Inhibition of MicroRNA-92a Prevents Endothelial Dysfunction and Atherosclerosis in Mice

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Rationale for Study: MicroRNAs (miRNAs) are small noncoding RNAs that regulate protein expression at post-transcriptional level. We hypothesized that a specific pool of endothelial miRNAs could be selectively regulated by flow conditions and inflammatory signals, and as such be involved in the development of atherosclerosis.

Objective: To identify miRNAs, called atheromiRs, which are selectively regulated by shear stress and oxidized low-density lipoproteins (oxLDL), and to determine their role in atherogenesis.

Methods and Results: Large-scale miRNA profiling in HUVECs identified miR-92a as an atheromiR candidate, whose expression is preferentially upregulated by the combination of low shear stress (SS) and atherogenic oxLDL. Ex vivo analysis of atheroprone and atheroprotected areas of mouse arteries and human atherosclerotic plaques demonstrated the preferential expression of miR-92a in atheroprone low SS regions. In Ldlr−/− mice, miR-92a expression was markedly enhanced by hypercholesterolemia, in particular in atheroprone areas of the aorta. Assessment of endothelial inflammation in gain- and loss-of-function experiments targeting miR-92a expression revealed that miR-92a regulated endothelial cell activation by oxLDL, more specifically under low SS conditions, which was associated with modulation of Kruppel-like factor 2 (KLF2), Kruppel-like factor 4 (KLF4), and suppressor of cytokine signaling 5. miR-92a expression was regulated by signal transducer and activator of transcription 3 in SS- and oxLDL-dependent manner. Furthermore, specific in vivo blockade of miR-92a expression in Ldlr−/− mice reduced endothelial inflammation and altered the development of atherosclerosis, decreasing plaque size and promoting a more stable lesion phenotype.

Conclusions: Upregulation of miR-92a by oxLDL in atheroprone areas promotes endothelial activation and the development of atherosclerotic lesions. Therefore, miR-92a antagonist seems as a new atheroprotective therapeutic strategy. (Circ Res. 2014;114:434-443.)

Key Words: atherosclerosis ■ endothelium ■ microRNAs

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by 2 main hallmarks. First, atherosclerosis develops preferentially at sites of branching, curvatures, and bifurcations in large arteries where flow conditions are disturbed with prevalence of low or oscillatory shear stress (SS).1 Second, subendothelial accumulation of low-density lipoprotein (LDL) and its subsequent modification at these atheroprone areas lead to further activation of the vascular wall and maintain vascular inflammation.2 In response to oxidized low-density lipoproteins (oxLDL), endothelial cells express a range of chemokines and adhesion molecules that contribute to leukocyte recruitment, adherence, and migration into the subendothelium,3 the first stage in the development of atherosclerosis.4 Endothelial activation is highly regulated by SS through mechanisms involving a mechanosensory complex constituted of platelet endothelial cell adhesion molecule 1, vascular endothelial-cadherin, and vascular endothelial growth factor receptor 2 at endothelial cell–cell junctions;5 and through transcription factors including Kruppel-like factor 2 (KLF2),6 Kruppel-like factor 4 (KLF4),7 and Nrf2.8

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An entirely new level of post-transcriptional gene regulation through microRNA (miR) expression has recently emerged as

Original received July 17, 2013; revision received November 15, 2013; accepted November 19, 2013. In October 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 12.81 days.


The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.114.302213/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.114.302213

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an important mechanism in the development and progression of several diseases. Yet, the role of miRs in atherosclerosis has not been fully elucidated. Recently, miR33a/b have been shown to act as post-transcriptional regulators of lipid metabolism, and their pharmacological inhibition diminished atherosclerosis by raising plasma high-density lipoprotein–cholesterol and lowering very-low-density lipoprotein triglycerides levels. Also, in vitro data indicate that the endothelial expression of miRs is regulated by SS and can modulate endothelial inflammation, suggesting that these miRs might play a role in atherosclerosis. However, to date there exists no direct in vivo evidence to support the involvement of SS-regulated miRs in the development of atherosclerosis. Here, we hypothesized that SS might control the inflammatory response of endothelial cells to oxLDL through the expression of a limited set of specific miRs, that we named atheromiRs. By using a large-scale microarray analysis of miR expression in endothelial cells exposed to low or high SS in the presence or absence of oxLDL, we identify miR-92a as an atheromiR candidate, and Suppressor of Cytokine Signaling 5 (SOCS5) as a novel miR-92a target. The present study shows that antagoniR-92a treatment prevents endothelial dysfunction and limits the development of atherosclerotic lesions in Ldlr−/− mice, therefore establishing a major in vivo role for miR-92a in the inflammatory endothelial dysfunction leading to atherosclerosis.

Methods

Cell Culture

Confluent human umbilical vein endothelial cells (HUVECs; passage 2–4; Promocell, Heidelberg, Germany) were cultured on 0.2% gelatin-coated slides (Menzel Glazer; Braunschweig, Germany) in endothelium cell basal medium containing growth factors, 1% fetal calf serum (Promocell), streptomycin (100 IU/mL), penicillin (100 IU/mL), and Amphotericin B (10 μg/mL). Human vascular smooth muscle cells (hVSMCs; ATCC, passage 2–5) were cultured in smooth muscle medium (Promocell) and macrophages in RPMI1640 Gutamax medium (Gibco). To activate macrophages, stimulation with IFNγ (100 U/mL; BD) and lipopolysaccharide (1 μg/mL; Sigma-Aldrich) was performed for 24 hours.

Shear Stress Experiments

A controlled level of laminar SS was applied to confluent HUVECs using a parallel plate chamber connected to a perfusion system driven by a peristaltic pump (Gilson) as previously described in the work by Ramkhelawon et al. The flow chamber was placed in a sterile 5% CO2 incubator set at 37°C. HUVECs were placed in the perfusion system for 24 hours and exposed either to low SS (low SS, 4 dyn/cm²) or high SS (high SS, 15 dyn/cm²). To mimic atheroprotein conditions, HUVECs were exposed to proatherogenic oxLDL (25 μg/mL; Biomedical Technologies). Degree of LDL oxidation was given by manufacturer and determined by thiobarbituric acid reactive substance content analysis using malondialdehyde quantification (26.9 nmol/mg proteins). Native LDL (Biomedical Technologies) were treated with 40 μmol/L 3,5-Di-nitro-4 butylhydroxytoluene (BHT, Sigma-Aldrich) and 0.25 mmol/L ethylenediaminetetraacetic acid to prevent oxidation.

miRNA Expression Analysis

Total RNAs were prepared using Trizol (Invitrogen). miRNA profiling was realized in Millteny. miRNA expression was determined using Taqman miRNA assay (Life Technologies) according to manufacturer protocols. U6 snRNA was used as endogenous control.

In Vivo miR-92a Inhibition in Ldlr−/−

All experiments were performed in accordance with the European Community Guidelines for the care and use of laboratory animals (No.07430). Eight-week-old male Ldlr−/− mice (Charles River) were fed a high-fat diet for 14 weeks. AntagomiR treatment started at week 4. We tested 2 AntagomiRs doses (8 and 16 mg/kg), and we found the 16 mg/kg dose to be more effective. AntagomiRs (VBC biotech, Vienna) were delivered by retro-orbital i.v. injections under brief anesthesia. A second injection was performed at week 9. A scramble antagomiR (AntagomiR-Control) was used as control. AntagomiR-Control (anti-Ctrl): 5′-AAGGCAAGCUGACCCUGAGUCAUU-3′ and antagomiR-92a (anti-92a): 5′-CAGGGCGGGAACAGUGCAAUAU-3′. PBS-treated mice were used as control of the scramble antagomiR. The study design is shown in Online Figure VIII.

SOCS5 3′ Untranslated Region Luciferase Assay

For validation of SOCS5 as a target of miR-92a, 3′ untranslated region of human SOCS5 was cloned into a mammalian expression vector with dual luciferase reporter system (GeneCopoeia). Human embryonic kidney 293 cells were transfected using Hiperfect (Qiagen). Transfections were performed using 1 μg dual luciferase reporter plasmids and a final concentration of 100 nmol/L synthetic miR-92a mimic, or miR-21 as an irrelevant miRNA mimic (Applied Biosystems). Twenty-four hours after transfection, dual luciferase assays were performed using Luciferase assay kit (GeneCopoeia) according to manufacturer instructions. Firefly luciferase activity was normalized to Renilla luciferase expression control. The QuikChange II XL site-directed mutagenesis kit (Agilent) was used to mutate miR-92a binding site at SOCS5 3′ untranslated region.

STAT3 Inhibition In Vitro and In Vivo

Cell cultures were preincubated with Stattic (2 μM/L; Calbiochem), a nonpeptitic specific signal transducer and activator of transcription 3 (STAT3) inhibitor, for 1 hour and then subjected to SS and oxLDL stimulation in the presence of Stattic for 24 hours. In vivo STAT3 inhibition was performed in Ldlr−/− mice as previously described.

Statistical Analysis

Data are mean±SEM unless otherwise stated. Statistical analysis was performed with the GraphPad software. For statistical comparison, we used ANOVA followed by Bonferroni post hoc test or otherwise stated in figure legends. Differences were considered significant when P<0.05.

For detailed experimental methods, please refer to the Online Data Supplement.

Results

Endothelial miR-92a Expression Is Differentially Regulated by Shear Stress and Hypercholesterolemia

HUVECs were subjected to either low SS or high SS in the presence or absence of oxLDL for 24 hours. RNAs were then isolated and miRNAs expression profiled. Data were expressed as delta (Δ) log2 values (Online Tables I and II). We defined atheromiRs as miRs fulfilling the following 2 criteria: (1) expression is changed by oxLDL under low SS, and (2) expression is not or barely affected by oxLDL under high SS (Online Table II). As shown in Figure 1A (right panel), we identified miR-92a as the most dysregulated microRNA. Next, we validated...
the microarray data by qPCR (Figure 1B). First, miR-92a expression was higher in HUVECs exposed to atheroprone low SS as compared with atheroprotective high SS. Second, oxLDL stimulation significantly increased miR-92a expression in low SS conditions but had no effect on cells exposed to high SS. The observed modulations were specific of oxLDL but for native LDL stimulation had no effect (Online Figure I). Because miR-92a is part of the miR-17 to 92 cluster, we next quantified...
the expression of this cluster to determine whether other orthologs could fulfill the atheromiR criteria (Online Figure II). Interestingly, only miR-92a fulfilled these criteria.

To confirm our findings in vivo, we investigated the relative expression of miR-92a in atheroprone (aortic arch) versus protected (descending thoracic aorta) regions of aorta in normocholesterolemic Ldlr+/+ and hypercholesterolemic Ldlr−/− mice. In normocholesterolemic C57Bl/6 mice, miR-92a expression was higher in the aortic arch as compared with the descending aorta (Figure 1C). Interestingly, we observed a significant increase of miR-92a expression in the aortic arch of Ldlr−/− mice compared with normocholesterolemic animals, whereas its expression remained unchanged in the descending aorta (Figure 1C). In agreement with our in vitro findings, miR-92a was the only miR within the miR-17 to 92 cluster to exhibit the atheromiR characteristics in vivo (Online Figure III). We also addressed the relevance of these findings to human atherosclerosis. We found that miR-92a was highly expressed in the downstream parts of human carotid plaques where low SS prevails23,24 as compared with the upstream parts, where its expression was similar to healthy parts of carotid arteries (Figure 1D).

The endothelial specificity of miR-92a was further confirmed by comparing its expression in endothelial cells, vascular smooth muscle cells, and macrophages on oxLDL or lipopolysaccharide/IFN-γ stimulation. Indeed, miR-92a expression was ≈15-fold higher in endothelial cells than in vascular smooth muscle cells or macrophages (Online Figure IV). Although macrophage expression of miR-92a was low, we assessed its potential role on foam cell formation by studying the effect of its overexpression or inhibition on oxLDL uptake in macrophages. We observed no effect of the modulation of miR-92a expression on lipid accumulation by macrophages (Online Figure V).

Moreover, in situ hybridization in mice showed that miR-92a expression was confined to the endothelium (Online Figure VI) and showed stronger expression in the inner curvature where low and oscillatory SS prevail compared with the outer curvature (Online Figure VI). Additionally, the pattern of miR-92a staining was similar to CD31 staining, indicating a preferential localization to endothelial cells.

In Vitro miR-92a Inhibition Protects Against Endothelial Inflammation in Response to Low Shear Stress and oxLDL Stimulation, and Prevents Monocyte Adhesion

To investigate the effects of miR-92a on endothelial inflammatory responses, we quantified IL-6 and MCP-1 release by ELISA after HUVEC transfection with specific miR-92a modulators (antimiR-92a or premiR-92a). AntimiR-92a efficiently blocked endothelial miR-92a expression under low and high SS in the presence or absence of oxLDL. PremiR-92a enhanced miR-92a expression in cells exposed to low SS but was much less efficient in cells under high SS likely because of potent

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**Figure 3.** miR-92a modulation affects leukocyte adhesion to endothelial cells in vitro. **A** and **C**, Relative adhesion of U937 to HUVEC in antimiR-control vs antimiR-92a transfected cells (**A**) or in premiR-control vs premiR-92a transfected cells (**C**) in response to shear stress (SS) with or without oxLDL stimulation for 24 hours. Data are expressed as relative values to low SS + antimiR-control and premiR-control. **B** and **D**, Intercellular adhesion molecule-1 (ICAM-1) expression in different SS conditions after miR-92a inhibition (**B**) or overexpression (**D**), with or without oxLDL stimulation. Data are represented as mean±SEM. N=3 to 4 independent experiments (each experiment assayed each condition in duplicate). *P<0.05 vs LSS+anti/premiR-Control without oxLDL, †P<0.05 vs LSS+anti/premiR-Control+oxLDL, ‡P<0.05 vs HSS+premiR-Control without oxLDL.
counter-regulatory mechanisms exerted by SS. Additionally, miR-92a modulation did not affect ortholog expression in response to SS and oxLDL (Online Figure VII). PremiR-92a similarly increased IL-6 and MCP-1 secretion, whereas miR-92a inhibition reduced it (Figure 2). We verified that the transfection itself had no effect on cytokine release (Online Table III).

OxLDL stimulation specifically increased IL-6 and MCP-1 secretion under low SS, which was partly reversed after miR-92a inhibition (Figure 2A and 2C). In contrast, miR-92a overexpression increased IL-6 and MCP-1 secretion in response to oxLDL, mainly under low SS (Figure 2B and 2D). These findings clearly indicate that miR-92a mediates endothelial cell activation by oxLDL, specifically when SS is low.

To analyze the functional consequences of miR-92a-mediated effects on endothelial activation, we performed a leukocyte-endothelial cell adhesion assay. As expected, adhesion of U937 to endothelial cells was higher under low than under high SS conditions (Figure 3A and 3C). miR-92a inhibition markedly decreased U937 adhesion under low SS in the presence or absence of oxLDL (Figure 3A). In contrast, miR-92a overexpression abrogated the protective effect of high SS and led to a significant increase of U937 adhesion under these conditions (Figure 3B).

To further investigate the link between monocyte adhesion and endothelial activation, we determined intercellular adhesion molecule-1 (ICAM-1) expression after miR-92a modulation. As shown in Figure 3C and 3D, ICAM-1 expression was much higher in low than in high SS conditions. oxLDL stimulation increased ICAM-1 expression both in low and high SS conditions, miR-92a inhibition reduced ICAM-1 expression when cells were exposed to low SS both in the presence or absence of oxLDL. Conversely, miR-92a overexpression induced a significant increase of ICAM-1 expression.

Our findings demonstrate that endothelial miR-92a controls oxLDL-induced endothelial cell adhesive properties in an SS-dependent manner.

In Vivo Inhibition of miR-92a Limits the Development of Atherosclerosis

To determine the in vivo role of miR-92a in the setting of progressing atherosclerosis, we specifically blocked miR-92a expression in Ldlr−/− mice fed a high-fat diet. Mice were fed for 4 weeks to initiate the development of atherosclerosis before starting the antagomir treatment for 10 additional weeks (Online Figure VIII). A scramble antagomir (Antagomir-Control) or PBS were used as controls. The efficacy of miR-92a inhibition was assessed in several tissues at the dose of 16 mg/kg, which was slightly more efficient than 8 mg/kg (Online Figure VIII) without affecting ortholog expression. miR-92a was significantly reduced up to 14 weeks after antagomir treatment in lungs, heart, liver, kidneys, and plasma (Online Figure IX). It is noteworthy that miR-92a plasma levels in antimiR-92a–treated Ldlr−/− mice were similar to those in C57B/6 mice (1.01±0.07 Arbitrary Unit). Antagomir treatment had no effect on body or liver weight, and did not alter the immune profile in blood and spleen (Online Table IV and Online Figure X). Plasma total cholesterol, high-density lipoprotein–cholesterol, and triglycerides levels were not altered by the antimiR-92a treatment (Online Table IV).

Quantification of atherosclerotic lesions in the thoracic aorta by en face analysis after 14 weeks of high fat diet showed that antimiR-92a treatment significantly decreased atherosclerosis by 32% compared with anti-Ctrl treatment or PBS (Figure 4A). When atherosclerotic lesions were analyzed according to their localization (aortic arch versus thoracic aorta), we found that antagomir-92a treatment significantly reduced lesions at aortic arch but much less in the thoracic aorta (Online Figure XI). Aortic sinus lesions also significantly decreased by 23% in antimiR-92a–treated Ldlr−/− mice compared with anti-Ctrl–treated or PBS-treated mice (Figure 4B) despite persistent high levels of plasma cholesterol.

Plaque composition analysis was determined by quantification of macrophage, lymphocyte, collagen content, and ICAM-1 expression. As shown in Figure 5, macrophage and T lymphocyte accumulation were significantly decreased by antimiR-92a treatment compared with PBS-treated or anti-Ctrl–treated mice. In contrast, the collagen content was increased, suggesting that antimiR-92a treatment led to more stable plaques (Figure 5C). We also investigated ICAM-1 expression, as a marker of endothelial inflammation, and found that ICAM-1 was markedly decreased by antimiR-92a treatment (Figure 5D).

miR-92a Targets In Vitro

Previous studies have shown that miR-92a negatively regulated the key endothelial transcription factors KLF2 and KLF4 through...
miR-92a recognition elements. Here, we investigated the effect of miR-92a on the expression of KLF2 and KLF4 in endothelial cells in response to flow and oxLDL stimulation. As shown in Online Figure XII, KLF2 and KLF4 expression levels were enhanced under high compared with low SS, both in the presence or absence of oxLDL. Under low SS, both KLF2 and KLF4 were decreased after stimulation with oxLDL. Specific blockade of miR-92a abrogated oxLDL effect and resulted in increased KLF2 and KLF4 expression. Blockade of miR-92a also markedly increased KLF2 and KLF4 expression under high SS. Next, we explored the regulation of NOS3 and NFκB, downstream of KLF2 and KLF4.25 As shown in Online Figure XII, NOS3 expression was much higher in high compared with low SS. miR-92a inhibition increased NOS3 expression independently of the SS level. The active form of NFκB, phospho-p65, was mainly expressed under low SS compared with high SS. miR-92a inhibition under low SS reduced phospho-p65 expression. Under high SS, blockade of miR-92a resulted in a moderate increase of phospho-p65 that was not affected by oxLDL. p65 expression was consistently more expressed under low SS compared with high SS and was modulated in parallel of KLF2 and KLF4 (Online Figure XIII). These findings confirm that KLF2 and KLF4 are targeted by miR-92a and show, for the first time, that KLF2 and KLF4 and their downstream elements can be modulated by miR-92a treatment after flow and oxLDL stimulation.

miR-92a Targets In Vivo

To translate our in vitro findings to the in vivo situation, the expression levels of KLF2, KLF4, NOS3, and p65 were determined in aortas of Ldlr−/− mice treated with PBS, anti-Ctrl, or anti-miR-92a. As shown in Figure 6, KLF2 and KLF4 expressions were markedly increased by anti-miR-92a treatment compared with PBS and anti-Ctrl treatment. NOS3 expression was increased by anti-miR-92a treatment, whereas phospho-p65 expression was decreased. p65 was also decreased by anti-miR-92a treatment (Online Figure XIII).

To further assess the protective effect of anti-miR-92a treatment against atherosclerosis-induced endothelial dysfunction, we determined plasma levels of endothelial microparticles (MPs) as a biomarker of endothelial dysfunction.26 As shown in Figure 6C, circulating endothelial CD144+ MPs levels were increased ≈3-fold in Ldlr−/− mice treated with PBS or anti-Ctrl as compared with age-matched C57BL/6 mice. Interestingly, anti-miR-92a treatment prevented endothelial dysfunction in Ldlr−/− mice as shown by the reduction of circulating CD144+ MPs to the level observed in normocholesterolemic mice. We also addressed the inflammatory status of treated mice by quantifying levels of circulating ICAM-1 as a marker of endothelial inflammation.27 As shown in Figure 6D, circulating endothelial CD144+ MPs levels were increased ≈3-fold in Ldlr−/− mice treated with PBS or anti-Ctrl as compared with age-matched C57BL/6 mice. Interestingly, anti-miR-92a treatment prevented endothelial dysfunction in Ldlr−/− mice as shown by the reduction of circulating CD144+ MPs to the level observed in normocholesterolemic mice. We also addressed the inflammatory status of treated mice by quantifying levels of circulating ICAM-1 as a marker of endothelial inflammation.27 As shown in Figure 6D, circulating ICAM-1 levels were decreased by 31% in mice treated with anti-miR-92a as compared with those treated with PBS or anti-Ctrl, indicating that blocking miR-92a protects from endothelial inflammation.

Identification of SOCS5 as a New Target of miR-92a in Response to Shear Stress

Large gene analysis in experimental conditions where miR-92a is highly expressed (low SS+oxLDL+anti-Ctrl)
compared with conditions where miR-92a is blocked (low SS+oxLDL+antimiR-92a) allowed us to determine potential new targets (Online Figure XIV). By using Gene Expression Arrays analysis, we identified 1 gene that was predicted to be a miR-92a target in human and mouse genomes, and was upregulated >25% in low SS+oxLDL+Anti-92a conditions compared with low SS+oxLDL+anti-Ctrl (Online Figure XIV). We found that SOCS5 expression was significantly increased and was validated by qPCR (Online Figure XV). 3′ untranslated region luciferase assay confirmed that miR-92a targeted SOCS5 as shown in Figure 7A. Protein expression level analysis of SOCS5 revealed that SOCS5 was expressed more strongly in high SS than in low SS in vitro. In response to oxLDL, SOCS5 decreased under low SS, whereas no change was observed under high SS. miR-92a inhibition increased, whereas miR-92a overexpression reduced SOCS5 expression in low SS (Figure 7B and 7C). Modulation of miR-92a had no effects in high SS conditions. SOCS5 function was assessed under high SS conditions where its expression was particularly high. We selectively blocked SOCS5 expression using siRNA (Online Figure XVI). SOCS5 inhibition on high SS and oxLDL stimulation increased IL-6 and MCP-1 release, indicating that SOCS5 protects against inflammation (Figure 7D and 7E). Silencing SOCS5 did not affect KLF2, KLF4, and NOS3 expression (Online Figure XVI).

In vivo, SOCS5 expression was similarly high in the aortic arch and descending aorta of normocholesterolemic mice but markedly decreased in the aortic arch of hypercholesterolemic mice (Figure 7F) where expression of miR-92a was the highest and remained highly expressed in the descending aorta of hypercholesterolemic mice where miR-92a was low. We also addressed the relevance of these findings to human atherosclerosis and found that SOCS5 gene expression was highly repressed in the downstream parts of human carotid plaques where low SS prevails23,24 and where miR-92a is highly expressed as compared with the upstream parts (Online Figure XVII).

miR-92a Expression Is Regulated by Stat3 in Atheroprone Conditions

Previous studies have shown that miR-92a expression was driven by several transcription factors (TFs), including STAT3 and c-Myc.28 On the contrary, these TFs may be regulated by SS.29–32 Therefore, we analyzed the expression of these TFs as a function of SS and hypercholesterolemia in vitro and in vivo. We found that STAT3 mRNA expression displayed the same expression pattern as miR-92a both in vitro and in vivo (Figure 8A and 8B) whereas c-Myc and miR-92a showed different expression patterns (data not shown). To examine the direct effect of STAT3 activation on miR-92a expression, we selectively blocked STAT3 activity using Stattic (see Methods) and found reduced miR-92a expression in response to low SS+oxLDL (Figure 8C). Moreover, in vivo blockade of STAT3 in Ldlr−/− mice prevented miR-92a expression in atheroprone areas (Figure 8D) and resulted in increased expression of SOCS5 (Online Figure XVIII). Altogether, these findings highlight the role of STAT3 in the regulation of miR-92a expression in response to SS and hypercholesterolemia and its consequences on endothelial inflammation.

Discussion

The results presented herein demonstrate for the first time the involvement of microRNA-92a in the development of atherosclerosis in vivo. The antagoniR-92a also represents a new strategy to treat atherosclerosis by preventing endothelial dysfunction in hypercholesterolemic state.

The newly emerging gene regulators, miRNAs, have been involved in a wide range of processes, which are linked to
atherosclerosis and cardiovascular diseases. We hypothesized that a restricted pool of endothelial miRNAs could be involved in the specific regulation of genes contributing to the development of atherosclerosis. These miRNAs, so-called atheromiRs, could represent targets for the development of new therapeutic strategies against atherosclerosis. Here, we provide strong evidence that miR-92a is an atheromiR, being preferentially expressed in endothelial cells and strongly stimulated by proatherogenic oxLDL, specifically in atheroprone areas where SS is low.

Endothelial activation is a primary event in the development of atherosclerosis. We demonstrated using both loss- and gain-of-function strategies that miR-92a acts as a proinflammatory regulator in endothelial cells by activating inflammatory cytokines and chemokines, and promoting monocyte adhesion. Moreover, NOS3 expression was restored by miR-92a inhibition in vitro. Our findings clearly indicate that miR-92a inhibition prevents in vitro endothelial activation. Additionally, measurement of endothelial MPs, a marker of endothelial dysfunction, and analysis of ICAM-1 expression in lesions demonstrated that antimiR-92a treatment protects against endothelial activation and dysfunction in vivo.

Atherosclerosis development is tightly controlled by both innate and adaptive immunity. Although miR-92a expression had been previously reported in T lymphocytes, we did not observe any effect of miR-92a inhibition on immune parameters, including monocyte, T, and B cell counts as well as immune cell activation. These observations reinforce our conclusion that the antiatherogenic effects observed after miR-92a blockade resulted from protection against endothelial dysfunction. However, T cell and macrophage contents in plaques were reduced by antimiR-92a treatment. This was likely because of the antiadhesive effect of miR-92a blockade in endothelial cells, possibly involving KLF2.

Previous studies showed that the key endothelial transcription factors, KLF2 and KLF4, are direct targets of miR-92a. Our data indicate that miR-92a inhibition resulted in increased expression of these factors, associated with reduction in activated NFκB, which likely accounted for the anti-inflammatory phenotype observed in vitro and in vivo after miR-92a blockade.
KLF4 is involved in the regulation of endothelial inflammation through blockade of NFκB pathway activation, and KLF2 is known to be protective and modulated by flow in endothelial cells. Moreover, deficiency in KLF2 augments experimental atherosclerosis. More recently, it has been established that endothelial KLF4 protects against atherosclerosis. In addition, we identified SOCS5 as a novel miR-92a target that is involved in the regulation of endothelial inflammation. SOCSs are key regulators of cytokine-induced responses in hematopoietic as well as nonhematopoietic cells. The specific functions of SOCS5 have not yet been determined. Recently, Zhuang et al reported that reduced SOCS5 levels in endothelial cells led to activation of JAK-STAT pathway. Here, we provide the first evidence that SOCS5 expression is induced by shear stress and confers anti-inflammatory properties to endothelial cells.

In the present study, we also demonstrate that miR-92a is expressed in human atherosclerotic lesions and differentially expressed as a function of SS. Preferential expression in downstream parts of human carotid lesions that are subjected to low shear stress is in agreement with our in vitro and in vivo data. miR-92a expression is controlled by a large variety of transcription factors including STAT3. Our in vitro and in vivo studies unveil a critical role for STAT3 in regulating miR-92a expression, which is in agreement with a recent report showing that STAT3 upregulates miR-92a in lung cancer cells.

In conclusion, we report here for the first time that miR-92a is a proatherogenic miR, which specifically promotes endothelial dysfunction and inflammation in a flow-dependent manner. Proatherogenic oxLDL activate miR-92a expression only in atheroprone low SS areas. High SS protects endothelial cells from elevated miR-92a expression but, more importantly, it hampers its induction by oxLDL. Hence, blockade of miR-92a seems as a novel therapeutic strategy to protect against hypercholesterolemia-induced endothelial dysfunction and atherosclerosis development.

Sources of Funding
This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM). We are grateful to Fondation pour la Recherche Médicale, Fondation Lefoulon-Delalande and ANR-11-META-004-02 for supporting Xavier Loyet. Stéphane Potteaux and Anne-Clémence Vion were supported by grants from the Région Ile-de-France CORDIM (Domaine d’Intérêt Majeur Cardiovasculaire-Obésité-Rein-Diabète).

Disclosures
None.

References


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Circ Res. 2014;114:434-443; originally published online November 19, 2013;

doi: 10.1161/CIRCRESAHA.114.302213

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/3/434

Data Supplement (unedited) at:
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**Supplemental Material**

**Online methods**

**miR-92a in vitro modulation**

miR-92a inhibition or overexpression were achieved in vitro by transfecting endothelial cells with either anti-miR-92a inhibitor or pre-miR-92a precursor, respectively, using Hiperfect transfection reagent (Qiagen). Anti-miR-Control#1 and pre-miR-Control#1 were used as control (All from Ambion, 50 nM).

**ELISA Assay**

The culture medium of cells exposed to different conditions of SS, with or without oxLDL, or after modulation of miR-92a (either inhibition or overexpression) were removed and centrifuged at 500g for 15 min, and stored at -80°C. Interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) levels were quantified using human IL-6 and human MCP-1 ELISA kit, respectively, provided by BD Pharmingen (San Diego, CA).

**Monocyte adhesion assay**

HUVECs were transfected with either miR-92a inhibitor or miR-92a precursor. U937 cells (ATCC) were labeled with fluorescence tracker (CFSE, Sigmaaldrich) for 30 min. After exposure to different conditions of SS for 24 hours, slides were removed from the flow chamber and incubated without flow with labeled U937 cells for 1h. After incubation, non-adherent cells were carefully removed by washing twice with 1X PBS, and cells were collected. Data were acquired on a BD LSR II Flow Cytometer and analyzed with FlowJo software.

**Regional isolation of aorta**

miR-92a expression patterns were determined in vivo in atheroprone and atheroprotected areas of the aorta in C57Bl6 (Charles River) and hypercholesterolemic mice (Ldlr⁻/⁻, Charles River) after 11 weeks of high fat diet (HFD) containing 15% fat, 1.25% cholesterol, and 0% cholate (Safe). Mice were euthanized and the aorta was dissected under binocular magnificator. The aortic arch (AA) and descending aorta (DA) were separated as previously described and kept on cold PBS ¹. Total RNAs were prepared using Trizol, and miR-92a was quantified as described above.

**miR-92a expression in human atherosclerotic lesions**

The study was approved by the local ethical committee, and all participants gave written informed consent. miR-92a expression level was assessed in human atherosclerotic plaques obtained from patients undergoing carotid endarterectomy as previously described ². Carotid samples were cut into 3 parts: healthy part, upstream part of atherosclerotic lesions and downstream part of atherosclerotic lesion. RNAs were prepared as described above and miR-92a expression was quantified.

**Tissues and blood collection**

Blood samples were collected and used for flow cytometry analysis, endothelial microparticule (MP) quantification and biochemical measurements. Serum total cholesterol, triglycerides and HDL-cholesterol were determined using commercial enzymatic kits on a DxC800 analyzer (Beckman-Coulter, Villepinte, France). The non-HDL cholesterol values were obtained by subtracting HDL-cholesterol from serum total cholesterol. For plasma
preparation and MP isolation, citrated blood was centrifuged at 1500 g for 15 min at room temperature, and the supernatant was centrifuged again at 13 000 g for 10 min to avoid platelet contamination. Plasma free of platelets was stored at -80°C until analysis. Just before analysis, plasma was centrifuged for 90 min at 20,500g at 4°C to pellet the MP fraction and avoid fat contamination between 0.1 and 1µm. MP pellet was resuspended in a volume of filtered PBS equal to the initial volume of plasma.

**Tissue preparation**

Spleen was collected for flow cytometry analysis. The heart and aorta were removed and fixed in 4% paraformaldehyde. Heart was embedded in OCT medium and frozen at -70°C. Aortas were open under a binocular magnificator and stained with Oil Red O (Sigma-Aldrich) to quantify atherosclerotic lesions in the ascending and descending aorta. Successive 10-µm sections of the aortic sinus were obtained from frozen heart. Atherosclerotic lesions were detected using Oil red O staining. Plaque composition was assessed by specific staining for macrophages, T lymphocytes, collagen and ICAM-1, using a monoclonal rat anti-mouse CD68 antibody (AbD Serotec), a polyclonal goat anti-CD3 antibody (DAKO), Sirius red and a monoclonal hamster anti-mouse ICAM-1/CD54 (BD Pharmingen) staining, respectively. Atherosclerotic lesions were quantified in en face total aorta. Lesion areas were determined by the ratio of lesion area to total aortic area. Morphometric studies were performed in the thoracic aorta and in aortic root sections using Histolab software (Microvisions). At least 4 sections per mouse were examined for each immunostaining, and appropriate negative controls were used.

**Flow cytometry analysis**

Leukocyte phenotyping was performed in blood, bone marrow, spleen and lymph nodes. Total leukocyte number was measured by automatic count (Z1 Coulter Particle Counter, Life Science Discovery) according to manufacturer’s instructions. The leukocyte populations were identified by staining with anti-B220 (RA3-6B2), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-NK1.1 (PK.36), CD11b (M1/70), CD11c (HL3), anti-MHCII (M5/114.15.2), anti-CD115 mAb (AFS98), and anti-Gr1 antibody (RB6-8C5). Data were acquired on a BD Fortessa Flow Cytometer and analyzed with FlowJo software.

**Microparticles analysis**

Twenty µl of MP resuspended pellet were incubated with 10µL anti-CD144 (BD Biosciences) at room temperature for 30 min in the dark, washed with 200µL PBS and then centrifuged at 20,500 g for 90 minutes. The resulting MP pellet was incubated with 4µL FITC-conjugated anti-rat IgG specific polyclonal antibody (Life technology) for 30 min in the dark. After incubation, samples were diluted in 200µL PBS and centrifuged at 20,500 g for 90 min. Pellet MP was resuspended in 200µL PBS prior to flow cytometry analysis (FORTESSA, BD). Flow-count fluorospheres (Beckman Coulter) were used to establish the MP concentration in the sample and data were analyzed using FlowJo software.

**Western Blot Analysis**

Cells and aortas were homogenized in RIPA buffer (50 mmol/L Tris-HCL [pH 7.4], 1 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L glycerophosphate, 0.1% SDS, 0.1% deoxycholate, 1% Triton, complete protease inhibitor cocktail tablet, Roche) as previously described in 1. Membranes were incubated with anti ICAM-1 (Santa Cruz and R&D), anti-KLF4 (Santa Cruz), anti-KLF2 (Santa Cruz), p65 (Cell Signaling), phospho-p65 (Cell Signaling), anti-NOS3 (Transduction Laboratories) and SOCS5 (Abcam). After secondary antibody incubation (Amersham), immunodetection proceeded using an enhanced chemiluminescence kit (ECL Plus, Amersham), and bands were revealed using the Las1000 imaging system and Image Gauge software (Fuji). After initial immunodetection, all
membranes were stripped of antibodies and reprobed with an anti-GAPDH antibody (Millipore).

**Gene expression analysis**

Total RNA was extracted from HUVECs, aortas or human biopsies with Trizol reagent according to manufacturer’s instructions (Invitrogen). DNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Polymerase chain reaction was performed on an ABI Prizm 7700 with the use of Power SYBR Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France). Mouse or Human GAPDH was used to normalize sample amplification. The following oligonucleotides served as primers: Mouse GAPDH forward: 5'-CGTCCCGTAGAAGGATGGTAA-3'; reverse: 5'-GCCGTAGAGTCATCGA-3'.

**SOCS5 silencing in vitro**

SOCS5 was silenced using ON-TARGETplus Human SOCS5 siRNA SMARTpool (Dharmacon). HUVECs were transfected with 50 nM ON-TARGETplus Human SOCS5 siRNA SMARTpool using Hiperfect following manufacturer’s instructions for 6 hours. Thereafter, medium was changed and SS applied for 24 hours with or without oxLDL.

**Taqman Gene Array analysis**

Gene arrays were performed using Taqman Gene Expression Assays (Applied Biosystems) according to manufacturer’s protocol. We used 8 different Applied Biosystem’s TaqMan Gene expression Arrays, specific for NFκB pathway, Extracellular matrix, Chemokines, Inflammation, SMAD pathway, Signal transduction, Apoptosis, and Cytokines. Briefly, 10 ng cDNA were used per well using Taqman Universal master mix (Applied Biosystems). RT-qPCR was performed on 7900HT System, and analysis was performed accordingly to manufacturer’s protocol.

**oxLDL uptake assay in macrophages**

Primary macrophages were derived from mouse bone marrow. Tibias and femurs of Ldlr−/− male mice were dissected, their marrow flushed out. Cells were grown for 7 days at 37°C in RPMI 1640 medium, 20% neonatal calf serum, and 20% Macrophage–Colony-Stimulating Factor (M-CSF)-rich L929-conditioned medium. Then, to assess oxLDL uptake, macrophages were transfected either with anti-miR-92a, pre-miR-92a, or with their respective controls (anti-miR-Ctrl or pre-miR-Ctrl) for 24 hours. Thereafter, cells were stimulated with either native LDL or oxLDL (25µM/L) for 72 hours. After incubation, cells were lysed in acetone and cholesterol content was measured using a commercial kit (Biomerieux). Data were expressed as ng cholesterol /μg of protein determined by BCA method (BioRad).

**In situ hybridization on aortas**

In situ hybridization was performed as previously described by Bonauer et al. Briefly, 7 μm-
thick paraffin-embedded aortic sections were cut and fixed in PFA 4% for 10 min. Then sections were washed with 1X PBS, acetylated for 10 min, and washed again before treatment with protein kinase K (Sigma-Aldrich) for 10 min at 37°C. After washing, sections were incubated with hybridization buffer for 5 h at room temperature. miRNA probes (miR-92a probe double-DIG labeled LNA probes, Exiqon, final concentration 20nM) were mixed with denaturation buffer and added to the sections followed by incubation overnight at 56 °C. U6snRNA probe (3'-DIG labeled LAN, probe, Exiqon) was used at 10 nM final concentration and as a positive control. The day after, sections were put in successive decreasing SSC buffers for 5 min (5X 1 time, 1X 2 times, 0.2X 3 times) and then washed. After incubation for 1 hour in blocking solution (B1 solution + 3% fetal calf serum+ 0.1% Tween-20), sections were incubated with anti-DIG AP antibody (Roche; 1:2000) overnight at 4°C. After washing, sections were incubated with NBT/BCIP (Promega) in NTMT + levamisole (0.2 mM/L) for 48 hours in the dark at RT. NBT/BCIP was changed every 12 hrs. After miR-92a detection, slides were counter-stained with CD31 as an endothelial marker (DIA-310 Histonova) following manufacturer’s protocols.

Online Tables

**Online Table I.** Top 10 miRNAs significantly modulated by shear stress. Data are delta (Δ) of Log₂ values.

**Online Table II.** Top 10 upregulated miRNAs in LSS + oxLDL vs HSS + oxLDL condition. Data are delta (Δ) of Log₂ values. Note that these miRNAs represent AtheromiR and that miR-92a appears at Rank #1.

**Online Table III.** Baseline values of IL-6 and MCP-1 release in response to different SS levels and oxLDL upon transfection. Data are mean ± s.e.m. N=3 independent experiments (each experiment assayed each condition in triplicate).

**Online Table IV.** Physiological parameters in *LdLr* mice treated with AntagomiRs. Data are mean ± s.e.m.

Online Figures legends

**Online Figure I.** miR-92a expression as a function of shear stress and LDL stimulation. microRNA-92a (miR-92a) expression was evaluated by Taqman Assay and normalized to U6snRNA in HUVECs exposed to low or high SS, and stimulated with native LDL or oxidized LDL (oxLDL). Data are mean ± s.e.m. N=3 independent experiments (each experiment assayed each condition in triplicate). **p<0.01 vs LSS control, † p<0.05 vs LSS + oxLDL.

**Online Figure II.** microRNA-17-92a cluster expression analysis in HUVECs exposed to low and high shear stress and stimulated with oxLDL. Expression of microRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) was evaluated by Taqman Assay and normalised to U6snRNA. Data are mean ± s.e.m.
N=3 independent experiments (each experiment assayed each condition in triplicate). *p<0.05 vs LSS without oxLDL and † p<0.05 vs LSS without and with oxLDL.

Online Figure III. microRNA-17-92a cluster expression analysis in atheroprotected areas in normocholesterolemic and hypercholesterolemic mice. Expression of microRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) was evaluated by Taqman Assay in aortic arch. And descending aorta of normocholesterolemic (C57Bl/6 – white column) and hypercholesterolemic (Ldlr−/− + High Fat Diet – Black column) mice and normalised to U6snRNA. Data are mean ± s.e.m. n=6 per group. *p<0.05 vs C57bl6 and † p<0.05 vs aortic arch C57Bl/6.

Online Figure IV. microRNA-92a expression in different cell types in vitro. microRNA-92a (miR-92a) expression was evaluated by Taqman Assay and normalized to U6snRNA in human umbilical venous endothelial cells (HUVECs), human vascular smooth muscle cells (hVSMC), and macrophages (Mφ), stimulated for 24 hours with oxLDL (25µg/mL), LPS/IFNγ (1µg/µL/100 Unit/mL). Data are mean ± s.e.m. N=3 independent experiments (each experiment assayed each condition in triplicate). *** p<0.001 vs HUVECs without stimulation.

Online Figure V. oxLDL uptake in Ldlr−/− macrophages after miR-92a modulation. oxLDL uptake was quantified in bone marrow-derived macrophages from Ldlr−/− mice after specific modulation of miR-92a (inhibition by anti-miR-92a or overexpression by pre-miR-92a transfection). Data are mean ± s.e.m. n= 6 per group. * p<0.05 vs unstimulated or native LDL treated cells.

Online Figure VI. miR-92a in situ hybridization in mouse aortic arch. A- Representative picture of the aortic arch with carotid branches (magnification x25, scale bar : 1 mm), B- Representative showing miR-92a staining (blue) in combinaison with CD31 (brown) (magnification x400; 25µm) C- Representative negative control picture (magnification x400, scale bar : 25µm).

Online Figure VII. microRNA-92a modulation in vitro. A- microRNA-92a expression in HUVECs after inhibition with anti-miR-92a (left pannel) or overexpression by pre-miR-92a transfection (right pannel). Cells were transfected with either pre-control (Pre-Ctrl) or Anti-control (Anti-Ctrl) for 24hrs and miR-92a expression was quantified by Taqman assay and normalized to U6snRNA. N=3 independent experiments (each experiment assayed each condition in duplicate). Data are mean± s.e.m. ** p<0.01 vs non treated cells and respective controls. B- miR-92a expression after miR-92a inhibition in cells exposed to low or high SS and stimulatied with oxLDL. Cells were transfected for 48 hours with either anti-miR-Ctrl or anti-miR-92a. C- microRNA-92a expression after miR-92a overexpression. Cells were transfected for 48 hours either with pre-miR-Ctrl or pre-miR-92a. 24 hours after transfection, cells were exposed to either low (LSS) or high (HSS) shear stress, in the presence or absence of oxLDL for 24 hours. D- miR-17 and miR-19a expression after miR-92a inhibition in HUVECs exposed to different shear stress levels and oxLDL stimulation. Data are mean ± s.e.m. N=4 independent experiments (each experiment assayed each condition in duplicate). * p<0.05 vs low SS + respective-miR-ctrl, † p<0.01 vs corresponding control HSS without oxLDL conditions.
Online Figure VIII. In vivo AntagomiR study design and miR-92a inhibition in aortas of LDLr<sup>-/-</sup> mice after antagomiR treatment. A- Study design of the in vivo experiments, B- miR-92a expression was evaluated by Taqman Assay and normalized to U6snRNA in aortas of LDLr<sup>-/-</sup> mice treated with PBS, Anti-Control (Anti-Ctrl) and Anti-miR-92a (Anti-92a). Anti-Ctrl and Anti-92a antagomiRs were used at 8 mg/kg or 16mg/kg. Data are mean ± s.e.m. N=6 per group. *p<0.05 vs corresponding Anti-Ctrl, † p<0.05 vs 8 mg/Kg Anti-92a. C- miR-17 and miR-19a expression in atheroproliferative aortas after in vivo miR-92a blockade with AntagomiR-92a (16mg/kg) for 10 weeks. D- AntagomiR efficiency as a function of regional aortas. miR-92a and miR-17 expressions were quantified by Taqman Assay and normalized to U6snRNA in aortas of LDLr<sup>-/-</sup> mice treated with AntagomiR-92a (Anti-92a). *** p<0.001 vs PBS or Anti-Ctrl. * p<0.05 vs PBS or Anti-Ctrl.

Online Figure IX. microRNA-92a inhibition in different organs after antagomiR treatment. miR-92a expression was evaluated by Taqman Assay and normalized to U6snRNA in A- lungs, B- heart, C- liver, D- kidney and E- plasma. Data are mean ± s.e.m. n=11-12 per group. PBS: Ldlr<sup>-/-</sup> mice treated with PBS; Anti-Ctrl: Ldlr<sup>-/-</sup> mice treated with AntagomiR-Control (Anti-Ctrl); Anti-92a: Ldlr<sup>-/-</sup> mice treated with AntagomiR-92a (Anti-92a).

Online Figure X. Immune profile analysis in LDLr<sup>-/-</sup> mice after blocking miR-92a. Percentage of A- B cells, B- T cells and C- CD11b<sup>+</sup> cells in spleen. Percentage of D- B cells, E- T cells and F- CD11b<sup>+</sup> cells in blood. Data are mean ± s.e.m. n=11-12 per group. PBS: Ldlr<sup>-/-</sup> mice treated with Phosphate Buffer Saline; Anti-Ctrl: Ldlr<sup>-/-</sup> mice treated with AntagomiR-Control (Anti-Ctrl); Anti-92a: Ldlr<sup>-/-</sup> mice treated with AntagomiR-92a (Anti-92a).

Online Figure XI. Regional atherosclerotic lesions quantification. A- Quantification of atherosclerotic lesions in the aortic arch in LDLr<sup>-/-</sup> mice after blocking miR-92a. B- Quantification of atherosclerotic lesions in the thoracic aorta in LDLr<sup>-/-</sup> mice after blocking miR-92a. Data are mean ± s.e.m. n=11-12 per group. PBS: Ldlr<sup>-/-</sup> mice treated with PBS; Anti-Ctrl: Ldlr<sup>-/-</sup> mice treated with AntagomiR-Control (Anti-Ctrl); Anti-92a: Ldlr<sup>-/-</sup> mice treated with AntagomiR-92a (Anti-92a). * p<0.05 vs PBS or Anti-Ctrl.

Online Figure XII. In vitro inhibition of miR-92a modulates endothelial signaling in response to shear stress and oxLDL. A- Representative western blots for KLF2, KLF4, NOS3 and phospho-p65 and GAPDH housekeeping gene expression, as a function of different shear stress levels and oxLDL stimulation after miR-92a inhibition B- Corresponding quantifications of western blots. Expression is normalized to GAPDH levels. Data are represented as mean +/- SEM. N=3-4 independent experiments (each experiment assayed each condition in triplicate). * p<0.05 vs low SS +Anti-miR-Ctrl; †p<0.05 vs high SS +Anti-miR-Ctrl.

Online Figure XIII. In vitro and in vivo p65 expression after miR-92a inhibition. A- Representative western blot for p65 in HUVECs exposed to low or high SS, with
or without oxLDL, and transfected with anti-Ctrl or anti-miR-92a. **- Corresponding quantification normalized to GAPDH expression. Data are mean ± s.e.m. N=3-4 independent experiments (each condition in triplicate). * P<0.05 vs LSS + Anti-Ctrl with or without oxLDL stimulation **- Representative western blot for p65 in aorta of Ldlr^/- mice treated with anti-Ctrl or anti-miR-92a antogomirs. D- Corresponding quantification normalized to GAPDH expression. n= 6 per group. ** p<0.01 vs PBS or Anti-Ctrl. PBS: Ldlr^/- mice treated with Phosphate Buffer Saline; Anti-Ctrl: Ldlr^/- mice treated with AntagomiR-Control (Anti-Ctrl); Anti-92a: Ldlr^/- mice treated with AntagomiR-92a (Anti-92a).

Online Figure XIV. Experimental approach used for determination of novel targets by Gene Array.

Online Figure XV. SOCS5 gene expression validation by qPCR in HUVECs. A- SOCS5 expression was evaluated by qPCR and normalized to GAPDH in HUVECs exposed to low or high SS, and stimulated with oxLDL after transfection with either Anti-Ctrl or Anti-miR-92a transfection. * P<0.05 vs LSS + Anti-Ctrl, † P<0.05 vs LSS + anti-miR-92a, ‡ P<0.05 vs LSS + Anti-Ctrl with or without oxLDL. B- SOCS5 expression was evaluated by qPCR and normalized to GAPDH in HUVECs exposed to low or high SS, and stimulated with oxLDL after transfection with either pre-Ctrl or pre-miR-92a transfection. * P<0.05 vs LSS + Anti-Ctrl with or without oxLDL, † P<0.05 vs LSS + Anti-Ctrl with or without oxLDL, ‡ P<0.05 vs Anti-miR-92a with or without oxLDL. Data are mean ± s.e.m. N=7 independent experiments (each condition in duplicate).

Online Figure XVI. SOCS5 expression in response to HSS and siRNA SOCS5. A- Representative immunoblot for SOCS5 in response to HSS after siSOCS5 in the presence or in absence of oxLDL for 24hrs and GAPDH housekeeping gene expression. B- Corresponding quantification of western blots. Expression is normalized to GAPDH. Data are given as mean +/- SEM. N=3 independent experiments (each experiment assayed each condition in duplicate). * p<0.05 vs high SS +siRNA-Ctrl; C- Representative immunoblot for KLF2, KL4 and NOS3 in response to HSS after silencing SOCS5 in the presence or in absence of oxLDL for 24hrs and GAPDH housekeeping gene expression, D- Corresponding quantification of western blots. Expression is normalized to GAPDH. Data are given as mean +/- SEM. N=3 independent experiments (each experiment assayed each condition in duplicate).

Online Figure XVII. SOCS5 gene in human atherosclerotic lesions. Healthy section, as well as the upstream and downstream area of atherosclerotic plaques were obtained from human carotid endarterectomy samples (n=4). * P<0.05 vs healthy area and upstream area of the lesion. Data are represented as mean +/- SEM.

Online Figure XVIII. SOCS5 gene expression in response to STAT3 inhibition in vivo. SOCS5 expression was evaluated by qPCR and normalized to GAPDH in atheroprone vs atheroprotected areas of Ldlr-/- +HFD treated with STAT3 inhibitor (Stattic 25mg/kg) for 14 days. Data are mean +/- SEM. n= 5 per group * p<0.05 vs C57Bl6 Aortic Arch.
**Online Table I. Top 10 miRNAs significantly modulated by shear stress.**

<table>
<thead>
<tr>
<th>Rank</th>
<th>miRNA ID</th>
<th>Δ Log(_2) LSS – HSS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MIR-552</td>
<td>1.781</td>
<td>0.042</td>
</tr>
<tr>
<td>2</td>
<td>MIR-765</td>
<td>1.773</td>
<td>0.017</td>
</tr>
<tr>
<td>3</td>
<td>MIR-362-5P</td>
<td>1.747</td>
<td>0.048</td>
</tr>
<tr>
<td>4</td>
<td>MIR-330-3P</td>
<td>1.706</td>
<td>0.038</td>
</tr>
<tr>
<td>5</td>
<td>MIR-629*</td>
<td>1.688</td>
<td>0.046</td>
</tr>
<tr>
<td>6</td>
<td>MIR-214</td>
<td>1.682</td>
<td>0.015</td>
</tr>
<tr>
<td>7</td>
<td>MIR-891A</td>
<td>1.648</td>
<td>0.027</td>
</tr>
<tr>
<td>8</td>
<td>MIR-549</td>
<td>1.632</td>
<td>0.039</td>
</tr>
<tr>
<td>9</td>
<td>MIR-425</td>
<td>1.629</td>
<td>0.035</td>
</tr>
<tr>
<td>10</td>
<td>MIR-631</td>
<td>1.580</td>
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Online Table II. Top 10 of upregulated miRNAs in response to LSS + oxLDL versus HSS + oxLDL

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<tr>
<th>Rank</th>
<th>miRNA ID</th>
<th>$\Delta \log_2 \text{LSS+oxLDL} - \text{HSS + oxLDL}$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MIR-92A</td>
<td>1.39</td>
<td>0.014</td>
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<tr>
<td>2</td>
<td>MIR-720</td>
<td>1.29</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>3</td>
<td>MIR-886-3P</td>
<td>1.17</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>4</td>
<td>MIR-212</td>
<td>1.13</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>5</td>
<td>MIR-154</td>
<td>1.10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>6</td>
<td>MIR-891A</td>
<td>1.10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>7</td>
<td>MIR-7</td>
<td>1.09</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>MIR-216A</td>
<td>1.07</td>
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</tr>
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<td>9</td>
<td>MIR-654-3P</td>
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<tr>
<td>10</td>
<td>MIR-223</td>
<td>1.06</td>
<td>&gt;0.05</td>
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Online Table III. Baseline level of IL-6 and MCP-1 release in response to different SS levels and oxLDL upon transfection

<table>
<thead>
<tr>
<th>IL-6 (ng/mL)</th>
<th>Baseline</th>
<th>Anti-Control</th>
<th>Pre-Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSS</td>
<td>25.69 ± 1.4</td>
<td>23.65 ± 1.1</td>
<td>25.57 ± 0.9</td>
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<tr>
<td>LSS + oxLDL</td>
<td>37.48 ± 2.4</td>
<td>38.3 ± 3.5</td>
<td>35.47 ± 2.1</td>
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<tr>
<td>HSS</td>
<td>13.49 ± 0.9</td>
<td>15.97 ± 0.9</td>
<td>12.24 ± 1.1</td>
</tr>
<tr>
<td>HSS+ oxLDL</td>
<td>14.48 ± 1.6</td>
<td>18.52 ± 2.7</td>
<td>11.38 ± 1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MCP-1 (ng/mL)</th>
<th>Baseline</th>
<th>Anti-Control</th>
<th>Pre-Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSS</td>
<td>92.05 ± 5.5</td>
<td>72.95 ± 2.5</td>
<td>80.7 ± 8</td>
</tr>
<tr>
<td>LSS + oxLDL</td>
<td>133.5 ± 5.7</td>
<td>110.8 ± 3.8</td>
<td>133.4 ± 3.8</td>
</tr>
<tr>
<td>HSS</td>
<td>53.7 ± 5.9</td>
<td>44.83 ± 2.7</td>
<td>47 ± 3.5</td>
</tr>
<tr>
<td>HSS+ oxLDL</td>
<td>61.3 ± 2.3</td>
<td>56.70 ± 3.7</td>
<td>64 ± 4.7</td>
</tr>
</tbody>
</table>

Data are represented as mean +/- SEM. N=3-4 independent experiments (each experiment assayed each condition in triplicate).
Online Table IV. Physiological parameters in LDLR-/- mice treated with AntagomiRs

<table>
<thead>
<tr>
<th></th>
<th>Ldlr&lt;sup&gt;+&lt;/sup&gt; + PBS</th>
<th>Ldlr&lt;sup&gt;+&lt;/sup&gt; + Anti-Ctrl</th>
<th>Ldlr&lt;sup&gt;+&lt;/sup&gt; + Anti-92a</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>27.8 ± 0.9</td>
<td>29.4 ± 0.7</td>
<td>28.3 ± 0.5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.48 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>1.37 ± 0.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>1564 ± 67</td>
<td>1360 ± 73</td>
<td>1416 ± 43</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dl)</td>
<td>230 ± 11</td>
<td>218 ± 14</td>
<td>236 ± 11</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>326 ± 48</td>
<td>311 ± 26</td>
<td>297 ± 25</td>
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</tbody>
</table>

Data are represented as mean +/- SEM
Online Figure I

![Graph showing miR-92a expression levels under different conditions.](image)
Online Figure III

![Graphs showing expression levels of miR-7, miR-8, miR-10a, and miR-23a in C57Bl6 and LdLr^+/− + HFD for aortic arch and descending aorta.](image-url)
Online Figure IV

![Bar graph showing miR-92a expression in different cell types and treatments.](image)

- **miR-92a expression (A.U.)**
- **HUVEC**
- **hVSMC**
- **Mφ**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HUVEC</th>
<th>hVSMC</th>
<th>Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxLDL</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LPS/IFNγ</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Online Figure VI
Online Figure VII

A

B

C

D
Online Figure VIII

A. Time (weeks)

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>High Fat Diet</th>
<th>Antagomir injections</th>
<th>Antagomir injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>17</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

B. miR-92a expression

<table>
<thead>
<tr>
<th>Condition</th>
<th>8 mg/kg</th>
<th>16 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Anti-Ctrl</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Anti-92a</td>
<td>1.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

C. mIg-17 expression

Aortic arch

- C57Bl6
- Ldlr-/- + HFD
- Ldlr-/- + Anti-92a

Descending aorta

- C57Bl6
- Ldlr-/- + HFD
- Ldlr-/- + HFD + Anti-Ctrl
- Ldlr-/- + HFD + Anti-92a

D. miR-92a expression

Aortic arch

- C57Bl6
- Ldlr-/- + HFD
- Ldlr-/- + HFD + Anti-Ctrl
- Ldlr-/- + HFD + Anti-92a

Descending aorta

- C57Bl6
- Ldlr-/- + HFD
- Ldlr-/- + HFD + Anti-Ctrl
- Ldlr-/- + HFD + Anti-92a
Online Figure X
Online Figure XI

A

B

% lesion in aortic arch

% lesion in thoracic aorta

PBS  Anti-Ctrl  Anti-92a

PBS  Anti-Ctrl  Anti-92a
Online Figure XII

### A

<table>
<thead>
<tr>
<th></th>
<th>LSS</th>
<th>HSS</th>
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<tbody>
<tr>
<td>KLF2</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>KLF4</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>NOS3</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Phospho-p65</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Antibodies:**
- Anti-miR-Ctrl: + + - - + + - -
- Anti-miR-92a: - - + + - - + +
- oxLDL: - + - + - + - +

### B

**KLF2 expression**
- Low SS: ![Image](image11.png) ![Image](image12.png)
- High SS: ![Image](image13.png) ![Image](image14.png)

**NOS3 expression**
- Low SS: ![Image](image15.png) ![Image](image16.png)
- High SS: ![Image](image17.png) ![Image](image18.png)

**Phospho-p65**
- Low SS: ![Image](image19.png) ![Image](image20.png)
- High SS: ![Image](image21.png) ![Image](image22.png)
Online Figure XIII

A

<table>
<thead>
<tr>
<th></th>
<th>p65</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-miR-Ctrl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-miR-92a</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>oxiLDL</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

C

<table>
<thead>
<tr>
<th></th>
<th>p65</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-Ctrl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-92a</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Online Figure XIV

Low SS + oxLDL + AC

→

TaqMan Gene Array
574 genes in 8 pathways

→

77 genes upregulated LSS oxA92 vs LSSox AC >1.25
188 genes downregulated LSS oxA92 vs LSSox AC < 0.8
200 genes not modulated between 0.8 and 1.25
109 genes not detected

→

38 genes predicted in Human AND Mouse

→

2 upregulated LSS oxA92 vs LSS ox AC >1.25

→

validation

→

SOCS5 as a potential new target
Online Figure XV

A.

B.

SOCS3 expression
Arbitrary Unit (AU)

Anti-miR-Ctrl  +  +  -  -  +  +  -  -  +  +  -  -  +  +
Anti-miR-92a   -  -  +  +  -  -  +  +  -  -  +  +  -  -
oxLDL        -  +  -  +  -  +  -  -  +  +  -  -  +  +

SOCS3 expression
Arbitrary Unit (AU)

Pre-miR-Ctrl  +  +  -  -  +  +  -  -  +  +  -  -  +  +
Pre-miR-92a   -  -  +  +  -  -  +  +  -  -  +  +  -  -
oxLDL        -  +  -  +  -  +  -  -  +  +  -  -  +  +
Online Figure XVI

A

SOCS5
GAPDH

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +

B

SOCS8 expression

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +

C

KLF2
KLF4
NOS3
GAPDH

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +

D

KLF2 expression

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +

E

KLF4 expression

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +

F

NOS3 expression

siRNA-Ctrl  +  +  -  -
siRNA-SOCS5 - -  +  +
oxLDL - +  -  +
Online Figure XVII

![SOCS5 expression diagram](image-url)

- Healthy area
- Upstream
- Downstream
- Lesion area
Online Figure XVIII

![Graph showing SOCS5 expression](image)

- **SOCS5 expression ( Arbitrary Unit (A.U.))**
- **Aortic arch**
- **Descending aorta**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Aortic arch</th>
<th>Descending aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stattic</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
References

