

The *microRNA-342-5p* Fosters Inflammatory Macrophage Activation Through an Akt1- and *microRNA-155*-Dependent Pathway During Atherosclerosis

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Background—Atherosclerosis is a chronic inflammatory vascular disease driven by the subendothelial accumulation of macrophages. The mechanism regulating the inflammatory response in macrophages during atherogenesis remains unclear. Because microRNAs (miRNAs) play a crucial role in cellular signaling by posttranscriptional regulation of gene expression, we studied the miRNA expression profiles during the progression of atherosclerosis.

Methods and Results—Using an miRNA real-time polymerase chain reaction array, we found that macrophage-derived *miR-342-5p* and *miR-155* are selectively upregulated in early atherosclerotic lesions in *Apoe*^{−/−} mice. *miR-342-5p* directly targets *Akt1* through its 3′-untranslated region. *Akt1* suppression by *miR-342-5p* induces proinflammatory mediators such as *Nos2* and *Il6* in macrophages via the upregulation of *miR-155*. The local application of an *miR-342-5p* antagomir inhibits the development of atherosclerosis in partially ligated carotid arteries. In atherosclerotic lesions, the *miR-342-5p* antagomir upregulated *Akt1* expression and suppressed the expression of *miR-155* and *Nos2*. This reduced *Nos2* expression was associated with a diminished generation of nitrotyrosine in the plaques. Furthermore, systemic treatment with an inhibitor of *miR-342-5p* reduced the progression of atherosclerosis in the aorta of *Apoe*^{−/−} mice.

Conclusions—Macrophage-derived *miR-342-5p* promotes atherosclerosis and enhances the inflammatory stimulation of macrophages by suppressing the *Akt1*-mediated inhibition of *miR-155* expression. Therefore, targeting *miR-342-5p* may offer a promising strategy to treat atherosclerotic vascular disease. (*Circulation*. 2013;127:1609-1619.)

Key Words: atherosclerosis ■ macrophages ■ microRNAs

Macrophage function is of central importance for the initiation and progression of atherosclerosis.¹ Inflammatory monocytes are recruited primarily to the arterial wall after endothelial cells are activated by the products of oxidatively modified lipoproteins and/or low shear stress. These inflammatory monocytes will subsequently differentiate into macrophages and dendritic-like cells.² In early atherosclerosis, macrophages accumulate cholesterol through the uptake of cytotoxic, modified lipoproteins deposited in the subendothelial space and partly reallocate cholesterol from the vessel wall to high-density lipoproteins.^{1,2} Minimally modified low-density lipoproteins, which can activate Toll-like receptor 4, induce the expression of inflammatory cytokines such as CCL2 in macrophages.³ Moreover, in a mouse model of early atherosclerosis, the vascular expression of proinflammatory factors such as *Nos2*, *Il-12*, and *Ccl2* steadily increases concomitant with the accumulation of leukocytes.⁴

Mechanistically, this inflammatory response amplifies monocyte recruitment, enhances the formation of oxidized lipoproteins, and may impair reverse cholesterol transport.⁵⁻⁷ Thus, the transition of early atherosclerotic lesions, which are potentially reversible and clinically silent, into advanced lesions is thought to arise from a defective resolution of vascular inflammation.⁸

Clinical Perspective on p 1619

The various phases of inflammation are modulated by distinct sets of microRNAs (miRNAs) that negatively regulate posttranscriptional gene expression in leukocyte subtypes.^{9,10} During the innate immune response, Toll-like receptor activation induces *miR-155* expression, which promotes inflammatory cytokine production by suppressing SOCS-1, as well as *miR-146a* and *miR-147*, which are involved in inflammation resolution.¹¹⁻¹⁴ Furthermore, miRNAs such as

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miR-223, *miR-155*, and *miR-146a* govern the proinflammatory activation of macrophages by regulating the nuclear factor- κ B signaling pathway.^{9,15} The atherogenic stimulation of monocytes and macrophages by oxidized low-density lipoprotein also alters the miRNA expression profile, including the expression of *miR-155* and *miR-146a*; this, in turn, affects lipid uptake and inflammatory cytokine secretion.¹⁶ Moreover, inhibition of *miR-33* causes an increase in reverse cholesterol transport, thereby reducing atherosclerosis and inflammatory gene expression.¹⁷ Thus, miRNAs may be crucial for the regulation of inflammatory and lipid-handling functions in lesional macrophages. However, the miRNAs that control the inflammatory response during atherosclerosis have not been identified.

In this study, we generated stage-specific miRNA expression profiles in atherosclerotic lesions from *Apoe*^{-/-} mice. During early atherosclerosis, the most prominently upregulated miRNA was *miR-342-5p*, which is expressed in lesional macrophages. On proinflammatory activation in macrophages in vitro, *miR-342-5p* promoted *Nos2* expression in an *miR-155*-dependent manner by targeting *Akt1*, an inhibitor of *miR-155* expression. Accordingly, the inhibition of *miR-342-5p* reduced atherosclerotic lesion formation and suppressed *Akt1*-dependent *Nos2* expression in lesional macrophages. Taken together, these data demonstrate a crucial role for *miR-342-5p* in the early inflammatory response in lesional macrophages.

Methods

Animal Models

Apoe^{-/-} mice (age, 6–8 weeks; The Jackson Laboratory, Bar Harbor, ME) were fed a high-cholesterol diet (HCD; Altromin, Germany) comprising 21% crude fat, 0.15% cholesterol, and 19.5% casein for 3 or 10 months. *LysM-Cre* mice were mated with *Dicer*^{fllox/flox}/*Apoe*^{-/-} mice (The Jackson Laboratory) to generate *Apoe*^{-/-} mice with a conditional deletion of *Dicer* in myeloid cells. Partial ligation of the carotid artery was performed as previously described.¹⁸ Then, the mice were fed an HCD for 42 days. All animal experiments were approved by the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen [LANUV NRW]) in accordance with German animal protection laws.

Laser Capture Microdissection

Serial sections (20 μ m thick) of aortic roots were mounted on membrane-mounted metal frame slides (Molecular Machines and Industries [MMI], Glattburg, Switzerland). Laser capture microdissection was performed with a laser microdissection system (MMI CellCut Plus laser system, MMI) assembled onto an inverted microscope (Olympus IX71, Olympus Optical Co Ltd, Tokyo, Japan).

miRNA Real-Time Polymerase Chain Reaction Array

The samples were loaded onto preconfigured 384-well microfluidic cards (TaqMan Array MicroRNA Cards) for the real-time analysis (7900HT RT-PCR System, Applied Biosystems, Foster City, CA) of 518 mouse miRNAs (Sanger miRBase version 10). The data were analyzed with StatMiner software (Integromics, Philadelphia, PA) according to the $\Delta\Delta$ Ct method, with multiple internal control genes.

Argonaut2 Immunoprecipitation

Unstimulated bone marrow-derived macrophages (BMDMs) were harvested and lysed 24 hours after the transfection of the *miR-342-5p* inhibitor. Protein A/G-conjugated magnetic beads (Millipore,

Billerica, MA) were preincubated with a mouse monoclonal anti-argonaut2 antibody (clone 2E12-1C9, Abnova, Heidelberg, Germany) or mouse IgG (Millipore). The antibody-conjugated beads were subsequently incubated with cell lysates, and the precipitate was immobilized with a magnetic separator (Millipore).

Antagomir Treatment

A chemically modified antisense RNA oligonucleotide against *miR-342-5p* and a negative control antagomir were generated (Dharmacon, Inc, Chicago, IL).¹⁹ Perivascular antagomir treatment of the carotid arteries was begun 3 weeks after the partial ligation of the carotid artery with 160 μ g antagomir dissolved in 35% pluronic gel. The application was repeated twice at weekly intervals.²⁰

Locked Nucleic Acid Inhibitor Treatment In Vivo

The locked nucleic acid (LNA) inhibitor of *miR-342-5p* (LNA-342-5p; ATCACAGATAGCACC) and the nonspecific control LNA oligonucleotide (LNA-control; ATCAAAGCTAGGACC) were synthesized by Exiqon, Inc (Vedbaek, Denmark). After 2 months of an HCD, *Apoe*^{-/-} mice were injected 4 times with LNA-control or LNA-342-5p (25 mg/kg IV), once per week.²¹ The aortas were harvested 1 week after the last injection.

Statistical Analysis

The data represent the mean \pm SEM and were compared by use of an unpaired or a paired *t* test or 1-way ANOVA followed by the Newman-Keuls post hoc test (StatMiner 4.2, Integromics; or Prism, GraphPad). A value of *P* < 0.05 was considered significant.

Results

miRNA Expression Profiles in Early and Advanced Atherosclerotic Lesions

During the HCD feeding period, atherosclerosis in the *Apoe*^{-/-} mice increased steadily, resulting in a 2-fold increase in lesion size between 3 and 10 months (Figure 1A in the online-only Data Supplement). This lesion progression was associated with the formation of a lipid core, demonstrating that early lesions (after 3 months) transformed into advanced lesions (after 10 months) in mice fed an HCD (Figure 1A in the online-only Data Supplement). Concomitantly, the lesional macrophage content, as detected by Mac-2-specific immunostaining, decreased during this progression (Figure 1B in the online-only Data Supplement). The smooth muscle cell content was marginal in both early and advanced lesions, as revealed by immunostaining for smooth muscle actin, and tended to decrease after 10 months of an HCD (Figure 1C in the online-only Data Supplement). Therefore, our results show that early and advanced atherosclerotic lesions were generated in mice fed an HCD after 3 and 10 months, respectively.

The miRNA expression profiles of laser-microdissected samples from early and advanced lesions were studied with an miRNA real-time polymerase chain reaction array (Figure 2 in the online-only Data Supplement). To ensure a more complete detection of differentially expressed miRNAs, statistical analysis of the miRNA array was performed without correction for multiple comparisons.²² In early lesions, 5 miRNAs (*miR-342-5p*, *miR-296-5p*, *miR-146b*, *miR-21**, and *miR-155*) were increased and 57 miRNAs were suppressed (Figure 1A and Table I in the online-only Data Supplement) compared

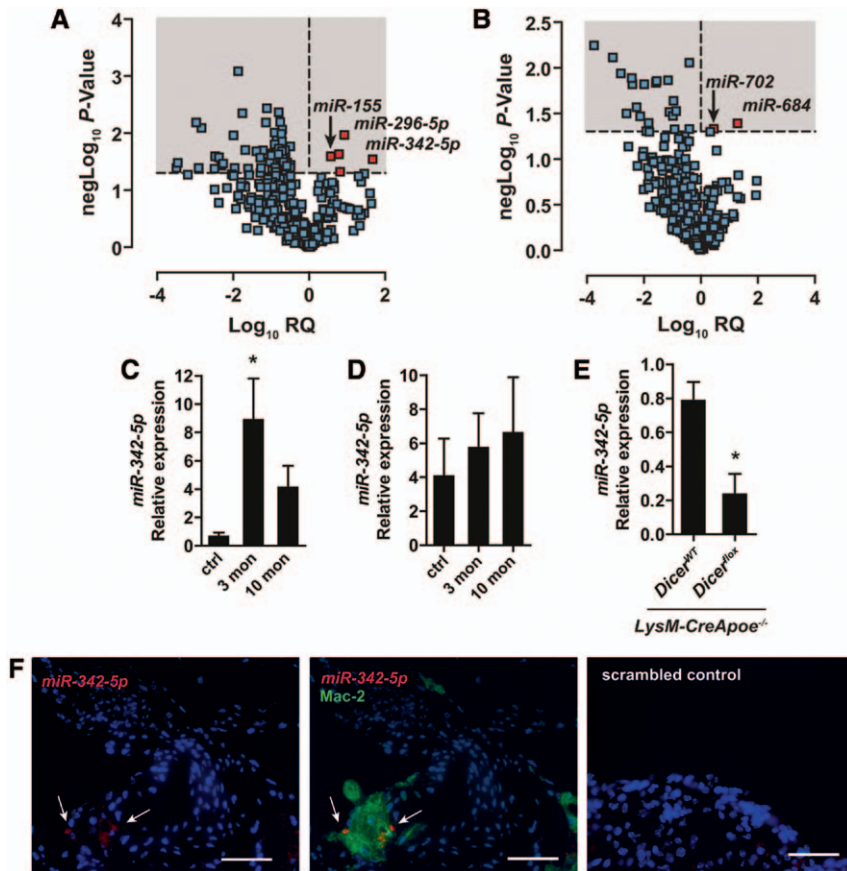


Figure 1. Increased expression of *miR-342-5p* in early atherosclerotic lesions is specific to macrophages. **A** and **B**, Differentially expressed miRNAs in lesions compared with healthy arterial tissue after (A) 3 months and (B) 10 months of a high-cholesterol diet (HCD; gray area). The upregulated miRNAs are labeled in red. $n=3$ or 4 mice per group. RQ indicates relative quantification value. **C** and **D**, Expression levels of *miR-342-5p* in (C) atherosclerotic lesions or (D) entire aorta after 3 months (3 mon) or 10 months (10 mon) on an HCD compared with levels in healthy vessel walls (control [ctrl]). * $P<0.05$ vs control; $n=3$ or 4 mice from each group. **E**, Expression of *miR-342-5p* in the aortic walls of *LysM-CreDicer^{lox}Apoe^{-/-}* mice maintained on an HCD for 3 months compared with that in *LysM-CreDicer^{WT}Apoe^{-/-}* mice. * $P<0.05$; $n=4$ or 5 mice from each group. **F**, In situ hybridization of *miR-342-5p* (red) combined with macrophage-specific Mac-2 staining (green) in atherosclerotic lesions from the aortic roots of *Apoe^{-/-}* mice after 3 months on an HCD. Arrows indicate *miR-342-5p*-positive macrophages. Nuclei were counterstained with DAPI. Scale bars=25 μm .

with nonatherosclerotic areas in the arterial walls of the same mice. Moreover, in advanced lesions, only 2 miRNAs (*miR-684* and *miR-702*) were elevated over those in nonatherosclerotic walls, and 21 miRNAs were reduced (Figure 1B and Figure IIIA in the online-only Data Supplement). Between the early- and late-staged lesions, 8 miRNAs were either upregulated or downregulated (Figure IIIB in the online-only Data Supplement). Even in the nonatherosclerotic vessel wall, miRNA deregulation was detectable during the HCD feeding period. After 10 months, 9 miRNAs, including *miR-145**, *miR-871*, and *miR-465a-5p*, were increased, and 5 miRNAs, including *miR-377* and *miR-665*, were downregulated relative to the respective levels in mice fed an HCD for only 3 months (Figure IIIC in the online-only Data Supplement).

miR-342-5p Is Expressed in Lesional Macrophages

In early, macrophage-rich lesions, *miR-342-5p* was one of the most highly upregulated miRNAs and was barely detectable in nonatherosclerotic arteries compared with the other upregulated miRNAs (Figure 1C), indicating that *miR-342-5p* plays a role in macrophages during atherosclerosis. Accordingly, in advanced lesions with lower macrophage content, the *miR-342-5p* expression declined compared with early lesions (Figure 1C). This increased expression of *miR-342-5p* was specific to lesioned regions of the arteries because its expression did not change significantly throughout the entire length of the aortic wall during high-cholesterol feeding (Figure 1D).

In the aortic walls of *Apoe^{-/-}* mice with a conditional knockout of the miRNA-processing enzyme *Dicer1* in myeloid

cells (*LysM-Cre/Dicer^{lox}*), the expression of *miR-342-5p* was reduced in mice after 3 months on an HCD compared with *LysM-Cre/Dicer^{WT}Apoe^{-/-}* mice, indicating that macrophages were likely to be the primary cellular source of *miR-342-5p* in atherosclerotic lesions (Figure 1E). To confirm this, we next performed in situ hybridization combined with Mac-2 immunostaining and found *miR-342-5p* to be expressed in the macrophages of early plaques (Figure 1F). Furthermore, *miR-342-5p* expression was low in BMDM precursor cells but increased during their differentiation into macrophages (Figure 2A). Macrophage stimulation with lipopolysaccharide/interferon- γ (IFN- γ) or interleukin-4, however, did not differentially regulate *miR-342-5p* expression after 6 and 12 hours (Figure 2B and Figure IVA in the online-only Data Supplement), with increases noted after 48 hours. Interestingly, lipopolysaccharide/IFN- γ alone slightly upregulated *miR-342-5p* as soon as 24 hours (Figure IVA in the online-only Data Supplement), and treatment with highly oxidized low-density lipoprotein increased *miR-342-5p* after 48 hours (Figure IVB in the online-only Data Supplement). These findings indicate that *miR-342-5p* expression is associated with mature and activated macrophages during early atherosclerosis.

miR-342-5p Promotes the Proinflammatory Activation of Macrophages

Early atherosclerotic lesions in *Apoe^{-/-}* mice are characterized by the increased expression of proinflammatory markers such as NOS2 and CCL2 and the enrichment of macrophages.⁴ Therefore, we hypothesized a role for *miR-342-5p* in macrophage

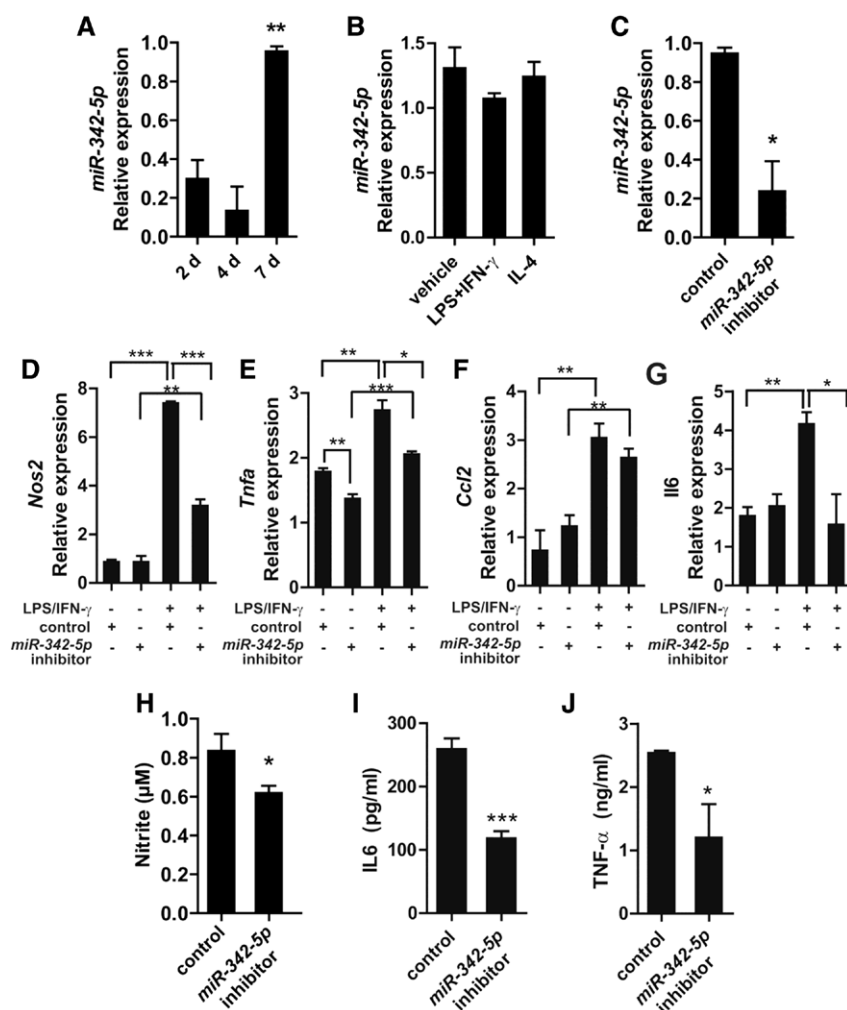


Figure 2. Effects of *miR-342-5p* on the proinflammatory activation of macrophages. **A**, The induction of *miR-342-5p* expression during the in vitro differentiation of murine bone marrow-derived macrophages (BMDMs) at days 2, 4, and 7. ** $P < 0.01$ vs all other groups; $n = 3$. **B**, Expression of *miR-342-5p* in BMDMs stimulated with lipopolysaccharide (LPS) and interferon- γ (IFN- γ) or interleukin (IL)-4. $n = 3$. **C**, *miR-342-5p* expression after BMDM treatment with the locked nucleic acid (LNA)-*miR-342-5p* inhibitor or nontargeting LNA oligonucleotides. * $P < 0.05$; $n = 3$ to 4. **D** through **G**, Effect of the *miR-342-5p* inhibitor on the mRNA expression of *Nos2* (**D**), *Tnfa* (**E**), *Ccl2* (**F**), and *Il6* (**G**) in unstimulated and LPS/IFN- γ -stimulated BMDMs. Nontargeting LNA oligonucleotides were used in the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 3$. **H** through **J**, Concentration of nitrite (**H**), tumor necrosis factor- α (TNF- α) protein (**I**), and IL6 protein (**J**) in the culture media of LPS/IFN- γ -stimulated BMDMs treated with the *miR-342-5p* LNA inhibitor or nontargeting LNA oligonucleotides. * $P < 0.05$, *** $P < 0.005$ vs control; $n = 3$ to 4.

activation. The expression of *miR-342-5p* in BMDMs was greatly reduced after transfection with an *miR-342-5p* inhibitor (Figure 2C). After lipopolysaccharide/IFN- γ stimulation, *Nos2* expression and nitrite generation by NOS2 were substantially reduced by the *miR-342-5p* inhibitor (Figure 2D and 2H and Figure VA in the online-only Data Supplement). The *miR-342-5p* inhibitor reduced *Tnfa* and *Il6* expression at both the mRNA and protein levels in lipopolysaccharide/IFN- γ -activated BMDMs, but a similar inhibition was not observed for *Ccl2* (Figure 2E–2G, 2I, and 2J and Figure V in the online-only Data Supplement). Interestingly, *Tnfa* expression was also decreased in unstimulated BMDMs by the *miR-342-5p* inhibitor (Figure 2E). The *miR-342-5p* inhibitor also diminished the degree of lipid accumulation in BMDMs incubated with acetylated low-density lipoprotein (Figure VI in the online-only Data Supplement). These results indicate that *miR-342-5p* promotes the proinflammatory activation of macrophages by enhancing nitric oxide production and the expression of *Tnfa* and *Il6*.

Bmpr2 and *Akt1* Are Direct Targets of *miR-342-5p*

To address the mechanism by which *miR-342-5p* affects macrophage activation, an in silico target prediction analysis was performed. Ten potential targets of *miR-342-5p* related to macrophage function were identified with 3 different target

prediction algorithms. The expression of these potential *miR-342-5p* targets was quantified in unstimulated BMDMs transfected with either the *miR-342-5p* mimic or the inhibitor. Of the 10 selected targets, only *Bmpr2* and *Akt1* were significantly increased by the *miR-342-5p* inhibitor and suppressed by the *miR-342-5p* mimic (Figure 3A and 3B). *Foxo3* was significantly upregulated only in macrophages treated with the *miR-342-5p* inhibitor, whereas *Nfkb2* and *Rara* expression was decreased by the *miR-342-5p* mimic (Figure 3A and 3B). To verify the direct repression of the differentially regulated genes by *miR-342-5p*, argonaute2 immunoprecipitations were performed. Only the messages of *Bmpr2*, *Akt1*, and *Nfkb2* were reduced in the argonaute2 immunoprecipitates from BMDMs treated with the *miR-342-5p* inhibitor (Figure 3C), indicating that *miR-342-5p* directly suppresses these 3 targets in macrophages.

Using luciferase reporter assays, we examined the binding sites for *miR-342-5p* in the 3'-untranslated regions (3'-UTRs) of *Bmpr2*, *Akt1*, and *Nfkb2*. Transfection with the *miR-342-5p* mimic repressed the luciferase activity of the *Bmpr2* and *Akt1* 3'-UTRs (Figure 3D and 3E) but not that of the *Nfkb2* 3'-UTR (Figure VII in the online-only Data Supplement). In contrast, the luciferase activities of these constructs bearing mutations in the *miR-342-5p* target sites in the 3'-UTRs of *Bmpr2* and

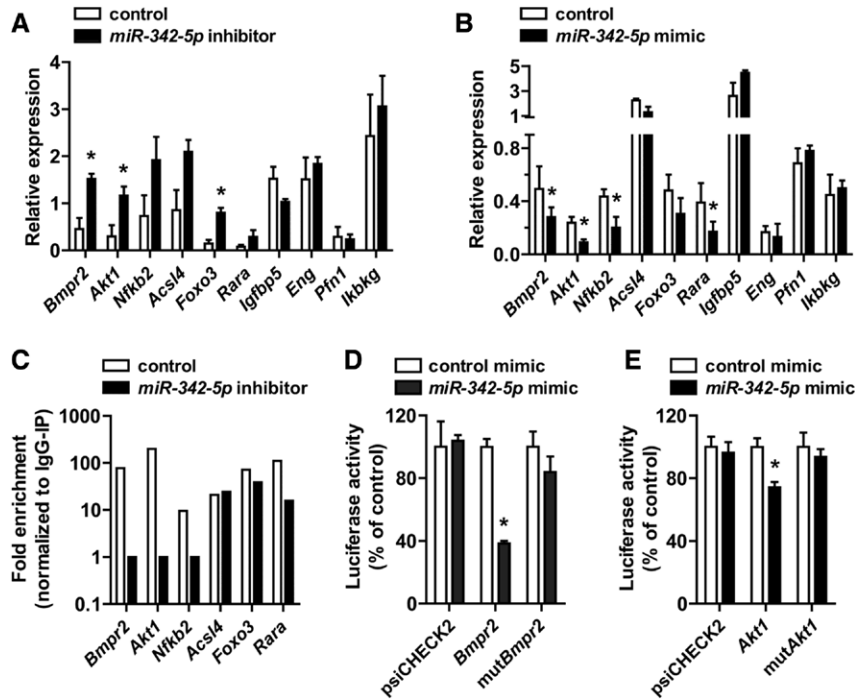


Figure 3. Identification of the mRNA targets of *miR-342-5p* in macrophages. **A** and **B**, Unstimulated bone marrow–derived macrophages (BMDMs) were transfected with the **(A)** *miR-342-5p* inhibitor or **(B)** an *miR-342-5p* mimic, and the mRNA expression of the predicted targets was quantified with quantitative real-time polymerase chain reaction 24 hours after transfection. Nontargeting locked nucleic acid (LNA) oligonucleotides and negative control mimic oligonucleotides were used in the control groups, respectively. * $P < 0.05$ vs control; $n = 3$ to 4. **C**, Enrichment of the predicted targets of *miR-342-5p* in argonaute2-coimmunoprecipitated RNA in unstimulated BMDMs transfected with the *miR-342-5p* inhibitor or nontargeting LNA oligonucleotides (control). The data are from 1 experiment that is representative of 3 independent experiments. **D** and **E**, Normalized luciferase activity in HEK293 cells transfected with the psiCHECK2 vector containing the 3'-untranslated region (UTR) of **(D)** *Bmpr2* with (mut*Bmpr2*) or without (*Bmpr2*) the mutated binding site of *miR-342-5p* and **(E)** the 3'-UTR of *Akt1* with (mut*Akt1*) or without (*Akt1*) the mutated binding site of *miR-342-5p* 48 hours after treatment with an *miR-342-5p* mimic or a negative control mimic. * $P < 0.05$ vs control; $n = 4$.

Akt1 were not affected by the *miR-342-5p* mimic (Figure 3D and 3E and Figure VIII in the online-only Data Supplement). Taken together, these results suggest that *miR-342-5p* directly targets *Bmpr2* and *Akt1* in macrophages via a recognition element in their 3'-UTRs. Although targeting of the 3'-UTR of *Nfkb2* by *miR-342-5p* was not observed, binding of *miR-342-5p* to the 5'-UTR or the coding region of the *Nfkb2* mRNA cannot be excluded.

Targeting of *Akt1* by *miR-342-5p* Mediates the Proinflammatory Activation of Macrophages

Next, we assessed the functional roles of *Akt1* and *Bmpr2* in *miR-342-5p*-mediated macrophage activation. In contrast to interleukin-4, lipopolysaccharide/IFN- γ treatment suppressed *Akt1* mRNA, which could be abolished with the *miR-342-5p* inhibitor (Figure 4A and Figure IX in the online-only Data Supplement). Accordingly, inhibition of *miR-342-5p* increased AKT1 protein expression and phosphorylation in lipopolysaccharide/IFN- γ -stimulated BMDMs (Figure 4B and 4C). Although treatment with an *miR-342-5p* inhibitor increased *Bmpr2* mRNA and protein expression in lipopolysaccharide/IFN- γ -stimulated macrophages, *Bmpr2* mRNA levels were still lower than those observed in unstimulated BMDMs (Figure 4A and 4D), suggesting additional mechanisms for *Bmpr2* suppression in inflammatory macrophages. This increase in *Akt1* and *Bmpr2* could be reduced by siRNAs specific to

Akt1 and *Bmpr2*, respectively (Figure X in the online-only Data Supplement). Furthermore, the silencing of *Akt1* but not *Bmpr2* in these macrophages treated with the *miR-342-5p* inhibitor upregulated *Nos2*, *Tnfa*, and *Il6* (Figure 4E). Overall, these results indicate that *miR-342-5p* primarily regulates the proinflammatory activation of macrophages by targeting *Akt1*.

Although *Akt1* suppression by *miR-342-5p* appeared to be enhanced after lipopolysaccharide/IFN- γ stimulation, the expression level of *miR-342-5p* was unaltered. To determine whether *Akt1* mRNA and *Bmpr2* mRNA compete for binding to *miR-342-5p* in unstimulated macrophages, we studied the effect of silencing *Bmpr2* on the expression of *Akt1*. Notably, *Bmpr2* silencing decreased *Akt1* mRNA expression in unstimulated macrophages (Figure 4F), which could be rescued by inhibiting *miR-342-5p* (Figure 4G). This indicated that an abundance of *Bmpr2* mRNA may regulate *Akt1* in unstimulated macrophages by competing with *Akt1* for *miR-342-5p* binding.

Role of *miR-155* in the *Akt1*-Dependent Regulation of Macrophage Activation by *miR-342-5p*

Akt1 negatively regulates *miR-155* expression in macrophages, which, in turn, impairs macrophage response to lipopolysaccharide.¹³ Therefore, we investigated the role of *miR-155* in *miR-342-5p*-mediated proinflammatory activation of macrophages. We found that lipopolysaccharide/IFN- γ stimulation increased *miR-155* expression (Figure 5A). This increase

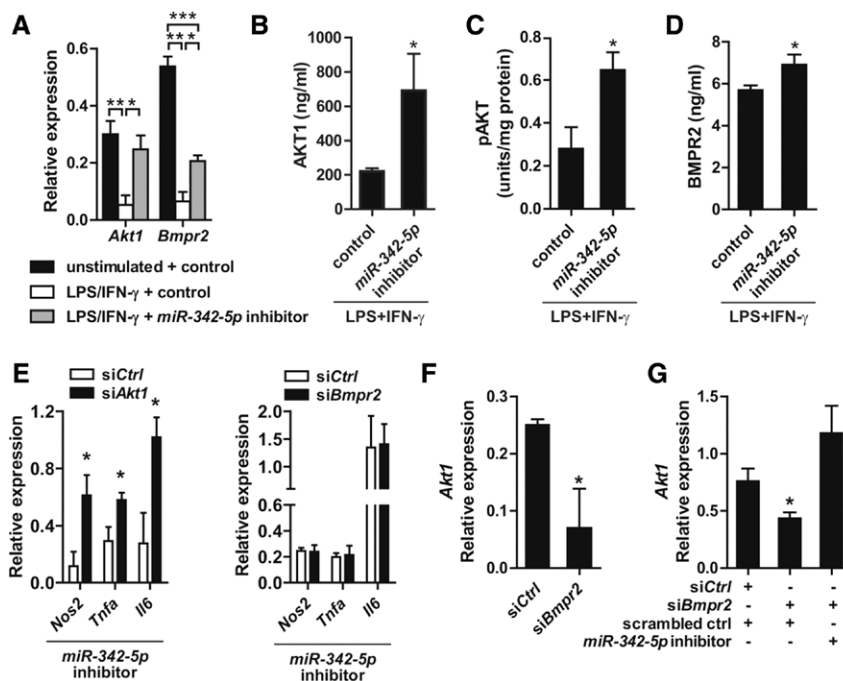


Figure 4. Roles of Akt1 and bone morphogenetic protein receptor, type II (Bmpr2) in *miR-342-5p*-mediated macrophage activation. **A**, *Akt1* and *Bmpr2* expression in bone marrow-derived macrophages (BMDMs) stimulated with or without lipopolysaccharide (LPS)/interferon- γ (IFN- γ) was analyzed after treatment with an *miR-342-5p* inhibitor or nontargeting locked nucleic acid (LNA; control) oligonucleotide. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; $n = 3$ to 4. **B** through **D**, Effects of the *miR-342-5p* inhibitor or nontargeting LNA (control) on AKT1 and BMPR2 protein (**B** and **D**) expression and the phosphorylation of Akt (**C**) in LPS/IFN- γ -stimulated BMDMs. * $P < 0.05$ vs control; $n = 3$ to 5. **E**, Effects of si*Akt1* or si*Bmpr2* treatment on *Nos2*, *Tnfa*, and *Il6* expression in LPS/IFN- γ -stimulated BMDMs treated with an *miR-342-5p* inhibitor or the nontargeting siRNA (siCtrl). * $P < 0.05$ vs siCtrl; $n = 3$ to 4. **F**, *Akt1* mRNA expression was determined in BMDMs treated with si*Bmpr2* or siCtrl. * $P < 0.05$; $n = 3$. **G**, *Akt1* mRNA expression levels in BMDMs treated with si*Bmpr2* or siCtrl with either an *miR-342-5p* inhibitor or a nontargeting LNA control oligonucleotide (scrambled control). * $P < 0.05$ vs all other groups; $n = 3$.

could be abolished by treatment with an *miR-342-5p* inhibitor (Figure 5B) but then subsequently restored by siRNA-mediated *Akt1* suppression (Figure 5C). These findings indicate that *miR-342-5p* upregulates *miR-155* via the suppression of *Akt1* in classically activated macrophages.

To verify the role of *miR-155* in this *miR-342-5p*-dependent proinflammatory pathway, we examined the effect of lipopolysaccharide/IFN- γ stimulation on BMDMs derived from *miR-155*^{-/-} mice. We found a reduced expression of *Nos2*, *Tnfa*, and *Il6* after lipopolysaccharide/IFN- γ stimulation in *miR-155*^{-/-} compared with *miR-155*^{+/+} macrophages (Figure 5D). Interestingly, treatment of *miR-155*^{-/-} macrophages with an *miR-342-5p* inhibitor increased *Nos2* expression after lipopolysaccharide/IFN- γ stimulation but had no effect on *Tnfa* and *Il6* expression (Figure 5E). This indicates that, in the absence of *miR-155*, *miR-342-5p* regulates *Nos2* via a different pathway. Bone morphogenetic protein 6 stimulation was previously shown to increase *Nos2* expression via *Bmpr2* in macrophages.²³ Therefore, we studied whether *miR-342-5p* affects *Nos2* expression in the absence of *miR-155* by targeting *Bmpr2*. Indeed, the siRNA-mediated suppression of *Bmpr2* prevented *Nos2* upregulation in lipopolysaccharide/IFN- γ -stimulated *miR-155*^{-/-} macrophages on treatment with the *miR-342-5p* inhibitor (Figure 5F). This suggests that the effect of *miR-342-5p* on *Nos2* expression in classically activated macrophages is reversed by targeting *Bmpr2* in the absence of the *Akt1*-*miR-155* pathway.

Inhibition of *miR-342-5p* Suppresses Atherosclerosis

To address the role of *miR-342-5p* in atherogenesis, atherosclerotic lesion formation was induced locally in the carotid artery 42 days after partial carotid ligation in hyperlipidemic *Apoe*^{-/-} mice. In this model, *miR-342-5p* was upregulated in the carotid artery as early as 7 days, remaining high compared with nonligated carotid arteries, until 42 days after ligation (Figure 6A). Perivascular treatment of the carotid arteries with the *miR-342-5p* inhibitor (*miR-342-5p* antagomir) suppressed *miR-342-5p* at 42 days compared with the nontargeting control antagomir (Figure 6B). Furthermore, the mean lesion size in the carotid arteries was significantly decreased after *miR-342-5p* antagomir treatment (Figure 6C), diminishing the lesional content of the macrophages and smooth muscle cells in the *miR-342-5p* antagomir-treated carotid arteries (Figure 6D and 6E and Figure XI in the online-only Data Supplement). Local treatment with the *miR-342-5p* antagomir did not affect serum cholesterol levels (Figure XII in the online-only Data Supplement). The inhibition of *miR-342-5p* increased *Akt1* expression and diminished *miR-155*, *Nos2*, and *Il6* expression in atherosclerotic lesions at 42 days (Figure 7A). In addition, NOS2 expression in lesional macrophages (Figure 7B) and peroxynitrite production (Figure 7C) were reduced after *miR-342-5p* inhibition. These results demonstrate that *miR-342-5p* promotes atherogenesis by increasing the accumulation of macrophages and smooth muscle cells in the lesions. This effect might be due to the *miR-342-5p*-mediated upregulation

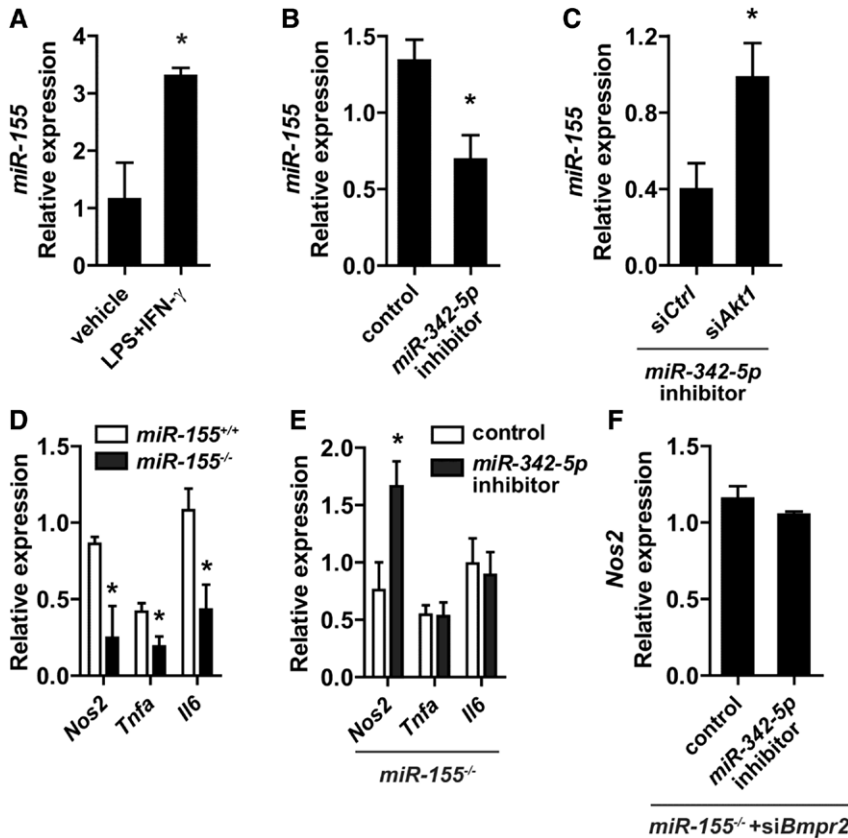


Figure 5. The role of *miR-155* in *miR-342-5p*-mediated macrophage activation. **A**, *miR-155* expression in bone marrow-derived macrophages (BMDMs) in response to lipopolysaccharide (LPS)/interferon- γ (IFN- γ) stimulation. * P <0.05; n =3 to 4. **B**, Effects of an *miR-342-5p* inhibitor on *miR-155* expression in LPS/IFN- γ -stimulated BMDMs compared with the effects of a nontargeting locked nucleic acid (LNA) oligonucleotide (control). * P <0.05; n =3 to 4. **C**, Silencing *Akt1* with RNA interference (si*Akt1*) increased the expression of *miR-155* in LPS/IFN- γ -stimulated BMDMs treated with an *miR-342-5p* inhibitor compared with those treated with a nontargeting siRNA (siCtrl). * P <0.05; n =3 to 4. **D**, Expression of *Nos2*, *Tnfa*, and *Il6* in *miR-155*^{-/-} BMDMs compared with their expression in *miR-155*^{+/+} LPS/IFN- γ -treated BMDMs. * P <0.05; n =3 to 4. **E**, Effects of treatment with an *miR-342-5p* inhibitor on the expression of *Nos2*, *Tnfa*, and *Il6* in LPS/IFN- γ -stimulated *miR-155*^{-/-} BMDMs compared with the effects of treatment with a nontargeting LNA oligonucleotide. * P <0.05; n =4. **F**, Expression of *Nos2* after treatment with an *miR-342-5p* inhibitor or a control LNA oligonucleotide in LPS/IFN- γ -stimulated *miR-155*^{-/-} BMDMs in which *Bmpr2* had been silenced by siRNA (si*Bmpr2*). n =3 to 4.

of *miR-155*, which, in turn, leads to an increase in *Nos2* activity in atherosclerotic lesions via *Akt1* suppression.

We next administered LNA-342-5p to *Apoe*^{-/-} mice fed an HCD for 2 months to investigate whether systemic treatment with the *miR-342-5p* inhibitor could inhibit the progression of atherosclerosis. One-month treatment with LNA-342-5p reduced lesion formation in both the aorta and the aortic root compared with a nontargeting control (LNA-control) (Figure 8A and 8B). Similar to the effect of local *miR-342-5p* inhibition on carotid lesion formation, systemic treatment with LNA-342-5p diminished the lesional macrophage and smooth muscle cell content (Figure 8C and 8D). Furthermore, silencing *miR-342-5p* enhanced AKT1 expression in lesional macrophages (Figure 8E) but reduced *miR-155*, *Nos2*, and *Il6* expression in the aortas of *Apoe*^{-/-} mice compared with the control (Figure 8F).

Discussion

Atherosclerotic lesion formation is associated with a distinct miRNA expression profile, but the functions of individual miRNAs in atherogenesis are unknown.²⁴ We found that the miRNA expression profile of murine atherosclerotic lesions is stage specific and that early lesions are characterized by the increased expression of *miR-342-5p* in macrophages. In vitro, *miR-342-5p* targets *Akt1* and thus turns off the repression of *miR-155*; this, in turn, mediates the upregulation of proinflammatory mediators such as *Nos2* and *Tnfa* in activated macrophages. In flow-induced atherosclerosis, the inhibition of *miR-342-5p* reduced lesion formation by impairing the

accumulation of macrophages and smooth muscle cells. Furthermore, blocking *miR-342-5p* in atherosclerotic lesions increased *Akt1* expression, suppressed the expression of *miR-155* and *Nos2*, and reduced protein nitrosylation. Systemic treatment with an inhibitor of *miR-342-5p* inhibited the progression of atherosclerosis in mice, demonstrating the therapeutic potential of oligonucleotide-based targeting of *miR-342-5p*.

During the progression of atherosclerosis, distinct, stage-specific lesional morphologies occur that range from early, macrophage-rich lesions to advanced lesions with extracellular lipid deposition and macrophage apoptosis. Whereas the inflammatory response driven by recruited macrophages prevails in early lesions, studies implicate impaired efferocytosis as the cause of defective resolution of inflammation in advanced atherosclerosis.⁸ miRNAs play a crucial role in the innate immune response by positively or negatively regulating inflammatory signaling pathways. However, the effects of lesion-specific miRNAs in the macrophage response during atherosclerosis have not been determined. Therefore, in a murine model of experimental atherosclerosis, we compared the miRNA expression profiles in early and advanced lesions with the respective non-lesion-bearing arterial wall from the same mice. Because macrophages exist only in the lesions, we were able to identify miRNAs involved in the development of lesional macrophages by comparing these lesioned tissues with normal arteries.

Our miRNA profiling demonstrated increased expression of 5 miRNAs, including *miR-342-5p* and *miR-155*, in early

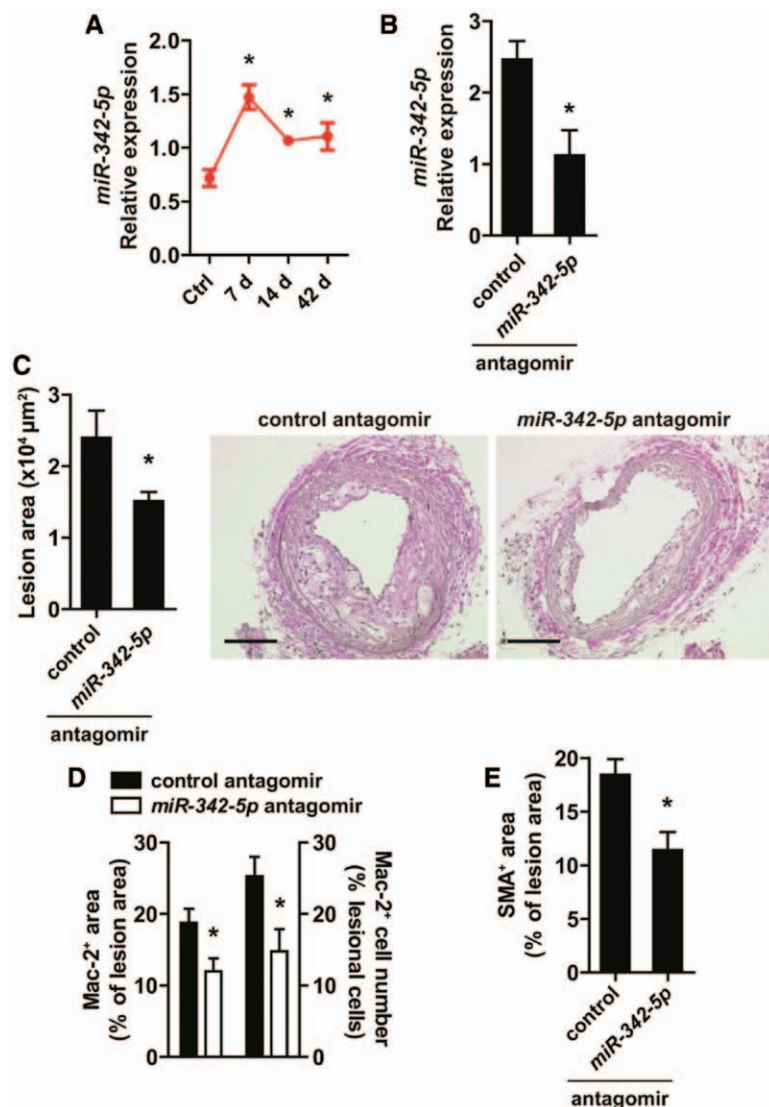


Figure 6. The role of *miR-342-5p* in atherosclerosis induced by acute flow disturbance. **A**, *miR-342-5p* expression during flow-induced lesion formation after the partial ligation of the carotid artery in *Apoe*^{-/-} mice fed a high-cholesterol diet (HCD). Carotid arteries without partial ligation were studied in the control group (Ctrl). **P*<0.05 vs control; n=4 to 5 mice from each group. **B**, *miR-342-5p* expression in carotid arteries from *Apoe*^{-/-} mice perivascularly treated with an *miR-342-5p* inhibitor (antagomir) or a nontargeting (control) antagomir 42 days after partial ligation. **P*<0.05; n=5 or 6 mice from each group. **C**, Effect of local treatment with the *miR-342-5p* antagomir or control antagomir on atherosclerotic lesion formation in the partially ligated carotid arteries of *Apoe*^{-/-} mice fed an HCD 42 days after partial ligation. Representative sections of carotid arteries stained with Elastic van Gieson stain are shown. **P*<0.05; n=5 or 6 mice per group. Scale bars=100 μm . **D**, Lesional macrophages accumulation, as determined by Mac-2 immunostaining, in partially ligated carotid arteries treated with the *miR-342-5p* antagomir or control antagomir. **P*<0.05; n=5 or 6 mice per group. **E**, Smooth muscle cell accumulation as determined by smooth muscle actin (SMA) immunostaining in partially ligated carotid arteries treated with the *miR-342-5p* antagomir or control antagomir. **P*<0.05; n=5 or 6 mice per group.

lesions. Although the sample size in this initial screening was limited and false discoveries were not controlled, we confirmed that *miR-342-5p* is expressed primarily in lesional macrophages and is a marker for mature macrophages. Moreover, *miR-155* is induced by the proinflammatory stimulation of macrophages and upregulated in atherosclerotic lesions.²⁵ Although *miR-342-5p* might be upregulated during the differentiation of monocytes into macrophages in atherosclerotic lesions, the increased expression of *miR-342-5p* in early lesions compared with advanced lesions is more likely due to the higher macrophage content in early lesions rather than transcriptional upregulation.

Intronic *miR-342* is expressed together with its host gene, *Evl* (Ena-vasodilator stimulated phosphoprotein), and the sister strand of *miR-342-5p*, *miR-342-3p*, is highly upregulated by the transcription factor PU.1 during macrophage differentiation.^{26,27} However, the functional role of *miR-342-5p* was unclear. We have shown that increased *miR-342-5p* expression sensitizes macrophages to proinflammatory stimulation by regulating *Nos2*. Lesional macrophages express high levels of *Nos2* in human and murine atherosclerosis, and a genetic deficiency

in *Nos2* in macrophages reduces atherogenesis in *Apoe*^{-/-} mice.^{28,29} Consistent with these results, we demonstrated that *miR-342-5p* inhibition in the vessel wall reduces atherosclerosis and inhibits the expression of *Nos2* in lesional macrophages. The proatherogenic effect of *Nos2* has been linked to the generation of peroxynitrite by the reaction of nitric oxide with superoxide in the vessel wall.^{28,30,31} Peroxynitrite is a strong oxidant species that induces highly atherogenic lipoprotein modifications and the dysregulation of various signaling pathways by protein nitration.³²⁻³⁴ Our findings suggest that *miR-342-5p* inhibition during atherosclerosis reduces *Nos2*-mediated peroxynitrite formation, which may be functionally linked to *miR-342-5p*-mediated lesion progression.

Several reports have demonstrated that *Nos2* and other proinflammatory mediators are negatively regulated by the activated PI3-kinase/AKT1 pathway.³⁵⁻³⁷ Accordingly, the serine/threonine kinase *Akt1* (also called protein kinase B) is essential for endotoxin tolerance and inhibits the expression of *Nos2*, *CCL2*, and tumor necrosis factor- α in lipopolysaccharide-stimulated macrophages.^{13,37} Moreover, *Akt1* expression in macrophages prevents their polarization to a proinflammatory

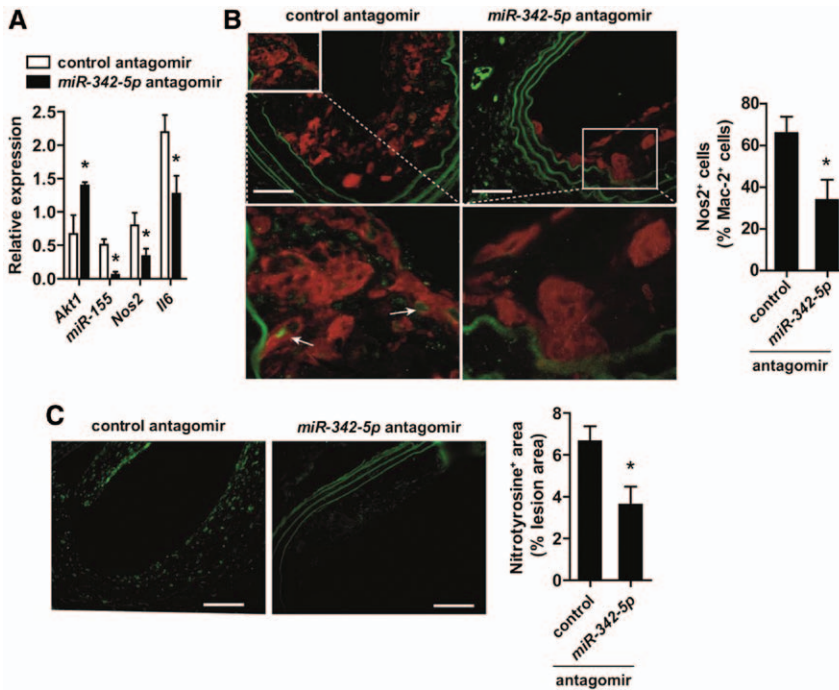


Figure 7. Effects of *miR-342-5p* inhibition on the expression of proinflammatory mediators in atherosclerotic lesions induced by acute flow disturbance. **A**, *Akt1*, *miR-155*, *Nos2*, and *Il6* expression was measured in carotid arteries treated with the *miR-342-5p* antagomir or the control antagomir 42 days after partial carotid ligation in *Apoe*^{-/-} mice fed a high-cholesterol diet. For all figures, **P* < 0.05; *n* = 5 or 6 mice per group. **B**, *Nos2* expression in lesional macrophages from partially ligated carotid arteries treated with the *miR-342-5p* antagomir or the control antagomir. Representative immunostaining shows combined immunostaining for *Nos2* (green) and *Mac-2* (red). Arrows indicate *Nos2*-expressing macrophages. Scale bars = 50 μ m. **C**, The nitrotyrosinylation in lesions of partially ligated carotid arteries treated with the *miR-342-5p* antagomir or the control antagomir. Representative immunostaining shows nitrotyrosine staining. Scale bars = 50 μ m.

M1 phenotype.³⁸ *AKT1* also activates nuclear factor- κ B, a key factor in the transcriptional regulation of *Nos2*, which appears, however, to be cell type specific and dependent on the availability of I kappa-B kinase alpha (*IKK α*).³⁹ Our results indicate that *miR-342-5p* suppresses *Akt1* in classically activated macrophages and thereby upregulates *Nos2*, supporting the concept of an anti-inflammatory role for *Akt1* in macrophages. In addition to the regulation of *AKT1* by PI3K-dependent phosphorylation, our data suggest that the posttranscriptional regulation of *Akt1* by miRNAs is crucial for the proinflammatory activation of macrophages. Compared with no stimulation, lipopolysaccharide/IFN- γ treatment enhanced the suppressive activity of *miR-342-5p* on *Akt1*. This “activation” of *miR-342-5p* appears to be due to the transcriptional downregulation of the alternative *miR-342-5p* target, *Bmpr2*, after stimulation, which might alleviate the competition between *Akt1* and *Bmpr2* for binding to *miR-342-5p* and thus promote the suppression of *Akt1* by *miR-342-5p*.⁴⁰

The increase in *Akt1* expression during atherosclerotic lesion formation resulting from the inhibition of *miR-342-5p* indicates that *Akt1* plays a protective role in atherosclerosis by impairing *Nos2*-dependent peroxynitrite generation. These findings are in agreement with previous reports showing increased lesion development in *Akt1*^{-/-} mice resulting from increased macrophage infiltration and enhanced expression of proinflammatory genes such as *Tnfa*.⁴¹

The effects of *Akt1* on lipopolysaccharide responsiveness are partly mediated by the suppression of *miR-155*, which targets *SOCS1*, an inhibitor of Toll-like receptor 4 signaling.¹³ In macrophages, *miR-155* expression is upregulated after the activation of various Toll-like receptors via either MyD88- or TRIF-dependent signaling pathways, which enhances the lipopolysaccharide-induced expression of *Nos2*.^{11,42} Moreover, *miR-155* can reduce macrophage apoptosis in infectious diseases, but only in stimulated macrophages.^{25,43} Although

miR-155 is reduced in the circulation of patients with coronary heart disease, microarray analyses have demonstrated an increase in the expression of *miR-155* in human atherosclerotic lesions.^{24,44} In line with a proatherogenic role of *miR-155*, the presence of advanced atherosclerotic lesions was reduced in *Apoe*^{-/-} mice harboring *miR-155*-deficient macrophages by removing the repression to the anti-inflammatory transcription factor *Bcl6*.²⁵ Interestingly, our findings indicate that *miR-342-5p* positively regulates *miR-155* expression in atherosclerotic lesions via *Akt1* suppression. Moreover, this *Akt1*–*miR-155* pathway may mediate the effects of *miR-342-5p* on lesion formation and nitro-oxidative stress because the *miR-342-5p*-mediated upregulation of *Nos2* depends on the expression of *miR-155*. Notably, the role of *miR-342-5p* in *Nos2* regulation in macrophages that are genetically deficient in *miR-155* was completely reversed by the targeting of *Bmpr2*. Therefore, *miR-342-5p* and *miR-155* may form a functional miRNA pair that promotes lesion inflammation and nitro-oxidative stress in atherosclerosis.

Conclusions

We found that the most significantly upregulated miRNA in murine atherosclerotic lesions, *miR-342-5p*, promotes atherosclerosis and increases nitro-oxidative stress during lesion formation. This effect may be due to the upregulation of *Nos2* in proinflammatory macrophages by removing the repression of *miR-155* by the targeting of *Akt1*. Thus, targeting *miR-342-5p* in atherogenesis may be a promising therapeutic strategy because it prevents the initiation of a cascade of molecular events that sensitize macrophages to inflammatory stimulation.

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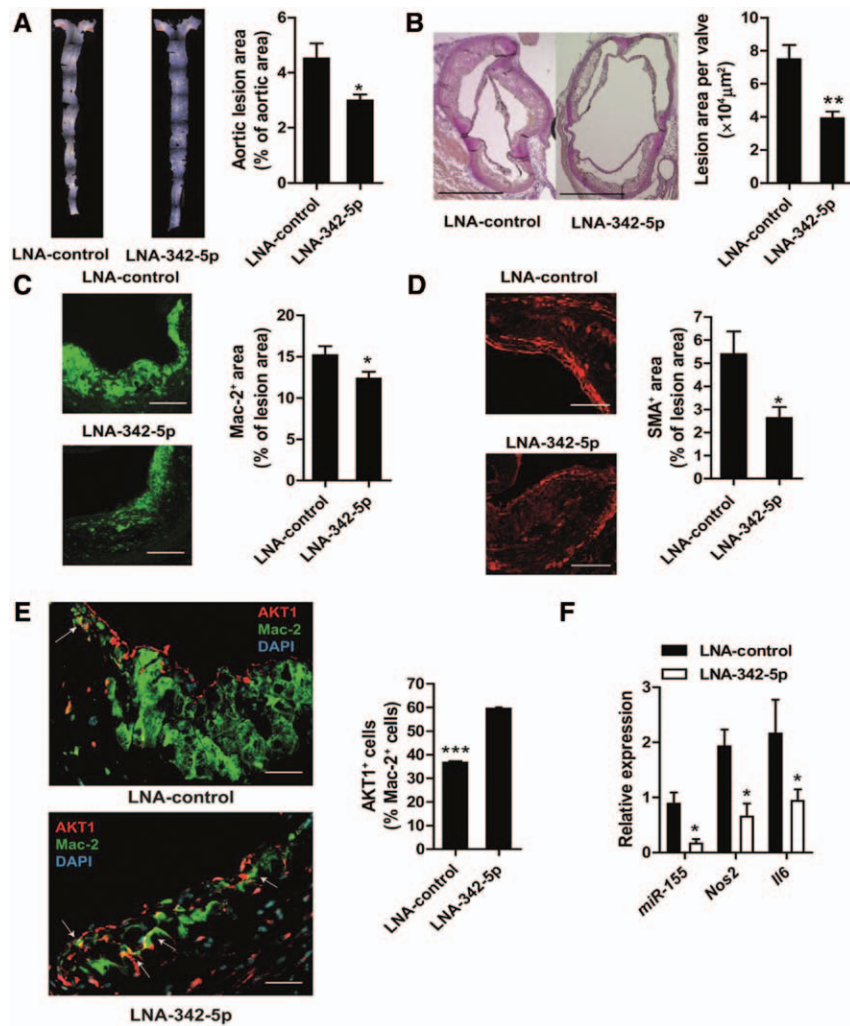


Figure 8. The effects of systemic administration of *miR-342-5p* inhibitors on diet-induced atherosclerosis. **A**, *Apoe*^{-/-} mice fed a high-cholesterol diet (HCD) for 2 months were treated with locked nucleic acid (LNA)-342-5p or LNA-control for 4 weeks. Lesion formation was determined in Oil red O-stained, en face prepared aortas. For all figures, **P*<0.05; ***P*<0.01, and ****P*<0.005. *n*=6 mice per group unless otherwise indicated. **B**, Representative sections of aortic roots stained with Elastic van Gieson stain from mice treated with LNA-342-5p or LNA-control. The lesion area was quantified by planimetry. Scale bars=500 μm . **C**, Accumulation of lesional macrophages, as determined by Mac-2 immunostaining, in aortic roots in LNA-342-5p-treated and LNA-control-treated *Apoe*^{-/-} mice. Scale bars=100 μm . **D**, Accumulation of smooth muscle cells in aortic root lesions after treatment with LNA-342-5p or LNA control as determined by smooth muscle actin (SMA) immunostaining. Scale bars=100 μm . **E**, Representative immunostaining for Akt1 in lesional macrophages in *Apoe*^{-/-} mice treated with LNA-342-5p or LNA control, Akt1 (red), and Mac-2 (green). Scale bars=25 μm . **F**, *miR-155*, *Nos2*, and *Il6* expression was quantified in the aortas of *Apoe*^{-/-} mice treated with LNA-342-5p or LNA control after 3 months of an HCD. *n*=3 or 4 mice per group.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerosis is a chronic inflammatory vascular disease and the leading cause of death worldwide. Deciphering the mechanisms of atherosclerosis is essential for developing novel and effective therapeutic strategies. microRNAs (miRNAs) are small RNA molecules that can fine-tune inflammatory responses through negative regulation of mRNA expression and may thereby control the pathogenesis of vascular diseases. Hence, studying the functional roles of miRNAs during atherogenesis may provide clues for the development of miRNA-based therapeutic strategies. Here, we demonstrated that miR-342-5p facilitates the inflammatory activation of macrophages by targeting Akt1 and promotes atherosclerotic lesions progression in mice. The suppression of Akt1 by miR-342-5p upregulates miR-155, which leads to increased lesional nitro-oxidative stress. Inhibition of miR-342-5p by local administration of antagomirs or systematic administration of locked nucleic acid antisense oligonucleotides greatly reduced the progression of atherosclerosis in *Apoe*^{−/−} mice. Thus, our data indicate that the progression of atherosclerosis can be suppressed by inhibiting miRNAs, which promote the inflammatory response in lesional macrophages. This treatment strategy may also be applied locally to the atherosclerotic vessel wall (eg, via drug-eluting stents) with presumably fewer side effects compared with systemic administration. Moreover, as a result of fine-tuning of the interactions of miRNAs in inflammatory macrophage activation, a more subtle manipulation of the chronic, nonresolving inflammation in atherosclerosis may be possible by inhibiting miRNAs without interfering with essential macrophage functions.