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miR-200b Mediates Endothelial-to-Mesenchymal Transition in Diabetic Cardiomyopathy

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Hyperglycemia-induced endothelial injury is a key pathogenetic factor in diabetic cardiomyopathy. Endothelial injury may lead to a phenotypic change (i.e., endothelialto-mesenchymal transition [EndMT]), causing cardiac fibrosis. Epigenetic mechanisms, through specific microRNA, may regulate such a process. We investigated the mechanisms for such changes in cardiac microvascular endothelial cells and in the heart of genetically engineered mice with chemically induced diabetes. Cardiac tissues and isolated mouse heart endothelial cells (MHECs) from animals with or without endothelialspecific overexpression of miR-200b, with or without streptozotocin-induced diabetes, were examined at the mRNA and protein levels for endothelial and mesenchymal markers. Expression of miR-200b and its targets was quantified. Cardiac functions and structures were analyzed. In the hearts of wild-type diabetic mice, EndMT was observed, which was prevented in the miR-200b transgenic diabetic mice. Expression of specific markers such as vascular endothelial growth factor, zinc finger E-box-binding homeobox, transforming growth factor- β 1, and p300 were increased in the hearts of diabetic mice and were prevented following miR-200b overexpression. MHECs showed similar changes. miR-200b overexpression also prevented diabetes-induced cardiac functional and structural changes. These data indicate that glucose-induced EndMT in vivo and in vitro in the hearts of diabetic mice is possibly mediated by miR-200b and p300.

Focal myocardial fibrosis is a structural hallmark of diabetic cardiomyopathy, manifesting functionally as defective

diastolic compliance and cardiac contractility (1–3). Myocardial fibrosis results from increased production and deposition of extracellular matrix (ECM) proteins, such as collagen. Endothelial cells (ECs) are initial targets of hyperglycemic damage and play a major role in the production of ECM proteins in all chronic diabetic complications. At the structural level in the heart, such changes are manifested as thickening of the capillary basement membrane, focal myocardial fibrosis, and so forth (3,4). A result of sustained injury in the fibrotic diseases is that ECs undergo a process of transdifferentiation (i.e., endothelial-to-mesenchymal transition [EndMT]) (5,6). Such changes in the ECs are manifested by a loss of endothelial markers and development of mesenchymal markers (7). Several reports have shown mesenchymal transition of ECs (i.e., EndMT during cardiac fibrosis) (5-7). This EndMT is similar to epithelial-to-mesenchymal transformation in cancer cells. EndMT is a key source of cardiac fibroblasts causing increased production of ECM proteins (5-7) and is characterized by a loss of cell junctions, vascular endothelial (VE)-cadherin, and CD31 expression and a gain of mesenchymal markers, such as vimentin (Vim) and α -smooth muscle actin (SMA) (5–7). Upregulation of p300 has been shown to be a key event in EndMT in the heart (5). The same study also demonstrated the alteration of several microRNAs (miRNAs) in EndMT of nondiabetic etiology (5). We previously demonstrated EndMT in the retina of diabetic animals (8) as well as a pathogenetic role of endothelin 1 (ET-1) in diabetic cardiomyopathy (3,4). Of note, ET-1 has been demonstrated to be of significance in EndMT in the diabetic heart (9). We also

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have demonstrated that p300 is upregulated in the cardiac tissues of diabetic animals (10). p300-Mediated histone acetylation is a key mechanism in regulating gene transcription. Such epigenetic mechanisms also influence miRNA production and vice versa (10,11).

miRNAs are small (~20-25-nucleotide) RNA molecules that significantly affect the regulation of gene expression (11,12). The transcription of miRNAs occurs through RNA polymerase II, and their processing to precursor miRNAs (70-100 nucleotides, hairpin-shaped) in the nucleus is mediated by RNAse III Drosha and DGCR8. Following synthesis, they are exported to the cytoplasm by exportin 5. In the cytoplasm, they are processed by Dicer into mature miRNA, the functionally active form. miRNAs bind to specific mRNA targets, causing their degradation or translational inhibition. The importance of miRNAs has been demonstrated in diverse cellular processes (11,12). We have previously demonstrated alterations of multiple miRNAs in ECs exposed to high glucose and in multiple tissues affected in chronic diabetic complications, including the heart. The list includes miR-133a, miR-1, miR-320, miR-146a, and miR-200b. Studies from our laboratory have demonstrated that miR-200b is a key mediator of EndMT in the diabetic retina (8,13-17).

The purpose of the current study was to investigate whether EndMT is of pathogenetic significance in diabetic cardiomyopathy and whether such changes are mediated through alteration of miR-200b and/or other epigenetic mechanisms, such as p300 alterations. We also investigated whether manipulation of miR-200b is a potential therapeutic approach to prevent EndMT. To test miR-200b-mediated EndMT in diabetic cardiomyopathy, we generated endothelial-specific (using a Tie2 promoter) miR-200b-overexpressing mice in which we chemically induced diabetes (8).

RESEARCH DESIGN AND METHODS

Animal Studies

We performed all animal experiments according to the Guiding Principles in the Care and Use of Animals. Experimental protocols were reviewed and approved by the Western University Council on Animal Care Committee. The experiments were also designed to conform with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication no. 85-23, revised 1996).

As described previously, we used transgenic mice with EC-specific overexpression of miR-200b generated by us (8). We cloned a cDNA fragment containing miR-200b into pg52pSPTg.T2FpAXK (pg52) plasmid containing a Tie2 promoter and enhancer and an SV40 PolyA signal (courtesy of T. Sato, Nara Institute of Science and Technology Graduate School of Biological Sciences, Ikoma, Japan) (18). We excised Tie2-miR-200b transgene by using *Sal*I and injected gel-purified transgene into pronuclei of fertilized eggs from C57BL/6 crossed with CBA/J mice. These were then transferred into pseudopregnant female

mice (18). PCR-based assays of the genomic DNA extracted from tail-tip biopsy specimens as a template were used to identify transgenic strains according to the previously described protocol (8). Phenotypic characterization revealed no alteration in the transgenic mice.

We divided male miR-200b transgenic mice and ageand sex-matched wild-type littermates (weighing 23-26 g) into diabetic and control groups. Diabetes was induced by five intraperitoneal injections of streptozotocin (STZ) 50 mg/kg in citrate buffer (pH 4.5) (controls received the same volume of buffer) on consecutive days as previously described (14-16). Hyperglycemia was confirmed by measuring blood glucose levels (at least twice) 3 days after the last STZ injection. Following confirmation of diabetes, the mice were maintained in a hyperglycemic state for 2 months (without exogenous insulin). At the end of the follow-up period, echocardiography was performed in all mice. The mice were then killed, and cardiac tissues and blood were collected. Most of the cardiac tissues were stored frozen $(-70^{\circ}C)$ for various analysis (see next sections). A small portion of ventricular tissue was fixed in 10% neutral-buffered formalin. These tissues were embedded in paraffin. For histologic studies, 5-µm sections with hematoxylin-eosin, immunofluorescence, and trichrome staining were used.

Echocardiography

Lightly anesthetized (1.5% inhaled isoflurane) mice were subjected to echocardiography on a warm handling platform. We used a 40-MHz linear array transducer (MS550D) and Vevo 2100 preclinical ultrasound system (VisualSonics, Toronto, ON, Canada) with nominal inplane spatial resolution of 40 (axial) \times 80 (lateral) μ m. M-mode and two-dimensional parasternal short-axis scans (133 frames/s) at the level of the papillary muscles were obtained to assess changes in left ventricular (LV) end-systolic inner diameter (LVIDs) and LV end-diastolic inner diameter (LVIDd). LV volumes at end diastole (LVEDV) and end systole (LVESV) were calculated by Eqs. 1 and 2:

$$LVEDV = [7/(2.4 + LVIDd)] \times [LVIDd^3]$$
 (Eq. 1)

$$LVESV = [7/(2.4 + LVIDs)] \times [LVIDs^3]$$
 (Eq. 2)

LV fractional shortening (FS) and ejection fraction (EF) were used as indexes of cardiac contractile function and were calculated from the inner diameters by Eqs. 3 and 4:

$$FS [\%] = (LVIDd - LVIDs)/LVIDd \times 100$$
 (Eq. 3)

$$EF[\%] = (LVEDV - LVESV)/LVEDV \times 100$$
 (Eq. 4)

Color flow-guided, pulsed wave Doppler recordings of the maximal early (E) and late (A) diastolic transmitral flow velocities and Doppler tissue imaging recordings of peak E' velocity and peak A' velocity were carried out. Mitral

inflow patterns (E/A ratio) and mitral annulus velocities (E'/A' ratio) were used to assess diastolic dysfunction.

Cell Isolation and Culture

We used isolated primary mouse heart ECs (MHECs) as previously described (19). Briefly, on the day before the experiment, 100 µL sheep anti-rat DynaBeads per litter (5-7 mice) were prepared by washing in 0.1% BSA-PBS and were resuspend 0.1% BSA-PBS. One hundred microliters of beads were mixed with 10 μ L of rat anti-mouse platelet EC adhesion molecule 1 monoclonal antibody (Invitrogen Canada, Burlington, ON, Canada) and incubated overnight at 4°C. The beads were then washed and resuspended in DMEM containing 10% FBS. After harvesting, the hearts were cut into small pieces and rinsed in Hanks' balanced salt solution buffer. They were then digested with collagenase A. Ten percent FBS-DMEM was added. The mixtures were filtered using nylon mesh (70-µm pores), pelleted by centrifugation, washed, and resuspended in the same solution. They were incubated with CD31 beads prepared by the same method. They were washed, and bound cells were cultured in 3.5-cm dishes precoated with 2% gelatin (Sigma, Oakville, ON, Canada) in endothelial basal medium 2 with 10% FBS. The remaining cells were incubated at 37°C for 1 h. The suspended cells (myocytes) and cells attached on the bottom of the flask (fibroblasts) were separated.

Isolated MHECs were plated at a density of 1×10^5 cells/mL. After 24 h of serum-free media incubation, MHECs were incubated with various levels of D-glucose for 48 h. Each experiment was performed with three or more samples.

mRNA Extraction and Quantitative PCR

Isolation of total RNA was performed using TRIzol reagent (Invitrogen Canada) according to the manufacturer's protocol and as described previously (13–17). The extracted RNA was used for cDNA synthesis by using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). Quantification of various transcripts was performed by real-time quantitative RT-PCR (LightCycler; Roche Diagnostics, Laval, QC, Canada). Primers for the PCR reactions were designed using Primer 5 software (Supplementary Table 1).

miRNA Analysis

For miRNA analyses, we used a *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX) for miRNA extraction from cardiac tissues collected as described previously (14,16). The cDNA was synthesized with TaqMan microRNA Assay Reverse Transcription Primer and MultiScribe Reverse Transcriptase (Life Technologies) by using the manufacturer's protocol. Real-time quantitative RT-PCR for miR-200b was performed with the TaqMan microRNA Assay (Light-Cycler 96) as described previously. The data were normalized to U6 small nuclear RNA to account for differences in reverse transcription efficiencies and the amount of template (13–16). miRNA from serum was extracted using the miRNeasy Serum/Plasma Kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer's instructions. The isolated serum miRNAs were analyzed as described earlier.

miRNA Mimic or Antagomir Transfection

ECs were transfected with mRNA-200b mimic or antagomir (20 nmol/L) using transfection reagent Lipofectamine 2000 (Invitrogen Canada). Scrambled control was used in parallel. miRNA transfection efficiency was determined by real-time RT-PCR by using established techniques (14,15).

Western Blotting and ELISA

The cells and tissues at the end of various treatments were lysed with radioimmunoprecipitation assay buffer. Total protein levels were quantified using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Briefly, 30 µg total protein from each sample was loaded to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat milk in PBS containing 0.05% Tween-20. The membranes were incubated with specific primary antibodies (anti-CD31 and anti-fibroblast-specific protein 1 [FSP1] antibodies [Abcam, Cambridge, MA], anti-zinc finger E-box-binding homeobox 1 [ZEB1] antibody [Santa Cruz Biotechnology, Dallas, TX], anti-SMAD/phospho-SMAD2/3 [Cell Signaling, Danvers, MA]). They were then incubated with corresponding horseradish peroxidase-conjugated anti-mouse (Invitrogen Canada) or anti-rabbit (Santa Cruz Biotechnology) secondary antibodies. After washing, the detections were carried out with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA). Blots were stripped and reprobed with β -actin antibody (Abcam) and analyzed by densitometry. ELISA for p300 was performed using commercially available kits (USCN Life Science Inc., San Diego, CA) according to the manufacturer's instructions.

Immunofluorescence

Cardiac tissue sections were deparaffinized with xylene and alcohol. After washing and incubation with 5% normal serum, the sections were incubated in one of the specific primary antibodies (mouse anti-CD31, rabbit anti-FSP1, rabbit anti-CD34 [Abcam] at 1:100 dilution; mouse anti- α -SMA [Sigma] at 1:400 dilution) followed by incubation in corresponding Alexa Fluor 488–conjugated secondary antibody (1:200 goat anti-mouse IgG or goat anti-rabbit IgG [Invitrogen Canada]). The sections were examined with a fluorescent microscope (Olympus BX51; Olympus America, Melville, NY) and analyzed with ImageJ software.

Statistical Analyses

Data are expressed as mean \pm SEM. Significant differences were determined using ANOVA and Student *t* test, with post hoc analyses as appropriate. Differences were considered significant at $P \leq 0.05$.



Figure 1—miR-200b expression analyses in mouse hearts (*A*) and in cardiac cellular components (*B*–*D*) showing reduced cardiac miR-200b in the wild-type animals with diabetes (D-B6) compared with controls (C-B6) (*A*). Analyses of isolated cells from the hearts showed that such reduction happened predominantly in the ECs (*C*) and not in the myocytes (*B*). Analyses of various cells from the EC-specific transgenic mice confirmed overexpression (~14-fold) in the ECs but not in the fibroblasts or myocytes from the transgenic mice compared with the controls (*D*). mRNA analyses of isolated ECs from the hearts showed glucose-induced downregulation of endothelial marker CD31 (*E*) and upregulation of mesenchymal marker Vim mRNA expression (*F*). These changes were associated with glucose-induced upregulation of VEGF (*G*) and ZEB1 (*H*) mRNA expression. These changes were prevented in the ECs of the diabetic mice with endothelial-specific miR-200b overexpression (200b) (ECs, following isolation, were incubated in 5 mmol/L glucose [NG] or 25 mmol/L glucose [HG]). mRNA levels are the ratio to β-actin and normalized to NG. miRNA levels are expressed as a ratio of U6 small nuclear RNA (U6) and normalized to controls. *Significantly different from NG B6 or C-B6 or other groups. **Significantly different from HG B6.

RESULTS

miR-200b Is Reduced in ECs in the Hearts of Diabetic Mice

We first examined miR-200b levels in the hearts of diabetic mice. Quantitative RT-PCR analyses showed that miR-200b levels are reduced in whole hearts of diabetic mice compared with age- and sex-matched nondiabetic controls (Fig. 1A). We then examined which cells are responsible for this reduction, hence, isolated ECs and cardiomyocytes. As expected, there were no significant changes of miR-200b levels in the cardiomyocytes (Fig. 1*B*), but the ECs showed a significant (~45%) decrease in miR-200b expression (Fig. 1C). Because cellular distribution had a specific implication in these studies, we also confirmed the cell type that overexpressed miR-200b in the transgenic mice. To this extent, we isolated myocytes, ECs, and fibroblasts from the transgenic and wild-type mice and found no significant differences in miR-200b levels in the fibroblasts and myocytes. The ECs, however, showed an \sim 14-fold overexpression of miR-200b in the transgenic mice compared with wild-type controls (Fig. 1D).

Cardiac ECs Show miR-200b–Dependent EndMT in High Glucose

We then explored whether cardiac ECs develop glucoseinduced miR-200b–dependent EndMT by isolating ECs from wild-type B6 mice and miR-200b transgenic mice, growing them in either 5 or 25 mmol/L glucose and measuring specific markers for EndMT. Glucose exposure of wild-type ECs showed reduced CD31 and increased Vim mRNA expression (Fig. 1*E* and *F*). In parallel, mRNA levels of vascular endothelial growth factor (VEGF) and ZEB1 (two additional targets of miR-200b) were increased following 25 mmol/L glucose exposure, but these changes were prevented in the transgenic mouse ECs (Fig. 1*G* and *H*).

Endothelial miR-200b Overexpression Prevents Diastolic Dysfunction in the Heart in Diabetes

We examined whether EndMT can produce functional deficits characteristic of diabetic cardiomyopathy and whether such changes are miR-200b dependent. To examine the functional deficits characteristic of diabetic cardiomyopathy, we used STZ-induced diabetic mice. The mice demonstrated hyperglycemia and reduced body weight gain after 2 months of poorly controlled diabetes (Supplementary Table 2) and further showed glucosuria and polyuria (data not shown). Echocardiographic examination showed a slight nonsignificant increase in EF and FS (Fig. 2A and B). However, E/A and E'/A' ratios were significantly reduced in the wild-type diabetic mice, indicating diastolic dysfunction. These changes were corrected in the transgenic animals (Fig. 2C and D). Additional parameters of echocardiographic data are provided in Supplementary Table 3.

In the Heart, Diabetes-Induced EndMT Is Mediated by miR-200b

EndMT is characterized by loss of endothelial markers and acquisition of mesenchymal markers (8). To examine

the potential role of miR-200b in this process, we examined endothelial and mesenchymal markers in cardiac tissues from miR-200b transgenic mice and littermate controls with and without STZ-induced diabetes. In keeping with our hypothesis, mRNA expression of endothelial markers CD31, VE-cadherin, and CD34 were reduced (\sim 45%, 35%, and 37%, respectively) in the hearts of wild-type mice with diabetes (Fig. 3A, D, and F) in association with increased mRNA expression of mesenchymal markers FSP1, Vim, α -SMA, and collagen 1 α 1 (COL1) and $4\alpha 1$ (COL4) (varying from ~20–60%), confirming EndMT in animals with diabetes (Figs. 4A, D, and F and 5A and B). The protein levels of endothelial markers, such as CD31 and CD34 (Fig. 3B, C, E, and G), as well as of mesenchymal markers, such as FSP1 and α -SMA (Fig. 4B, C, E, and G), showed similar changes. Additional immunofluorescence stain for CD31, CD34, FSP1, and α -SMA confirmed such alterations in the cardiac microvascular ECs (Figs. 3E and G and 4E and G). Of note, no alterations of collagen $3\alpha 1$ (COL3) were seen (Fig. 5C).

All such abnormalities were prevented in the miR-200b transgenic mice with diabetes, further establishing a pathogenetic mechanism involving miR-200b in cardiac EndMT in diabetes (Figs. 3-5). However, the level of prevention varied among the transcripts (Figs. 3-5). In parallel, focal myocardial fibrosis was present in the hearts of wild-type diabetic animals (Fig. 5E). Such foci (one to three per animal) were identified in the wild-type diabetic mice and were prevented in the transgenic mice with diabetes. To understand whether circulating miR-200b played a role in the generation or prevention of cardiac EndMT in diabetes, we measured serum miR-200b levels. The results showed no significant differences in the serum miR-200b levels among the various groups, indicating that the observed effects were mediated by autocrine/paracrine effects of miR-200b (Fig. 5D).

miR-200b Prevents EndMT Through Multiple Mechanisms

All miRNAs, including miR-200b, have multiple targets. Hence, we examined other miR-200b targets modulated by glucose exposure. We have previously demonstrated that miR-200b has additional effects on other molecules of interest in chronic diabetic complications (15). To this extent, we examined VEGF, p300, ZEB1, ZEB2 (all targets of miR-200b), and transforming growth factor- β (TGF- β), molecules that have been demonstrated to play key roles in EndMT in other situations (5-8,15). We measured RNA by RT-PCR and proteins by ELISA or by Western blot. In the hearts of diabetic mice, all these transcripts and the corresponding proteins were elevated, but they were prevented in the miR-200b transgenic animals with diabetes (Fig. 6A–H). We have previously shown that VEGF and p300 are direct targets of miR-200b (8,15). Here, we carried out additional





Figure 2—Echocardiographic analyses of mice showing a nonsignificant increase in the EF (*A*) and FS (*B*) in wild-type (B6) diabetic (D) mice compared with controls (C). There was a significant decrease in the E/A ratio (*C*) and E'/A' ratio (*D*) in the wild-type diabetic animals. Such diabetes-induced changes were corrected in the transgenic mice with endothelial-specific miR-200b overexpression (200b). *E*: Representative tracings of acquisition and quantification of echocardiography and Doppler data in mice showing an M-mode tracing indicating end-systolic diameter (ESD) and end-diastolic diameter (EDD), tissue Doppler imaging of the LV posterior wall, and a pulsed wave Doppler recording of mitral valve leaflet tips showing mitral inflow velocity patterns from which E and A are calculated. *Significantly different from C-B6 ($n \ge 8$ /group). Additional echocardiographic data are shown in Supplementary Table 3.

experiments in the ECs by using miR-200b mimics and miR-200b antagomirs, which suggest that these are also directly modulated by miR-200b (Supplementary Fig. 1). To further investigate downstream mediators of TGF- β signaling, we performed Western blot analyses SMAD2/3. These assays showed upregulation of SMAD2/3, an miR-200b target. These changes were also prevented in the miR-200b transgenic mice (Fig. 6*I* and *J*).

DISCUSSION

In the current study, we show that diabetes-induced endothelial phenotypic change (i.e., EndMT) in the heart is responsible for increased ECM protein production. We demonstrated these changes in the glucoseexposed ECs isolated from wild-type and transgenic mice with endothelial-specific miR-200b overexpression and confirmed that miR-200b regulates such processes



Figure 3—Effect of diabetes and endothelial-specific miR-200b overexpression on mRNA expression of EndMT-associated endothelial markers. mRNA expression of CD31 (*A*), VE-cadherin (VE-cad) (*D*), and CD34 (*F*) and protein expression of CD31 (*B* and *C*) were significantly reduced (representative Western blot and densitometry, respectively) in the hearts of wild-type (B6) diabetic (D) animals compared with controls (C). Immunofluorescence stain confirmed decreased CD31 protein (*E*) and decreased CD34 protein (*G*) expression in the cardiac microvascular endothelial cells (arrows) in the hearts of wild-type diabetic animals compared with controls. Such alterations were prevented in the hearts of the transgenic mice with endothelial-specific miR-200b overexpression (200b). *Significantly different from C-B6. mRNA and protein levels are expressed as a ratio to β -actin and normalized to C ($n \ge 8$ /group; bar = 50 μ m [same magnification for all micrographs in each panel]).

and that endothelial-specific miR-200b overexpression prevents diabetes-induced cardiac functional and structural abnormalities.

High glucose levels in diabetes is widely recognized as a key initiating factor for multiple chronic diabetic complications (2,8,20,21). ECs are one of the first cell types exposed to hyperglycemia. Elevated intracellular glucose levels may initiate multiple interlinked metabolic abnormalities, such as oxidative stress, protein kinase C activation, and increased nonenzymatic glycation (20,21). These metabolic alterations ultimately converge on the endothelial nucleus. Possibly as a defensive and/or compensatory response to cellular damage, ECs start producing altered amounts of multiple proteins. Such gene transcriptions at several levels are regulated by epigenetic mechanisms, such as acetylation and methylation, at the transcription or posttranscription level through miRNAs (11,12,22). Abnormal protein production may result in cellular phenotypic changes. Although we and others have previously demonstrated some aspects of EndMT (e.g.,



Figure 4—Effect of diabetes and endothelial-specific miR-200b overexpression on mRNA expression of EndMT-associated mesenchymal markers. mRNA expression of FSP1 (*A*), Vim (*D*), and α -SMA (*F*) and protein expression of FSP1 (*B* and *C*) were significantly increased (representative Western blot and densitometry, respectively) in the hearts of wild-type (B6) diabetic (D) animals compared with controls (C). Immunofluorescence stain confirmed increased FSP1 protein (*E*) and increased α -SMA protein (*G*) expression in the cardiac microvascular endothelial cells (arrows) in the hearts of wild-type diabetic animals compared with controls. Such alterations were prevented in the hearts of transgenic mice with endothelial-specific miR-200b overexpression (200b). *Significantly different from C-B6. **Significantly different from D-B6. mRNA levels are expressed as a ratio to β -actin and normalized to C ($n \ge 8$ /group; bar = 50 μ m [same magnification for all micrographs in each panel]).



Figure 5—Effect of diabetes and endothelial-specific miR-200b overexpression on cardiac mRNA expression of ECM proteins associated with EndMT. mRNA expression of COL1 (*A*) and COL4 (*B*) were increased in the hearts of wild-type (B6) diabetic (D) animals compared with controls (C). No significant alterations in COL3 mRNA were seen (C). Such changes were not associated with alterations of serum miR-200b among various groups (*D*). Furthermore, trichrome stain (*E*) showed increased focal fibrosis (arrow, green stain) in the hearts of diabetic animals compared with control animals. Such diabetes-induced changes were corrected in the transgenic (TG) mice with endothelial-specific miR-200b overexpression (200b). *Significantly different from C-B6. **Significantly different from D-B6. mRNA levels are expressed as a ratio to β -actin and normalized to C ($n \ge 8$ /group; bar = 50 µm [same magnification for all micrographs]).



Figure 6—Effect of diabetes and endothelial-specific miR-200b overexpression on the expression of markers associated in the pathogenesis of EndMT. mRNA expression of ZEB1 (*A*) and ZEB2 (*D*) and protein expression ZEB1 (*B* and *C*) (representative Western blot and densitometric quantification, respectively) showing that ZEB1 and ZEB2 levels were significantly increased in the hearts of wild-type (B6) diabetic (D) animals compared with the controls (C). In addition, mRNA expression of p300 (*E*), VEGF (*G*), and TGF- β (*H*) and protein expression of p300 (*F*) (measured by ELISA) were significantly increased in the hearts of D-B6 mice compared with the controls. Similar upregulation of SMAD2/3 was also seen in the heart of wild-type diabetic mice (*I* and *J*) (representative Western blot and densitometric quantification, respectively). Such diabetes-induced changes were corrected in the transgenic mice with endothelial-specific miR-200b overexpression (200b). Significantly different from C-B6. **Significantly different from D-B6. mRNA and protein levels are expressed as a ratio to β -actin (except for SMAD2/3, where a phospho-SMAD2/3/SMAD2/3 [P-Smad2/3/Smad2/3] ratio was used) and normalized to C ($n \ge 8$ /group for all experiments).

increased ECM protein production in diabetic cardiomyopathy and in other chronic diabetic complications [8,9,23,24]), the role of specific miRNA has not been demonstrated previously. In the current study, we show glucose-induced reduced expression of EC markers, such as CD31, CD34, and VE-cadherin, and increased expression of multiple mesenchymal markers, such as FSP1, COL4, COL1, SMA, and Vim, confirming EndMT. We further show that miR-200b regulates such processes. Of note, the amount of regulation varied among the transcripts. Overall, 30-40% of EndMT-related transcripts appeared to be influenced by miR-200b, but in the case of some mesenchymal transcripts, miR-200b appeared to have pronounced effects, the exact reasons for which are not clear. In keeping with the current findings, in pressure-induced cardiac damage, EndMT has been shown to contribute significantly to cardiac fibrosis and heart failure (24). However, miR-200b also regulates p300 production (8). As shown in this and our previous reports, a large number of molecules that cause EndMT may further be regulated through p300-dependant histone acetylation (8,10,15,25). Further experiments are needed to decipher the specific contribution of each pathway.

EndMT may happen in several chronic diseases. In the heart, physiologic development of cardiac valves depends on EndMT (5,26,27). In keeping with the current study, EndMT has been demonstrated in various diseases that lead to cardiac fibrosis (24,26,27). Oxidative stress may also be a key factor in such processes (27,28). Increased production of ECM proteins and endothelial dysfunctions are well established features of diabetic cardiomyopathy (10,14,29,30). The data generated from the current study indicate phenotypic change in the ECs linking such processes. We have previously demonstrated the role of miR-200b in augmented VEGF production in diabetes and its role in EndMT in the retina (8,15) and demonstrate similar findings in the current study. VEGF upregulation is known in several chronic diabetic complications (15,21). Furthermore, VEGF alteration has been demonstrated in pressure-induced EndMT (24).

In keeping with our previous finding in diabetic retinopathy, we show that miR-200b also modulates EndMT through modulation of transcription coactivator p300 (8,15). Alterations of p300 have previously been shown in cardiac EndMT (26), and p300 is known to regulate a large number of transcripts (10,25,31). We have also shown that p300 production is regulated by miR-200b (15). Conceptually, through p300, miR-200b may regulate multiple molecules, such as ET-1 and TGF-B. ET-1 has been shown previously to be involved in EndMT in the diabetic heart (9). In keeping with our previous retinal data, this study also showed that TGF-\beta-mediated SMAD signaling plays a key role in cardiac EndMT (8). We have previously demonstrated that other members of the miR-200 cluster (miR-200a, miR-200c, and miR-429) are not significantly altered by glucose and may not have pathogenetic significance (15). In addition, this study supports the important role ECs play in the pathogenesis of diabetic cardiomyopathy (15,21,30), indicating that alterations in ECs may be a primary initiating factor for the development of diabetic complications and further opening up new targets to block EndMT and its consequences. Such notions need to be further validated by carefully conducted long-term studies.

We also recognize that several other miRNAs may be altered in EndMT (5,14,16,26). In a previous study, miR-125b has been implicated in TGF- β 2-mediated cardiac EndMT (5). Whether similar changes or additional miRNA changes occur specifically in diabetic cardiomyopathy would be of interest for future examination.

In summary, the current data indicate an miR-200bmediated mechanism for the development of EndMT in the context of diabetic cardiomyopathy. Such findings open up the possibility of future RNA-based therapeutic strategies.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

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References

1. LeWinter MM. Diabetic cardiomyopathy: an overview. Coron Artery Dis 1996;7:95–98

2. Shehadeh A, Regan TJ. Cardiac consequences of diabetes mellitus. Clin Cardiol 1995;18:301–305

 Chen S, Evans T, Mukherjee K, Karmazyn M, Chakrabarti S. Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. J Mol Cell Cardiol 2000;32:1621–1629

4. Hileeto D, Cukiernik M, Mukherjee S, et al. Contributions of endothelin-1 and sodium hydrogen exchanger-1 in the diabetic myocardium. Diabetes Metab Res Rev 2002;18:386–394

5. Ghosh AK, Nagpal V, Covington JW, Michaels MA, Vaughan DE. Molecular basis of cardiac endothelial-to-mesenchymal transition (EndMT): differential expression of microRNAs during EndMT. Cell Signal 2012;24:1031–1036

6. Yoshimatsu Y, Watabe T. Roles of TGF- β signals in endothelial-mesenchymal transition during cardiac fibrosis. Int J Inflam 2011;2011:724080

7. Zeisberg EM, Tarnavski O, Zeisberg M, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007;13:952–961

8. Cao Y, Feng B, Chen S, Chu Y, Chakrabarti S. Mechanisms of endothelial to mesenchymal transition in the retina in diabetes. Invest Ophthalmol Vis Sci 2014; 55:7321–7331

9. Widyantoro B, Emoto N, Nakayama K, et al. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. Circulation 2010;121:2407–2418

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10. Kaur H, Chen S, Xin X, Chiu J, Khan ZA, Chakrabarti S. Diabetes-induced extracellular matrix protein expression is mediated by transcription coactivator p300. Diabetes 2006;55:3104–3111

11. Chuang JC, Jones PA. Epigenetics and microRNAs. Pediatr Res 2007;61: 24R-29R

12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297

13. Feng B, Cao Y, Chen S, Ruiz M, Chakrabarti S. miRNA-1 regulates endothelin-1 in diabetes. Life Sci 2014;98:18–23

14. Feng B, Chen S, McArthur K, et al. miR-146a-Mediated extracellular matrix protein production in chronic diabetes complications. Diabetes 2011;60: 2975–2984

15. McArthur K, Feng B, Wu Y, Chen S, Chakrabarti S. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes 2011;60:1314–1323

 Chen S, Puthanveetil P, Feng B, Matkovich SJ, Dorn GW 2nd, Chakrabarti S. Cardiac miR-133a overexpression prevents early cardiac fibrosis in diabetes. J Cell Mol Med 2014;18:415–421

17. Feng B, Chakrabarti S. miR-320 regulates glucose-induced gene expression in diabetes. ISRN Endocrinol 2012;2012:549875

 Schlaeger TM, Qin Y, Fujiwara Y, Magram J, Sato TN. Vascular endothelial cell lineage-specific promoter in transgenic mice. Development 1995;121: 1089–1098

19. Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, Lechler RI. Isolation of endothelial cells from murine tissue. J Immunol Methods 2000;244:205–215

20. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes: the role of reparatory mechanisms. Diabetes Care 2011;34(Suppl. 2):S285–S290 21. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615–1625

22. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–531

23. Li J, Qu X, Bertram JF. Endothelial-myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in streptozotocininduced diabetic mice. Am J Pathol 2009;175:1380–1388

24. Gogiraju R, Xu X, Bochenek ML, et al. Endothelial p53 deletion improves angiogenesis and prevents cardiac fibrosis and heart failure induced by pressure overload in mice. J Am Heart Assoc 2015;4:e001770

25. Chen S, Feng B, George B, Chakrabarti R, Chen M, Chakrabarti S. Transcriptional coactivator p300 regulates glucose-induced gene expression in endothelial cells. Am J Physiol Endocrinol Metab 2010;298:E127–E137

26. Eisenberg LM, Markwald RR. Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res 1995;77:1-6

27. Piera-Velazquez S, Jimenez SA. Molecular mechanisms of endothelial to mesenchymal cell transition (EndoMT) in experimentally induced fibrotic diseases. Fibrogenesis Tissue Repair 2012;5(Suppl. 1):S7

28. Liu RM, Gaston Pravia KA. Oxidative stress and glutathione in TGFbeta-mediated fibrogenesis. Free Radic Biol Med 2010;48:1-15

29. Feng B, Ruiz MA, Chakrabarti S. Oxidative-stress-induced epigenetic changes in chronic diabetic complications. Can J Physiol Pharmacol 2013;91: 213–220

30. Farhangkhoee H, Khan ZA, Kaur H, Xin X, Chen S, Chakrabarti S. Vascular endothelial dysfunction in diabetic cardiomyopathy: pathogenesis and potential treatment targets. Pharmacol Ther 2006;111:384–399

31. Ghosh AK, Varga J. The transcriptional coactivator and acetyltransferase p300 in fibroblast biology and fibrosis. J Cell Physiol 2007;213:663–671