesions in the aortic root, aortic arch, and brachiocephalic artery. Use of a sense oligonucleotide replicating the targeted sequence region of the LDLR mRNA rapidly reduced circulating cholesterol levels because of recovery of hepatic LDLR expression. This led to a decrease in macrophages within the aortic root plaques and brachiocephalic artery, that is, regression of inflammatory cell content, after a period of 2 to 3 weeks.**

Conclusions: **We have developed an inducible and reversible hepatic LDLR knockdown mouse model of atherosclerosis regression. Although cholesterol reduction decreased early en face lesions in the aortic arches, macrophage area was reduced in both early and late lesions within the aortic sinus after reversal of hypercholesterolemia. Our model circumvents many of the challenges associated with current mouse models of regression. The use of this technology will potentially expedite studies of atherosclerosis and regression without use of mice with genetic defects in lipid metabolism. (*Circ Res*. 2018;122:560-567. DOI: 10.1161/CIRCRESAHA.117.311361.)**

Key Words: cholesterol, LDL ■ lipids ■ lipoprotein ■ macrophage ■ metabolism

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With the development of mice with genetic loss of apo (apolipoprotein) E or the LDLR (low-density lipoprotein receptor),[1,2] investigators have been able to create atherosclerosis in mice, a species that normally has relatively low circulating levels of apoB-containing lipoproteins. These models proved the low-density lipoprotein (LDL) hypothesis, as a single mutation changing lipid metabolism led to atherosclerotic disease in previously disease-free animals. ApoE-deficient and LDLR-deficient mice have become the standard models for investigating how lipoprotein alterations, diet, and genetic changes in white blood cells and other arterial cells modulate atherosclerosis.

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Although vascular biologists have primarily studied atherogenesis, increasingly, both clinical medicine and investigative models now focus on changes in lesion size and loss of inflammatory macrophages that occur with cholesterol lowering. The conversion of inflamed atherosclerotic lesions into a more benign fibrotic scar-like histology is often referred to as regression. In humans, intravascular ultrasound studies found that ≈2/3 of patients have some lesion regression with high-dose statin therapy,[3] and more recently, with use of PCSK9 (proprotein convertase subtilisin kexin-like 9) antibodies.[4] Additionally,

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Novelty and Significance

What Is Known?

- Atherosclerosis regression is defined as the reduction of macrophage and lipid content of plaques when plasma low-density lipoprotein cholesterol is markedly decreased using drugs like statins.
- Studies of regression entails technically difficult aortic transplants or time-consuming breeding strategies.
- LDLR (low-density lipoprotein receptor) is the major receptor responsible for the removal of low-density lipoprotein cholesterol from circulation.

What New Information Does This Article Contribute?

- A new method to create atherosclerosis and its regression in C57Bl/6 mice is described, where hepatic LDLRs were knocked down with antisense oligonucleotides, leading to plaques in the aortic root and brachiocephalic artery.
- The expression of the receptors was restored by sense oligonucleotide treatment, which led to reversal of hypercholesterolemia and fewer plaque macrophages in the aortic root and brachiocephalic artery.
- This model of regression can be used with any genetically modified mouse strain and obviates the need for extensive breeding.

Several genetic mouse models and a surgical model have been used to study the pathophysiology of atherosclerosis regression in mice. However, these involve time-consuming breeding strategies or technically demanding surgeries. To overcome these challenges, we have created hypercholesterolemia and atherosclerosis in mice by transient knockdown of hepatic LDLR using an antisense oligonucleotides. Early fatty-streak lesions developed after 9 weeks, whereas more advanced lesions were observed in mice after 16 weeks. Subsequently, plasma low-density lipoprotein cholesterol was reduced using sense oligonucleotide to block the residual antisense oligonucleotides and restore hepatic LDLR availability. Regression of lesional macrophages was observed in 2 different vascular beds, namely, aortic root and brachiocephalic artery. This novel in vivo strategy is readily transferable and should expedite studies of atherosclerosis and regression.

Nonstandard Abbreviations and Acronyms

Apo	apolipoprotein
ASO	antisense oligonucleotides
BCA	brachiocephalic artery
CD	chow diet
IDOL	inducible degrader of the LDL receptor
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
MTP	microsomal triglyceride transfer protein
PCSK9	proprotein convertase subtilisin kexin-like 9
SO	sense oligonucleotides
TC	total cholesterol
vldlr	very low-density lipoprotein receptor
WD	Western diet
WT	wild type

simvastatin treatment reduced plaque inflammation in humans, measured by ^{18}F-fluorodeoxyglucose accumulation, which corresponds to the macrophage-rich area of the plaque.[5] The biology of this remodeling has also been studied in mice, and investigators have delineated many of the cellular requirements for regression.[6] For example, we and others have reproduced the defects in atherosclerosis regression found with diabetes mellitus[7,8] and have shown the importance of changes in macrophage number and gene expression with cholesterol reduction.

Atherosclerosis regression requires reduced arterial exposure to high circulating lipoprotein levels. Marked reductions in circulating apoB-cholesterol have been achieved using the following methods: (1) arteries have been transplanted from hypercholesterolemic *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice to normolipidemic wild-type (WT) C57BL/6 mice[9–12]; (2) *Apoe*, *Ldlr*, or *Vldlr* (very low-density lipoprotein receptor) have been reintroduced using adenoviruses[13–18]; (3) MTP (microsomal triglyceride transfer protein) has been deleted in the Reversa mouse or pharmacologically inhibited[19–21]; or (4) apoB synthesis has been blocked using antisense oligonucleotides (ASOs).[22] These models, reviewed in detail elsewhere,[23,24] have certain limitations. Arterial transplantation is technically demanding, while reduction of hepatic production of apoB-containing lipoproteins results in fatty liver and altered triglyceride-rich lipoprotein metabolism. Finally, the need to use *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice to induce lesions is costly and time consuming because genes of interest have to be crossed onto these mouse models of atherosclerosis. Recently, adeno-associated virus–mediated overexpression of PCSK9 or IDOL (inducible degrader of the LDL receptor) have been used to induce atherosclerosis.[25–28] However, reversal of hypercholesterolemia requires MTP inhibition or long periods of chow diet (CD) feeding.[29]

Here, we describe the use of an ASO targeted to LDLR mRNA to create hypercholesterolemia in WT C57BL/6 mice. As expected, this resulted in atherosclerosis development in the aortic arch, aortic sinus, and brachiocephalic artery (BCA). We then describe methods to reduce cholesterol by gradual decay of LDLR ASO after its withdrawal or by more rapid reversal with injection of LDLR sense oligonucleotides (SOs). This novel methodology is expected to markedly facilitate mechanistic studies on atherosclerosis regression.

Methods

The authors declare that all supporting data are available within the article (and its Online Data Supplement).

Mice and Diets

All procedures were approved by the Institutional Animal Care and Use Committees at New York University Langone Health and University Clinic Freiburg, Germany. Twelve to 14-week-old, male and female C57BL/6J mice were maintained in a temperature-controlled (25°C) facility with a 12-hour light/dark cycle. Male mice were used for a majority of experiments to avoid introducing variability attributable to hormonal changes in female mice. Mice were given free access to water and food, except when fasting blood specimens

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were obtained. Mice were either fed a rodent CD or an atherogenic Western diet (WD; Dyets, Inc, catalog number: 101977; 0.3% cholesterol), as indicated. For the data shown in Online Figure IV, modified WD (Research Diet D12108; 1.25% cholesterol) was used.

Oligonucleotide Treatments

GalNAc-conjugated Gen 2.5 ASO targeting mouse LDLR was developed and provided by Ionis Pharmaceuticals. Previous reports have demonstrated ≤30-fold improvements in liver ASO potency with GalNAc conjugation together with Gen 2.5 (cET) ASO modifications.[30,31] LDLR ASO was injected intraperitoneally at a dose of 5 or 2.5 mg/kg body weight (Online Figure IV) once a week for 9 or 16 weeks, as indicated. GalNAc-conjugated SO designed to bind and inactivate the LDLR ASO was injected once intraperitoneally at a dose of 20 mg/kg body weight.

Blood Sampling

All blood samples were collected after 4 hours of fasting. Blood was collected from the retro-orbital plexus of mice using heparinized micro capillary tubes. Blood was centrifuged at 10 000*g* for 10 minutes for cell removal and collection of the plasma, which was then used for lipid measurements and frozen at −80°C.

Tissue Collection

Mice were deeply anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg) and then perfused by heart puncture with 10 mL of PBS or until the livers blanched. Tissues were rapidly excised and snap frozen in liquid nitrogen unless otherwise noted.

Lipid Measurements

Total cholesterol (TC) was measured using Infinity Total Cholesterol Reagent (#TR13521; Thermo Scientific, Waltham, MA).

Lipoprotein Fractionation

Equal amounts of mouse plasma (70–100 µL) were used for sequential density ultracentrifugation to separate very-low-density lipoprotein (d<1.006 g/mL), LDL (d=1.006–1.063 g/mL), and high-density lipoprotein (d=1.063–1.21 g/mL) in a TLA 100 rotor (Beckmann Instruments, Palo Alto, CA). Fractions were used to measure TC, as described above.

Western Blot Analysis

Snap-frozen liver samples were homogenized using a RIPA (radioimmunoprecipitation) buffer with protease inhibitors. Protein concentrations were determined using the Pierce BCA Protein kit (#23227; Thermo Scientific, Rockford, IL). Forty microgram samples of total protein were subjected to Western blot analysis with a mouse LDLR antibody (R&D AF2255). GAPDH antibody (#9484; Abcam) was used for control of protein loading.

Tissue Gene Expression

Total RNA was prepared using a GeneJET RNA Purification Kit (Thermo Scientific). One microgram of RNA was used for reverse transcription using the Verso cDNA synthesis kit (Thermo Scientific). Real-time quantitative polymerase chain reaction was performed using an ABI 7700 (Applied Biosystems). Amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers used for polymerase chain reaction amplification were obtained from Primer Bank. Analysis was performed using Sequence Detection Software (Applied Biosciences). Data were normalized to *Rn18s*.

Atherosclerotic Lesion Analysis

Each anesthetized mouse was perfused with PBS. The aorta was then exposed, and fat was carefully cleaned under a binocular microscope. Pictures of the aortic arch and BCA were taken using a camera fitted to the binocular microscope. The BCA was collected in 10% formalin, kept overnight at 4°C, and then stored in 70% ethanol at 4°C for further processing. The root of the heart was cut and embedded in TissueTek optimal cutting temperature, frozen, and stored at −80°C.

Histological and Morphometric Analysis of Aortic Roots

Serial sections (6 µm) were obtained by cryosectioning the frozen aortic roots that were embedded in optimal cutting temperature. The sections were then stained with Picrosirius Red and analyzed for lesion size using brightfield microscopy. Collagen content was measured under polarized light. Mac-2 or CD68 immunostaining was used to determine macrophage content in the aortic root sections, as described previously.[32–34]

Staining and Analysis of BCAs

BCAs embedded in paraffin were sectioned (5 µm). Every fifth cross section was stained using the Movat pentachrome method to visualize atherosclerotic lesions. The maximal lesion site was determined, and adjacent sections were immunostained using a Mac-2 antibody to detect lesion macrophages, as described previously.[35]

Morphometric Measurements

Morphometric measurements were performed on digitized images of stained serial sections by using IMAGEPRO PLUS software. At least 12 BCA sections per mouse were analyzed (60 sections/mouse with every fifth section used for Movat stain and morphometry), and the maximal lesion area value for each mouse was used as the summary parameter. At least 5 sections per root were analyzed, and the mean value used as the summary parameter.

Statistical Analysis

Data are presented as means±SD. Statistical differences were assessed via unpaired Student *t* test or a 1-way or 2-way ANOVA using GraphPad Prism 7 where appropriate. A *P* value of <0.05 was considered as significant.

Results

Effect of LDLR ASO on Plasma Cholesterol in WT Mice

Male C57BL/6J mice were treated with intraperitoneal injections of LDLR ASO or control ASO once a week for 9 weeks. The mice were fed an atherogenic WD throughout the study (Figure 1A). Plasma TC levels increased within a week of ASO treatment and stayed high (≈400–800 mg/dL) throughout the 9 weeks (Figure 1B) compared with control ASO-treated mice. LDLR ASO treatment led to hypercholesterolemia in both male (Figure 1B) and female mice (Online Figure I). Hepatic LDLR knockdown was confirmed (Figure 1D) at the end of the study. One group of mice, denoted baseline, was euthanized after 9 weeks of LDLR ASO treatment. The rest of the mice were divided into 2 groups. The first group, denoted SO, was treated with SOs antagonizing the residual LDLR ASO, whereas the second group, denoted None, received no further intervention. Mice in both the groups were kept on WD to monitor changes in plasma TC as a function of hepatic LDLR recovery. Plasma TC dropped substantially in both SO and None groups by 2 weeks (week 11); ≈ 135 mg/dL in SO group and ≈430 mg/dL in None group. After another week (week 12), both SO and None groups had similar TC levels (≈200 mg/dL; Figure 1C). LDLR expression recovered in both SO and None groups, with higher levels in the SO group, as shown by Western blot (Figure 1D) and ELISA (Online Figure II).

Atherosclerosis in LDLR ASO-Treated WT Mice

Direct visualization of aortae in the baseline group of mice showed fatty-streak–type early lesions in the aortic arch and adjacent BCA (Figure 1E, baseline, n=4–5). Remarkably,

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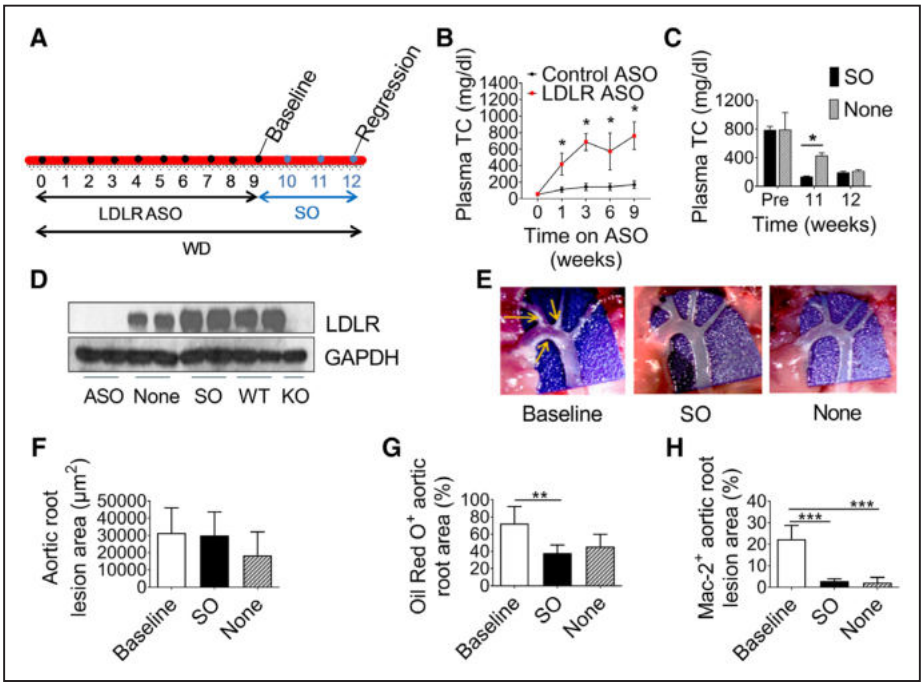

Figure 1. Model of atherosclerosis regression using oligonucleotide-mediated hepatic LDLR (low-density lipoprotein receptor) modulation. A, Experimental outline. **B**, Time course of plasma total cholesterol (TC) in 12-wk-old male mice treated with control antisense oligonucleotide (ASO) and LDLR ASO. All mice were fed Western diet (WD) throughout, n=18. **C**, Plasma TC levels after 9 wk of ASO treatment and at week 11 (2 wk after sense oligonucleotide [SO]) and week 12 (3 wk after SO), n=4 to 5/group. **D**, Western blot of hepatic LDLR at the end of the study. Controls for the Western blot are positive control—WT (C57BL/6J) mice on chow diet (CD) and negative control—KO (*Ldlr*$^{-/-}$) mouse on CD. **E**, Representative images of aortic arch lesions in the 3 groups, n=4 to 6/group. **F**, Quantification of aortic root lesion area after staining with Picrosirius Red. **G**, Quantification of Oil Red O^{+} lesion area. **H**, Quantification showing percentage of macrophage content in aortic root lesions, visualized by Mac-2 immunofluorescence in baseline mice and 2 regression groups of SO and None, n=4 to 5/group. **P*<0.05, ***P*<0.01, ****P*<0.001, data presented as means±SD.

mice in both SO and None groups showed drastic declines in en face visible lesions in the aortic arch and adjacent BCA (Figure 1E, SO and None, n=4–5/group). Next, lesions in the aortic roots were analyzed. Although total plaque areas were not different among the groups (Figure 1F), Oil Red-O–stained lesion areas were significantly reduced with SO treatment and

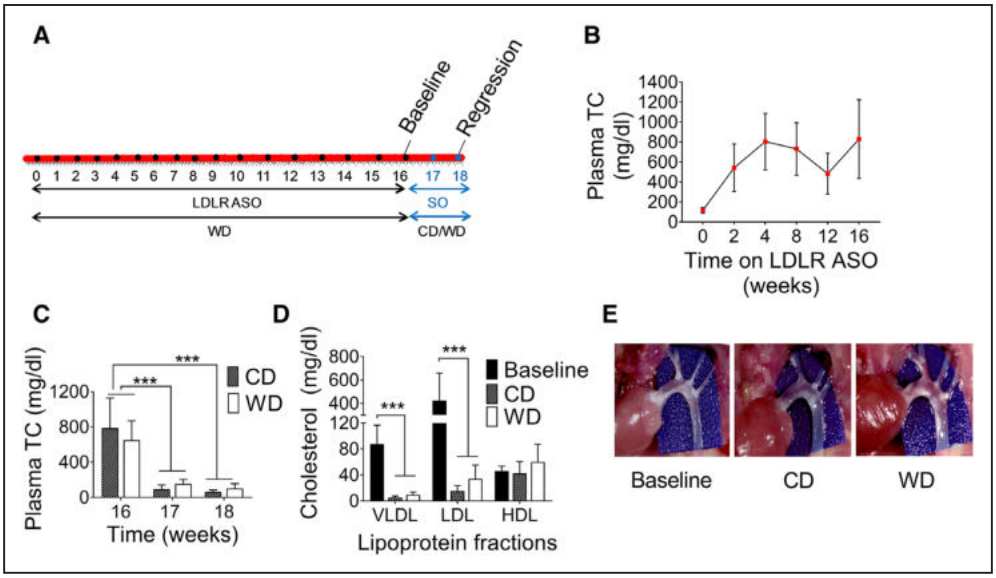

Figure 2. Regression of advanced atherosclerotic lesions. A, Experimental outline. **B**, Time course of plasma total cholesterol (TC) in 12- to 14-wk-old male mice treated with LDLR (low-density lipoprotein receptor) antisense oligonucleotide (ASO) and fed Western diet (WD). **C**, Plasma TC levels before sense oligonucleotide (SO) treatment (week 16), after 1 wk (week 17), and after 2 wk of SO (week 18); 1 group of mice was kept on WD and another was switched to chow diet (CD) after SO treatment. **D**, Cholesterol content in very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions of plasma in baseline group and the 2 regression groups of CD and WD at the end of the study. **E**, Representative images of aortic arch lesions in the 3 groups. n=15 to 16 per group ****P*<0.001, data presented as means±SD.

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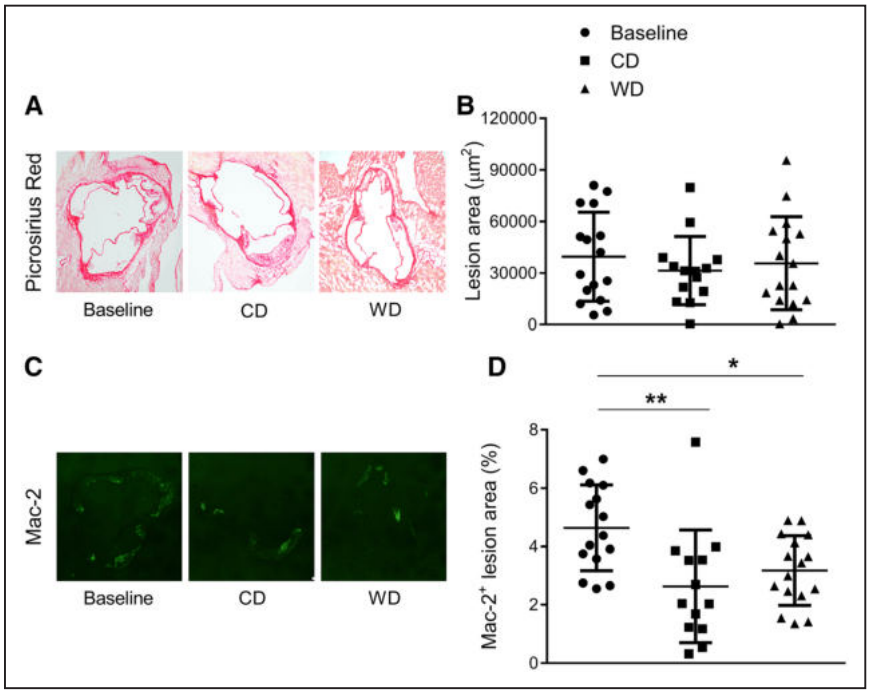

Figure 3. Assessment of atherosclerosis lesions in aortic roots of mice at baseline and after 2 wk of cholesterol reduction. A, Morphology of aortic root lesions in baseline mice and 2 regression groups of chow diet (CD) and Western diet (WD) visualized by Picrosirius Red brightfield microscopy and its (**B**) quantification. **C**, Macrophage content of aortic root lesions visualized by Mac-2 immunofluorescence and its (**D**) quantification showing Mac-2 positive lesion area (%). Representative images are shown. **P*<0.05, ***P*<0.01. n=15 to 16 per group, data presented as means±SD.

showed a trend toward a decrease in None group, compared with baseline (Figure 1G). Importantly, there was an extensive reduction of macrophage content within the aortic root lesions of both SO and None groups (Figure 1H).

These initial studies confirm that LDLR ASO-driven cholesterol increases (≈800 mg/dL) lead to atherosclerosis, which can then be reduced by lowering cholesterol levels (≈200 mg/dL).

Effect of LDLR SO and Diet on Complex Atherosclerotic Lesions

In our previous studies, we used longer hypercholesterolemic exposure to create advanced lesions relevant to human disease.[17,19,36] This allowed us to better assess the mechanisms responsible for monocyte recruitment and macrophage accumulation and to quantify reduced regression with diabetes mellitus.[7,8,17] For the current experiment, LDLR ASO treatment was extended to 16 weeks before initiating cholesterol reduction. The effect of CD versus WD on lesion regression was also assessed (Figure 2A). As before, plasma TC increased to ≈500 mg/dL within 2 weeks of LDLR ASO treatment and was maintained at high levels (≈800 mg/dL) until 16 weeks (Figure 2B). One group of mice was euthanized for baseline analysis at the 16-week time point when LDLR ASO treatments were stopped. From our initial study (Figure 1), we learned that including LDLR SO in our regression model allowed us to shorten the regression time. In this long-term study, cholesterol reduction was, therefore, induced by SO in all the mice, as we predicted that SO treatment would restore hepatic LDLR levels and lower plasma cholesterol faster than no treatment. TC levels were significantly lowered in both the CD- and WD-fed groups by 1 week of SO treatment; these levels remained low until the end of the study at week 18

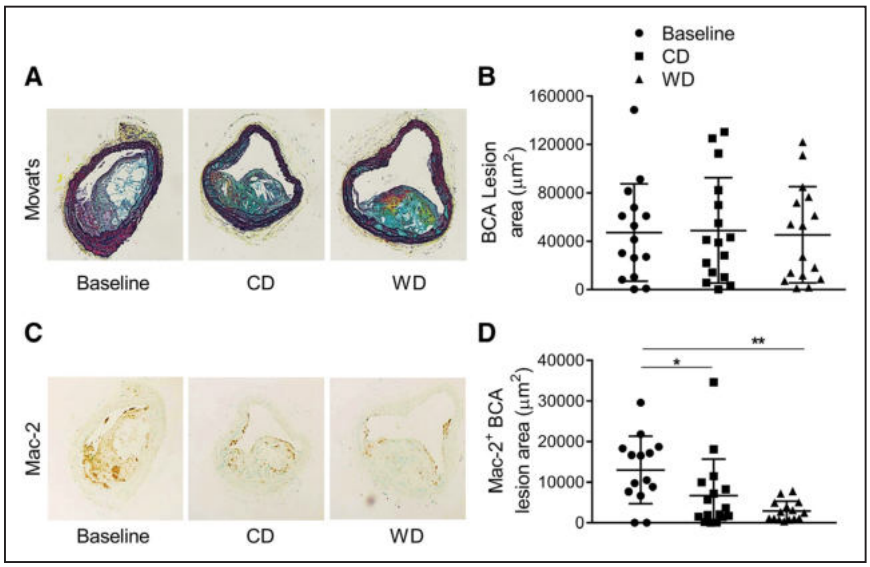

Figure 4. Assessment of atherosclerosis lesions in brachiocephalic artery (BCA) at baseline and after 2 wk of cholesterol reduction. A, Lesions in the BCA visualized by Movat stain. **B**, Quantification of total lesion area. **C**, Mac-2–positive area showing lesion macrophages and (**D**) its quantification, in the baseline group mice and the 2 regression groups of chow diet (CD) and Western diet (WD). Representative pictures of Movat staining and Mac-2 staining are shown. **P*<0.05, ***P*<0.01. n=15 to 16/group; data presented as means±SD.

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(Figure 2C). Plasma lipoprotein fractionation showed that decreased plasma TC after SO treatment was mainly caused by a reduction in LDL cholesterol (Figure 2D). The very-low-density lipoprotein cholesterol fraction was also significantly reduced in both regression groups, whereas high-density lipoprotein cholesterol was unaffected (Figure 2D). In contrast to the previous short-term study, no obvious changes in en face aortic arch or BCA plaque density were found between the 3 groups, on direct visualization (Figure 2E).

The continued opacity of the aorta despite cholesterol reduction has been shown in a prior study[17] and is most likely explained by the conversion of the plaques to fibrous lesions. To test this, we assessed the histology of atherosclerotic lesions in the aortic sinus. Picrosirius Red brightfield microscopy showed that total lesion area was not significantly altered in the baseline and the 2 regression groups (Figure 3A and 3B). Lesion collagen content, an indicator of vascular remodeling toward stable lesions, tended to increase in the regression groups (Online Figure III), but this did not reach statistical significance. However, macrophage content of lesions, as measured by Mac-2 immunofluorescence, was significantly reduced in the lesions of both the regression groups compared with the baseline (Figure 3C and 3D).

Lesions within the BCA were also characterized, as this site develops complex lesions more representative of certain human plaque characteristics. Similar to the observations in aortic root lesions, there were significantly fewer macrophages within BCA lesions of both CD and WD groups, compared with baseline (Figure 4C and 4D). Total lesion area, visualized by Movat staining, was unchanged among all 3 groups (Figure 4A and 4B).

Effect of a Lower Dose of LDLR ASO on Atherosclerosis

Finally, to determine whether the current protocol could be altered to allow use of less LDLR ASO, C57BL/6J WT mice were treated with half the dose of LDLR ASO (2.5 mg/kg) for 16 weeks (Online Figure IVA). This dose was sufficient to raise plasma TC to ≈500 mg/dL when the mice were fed a modified WD. When these mice were treated with SO and switched to CD, plasma cholesterol dropped <200 mg/dL (Online Figure IVB). Aortic root lesion analysis showed no changes in total plaque size and lipid content between baseline and SO groups (Online Figure IVC through IVE). However, macrophage content of lesions was reduced in the SO-treated mice compared with baseline (Online Figure IVF), similar to what was seen in our previous experiments.

Discussion

The development of atherosclerosis-prone mice nearly 25 years ago led to major advances in our understanding of the roles of circulating lipoproteins and inflammatory factors in the creation of arterial lesions. More recently, many investigators have focused on the regression of lesions in an effort to understand the biology that occurs when patients with atherosclerosis are treated with potent cholesterol-reducing medications. Clinical studies have shown that this intervention reduces the incidence of cardiovascular events,[37] even though the changes in atherosclerosis burden found using intravascular ultrasound is <2%.[4] This disconnect between events and lesion size has been explained by studies in mice that have shown that cholesterol reduction causes a more dramatic reduction in plaque macrophages and increase in collagen content than overall lesion size.[6] Thus, what others and we have observed is more properly the regression of the inflammatory state of the plaque, which in humans would be expected to decrease plaque vulnerability and increase stability. Defining the many cellular and circulating factors that mediate vascular remodeling, and determining how they are defective in diseases such as diabetes mellitus, is an active area of investigation.

In this report, we describe methods to both create atherosclerosis and then assess the biology of regression after cholesterol reduction. Using the ASO approach, hepatic LDLR expression was reduced followed by its reversal with introduction of LDLR SOs. This protocol allowed for the creation of atherosclerosis and its resolution, without the time-intensive and costly process of crossing mice onto a genetically hypercholesterolemic background. Early fatty-streak lesions developed after 9 weeks, whereas more advanced lesions were observed in mice after 16 weeks. Regression was induced without the need for transplantation or additional genetic or pharmacological treatments to reduce cholesterol. Although cessation of use of the LDLR ASO led to reduced cholesterol, the cholesterol reduction was more rapid using the SOs. Moreover, using the SOs, we could even achieve cholesterol lowering and regression of lesional macrophages while the mice continued to eat a high-cholesterol atherogenic diet (WD). We analyzed atherosclerosis lesions in 2 different vascular beds, namely, aortic root and BCA, both of which showed decreased macrophage content within lesions after cholesterol reduction.

We did not compare the effects of the LDLR ASO to the atherosclerosis that occurs in LDLR knockout (*Ldlr*$^{-/-}$) mice, although grossly, the characteristics of the lesions seemed similar to our historical experience with these mice. We and others have noted the marked variability in cholesterol levels of *Ldlr*$^{-/-}$ mice as a function of strain, diet, and perhaps even the microbiome.[38] We found similar variability in plasma cholesterol and lesions between LDLR ASO and *Ldlr*$^{-/-}$ mice.[39] As has been found in humans,[40] it is possible that genetic deficiency of LDLRs might lead to increased atherosclerosis over and above that which is expected from adult levels of LDL cholesterol levels. This might occur either because even on chow the LDLR deficiency is associated with small increases in cholesterol above normal or because the LDLR mutation leads to additional vascular toxicity. Also, reports have shown a significant contribution of macrophage LDLR toward lesion development, so our model described in this study would be expected to have less atherosclerosis compared with *Ldlr*$^{-/-}$ mice.[41,42] At the doses used (5 and 2.5 mg/kg), we would not have expected to see much activity of LDLR ASO outside the liver. Use of 2 different doses of LDLR ASO pointed to dose-dependent effect on cholesterol and possibly lesion size as well.

In summary, we describe a new method to create both atherosclerosis and regression in mice that do not have genetic alterations in lipoprotein metabolism. This method is readily transferable to use for assessment of atherosclerotic plaques and their repair in animals and will allow investigators to more

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conveniently test whether changes in leukocytes, smooth muscle cells, endothelial cells, or nonarterial cells affect atherogenesis.

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Disclosures

A.E. Mullick and M.J. Graham are employees of Ionis Pharmaceuticals and could be contacted by investigators who wish to obtain LDLR ASO for atherosclerosis studies. The other authors report no conflicts.

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Novel Reversible Model of Atherosclerosis and Regression Using Oligonucleotide Regulation of the LDL Receptor

Debapriya Basu, Yunying Hu, Lesley-Ann Huggins, Adam E. Mullick, Mark J. Graham, Tomasz Wietecha, Shelley Barnhart, Allison Mogul, Katharina Pfeiffer, Andreas Zirlik, Edward A. Fisher, Karin E. Bornfeldt, Florian Willecke and Ira J. Goldberg

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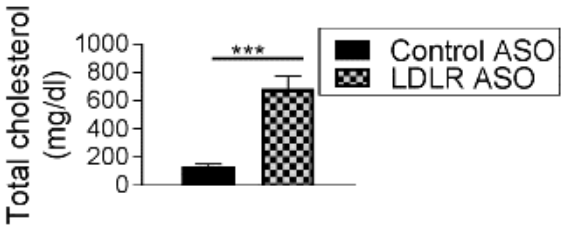
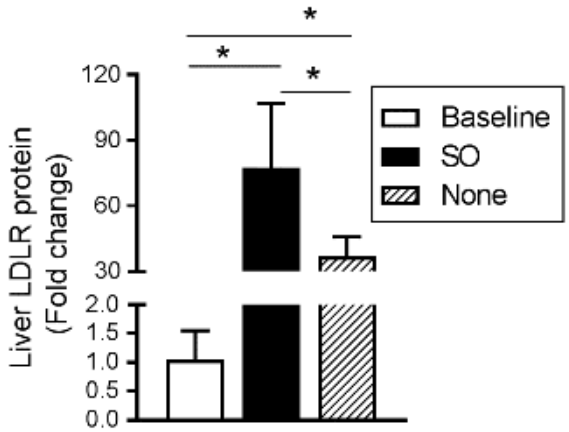
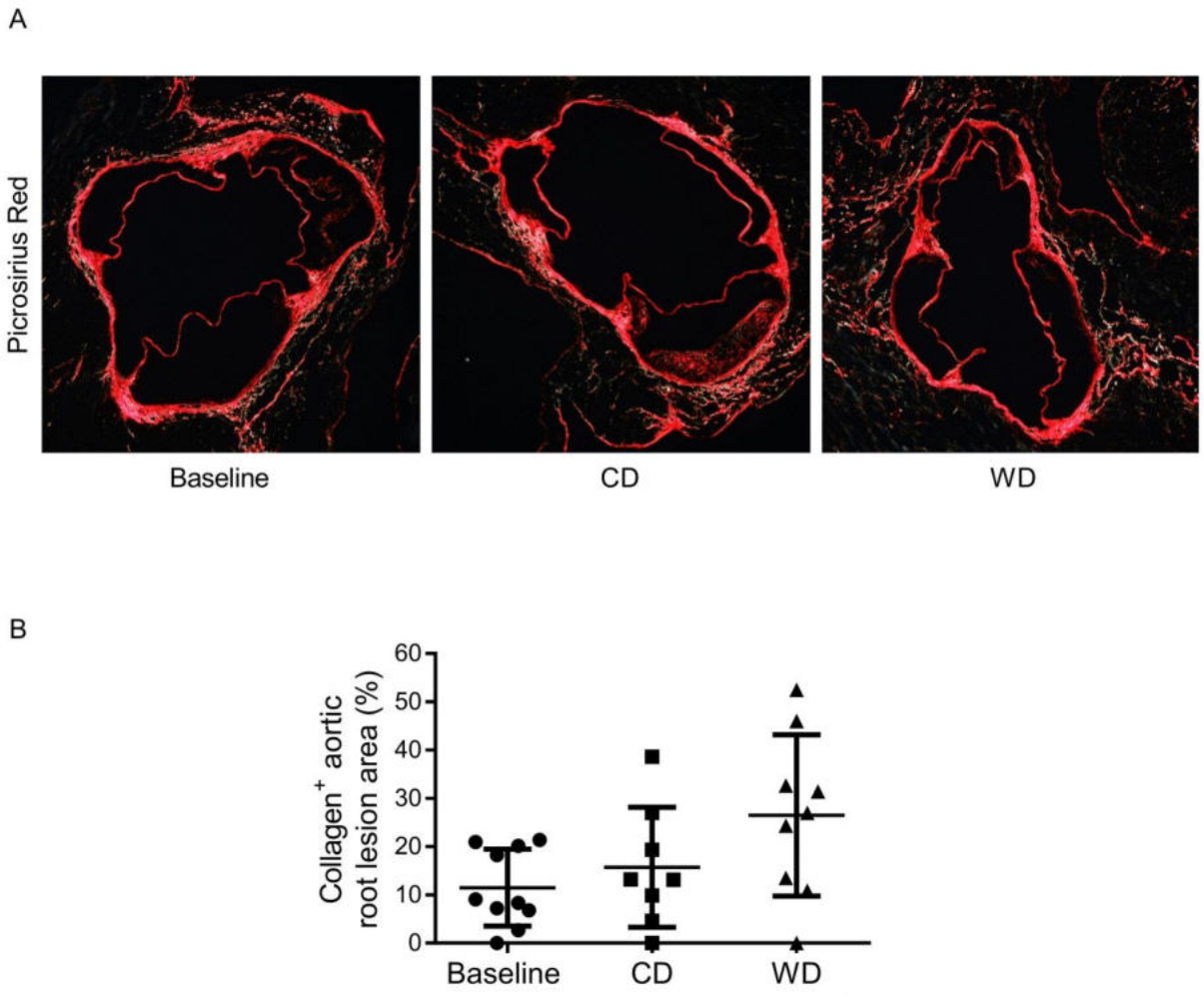


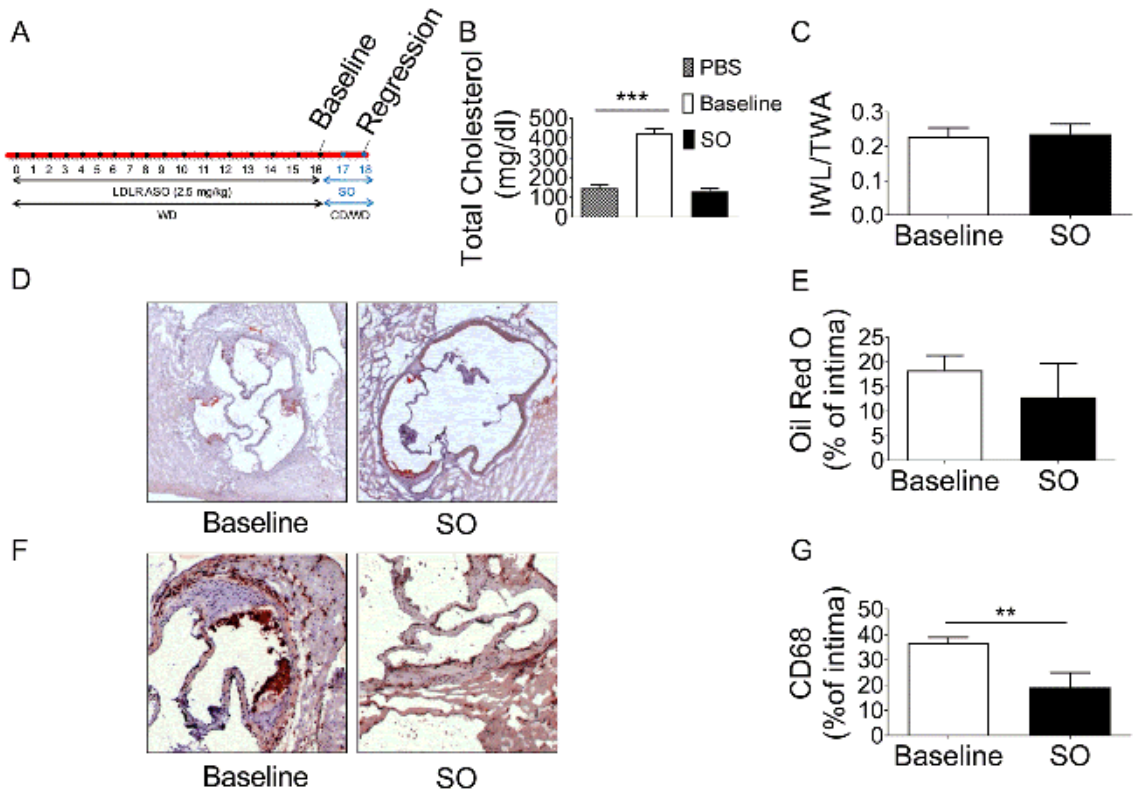
Online Figure I. LDLR ASO raises plasma cholesterol in female WT mice. Fasting plasma TC in 12 week old female mice treated with Control ASO or LDLR ASO. All mice were fed with Western diet (WD) for 6 weeks, n=4-6, *** denotes $p<0.001$, data presented as means ± SD.



Online Figure II. Restoration of hepatic LDLR expression. Analysis of hepatic LDLR protein using ELISA at the end of the study, n=4-6/group; *p<0.05, data presented as means ± SD.



Online Figure III. Collagen content in aortic root lesions. (A) Collagen-stained aortic root sections visualized by Picrosirius Red polarized light microscopy; (B) quantification showing percentage of collagen positive area within the lesion. Representative images are shown. n=9-10/group, data presented as means ± SD.



Online Figure IV. Effect of low-dose LDLR ASO on atherosclerosis and regression. (A) Plasma TC in mice fed with modified WD and treated with either PBS or LDLR ASO (low dose) alone for 16 weeks or LDLR ASO for 16 weeks and then SO injection, with TC measured after 3 weeks; (B) hepatic LDLR gene expression assessed by RT-PCR in baseline ASO and SO mice; (C) quantification of intimal wall lesion size; (D) lipid accumulation in lesions stained by Oil Red O and (E) its quantification; (F) macrophage accumulation in aortic root lesions visualized by CD68 immunostaining and (G) its quantification in the aortic roots of baseline ASO and SO treated mice. Representative pictures are shown, n = 5-10/group ***p<0.001, data presented as means ± SD.