Protective Effect of let-7 miRNA Family in Regulating Inflammation in Diabetes-Associated Atherosclerosis

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COMPLICATIONS

The let-7 miRNA family plays a key role in modulating inflammatory responses. Vascular smooth muscle cell (SMC) proliferation and endothelial cell (EC) dysfunction are critical in the pathogenesis of atherosclerosis, including in the setting of diabetes. Here we report that let-7 levels are decreased in diabetic human carotid plaques and in a model of diabetes-associated atherosclerosis, the diabetic ApoE$^{-/-}$ mouse. In vitro platelet-derived growth factor (PDGF)– and tumor necrosis factor-α (TNF-α)–induced vascular SMC and EC activation was associated with reduced let-7 miRNA expression via Lin28b, a negative regulator of let-7 biogenesis. Ectopic overexpression of let-7 in SMCs inhibited inflammatory responses including proliferation, migration, monocyte adhesion, and nuclear factor-κB activation. The therapeutic potential of restoring let-7 levels using a let-7 mimic was tested: in vitro in SMCs using an endogenous anti-inflammatory lipid (lipoxin A_4), ex vivo in murine aortas, and in vivo via tail vein injection in a 24-h murine model. Furthermore, we delivered let-7 mimic to human carotid plaque ex vivo and observed significant changes to the secretome in response to let-7 therapy. Restoration of let-7 expression could provide a new target for an anti-inflammatory approach in diabetic vascular disease.

Mortality from cardiovascular disease (CVD) in patients with diabetes is more than double that in the general population, with increased atherosclerosis and higher rates of restenosis after angioplasty and in-stent restenosis (1–3). At the cellular level, atherosclerosis reflects activation of vascular smooth muscle cells (SMCs) and endothelial cells (ECs) and monocyte recruitment to the site of inflammation. The excessive proinflammatory phenotype observed in atherosclerotic lesions, which is enhanced in diabetes, is driven by multiple factors, including proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and growth factors such as platelet-derived growth factor (PDGF) (4).

microRNAs (miRNAs) are implicated in the development of a wide range of diseases, including atherosclerosis, obesity, and diabetes (5–8). Among these miRNAs, aberrant let-7 expression is associated with diseases including liver and lung fibrosis and cancer (9,10). In the context of diabetes-associated atherosclerosis (DAA), previous reports indicate that circulating let-7 levels are lower in patients with CVD and type 2 diabetes. let-7 levels can be restored to normal levels after therapies frequently used in patients to reduce the risk or progression of CVD, specifically statins (11) or glucose-lowering agents such as metformin (12) or DPP4-inhibitor (13). The let-7 family was one of the first-described miRNA families, with 12 highly conserved let-7 isoforms (14,15). Mature let-7 family members contain identical sequences and, as a result, act to suppress the expression of a common set of target mRNAs (15). let-7

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family members have a distinct expression pattern in animal development, largely regulated by the RNA-binding protein Lin28, with let-7 expression repressed in the embryonic stages of development (16–18). The let-7/lin28 axis has been implicated as a master regulator of cell proliferation and differentiation, with reduced let-7 miRNA expression reported in epithelial-to-mesenchymal transition and enhanced cell migration/invasion (19,20).

We have identified a role for the let-7 miRNA family in renal fibrosis, with let-7 levels reduced in vitro and in experimental models of diabetic and nondiabetic kidney disease (21,22). Recent miRNA profiling of human tissues has implicated let-7 miRNAs in CVD (11,23–27). Here, we examined the role of let-7 miRNA in the development and progression of DAA. Our findings reveal that let-7 miRNAs are dysregulated in clinical and experimental DAA, and modulate SMC and EC activation and inflammation, via regulation of PDGF and TNF-α signaling. Furthermore, we demonstrate that restoration of let-7 levels can suppress mediators of vascular inflammation, including interleukin (IL)-6, IL-1β, and nuclear factor-κB (NF-κB). The translational potential of these findings was confirmed by ex vivo delivery of let-7 mimic to human carotid plaque biopsy tissue. These results provide insights into the pathophysiology of vascular damage as observed in atherosclerosis, particularly in the diabetes setting, and identify let-7 as a novel therapeutic target for diabetes complications.

RESEARCH DESIGN AND METHODS

Cell Culture

Mouse primary vascular SMCs were cultured in DMEM (Life Technologies) supplemented with 25 mmol/L glucose and 10% (v/v) FBS. For treatments, media contained only 1% FBS. Mouse primary aortic ECs were maintained and passaged in 1:1 DMEM (Gibco) and Ham's F12 (Gibco) and supplemented with 10% (v/v) heat-inactivated FBS, EC growth supplement (Sigma), heparin, and 5 mmol/L D-glucose. After serum restriction for 24 h, cells were stimulated with PDGF or TNF-α (R&D systems) as indicated. For lipoxin (LX) cotreatments, cells were stimulated with vehicle (0.1% ethanol) or LXA_4 (0.1 nmol/L; Calbiochem, Merck Millipore) as indicated. The formyl peptide receptor 2 (ALX/FPR2) antagonist Boc-FLFLF peptide (10 μmol/L; GL Biochem, Shanghai, China) was used. The PDGF inhibitor (imatinib, category no. STI-571; Novartis Pharmaceuticals, Basel, Switzerland) was used as indicated. All cell culture experiments were performed 3–6 times.

miRNA, Small Interfering RNA, and Plasmid Transfection

SMCs were transfected with 20 nmol/L miR mimic (Life Technologies) or 50 nmol/L locked nucleic acids (Exiqon) in Opti-MEM (Life Technologies) using RNAiMAX (Life Technologies). Negative control pre-miR and locked nucleic acids were used at 20 nmol/L and 50 nmol/L, respectively. Cells were transfected with 25 nmol/L Lin28b small interfering RNA (siRNA) or scrambled control siRNA (Sigma-Aldrich). Media was changed to 1% FBS DMEM after 16 h, and stimuli were added as indicated. NF-κB activity was assessed by cotransfection with miR mimic and an NF-κB reporter plasmid (pNFκB-SEAP Vector; Takara Bio/Clontech) for 24 h and stimulation with TNF-α. NF-κB activity was determined using the SEAP Reporter Gene Assay System (Roche).

miRNA-Target Analysis

Bioinformatic prediction of miRNA-mRNA interactions was performed using TargetScan (http://www.targetscan.org/vert_71/). Supplementary Table 1 outlines direct targets of let-7 miRNAs. Pathway analysis was performed using DIANA-mirPath v.3.5 (http://snf-515788.vm.okeanos.grnet.gr/) (28) (Supplementary Table 2).

RNA Extraction and Quantitative RT-PCR

RNA was extracted using TRIzol (Ambion). DNase treatment and cDNA synthesis were performed as previously described (29,30). Gene expression was determined utilizing TaqMan reagents (Life Technologies) with fluorescence signals normalized to 18S rRNA utilizing the ΔΔCt method. miRNA expression was measured using TaqMan miRNA assays (Life Technologies). Fluorescence was normalized to U87, SnoRNA-135 small-RNA, or RNU6B.

Western Blotting

Denatured cell lysates (40 μg protein) were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Lin28b primary antibody (Cell Signaling) was applied overnight with 0.5% Tris-buffered saline, Tween. Secondary antibody was incubated with membranes for 1 h with Tris-buffered saline, Tween. Antibody hybridization was detected using the Odyssey imaging system (LI-COR).

Transwell Migration Assay

SMCs were transfected with let-7 mimic (see above), trypsinized (0.05% trypsin), and applied to gelatin-coated transwell inserts (10-μm pore size; Corning). PDGF (10 ng/mL) was added for 6 h. SMCs were removed from the upper side of the membrane, and migrated SMCs on the underside were stained with 1% crystal violet. The number of migrated cells was counted under microscope (×20). Twelve representative images were taken per transwell. Migration assays were performed three times.

Monocyte Adhesion Assay

SMCs were cultured as described above for 24 h, after which cells were maintained in serum-restricted media (1% FBS) for 24 h and transfected with miRNA mimic (24 h). Human monocytic cells (THP-1) were stained with the CellVue Burgundy Fluorescent Cell Labeling Kit (LI-COR). Labeled THP-1 cells were cocultured with SMCs (20 min at 37°C), nonadherent cells were gently washed, and adherent cells were fixed with 10% neutral buffered formalin and counted using the Odyssey imaging system (LI-COR). Adhesion assays were performed three times.

Ex Vivo Aorta Studies

Murine aortas were removed, cleaned of adipose tissue in Krebs buffer, and transfected with 50 nmol/L miR mimic

or negative control miR mimic (Life Technologies) using lipofectamine (Life Technologies) for 5 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After transfection, aortas were washed with Krebs buffer and RNA extracted. Ex vivo aorta experiments were performed 6–10 times.

Animal Studies and In Vivo let-7d Overexpression

Animals were housed at the Baker IDI Heart and Diabetes Research Institute according to National Health and Medical Research Council guidelines. Animals had unrestricted access to water and feed and were maintained on a 12-h light/dark cycle on standard mouse chow. Six-week-old *ApoE*$^{-/-}$ male mice were rendered diabetic with streptozotocin as previously described (31). For in vivo delivery, let-7d mimic or an miRNA negative control (1 nmol/mouse; Life Technologies) was mixed with atelocollagen (AteloGene; KOKEN, Tokyo, Japan) and tail vein–injected into male C57BL/6 mice (n = 6) at 6–8 weeks of age for 24 h.

Murine Atherosclerotic Plaque Analysis

Assessment of plaque was undertaken using en face analysis after staining with Sudan IV-Herxheimer's solution (BDH, Poole, U.K.) as previously described (32). Digital photographs of opened aortas were obtained using a dissecting microscope (Olympus SZX9; Olympus Optical, Tokyo, Japan) and camera (AxioCam; Carl Zeiss, North Ryde, New South Wales, Australia).

Human Carotid Endarterectomy

Recruitment of individuals without and with diabetes for carotid endarterectomies was carried out at the Alfred Hospital, Melbourne, Victoria, Australia. Ethics approval was obtained from the Alfred Human Research Ethics Committee (authorization no. 24/07). Carotid endarterectomy specimens from patients with asymptomatic and symptomatic atherosclerosis were obtained after informed consent and approval of the ethics committee of St. Vincent's University Hospital. For ex vivo studies, diseased tissue was removed during the carotid endarterectomy procedure from patients (n = 4 males age 71.25 ± 2.9 years) with significant arterial stenosis and immediately stored in saline. Plaque processing and dissection were performed as previously described (33,34). Plaque tissue was transfected with let-7d or an miRNA negative control (20 μmol/L; Life Technologies) for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Treatments were performed in triplicate for each plaque specimen. The Proteome Profiler Human XL Cytokine Array Kit (R&D Systems) was used to detect the secreted levels of 105 proteins. A pooling strategy using equal volumes of supernatant (300 μL) from four carotid plaques was used to determine cytokine levels released from plaques. Images were quantified using ImageJ (National Institutes of Health). Mean pixel density of reference spots was set to 100, to which all other values given are relative. Heat maps were generated using Morpheus (Broad Institute).

Statistical Analysis

All statistical analyses were performed utilizing GraphPad Prism software. All parameters were checked for normal distribution using the Kolmogorov-Smirnov test (Supplementary Table 3). Experiments with only one treatment were assessed by Student t test. Experiments with multiple treatment groups were analyzed by one-way ANOVA with post hoc comparisons of group means performed by the Fisher least significant differences test. Where nonnormal variable distribution was noted, equivalent nonparametric tests were applied (Mann-Whitney U test). A P value ≤0.05 was considered statistically significant. Supplementary Table 4 outlines results from all statistical tests used and calculated P values. Significance between groups is indicated for each figure. Unless otherwise specified, data are shown as means ± SEM.

RESULTS

let-7 miRNAs Are Dysregulated in Atherosclerosis

We have recently characterized atherosclerotic plaques from symptomatic and asymptomatic patients with coronary artery disease (34). Here we measured let-7b and let-7d expression in human carotid plaque tissue and observed significantly lower expression levels in plaque tissue from patients with symptomatic versus asymptomatic atherosclerosis (Fig. 1*A*). Given the role that diabetes plays in accelerating atherosclerosis, let-7b levels were also measured in diabetic and nondiabetic human carotid plaque tissue. We observed significantly lower levels of let-7b in diabetic plaque tissue, and this was associated with increased expression of markers of vascular inflammation (Fig. 1*B* and *C*). Consistent with these clinical findings, let-7b and let-7d levels were significantly lower in aortic tissue isolated from a well-characterized animal model of DAA, the streptozotocin diabetic apolipoprotein E knockout (ApoE$^{-/-}$) mouse. With use of this model, at 10 weeks of age plaque progression has initiated, as indicated by enhanced Sudan IV–positive staining within the aortic arch (Fig. 1*D*), and this was associated with reduced let-7b and -7d levels in aortic tissue isolated from diabetic versus nondiabetic ApoE$^{-/-}$ mice (Fig. 1*E*).

let-7 miRNAs Regulate PDGF-Mediated Proliferation and Migration in Vascular SMCs

PDGF plays a critical role in the regulation of SMC proliferation in DAA (35). Here, we investigated the role of the let-7 miRNA family in PDGF-mediated SMC activation. PDGF treatment (10 ng/mL for 24 h) of SMCs induced an upregulation of several genes relevant to atherosclerosis, including the proliferation marker PCNA, cell cycle regulators p21 and p53, and PDGF receptor (PDGFR) and transforming growth factor (TGF)βR1 (Fig. 2*A*). PDGF treatment of SMCs led to reduced expression of let-7b and let-7d by 45% and 40%, respectively (Fig. 2*B*).

We previously demonstrated that a PDGF receptor, tyrosine kinase inhibitor (imatinib), attenuates DAA (35). Here we report that imatinib blocked PDGF-mediated induction

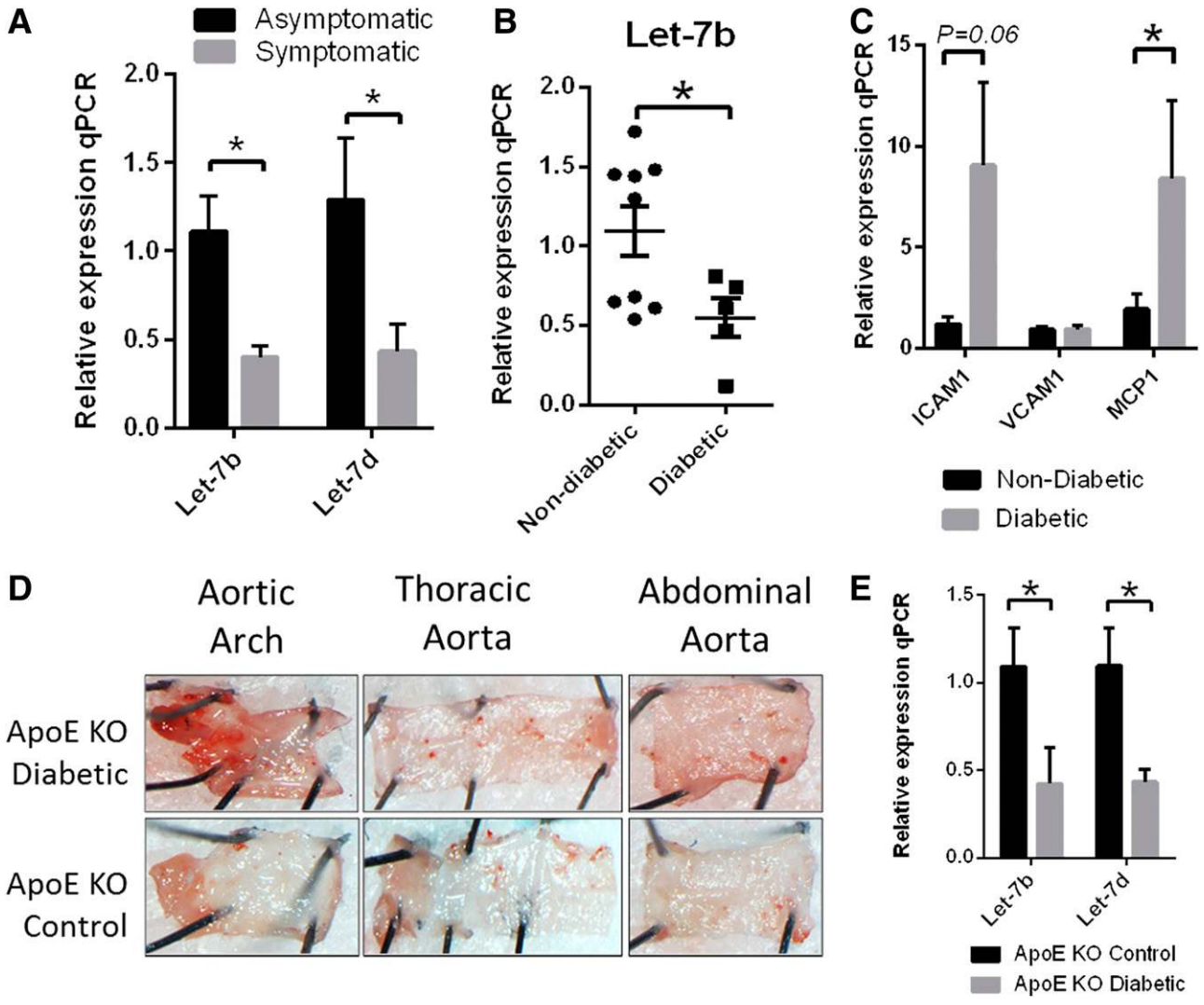

Figure 1—let-7 miRs are dysregulated in atherosclerosis. *A*: let-7b and -7d expression in carotid endarterectomy specimens from patients with symptomatic and asymptomatic atherosclerosis (n = 3–4) (±SEM). let-7b expression (*B*) and expression analysis of proinflammatory genes (*C*) in carotid endarterectomy specimens from patients without and with diabetes (n = 5–9) (±SEM). *D*: Representative Sudan IV staining of aortas isolated from 10-week-old diabetic and nondiabetic ApoE$^{-/-}$ mice. Red stain indicates positive stain for lipid accumulation. *E*: let-7b and -7d expression in aortic tissue isolated from 10-week-old ApoE$^{-/-}$ control and diabetic mice (n = 6) (±SEM). Expression was normalized to 18s for gene analysis and RNU6B for miRNA analysis. *$P \leq 0.05$. KO, knockout; qPCR, quantitative PCR.

of p21 and PCNA in SMCs and downregulation of let-7b expression (Fig. 2*C* and *D*). For determination of the role of let-7b in SMC activation, SMCs were transfected with let-7b or control miRNA, demonstrating that the PDGF-mediated induction of p21, PCNA, P53, and TGFβR1 was significantly attenuated by let-7b (Fig. 2*E*). SMC proliferation and migration were also modulated by transfection with let-7b mimic, resulting in reduced proliferation, even in the presence of PDGF, compared with control miRNA (Fig. 2*F*). Furthermore, with use of a transwell migration assay, SMC migration was increased by ~60% in response to PDGF, and this could be prevented by transfection of let-7b mimic into SMCs (Fig. 2*G* and *H*). Bioinformatic analysis of the PDGF signaling pathway identifies the let-7 miRNAs among the most significant regulators of this pathway, targeting PDGF and PDGFR transcripts (Supplementary Fig. 1 and Supplementary Table 2). Taken together, these data implicate the let-7 miRNA family as important regulators of SMC function.

let-7 miRNAs Regulate TNF-α–Mediated Inflammation in Vascular SMCs

In response to proinflammatory cytokines such as TNF-α released at the site of inflammation, SMCs produce an array of cellular adhesion molecules (intracellular adhesion molecule [ICAM]-1 and vascular cell adhesion molecule [VCAM]-1) and cytokines that promote plaque progression. For determination of the importance of the let-7 family in this context, SMCs were exposed to TNF-α (1 ng/mL for 24 h). Significant upregulation of VCAM-1, ICAM-1, IL-6, and TNF-α receptor (TNFR-α) was observed (Fig. 3*A*). With use of this model,

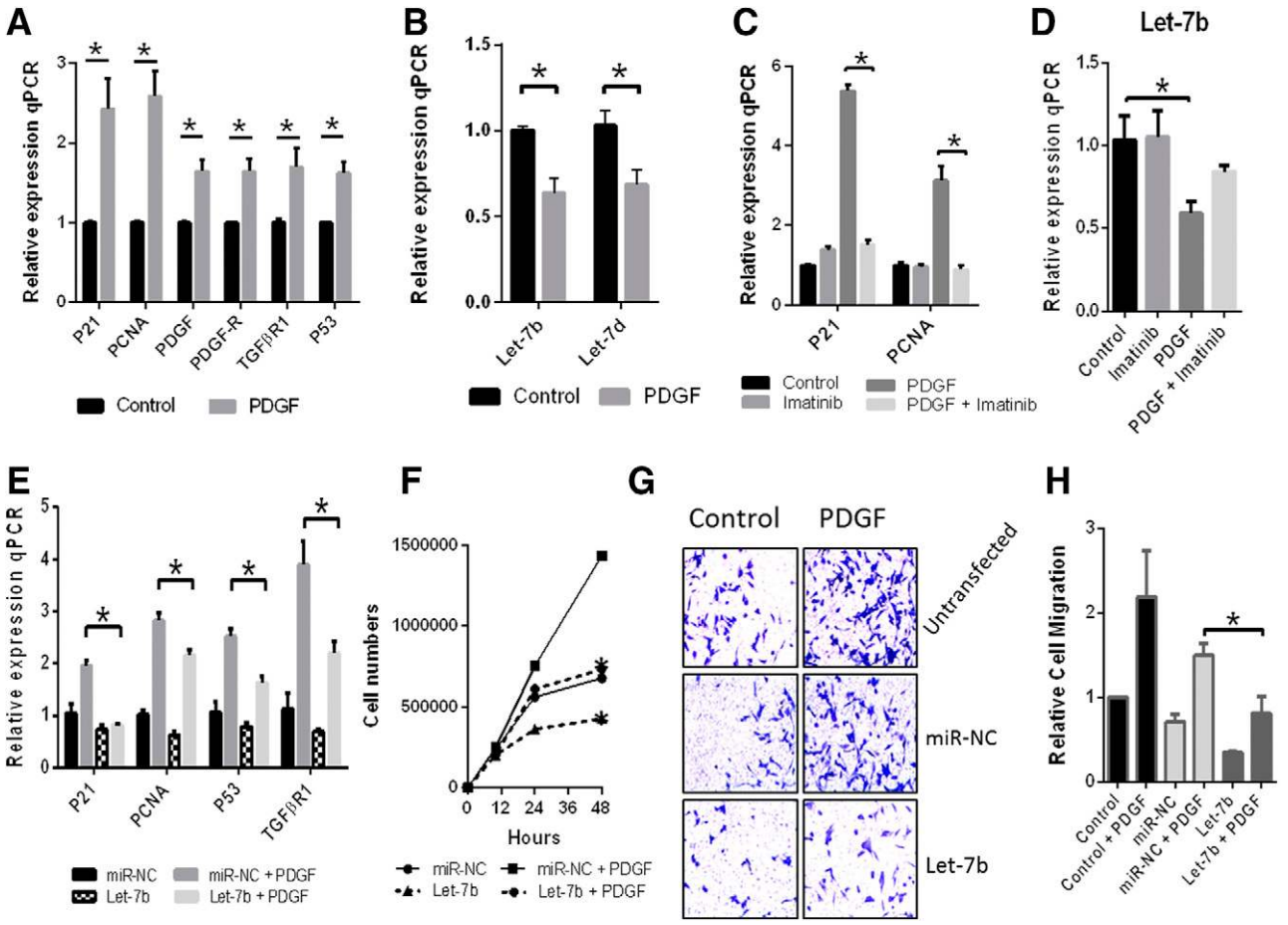

Figure 2—let-7 miRs regulate SMC activation and proliferation. *A*: Gene expression of markers of SMC activation after PDGF treatment (10 ng/mL for 24 h). *B*: Expression of let-7 miRNAs in SMCs treated with PDGF (10 ng/mL for 24 h). *C*: Expression of P21 and PCNA in SMCs treated with the PDGF inhibitor imatinib, followed by treatment with PDGF (10 ng/mL for 24 h). *D*: Expression of let-7b in SMCs treated with the PDGF inhibitor imatinib, followed by treatment with PDGF (10 ng/mL for 24 h). *E*: Analysis of P21, PCNA, P53, and TGFβR1 gene expression in SMCs transfected with let-7b miRNA or control miRNA (miR-NC), followed by PDGF treatment for 24 h (10 ng/mL). *F*: Cell proliferation as measured by cell number count in SMCs transfected with let-7b or miR-NC followed by stimulation with PDGF (10 ng/mL) (0–48 h). *G* and *H*: Representative images and quantification of crystal violet–stained SMC migratory cells transfected with let-7b or miR-NC, followed by stimulation with PDGF (10 ng/mL for 6 h). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis ($n = 3$) (±SEM). *$P \leq 0.05$. qPCR, quantitative PCR.

let-7d expression was reduced by ~30% in response to TNF-α, whereas let-7b was unchanged (Fig. 3*B*). To assess the potential role of let-7 in TNF-α signaling in SMCs, we examined the effect of let-7d overexpression. Here, transfection of let-7d mimic into SMCs modulated TNF-α effects on IL-1β and IL-6 (Fig. 3*C*). Interestingly, overexpression of let-7d in SMCs also modulated TNF-α–induced NF-κB activity. Using a luciferase NF-κB reporter activity assay, we observed a fourfold increase in NF-κB activity in SMCs in response to TNF-α, which was prevented by overexpression of let-7d (Fig. 3*D*). These data indicate that one of the main mechanisms through which let-7 may regulate inflammation in SMCs is via activation of NF-κB. Within the atherosclerotic plaque, the interaction between SMCs and monocytes promotes monocyte retention and foam-cell formation (36). Our findings indicate that TNF-α significantly increases monocyte adhesion to SMCs. Furthermore, this interaction can be prevented by overexpression of let-7d miRNA (Fig. 3*E* and *F*). These data indicate that restoration of let-7 levels in SMCs can suppress key inflammatory processes that contribute to atherogenesis. In silico analyses of the TNF-α and NF-κB signaling pathways validate these findings, identifying the let-7 miRNA family among the top ranked miRNA regulators of these key inflammatory pathways (Supplementary Figs. 2 and 3 and Supplementary Table 2). Here, let-7 miRNAs target 29 and 18 genes within the TNF-α and NF-kB pathways, respectively.

TNF-α and PDGF Regulate let-7 Expression via Lin28b

The RNA-binding protein Lin28b blocks let-7 biogenesis (16,17), and the Lin28b 3′-untranslated region contains several highly conserved let-7 miRNA binding sites (Supplementary Fig. 4). Here, we observed increased Lin28b protein expression in SMCs after treatment with PDGF and TNF-α, with a twofold induction observed 24 h post-treatment (Fig. 4*A*). Lin28b gene expression was also

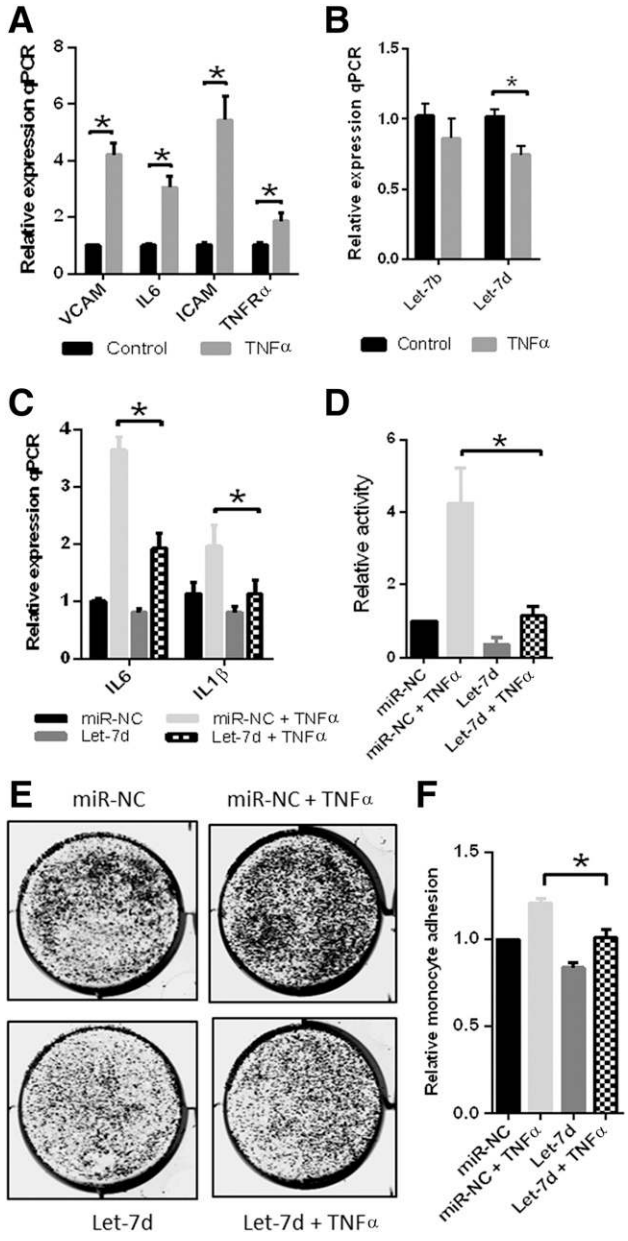

Figure 3—let-7 miRs regulate NF-κB activity and SMC-monocyte interactions. *A*: Gene expression analysis of markers of vascular inflammation in SMCs in response to TNF-α (1 ng/mL for 24 h). *B*: Expression of let-7 miRNAs in SMCs treated with TNF-α (1 ng/mL for 24 h). *C*: Analysis of IL-6 and IL-1β gene expression in SMCs transfected with let-7d miRNA or control miRNA (miR-NC) followed by TNF-α treatment for 24 h (1 ng/mL). *D*: Luciferase/Renilla ratio results for SMCs cotransfected with let-7d or miR-NC together with an NF-κB activity reporter plasmid and subsequently stimulated with TNF-α (1 ng/mL for 24 h). *E* and *F*: Representative images and quantification of labeled THP-1 monocytes adhered to SMCs transfected with let-7d or miR-NC and stimulated with TNF-α (1 ng/mL for 24 h). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis (n = 3–4) (±SEM). *$P \leq 0.05$. qPCR, quantitative PCR.

significantly higher in aortic tissue isolated from 10-week-old diabetic ApoE$^{-/-}$ mice in contrast with nondiabetic control ApoE$^{-/-}$ mice (Fig. 4*B*). Based on our findings in mouse studies, we assessed Lin28b expression in human carotid endarterectomy specimens and observed a fourfold increase in Lin28b gene expression in diabetic versus nondiabetic tissue; however, it was noted that this difference did not achieve statistical significance (Supplementary Fig. 5).

For determination of the importance of Lin28b for PDGF and TNF-α signaling, silencing experiments were performed (Fig. 4*C*). In SMCs, Lin28b depletion led to a suppression of TNF-α–mediated upregulation of IL-6 and PDGFR (Fig. 4*D*). Similarly, induction of PDGFR and TGFβR1 by PDGF was suppressed via silencing of Lin28b (Fig. 4*E*). In SMCs transfected with Lin28b siRNA, TNF-α and PDGF were no longer able to suppress let-7d and let-7b expression, respectively. This would suggest that TNF-α and PDGF require an upregulation of Lin28b to suppress let-7 miRNAs (Fig. 4*G* and *H*).

TNF-α and PDGF Regulate let-7 miRNA Expression in Aortic ECs

In response to inflammatory stimuli, the vascular endothelium expresses adhesion molecules (e.g., VCAM-1, ICAM-1), which play key roles in the recruitment of leukocytes to sites of inflammation. For identification of how proinflammatory stimuli regulate endothelial dysfunction, mouse primary aortic ECs were exposed to the proinflammatory cytokine TNF-α (1 ng/mL for 24 h). Here, induction of VCAM-1, ICAM-1, IL-6, and MCP-1 was noted (Fig. 5*A*). With use of this model, expression levels of let-7b and -7d were reduced by ~25% in response to TNF-α (Fig. 5*B*). We also investigated the role of the let-7 miRNA family in PDGF-mediated endothelial dysfunction. PDGF treatment of aortic ECs induced an upregulation of several genes relevant to atherosclerosis, including MCP-1 and PDGFR (Fig. 5*C*). Interestingly, whereas PDGF suppressed let-7b and -7d expression levels in SMCs (Fig. 2*B*), PDGF failed to suppress the expression of these let-7 miRs in ECs (Fig. 5*D*).

LXs Restore let-7 miRNA Levels and Reduce Inflammatory Markers

Therapeutic strategies based on resolution of inflammation in the arterial wall represent a novel approach to treat atherosclerosis. There is a growing appreciation of the role of endogenous lipid mediators including LXs in promoting the resolution of inflammation (37–39). We have identified a novel let-7–mediated mechanism of action for LXs whereby LXA_4 restored the loss of let-7 induced by the profibrotic cytokine TGF-β1 in renal epithelial cells (21). Here we determined whether a similar mechanism of action for LXs exists in the context of PDGF and TNF-α signaling in SMC activation and EC dysfunction.

LXA_4 (0.1 nmol/L) significantly attenuated PDGF-induced gene expression of p21 and PCNA in SMCs (Fig. 6*A*). LXA_4 also significantly attenuated TNF-α–induced IL-6 expression (Fig. 6*C*). For determination of whether the proresolution action of LXA_4 in SMCs was associated with any changes in let-7 expression, let-7 levels were assayed. Whereas LXA_4 failed to restore the PDGF-driven loss of let-7b and -7d in SMCs (Fig. 6*B*), LXA_4 did prevent the loss of let-7d

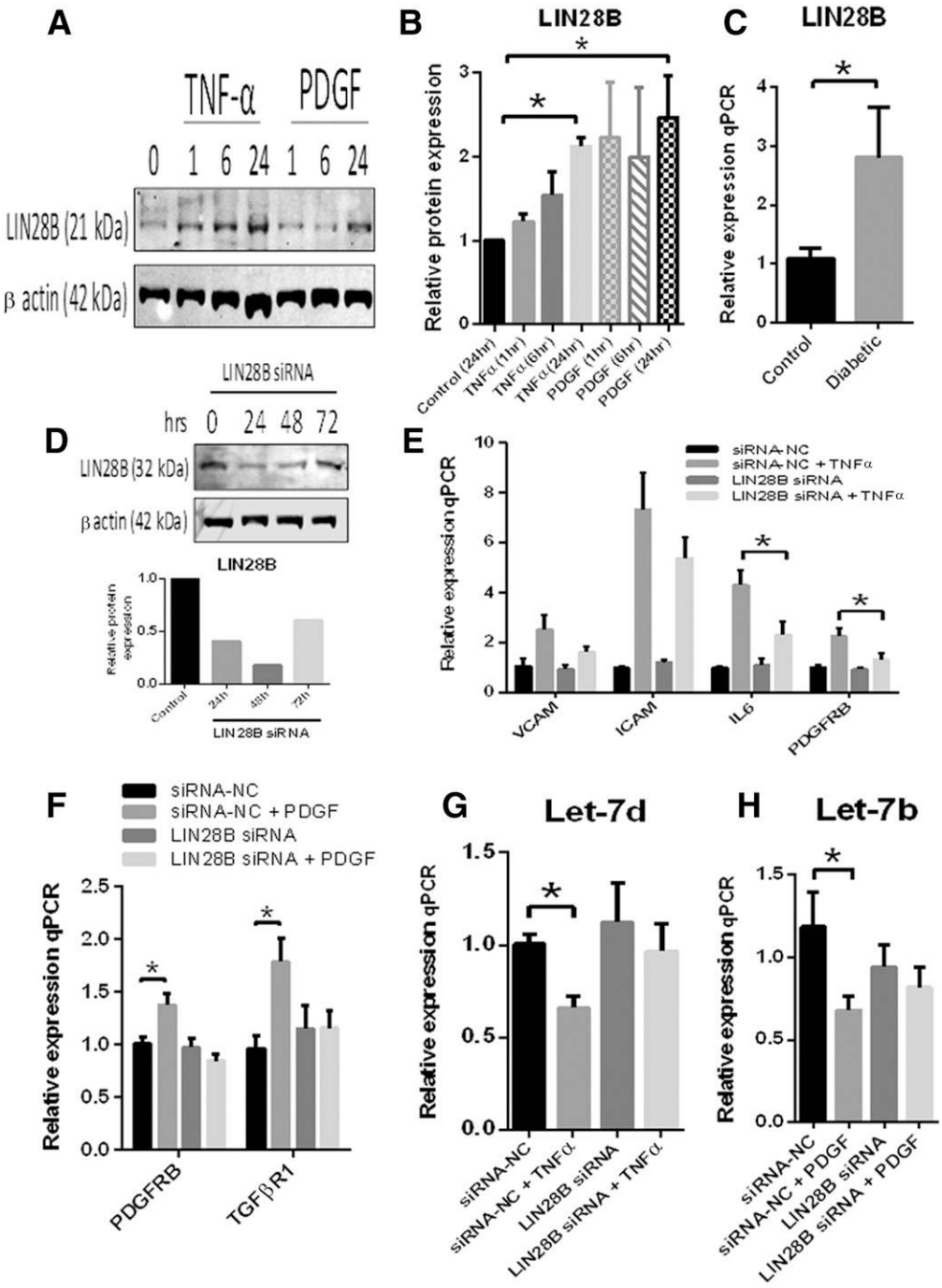

Figure 4—TNF-α and PDGF regulate the let-7 repressor Lin28b in SMCs. *A* and *B*: Representative Western blot and densitometry of Lin28b protein expression in SMCs stimulated with TNF-α (1 ng/mL) and PDGF (10 ng/mL) for 1, 6, and 24 h ($n = 3$) (±SEM). *C*: Lin28b gene expression in aortas from 10-week-old diabetic and nondiabetic ApoE$^{-/-}$ mice ($n = 4$) (±SEM). *D*: Representative Western blot of Lin28b protein expression in SMCs transfected with Lin28b siRNA for 0–72 h. *E*: Gene expression of markers of SMC activation and inflammation after transfection with Lin28b siRNA or control siRNA (siRNA-NC), and subsequent TNF-α treatment for 24 h (1 ng/mL). *F*: Gene expression of markers of SMC activation after transfection with Lin28b siRNA or siRNA-NC and PDGF treatment for 24 h (10 ng/mL). *G*: Expression of let-7d in SMCs transfected with Lin28b siRNA or siRNA-NC and treated with TNF-α (1 ng/mL for 24 h). *H*: Expression of let-7b in SMCs transfected with Lin28b siRNA or siRNA-NC and treated with PDGF (10 ng/mL for 24 h). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis ($n = 3$–4) (±SEM). *$P \leq 0.05$. qPCR, quantitative PCR.

mediated by TNF-α (Fig. 6*D*). Importantly, this effect of LXA_4 on let-7d expression was inhibited by Boc-2, an antagonist of the ALX/FPR2 receptor (Supplementary Fig. 6). These data indicate that PDGF and TNF-α regulate let-7d levels via distinct mechanisms in SMCs, and restoration of let-7d levels by LXA_4 is mediated via ALX/FPR2. Finally, consistent with findings in SMCs, LXA_4 significantly suppressed TNF-α–induced upregulation of IL-6 in ECs

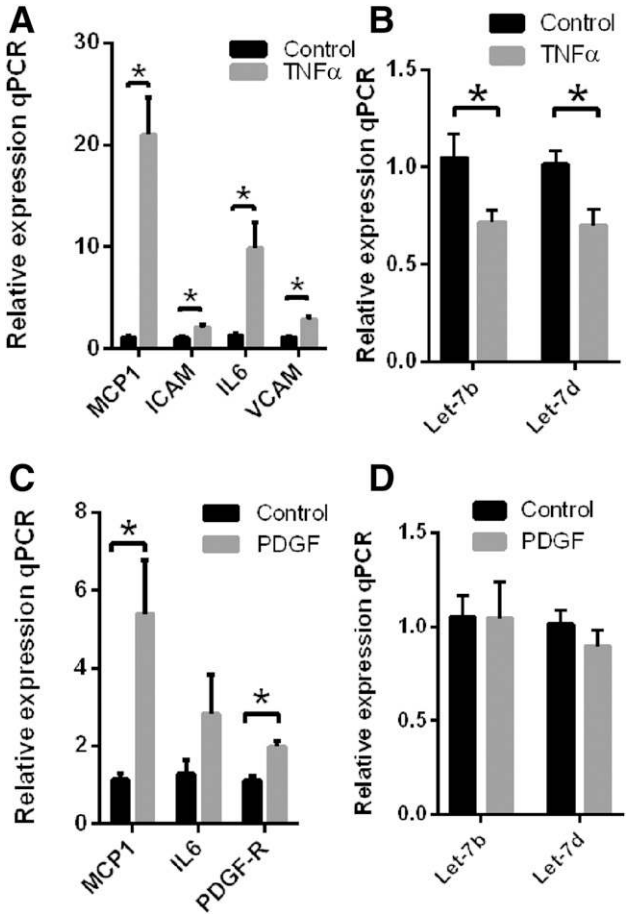

Figure 5—Reduced let-7 levels are associated with enhanced proinflammatory gene expression in ECs. *A*: Expression analysis of proinflammatory genes in ECs in response to TNF-α (1 ng/mL for 24 h). *B*: Expression of let-7 miRNAs in ECs treated with TNF-α (1 ng/mL for 24 h). *C*: Gene expression analysis of markers of vascular inflammation in ECs in response to PDGF (10 ng/mL for 24 h). *D*: Expression of let-7 miRNAs in ECs treated with PDGF (1 ng/mL for 24 h). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis (n = 3–4) (±SEM). *$P \leq 0.05$. qPCR, quantitative PCR.

(Fig. 6*E*). Here, the cotreatment of LXA_4 with TNF-α was associated with restored levels of let-7b and -7d (Fig. 6*F*).

Restoration of let-7 Levels Ex Vivo and In Vivo Reduces Expression of Markers of Inflammation

To determine whether delivery of let-7 mimic to aortic tissue could repress markers of vascular dysfunction, we performed ex vivo and in vivo delivery studies. Here, ex vivo transfection of let-7d mimic into mouse aortas led to a significant reduction in expression of both predicted targets of let-7, i.e., IL-6 and TGFβR1, as well as the indirect target, VCAM-1 (Fig. 7*A*). Delivery of let-7d mimic to aortas from diabetic mice, where atherosclerosis is established and the expression of proinflammatory genes is upregulated, led to a reduction in the expression of ICAM-1 and IL-6 (Fig. 7*B*). A significant reduction in the expression of the let-7 regulator, and let-7 target, Lin28b, was also noted in response to let-7d overexpression (Fig. 7*B*).

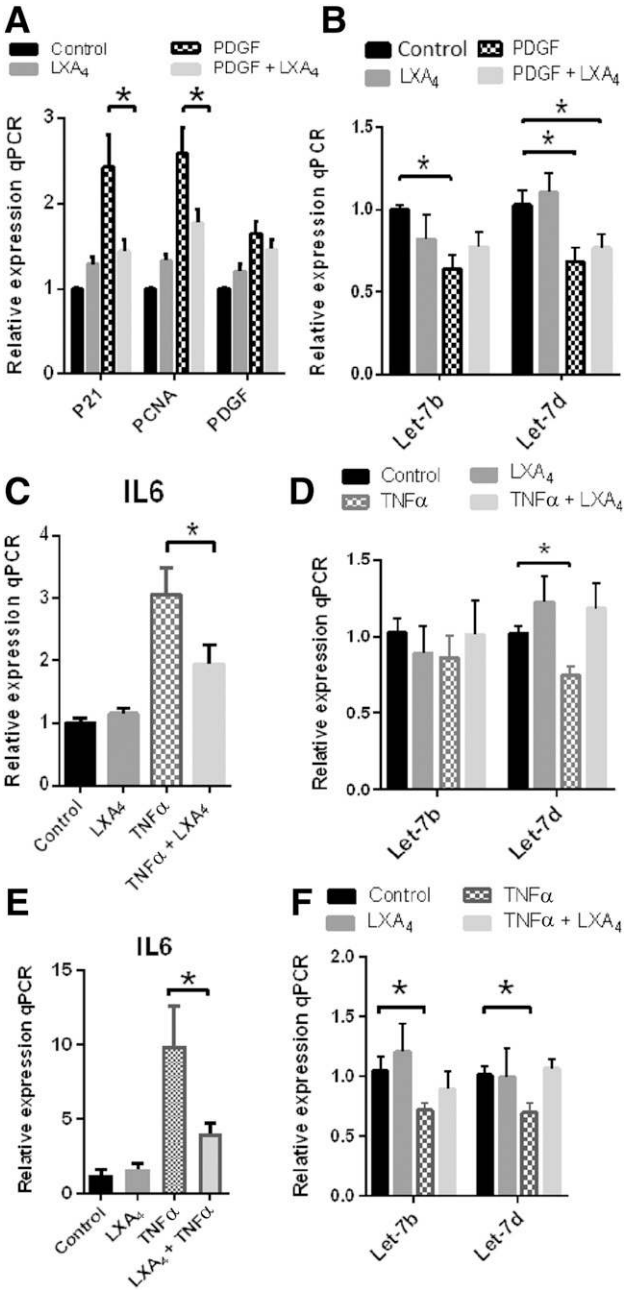

Figure 6—LXs can restore let-7 levels in SMCs and ECs. *A*: Gene expression of markers of SMC activation after PDGF stimulation (10 ng/mL for 24 h) and pretreatment with or without LXA_4 (1 nmol/L for 30 min). *B*: let-7 expression in SMCs after PDGF stimulation (10 ng/mL for 24 h) and pretreatment with or without LXA_4 (1 nmol/L for 30 min). *C* and *D*: IL-6 and let-7 expression in SMCs stimulated with TNF-α (1 ng/mL for 24 h) and pretreatment with or without LXA_4 (1 nmol/L for 30 min). *E* and *F*: IL-6 and let-7 expression in ECs stimulated with TNF-α (1 ng/mL for 24 h) and pretreatment with or without LXA_4 (1 nmol/L for 30 min). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis (n = 3) (±SEM). *$P \leq 0.05$. qPCR, quantitative PCR.

Finally, systemic administration of let-7d in vivo was performed to determine the impact on expression of markers of vascular dysfunction. After tail-vein injection with let-7d mimic, let-7d levels were increased by ~60% in

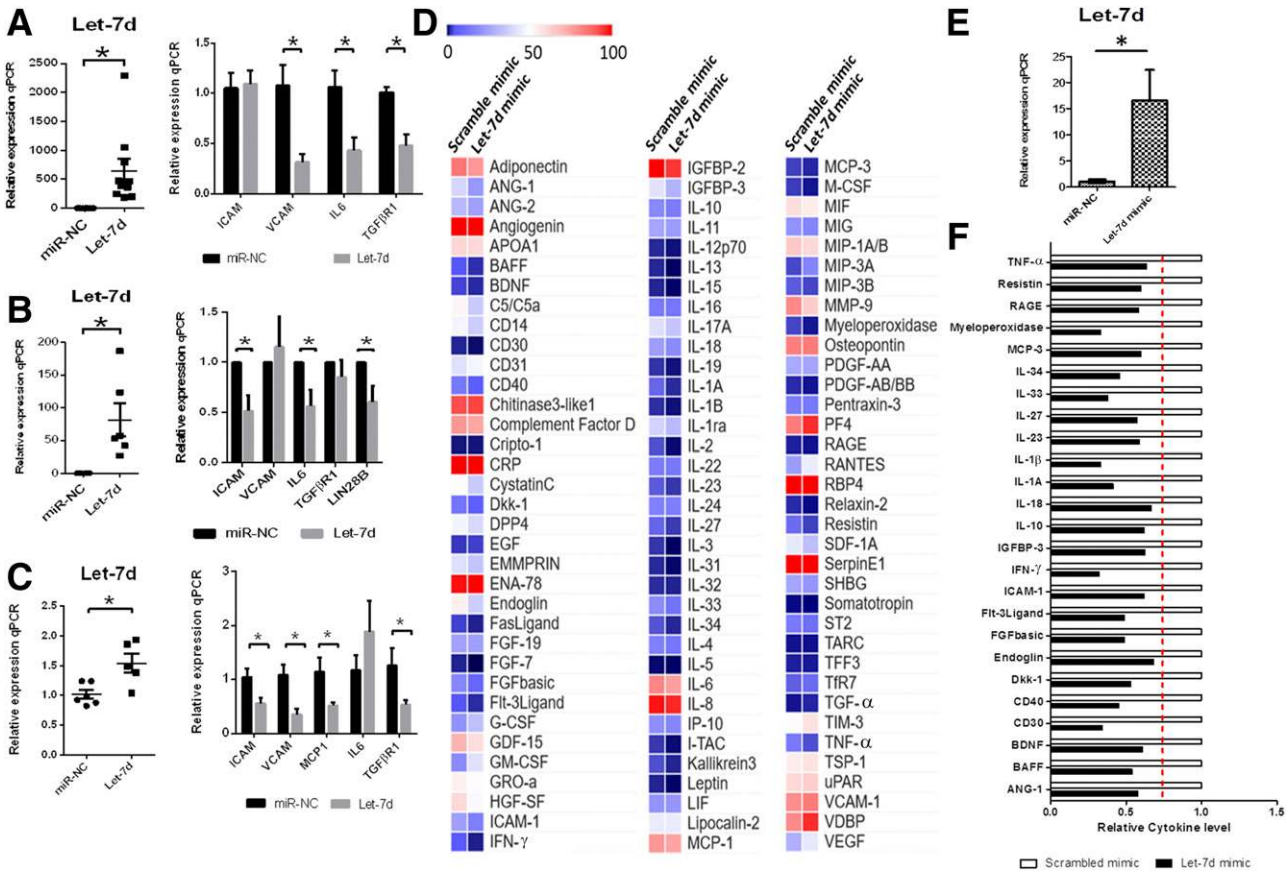

Figure 7—Restoration of let-7 levels reduces expression of proinflammatory genes. *A*: Expression levels of let-7d and direct/indirect target genes in isolated C57BL/6 aortas transfected ex vivo with let-7d mimic or control miRNA (miR-NC) for 5 h (n = 7–10) (±SEM). *B*: Expression levels of let-7d and direct/indirect target genes in isolated 10-week-old diabetic ApoE$^{-/-}$ aortas transfected ex vivo with let-7d mimic or control miRNA for 5 h (n = 6) (±SEM). *C*: Expression levels of let-7d and direct/indirect target genes in C57BL/6 mice that received a single tail-vein injection of let-7d mimic or control miRNA for 24 h (n = 6) (±SEM). Expression was normalized to 18S for gene expression analysis and U87 for miRNA analysis. *$P \leq 0.05$. *D*: Heat map indicating secretory protein abundance of human carotid plaques (n = 4) in response to let-7d mimic or control miRNA. The heat map was created by setting the maximal pixel intensity of the reference spots on the array arbitrarily to 100 (red), to which the abundance of all other analytes is relative. Minimal abundance (0) is encoded by blue and mean abundance (50) by white. *E*: Expression levels of let-7d in human carotid plaque tissue explants after transfection with let-7d mimic or control miRNA for 24 h (n = 4) (mean ± SEM shown). Expression was normalized to U87 for miRNA analysis. *$P \leq 0.05$. *F*: Quantification of *D*, indicating cytokines secreted from human plaques after transfection with control miRNA or let-7d mimic. Cytokines displaying ≥30% reduction in response to let-7d mimic transfection, relative to control miRNA transfection (set = 1), are shown. qPCR, quantitative PCR.

aortic tissues in comparison with miRNA negative control (Fig. 7*C*). Indeed, systemic administration of let-7d mimic reduced the expression levels of the predicted let-7 target TGFβR1, as well as various indirect targets, MCP-1, VCAM-1, and ICAM-1 (Fig. 7*C*), which are implicated in DAA. With the exception of COL3A1, let-7d mimic delivery had no effects on genes associated with extracellular matrix integrity or fibrosis (MMP2, MMP9, MMP12, PAI1, COL1A1, or COL4A1) (Supplementary Fig. 7).

Delivery of let-7 Mimic to Human Carotid Plaque Explants Modulates Proinflammatory Cytokines

To investigate the potential of let-7 therapeutics in atherosclerosis, we performed ex vivo transfection experiments of human carotid plaque tissue using a let-7d mimic. Plaque tissue from freshly isolated specimens (n = 4) was transfected with either a let-7d or a scrambled control miRNA mimic. After transfection with the let-7d mimic, let-7d miRNA levels were ∼30-fold higher in comparison with the scrambled control miRNA (Fig. 7*E*). To expand on these data, we used a proinflammatory cytokine array kit for determination of the secreted pattern of 105 cytokines from carotid plaques in response to transfection with let-7d or control miRNA mimics. A densitometric assessment of the secreted cytokines identified several cytokines suppressed by let-7d mimic (Fig. 7*D* and *F* and Supplementary Figs. 8 and 9). In particular, levels of TNF-α, RAGE, MCP3/CCL7, IL-1β, IFN-γ, and ICAM-1 were secreted at lower levels in response to the let-7d mimic. These data suggest the atheroprotective potential of restoring let-7d levels as a novel therapeutic strategy.

DISCUSSION

Vascular SMC and EC activation is critical to the development and progression of atherosclerosis, and these processes

are exacerbated in the diabetic milieu (40–44). Current approaches to treating CVD in diabetes typically target risk factors for atherosclerosis, including hypertension, hyperglycemia, and dyslipidemia. Here, we have identified let-7b and let-7d as important regulators of atherogenesis (Fig. 8), with a particular emphasis on combating proinflammatory and proliferative pathways that are activated in the diabetic blood vessels at high risk of accelerated atherosclerosis. Based on positive findings indicating that reduced let-7 expression is linked to vascular injury, and is reduced at other sites of diabetes complications (21,22), we explored the role of the let-7 miRNA family in PDGF- and TNF-α–mediated fibrosis and proinflammatory gene expression in DAA. The translational potential of let-7 therapy in atherosclerosis was demonstrated by ex vivo delivery of let-7d mimic to human carotid plaque tissue. Indeed, our positive results indicate that restoration of let-7 levels is potentially a novel therapeutic strategy to prevent or retard the development and progression of DAA.

In recent years, several studies have implicated let-7 miRNAs in CVD, albeit not in the context of diabetes (11,23–27). Furthermore, our previous findings suggest an important role for the let-7 miRNA family in renal fibrosis (21,22). In the current study, decreased let-7 miRNA expression was detected in human carotid plaque specimens from participants with symptomatic versus asymptomatic atherosclerosis and also in patients with DAA. Consistent with these findings in human atherosclerosis, decreased let-7 expression was observed in aortic tissue from diabetic ApoE$^{-/-}$ mice with evidence of plaque formation.

Our data indicate an important role for the let-7 miRNA family in regulating PDGF and TNF-α signaling in vascular SMCs and ECs, with direct consequences on SMC proliferation and migration, monocyte adhesion, and NF-κB activation. These findings are of relevance to DAA, since these pathological events are hallmarks of atherosclerosis including in the setting of concomitant diabetes. In silico analysis of miRNA interactions with signaling pathways implicated in inflammation and fibrosis (PDGF, TNF-α, and NF-κB pathways) identified the let-7 miRNA family as among the strongest predicted regulators of such pathways. Interestingly, let-7 miRNAs target several key transmembrane receptors implicated in these processes (TNFR1, TNFR2, IL1R, and PDGFR). It is possible that reduced receptor gene expression in the presence of let-7 miRNA may be a potential mechanism through which let-7 miRNAs can modulate such pathways. This is consistent with our previous findings demonstrating an interaction between let-7 miRNA and TGFβR1 (21,22).

Our data implicate the let-7–Lin28 axis in SMC and EC responses to PDGF and TNF-α. Lin28 is well established as a regulator of let-7 biogenesis and has been widely investigated as an oncogene (45). Although mostly downregulated in adult tissue cells, overexpression of Lin28 has been observed in numerous advanced stage tumors, including ovarian and prostate cancer (45). Interestingly, a recent study investigating Lin28b in the context of renal fibrosis has demonstrated that TGF-β–driven downregulation of let-7 in renal mesangial cells was associated with an increase in the expression of Lin28b (46). Consistent with these data, we have extended these findings to the vascular setting where Lin28b levels were increased in SMCs in response to PDGF and TNF-α and also in aortic tissue from diabetic ApoE$^{-/-}$ mice in comparison with nondiabetic mice. Importantly, silencing of Lin28b in SMCs attenuated PDGF and TNF-α signaling in these cells, including the regulation

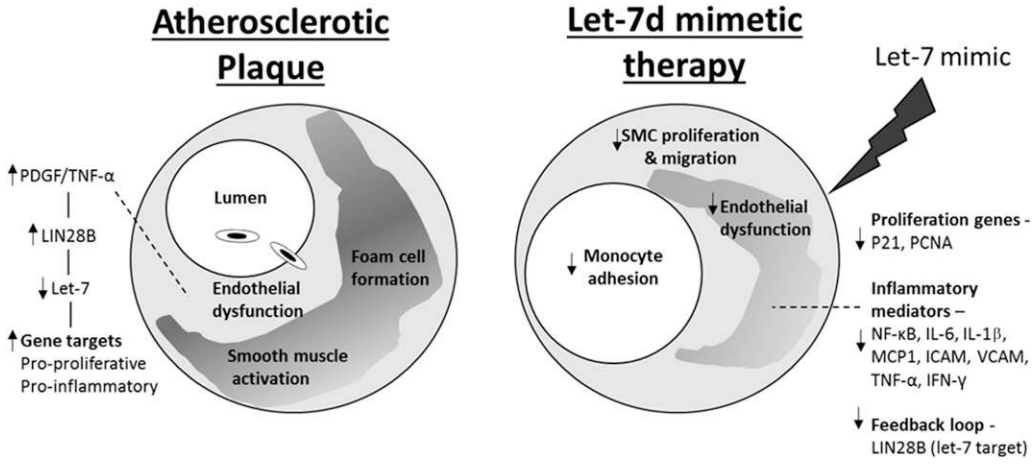

Figure 8—A conceptual model of let-7 mimetic as a therapeutic strategy in atherosclerosis as seen in diabetes. During the course of plaque progression, the levels of proproliferative and proinflammatory cytokines and growth factors (e.g., PDGF and TNF-α) are increased in the atherosclerotic milieu. Consequently, this leads to increased levels of the let-7 regulator Lin28b and a corresponding decrease in let-7 miRNA expression. Reduction of let-7 levels leads to enhanced expression of multiple proproliferative and proinflammatory genes implicated in plaque formation. Delivery of let-7 mimic to atherosclerotic plaques leads to a reduction in levels of key genes implicated in proliferation and inflammation. Ultimately this may lead to reduced SMC proliferation and migration and reduced monocyte adhesion to the vascular wall, thereby attenuating atherosclerosis.

of let-7 miRs. These specific findings suggest an important role for Lin28b in PDGF and TNF-α signaling in SMCs. Interestingly, it is noteworthy that Lin28b silencing in SMCs affected TNF-α–mediated modulation of some genes, whereas other genes were unaffected such as VCAM and ICAM. This would suggest that TNF-α drives the upregulation of these genes through multiple pathways and that targeting the Lin28b pathway may be compensated for by alternative pathways.

We have used multiple approaches including in vitro, ex vivo, and in vivo experiments in order to investigate whether restoration of let-7 levels could suppress atherosclerotic processes. Previously, we performed renal studies and showed that the endogenous proresolution lipid LXA_4 can inhibit PDGF-stimulated renal mesangial cell proliferation via modulation of the phosphatidylinositol 3-kinase pathway (47) and act to restore let-7 levels in renal epithelial cells stimulated with TGF-β as well as in vivo in a model of renal fibrosis (21). Here we hypothesized that such processes are also operating in the microvasculature and report that LXA_4 can attenuate PDGF and TNF-α signaling in SMCs and ECs and that this was associated with a restoration of let-7 levels in these cells. Interestingly, our data indicate that the regulation of let-7 expression by LXA_4 appeared to be cell specific and cytokine specific. Here, LXA_4 could modulate the regulation of let-7 miRNA by TNF-α but not PDGF. Importantly, our ex vivo studies demonstrated that delivery of let-7d mimic to diabetic mouse aorta was sufficient to modulate the expression of key adhesion molecules (VCAM-1 and ICAM-1) and markers of inflammation (IL-6). Systemic administration of let-7d mimic for 24 h was also sufficient to modulate the expression of markers of inflammation and atherosclerosis. Indeed, a significant attenuation of ICAM-1, VCAM-1, MCP-1, and TGFβR1 expression in aortas from mice administered let-7d mimic was identified.

Finally, to investigate the potential clinical translation of these findings, we performed ex vivo delivery studies of let-7d mimic in human carotid plaque biopsies and assessed the effects on secreted proinflammatory cytokines. Using this approach, we identified a number of secreted proteins modulated by let-7 delivery, including ICAM1, IL-1β, TNF-α, and IFN-γ. These are the first such studies to comprehensively assess the direct effect of a let-7 mimic on human plaque biopsies, demonstrating important atheroprotective potential.

The let-7 miRNA family comprises multiple family members under the regulation of potentially distinct promoters. Our data further highlight the complex nature of regulating this miRNA family, and it is likely that different let-7 miRs will be regulated by different growth factors in a cell-specific manner. It is noteworthy that we have focused on two members of the let-7 miRNA family that are abundantly expressed in SMCs and ECs. However, it is likely that additional family members may regulate vascular function and are under distinct mechanisms of control. Future let-7 mimic delivery studies using chronic models of atherosclerosis and vascular dysfunction are required to determine the long-term consequences of let-7 therapy. Our data indicate that let-7 mimic can suppress SMC proliferation and migration, key features of atherosclerosis formation, but also essential to fibrous cap formation and maintaining plaque stability. Therefore, the long-term effect of let-7 mimic on plaque stability is an important question. Further considerations include the mechanism of delivery of safe, effective let-7 mimic to restore deficiencies in diabetic vascular lesions.

It appears likely that the restoration of let-7 levels represents a novel therapeutic approach to prevent upregulation of key proteins implicated in inflammation, vascular adhesion, and fibrosis, key pathological hallmarks of the atherosclerotic process, as seen particularly in diabetes.

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