Pro-Inflammatory Role of MicroRNA-200 in Vascular Smooth Muscle Cells From Diabetic Mice


Objective—Vascular smooth muscle cells (VSMC) from type 2 diabetic db/db mice exhibit enhanced proinflammatory responses implicated in accelerated vascular complications. We examined the role of microRNA (miR)-200 family members and their target Zeb1, an E-box binding transcriptional repressor, in these events.

Methods and Results—The expression levels of miR-200b, miR-200c, and miR-429 were increased, although protein levels of Zeb1 were decreased in VSMC and aortas from db/db mice relative to control db/+ mice. Transfection of miR-200 mimics into VSMC downregulated Zeb1 by targeting its 3'UTR, upregulated the inflammatory genes cyclooxygenase-2 and monocyte chemoattractant protein-1, and promoted monocyte binding in db/+ VSMC. In contrast, miR-200 inhibitors reversed the enhanced monocyte binding of db/db VSMC. Zeb1 gene silencing with siRNAs also increased these proinflammatory responses in db/+ VSMC confirming negative regulatory role of Zeb1. Both miR-200 mimics and Zeb1 siRNAs increased cyclooxygenase-2 promoter transcriptional activity. Chromatin immunoprecipitation showed that Zeb1 occupancy at inflammatory gene promoters was reduced in type 2 diabetic db/db mice. Furthermore, Zeb1 knockdown increased miR-200 levels demonstrating a feedback regulatory loop.

Conclusion—Disruption of the reciprocal negative regulatory loop between miR-200 and Zeb1 under diabetic conditions enhances proinflammatory responses of VSMC implicated in vascular complications.

Key Words: diabetes mellitus ■ Zeb1 ■ inflammatory genes ■ miR-200 ■ vascular complications

Proinflammatory and proatherogenic responses in vascular smooth muscle cells (VSMC) play key roles in the development of atherosclerosis.1–3 Diabetic conditions increase the expression of proinflammatory mediators such as receptor for advanced glycation end products, cyclooxygenase-2 (COX-2), cytokines such as tumor necrosis factor-α and interleukin-6, and chemokines such as monocyte chemoattractant protein-1 (MCP-1) and Fractalkine (CX3CL1) in VSMC and other inflammatory cells.4–8 Inflammatory mediators promote proliferation, oxidant stress, migration, and monocyte-VSMC binding leading to VSMC dysfunction implicated in accelerated vascular complications of diabetes.9–10 Inflammatory gene expression is regulated by multiple signal transduction pathways leading to the activation of proinflammatory transcription factors such as nuclear factor-kappaB (NF-κB) and cAMP responsive element binding protein under diabetic conditions in VSMC.3,5,11 Recent studies have also demonstrated the role of epigenetic mechanisms, including histone posttranslational modifications, in the upregulation of inflammatory genes in endothelial cells and VSMC under diabetic conditions.3,12,13 Enhanced expression of interleukin-6, MCP-1, and colony stimulating factor-1 in VSMC derived from type 2 diabetic db/db mice (db/db VSMC) was associated with reduced levels of the histone methyltransferase Suv39h1 and the corresponding repressive histone modification H3 lysine-9 trimethylation at their promoters,11 suggesting that reversal of transcription repression could be a key mechanism for the enhanced expression of inflammatory genes in diabetes. In this study, we have uncovered a new cross talk mechanism between key miRNAs and their targets that further increases our understanding of molecular mechanisms involved in the derepression of pathological genes in VSMC under diabetic conditions.

Zeb1 (also known as deltaEF1, TCF8, or Zfx1a) is a zinc finger transcription factor expressed in various cell types including VSMC.14 It contains multiple functional domains including 2 zinc fingers, a centrally located homeodomain, and others that interact with various transcription regulators. Zeb1 binds to canonical E-box elements [CACCT(G)] in target gene promoters and mediates gene repression via interaction with corepressors including C-terminal-binding protein (CtBP) 1 (CtBP1), CtBP2, and HDAC1. Zeb1 and its
family member Zeb2 (also known as SIP1 or Zfx1b) regulate genes associated with epithelial-mesenchymal transition (EMT), fibrogenesis, chondrogenesis, T-cell development, and skeletal muscle differentiation, and the pathogenesis of cancer and renal complications such as diabetic nephropathy. However, the role of Zeb1 in inflammatory gene expression associated with diabetic vascular complications is not known.

Emerging evidence supports a role for microRNAs (miRNAs) in cardiovascular disease and diabetic complications. miRNAs are 22 to 25 nucleotide noncoding RNAs that can repress target gene expression by posttranscriptional mechanisms. They act primarily through interaction with 3'-UTR of target mRNAs leading to downregulation or translation inhibition. We recently showed that inhibition of Suv39h1 protein levels by miR-125b was one of the mechanisms underlying enhanced proinflammatory responses in db/dbVSMC. However, the role of other miRNAs in these processes has not been examined. Lately, miR-200 family members have gained increased attention due to reports that they are downregulated in EMT implicated by targeting transcription repressors Zeb1 and Zeb2 that repress E-cadherin. Zeb1/Zeb2 in turn negatively regulate promoters. Dysregulation of this double negative feedback loop has been suggested to be a key mechanism in EMT and cancer. The miR-200 family consists of 5 members subdivided into Group I (miR-200a and miR-141) and Group II (miR-200b, miR200c, and miR-429). They share common targets because the seed sequence between the two groups differs only by a single nucleotide. In mice, miR-200b, miR-200a, and miR-429 are expressed as a single polycistronic transcript on chromosome 4 (chromosome 1 in humans), whereas miR-200c and miR-141 are expressed as a single transcript on chromosome 6 (chromosome 12 in humans). These miRNAs maintain epithelial cell phenotype by targeting transcription repressors Zeb1 and Zeb2 that repress E-cadherin. Zeb1/Zeb2 in turn negatively regulate miR-200 family members via E-box binding sites in their promoters. Dysregulation of this double negative feedback loop has been suggested to be a key mechanism in EMT and cancer.

To examine the functional role of miR-200 family members, we first determined their expression levels by RT-QPCR analysis. Results showed that miR-200b, miR-429, and miR-200c levels were significantly increased in db/dbVSMC, we first determined their expression levels by RT-QPCR analysis. Results showed that miR-200b, miR-429, and miR-200c levels were significantly increased in db/dbVSMC compared to db/+VSMC (Figure 1A–C). On the other hand, miR-200a that is cotranscribed along with miR200b/429 was not detected in either db/+ or db/dbVSMC (not shown). Levels of miR-15b, unrelated to the miR-200 family, were not changed (Figure 1D), indicating specificity. Expression of miR-200b and miR-429 was also significantly increased in aortas derived from db/db mice relative to db/+ mice (Figure 1E and 1F) establishing in vivo relevance.

Here we examined the role of miR-200 and its target Zeb1 in the enhanced inflammatory responses of db/dbVSMC relative to VSMC from db/+ mice (db/+VSMC). Our results showed that miR-200b, miR-200c, and miR-429 levels are upregulated in db/dbVSMC leading to Zeb1 downregulation and enhanced inflammatory gene expression, miR-200 mimics directly targeted Zeb1 and promoted inflammatory responses in nondiabetic db/+VSMC. Furthermore, Zeb1 silencing also increased proinflammatory responses and expression of miR-200 members. Thus our studies identified novel proinflammatory effects associated with increased miR-200 and dysregulation of the reciprocal repression loop between Zeb1 and miR-200 in VSMC under diabetic conditions.

Materials and Methods
Expanded Materials and methods are available in the online-only Data Supplement.

VSMC Isolation
All animal protocols used were approved by the Institutional Animal Use and Care Committee. VSMC from thoracic aortas of Type 2 diabetic male db/db mice (10–12 weeks old) and age matched control littermates db/+ mice (Jackson Laboratories) were isolated by enzymatic digestion and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum. HeLa cells were cultured in DMEM supplemented with 10% serum.

Other Methods
Western blotting, analyses of miRNA and mRNA by reverse transcriptase-real time quantitative PCR (RT-QPCR), transient transfections, luciferase assays, immunohistochemical staining, and monocyte-VSMC binding assays were performed as described in the online-only Data Supplement.

Chromatin Immunoprecipitation Assays
Chromatin immunoprecipitation (ChIP) assays were performed as described in the online-only Data Supplement. ChIP-enriched DNA was amplified by SYBR green based QPCR using promoter specific primers (Table I in the online-only Data Supplement), analyzed by $^{2}^{-\Delta \Delta C_{T}}$ method and results normalized to input were expressed as % of db/+VSMC.

Statistical Analysis
Statistical significance was calculated with Prism software (Graphpad, San Diego, CA) by $t$-tests when comparing 2 groups, or ANOVA for multiple groups. Data were presented as means±SEM.

Results
Levels of Key miR-200 Family Members Are Increased in VSMC and Aortas From db/db Mice
To examine the functional role of miR-200 family members in db/dbVSMC, we first determined their expression levels by RT-QPCR analysis. Results showed that miR-200b, miR-429, and miR-200c levels were significantly increased in db/dbVSMC compared to db/+VSMC (Figure 1A–C). On the other hand, miR-200a that is cotranscribed along with miR200b/429 was not detected in either db/+ or db/dbVSMC (not shown). Levels of miR-15b, unrelated to the miR-200 family, were not changed (Figure 1D), indicating specificity. Expression of miR-200b and miR-429 was also significantly increased in aortas derived from db/db mice relative to db/+ mice (Figure 1E and 1F) establishing in vivo relevance.

Zeb1 Levels Are Decreased in VSMC and Aortas From db/db Mice
Zeb1 3'-UTR contains multiple miR-200 binding sites (Figure I in the online-only Data Supplement). Studies in other cell types showed that miR-200 family members target E-box binding transcription repressor Zeb1 and modulate genes associated with cancer and diabetic nephropathy. However, this inverse relationship has not been studied in VSMC and whether it can modulate processes associated with vascular complications has not been determined. We first examined whether the increased miR-200b and miR-429 noted in db/db mice was associated with downregulation of Zeb1 levels. Immunohistochemical staining with Zeb1 antibody revealed a significant decrease in Zeb1 positive nuclei in VSMC of aortas derived from db/db mice compared to db/+ (Figure 1G and 1H). Furthermore, immunoblotting of cell lysates showed that Zeb1 protein levels were significantly reduced in VSMC cultured from db/db mice compared to db/+VSMC. This inverse relationship has not been studied in VSMC and whether it can modulate processes associated with vascular complications has not been determined. We first examined whether the increased miR-200b and miR-429 noted in db/db mice was associated with downregulation of Zeb1 levels. Immunohistochemical staining with Zeb1 antibody revealed a significant decrease in Zeb1 positive nuclei in VSMC of aortas derived from db/db mice compared to db/+ (Figure 1G and 1H). Furthermore, immunoblotting of cell lysates showed that Zeb1 protein levels were significantly reduced in VSMC cultured from db/db mice compared to db/+VSMC.

Materials and Methods
Expanded Materials and methods are available in the online-only Data Supplement.
to db/+ mice (Figure II and IJ). These results clearly demonstrated that Zeb1 expression is downregulated in VSMC of diabetic mice and established the reciprocal relation with increased miR-200 expression.

Zeb1 Downregulation by miR-200b and miR-429
We next tested if Zeb1 is a direct target of these miRNAs in VSMC. We transfected nondiabetic db/+VSMC with miR-200b mimic (200b-M) and negative control mimic (NC-M) oligonucleotides and examined endogenous Zeb1 protein levels 48 hours post transfection. Results showed that 200b-M significantly inhibited Zeb1 protein levels in db/+VSMC (Figure 2A and 2B) compared to NC-M. Similarly, 429-mol/L transfection also reduced Zeb1 protein levels relative to NC-M (Figure II, Online Supplement). Next, we checked whether Zeb1 is targeted by miR-200b and miR-429 in VSMC by using luciferase reporters containing wild type Zeb1 3′-UTR with 2 miR-200b/429 binding sites and a Zeb1 mutant 3′-UTR (mUTR) harboring mutations in both miR-200b/429 binding sites.16 These reporter plasmids were cotransfected with NC-M or 200b-M, and luciferase assays performed 48 hours post-transfection. As shown in Figure 2C, 200b-M significantly inhibited luciferase activity of Zeb1 wild type 3′-UTR (UTR) construct, and this inhibition was abolished in mUTR. Similar results were obtained with 429-mol/L (Figure 2D). These results clearly demonstrate that Zeb1 is a direct target of miR-200b and miR-429 in VSMC, and that increased levels of these miRNAs could be a mechanism for downregulation of Zeb1 in db/dbVSMC.

Upregulation of Inflammatory Genes by miR-200b and miR-429
Because we noted the presence of E-boxes in the promoters of some inflammatory genes such as COX-2 that are upregulated in db/dbVSMC, we next hypothesized that loss of Zeb1 might be a mechanism for derepression of these genes. We therefore examined the potential functional roles of miR-200b and miR-429 in VSMC by transfecting db/+VSMC with NC-M and 200b-M or 429-mol/L oligonucleotides. Inflammatory gene expression was analyzed 48 hours post transfection. Results showed that Zeb1 mRNA expression was downregulated whereas levels of COX-2 and MCP-1 miRNAs were significantly upregulated in db/+VSMC transfected with 200b-M compared to NC-M (Figure 2E). However, 200b-M had no effect on other inflammatory genes such as CX3CL1 and receptor for advanced glycation end products that were also upregulated in db/+VSMC (data not shown). Transfection of 429-mol/L also inhibited Zeb1 and increased COX-2 and MCP-1 in db/+VSMC (Figure 2F). Next, we examined whether miR-200b regulates the transcriptional activation of inflammatory gene promoters. COX-2 promoter contains cis-elements such as E-box, cyclic-AMP response element and NF-κB upstream of transcription start site (Figure 2G). We used plasmids containing luciferase reporter downstream of COX-2 wild type (WT) promoter or promoter with mutations in these binding sites to determine if miR-200 affects their transcriptional activation. Cotransfection experiments showed that 200b-M significantly increased the luciferase activity of COX-2 WT promoter compared to NC-M, but failed to activate COX-2 promoter constructs with mutations in E-box, NF-κB (KBm) or cyclic-AMP response element (CREm) binding sites (Figure 2H) suggesting potential crosstalk between these 3 sites examined. These results demonstrate that miR-200 family has proinflammatory roles in VSMC and that downregulation of Zeb1 could be a key mechanism involved, at least in part.
Zeb1 Gene Silencing Upregulates Inflammatory Genes in db/+VSMC

To examine the direct role of Zeb1 in inflammatory gene expression, we transfected db/+VSMC with 2 siRNAs targeting Zeb1 (siZeb1a and siZeb1b) or a nontargeting control (siNTC) oligonucleotides. Zeb1 siRNAs significantly inhibited Zeb1 mRNA levels (Figure 3A) and also increased the expression of COX-2 (Figure 3B) and MCP-1 (Figure 3C) compared to siNTC. However, the expression of receptor for advanced glycation end products and another chemokine CX3CL1, which were shown to be upregulated in db/dbVSMC, was not affected (data not shown). Furthermore, cotransfection of COX-2 WT promoter luciferase reporter plasmid with siZeb1 (1:1 mixture of siZeb1a and siZeb1b) significantly increased COX2-WT (WT) promoter activity relative to siNTC (Figure 3D). Furthermore, COX-2 E-box mutant promoter activity was elevated relative to WT promoter in siNTC transfected cells, and this was further increased by siZeb1 (Figure 3D). These results demonstrate that Zeb1 can repress these inflammatory genes in VSMC under normal conditions and that its downregulation in diabetes (via miR-200 or Zeb1 siRNAs) can relieve this repression to augment expression.

Zeb1 Binding to Inflammatory Gene Promoters is Reduced in db/dbVSMC

In order to further verify that a loss of Zeb1 at the MCP-1 and COX-2 promoters is associated with the increased expression of these genes in diabetic db/dbVSMC, we next used ChIP assays to examine Zeb1 occupancy at these promoters. ChIP assays were performed with Zeb1 antibody, and ChIP DNA was analyzed by QPCR with promoter specific primers (Figure III in the online-only supplement). Results showed that Zeb1 occupancy was significantly reduced at both COX-2 (Figure 3E) and MCP-1 promoters (Figure 3F), with no significant change at the Cyclophilin A promoter (Figure 3G) in db/dbVSMC relative to control db/VSMC.4,6 Because transfection of 200b-M downregulates Zeb1 and increases inflammatory gene expression which mimics the proinflammatory db/db phenotype, we examined if it also leads to reduced Zeb1 occupancy at inflammatory gene promoters. We performed ChIP assays with cell lysates from NC-M and 200b-M transfected db/+VSMC using Zeb1 antibody. Results showed that Zeb1 occupancy was greatly reduced at COX-2 and MCP-1 gene promoters in 200b-M transfected cells demonstrating that 200b-M effects can be mediated through Zeb1 downregulation (Figure 3H). These results further support our hypothesis that enhanced expression of key VSMC inflammatory genes in diabetes could be due to reduced Zeb1 occupancy at their promoters.

COX-2 Plays a Role in Enhanced Monocyte Binding in db/dbVSMC

Next we examined the functional relevance of miR-200b and Zeb1 mediated gene expression in proinflammatory responses such as monocyte-VSMC binding. Previous studies showed the role of MCP-1 in enhanced monocyte binding in db/dbVSMC relative to control db/+VSMC. However, the involvement of increased COX-2 was not studied. We first confirmed that COX-2 mRNA (Figure 4A) and protein (Figure...
Figure 3. Regulation of inflammatory gene expression by Zeb1 in vascular smooth muscle cells (VSMC). **A-C**, Expression of Zeb1 (A), COX-2 (B), and MCP-1 (C) mRNAs in db/+VSMC transfected with siNTC (non-targeting control) or 2 separate siRNA oligonucleotides targeting Zeb1 (siZeb1a or siZeb1b) was determined by reverse transcriptase-real time quantitative PCR (QPCR) 72 hours posttransfection. Results normalized to internal control were expressed as % of siNTC (*P<0.05, **P<0.005, ***P<0.0002 vs siNTC, n=7). **D**, COX-2 wild type promoter (WT) and an E-box mutant promoter (EBm) were cotransfected with siNTC (non-targeting control) or 2 separate siRNA oligonucleotides targeting Zeb1 (siZeb1a or siZeb1b) was determined by reverse transcriptase-real time quantitative PCR (QPCR) 72 hours posttransfection. Results normalized to internal control were expressed as % of siNTC (*P<0.05, **P<0.005, ***P<0.0002 vs siNTC, n=7). **E**, Protein expression of COX-2 and MCP-1 in db/+VSMC transfected with siNTC or 2 separate siRNA oligonucleotides targeting Zeb1 (siZeb1a or siZeb1b). Western blotting was performed 72 hours posttransfection. Results normalized to input DNA were expressed as fold over siNTC (*P<0.05, **P<0.005, ***P<0.0002, n=8).

Figure 4. Increased COX-2 levels regulate enhanced monocyte binding in db/dbvascular smooth muscle cells (VSMC). **A**, COX-2 mRNA expression was determined by reverse transcriptase-real time quantitative PCR in db/+VSMC (db/+) and db/dbVSMC (db/db) (**P<0.05 vs db/+, n=8). **B**, Representative immunoblots of cell lysates from db/+VSMC (db/) and db/dbVSMC (db/db) performed with indicated antibodies. **C**, Intensity of COX-2 protein bands determined using a calibrated densitometer and expressed as fold over db/+VSMC (**P<0.0002 vs db/+, n=6). **D**, **E**, COX-2 inhibitor Celecoxib blocks the enhanced monocyte binding in db/dbVSMC. Both db/+VSMC and db/dbVSMC were pretreated with vehicle (Ctrl) or indicated concentrations of Celecoxib for 1 hour, and monocyte-VSMC binding assays performed using fluorescently labeled WEHI mouse monocytes as described in the Methods section in the online-only Supplement. Images of monocytes bound to VSMC monolayers were collected using a fluorescence microscope (D) and number of bound monocytes per field determined (E). Results were expressed as % of vehicle (control [Ctrl]) treated db/+VSMC (**P<0.005, n=4). 4B and 4C) levels were significantly increased in db/dbVSMC relative to db/+VSMC. Then, both db/+VSMC and db/db-VSMC were pretreated with COX-2 specific inhibitor Celecoxib (1 and 10 μmol/L) or the vehicle DMSO (control) for 1 hour. Monocyte-VSMC binding assays were performed with fluorescently labeled mouse WEHI monocytes. Results showed that vehicle treated db/dbVSMC exhibited significantly enhanced monocyte binding compared to db/+VSMC, and this was blocked by pretreatment with Celecoxib (Figure 4D and 4E). These results demonstrate a key role for COX-2 in the enhanced monocyte binding displayed by the diabetic db/dbVSMC relative to control db/+VSMC.
VSMC Dysfunction

VSMC. Representations of miR-200b increases monocyte binding to db/dbVSMC interactions by microRNA (miR)-200b. Since Zeb1 also negatively regulates COX-2 and MCP-1, we studied the direct effect of Zeb1 knockdown on monocyte binding in diabetic db/dbVSMC. Results demonstrated that increased miR-200b can promote, at least in part, the inflammatory responses in VSMC under diabetic conditions. Furthermore, transfection of db/dbVSMC with miR-200b inhibitor significantly increased the binding of WEHI mouse monocytes to db/dbVSMC compared to NC-M transfected cells. As controls, db/+VSMC were also transfected with NC-I. Monocyte binding assays were performed and results expressed as % of db/+VSMC transfected with NC-I (**P<0.05, ***P<0.0002 vs NC-I, n=8).

Regulation of Monocyte-VSMC Binding by miR-200b in Diabetes

To evaluate the functional role of miR-200b, we transfected db/+ VSMC with 200b-M or NC-M and performed monocyte binding 48 hours posttransfection. Results showed that 200b-M significantly increased the binding of WEHI mouse monocytes to db/+VSMC compared to NC-M transfected cells (Figure 5A and 5B). Next, we tested if the enhanced monocyte binding in diabetic db/dbVSMC can be blocked by miR-200b inhibitors. We transfected db/dbVSMC with miR-200b inhibitor (200b-I) that targets miR-200b hairpin, or negative control inhibitor (NC-I). As controls, db/+VSMC were also transfected with NC-I. Monocyte binding assays were performed and results expressed as % of db/+VSMC transfected with NC-I (**P<0.05, ***P<0.0002 vs NC-I, n=8).

Zeb1 Knockdown Increases Monocyte-VSMC Binding in db/+VSMC

Since Zeb1 also negatively regulates COX-2 and MCP-1, we studied the direct effect of Zeb1 knockdown on monocyte-VSMC binding. As shown in Figure 6A and 6B, transfection of siZeb1a or siZeb1b significantly increased monocyte-VSMC binding relative to siNTC confirming that Zeb1 is an endogenous repressor of key genes such as MCP-1 and COX-2 involved in VSMC-monocyte interactions.

Zeb1 Knockdown Upregulates miR-200b and miR-429

Studies in epithelial cells showed that Zeb1 can also negatively regulate miR-200 expression. Therefore, we examined if Zeb1 downregulation can upregulate miR-200b and miR-429 levels in a feedback mechanism in VSMC. Results showed that Zeb1 knockdown by siZeb1 (1:1 mixture of
Discussion

In this study, we demonstrated that miR-200 family members miR-200b, miR-200c, and miR-429 were upregulated, while conversely protein levels of their target Zeb1 were downregulated in VSMC and aortas from type 2 diabetic db/db mice. Overexpression of miR-200 inhibited Zeb1 expression, whereas Zeb1 gene silencing increased miR-200b and miR-429, demonstrating the reciprocal negative regulation of each other in VSMC. Interestingly, miR-200 mimics and Zeb1 siRNAs increased the expression of inflammatory genes COX-2 and MCP-1 as well as monocyte binding in nondiabetic db/+VSMC, mimicking the enhanced proinflammatory phenotype of db/dbVSMC. Inhibition of miR-200 blocked enhanced proinflammatory responses in db/dbVSMC further supporting the key role of miR-200 in these events associated with diabetic vasculopathy.

Downregulation of miR-200 family members in EMT in cancer cells and renal epithelial cells is widely described.17,22–23 However, only recently the role of miR-200 in vascular and renal cells has been investigated. Downregulation of miR-200b in endothelial cells by hypoxia22 and streptozotocin induced diabetic retinopathy23 increased the expression of its targets Ets-1 and VEGF that promote angiogenesis. In contrast, increased levels of miR-200b and miR-200c were seen in diabetic mouse glomeruli16,29 and in renal mesangial cells treated with TGF-β, resulting in Zeb1 and Zeb2 downregulation and increased fibrotic gene expression,16 a key mechanism involved in diabetic nephropathy. Our results demonstrate that increased levels of miR-200b, miR-200c and miR-429 are present in VSMC of type 2 diabetic mice along with Zeb1 downregulation and enhanced inflammatory gene expression. These studies reveal the cell and disease-specific differences and complexities in the regulation of miR-200 family members, as well as the diverse roles played by them in various pathophysiological conditions.

Inflammatory genes are regulated by transcription factors such as NF-κB and cAMP responsive element binding protein in VSMC, and activities of these are enhanced under diabetic conditions.3–5,13 We found that COX-2 promoter activation by miR-200b required cis- elements NF-κB and cyclic-AMP response element suggesting its interaction with pathways involved in the activation of NF-κB and cAMP responsive element binding protein in VSMC. Furthermore, mutations in E-box increased the basal activity of the COX-2 promoter demonstrating that binding of repressors such as Zeb1 might inhibit its activity. However, miR-200b did not further enhance E-box mutant activity suggesting that E-box element in COX-2 promoter binds to both positive and negative regulatory transcription factors. Indeed, studies in other cell types showed that transcription factors USF-1 and USF-2 activate COX-2 promoter through binding to this E-box.30–31 This suggests that any alterations in the fine balance between activating and repressive transcription factors at promoters of pathological genes might be one of mechanisms underlying enhanced inflammatory gene expression mediated by miR-200b in VSMC.

Zeb1 gene silencing (by siRNAs) also increased inflammatory genes and increased COX-2 WT promoter activation in VSMC. In addition, E-box mutant COX-2 promoter showed increased activity relative to WT in siNTC transfected cells, which was further enhanced by Zeb1 knockdown, demonstrating negative regulatory role of Zeb1 and E-box elements in VSMC. Furthermore, ChIP assays demonstrated reduced Zeb1 occupancy at both COX-2 and MCP-1 promoter in db/dbVSMC and in db/+ VSMC transfected with 200b-M, further supporting the notion that Zeb1 inhibition can contribute to the enhanced inflammatory gene expression in db/dbVSMC. Interestingly, the MCP-1 proximal promoter region amplified in Zeb1 ChIP assays did not have a canonical E-box element, yet Zeb1 knockdown increased MCP-1 expression. This suggests that the MCP-1 promoter may have unidentified putative Zeb1 binding sites in its proximal promoter or away from the promoter. Previous studies have shown that Zeb1 binding as far as 16 kb away from transcription start site can influence Colla2 gene expression.15,22 Alternately, Zeb1 may interact with the MCP-1 promoter indirectly through protein–protein interactions with transcription repressors such as CtBP1 and CtBP2.33 Furthermore, Zeb1 can associate with repressor complexes formed by CtBP at gene promoters, which also includes other transcription repressors such as histone deacetylase 1 and lysine specific demethylase 1, a histone H3 lysine 4 demethylase.14,15,22 In addition, increases in miR-200 levels in Zeb1 knockdown VSMC (Figure 6D and 6E) can further amplify inflammatory gene expression. Overall, our results suggest that Zeb1 may exert repressive effects at inflammatory gene promoters through multiple mechanisms in VSMC.

Evidence shows that Zeb1 negatively regulates the expression of miR-200 members through binding to E-box elements located upstream of their promoters in epithelial cells, whereas miR-200 in turn downregulates Zeb1 through 3′-UTR inhibition.23 This reciprocal negative regulatory loop has been implicated in pathological conditions including EMT,22–24 renal fibrosis and diabetic nephropathy.15–17,19 Our current studies demonstrate the operation of this reciprocal regulation and a novel proinflammatory function for miR-200 family members in VSMC. In contrast, Zeb1 knockdown by siRNAs or miR-200 transfection alone did not increase ECM genes such as Col1a2 and Col4a1 expression in VSMC (not shown) suggesting the requirement of additional transcription factors as shown previously in VSMC32 and MC.16 Thus, a balance between Zeb1 and miR-200 members seems to regulate or fine tune the expression of inflammatory genes in VSMC while a dysregulation of this balance under diabetic conditions leads to inappropriate expression (Figure 6G). However, from these studies it is not clear how Zeb1 and miR-200b expression is regulated in the diabetic state. Exposure of VSMC to high glucose (25 mmol/L) alone did not alter miR-200 and Zeb1 levels in VSMC (data not shown). This is in contrast to miR-200b downregulation by high
glucose in human umbilical vein ECs and bovine retinal capillary ECs, mimicking miR-200b downregulation in retinas from streptozotocin induced diabetic rats, further suggesting cell and tissue specific differences in miRNA regulation. The observed changes in miRNAs in diabetic db/dbVSMC could be a cumulative effect of hyperglycemia, dyslipidemia, hyperinsulinemia, and insulin resistance associated with this Type 2 diabetes model. Furthermore, diabetes activates multiple signaling pathways including oxidant stress, protein kinase C, tyrosine kinases, MAPKs, and AGES, many of which are altered in this model. Involvement of these and other potential mechanisms including increased expression of growth factors such as TGF-β1 and PDGF-D in disrupting the miR-200-Zeb1 loop in VSMC awaits further investigation.

Inflammatory genes in VSMC play key roles in the development of vascular complications by promoting VSMC migration, proliferation, and monocyte binding. Previous results from in vitro and ex vivo studies showed that MCP-1 and CX3CL1, which are increased under diabetic conditions, mediate enhanced monocyte-VSMC binding and foam cell formation, key events in the pathogenesis of atherosclerosis. Thus, miR-200–Zeb1 regulation of inflammatory genes such as MCP-1 and COX-2 could promote monocyte binding and subsequent differentiation in the subendothelial space and thereby contribute to accelerated vascular complications. Reports show that elevated COX-2 in db/dbVSMC was also associated with vascular smooth muscle hypercontractility related to hypertension. Therefore, miR-200 mediated COX-2 expression might also be involved in accelerated hypertension associated with diabetes.

In summary, we have identified a novel proinflammatory role for the negative feedback loop between miR-200 and Zeb1 in VSMC. Our previous studies showed the role of miR-125b in promoting inflammatory gene expression through downregulation of the epigenetic repressive histone methyltransferase Suv39h1 in db/dbVSMC. Together, these studies suggest that miRNA mediated targeting and down-regulation of repressive mechanisms regulated by key epigenetic and transcription factors might augment proinflammatory responses of VSMC. These results could lead to the identification of potential new therapeutic targets for the accelerated vascular complications in diabetes.

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None.

References


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SUPPLEMENT MATERIAL

MATERIALS AND METHODS

VSMC isolation: All animal protocols used were approved by the Institutional Animal Use and Care Committee (IACUC). VSMC from thoracic aortas of Type 2 diabetic 10-12 weeks old male \textit{db/db} mice ((BKS.Cg-m+/-leprdb/J) and age matched control littermates \textit{db/+} mice (Jackson laboratories, Bar Harbor, Maine) were isolated by enzymatic digestion as described earlier\(^1\). VSMC were cultured in DMEM/F12 medium supplemented with penicillin/streptomycin, plasmocin and 10% fetal bovine serum. Mouse WEHI monocytes were cultured in DMEM supplemented with antibiotics, 10% FBS and 50 \(\mu\text{mol/L}\) \(\beta\)-mercaptoethanol. HeLa cells were cultured in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum.

Total RNA extraction and RT-PCR: Total RNA including miRNA fraction was extracted using miRNAeasy columns (Qiagen, Valencia, CA), with an on column DNAse I digestion using manufacturer supplied protocols. To prepare cDNAs, 0.5-1.0 \(\mu\text{g}\) of RNA was incubated miScript Reverse Transcriptase Kit reagents (Qiagen, Valencia, CA) in a 20 \(\mu\text{l}\) reaction volume at 37 \(^\circ\text{C}\) for 60 min. Reaction was terminated by incubation at 95 \(^\circ\text{C}\) for 5 min and cDNA was diluted to 100 \(\mu\text{l}\). Levels of miRNAs were determined by Realtime quantitative PCR (QPCR) using miScript SYBR Green PCR Kits with miRNA specific primers provided by the manufacturer (Qiagen, Valencia, CA). The levels of inflammatory gene mRNAs were determined by QPCR using SYBR green master mix (Applied Biosystems, Foster City, CA) with gene specific primers (Table S1). PCR reactions were performed on 7300 or 7500 Realtime PCR systems (Applied Biosystems) in 20 \(\mu\text{l}\) reaction volumes. As internal controls U6 (for miRNAs) and \(\beta\)-Actin
Reddy, Pro-inflammatory function of miR-200 in VSMC

(mRNAs) were used. QPCR data was analyzed by $2^{-\Delta\Delta Ct}$ method and results normalized with internal control genes were expressed % of control or Fold over control$^2$.

**Preparation of cell lysates and Western blotting:** Whole cell lysates from VSMC were prepared by lysing cells in 1.5X Laemmli sample buffer without reducing agents or dyes. Lysates were boiled for 5 min and sonicated briefly to reduce the viscosity. Protein concentration was determined using Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins were mixed with b-mercaptoethanol and bromophenol blue dye and boiled for 5 min. Samples were fractionated on 4-15% Criterion gradient gels and transferred to nitocellulaose membranes using semi-dry blotting apparatus. Blots were blocked with 5% milk in Tris buffered saline containing Triton-X100 (TBST), incubated with indicated antibodies overnight at 4°C. Next day, blots were washed 4X with TBST, incubated with appropriate horse radish peroxidase conjugated secondary antibodies for one h at room temperature. Blots were washed 4X with TBST and protein bands visualized by Chemiluminescence kit (Perkin Elmer, Waltham, MA). Blots were stripped using Restore Western Blot Stripping Buffer (Pierce) and probed with internal control β-Actin antibody (Sigma, St. Louis, MO). Zeb1 antibodies were from Santa Cruz Biotech (Santa Cruz, CA) and Bethyl Laboratories (Montgomery, TX). The protein bands on the developed films were quantified using a calibrated densitometer GS-800 and Quantity One software (Bio-Rad, Hercules, CA).

**Transient transfections and luciferase assays:** VSMC were transfected using Nucleofection technique in Nucleofector equipment (Lonza). VSMC were trypsinized and resuspended in Basic Nucleofector® solution at 1X10$^7$ cells/ml. Indicated plasmid DNAs (0.1-1 μg) or miRNA mimics (1-2 μl of 25 μmol/L) or siRNA (300 ng) oligonucleotides (both obtained from Thermo Scientific) were mixed with 1X10$^6$ cells and transfected in Nucleofector equipment using
program D-33 optimized for VSMC. Cells were plated in 6 well plates for RNA extraction, 65 mm dishes for protein extraction and 24 wells for monocyte binding assays or luciferase assays. HeLa cells were co-transfected with COX-2 reporter plasmids and miRNA mimic oligonucleotides using Xtreme gene (Roche) according to protocols supplied by the manufacturer. Cells transfected with miRNAs were harvested 48 h after transfection and those transfected with siRNAs were harvested 72 h post transfection. Luciferase assays were performed using Dual luciferase assay kit (Promega, Madison, WI) on a Veritas microplate luminometer (Turner BioSystems). To perform ChIP assays, db/+VSMC were transfected with NC-M or 200b-M as indicated above, and cells from multiple transfections were pooled to obtain sufficient amount of cell lysates for ChIP assays which were performed in triplicate.

Chromatin Immunoprecipitation (ChIP) Assays: VSMC were cross-linked with 1% formaldehyde for 10 min at 37 °C and lysed in Immunoprecipitation buffer (IPB) containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5%), Triton X-100 (1.0%), 1 mM PMSF and, 1X Complete Protease mix (Roche). Lysates were centrifuged and chromatin pellets were resuspended in IPB and genomic DNA was fragmented by sonication. Sonicated lysates were immunoprecipitated with Zeb1 antibodies (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4 °C. Immune complexes were collected on Protein A coated magnetic beads (Invitrogen, Carlsbad, CA) for 2 h, washed successively with IPB, IPB+0.35 mmol/L NaCl, IPB+250 mmol/L LiCl at 4 °C and TE (10 mM Tris, pH 8.0 and 1 mM EDTA) at room temperature. ChIP DNA from magnetic beads was eluted and reverse cross linked in elution buffer containing 100 μg/mL of Proteinase K on an Eppendorf vortex mixer at 1200 rpm at 65 °C for 2 hours. DNA was purified by phenol:chloroform extraction and ethanol precipitation. ChIP DNA was amplified by SYBR green based QPCR using promoter specific primers (Table 1) on 7300 or
7500 Realtime PCR systems (Applied Biosystems) analyzed by $2^{-}\Delta\Delta Ct$ method and results normalized to input were expressed as fold over db/+VSMC

**Monocyte-VSMC binding Assays:** Monocyte-VSMC binding assays were performed as described before with some modifications. Briefly, mouse WEHI monocytes were labeled with fluorescent dye BCECF/AM (10 μg/ml) for 1 h at and excess dye was removed by washing cells three times with DMEM containing 0.5% FBS. Fluorescently labeled cells were added to VSMC monolayers plated in quadruplicate in 24 well plates. After incubation for one h at 37°C, unbound monocytes were removed by gently washing VSMC with DMEM+0.5% FBS four times. Then, bound monocytes were observed with an Olympus IX50 fluorescent microscope at ×10 magnification and images from 3-4 different areas in each well were collected using a DP70 camera and Olympus Microsuite software. Number of monocytes bound to VSMC in each image was counted using Image J or Adobe Photoshop CS3 Extended softwares.

**Immunohistochemistry:** Aortas from db/+ and db/db mouse were fixed with formalin and paraffin embedded sections were stained with Zeb1 antibody and counterstained with hematoxylin as described before. Images of stained aortas were collected using Olympus BX51 microscope equipped with a 60x objective, a Pixera 600 camera and InStudio (Pixera Corp) software. Total nuclei and ZEB1 positive nuclei were counted in 5 fields per sample using Adobe Photoshop CS3 software. Results were expressed as % ZEB1 positive nuclei per field.
Figure I: Binding sites for miR-200 family members in the Zeb1 3’-UTR. miR-200 binding sites in Zeb1 3’-UTR predicted by TargetScan.
Figure II: Zeb1 protein inhibition by mR-429 mimics: db/+ VSMC were transfected with negative control mimics (NC-M) or miR-429 mimics (429-M) and cell lysates were collected 48 h post transfection. Lysates were immunoblotted with Zeb1 antibody, stripped and probed with β-Actin antibody.
Figure III: Location of ChIP-QPCR primers used to amplify the indicated gene promoters in ChIP enriched DNA samples. Diagram showing the location of primers (small arrows) in the indicated gene promoters used for the PCR amplification of ChIP DNA samples. Not drawn to scale.

**COX-2 Promoter**

- NF-κB
- CRE
- EB
- -392
- -51-47

**MCP-1 Promoter**

- NF-κB
- SP1
- -156
- -43
### Table I: List of primers used:

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<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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*CypA: Cyclophilin A (peptidylprolyl isomerase A, Ppia)*
REFERENCES


