Systemic Delivery of MicroRNA-181b Inhibits Nuclear Factor-κB Activation, Vascular Inflammation, and Atherosclerosis in Apolipoprotein E–Deficient Mice

Xinghui Sun,* Shaolin He,* A.K.M. Wara, Basak Icli, Eugenia Shvartz, Yevgenia Tesmenitsky, Nathan Belkin, Dazhu Li, Timothy S. Blackwell, Galina K. Sukhova, Kevin Croce, Mark W. Feinberg

Rationale: Activated nuclear factor (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 apolipoprotein E–deficient 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 apolipoprotein E–deficient mice and suppressed NF-κB signaling revealed by bioluminescence imaging and reduced target gene expression in the aortic arch in apolipoprotein E–deficient/NF-κB-luciferase transgenic mice. MiR-181b significantly inhibited atherosclerotic lesion formation, proinflammatory 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.

Conclusions: 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. (Circ Res. 2014;114:32-40.)

Key Words: atherosclerosis ■ endothelial cells ■ inflammation ■ karyopherins ■ microRNAs ■ NF-κB

Atherosclerosis is recognized as a chronic inflammatory disease of the arterial wall.1,2 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 mellitus, oxidized low-density lipoprotein, angiotensin II, and hemodynamic forces.3,4 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. On stimulus-mediated activation, the IκB kinase (IKK) complex rapidly phosphorylates IκBα, which results in IκBα degradation by the proteasome.9,10 Once NF-κB heterodimers are released from IκBα, importin proteins (also known as karyopherins) direct NF-κB translocation to the nucleus where it controls a wide range of gene expression by binding to various κB elements. In the vascular endothelium, NF-κB activation induces the expression of proinflammatory genes, including those encoding adhesion molecules, cytokines, and chemotactrant proteins that collectively play critical roles in the initiation and progression of atherosclerosis.8,11,12 Consistent with this premise, endothelial cell (EC)–specific
NF-κB inhibition reduces atherosclerosis in 3 different mouse models, IKKγ EC knockout, IKKγ EC–inducible knockout, and dominant-negative IκBα EC transgenic, in apolipoprotein E–deficient (ApoE−/−) mice.13 Furthermore, genetic inhibition of several NF-κB target genes, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, E- and P-selectins, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β also reduces various aspects of atherosclerotic lesion formation.14–16 Thus, targeting NF-κB–mediated EC activation holds promise for the development of novel anti-inflammatory therapies for acute and chronic inflammatory diseases.

In This Issue, see p 2

Editorial, see p 3

MicroRNAs (miRNAs) are single-stranded, noncoding, small RNAs that regulate gene expression by destabilizing target mRNAs 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 vascularendothelial growth factor (VEGF), S100A8, and matrix metalloproteinase (MMP) family members in ECs.17–19 MiR-146a can repress the proinflammatory NF-κB pathway and the MAP kinase pathway in ECs by targeting TNF receptor–associated factor 6 and HuR.20 Recently, we previously demonstrated that miR-181b expression is reduced in the aortic intima of ApoE−/− mice after HFD for 4 or 6 weeks. As shown in Figure 1A, miR-181b expression was reduced by ≈59% and ≈53% in the aortic intima of ApoE−/− mice after HFD for 4 or 6 weeks. 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 4 or 6 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, circulating miR-181b levels were also reduced by ≈76% in plasma compared with 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 4 weeks of HFD (Online Figure 1A). In contrast, circulating miR-146a levels increased by 3.5- and 3.1-fold after 4 and 6 weeks of HFD, respectively (Online Figure 1B).

Methods

Pre-miR miRNA precursor molecules negative (nonspecific) control #1 (AM17110) and Hsa-miR-181b-5p Pre-miR miRNA precursor (PM12442) were used from Ambion. Real-time quantitative polymerase chain reaction was performed with the Mx3000P real-time polymerase chain reaction system (Stratagene) following the manufacturer’s instructions. NF-κB promoter with GFP/luciferase fusion reporter (NGL) mice fully backcrossed into C57BL/6 were crossed with homozygous ApoE−/− mice to generate ApoE−/−/NGL transgenic mice. To induce atherosclerosis, 8-week-old male ApoE−/− mice were fed a high-fat diet (HFD) from Research Diets Inc (D12108C) for 12 weeks. Aortas were carefully excised from mice and examined for immunohistology and characterization of atherosclerotic lesions.

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 proinflammatory stimuli (eg, TNF-α or lipopolysaccharide for 4 hours), we previously demonstrated that miR-181b expression is reduced in the aortic intima of mice.21 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 4 or 6 weeks. 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 4 or 6 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, circulating miR-181b levels were also reduced by ≈76% in plasma compared with 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 4 weeks of HFD (Online Figure 1A). In contrast, circulating miR-146a levels increased by 3.5- and 3.1-fold after 4 and 6 weeks of HFD, respectively (Online Figure 1B).

Figure 1. MiR-181b expression is reduced in the aortic intima of apolipoprotein E–deficient mice (ApoE−/−) mice fed a high-fat diet (HFD) or in plasma from human subjects with coronary artery disease. A, MiR-181a, miR-181b, and miR-181c expression was detected by quantitative polymerase chain reaction (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 with the expression of miR-181c at 1 week of HFD that was subsequently set to a value of 1. 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 means±SEM. *P<0.05; **P<0.01; and ***P<0.001. CAD indicates coronary artery disease.
suggested a specific effect for the miR-181b reduction. To characterize the components of the aortic intima, quantitative polymerase chain reaction analysis was performed for cell-specific markers. As shown in Online Figure IIA, endothelial mRNA markers von Willebrand factor and Tie-2 were robustly enriched in the intima and were barely detectable in the media plus adventitia (2.2% and 2.3% compared with the intima, respectively). Conversely, the expression of smooth muscle cell myosin heavy chain mRNA was much lower (1.4%) in the aortic intima compared with 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 with 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 nonspecific control mimics 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 nonspecific control (Figure 2A). No overexpression 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 nonspecific 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 nonspecific 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 exogenous 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 play a critical role in the initiation and progression of atherosclerosis.13 MiR-181b has been shown to inhibit these events in an acute inflammatory disease state such as sepsis.23 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 NGL reporter mice. After 4 weekly injections (IV) of 181b-m, miRNA nonspecific control mimics, or vehicle control in the ApoE−/−/NGL and NGL mice, NF-κB activity in the aortic

Figure 2. Systemic delivery of miR-181b reduces nuclear factor (NF)-κB activity in the aortic arch of apolipoprotein E-deficient (ApoE−/−)/NF-κB GFP-luciferase reporter (NGL) mice. A to E, ApoE−/−/NGL or NGL transgenic (Tg) mice (NF-κB promoter with GFP/luciferase fusion reporter) were fed a high-fat diet and tail vein injected with miRNA nonspecific control (NS-m), miR-181b (181b-m) mimics, or vehicle (Veh) twice a week for 4 weeks as described in the Methods section. MiR-181a, miR-181b, and miR-181c expression was examined by quantitative polymerase chain reaction (qPCR) in the aortic intima (A), media/adventitia (B), and peripheral blood mononuclear cells (PBMCs; C). Data shown are mean±SEM (n=3). The expression of miR-181 was normalized to small RNA U6 expression and compared with the expression of miR-181a in mice injected with vehicle that was subsequently set to a value of 1. 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 with its expression in mice received NS-m that was subsequently set to a value of one hundred. Data shown are mean±SEM (n=3–12). **P<0.01. ICAM indicates intercellular adhesion molecule; NS, nonsignificant; and VCAM, vascular cell adhesion molecule.
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 with 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 the nonspecific control mimics. In addition, the induction of expression of NF-κB target genes, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-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 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 181b-m or miRNA nonspecific control as shown in Figure 3A. After 12 weeks on HFD, there were no differences observed in body weight, total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglyceride levels between ApoE−/− mice injected with miR-181b mimics or miRNA nonspecific control (Table). 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 proinflammatory markers, including adhesion molecules intercellular adhesion molecule-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 2 groups (Figure 3F). As shown by immunohistochemistry in Figure 3G, ApoE−/− mice that received miR-181b mimics...
exhibited a reduction in vascular cell adhesion molecule-1 expression by 36% compared with miRNA nonspecific 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 and alanine transaminase (Online Figure IV). No differences were observed in liver NF-κB activity or plasma alanine transaminase and aspartate aminotransferase concentrations between miRNA nonspecific 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 proinflammatory 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 IPOA3 and NF-κB p65 Nuclear Translocation in the Vascular Endothelium of Lesions

We previously demonstrated that miR-181b directly targeted IPOA3 and reduced its expression and subsequently inhibited NF-κB nuclear translocation.23 Interestingly, IPOA3 mRNA expression increased in the aortic intima by ≈20% in ApoE−/− mice after 4 weeks of HFD (Online Figure V). To further validate the hypothesis that miR-181b inhibits endothelial NF-κB in the context of atherosclerosis, IPOA3 expression and nuclear translocation of NF-κB were directly evaluated in the endothelium of atherosclerotic lesions by immunostaining with antibodies against IPOA3 and CD31, or p65 and CD31, in sections from the aortic sinus. As shown in Figure 4A, ApoE−/− mice that received 181b-m exhibited a reduction in IPOA3 expression by 35% in the endothelium compared with miRNA nonspecific control–treated ApoE−/− mice. Multiple genes have been identified as direct targets of miR-181a or miR-181b in different cell types.24–31 In addition to IPOA3, 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, but not other reported target genes. Consistent with reduced expression of IPOA3 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 with mice injected with miRNA nonspecific 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 IPOA3 expression and NF-κB p65 nuclear translocation in vascular endothelium, but not in lesional macrophages.

**Differential Expression of IPOA3 and Importin-α5 in ECs and Leukocytes Account for the Cell-Specific Effect of MiR-181b on NF-κB Inhibition**

The role of myeloid NF-κB signaling in atherogenesis remains controversial, which prompted us to ask whether systemic delivery of miR-181b regulates NF-κB signaling in leukocytes. We found that 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 with mice injected with the miRNA nonspecific 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 isolated from ApoE−/−/NGL mice (Figure 5D) or p65 nuclear translocation in peritoneal macrophages in response to lipopolysaccharide (Online Figure VII). Previous studies demonstrate that several importin-α molecules, including IPOA3 and importin-α5 (IPOA5), may be involved in NF-κB nuclear translocation. We previously identified that miR-181b directly targets IPOA3, a protein that is critical for nuclear

Figure 5. MiR-181b does not inhibit nuclear factor (NF-κB) activation in leukocytes because of dominant expression of importin-α5. A to C, Apolipoprotein E–deficient (ApoE−/−) or ApoE−/−/NF-κB GFP-luciferase reporter Tg mice were fed a high-fat diet and intravenously injected with miRNA nonspecific control (NS-m) or miR-181b (181b-m) mimics once a week for 12 weeks. A, Real-time quantitative polymerase chain reaction (qPCR) analysis of miR-181b in peripheral blood mononuclear cells (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 lipopolysaccharide (LPS) for 12 hours. 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 with importin-α1 gene expression in PBMCs that was subsequently set to a value of 1 (n=3). H, Western blot analysis of IPOA3, importin-α4 (IPOA4), and importin-α5 (IPOA5) in endothelial cells and PBMCs. I and J, PBMCs from ApoE−/−/NGL mice were transfected with c3i 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 interleukin (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 c3i siRNA or IPOA5 siRNA (n=4–5). Data shown are means±SEM. *P <0.05; **P <0.01; and ***P <0.001. HAECs indicates human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; MAEC, mouse aortic endothelial cell; and NS, nonsignificant.
translocation of NF-κB in ECs.\(^\text{23}\) As shown in Figure 5E, the expression of IPOA3 was also reduced in mouse PBMCs by systemically delivered miR-181b. In contrast, the expression of IPOA5 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 IPOA3 by miR-181b in both cell types, we assessed the relative expression of the miR-181b target IPOA3 and other importin-α proteins in the aortic intima, human and mouse ECs, and mouse PBMCs. As shown in Figure 5G, IPOA3 mRNA expression was 4.4-fold higher in the aortic intima compared with PBMCs. IPOA3 was also the dominantly expressed member of the importin-α family in the aortic intima at the mRNA level and is \(\approx 3\)-fold higher than the second most abundant importin-α molecule (importin-α7). Consistently, the expression of IPOA3 was higher in ECs (8-fold in mouse aortic endothelial cells) at the protein level compared with 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 IPOA5 in PBMCs was \(\approx 3\)-fold higher compared with the aortic intima at the mRNA level and \(\approx 4\)-fold higher compared with mouse aortic endothelial cells at the protein level, respectively (Figure 5G and 5H). IPOA5 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 IPOA5\(^\text{23}\) (Figure 5F), and knockdown of IPOA5 expression by siRNA transfection (Figure 5I) was able to inhibit lipopolysaccharide-induced NF-κB activation (Figure 5J) and NF-κB target gene expression (Figure 5K and 5L) in cultured PBMCs. Furthermore, systemic delivery of IPOA5 siRNA inhibited its expression in vivo in PBMCs (Figure 5M) and inhibited lipopolysaccharide-induced NF-κB activation by 31% (Figure 5N). In summary, these data demonstrate that systemic delivery of miR-181b 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—IPOA3 (a miR-181b target) is expressed significantly higher than IPOA5 in ECs, whereas IPOA5 (a non-miR-181b target) is expressed higher than IPOA3 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 proinflammatory gene expression, leukocyte accumulation, and atherosclerotic lesion formation. Furthermore, these studies highlight for the first time to our knowledge that miRNA mimetics penetrated 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 ECs (by ablating IKKγ/ NEMO or expression of a dominant-negative IκBα) confers an atheroprotective effect in ApoE\(^{-/-}\) mice.\(^\text{13}\)

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, although EC-specific inhibition of NF-κB resulted in reduced lesion formation in ApoE\(^{-/-}\) mice,\(^\text{13}\) 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.\(^\text{34}\) In contrast, a recent study showed that myeloid-specific IKKβ deficiency decreases atherosclerosis in low-density lipoprotein receptor–deficient mice.\(^\text{35}\) In another study, myeloid IκBα deficiency promoted atherogenesis by enhancing leukocyte recruitment to the developing plaques.\(^\text{32}\) Furthermore, bone marrow deficiency of NF-κB1 resulted in reduced atherosclerotic lesion size and macrophage foam cells but caused increased plaque inflammation.\(^\text{33}\) Finally, conditional targeting of TNF receptor–associated factor 6 revealed opposing functions of Toll-like receptor signaling in endothelial and myeloid cells in a mouse model of atherosclerosis.\(^\text{36}\) 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 \(\approx 7\)- to 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 overexpression 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, IPOA3 is a protein involved in NF-κB translocation from the cytoplasm to nucleus.\(^\text{37,38}\) We previously demonstrated that IPOA3 is a bona fide direct target of miR-181b in ECs.\(^\text{23}\) Indeed, systemic delivery of miR-181b significantly reduced the expression of IPOA3 in the aortic intima of lesions by immunohistochemical staining (Figure 4A). Thus, miR-181b seems to suppress NF-κB activation and target gene expression in the vascular wall by reducing IPOA3 expression in the vascular endothelium. In addition to IPOA3, the expression of neuropilin-1, but not other previously identified miR-181b target genes, was reduced by miR-181b in the aortic arch (Online Figure VI). Neuropilin-1 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.\(^\text{34}\) It remains unknown whether neuropilin-1 exerts anti-inflammatory effects on NF-κB signaling or other relevant proinflammatory 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 IPOA3 is higher in the aortic intima (4.4-fold at the mRNA level) and ECs (8-fold in mouse aortic endothelial cells at the protein level) compared with PBMCs (Figure 5G and 5H). Interestingly, a similar gradient of cellular expression was noted in the investigation that originally reported cloning for the IPOA3 gene. Surprisingly, the expression pattern of importin-α molecules is strikingly different in PBMCs. The expression of IPOA5 is higher in PBMCs than in ECs (3- and 4-fold at the mRNA and protein levels, respectively; Figure 5G and 5H). Furthermore, IPOA5 participates in NF-κB activation in PBMCs as verified by siRNA knockdown of IPOA5 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 IPOA3 in ECs and IPOA5 in leukocytes likely accounts for the cell-specific effects of miR-181b on NF-κB signaling. As miR-181b cannot inhibit IPOA5 expression (Figure 5F) or its 3′-untranslated region, 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 interdependent signaling pathways they may affect (eg, MAPK signaling, insulin signaling, and p53). Second, miR-181b–mediated inhibition of IPOA3 provides targeting of a focused downstream event of preventing nuclear translocation of NF-κB heterodimers. Third, because of the differential expression of IPOA3 and IPOA5 in ECs and leukocytes, respectively, miR-181–mediated targeting of IPOA3 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 to maintain protection in response to various infectious pathogens.

In summary, our study in ApoE−/− 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 because of the use of different importin-α isoforms in ECs (IPOA3) and PBMCs (IPOA5; 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
This work was supported by the National Institutes of Health (HL091076, HL115141, and HL177994 to M.W. Feinberg), a Watkins Cardiovascular Medicine Discovery Award (to M.W. Feinberg), a State Scholarship Fund of the China Scholarship Council (to S. He), and a Jonathan Levy Research Fund (to M.W. Feinberg).

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. The other authors report no conflicts.

References
Intravenous delivery of miR-181b inhibited NF-κB.

Atherosclerosis is a chronic inflammatory disease of the arterial wall. MiR-181b expression in the aortic intima is markedly reduced after a high-fat diet feeding in ApoE−/− mice. Intravenous delivery of miR-181b inhibited NF-κB activation, atherosclerotic lesion formation. These findings suggest that miR-181b mimetics may provide a novel therapeutic approach to treat chronic inflammatory diseases such as atherosclerosis.
Systemic Delivery of MicroRNA-181b Inhibits Nuclear Factor-κB Activation, Vascular Inflammation, and Atherosclerosis in Apolipoprotein E−Deficient Mice

Xinghui Sun, Shaolin He, A.K.M. Wara, Basak Icli, Eugenia Shvartz, Yevgenia Tesmenitsky, Nathan Belkin, Dazhu Li, Timothy S. Blackwell, Galina K. Sukhova, Kevin Croce and Mark W. Feinberg

Circ Res. 2014;114:32-40; originally published online October 1, 2013; doi: 10.1161/CIRCRESAHA.113.302089

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
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/114/1/32

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/10/01/CIRCRESAHA.113.302089.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

METHODS AND MATERIALS

Reagents
Pre-miR™ miRNA precursor molecules-negative (non-specific) control #1 (AM17110) and Hsa-miR-181b-5p Pre-miR™ miRNA precursor (PM12442) were ordered from Ambion. They are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs. The mature miR-181b sequence is 5'-AACAUUCAUUGCUUGCGUGGGU-3', and its miRBase Accession# is MIMAT0000257. For in vivo studies, oligomers with the same sequence were synthesized on a larger scale by Ambion. Lipofectamine™ 2000 reagent was from Invitrogen.

Real-time qPCR
Tissues were homogenized using TissueLyser II (QIAGEN) according to the manufacture’s instruction. Total RNA was isolated using TRizol® reagent (Invitrogen) from homogenized tissues or cells. QuantiTect Reverse Transcription Kit (QIAGEN) was used to generate cDNA and QuantiFast SYBR Green PCR Kit was used for real-time qPCR with the Mx3000P Real-time PCR system (Stratagene) following the manufacturer’s instructions. Primers for mouse VCAM-1, E-selectin, ICAM-1, TNF-α, IL-1β, and others are listed in Online Table I. To compare importin-α expression, the standard curve method was used to quantify the absolute copy numbers; subsequently, relative expression was calculated after normalization with mouse β-actin. To amplify mature miRNA sequences, TaqMan® MicroRNA Assays hsa-miR-181b (Assay ID 001098), TaqMan® MicroRNA Assays hsa-miR-181a (Assay ID 000480), TaqMan® MicroRNA Assays hsa-miR-181c (Assay ID 000482), TaqMan® MicroRNA Assays hsa-miR-146a (Assay ID 000468), RNU6B (Assay ID 001093), U6 snRNA (Assay ID 001973), TaqMan® MicroRNA Reverse Transcription Kit (PN4366596), and TaqMan® Universal PCR Master Mix No AmpErase® UNG (PN4324018) were used.

Bioluminescence imaging
NGL mice (NF-κB promoter with GFP/luciferase fusion reporter) fully backcrossed into C57BL/6 as previously reported1 were crossed with homozygous ApoE-deficient mice to generate ApoE-/-/NGL transgenic mice. ApoE-/-/NGL mice were injected with miR-181b, miRNA non-specific control, or vehicle twice on two consecutive days followed by twice a week for 4 weeks and fed a HFD. NGL mice were also injected with vehicle in the same manner and fed a HFD. After 4 weeks, aortas were carefully excised, kept in ice-cold 1 x PBS, and incubated with 1 x PBS containing 1.5 mg/ml D-luciferin for bioluminescence imaging using a Xenogen IVIS System 100 (Xenogen Corp.,
Alameda). Serial images were taken and luminescence was quantitated at the plateau of the luminescent signal (about 40 – 45 min after incubation).

**Systemic delivery of miR-181b or siRNAs in mice**

Mice were injected with miR-181b or miRNA non-specific control twice a week for 4 weeks or once a week for 12 weeks through tail vein as previously described. In brief, 1 µl of 1 nmol/µl miRNA non-specific control or miR-181b mimics was mixed with 100 µl dPBS (solution 1). Lipofectamine 2000 (30 µl) was mixed with dPBS (70 µl) by pipetting up and down 50 times (solution 2), and placed at room temperature for 15 min. Solution 1 and solution 2 were then mixed with pipetting up and down 100 times. After incubating at room temperature for 30 min, the mixture was tail-vein injected into mice using an insulin syringe (1/2 mL), 200 µl per mouse. Injection of negative control siRNA or importin-α5 siRNA was followed by the same procedures except that 2 nmol of siRNA was mixed with 100 µl dPBS for solution 1. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at Harvard Medical School, Boston, MA and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Immunohistology and characterization of atherosclerotic lesions**

To induce atherosclerosis, we fed 8-week-old male ApoE-/- mice a HFD from Research Diets Inc. (D12108Ci) for 12 weeks. Aortas were carefully excised from mice, and the basal portion of the heart and aortic roots were embedded in OCT compound and frozen at -80°C. Serial cryostat sections (6 µm) were prepared using a Lab-Tek tissue processor Leica CM3050. Lesion characterizations, including Oil red O staining of the thoracic-abdominal aorta and aortic root and staining for macrophages (Mac-3) and T cells (CD4), were performed as previously described. In addition, immunostaining was performed on the aortic root for expression of VCAM-1 (Santa Cruz). Images were captured by a digital system, the staining area was measured using computer-assisted image quantification (Image-Pro Plus software, Media Cybernetics), and CD4-positive cells were counted manually. The quantification of VCAM-1 staining was performed as previously described. For immunofluorescence staining, frozen sections of aortic roots were stained with: 1) DAPI, rat monoclonal anti-mouse CD31 (Dianova), and anti-p65 (Abcam); 2) DAPI, rat monoclonal anti-mouse CD31, and anti-importin-α3 (Abcam); or 3) DAPI, rat monoclonal anti-mouse Mac3, and rabbit polyclonal anti-p65, followed by corresponding secondary antibodies. Images were acquired on an upright Carl Zeiss LSM 510 confocal microscope equipped with Plan-Neofluar 40×/1.3 oil-immersion objective using the 405 nm diode laser, the 543 nm line of a HeNe543 laser, and the 633 nm line of a HeNe633 laser. Fluorescence intensity of importin-α3 and nuclear p65 was measured using Image J software. Data were analyzed in a blinded fashion, by two independent observers. The data were calculated from at least 40 cells for each group
with 8 – 10 mice. For each mouse, 2 – 5 cells randomly selected from 2 sections were used for quantification.

**Lipid profile analysis**

Triglyceride levels were determined using Infinity™ Triglycerides Liquid Stable Reagent (Thermo Scientific) as described by the protocol. Total cholesterol was measured using the Infinity™ Cholesterol Reagent (Thermo Scientific) and HDL cholesterol was measured by colorimetric assay using EnzyChrom™ HDL Assay Kit (BioAssay Systems). LDL cholesterol levels were calculated using the following formula: 

\[
LDL = \frac{Total \ Cholesterol - HDL \ Cholesterol - Triglycerides}{5}
\]

Cholesterol standard, Triglyceride standard, HDL standard were from Pointe Scientific, Inc.

**Intimal RNA isolation from aorta tissue**

Isolation of intimal RNA from aorta was described as previously reported.\(^2\) Six-week old ApoE-/ mice were fed a HFD for 1, 4, and 6 weeks before aortic intimal isolation and blood collection.

**Cell culture**

Human umbilical vein endothelial cells (HUVECs) (cc-2159) and human aortic endothelial cells (HAECs) (cc-2535) were obtained from Lonza and cultured in endothelial cell growth medium EGM®-2 (cc-3162). C57BL/6 mouse aortic endothelial cells (MAECs) (C57-6052) were purchased from Cell Biologics, Inc., and cultured in endothelial cell medium (Cell Biologics, M1168). Cells passaged less than five times were used for all experiments.

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by using LSM® Lymphocyte Separation Medium (MP Biomedicals, LLC) according to the manufacturer’s instructions.

Bone marrow-derived macrophages were isolated as previously described.\(^6\) Briefly, femurs were removed from 6 to 11-week old mice. Bone marrow cells were flushed out using a 27-gauge needle attached to a syringe containing 10 mL culture medium. Cells were resuspended by gentle pipetting, passed through a 70 µm filter, and spun down at 300 x g for 10 min at 4 °C. Cells were plated into 12-well plates at 1 x 10^6 cells per well and allowed to grow until they were ready for transfection. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, GlutaMAX, and recombinant 10 ng/ml M-CSF. For isolation of peritoneal macrophages, mice were injected with 3% thioglycollate medium (3 ml each mouse) into the peritoneum. After 4 days, cells were harvested and plated on 12-well plates (1x10^6 cells per well), and allowed to grown for 3 days before transfection. Cells were cultured in high glucose DMEM with 10% fetal bovine serum.
Lipofectamine™ 2000 transfection reagent from Invitrogen was used for transfection, following manufacturer's instructions. MiRNAs or siRNAs were transfected at 10 nM or 30 nM concentration, respectively.

Western blot analysis

Cultured cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail tablets (Roche). Cell debris was removed by centrifugation at 12,000 rpm for 10 min. Lysates were separated by SDS-PAGE gels, transferred to PVDF membranes (Bio-Rad), and incubated with the relevant antibodies as indicated. Proteins were visualized by ECL Plus Western blotting detection reagents (RPN2132; GE Healthcare). Densitometry scanning of the blots with ImageJ software was used to calculate the abundance of protein. Antibody against p65 (sc-372), USF-2 (sc-862) were purchased from Santa Cruz Biotechnology, goat polyclonal to KPNA4/importin-α3 (ab6039), goat polyclonal to KPNA3/importin-α4 (ab6038), and rabbit polyclonal to SRP1/importin-α5 (ab154399) were purchased from Abcam.

Human and mouse plasma samples and real-time qPCR analysis

Human plasma samples were obtained from the cardiac catheterization laboratory in accordance with the Institutional Review Board-approved protocol at Brigham and Women's Hospital. Written informed consent was obtained from all participants or their appropriate surrogates. Enrolled subjects were characterized as “coronary artery disease”, or “control” subjects by a group of blinded physicians based on the presence of >70% stenosis on coronary angiogram. Anonymized plasma samples were generated from blood collected in EDTA-containing tubes obtained from patients at the time of coronary angiography and stored at −80°C. Mouse blood was collected via heart puncture and transferred to EDTA-containing tubes. Plasma was isolated from whole blood at 1500g for 15 minutes at room temperature. Total RNA was isolated from plasma by using total RNA purification kit from Norgen Biotek Corporation and reverse transcription and real time qPCR was performed as described.

Plasma levels of aspartate and alanine aminotransferases were measured using the VITROS DT60 II Chemistry System from Ortho-Clinical Diagnostics, Inc. according to the manufacturer's instruction.

Statistical analysis

Student t test was used to determine statistical significance between the groups. A P<0.05 was considered significant.
Reference


Online Figure I. Circulating miR-181b and miR-146a in the plasma of ApoE-/- mice fed a high-fat diet. The expression of circulating miR-181b and miR-146a was detected by qPCR in mouse plasma samples from ApoE-/- mice fed a HFD for 1, 4, or 6 weeks (n=6-8 per group). The expression of miR-181b (A) or miR-146a (B) was normalized to small RNA U6 expression and compared to the expression at 1 week of HFD that was set to a value of one hundred or one, respectively. Data show mean ± SEM. *, P < 0.05.
Online Figure II. Characterization of aortic intima. QPCR analysis of EC markers vWF and Tie2 (A), smooth muscle marker smMHC (B), macrophage marker CD68 (C), and T cell markers CD3 and CD4 (D) in the aortic intima, media, and adventitia. Data show mean ± SEM, n=3.
Online Figure III. MiR-181b expression in the aortic intima, media/adventitia, and PBMCs from ApoE-/ mouse. MiR-181b expression was detected by qPCR (n=5-6 per group). The expression of miR-181b was normalized to small RNA U6 expression and compared to its expression in aortic intima that was set to a value of one. Data show mean ± SEM. **, P < 0.01; #, P < 0.001.
Online Figure IV. Systemic delivery of miR-181b does not lead to changes in liver function. **A**, Luciferase activity (represents NF-κB activity) is shown in livers from ApoE-/-/NGL Tg mice fed a high-fat diet for 12 weeks and received NS-m (n=9) or 181b-m (n=8). **B** and **C**, Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of ApoE-/ mice fed a high-fat diet for 12 weeks and received NS-m (n=5) or 181b-m (n=5). Data show mean ± SEM, N.S., non-significant.
Online Figure V. Importin-α3 expression increases in the aortic intima of ApoE-/- mice fed a high-fat diet. Importin-α3 expression was detected by qPCR in the aortic intima from ApoE-/- mice fed a HFD for 1, 4, or 6 weeks (n=6-8 per group). The expression of importin-α3 was normalized to β-actin expression and compared to its expression at 1 week of HFD that was set to a value of one. Data show mean ± SEM. *, P < 0.05.
Online Figure VI. qPCR analysis of potential miR-181b target genes in the aortic arch. The expression of each gene was normalized to mouse β-actin expression and compared to its expression in mice that received NS-m and was subsequently set to a value of one hundred. Data show mean ± SEM, n=7-11. *, P < 0.05. N.S., non-significant.
Online Figure VII. MiR-181b does not reduce NF-κB p65 nuclear translocation in macrophages. Western blot analysis of p65 in the nuclear fraction from macrophages transfected with NS-m or 181b-m, and treated with 100 ng/ml LPS for 90 min.
Online Figure VIII. MiR-181b inhibits vascular endothelial NF-κB activation, the recruitment of leukocytes into lesions, and atherosclerotic lesion formation without altering NF-κB activation in leukocytes. Systemic delivery of miR-181b suppresses NF-κB activation in the vascular endothelium by reducing the expression of importin-α3, a protein that plays a critical role in NF-κB nuclear translocation. In contrast, importin-α5 is the dominantly expressed importin isoform in leukocytes that mediates NF-κB translocation. Subsequently, miR-181b selectively inhibits endothelial NF-κB target gene expression including adhesion molecules VCAM-1, E-selectin, ICAM-1, and cytokines TNF-α and IL-1β. Consequently, the influx of lesional macrophages and CD4+ T cells, and atherosclerotic lesion formation are reduced due to reduced endothelial NF-κB activation. EC, endothelial cell; T, T cell; MØ, macrophage; SMC, smooth muscle cell.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' −&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse VCAM-1 forward:</td>
<td>GTTCCAGCGAGGGTCTACC</td>
</tr>
<tr>
<td>mouse VCAM-1 reverse:</td>
<td>AACTCTTGCGAAGATTAGGTGT</td>
</tr>
<tr>
<td>mouse E-selectin forward:</td>
<td>ATGCCTCGCGGTTTCTCTC</td>
</tr>
<tr>
<td>mouse E-selectin reverse:</td>
<td>GTAGTCCCGCTGACAGTATGC</td>
</tr>
<tr>
<td>mouse ICAM-1 forward:</td>
<td>GTGATGCTCAGGTATCCATCA</td>
</tr>
<tr>
<td>mouse ICAM-1 reverse:</td>
<td>CACAGTTCTCAAGACACAGC</td>
</tr>
<tr>
<td>mouse TNF-alpha forward:</td>
<td>CCTCACACTCAGATCATCCTTCT</td>
</tr>
<tr>
<td>mouse TNF-alpha reverse:</td>
<td>GCTACGACGTGGGCTACAG</td>
</tr>
<tr>
<td>mouse IL-1beta forward:</td>
<td>GCAACTGGTTCTGAACTCAACT</td>
</tr>
<tr>
<td>mouse IL-1beta reverse:</td>
<td>ATCTTTTGAGGTCGGCTCAACT</td>
</tr>
<tr>
<td>mouse beta-actin forward:</td>
<td>GAAATCGTGCGTACATCAAAAG</td>
</tr>
<tr>
<td>mouse beta-actin reverse:</td>
<td>TGTAATTTCTAGTGGACACAGC</td>
</tr>
<tr>
<td>mouse IL10 forward:</td>
<td>GCTTCTACTGACTGGCATG</td>
</tr>
<tr>
<td>mouse IL10 reverse:</td>
<td>CGCAGCTCTAGAGCATGTG</td>
</tr>
<tr>
<td>mouse Cd3 forward:</td>
<td>ATGCGGTGGAACACTTTTCTG</td>
</tr>
<tr>
<td>mouse Cd3 reverse:</td>
<td>GCACGTCAGCTCAGCTACTTG</td>
</tr>
<tr>
<td>mouse Cd4 forward:</td>
<td>TCCTAGCTGTCAGCTAACAGG</td>
</tr>
<tr>
<td>mouse Cd4 reverse:</td>
<td>TCAGAGAATCTCCAGGTGAAGA</td>
</tr>
<tr>
<td>mouse Cox2 forward:</td>
<td>TGAGCAACTATTCCAAACAGC</td>
</tr>
<tr>
<td>mouse Cox2 reverse:</td>
<td>GACGCTAGTCTCCAGTACTATC</td>
</tr>
<tr>
<td>mouse Cd68 forward:</td>
<td>TGCTGATCTTGTGGAGACAG</td>
</tr>
<tr>
<td>mouse Cd68 reverse:</td>
<td>GAGAGTAACGGCCTTTTTTGTA</td>
</tr>
<tr>
<td>mouse vWF forward:</td>
<td>CTTCTGTACGCTACAGCTATG</td>
</tr>
<tr>
<td>mouse vWF reverse:</td>
<td>GCGTGTGTAATTCCACACAG</td>
</tr>
<tr>
<td>mouse Tie-2 forward:</td>
<td>GAGTCAGCTTTGCTCCTTTATGG</td>
</tr>
<tr>
<td>mouse Tie-2 reverse:</td>
<td>AGACACAAAGAGGTAGGAATTGA</td>
</tr>
<tr>
<td>mouse smMHC forward:</td>
<td>AAGCTGCGGCTAGAGGTA</td>
</tr>
<tr>
<td>mouse smMHC reverse:</td>
<td>CCCTCCCTTTGATGGCTG</td>
</tr>
<tr>
<td>mouse IPOA1 forward:</td>
<td>ATGTCACGAAGAGAAAGATCT</td>
</tr>
<tr>
<td>mouse IPOA1 reverse:</td>
<td>AGAGAAGCTGACATTCTCTTCTT</td>
</tr>
<tr>
<td>mouse IPOA3 forward:</td>
<td>CCAGTGATCGAAATCCCAACAA</td>
</tr>
<tr>
<td>mouse IPOA3 reverse:</td>
<td>CGTTTGTCTCAGCAGTAGTCT</td>
</tr>
<tr>
<td>mouse IPOA4 forward:</td>
<td>TCAGGGACTTCTGACCAGAC</td>
</tr>
<tr>
<td>mouse IPOA4 reverse:</td>
<td>ACACCGTGTTCCAGACAAAACATT</td>
</tr>
<tr>
<td>mouse IPOA5 forward:</td>
<td>ACCAGGAAAGGAGAATCTTCTCGC</td>
</tr>
<tr>
<td>mouse IPOA5 reverse:</td>
<td>GTGAGAAGGTGATGACACCC</td>
</tr>
<tr>
<td>mouse IPOA7 forward:</td>
<td>AGAAGAACATGCTTTAACCCTGTA</td>
</tr>
<tr>
<td>mouse IPOA7 reverse:</td>
<td>AGCAGACTATCAAAACATGGCAG</td>
</tr>
<tr>
<td>mouse PROX1 forward:</td>
<td>AGAAGGGTTGACATTTGGAGTGA</td>
</tr>
<tr>
<td>mouse PROX1 reverse:</td>
<td>TGCCTGTGACACACAGATA</td>
</tr>
<tr>
<td>mouse Aid forward:</td>
<td>ACCTTCCGGAACAGTCTGGCT</td>
</tr>
<tr>
<td>mouse Aid reverse:</td>
<td>AGCCTTGGCTTCCACAGAA</td>
</tr>
<tr>
<td>mouse NRP1 forward:</td>
<td>GACAAATGTCGGGCAAGCATA</td>
</tr>
<tr>
<td>mouse NRP1 reverse:</td>
<td>TGGATTAGCCATTCAACTTCTC</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>mouse PTEN</td>
<td>TGGATTTCGACTTAGACTTGACCT</td>
</tr>
<tr>
<td>mouse Ptpn22</td>
<td>CAGCAACTACTGAAAGAAGCCC</td>
</tr>
<tr>
<td>mouse Ptpn11</td>
<td>AGAGGAAGAGCAAATGTGTCA</td>
</tr>
<tr>
<td>mouse DUSP6</td>
<td>ATAGATACGCTCGACCCGTG</td>
</tr>
<tr>
<td>mouse CYLD</td>
<td>ATTTCCAGGAGTTGTACGCTTC</td>
</tr>
</tbody>
</table>