Systemic Delivery of MicroRNA-181b Inhibits NF-κB Activation, Vascular Inflammation, and Atherosclerosis in Apoe-/- Mice

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ABSTRACT

Rationale: Activated NF-κB signaling in the vascular endothelium promotes the initiation and progression of atherosclerosis. Targeting endothelial NF-κB may provide a novel strategy to limit chronic inflammation.

Objective: To examine the role of microRNA-181b (miR-181b) in endothelial NF-κB signaling and effects on atherosclerosis.

Methods and Results: MiR-181b expression was reduced in the aortic intima and plasma in ApoE-/- mice fed a high-fat diet. Correspondingly, circulating miR-181b in the plasma was markedly reduced in human subjects with coronary artery disease. Systemic delivery of miR-181b resulted in a 2.3-fold overexpression of miR-181b in the aortic intima of ApoE-/- mice and suppressed NF-κB signaling revealed by bioluminescence imaging and reduced target gene expression in the aortic arch in ApoE-/-/NF-κB-luciferase transgenic mice. MiR-181b significantly inhibited atherosclerotic lesion formation, pro-inflammatory gene expression, and the influx of lesional macrophages and CD4+ T cells in the vessel wall. Mechanistically, miR-181b inhibited the expression of the target gene importin-α3, an effect that reduced NF-κB nuclear translocation specifically in the vascular endothelium of lesions, whereas surprisingly leukocyte NF-κB signaling was unaffected despite a 7-fold overexpression of miR-181b. Our findings uncover that NF-κB nuclear translocation in leukocytes does not involve importin-α3, but rather importin-α5 which miR-181b does not target, highlighting that inhibition of NF-κB signaling in the endothelium is sufficient to mediate miR-181b’s protective effects.

Conclusion: Systemic delivery of miR-181b inhibits the activation of NF-κB and atherosclerosis through cell-specific mechanisms in the vascular endothelium. These findings support the rationale that delivery of miR-181b may provide a novel therapeutic approach to treat chronic inflammatory diseases such as atherosclerosis.

Keywords: microRNA-181b, NF-κB, atherosclerosis, chronic inflammation, importins, endothelial cell

Nonstandard Abbreviations and Acronyms:

EC  endothelial cell
HFD  high fat diet
miRNA microRNA
miR-181b microRNA-181b
3’UTR 3’-untranslated region
PBMCs peripheral blood mononuclear cells
NGL Tg NF-κB GFP-luciferase reporter transgenic
NS-m miRNA negative control mimics
181b-m microRNA-181 mimics
CAD coronary artery disease
HUVECs human umbilical vein endothelial cells
HAECs human aortic endothelial cells
vWF von Willebrand factor
smMHC smooth muscle cell myosin heavy chain
IPOA3 importin-α3
IPOA5 importin-α5
INTRODUCTION

Atherosclerosis is recognized as a chronic inflammatory disease of the arterial wall.\(^1\) Nuclear factor-κB (NF-κB)-mediated vascular inflammation plays a critical role in the initiation and progression of atherosclerosis. The transcriptional activity of NF-κB can be induced by a variety of atherogenic stimuli, including inflammatory cytokines, type 2 diabetes, oxidized LDL, angiotensin II, and hemodynamic forces.\(^3\) In the canonical NF-κB signaling pathway, NF-κB heterodimers exist in an inactive form in the cytoplasm bound to an inhibitor such as IκBα. Upon stimulus-mediated activation, the IκB kinase (IKK) complex rapidly phosphorylates IκBα which results in IκBα degradation by the proteasome.\(^9\) Once NF-κB heterodimers are released from IκBα, importin proteins direct NFκB translocation to the nucleus where it controls a wide range of gene expression by binding to various κB elements. In the vascular endothelium, NFκB activation induces the expression of proinflammatory genes, including those encoding adhesion molecules, cytokines, and chemoattractant proteins that collectively play critical roles in the initiation and progression of atherosclerosis.\(^8\)\(^,\)\(^11\)\(^,\)\(^12\) Consistent with this premise, endothelial cell (EC)-specific NFκB inhibition reduces atherosclerosis in three different mouse models, IKKγ EC knockout, IKKγ EC inducible knockout, and dominant negative-IκBα EC transgenic, in ApoE-/- mice.\(^13\) Furthermore, genetic inhibition of several NF-κB target genes, including VCAM-1, ICAM-1, E- and P-selectins, TNF-α, and IL-1β, also reduces various aspects of atherosclerotic lesion formation.\(^14\)\(^-\)\(^18\) Thus, targeting NF-κB–mediated EC activation holds promise for the development of novel anti-inflammatory therapies for acute and chronic inflammatory diseases.

MicroRNAs (miRNAs) are single-stranded, non-coding, small RNAs which regulate gene expression by destabilizing target mRNAs and/or inhibiting translation. For example, in the context of vascular inflammation, miR-126, miR-31, and miR-17-3p were reported to reduce the expression of VCAM-1, E-selectin, and ICAM-1, respectively, by directly targeting the 3’ untranslated region (3’UTR) of these genes.\(^19\)\(^,\)\(^20\) MiR-10a targeted two proteins MAP3K7 (TAK1) and β-TRC that regulate IκBα degradation.\(^21\) MiR-146a can repress the pro-inflammatory NFκB pathway as well as the MAP kinase pathway in ECs by targeting TNF receptor-associated factor 6 and HuR.\(^22\) Recently, we identified that miR-181b inhibits NFκB-mediated endothelial activation by reducing the expression of importin-α3, a protein critical for NFκB translocation from cytoplasm to nucleus.\(^23\) However, the role of miRNA-181b in chronic inflammatory disease states such as atherosclerosis has not been examined.

In this study, we investigate the role of miR-181b in the development of atherosclerosis in ApoE-/- mice. Our findings reveal cell-specific mechanisms by which miR-181b exerts its protective effect in the vascular endothelium via importin isoform targeting and provide the rationale for the potential clinical use of miR-181b mimetics to treat chronic vascular inflammatory diseases such as atherosclerosis.

METHODS

Pre-miR™ miRNA precursor molecules-negative (non-specific) control #1 (AM17110), and Hsa-miR-181b-5p Pre-miR™ miRNA precursor (PM12442) were used from Ambion. Real-time qPCR was performed with the Mx3000P Real-time PCR system (Stratagene) following the manufacturer’s instructions. NGL mice (NF-κB promoter with GFP/luciferase fusion reporter) fully backcrossed into C57BL/6 were crossed with homozygous ApoE-deficient mice to generate ApoE-/-/NGL transgenic mice. To induce atherosclerosis, 8-week-old male ApoE-/- mice were fed a HFD from Research Diets Inc. (D12108Ci) for 12 weeks. Aortas were carefully excised from mice, and examined for Immunohistology and characterization of atherosclerotic lesions.

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

MiR-181b expression is reduced in aortic intima of ApoE-/- mice or in human plasma from patients with coronary artery disease.

In response to acute pro-inflammatory stimuli (e.g. TNF-α or LPS for 4 hours.), we previously demonstrated that miR-181b expression is reduced in the aortic intima of mice. To examine whether miR-181b expression is reduced in chronic inflammation, aortic intima was harvested from ApoE-/- mice fed a HFD. As shown in Figure 1A, miR-181b expression was reduced by ~59% and ~53% in the aortic intima of ApoE-/- mice after HFD for four or six weeks, respectively. The second most dominantly expressed miR-181 family member in the aortic intima is miR-181a, which was also reduced in aortic intima at the time of four or six weeks of HFD (~74% and ~69%). The level of miR-181c is much lower than that of miR-181a and miR-181b and was not significantly changed. In human subjects with coronary artery disease (CAD), circulating miR-181b levels were also reduced by ~76% in plasma compared to subjects without angiographically defined focal obstructive coronary artery disease (Figure 1B). Consistently, circulating plasma miR-181b levels were reduced by ~44% in ApoE-/- mice after four weeks of HFD (Online Figure IA). In contrast, circulating miR-146a levels increased by 3.5- and 3.1-fold after four and six weeks of HFD, respectively (Online Figure IB), suggesting a specific effect for the miR-181b reduction. To characterize the components of the aortic intima, qPCR analysis was performed for cell-specific markers. As shown in Online Figure IIA, endothelial mRNA markers Von Willebrand factor (vWF) and Tie-2 were robustly enriched in the intima and were barely detectable in the media plus adventitia (2.2% and 2.3% compared to the intima, respectively). Conversely, the expression of smooth muscle cell myosin heavy chain (smMHC) mRNA was much lower (1.4%) in the aortic intima compared to the media plus adventitia (Online Figure IIB). Finally, the macrophage marker CD68 and T cell markers CD3 and CD4 were also detected at very low levels (1.8%, 4.8%, and 3.1%, respectively) in the aortic intima (Online Figure IIC and IID) compared to peripheral blood-derived macrophages and T cells, respectively. These data indicate that the isolated aortic intima contained >90% RNA enriched from ECs.

These data demonstrate that miR-181b is reduced by chronic inflammatory stimuli in the vascular endothelium and plasma of mice suggesting it may be involved in the early pathogenesis of atherosclerosis.

Rescue of miR-181b expression in the aortic intima.

To rescue the expression of miR-181b in the aortic intima under chronic inflammatory conditions, we systemically delivered liposomally encapsulated miR-181b mimics (181b-m) or non-specific (NS) control mimics (NS-m) by tail vein injection. As shown in Figure 2A, the expression of miR-181b in the aortic intima from mice injected with miR-181b was 2.3-fold higher than that in mice injected with the miRNA non-specific control (Figure 2A). No over-expression of miR-181b was observed in the aortic media/adventitia (Figure 2B). However, the expression of miR-181b in peripheral blood mononuclear cells (PBMCs) from mice injected with miR-181b was ~10.6 fold higher than that in PBMCs from mice injected with the miRNA non-specific control (Figure 2C). Systemic delivery of miR-181b did not alter the endogenous expression of miR-181a and miR-181c in the aortic intima, media/adventitia, or PBMCs (Figure 2A-2C). Basal endogenous levels of miR-181b expression in the media/adventitia and PBMCs were 3.2-fold and 5-fold higher, respectively, than that in the aortic intima (Online Figure III). However, intravenous injection of miRNA non-specific control did not change endogenous miR-181b expression (Figure 2A-2C), indicating that the fold differences in the aortic intima, media/adventitia, and PBMCs represent endogenous miR-181b. These data indicate that exogenous miR-181b is able to accumulate in the aortic intima and PBMCs but not the aortic media/adventitia after intravenous administration.

Systemic delivery of miR-181b inhibits NF-κB signaling and gene expression.

NF-κB-mediated EC activation and vascular inflammation plays a critical role in the initiation and progression of atherosclerosis. MiR-181b has been shown to inhibit these events in an acute inflammatory disease state such as sepsis. To explore whether miR-181b is able to suppress NF-κB
signaling in the context of atherosclerosis, we generated compound ApoE-/-/NGL mice by crossing ApoE-/- mice with transgenic NF-κB GFP-luciferase (NGL) reporter mice. After 4 weekly injections (i.v.) of miR-181b mimics (181b-m), miRNA non-specific control mimics (NS-m), or vehicle control in the ApoE-/-/NGL and NGL mice, NF-κB activity in the aortic arch was quantified by bioluminescence imaging. As shown in Figure 2D, the intensity of NF-κB activity increased by ~3-fold in the aortic arch in ApoE-/-/NGL mice compared to NGL mice after 4 weeks of HFD. However, in the presence of exogenous miR-181b, NF-κB activity was reduced by 31%. There were no differences observed in ApoE-/-/NGL mice treated with either the vehicle control or non-specific control mimics. In addition, the induction of expression of NF-κB target genes such as VCAM-1, ICAM-1, and E-selectin were significantly reduced in the aortic arch in ApoE-/-/NGL mice by 32%, 20%, and 43%, respectively, in the presence of exogenous miR-181b (181b-m group) (Figure 2E). Collectively, these data demonstrate that systemic delivery of miR-181b inhibits NF-κB activation and target gene expression primarily in the vessel wall of ApoE-/-/NGL mice.

**Systemic delivery of miR-181b protects ApoE-/- mice from atherosclerosis.**

To explore the role of systemically delivered miR-181b in atherosclerosis, ApoE-/- mice were fed a HFD for 12 weeks and administered weekly injections of miR-181b mimics (181b-m) or miRNA non-specific control (NS-m) as shown in Figure 3A. Following 12 weeks on HFD, there were no differences observed in body weight, total cholesterol, LDL, HDL, and triglyceride levels between ApoE-/- mice injected with miR-181b mimics or miRNA non-specific control (Table 1). Analyses of atherosclerotic lesion formation by Oil red O staining revealed a 44% reduction in lesion area in the descending thoracic and abdominal aorta (Figure 3B) and a 25% reduction in lesion size at the level of the aortic sinus (Figure 3C). Histological assessment of atherosclerotic lesions at the aortic sinus revealed a 21% reduction of macrophages by Mac3 staining (Figure 3D) and a 50% reduction of CD4+ T cells by CD4 staining (Figure 3E). The miR-181b-mediated inhibition of atherosclerotic lesion formation was associated with decreased expression of pro-inflammatory markers including adhesion molecules ICAM-1 and E-selectin by 41% and 53%, respectively, and cytokines TNF-α and IL-β by 42% and 48%, respectively, in the aortic arch of ApoE-/- mice (Figure 3F). The expression of IL-10 in the aortic arch was not different between two groups (Figure 3F). As shown by immunohistochemistry in Figure 3G, ApoE-/- mice that received miR-181b mimics exhibited a reduction in VCAM-1 expression by 36% compared to miRNA non-specific control treated ApoE-/- mice. To test whether systemic delivery of miR-181b would lead to changes in liver function, we examined liver NF-κB activity and measured blood levels of aspartate aminotransferase (AST) and alanine transaminase (ALT) (Online Figure IV). No differences were observed in liver NF-κB activity or plasma ALT and AST concentrations between miRNA non-specific control mimics and miR-181b treatment (Online Figure IV). These data suggest that liver NF-κB activity or liver toxicity is not likely contributory in the miR-181b-mediated inhibition of atherosclerotic lesion formation. Taken together, these data indicate that systemic delivery of miR-181b inhibits NF-κB activity and pro-inflammatory gene expression in the vessel wall, and results in reduced leukocyte accumulation and atherosclerotic lesion formation in ApoE-/- mice.

**MiR-181b reduces the expression of importin-α3 and NF-κB p65 nuclear translocation in the vascular endothelium of lesions.**

We previously demonstrated that miR-181b directly targeted importin-α3 and reduced its expression, and subsequently inhibited NF-κB nuclear translocation. Interestingly, importin-α3 mRNA expression increased in the aortic intima by ~20% in ApoE-/- mice after four weeks of HFD (Online Figure V). To further validate the hypothesis that miR-181b inhibits endothelial NF-κB in the context of atherosclerosis, importin-α3 expression and nuclear translocation of NF-κB was directly evaluated in the endothelium of atherosclerotic lesions by immunostaining with antibodies against importin-α3 and CD31, or p65 and CD31, in sections from the aortic sinus. As shown in Figure 4A, ApoE-/- mice that received miR-181b mimics (181b-m) exhibited a reduction in importin-α3 expression by 35% in the endothelium.
compared to miRNA non-specific control (NS-m) treated ApoE-/- mice. Multiple genes have been identified as direct targets of miR-181a or miR-181b in different cell types. In addition to importin-α3, the expression of these genes was also examined in the aortic arch (Online Figure VI). Systemic delivery of miR-181b significantly reduced the expression of neuropilin-1 (NRP1), but not other reported target genes. Consistent with reduced expression of importin-α3 expression in the vascular endothelium in the presence of systemically delivered miR-181b, the accumulation of NF-κB p65 in endothelial nuclei within lesions was reduced by 33% compared to mice injected with miRNA non-specific control (Figure 4B). Interestingly, we found that systemically delivered miR-181b did not reduce NF-κB p65 nuclear accumulation in lesional Mac3-positive macrophages by immunofluorescent staining (Figure 4C). Taken together, these data indicate that systemic delivery of miR-181b inhibits importin-α3 expression and NF-κB p65 nuclear translocation in vascular endothelium but not in lesional macrophages.

**Differential expression of importin-α3 and importin-α5 in endothelial cells and leukocytes account for the cell-specific effect of miR-181b on NF-κB inhibition.**

The role of myeloid NF-κB signaling in the atherogenesis remains controversial, which prompted us to ask whether systemic delivery of miR-181b regulates NF-κB signaling in leukocytes. We found systemic delivery of miR-181b does not inhibit p65 nuclear translocation in lesional macrophages by immunostaining of sections at the aortic sinus from ApoE-/- mice fed a HFD for 12 weeks (Figure 4C). Further examination revealed that miR-181b was ~7.2 fold higher in PBMCs of ApoE-/- mice injected with miR-181b compared to mice injected with the miRNA non-specific control (Figure 5A). Surprisingly, systemic delivery of miR-181b had no significant effect on NF-κB activity in PBMCs (Figure 5B) or on the expression of NF-κB target genes in PBMCs such as COX-2, IL-1β, and IL-10 (Figure 5C). Overexpression of miR-181b was not able to reduce NF-κB activation as measured by NF-κB-induced luciferase activity in bone marrow-derived macrophages (BMDMs) isolated from ApoE-/-/NGL mice (Figure 5D) or p65 nuclear translocation in peritoneal macrophages in response to LPS (Online Figure VII). Previous studies demonstrate that several importin-α molecules including importin-α3 and importin-α5 may be involved in NF-κB nuclear translocation. We previously identified that miR-181b directly targets importin-α3, a protein that is critical for nuclear translocation of NF-κB in ECs. As shown in Figure 5E, the expression of importin-α3 was also reduced in mouse PBMCs by systemically delivered miR-181b. In contrast, the expression of importin-α5 was not changed (Figure 5F). To examine why miR-181b was capable of inhibiting NF-κB in ECs and aortic intima, but not in PBMCs despite the reduction of importin-α3 by miR-181b in both cell types, we assessed the relative expression of the miR-181b target importin-α3 and other importin-α proteins in the aortic intima, human and mouse ECs, and mouse PBMCs. As shown in Figure 5G, importin-α3 mRNA expression was 4.4-fold higher in the aortic intima compared to PBMCs. Importin-α3 was also the dominantly expressed member of the importin-α family in the aortic intima at the mRNA level and is about 3-fold higher than the second most abundant importin-α molecule (importin-α7). Consistently, the expression of importin-α3 was higher in ECs (8-fold in MAECs) at the protein level compared to mouse PBMCs (Figure 5H). The expression of importin-α4 was also higher in ECs than in PBMCs at the protein level. In contrast, the expression of importin-α5 in PBMCs was ~3-fold higher compared to the aortic intima at the mRNA level and ~4-fold higher compared to MAECs at the protein level, respectively (Figure 5G and 5H). Importin-α5 was the dominantly expressed member of the importin-α family in mouse PBMCs at the mRNA level and is 3.5-fold higher than the second most abundant importin-α molecule (importin-α7). Importantly, miR-181b does not target importin-α5 (Figure 5F) and knockdown of importin-α5 expression by siRNA transfection (Figure 5I) was able to inhibit LPS-induced NF-κB activation (Figure 5J) and NF-κB target gene expression (Figure 5K and 5L) in cultured PBMCs. Furthermore, systemic delivery of importin-α5 siRNA inhibited its expression in vivo in PBMCs (Figure 5M) and inhibited LPS-induced NF-κB activation by 31% (Figure 5N). In summary, these data demonstrate that systemic delivery of miR-181b

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specifically inhibited NF-κB activation and NF-κB target gene expression in the aortic intima of the vessel wall but not in leukocytes of ApoE-/- mice likely because of distinct expression patterns of importin-α molecules—importin-α3 (a miR-181b target) is expressed significantly higher than importin-α5 in endothelial cells, whereas importin-α5 (a non-miR-181b target) is expressed higher than importin-α3 in leukocytes thereby allowing for NF-κB activation to proceed in this cell type. Furthermore, these findings highlight that inhibition of NF-κB activity in the vascular endothelium is sufficient to confer miR-181b’s protective effects on atherosclerotic lesion formation.

DISCUSSION

The current study demonstrates that systemic delivery of liposomally encapsulated miR-181b mimetics in ApoE-/- mice inhibits NF-κB activation, NF-κB-responsive pro-inflammatory gene expression, leukocyte accumulation, and atherosclerotic lesion formation. Furthermore, these studies highlight for the first time to our knowledge that miRNA mimetics penetrate the intima lining of the atherosclerotic plaque in amounts sufficient to limit NF-κB activation, inflammatory gene expression, and leukocyte accumulation. Moreover, these miR-181b-mediated effects occurred independent of NF-κB inhibition in lesional macrophages or PBMCs. These findings are consistent with prior studies showing that inhibition of NF-κB signaling specifically in endothelial cells (by ablating IKKγ/NEMO or expression of a dominant-negative IκBα) confers an atheroprotective effect in ApoE-/-mice.13

The NF-κB signaling pathway centrally integrates multiple signal inputs in the pathogenesis of atherosclerosis and emerging studies suggest that inhibition of NF-κB in specific cell types may have divergent effects. For example, while EC-specific inhibition of NF-κB resulted in reduced lesion formation in ApoE-/- mice,13 the effect of altering NF-κB activation in myeloid cells is more complex. For example, low-density lipoprotein receptor-deficient mice transplanted with IKK2/IKKβ-deficient macrophages had increased atherosclerosis associated with higher numbers of apoptotic cells within the plaque.34 In contrast, a recent study showed that myeloid-specific IKKβ deficiency decreases atherosclerosis in low-density lipoprotein receptor-deficient mice.35 In another study, myeloid IκBα deficiency promoted atherogenesis by enhancing leukocyte recruitment to the developing plaques.32 Furthermore, bone marrow-deficiency of NF-κB1 resulted in reduced atherosclerotic lesion size and macrophage foam cells, but caused increased plaque inflammation.33 Finally, conditional targeting of tumor necrosis factor receptor-associated factor 6 revealed opposing functions of Toll-like receptor signaling in endothelial and myeloid cells in a mouse model of atherosclerosis.36 Collectively, these studies illustrate that cell-type specific inhibition of upstream NF-κB effectors may exert varying effects on plaque composition and atherosclerotic lesion formation.

In our study, miR-181b was overexpressed 2.3-fold in the aortic intima and ~7-10 fold in PBMCs of ApoE-/- mice after intravenous injection of miR-181b mimics for up to 12 weeks. Despite the higher levels achieved for miR-181b over-expression in PBMCs, there were no significant effects on PBMC expression of the NF-κB-regulated inflammatory genes COX-2, IL-1β, or IL-10 (Figure 5C). MiR-181b also had no effect of NF-κB activity in the liver (Online Figure IV). These data suggest that the protective role of miR-181b on vascular inflammation may be independent of effects of miR-181b in PBMCs or liver. As outlined above, importin-α3 is a protein involved in NF-κB translocation from the cytoplasm to nucleus.37, 38 We previously demonstrated that importin-α3 is a bona fide direct target of miR-181b in ECs.23 Indeed, systemic delivery of miR-181b significantly reduced the expression of importin-α3 in the aortic intima of lesions by immunohistochemical staining (Figure 4A). Thus, miR-181b appears to suppress NF-κB activation and target gene expression in the vascular wall by reducing importin-α3 expression in the vascular endothelium. In addition to importin-α3, the expression of NRP1, but not other previously identified miR-181b target genes, was reduced by miR-181b in the aortic arch (Online Figure VI). NRP1 is a single spanning transmembrane glycoprotein, which plays versatile roles in angiogenesis,
cell survival, migration, and has been identified as a direct target of miR-181b in ECs in the context of arsenic-induced angiogenesis. It remains unknown whether NRP1 exerts anti-inflammatory effects on NF-κB signaling or other relevant pro-inflammatory pathways in ECs or atherosclerosis.

An interesting question arising from these studies is—why did miR-181b overexpression in PBMCs fail to inhibit NF-κB activity and NF-κB-regulated gene expression? An emerging paradigm from several studies indicates that miRNA-mediated effects in a specific cell type are dependent on the relative expression of the proteins that are regulated by the miRNAs. Consistent with this premise is the finding that the expression of importin-α3 is higher in the aortic intima (4.4-fold at the mRNA level) and ECs (8-fold in MAECs at the protein level) compared to PBMCs (Figure 5G and 5H). Interestingly, a similar gradient of cellular expression was noted in the investigation that originally reported cloning for the importin-α3 gene. Surprisingly, the expression pattern of importin-α molecules is strikingly different in PBMCs. The expression of importin-α5 is higher in PBMCs than in ECs (3-fold and 4-fold at the mRNA and protein levels, respectively) (Figure 5G and 5H). Furthermore, importin-α5 participates in NF-κB activation in PBMCs as verified by siRNA knockdown of importin-α5 in PBMCs both in vitro and in vivo (Figure 5I-5N). Therefore, the distinct expression pattern of importin-α molecules in ECs and leukocytes with a dominant expression pattern of importin-α3 in ECs and importin-α5 in leukocytes likely accounts for the cell-specific effects of miR-181b on NF-κB signaling. As miR-181b cannot inhibit importin-α5 expression (Figure 5F) or its 3′-UTR, these findings further support other studies demonstrating that endothelial-specific NF-κB inhibition is sufficient to confer atheroprotection.

MiR-181b-mediated targeting of downstream NF-κB signaling in the vascular endothelium may offer several advantages to inhibit this pathway. First, previous targeting of upstream NF-κB signaling effectors including IKKs or IκBα may lead to off-target effects by virtue of the large number of inter-dependent signaling pathways that they may affect (e.g. MAPK signaling, insulin signaling, and p53). Second, miR-181b-mediated inhibition of importin-α3 provides targeting of a focused downstream event of preventing nuclear translocation of NF-κB heterodimers. Third, because of the differential expression of importin-α3 and importin-α5 in endothelial cells and leukocytes, respectively, miR-181-mediated targeting of importin-α3 only limits NF-κB activation in the vascular endothelium of lesions, thereby providing a means of cell-specific targeting of inflammation. For example, it may be advantageous to avoid inhibition of myeloid NF-κB in order to maintain protection in response to various infectious pathogens.

In summary, our study in ApoE−/− and diet-induced obese mice, demonstrates that miR-181b mimetics decrease arterial NF-κB activation and NF-κB-regulated gene expression in the vascular endothelium resulting in reduced leukocyte accumulation and atherosclerosis. MiR-181b-mediated effects occurred primarily in the vascular endothelium and independent of NF-κB inhibition in lesional macrophages or PBMCs due to utilization of different importin-α isoforms in ECs (importin-α3) and PBMCs (importin-α5) (Online Figure VIII). These data indicate that strategies aimed at restoring miR-181b expression may provide a novel therapeutic approach for chronic inflammatory disease states such as atherosclerosis.
**SOURCES OF FUNDING**
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**DISCLOSURES**
Mark W. Feinberg, Xinghui Sun, and The Brigham and Women’s Hospital have a patent pending related to the work that is described in the present study.

**REFERENCES**


Table 1. Systemic delivery of miR-181b did not affect lipid profiles or body weight of ApoE-/- mice fed a HFD.

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<tr>
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<th>Ctl mimics</th>
<th>miR-181b</th>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>1076.8 ± 93.6</td>
<td>994.7 ± 41.3</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>120.3 ± 10.2</td>
<td>108.8 ± 6.9</td>
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<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>993.2 ± 97.3</td>
<td>905.9 ± 40.1</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>51.6 ± 6.7</td>
<td>63.7 ± 7.5</td>
</tr>
<tr>
<td>Body weight (gram)</td>
<td>31.8 ± 1.0</td>
<td>29.9 ± 0.7</td>
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All values Ctl mimics vs. miR-181b, P=N.S., Mean ± SEM, n=10-12.
FIGURE LEGENDS

Figure 1. MiR-181b expression is reduced in the aortic intima of ApoE-/- mice fed a high-fat diet or in plasma from human subjects with coronary artery disease. A, MiR-181a, miR-181b, and miR-181c expression were detected by qPCR in the aortic intima from ApoE-/- mice fed a HFD for 1, 4, or 6 weeks (n=5-8 per group). The expression of miR-181 was normalized to small RNA U6 expression and compared to the expression of miR-181c at 1 week of HFD that was subsequently set to a value of one. B, Circulating miR-181b expression was detected by qPCR in human plasma samples from control human subjects without (n=14) or with coronary artery disease (n=26). Data show mean ± SEM. *, P < 0.05; **, P < 0.01; #, P < 0.001.

Figure 2. Systemic delivery of miR-181b reduces NF-κB activity in the aortic arch of ApoE-/-/NGL mice. A-E, ApoE-/-/NGL or NGL transgenic (Tg) mice (NF-κB promoter with GFP/luciferase fusion reporter) were fed a HFD and tail vein injected with miRNA non-specific control (NS-m), miR-181b (181b-m) mimics, or vehicle twice a week for 4 weeks as described in the Methods Section. MiR-181a, miR-181b, and miR-181c expression was examined by qPCR in the aortic intima (A), media/adventitia (B), and peripheral blood mononuclear cells (PBMCs) (C). Data show mean ± SEM, n=3. The expression of miR-181 was normalized to small RNA U6 expression and compared to the expression of miR-181a in mice injected with vehicle that was subsequently set to a value of one. D, Bioluminescence imaging of luciferase activity (represents NF-κB activity) is shown in excised aortas from NGL Tg mice that received vehicle (n=8) and from ApoE-/-/NGL Tg mice that received vehicle (n=5), NS-m (n=8), or 181b-m (n=12), respectively. E, qPCR analysis of NF-κB target gene expression in the aortic arch. The expression of NF-κB target gene was normalized to mouse β-actin expression and compared to its expression in mice received NS-m that was subsequently set to a value of one hundred. Data show mean ± SEM, n=3-12. *, P < 0.05; **, P < 0.01. N.S., non-significant.

Figure 3. Systemic delivery of miR-181b mimics inhibits atherosclerosis in ApoE-/- mice. ApoE-/- mice were fed a HFD and received weekly tail vein injections of miRNA non-specific control (NS-m) or miR-181b (181b-m) mimics for 12 weeks as described in the Method Section. A, Schema of experimental procedure. B, Lesion areas shown were quantified using Oil-red O (ORO) staining of the thoracoabdominal aorta. Data represent ORO – stained areas as a percentage of aorta areas. C, Lesion areas shown were quantified as areas between the lumen and tunica media on ORO stained aortic sinus sections. D and E, Representative images and quantification show Mac-3-positive macrophages (D) and CD4-positive T cells (E) in the aortic root lesions. Mac3-stained areas as a percentage of lesion area were shown in (D). F, qPCR analysis of the indicated pro-inflammatory genes in the aortic arch. The expression of inflammatory genes was normalized to mouse β-actin expression and compared to its expression in mice received NS-m that was subsequently set to a value of one hundred. G, Images and quantification of VCAM-1 staining in the intima layer at the aortic root. Mean ± SEM, n=6-14 mice per group. *, P < 0.05. N.S., non-significant.

Figure 4. MiR-181b inhibits importin-α3 expression and NF-κB activation in aortic endothelial cells in lesions. ApoE-/- mice were fed a HFD and weekly tail vein injected with miRNA non-specific control (NS-m) or miR-181b (181b-m) mimics for 12 weeks. Frozen sections of aortic root were stained for anti-importin-α3 (red) and CD31 (green) (A), or anti-p65 (red), anti-CD31 (green), and DAPI (blue) (B), or anti-importin-α3 (red), Mac3 (green) and DAPI (blue) (C). Arrows indicate differential importin-α3 expression (A), p65 accumulation in nuclei of ECs (B), or nuclear p65 in macrophage (C). Importin-α3 expression and nuclear p65 expression were quantified in vascular ECs reflecting NS-m (n=40 or 42 ECs) and 181b-m (n=41 or 43 ECs). Nuclear p65 expression in lesional macrophages was quantified reflecting NS-m (n=41 cells) and 181b-m (n=42 cells). Mean ± SEM, n= 8 - 10 mice each group. * P < 0.05. N.S., non-significant.
Figure 5. MiR-181b does not inhibit NF-κB activation in leukocytes due to dominant expression of importin-α5. A-C, ApoE/- or ApoE/-/NGL Tg mice were fed a HFD and intravenously injected with miRNA non-specific control (NS-m) or miR-181b (181b-m) mimics once a week for 12 weeks. A, Real-time qPCR analysis of miR-181b in PBMCs. B, Luciferase activity (reflecting NF-κB activity) was measured in PBMCs. N=4-6 per group. C, Real-time qPCR analysis of NF-κB target genes in PBMCs. D, Luciferase activity in bone marrow-derived macrophages (BMDM) from ApoE/-/NGL mice transfected with NS-m or 181b-m, and treated with 10 ng/ml LPS for 12 hrs. E and F, Western blot analysis of importin-α3 (IPOA3) and importin-α5 (IPOA5) in PBMCs from mice injected with NS-m or 181b-m. G, qPCR analysis of importin-α molecules in PBMCs or aortic intima. The expression of importin-α genes was normalized to mouse β-actin expression and compared to importin-α1 gene expression in PBMCs that was subsequently set to a value of one. N=3. H, Western blot analysis of IPOA3, importin-α4 (IPOA4), and importin-α5 (IPOA5) in ECs and PBMCs. I and J, PBMCs from ApoE/-/NGL mice were transfected with ctl siRNA or IPOA5 siRNA. Western blot analysis of IPOA5 is shown in (I) and NF-κB luciferase activity in (J). K and L, Real time qPCR analysis of COX-2 (K) and IL-1β (L) expression in PBMCs. N=3. M and N, qPCR analysis of IPOA5 and NF-κB luciferase activity in PBMCs from ApoE/-/NGL mice tail vein-injected with ctl siRNA or IPOA5 siRNA. N=4-5. Data show mean ± SEM. *, P < 0.05; **, P < 0.01; # P < 0.001. N.S., non-significant.
Novelty and Significance

What Is Known?

- Atherosclerosis is a chronic inflammatory disease of the arterial wall.
- Endothelial NF-κB activation contributes to the initiation and progression of atherosclerosis.
- We previously showed that microRNA181b inhibits NF-κB-mediated endothelial activation during endotoxin-induced acute inflammation by reducing the expression of importin-α3, a protein critical for NF-κB translocation from cytoplasm to nucleus. However, the role of miRNA-181b in chronic inflammatory disease states such as atherosclerosis has not been examined.

What New Information Does This Article Contribute?

- MiR-181b expression in the aortic intima is markedly reduced after high-fat diet feeding in Apoe<sup>−/−</sup> mice.
- Intravenous delivery of miR-181b inhibited NF-κB activation, NF-κB-responsive pro-inflammatory gene expression, leukocyte accumulation, and atherosclerotic lesion formation.
- MiR-181b inhibited NF-κB only in the vascular endothelium of atherosclerotic lesions, and not in leukocytes, reflecting high expression of importin-α3 in endothelial cells but not leukocytes.

NF-κB activation in the vascular endothelium promotes the initiation and development of atherosclerosis. In this study, we found that miR-181b expression is reduced in Apoe<sup>−/−</sup> mice and in humans with coronary artery disease. Rescue of miR-181b expression by systemic delivery of miR-181b mimetics to the vessel wall inhibited NF-κB activation in vascular ECs, but not leukocytes. This reduced leukocyte accumulation and atherosclerotic lesion formation. These findings suggest that miR-181b mimetics may provide a novel therapeutic approach to treat chronic inflammatory diseases such as atherosclerosis.
Figure 1

A. Aortic intima

qPCR (fold change)

High fat diet (weeks)

B. Human plasma

miR-181b

qPCR (%)

Healthy subjects  CAD patients
Figure 2

A. In aortic intima

<table>
<thead>
<tr>
<th>miR-181a</th>
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<tr>
<td>Veh</td>
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B. In aortic media/adventitia

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C. In PBMCs

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D. Aortic arch NF-κB activity

- NGL veh
- ApoE-/-/NGL veh
- ApoE-/-/NGL NS-m
- ApoE-/-/NGL 181b-m

E. Expression of VCAM-1, ICAM-1, and E-selectin