Circulation Research

ORIGINAL RESEARCH

miR-223 Exerts Translational Control of Proatherogenic Genes in Macrophages

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BACKGROUND: A significant burden of atherosclerotic disease is driven by inflammation. Recently, microRNAs (miRNAs) have emerged as important factors driving and protecting from atherosclerosis. miR-223 regulates cholesterol metabolism and inflammation via targeting both cholesterol biosynthesis pathway and NF_kB signaling pathways; however, its role in atherosclerosis has not been investigated. We hypothesize that miR-223 globally regulates core inflammatory pathways in macrophages in response to inflammatory and atherogenic stimuli thus limiting the progression of atherosclerosis.

METHODS AND RESULTS: Loss of miR-223 in macrophages decreases *Abca1* gene and protein expression as well as cholesterol efflux to apoA1 (Apolipoprotein A1) and enhances proinflammatory gene expression. In contrast, overexpression of miR-223 promotes the efflux of cholesterol and macrophage polarization toward an anti-inflammatory phenotype. These beneficial effects of miR-223 are dependent on its target gene, the transcription factor *Sp3*. Consistent with the antiatherogenic effects of miR-223 in vitro, mice receiving *miR223*$^{-/-}$ bone marrow exhibit increased plaque size, lipid content, and circulating inflammatory cytokines (ie, IL-1β). Deficiency of miR-223 in bone marrow–derived cells also results in an increase in circulating pro-atherogenic cells (total monocytes and neutrophils) compared with control mice. Furthermore, the expression of miR-223 target gene (*Sp3*) and pro-inflammatory marker (*Il-6*) are enhanced whereas the expression of *Abca1* and anti-inflammatory marker (*Retnla*) are reduced in aortic arches from mice lacking miR-223 in bone marrow–derived cells. In mice fed a high-cholesterol diet and in humans with unstable carotid atherosclerosis, the expression of miR-223 is increased. To further understand the molecular mechanisms underlying the effect of miR-223 on atherosclerosis in vivo, we characterized global RNA translation profile of macrophages isolated from mice receiving wild-type or *miR223*$^{-/-}$ bone marrow. Using ribosome profiling, we reveal a notable upregulation of inflammatory signaling and lipid metabolism at the translation level but less significant at the transcription level. Analysis of upregulated genes at the translation level reveal an enrichment of miR-223-binding sites, confirming that miR-223 exerts significant changes in target genes in atherogenic macrophages via altering their translation.

CONCLUSIONS: Our study demonstrates that miR-223 can protect against atherosclerosis by acting as a global regulator of RNA translation of cholesterol efflux and inflammation pathways.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: animal models of human disease ■ cholesterol ■ inflammation ■ lipids ■ metabolism ■ vascular biology

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Atherosclerosis is a chronic inflammatory disease that is driven by the interplay of excess cholesterol accumulation in the vessel wall and a maladaptive immune response. It is characterized by retention of cholesterol-rich LPs (lipoproteins) in susceptible areas of the arterial vasculature, followed by chronic endothelial activation and recruitment of circulating monocytes into the vascular intima.[1–3] In the subendothelial space, the majority of recruited monocytes differentiate into macrophages, where they scavenge retained modified LPs,

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.121.319120.
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Novelty and Significance

What Is Known?

- Thousands of small, noncoding RNAs are present in the human genome, and some are highly conserved between humans and mice.
- MicroRNAs (miRNA) inhibit their target gene(s) either by inducing degradation of the transcript levels (mRNA) and/or by blocking translation of the target protein. Many miRNAs target multiple genes within the same biological pathway.
- Most studies examining miRNA contribution to atherosclerosis only measure mRNA target levels, and overlook protein translation to understand miRNA mechanisms.
- miR-223 has previously been shown in liver cells to control genes involved in cholesterol synthesis and uptake, and in immune cells to control NFkB activation, but the contribution of miR-223 to the development of atherosclerosis, which combines both cholesterol and inflammatory responses, had never been investigated.

What New Information Does This Article Contribute?

- The current work explores the positive impact of miR-223 in atherosclerosis development and proposes that boosting miR-223 expression or activity may slow atherosclerosis progression in animal models.
- SP3 is identified as key factor controlling miR-223 expression during inflammation.
- miR-223 suppresses inflammation largely via controlling translation of its target genes, rather than levels of target gene transcripts.

These data support a role for miR-223 in atherosclerosis development by exerting control over 2 main pathways—cholesterol efflux and inflammation—that drive plaque formation in the vessel wall. Boosting miR-223 levels may protect from atherosclerotic disease. These findings highlight the need to extend miRNA studies beyond target mRNA expression to fully understand miRNA-regulated biology and pathobiology.

Nonstandard Abbreviations and Acronyms

ABCA1	ATP-binding cassette transporter A1
ApoA1	apolipoprotein A1
ApoE	apolipoprotein E
BM	bone marrow
HCD	high cholesterol diet
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
LP	lipoprotein
miRNA	microRNA
NLRP3	NOD-, LRR-, and pyrin domain-containing protein 3
RPF	ribosome-protected fragment
SC4MOL	methylsterol monooxygenase 1
SP3	specificity protein 3
TLR	toll-like receptor
TRAF6	TNF receptor associated factor 6
UTR	Untranslated region
WT	Wild-type

which eventually transform them to cholesterol-laden foam cells.[4] However, excessive cholesterol uptake, leading to free cholesterol accumulation in macrophage-derived foam cells, triggers the activation of downstream cascades including NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome, TLR (toll-like receptor), and NFκB signaling pathways, resulting in the secretion of pro-inflammatory mediators (cytokines, chemokines, and reactive oxygen and nitrogen species). These inflammatory signals induce the transmigration of additional inflammatory cells including monocytes into the intima, further aggravating plaque inflammation.[5–8] Traditionally, lipid lowering has been the gold-standard therapy for atherosclerosis; yet, a significant burden of atherosclerotic disease remains even in the setting of low plasma cholesterol, likely as a result of residual inflammatory risk.[9,10] Thus, there is an urgent need to better understand the molecular mechanisms that govern atherogenesis particularly those that drive inflammation.

Over the past decade, microRNAs (miRNAs) have emerged as key modulators and fine-tuners of multiple signaling pathways involved in atherosclerosis.[11,12] miRNAs are defined as highly conserved small RNA sequences of 20 to 23 nucleotides that contain complementary sequences for specific target messenger RNAs (mRNAs). Via binding to the 3'-untranslated regions (UTR), miRNAs post-transcriptionally regulate gene expression by degradation and/or translational inhibition of their bound targets. Notably, 1 miRNA can simultaneously repress multiple target genes through seed-based targeting. In addition, one functional gene network can be regulated by a group of miRNAs, providing a mechanism to coordinate complex gene expression programs and thereby modulate many aspects of cellular homeostasis and physiology.[13–15] Indeed, accumulating evidence has highlighted the importance of miRNAs in regulating lipoprotein homeostasis, vascular inflammation, leukocyte recruitment/activation, and vascular smooth muscle function,[16,17] thus controlling each stage of atherosclerosis from development, progression to disruption. One of the first miRNAs to be studied for its role in

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cholesterol regulation was miR-223-3p (hereafter referred to as miR-223), which controls intracellular cholesterol levels through directly repressing genes involved in cholesterol biosynthesis (HMGCS1 [3-hydroxy-3-methylglutaryl-CoA synthase 1]/and methylsterol monooxygenase 1/SC4MOL) and cholesterol uptake (scavenger receptor class B member 1) in hepatocytes and coronary arterial endothelial cells.[18] In addition to cholesterol metabolism, miR-223 suppresses the TLR4-NFκB pathway leading to a reduction in pro-inflammatory cytokine production in LPS activated macrophages[19] possibly as a result of its direct targeting of IKKα and reduction in noncanonical NFκB activation.[20] This feedback likely prevents macrophages from becoming overactivated while priming them for future NFκB signaling events.[20] Finally, genome-wide miRNA screens have identified miR-223 as a negative regulator of NLRP3 inflammasome activation by directly suppressing NLRP3 expression and reducing NLRP3-dependent induction of IL-1β in primary neutrophils.[21,22] Together, these studies have established the importance of miR-223 in cholesterol metabolism and inflammation in vitro, yet its role in lipid-driven inflammatory diseases in vivo has not been established.

Given the intersection of cholesterol and inflammation in the pathogenesis of atherosclerosis, we set out to determine the contribution of miR-223 in atherosclerosis development, particularly its role in myeloid-derived cells that perpetuate inflammation in the plaque. We find that loss of miR-223 in macrophages promotes a pro-inflammatory phenotype and dampens macrophage cholesterol efflux in vitro. These effects of miR-223 are largely dependent on the inhibition of its target gene, the transcription factor Specificity Protein 3 (referred as *Sp3* in mouse and *SP3* in human). Consistent with pro-atherogenic effects in vitro, deletion of miR-223 in bone marrow (BM)-derived cells in low density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice fed an atherogenic diet results in an increase in plaque size as well as lipid content, plasma inflammatory cytokines, and circulating proatherogenic cells compared with wild-type (WT) controls. Furthermore, the expression of pro-inflammatory markers is enhanced whereas the expression of anti-inflammatory markers is reduced in aortic arches from mice lacking miR-223 in BM-derived cells. Interestingly, we find that miR-223 exerts its anti-atherogenic effects by shifting the overall ribosomal profile of inflammatory and lipid metabolism genes, suggesting that miR-223 acts to globally alter macrophage translational activity in the atherogenic environment. Thus, our study demonstrates that miR-223 can protect against atherosclerosis by acting as a central modulator of macrophage cholesterol efflux and inflammation.

METHODS

Materials and methods can be found in the Supplemental Material.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

miR-223 Positively Regulates ABCA1 Expression and Promotes Macrophage Cholesterol Efflux In Vitro

ABCA1 (ATP-binding cassette transporters A1) plays a key role in stimulating the efflux of cholesterol from macrophages, thereby reducing foam cell formation and the development of atherosclerosis.[23–25] miR-223 has been found to indirectly induce ABCA1 expression and cholesterol efflux in hepatocytes via directly targeting the transcription factor SP3 (Specificity protein 3), which acts as a repressor of ABCA1 expression.[18,26,27] To confirm whether miR-223 similarly regulates cholesterol efflux in macrophages, we first examined the expression of ABCA1 and *Sp3* in bone marrow-derived macrophages (BMDMs) transfected with miR-223 or isolated from *miR223*$^{-/-}$ mice. As expected, loss of miR-223 elevated, whereas over-expression of miR-223 suppressed, *Sp3* expression (Figure 1A and 1B). In contrast, the expression of ABCA1 decreased or increased when miR-223 was lost or over-expressed, respectively (Figure 1C). We next investigated the effects of this miR-223 on macrophage cholesterol efflux and indicated that macrophages transfected with miR-223 exhibited enhanced cholesterol efflux to apoA1 (apolipoprotein A1) relative to control (25.3±3.2% for control mimic versus 34.6±2.0% for miR-223; $P \leq 0.05$; Figure 1D).

Atherosclerotic lesions have a highly heterogeneous phenotype as indicated by the simultaneous co-existence of various macrophage subpopulations.[28] To determine whether or not macrophage polarization to a pro-inflammatory M1 or alternative M2 state impacts the effect of miR-223 on cholesterol efflux, we performed cholesterol efflux assays on Mo, M1, or M2 macrophages transfected with miR-223 or isolated from *miR223*$^{-/-}$ mice. We confirmed that miR-223 over-expression increased apoA1-mediated cholesterol efflux from different macrophage subtypes including resting (Mo) macrophages, pro-inflammatory (treated with LPS+IFN-γ, M1) macrophages and anti-inflammatory (treated with IL-4, M2) macrophages. Conversely, Mo macrophages and M2 macrophages, but not M1 macrophages, from *miR223*$^{-/-}$ mice showed decreased cholesterol efflux to apoA1 (Figure 1E). Supporting the reduced cholesterol efflux capacity of different macrophage subtypes isolated from *miR223*$^{-/-}$ mice was a significant reduction in *Abca1* expression at the mRNA level (Figure S1A). To further confirm whether miR-223 regulates cholesterol efflux gene expression, we also examined the expression of *Sp3* and *Abca1* in BMDMs transfected with control anti-miR or anti-miR223. Indeed, anti-miR223 treatment increased Sp3

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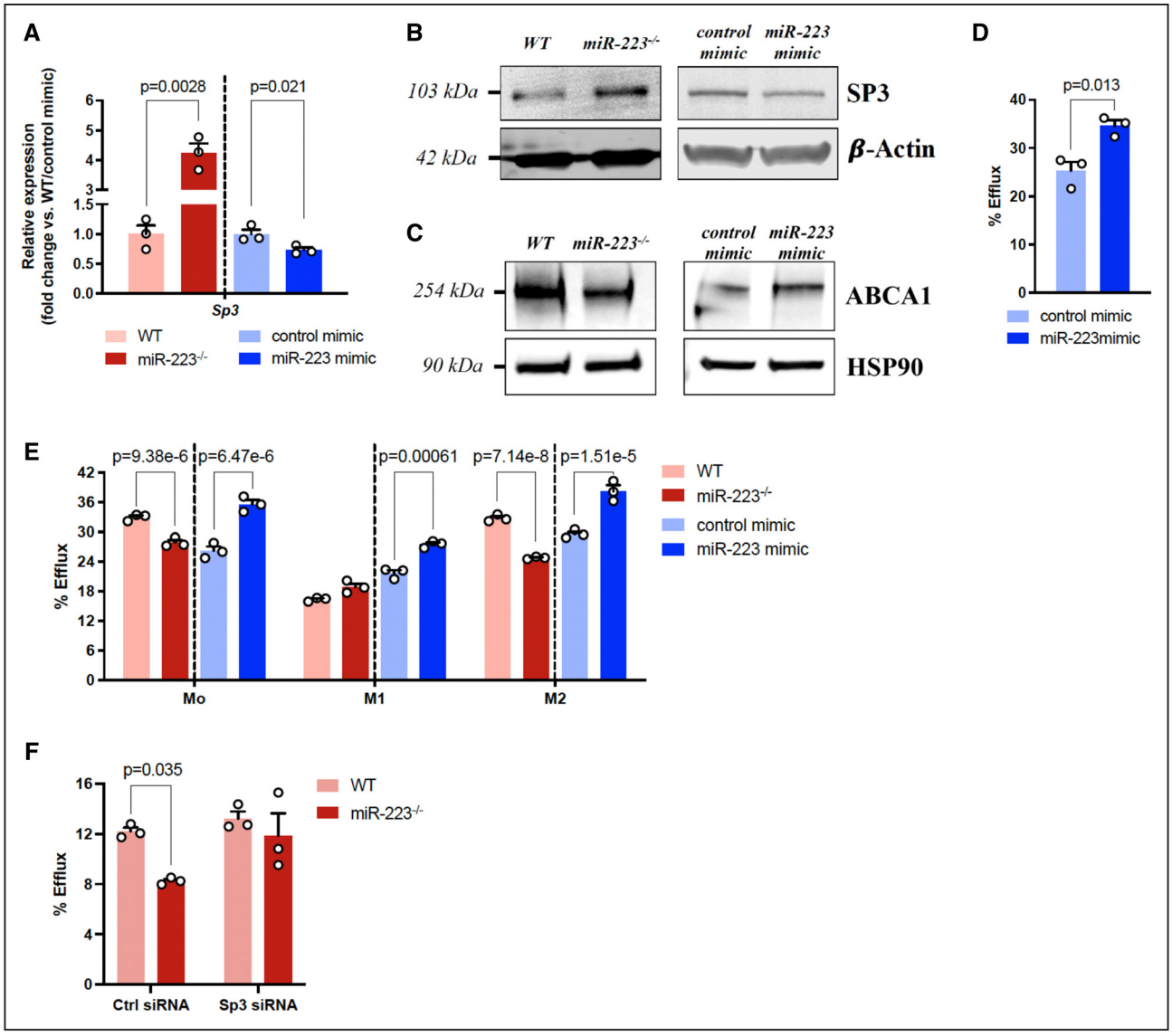

Figure 1. miR-223 positively regulates ABCA1 (ATP-binding cassette transporter A1) expression and promotes macrophage cholesterol efflux.

A–**C**, SP3 mRNA (**A**) and protein (**B**) expression, as well as ABCA1 protein expression (**C**), in BMDMs transfected with control mimic/miR-223 mimic (100 nM) or isolated from WT/miR-223$^{-/-}$ mice. Data are the means of n=3 independent experiments±SEM. Western blot is representative of a single experiment done in triplicate (n=3). **D**, Cholesterol efflux to apoA1 (25 μg/mL) was measured for 24 h. Graphs represent the means±SEM from n=3 independent experiments. **E**, BMDMs were cholesterol-loaded and polarized into M1 macrophages or M2 macrophages by induction with LPS (100 ng/ml)/IFN-γ (100 ng/mL) or IL-4 (10 ng/mL), respectively, for 24 h. Cholesterol efflux to apoA1 (25 μg/mL) was measured for 24 h. Graphs represent the experiments performed in triplicate (n=3). **F**, BMDMs isolated from WT/miR-223$^{-/-}$ mice were transfected with control siRNA or *Sp3* siRNA (25 nM). Following siRNA transfection, BMDMs were cholesterol-loaded for 24 h, and cholesterol efflux to apoA1 (25 μg/mL) was measured for 24 h. Graphs represent the experiments performed in triplicate (n=3). For comparisons between 2 groups (**A**–**D**), a *t* test was used. For comparisons between 2 groups in different conditions (**E** and **F**), a 2-way ANOVA with a Sidak post hoc test for multiple comparisons was used (**E** was corrected for 3 tests and **F** was corrected for 2 tests). Ctrl siRNA indicates control short interfering RNA; HSP90, heat shock protein 90 kDa; SP3, specificity protein 3; and WT, wild-type.

expression while decreased Abca1 expression (Figure S1B). Interestingly, knockdown of *Sp3* using short interfering RNA relieved the inhibitory effect of miR-223 deficiency on cholesterol efflux (Figure 1F), indicating that miR-223 regulation of macrophage cholesterol efflux is dependent on *Sp3*. Taken together, these data confirm that miR-223 can indirectly enhance macrophage ABCA1 expression and cholesterol efflux through *Sp3*.

miR-223 Suppresses Macrophage Pro-inflammatory Activation and Promotes Macrophage Polarization Toward the Anti-inflammatory Phenotype In Vitro

Given the important role of miR-223 in regulating the NFκB signaling pathway,[19,20] we sought to determine if and how miR-223 instructs macrophage inflammatory

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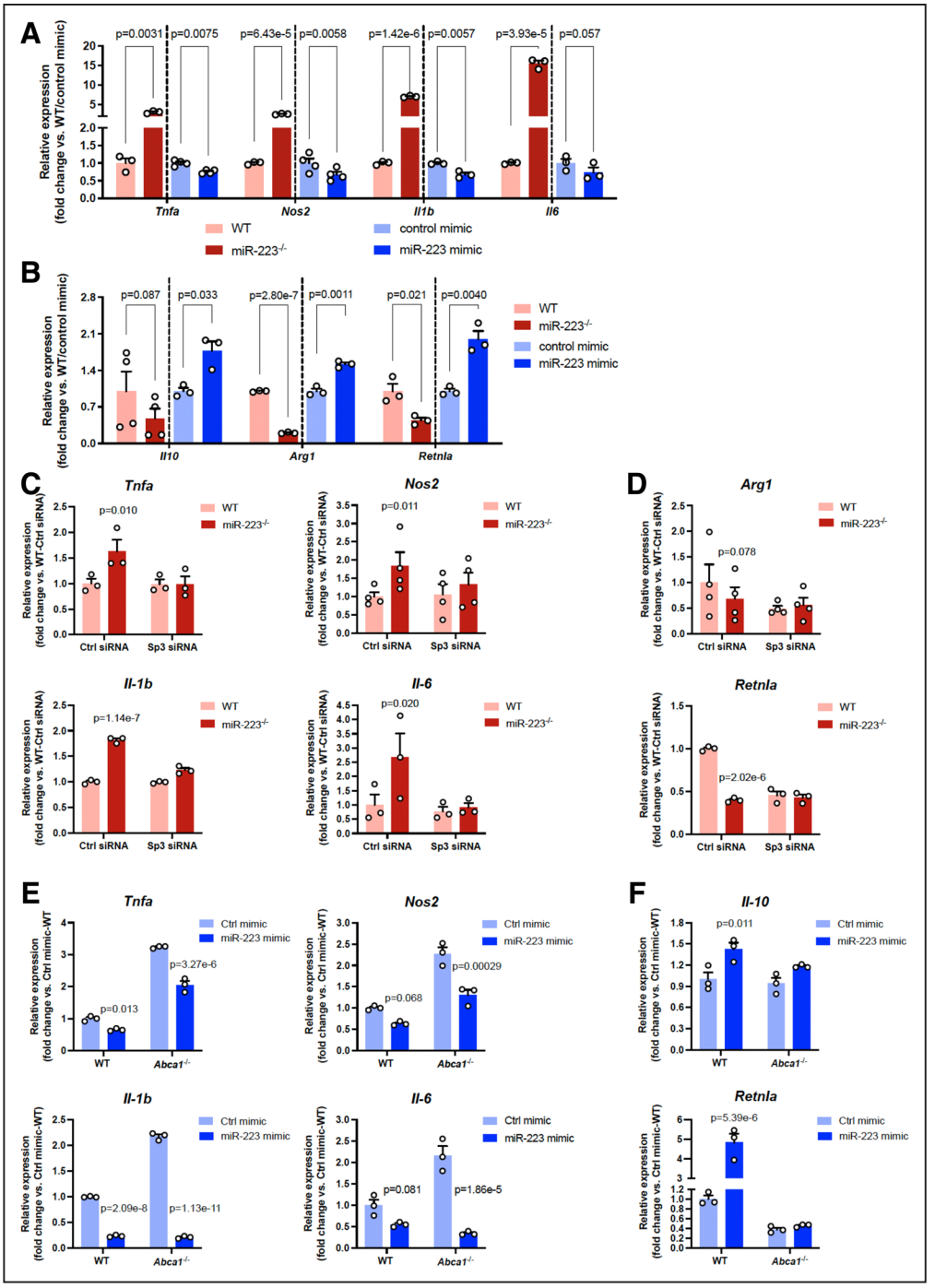

Figure 2. miR-223 suppresses macrophage pro-inflammatory activation and promotes macrophage polarization toward the anti-inflammatory phenotype.

A and **B**, BMDMs transfected with control mimic/miR-223 mimic (100 nM) or isolated from WT/miR-223$^{-/-}$ mice were polarized into either M1 macrophages by induction with LPS (100 ng/mL)/IFN-γ (100 ng/mL) or M2 macrophages by induction with IL-4 (10 ng/mL) for 24 h. Expression levels of (**A**) M1 markers (*Tnfa*, *Nos2*, *Il-1b*, *Il-6*) and (**B**) M2 markers (*Il-10*, *Arg1*, *Retnla*) were measured by qPCR. Graphs represent the means±SEM from at least n=3 technical or biological replicates. **C** and **D**, Inhibition of SP3 partially relieves the effects of miR-223 on macrophage polarization. BMDMs isolated from WT/miR-223$^{-/-}$ mice were transfected with control siRNA or *Sp3* siRNA (25 nM). Expression levels of (**C**) M1 markers (*Tnfa*, *Nos2*, *Il-1b*, *Il-6*) and (**D**) M2 markers (*Arg1*, *Retnla*) were measured by qPCR. Data are the means of n=3 (*Tnfa*, *Il-1b*, *Il-6*, *Retnla*) or n=4 (*Nos2*, *Arg1*) technical/biological replicates±SEM. **E** and **F**, miR-223 alters M2 activation but not M1 activation dependently of ABCA1 (ATP-binding cassette subfamily A member 1). BMDMs isolated from WT/*Abca1*$^{-/-}$ mice were transfected with control mimic or miR-223 mimic (100 nM). Expression levels of (**E**) M1 markers (*Tnfa*, *Nos2*, *Il-1b*, *Il-6*) and (**F**) M2 markers (*Il-10*, *Retnla*) were measured by qPCR. Graphs represent the experiments performed in triplicate (n=3). For comparisons between 2 groups (**A** and **B**), a *t* test was used. For comparisons between 2 groups in different conditions (**C**–**F**), a 2-way ANOVA with a Sidak post hoc test for multiple comparisons (corrected for 2 tests) was used. Arg1 indicates arginase 1; Ctrl siRNA, control short interfering RNA; Il, interleukin; Nos2, nitric oxide synthase 2; Retnla, resistin-like alpha; Sp3, specificity protein 3; Tnfa, tumor necrosis factor alpha; and WT, wild-type.

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polarization. We treated BMDMs isolated from *miR223*$^{-/-}$ mice with either LPS + IFN-γ (to induce M1) or IL-4 (to induce M2). qRT-PCR analysis showed that pro-inflammatory cytokines *Tnfa*, *Il-1b*, *Il-6*, and *Nos2* were significantly increased in *miR223*$^{-/-}$ macrophages compared with WT cells. In contrast, overexpression of miR-223 in BMDMs repressed the expression of these M1 macrophage markers (Figure 2A). Expression of M2-associated genes *Il-10*, *Arg1*, and *Retnla*, on the other hand, was decreased in macrophages isolated from *miR223*$^{-/-}$ mice and elevated in macrophages transfected with miR-223 mimic, as compared with control cells (Figure 2B). Similarly, we observed a de-repression of pro-inflammatory genes *Tnfa*, *Nos2*, *Il-1b*, as well as *Il-6* (Figure S1C), and a suppression of anti-inflammatory genes *Il-10*, *Arg1*, and *Retnla* in macrophages transfected with anti-miR223 (Figure S1D). Thus, miR-223 can suppress macrophage pro-inflammatory activation while promoting macrophage polarization toward the anti-inflammatory M2 phenotype in vitro, in consistent with a previous study.[29]

SP3 has been shown to drive the production of inflammatory cytokines in cancer cells via stimulating NFκB-mediated transcription activation.[30] We, therefore, postulated that SP3 might mediate the effects exerted by miR-223 on macrophage polarization. To address this, we first measured the expression of M1 and M2 markers in macrophages where *Sp3* was knocked down by short interfering RNA. Inhibition of *Sp3* resulted in a phenotype similar to that of miR-223 overexpression, with decreased expression levels of M1-associated genes such as *Nos2* and *Tnfa* (Figure S1E) and increased expression levels of M2-associated genes such as *Arg1* and *Retnla* (Figure S1F), suggesting that *Sp3* may mediate the effects of miR-223 on macrophage activation. To further confirm the importance of Sp3 in modulating miR-223-controlled macrophage polarization, we performed loss-of-function analyses in *miR223*$^{-/-}$ macrophages by knocking down *Sp3*. In the absence of *Sp3*, miR-223 deficiency did not alter the expression of M1 markers (*Tnfa*, *Il-1b*, *Il-6*, and *Nos2*, Figure 2C) or M2 markers (*Arg1* and *Retnla*, Figure 2D). These data confirm that the effect of miR-223 on macrophage polarization is partially attributable to *Sp3*. Because miR-223 can regulate ABCA1 expression, and ABCA1 has been shown to control macrophage inflammatory response by regulating lipid raft, membrane cholesterol[31–35] and inflammasome activation,[36] we next tested whether miR-223 can also modulate macrophage inflammation via *Abca1*. Interestingly, the effects of miR-223 overexpression on M2 polarization were blunted in *Abca1*$^{-/-}$ macrophages (Figure 2E); yet, the inhibitory effects on M1 responses were still maintained in the absence of *Abca1* (Figure 2F). Taken together, these data demonstrate that miR-223 modulates the inflammatory response in both M1 and M2 macrophages primarily *via* regulation of *Sp3* expression.

Deletion of miR-223 in BM-Derived Cells Promotes Atherogenesis and Enhances Inflammatory Signaling

Considering that miR-223 plays a key role in promoting macrophage cholesterol efflux and reducing inflammation in vitro, we examined its expression in both human and mouse atherosclerosis. In lesions from apolipoprotein E knockout (*ApoE*$^{-/-}$) mice fed a high-cholesterol diet, there was a significant upregulation in the expression of miR-223 compared with mice fed a chow diet (Figure 3A). To extend these findings to humans, we also analyzed miR-223 expression in carotid atherosclerotic plaques. miRNA expression analysis of unstable plaques (fibrous cap <200 μm) from patients with symptomatic carotid disease (defined as transitory ischemic attack, minor stroke, or retinal stroke) showed a significant increase of miR-223 expression compared with stable plaques (fibrous cap >200 μm) from asymptomatic patients (Figure 3B), in agreement with previous studies.[37,38]

To elucidate the role of miR-223 in immune cells in the development of atherosclerosis, we lethally irradiated *Ldlr*$^{-/-}$ mice and reconstituted them with BM from WT or *miR223*$^{-/-}$ mice. BM-transplanted mice were then fed a Western-type high cholesterol diet (HCD) for 12 weeks (Figure 3C). Reconstitution of the hematopoietic compartment after transplantation of WT or *miR223*$^{-/-}$ BM cells appeared normal as circulating levels of leukocytes and lymphocytes were similar between the 2 groups at 6 weeks after BM transplantation, before start of the HCD (Figure S2A). Aortic root lesions from male mice lacking miR-223 in BM-derived cells showed a 51.3% increase in plaque size relative to control mice (n=6/group, P=0.0729), whereas plaque burden in the aortic root of female mice receiving *miR223*$^{-/-}$ BM was statistically unchanged (Figure 3D). Quantification of lipid accumulation in aortic sinus lesions by oil red O staining demonstrated a corresponding 20% increase in lipid plaque in mice receiving *miR223*$^{-/-}$ BM compared with that of controls (Figure 3E, P≤0.05). To further evaluate lesion characteristics, we also quantified necrotic core, macrophage, as well as smooth muscle cell area and detected no statistical differences in these contents (Figure S2B). In addition, there were no statistically significant differences in total plasma cholesterol or body weight before HCD administration, at early stages (ie, 6-week HCD), or after 12 weeks of HCD (Figure S2C and S2D).

We next assessed systemic inflammation using multiplex immunoassays. We detected higher levels

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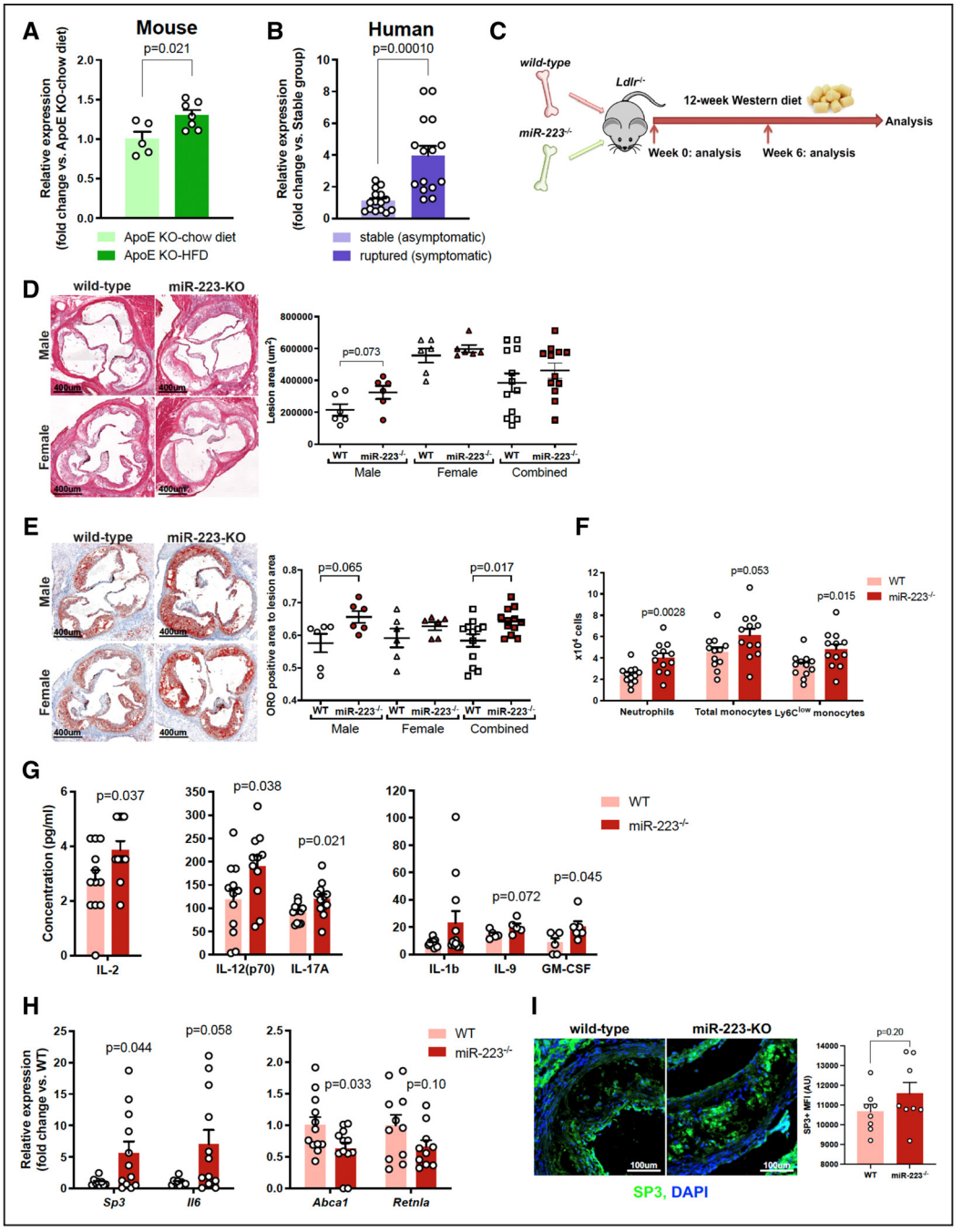

Figure 3. Deletion of miR-223 in bone marrow (BM) cells promotes atherogenesis and enhances inflammatory signaling.
A, Aortic expression of miR-223 in *ApoE*$^{-/-}$ mice fed a chow (n=5) or western diet (21% fat, 0.2% cholesterol) for 12 wk (n=7). The graph represents the means±SEM. **B**, Human miR-223 expression in plaque samples from asymptomatic (stable) (n=15) or symptomatic (ruptured) (n=15) patients. Data are the means±SEM. **C**, *Ldlr*$^{-/-}$ mice (n=6 male and 6 female/group) were lethally irradiated and given BM transplantation from WT or miR-223$^{-/-}$ donors, followed by high-cholesterol diet (HCD) for 12 wk. **D** and **E**, miR-223 deficiency in BM cells increases atherosclerotic plaque (**D**) size and (**E**) lipid content. Aortic sinus lesion areas across the entire aortic root from H&E-stained sections were quantified using ImageJ whereas lipid accumulation in aortic sinus lesions was measured by oil red O staining. Images show representative sections from *Ldlr*$^{-/-}$ mice with WT BM (left) and miR-223$^{-/-}$ BM (right). Data are the means±SEM. **F**, Circulating levels of leukocytes after 12 wk of HCD were analyzed by FACS. Data are the mean levels of 12 mice per group ± SEM. **G**, Circulating cytokine levels in mice receiving WT BM or miR-223$^{-/-}$ BM (n=12 mice per group for IL-2, IL-12(p70), IL-17A, IL-1b; n=6 mice per group for IL-9, GM-CSF) were determined by the Bio-Plex Pro Mouse Cytokine 23-plex assay at the end of the 12-wk study. Data are the means±SEM. **H**, Gene expression in the aortas of *Ldlr*$^{-/-}$ mice receiving WT BM or miR-223$^{-/-}$ BM. The aorta was homogenized using microbeads along with the Bullet Blender. Total RNA was isolated and the expression levels of *Sp3*, *Il6*, *Abca1*, and *Retnla* were measured by ddRT-PCR. Graphs represent the means±SEM from n=10–12 mice per group. **I**, Immunofluorescence staining for SP3 in lesions from mice receiving WT BM or miR-223$^{-/-}$ BM. Data are the mean levels of 8 mice per group±SEM. A *t* test was used to determine statistical significance for comparisons between 2 groups (**A**–**I**), except **E**—data of male mice, **G**—IL-1b data, and **H**—Il6 data (that did not pass normality testing) where a Mann-Whitney *U* test was used. *Abca1* indicates ATP-binding cassette subfamily A member 1; ApoE, apolipoprotein E; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; *Ldlr*, low-density lipoprotein receptor; MFI, mean fluorescence intensity; ORO, oil red O; *Retnla*, resistin like alpha; *Sp3*, specificity protein 3; and WT, wild-type.

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of circulating pro-inflammatory cytokines IL-1β, IL-2, IL-9, IL-12(p70), IL-17A, and GM-CSF in mice receiving *miR223*$^{-/-}$ BM after 12 weeks of HCD (Figure 3G), while other cytokines such as IL-1α, IL-4, Eotaxin, G-CSF, and MIP-1α remained statistically unchanged (data not shown). Mice lacking miR-223 in BM-derived cells also had increased levels of circulating neutrophils, total monocytes, and Ly6C^{low} monocytes after 12 weeks of HCD (Figure 3F) whereas there were no statistical differences in circulating levels of other immune cell subsets, including Ly6C^{hi} (Figure S2E). Assessing circulating levels of leukocytes after 6-week HCD showed similar results (Figure S2F). These data indicate that the absence of miR-223 from the hematopoietic compartment not only resulted in an increase in lesion lipid accumulation in the aortic sinus but was also accompanied by signs of systemic inflammation.

To further evaluate the inflammatory profile of mice lacking hematopoietic miR-223, we analyzed gene expression in the aortas of mice receiving WT or *miR223*$^{-/-}$ BM after 12 weeks of HCD. Consistent with the in vitro data, qRT-PCR analysis demonstrated that the expression *Sp3* and *Il-6* were up-regulated whereas the expression of *Abca1* and *Retnla* were down-regulated in the aortas of mice lacking miR-223 in BM-derived cells (Figure 3H). At the protein level, Sp3 expression showed a nonstatistically significant increase in plaques from *miR223*$^{-/-}$ BM recipients compared with controls (Figure 3I). On the other hand, other target genes of miR-223 including *Pknox1*,[29] *Rasa1,* and *Nfat5*[39] did not show statistically significant changes or showed a reduction in mice receiving *miR223*$^{-/-}$ BM (Figure S2G). Deficiency of miR-223 in the aortas of mice receiving *miR223*$^{-/-}$ BM was also confirmed (Figure S2H).

To further characterize the pathways impacted by miR-223, we compared gene expression of macrophages from WT and *miR223*$^{-/-}$ mice using an atherosclerosis pathway-focused PCR array. We compared the expression of genes involved in the stress response, apoptosis, cell adhesion molecules, lipid transportation and metabolism, as well as cell growth and proliferation in Mo, M1, and M2 macrophages from WT and *miR223*$^{-/-}$ mice. Lack of miR-223 significantly increased the expression of *Cd44*, *Fga*, *Mmp1a*, *Sod1,* and *Tnfaip3* and decreased the expression of *Bid*, *Csf2*, *Cxcl1*, *Eln*, *Hbegf*, *Icam1*, *Lif*, *Lpl*, *Plin2*, *Serpinb2*, *Tgfb2,* and *Tnc* in all macrophage subtypes (Figure S3; Table 1). Interestingly, we searched for the presence of a miR-223-binding site in the 3'UTR of this group of altered genes using miRSystem,[40] but none were identified. This suggests that miR-223 controls the expression of multiple pro-inflammatory genes indirectly and not *via* direct repression of the 3'UTR, possibly via its regulation of a common regulatory factor such as *Sp3*.

Table 1. Most Significantly Dysregulated Genes (Up- or Downregulated) in miR-223$^{-/-}$ Macrophages

Downregulated (*miR-223*$^{-/-}$ vs WT)		Upregulated (*miR-223*$^{-/-}$ vs WT)
Bid	Lif	Cd44
Csf2	Lpl	Fga
Cxcl1	Plin2	Mmp1a
Eln	Serpinb2	Sod1
Hbegf	Tgfb2	Tnfaip3
Icam1	Tnc	

Atherosclerosis RT^2 Profiler PCR array was performed using RNA isolated from BMDMs of WT mice or miR-223$^{-/-}$ mice and normalized to housekeeping genes using the ΔΔCt method. WT indicates wild-type.

Global Analysis of Transcript Levels and Translation Regulation by miR-223 in Macrophages

To further understand the molecular mechanisms underlying how miR-223 controls the development of atherosclerosis in vivo, we employed ribosome profiling to characterize the global translation profile of macrophages isolated from mice receiving WT or *miR223*$^{-/-}$ BM. Because we found that deletion of miR-223 in BM-derived cells aggravated inflammation and atherosclerosis progression, we compared pro-inflammatory M1 macrophages to resting macrophages (Mo) to better understand the inflammatory pathways that may be activated uniquely in progressing plaques.[41] In this experiment, BMDMs isolated and differentiated from atherogenic mice were lysed to collect cytosolic total RNA, and a fraction of which was used for RNA-seq and the other fraction was used to isolate ribosome-protected fragments (RPF).[42–44] Analysis of the cytosolic lysates from BMDMs (optimization shown in Figure S4A) demonstrates a strong correlation between biological replicates for both RNA-Seq (RNA) and Ribo-Seq (RPF) gene expression (Figure S4B). Metagene analysis indicates that RPF densities were significantly higher on the coding regions compared with the 5'-UTR or 3'-UTR, corresponding to the expected position of translating ribosomes (Figure S4C). Interestingly, we find a poor correlation between gene expression at the transcription level (RNA-Seq) and the translation level (Ribo-Seq; Figure S4D). Specifically, we observe a stronger correlation between RNA and RPF read densities in WT Mo macrophages (Pearson correlation r=0.67) compared with WT M1 (Pearson correlation r=0.46; Figure 4A) suggesting that mRNA expression alone does not faithfully reflect protein expression and that translation is more active in M1 macrophages. This disturbed translation activity is consistent with previous studies that described the modulation effect of IFN-γ or LPS, used during M1 activation, on translation.[45,46] Intriguingly, RNA and RPF densities showed less correlation in the absence of miR-223, as compared with WT, in both Mo and M1 macrophages

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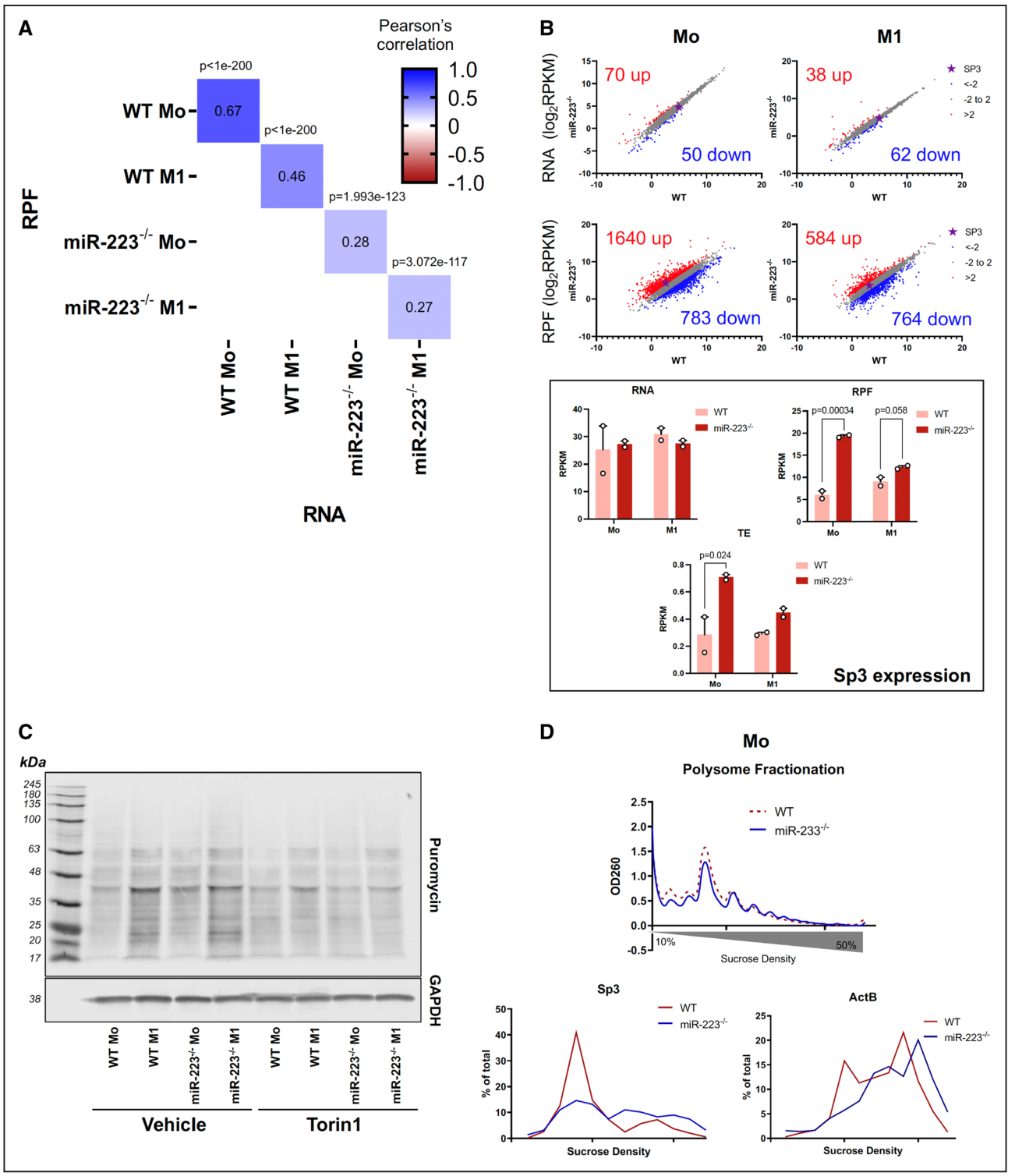

Figure 4. Global analysis of transcription and translation regulation in miR-223$^{-/-}$ BMDMs.
A, Correlation matrix of ribosome-protected fragment (RPF; *y*-axis) and mRNA (*x*-axis) expression. Numbers show Pearson's correlation for each pair of RPF-RNA. *P* for each comparison is shown on top of each cell. Average expression level of each gene was used. **B**, Upper panels: scatter plots showing the expression levels of individual genes in miR-223$^{-/-}$ (*y*-axis) vs WT (*x*-axis) BMDMs at the mRNA or RPF level as indicated. Lower panels: graphs of *Sp3* expression measured at the RNA or RPF level, or *Sp3* translation efficiency (RPF/RNA). A 2-way ANOVA with a Sidak post hoc test for multiple comparisons was used (corrected for 2 tests). **C**, Puromycin metabolic labeling of newly synthesized proteins of WT or miR-223$^{-/-}$ BMDMs. Torin1 (300 nmol/L) was used to inhibit mTORC1-dependent protein synthesis as a control. **D**, Polysome fractionation analysis of translation activity in WT vs miR-223$^{-/-}$ Mo BMDMs. Left panel: polysome traces of cytoplasmic RNA extract on a linear 10% to 50% sucrose gradient. Middle and right graphs: distribution of *Sp3* or *ActB* mRNA across sucrose gradient, quantified by RT-qPCR. ActB indicates beta-actin; RPF, ribosome protected fragment; RPKM, read per kilobase million; Sp3, specificity protein 3; TE, translation efficiency; and WT, wild-type.

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(r=0.28 and 0.27 for Mo and M1, respectively), suggesting that global translation control is more active in the absence of miR-223 (Figure 4A). In agreement with the correlation analysis, we found that miR-223 deficiency resulted in modest transcript-level disturbance in Mo (70 genes upregulated, 50 genes downregulated) and M1 (38 genes upregulated, 62 genes downregulated) macrophages, but induced more widespread translation dysregulation (1640 genes up- and 783 genes downregulated in Mo macrophages, 584 genes up- and 764 genes downregulated in M1 macrophages; Figure 4B, Figure S4E). Quantification of global protein synthesis rate by metabolic labeling of newly synthesized protein with puromycin demonstrates an increase in global protein synthesis in M1 compared with Mo BMDMs, but that protein synthesis is more active in *miR223*$^{-/-}$ compared with WT BMDMs (Figure 4C). Together, these data show that miR-223 alters gene expression in Mo and M1 macrophages primarily via control of translation rather than control of transcript levels of genes.

Because our Ribo-Seq and RNA-Seq data indicated that changes in WT and *miR223*$^{-/-}$ macrophages were mainly at the translation level, we examined *Sp3* as a specific miR-223 target and investigated its relative translation efficiency in macrophages. Deficiency of miR-223 enhanced the expression of *Sp3* predominantly at the translation level compared with the transcription level (RNA, RPF and translation efficiency, right panel of Figure 4C). To confirm the translational regulation of *Sp3* by miR-223, we performed polysome fractionation to resolve polysome-bound mRNAs, where translating ribosomes bound to transcripts increase their overall polysomal density on a sucrose gradient. Consistent with a disturbance in global translation and enhanced global protein synthesis, the absorbance trace at 260 nm (A260) indicates an increased accumulation of 80S ribosomes in WT compared with *miR223*$^{-/-}$ macrophages (Figure 4D, left). We also find *Sp3* mRNA distributed predominantly to the monosome fraction in WT macrophages, while shifted more toward polysome fractions in *miR223*$^{-/-}$ macrophages, indicating augmented translation in the absence of miR-223 (Figure 4D, middle). In comparison, the translationally active *ActB* mRNA was primarily distributed to the polysome fractions, although slightly shifted to the higher polysome fractions in *miR223*$^{-/-}$ macrophages (Figure 4D, right panel), perhaps as a consequence of the changes in global translation observed.

Next, we sought to understand the putative consequences of this global change in translation upon miR-223 ablation using ingenuity pathway analysis on differentially expressed genes (DEGs) captured at the transcriptome and translatome level. Ingenuity pathway analysis revealed that the most significantly altered processes/functions (ranked by Benjamini-Hochberg adjusted *P*) are related to hematopoietic development and function, including Development of hematopoietic system, Development of bone marrow for DEGs at the RNA level and Quantity of blood cells, Quantity of leukocyte for DEGs at the RPF level (Figure S5A), consistent with the hematopoietic-specific role of miR-223 described previously.[47,48] We focused on processes and functions in Disease and Disorder to detect potential pathological effects of miR-223 deficiency in macrophages and found that the top enriched annotation associated with processes/functions related to inflammatory response or inflammation-related processes (Figure 5A). When comparing the degree and directionality (activation or inhibition) of changes in inflammatory response networks (as measured by B-H adjusted *P* and activation z-score by ingenuity pathway analysis), activation of the inflammatory response in *miR223*$^{-/-}$ macrophages was more pronounced at the RPF (translation) level than at the RNA (transcription) level, especially in Mo macrophages (Figure 5B). Individual genes associated with inflammatory response also showed more profound changes in expression at the RPF level than at the RNA level (Figure 5C). In addition, we noted genes categorized as Lipid metabolism were also altered at the translation level by miR-223 deficiency in Mo macrophages (Figure S5B). When we examined the list of upregulated genes from the Ribo-Seq dataset for, we found a number of genes in both M0 and M1 macrophages that contained predicted miR-223 target sites in the UTR (Table 2). Further analysis of these upregulated genes reveals an enrichment of AU-rich elements (AREs), a cis-element commonly found in the 3'UTR of cytokine and inflammatory transcripts,[49] in *miR223*$^{-/-}$ macrophages compared with WT (Figure 5D). Thus, data from our transcriptional and translational profiling confirmed that miR-223 knockout aggravates inflammatory signaling in macrophages and identifies miR-223 as a global regulator of translational control of inflammatory genes.

DISCUSSION

Since their discovery in *C. elegans*, miRNAs have shown to play important roles in modulating atherogenesis, with many miRNAs dysregulated in different disease states and alterations in miRNA expression associated with atherosclerosis progression.[16,17] Previous studies demonstrated that miR-223 is key to the regulation of the inflammatory response.[19–22] In addition, miR-223 serves as a critical regulator of hepatic cholesterol metabolism.[18] Nonetheless, the contribution of miR-223, particularly macrophage miR-223, to the development of atherosclerosis has not been investigated. In this study, we demonstrate that in vitro, miR-223 indeed promotes macrophage cholesterol efflux and macrophage polarization toward the anti-inflammatory M2 phenotype; in contrast, loss of miR-223 attenuates cholesterol efflux and enhances the pro-inflammatory responses.

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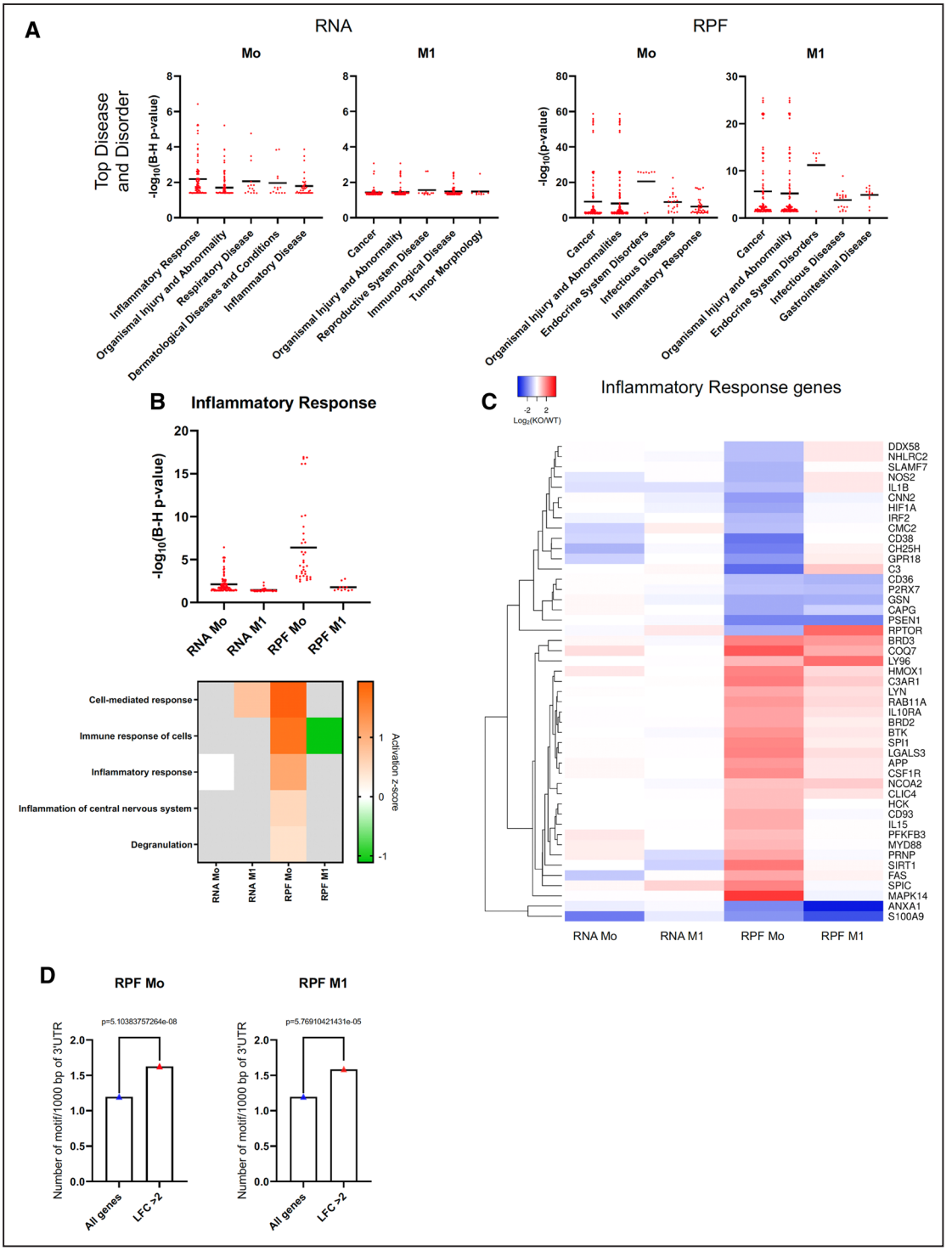

Figure 5. Ingenuity pathway analysis reveals activation of inflammatory signaling at the transcription and translation level in BMDMs. **A**, Scatter plot of Benjamini-Hochberg (B–H) adjusted *P* of significantly dysregulated processes/functions in Disease and Disorder analysis of the transcriptome and translatome of miR-223$^{-/-}$ BMDMs compared with WT BMDMs. A B-H adjusted *P* cutoff of 0.05 (–$\log_{10}$[B-H adjusted *P*] >1.3) was applied and all accepted processes/functions were shown as individual dots. **B**, Top plot: scatter plot of B-H adjusted *P* of significantly dysregulated processes/functions in the Inflammatory Response sub-category of miR-223$^{-/-}$ BMDMs compared with that of WT BMDMs. A B-H adjusted *P* cut-off of 0.05 (–$\log_{10}$[B-H adjusted *P*] >1.3) was applied, and all accepted processes/functions were shown as individual dots. Bottom plot: activation z-score of top 5 differentially regulated pathways in the Inflammatory Response sub-category of miR-223$^{-/-}$ BMDMs compared with that of WT BMDMs. The cell is gray when an activation z-score cannot be determined by IPA. **C**, Heatmap of average miR-233$^{-/-}$/WT fold change of representative inflammatory response genes at the RNA and ribosome protected fragment (RPF) level. All genes associated with all processes/functions in **B** were combined, then their clustering was calculated based on their fold change (miR-233$^{-/-}$/WT) values using the Average Linkage method. **D**, Occurrence of AUUUA motif in the 3'UTR of all genes vs upregulated genes in miR-223$^{-/-}$ BMDMs. Normalized count values were compared using Mann-Whitney *U* test. RPF indicates ribosome protected fragment.

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Table 2. Upregulated genes with predicted miR-223 target sites in M0 and M1 macrophage translatome (RPF)

M0		M1
Plagl2	Eif5b	Edc3
Apool	Gstz1	Treml2
Sp3	Zbtb18	Apool
Vmp1	Usp6nl	Spata13
Trim3	Pknox1	Vmp1
Cdk17	Lmo2	Prpf38a
Gmpr	Adcy7	Ypel1
Rap2a	Jmy	Csnk1g1
Derl1	Ankrd40	Cnep1r1
Ptbp2	Tmem170	Pkn2
Fubp3	Sft2d2	Pknox1
Rab8b	Mcmbp	Lmo2
Pik3c2a	Slc39a8	Msi2
Smarcd1	Ube2q2	Fubp3
Srp19	Ctsl	Pou2f1
Vamp2	Fam199x	Trmt5
Mbnl1	Dcn	Tnfsf15
Dera	Abhd13	
Naa50	Arhgap5	
Phactr4	Kpna3	
Snx12	Zfand5	
Arpp19	Cnep1r1	
Mafb	Orc4	
Pag1	Prdx2	
Pou2f1	Mpv17l2	
Rab22a	Fbxo8	
Cops2	Hhex	

Data represents the comparison between WT and *miR223*$^{-/-}$ BMDM RPF. Upregulated genes were identified using the fold-change threshold of 2 (log2(FC)>1).Target sites for miR-223 were identified using TargetScan (www.targetscan.org). RPF indicates ribosome-protected fragment; and WT, wild-type.

Remarkably, miR-223 appears to control inflammatory responses and lipid homeostasis primarily via regulation of translation, rather than regulation of transcript levels, as revealed by major changes in the ribosomal profile of miR-223-deficient cells. These beneficial effects of miR-223 seem to be partially dependent on the inhibition of its target gene, the transcription factor *Sp3*. In vivo, deficiency of miR-223 in BM-derived cells can promote vascular lipid accumulation and exaggerating the inflammatory response to a HCD. Our study places miR-223 as a translational hub of cholesterol and inflammatory responses in atherosclerosis.

The efficacy of macrophages to efflux and remove intracellular cholesterol is crucial to attenuate lipid accumulation and atherosclerosis formation,[24] and as such, targeting ABCA1 and macrophage cholesterol efflux has been a major focus for the prevention and treatment of atherosclerosis. Multiple miRNAs such as miR-33,[50,51] miR-144,[52,53] miR-148a,[54,55] and miR-302a[56] have been found to regulate cholesterol efflux. However, most of them suppress *Abca1* expression and inhibit macrophage cholesterol efflux, thereby accelerating atherosclerosis progression. miR-223 is one of a few miRNAs whose activation, rather than inhibition, can increase *Abca1* expression, promote cholesterol efflux and protect against atherosclerosis. Consistent with studies in hepatocytes,[18] we show that miR-223 can enhance the ability of macrophages to efflux intracellular cholesterol *via* up-regulating *Abca1*. More importantly, miR-223 can promote *Abca1* and cholesterol efflux independently of macrophage polarization status. In vivo, mice lacking miR-223 in BM-derived cells exhibited increased lipid accumulation in the aortic sinus and decreased *Abca1* gene expression in the aorta arch, further confirming the role of miR-223 in regulating macrophage cholesterol efflux. Interestingly, recent work from our laboratory indicates that miR-223 delivered via nanoparticles can promote cholesterol removal from macrophage-derived foam cells to the liver or intestine for excretion,[57] again highlighting the therapeutic potential of this miRNA in reducing vascular lipid accumulation and inflammation during atherosclerosis.

Our study also shows that deficiency of miR-223 in BM-derived cells alter lipid accumulation in plaques of male mice, but less so in female mice. Our data align with various studies that observed the male-specific effect of modulating inflammation responses on atherosclerosis development.[58–64] Interestingly, macrophage-specific deletion of *Abca1* have also been shown to exert the sex-specific effect on atherogenesis, although in the opposite direction, where *Abca1* deficiency resulted in an increase in atherosclerosis mainly in female mice.[65–67] The reasons for the difference between male and female mice lacking miR-223 in BM cells are not clear, although the gene encoding miR-223 is located within the q12 locus of the X chromosome, suggesting a role for sex chromosomes on miR-223 function.[68] In addition, sex hormones are known to affect endothelial and macrophage function during atherogenesis.[69–71] One caveat of our study is the relatively small number of mice of each sex, which may be masking the effect of sex on the phenotypes observed. Further studies are needed to dissect the mechanism for these sex-specific differences in miR-223 in atherosclerosis.

Macrophages are central players in the development of atherosclerosis. Although macrophages have a wide phenotypic range, for simplicity, they can be classified into 2 groups: classically activated M1 macrophages and alternatively activated M2 macrophages.[72,73] Macrophage polarization in vitro and in mouse models of atherosclerosis have indicated that increasing macrophage activation toward the M1 phenotype or inhibiting macrophage activation toward M2 can promote plaque inflammation and potentiate atherosclerosis.[60,62,74–77] In contrast, driving plaque macrophages to M2-like cells

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by the administration of IL-13 can attenuate atherosclerosis progression.[78] Therefore, targeting macrophage polarization would be beneficial for the prevention of atherosclerosis. Our study places miR-223 as a central regulator that instructs macrophage polarization. Loss of miR-223 promotes macrophage activation toward the pro-inflammatory phenotype (M1) and suppress macrophage anti-inflammatory activation (M2) in vitro and in vivo, which is supported by an increase and a decrease in the expression of the M1 marker *Il-6* and the M2 marker *Retnla*, respectively. Our findings are consistent with the observation that ablation of miR-223 expression in mice fed a high-fat diet exacerbated obesity-associated adipose tissue inflammation by enhancing classic proinflammatory responses.[29] While previous work has shown that miR-223 directly targets *Nlrp3* to regulate IL-1β expression, we saw only a modest change in Nlrp3 at the translational (RPF) level, and no statistical changes at the RNA level (data not shown). This may indicate that regulation of inflammation in atherogenic macrophages by miR-223 is dependent less on NLRP3 and more on Sp3. We also found that miR-223 loss led to higher levels of cytokines IL-1β,[58,79] IL-2,[80] IL-9,[81,82] IL-12(p70),[83,84] IL-17A,[85,86] and GM-CSF,[87] which are known to exert pro-inflammatory and pro-atherogenic effects, and enhanced levels of circulating leukocytes (ie, neutrophils, total monocytes). Several studies have shown that increased lipid raft formation and membrane cholesterol accumulation in ABCA1-deficient macrophages can account for the enhanced inflammatory responses in response to LPS or other TLR ligands,[31–34,36] thereby indicating a role of ABCA1 in modulating macrophage inflammatory response. Here, we determine that miR-223 deficiency indeed promotes the pro-inflammatory responses in vitro and in vivo via both of enhancing the expression of its target gene *Sp3* and *via* suppressing the expression of *Abca1*. It was shown that miR-223 can regulate macrophage responses via directly targeting *Pknox1*, *Rasa1*, and *Nfat5*.[29,39] However, we observed no statistical differences in the expression of *Pknox1*, *Rasa1*, and *Nfat5* in aortic plaque from mice lacking miR-223 in BM-derived cells, suggesting that these genes may not mediate the effects of miR-223 on macrophage activation directly the atherosclerosis plaque. This may be partly explained by the expression of different miR-223 target genes in different cell types in the plaque, and/or their cell-specific regulatory mechanisms that may or may not be active during atherosclerosis. On the other hand, the expression of *Sp3*, another direct target of miR-223,[18] was increased in aortic arches from mice receiving *miR223*$^{-/-}$ BM. SP3 was shown to drive the production of inflammatory cytokines including TNFα in cancer cells by promoting NFκB-mediated transcription activation.[30] In addition, knockdown of *Sp3* using RNA interference resulted in a phenotype similar to that of miR-223 overexpression, with decreased expression levels of M1-associated genes and increased expression levels of M2-associated genes, suggesting that the effects of miR-223 on macrophage polarization may be dependent on the inhibition of *Sp3* in our model.

The expression of miR-223 is known to be altered during the course of myeloid cell differentiation and contributes to myeloid differentiation in human leukemia cells.[47,88] We did not observe any statistical differences in circulating immune cells (monocytes, neutrophils, T- and B-cells) at baseline, before the start of atherogenic diet, between mice receiving *miR223*$^{-/-}$ BM compared with WT. These data suggest that deletion of miR-223 from the hematopoietic compartment does not dramatically alter the profile of immune cells in the circulation at rest. However, after 12 weeks of atherogenic diet feeding, we see increased levels of circulating neutrophils, total monocytes, and Ly6C^{lo} monocytes, suggesting that during hypercholesterolemia, miR-223 indeed influences immune cells in the circulation, particularly those of myeloid lineage, and these changes could impact atherosclerosis progression. Although miR-223 deletion only occurred in the hematopoietic compartment, miR-223 expression was barely detected in the aorta of mice receiving *miR223*$^{-/-}$ BM. Therefore, within the atherosclerotic plaque, miR-223 expression is largely dictated by immune cells rather than vascular cells (smooth muscle cell, EC) that are minimally impacted by irradiation and BMT. Together, these data indicate that hematopoietic miR-223 expression dictates immune cells in the circulation and is the major source of miR-223 levels within the plaque.

miRNAs can control gene expression at the translation level via inhibition of translation initiation, followed by induction of transcript decay.[89–91] Our study incorporated genome-wide profiling of translation control using ribosome profiling, which revealed that miR-223 regulates inflammation extensively at the translation level, thus emphasizing the importance of characterizing the effect of miRNAs at both transcription and translation level to fully capture their impact on gene expression. While the precise mechanisms for this global control of inflammatory gene translation is not known, it was suggested that the presence of AREs, a cis-element commonly found in the 3'UTR of cytokine and inflammatory transcripts,[49] synergizes with miR-223 to inhibit translation.[92] It suggests a potential sequence-dependent mode for miR-223 to favor direct translation inhibition of inflammatory transcripts. Indeed, in *miR223*$^{-/-}$ macrophages the translationally up-regulated genes compared to WT contain an overrepresentation of AREs in the UTR, supporting the idea that miR-223 controls sequence-dependent translation of its target genes. miR-223 also inhibits mTORC1,[93] the master regulator of mammalian cap-dependent translation, which has been shown to be crucial for macrophage polarization,[45,94] as well as significantly

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contribute to the regulation of inflammation signaling.[95,96] Among the known targets of miR-223, there was an increase in the translation of *Mafb* and *Slc39a1* in the absence of miR-223, but no statistical change in other targets *RhoB* and *Rnf4*. *MafB* has been shown to regulate macrophage survival, cholesterol efflux and M2 polarization phenotype in the atherosclerotic plaque.[97,98] *RhoB* is a signaling mediator of stress and has been shown to participate in vascular activation, development and response to hypoxia, but the contribution of macrophage *RhoB* in atherosclerosis progression is unknown. Similarly, the contribution of *Slc39a1* (a zinc transporter) and *Rnf4* (an E3-ubiquitin ligase) in macrophages or in the progression of atherosclerosis has not been tested. Therefore, although it is clear that miR-223 exerts strong translational control over many genes, there appears to be some specificity regarding which targets are translationally controlled in the atherosclerotic macrophage environment. Moreover, given that not all genes altered at the translational level by miR-223 contain miR-223 seed sites, miR-223 must be acting via other mechanisms. The current data as well as previous studies strongly suggest that miR-223 acts as a key component controlling the translation of many inflammatory signaling components in atherosclerotic macrophages, although additional studies regarding the target specificity and the mechanisms of translational control are needed.

Interestingly, we found that miR-223 expression is increased in both mice fed a high-cholesterol diet and in humans with unstable carotid atherosclerosis. This induction might be a part of the negative feedback mechanism wherein inflamed and atherosclerotic tissues up-regulate an anti-inflammatory gene expression program in an attempt to suppress inflammation and/or atherosclerotic plaque progression. This is in line with other negative feedback miRNA circuits in inflammatory signaling, such as the case of miR-146. In response to cytokines or LPS stimulation, miR-146 expression is induced in macrophages and is dependent on NF-κB. However, up-regulated miR-146 in turn targets components of the NF-κB pathway like IRAK1/IRAK2 (interleukin 1 receptor associated kinase 1/2) and TRAF6 (TNF receptor associated factor 6), thereby suppressing the actions of NF-κB.[99] Thus, in an attempt to restore cholesterol balance and reduce inflammation in lesional foam cells, miR-223 expression may be upregulated to suppress Sp3 and allow for maximal ABCA1 activity and anti-inflammatory signaling. Although there is little known about the full spectrum of cellular expression of miR-223 in the plaque, it has been found to be induced in vascular smooth muscle cells in response to calcification media, where it controls smooth muscle cell migration and proliferation.[100] Therefore, it is possible that elevated miR-223 expression in atherosclerotic plaques reflects both the presence of differentiated myeloid cells (ie, macrophages) and the presence of SMCs undergoing transdifferentiation. Further experiments are needed to elucidate the cell-specific roles of miR-223, as well as a possible negative feedback circuit involving miR-223 in the plaque.

In conclusion, our study provides evidence that miR-223 can reduce susceptibility to atherosclerosis by acting as a crucial modulator of macrophage cholesterol efflux and inflammation via globally controlling cholesterol and inflammatory gene expression. As such, systemic delivery or macrophage-specific delivery of miR-223 using viral-based vectors, nanoparticles, or extracellular vesicles could be a promising therapeutic strategy to combat inflammation in the atherosclerotic plaque.

ARTICLE INFORMATION

Received May 12, 2021; revision received May 10, 2022; accepted May 12, 2022.

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Sources of Funding

This study was supported with funding from the following organizations: Canadian Institutes of Health Research (to K.J. Rayner, T. Alain, E.E. Mulvihill), NIH (R01 HL119047 to K.J. Rayner), European Research Area Network on Cardiovascular Diseases (to K.J. Rayner) and Diabetes Canada (to E.E. Mulvihill). The Scientific and Technological Research Council of Turkey (118S930 to H. Kazan) and Health Institutes of Turkey (2019-TA-01-4069 to H. Kazan).

Disclosures

None.

Supplemental Material

Materials and Methods
Figures S1–S5
References 42–44,50,51,101–109

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