Identification of MicroRNA-93 As A Novel Regulator of Vascular Endothelial Growth Factor (VEGF) In Hyperglycemic Conditions

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Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein with a central role in angiogenesis, vascular homeostasis, and the maintenance of capillary integrity, particularly in the kidney glomerulus (1,2). Several lines of evidence have recently suggested that precise regulation of glomerular VEGF expression in the kidney is required for the proper development and function of glomerulus (2,3). First, pharmacological and genetic disruptions of VEGF were shown to result in significant proteinuria and glomerular endotheliosis (2,4,5). Second, the administration of an adenovirus expressing sFlt (soluble fms-like tyrosine kinase) in animal models also caused endotheliosis and proteinuria (6). Finally, targeted deletion of VEGF in podocytes, the major source of VEGF production in the kidneys, resulted in thrombotic glomerular injury (2). While these observations suggest a detrimental effect of low levels of VEGF in the kidney, high levels of VEGF have also been implicated in the pathogenesis of a variety of inflammatory diseases, particularly in microvascular complications of diabetes (2,3,7). Indeed, VEGF has emerged as a major mediator of diabetic retinopathy and nephropathy (7-9). A pathogenic role for VEGF in diabetic nephropathy (DN) was inferred from reports indicating that VEGF-A was upregulated in the kidneys of animal models of DN. The use of anti-VEGF in streptozotocin (STZ)-induced type-1 diabetic animals or in db/db type-2 diabetic mice was shown to result in significant improvement in the kidney function in animal models of DN (10,11). However, the regulatory mechanisms by which glomerular VEGF expression is tightly maintained in the kidneys are unknown.

A potential mechanism for the precise regulation of VEGF in the kidneys might be through its regulation by microRNAs (miRNAs).
miRNAs are a class of short (21~24 nucleotides), non-coding RNA molecules that are evolutionarily conserved, and function as negative regulators of gene expression (12,13). The involvement of miRNAs as novel regulators of a variety of biological processes in the cell as well as their roles in diverse pathologies is increasingly reported (14,15). Of particular interest, several recent reports have implicated miRNAs in the regulation of kidney development and angiogenesis (16,17).

Despite the critical role of VEGF in microvascular complications of diabetes, the regulatory role of miRNAs on VEGF remains unknown. Here, we investigated the role of miRNAs on VEGF expression in the hyperglycemic environment using an integrated in vitro and in vivo approach. We generated miRNA expression profiles from glomerular kidney samples from diabetic db/db mice as well as from cultured podocytes and kidney microvascular endothelial cells exposed to elevated glucose concentrations. We identified and validated miR-93 as a key regulator of VEGF signaling in the kidneys. Using a combination of gain of function and silencing experiments, we showed that miR-93 is a critical regulator of VEGF expression in the diabetic milieu both in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Morpholino Oligomers**—The 3'-UTR of the mouse vegfa gene (NM_001025250) was amplified from podocytes genomic DNA by PCR using HotMaster Taq DNA Polymerase (5PRIME, Gaithersburg, MD), with the following primers: GTCTCGAGGGTTTCGGGAACCAGACCTCTCA (forward), CAGAATTC CAGAAACAACCCTAATCTTCCGGG (reverse). PCR product was cloned between XhoI and EcoRI sites of luciferase reporter vector 3.1-luc, kindly provided by Dr. Ralph Nicholas (Dartmouth Medical School, Hanover, NH) (18). Putative miR-93 binding site GCACUUU (nt 162-168) was mutated into UGUAGCG by oligo-directed PCR. All constructs were verified by sequencing. The pWZL Blast VEGF expression plasmid was purchased from Addgene (Cambridge, MA). The pEGP-miR-93 was obtained from Cell Biolabs (San Diego, CA). The luciferase reporter vector pGL4.10 [luc2] was from Promega (Madison, WI). The mmu-miR-93 antisense morpholino was synthesized by Gene Tools (Philomath, OR) using the sequence 5’-CAGTACCTCGACAGACAGCA CTTTG-3’. For experiments using 3.1-luc luciferase reporter constructs in vitro, 1.5x10⁵ HeLa cells were plated in 12-well plates. 1.5µg of 3.1-luc luciferase construct, 50 ng of pSV-β-gal control vector (Promega, Madison, WI) and 30nM of miRNA mimics (Ambion, Austin, TX) were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Following 48 hrs of transfections, luciferase and β-galactosidase activity were assayed as previously reported (19).

**Tissue Culture**—Conditionally immortalized renal microvascular endothelial cell line was a kind gift of Dr. Robert Langley (M.D. Anderson Cancer Center, University of Texas, Houston, TX) (20). Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (University of Miami, Miami, FL) and cultured as previously reported (21). Briefly, podocytes were cultured on BD BioCoat Collagen I plates (BD Biosciences, San Jose, CA) at 33°C in the presence of 20 U/ml mouse recombinant IFN-γ (Invitrogen, Carlsbad, CA) to enhance expression of a thermosensitive T antigen. To induce differentiation, podocytes were maintained at 37°C without IFN-γ for 10-12 days.

**Animal Studies**—All animal studies were conducted according to the “Principles of Laboratory Animal Care” (NIH publication No. 85023, revised 1985) and the guidelines of the IACUC of Baylor College of Medicine. The diabetic db/db mice and their control littermates db/m mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were maintained on a normal chow diet and housed in a room with a 12:12-hour light/dark cycle and an ambient temperature of 22°C. The Vegf-lacZ-KI/+ mice were kindly provided by Dr. Andras Nagy (Samuel Lunenfeld Research Institute, Toronto, ON, Canada) (22). Kidney glomeruli were isolated by perfusion using Dynabeads (Invitrogen, Carlsbad, CA) as previously described (23). miR-93 knock-out mice (Mmir106b-25tm1.1Tyj/J) were purchased from Jackson Laboratories (Bar Harbor, ME).

**miRNA Extraction and Microarray Analysis**—miRNAs were extracted using miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. miRNA microarray was performed using Micro
Paraflo microfluidic chips (LC Sciences, Houston, TX). Data were analyzed by normalizing the signals using a LOWESS filter (Locally-weighted Regression) (24). For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p values of the t-test were calculated. Those with \( p < 0.05 \) were considered as differentially expressed miRNAs.

Computational Targeted Gene Predictions of miR-93—The full-length mRNAs of mouse VEGF (NM 001025250) was obtained from the NCBI Database. The miRNA sequence database (miRBase) was obtained from the University of Manchester. Three separate algorithms (miRanda, TargetScan, and PicTar) were used to find potential targets sites for miR-93. The websites for these programs are as follows: TargetScan http://www.targetscan.org/, PicTar http://pictar.mdc-berlin.de/ and miRanda http://www.microrna.org/microrna/home.do. The RNA Hybrid program (http://bibiserv.techfak.uni-bielefeld.de/ghaybrid) (25) was used to predict the secondary structure of the RNA/miRNA duplex.

Real Time RT-PCR, Northern Blot, and In Situ Hybridization for miRNAs—miRCURY LNA microRNA PCR System (Exiqon, Woburn, MA) was used in conjunction with qPCR and SYBR Green Supermix (Bio-Rad, Hercules, CA) for quantification of miRNA transcripts according to the manufacturer’s instructions. U6 snRNA was used as an internal control with the following primers: 5’-CGCTTCGGCAGCACATATAC-3’ (forward), 5’-TTCACGAATTTGCGTGTCAT-3’ (reverse). The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec and 60 °C for 1 min. Individual samples were run in triplicate, and each experiment was repeated at least 3 times. Relative gene expression was calculated using the 2^(-ΔΔCt) method (26). Northern blots were carried out using γ-32P-ATP (Perkin Elmer, Waltham, MA) end-labeled miRNA locked nucleic acid (LNA) probes (Exiqon, Woburn, MA) (27). Signals were quantitated using software NIH Image J ver. 1.42q.

In situ hybridization was performed using LNA miR-93 probe (5’-CTACCTGGACAAGCCACGTGCATT-3’ (forward) and 5’-TCCACGAATTTGCGTGTCAT-3’ (reverse). The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec and 60 °C for 1 min. Individual samples were run in triplicate, and each experiment was repeated at least 3 times. Relative gene expression was calculated using the 2^(-ΔΔCt) method (26). Northern blots were carried out using γ-32P-ATP (Perkin Elmer, Waltham, MA) end-labeled miRNA locked nucleic acid (LNA) probes (Exiqon, Woburn, MA) (27). Signals were quantitated using software NIH Image J ver. 1.42q.

miRNA Mimics and Inhibitors—PremiRNA precursor molecules and anti-miRNA miRNAs were purchased from Ambion (Austin, TX). They were introduced into podocytes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a final concentration of 30nM. These Pre-miRNA mimics are small, double-strand RNAs that mimic endogenous precursor miRNAs, and can be uptaken and activated by the miRNA processing pathway. The anti-miR miRNA inhibitors are chemically modified RNAs that can bind to and inhibit the activity of target miRNAs.

RT-qPCR—Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were generated using Superscript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). SYBR Green-based qPCR on a DNAEngine OPTICON (Bio-Rad, Hercules, CA) were used to analyze the relative expression levels of the following genes with the different primer sets: Collagen type IV alpha 3 (COL4A3): 5’-AG AGGGGACGAGGGCGGAAC-3’ (forward) and 5’-TCCCCGGGCAGCAACAGATT-3’ (reverse); Fibronectin (FN1): 5’-GCGGTTGTCTGACGCTGCTC-3’ (forward) and 5’-TGGGTTCAGCAGCCGTGC-3’ (reverse) and 5’-AGGGTGTTGAAGGTCTCAAA-3’ (reverse).

Measurement of VEGF by ELISA and Western Blot—VEGF concentrations in the supernatant were measured by ELISA (Exiqon, Woburn, MA) using Quantikine Mouse VEGF Immunoassay (R&D Systems, Minneapolis, MN). Protein levels of VEGF-A were analyzed by Western blot in whole cell lysates using anti-VEGF antibody (Thermo Scientific, Fremont, CA). Signals were quantitated by Odyssey Infrared Imager (Li-CoR Biosciences, Lincoln, NE).

Generation of miR-93 Stable Podocytes—Undifferentiated podocytes were stably transfected with pEGP-miR-93 precursor using Lipofectamine 2000 and selected with 1µg/ml puromycin (Sigma, St. Louis, MO) in the presence of 20 U/ml IFN-γ at 33°C.
Immunofluorescence Microscopy—Immunostaining was performed as previously described (29,30). Briefly, expression of miR-93 and endogenous VEGF in the stable miR-93-GFP podocytes were detected using GFP and anti-VEGF antibodies (Thermo Scientific, Fremont, CA). Coverslips were imaged on an Applied Precision SoftWoRx Image Restoration Microscope (deconvolution). For immunostaining in the kidneys, goat anti-nephrin (R&D Systems, Minneapolis, MN) and mouse anti-VEGF antibodies were used. Sections were incubated at 4°C overnight. Sections were imaged on a Zeiss LSM 510 inverted Laser Scanning Microscope.

Whole-Mount X-Gal Staining of Kidney Organ Culture—Adult kidney organ culture and tissue delivery of antisense morpholino oligomers were carried out as previously reported (31,32). Briefly, kidneys from VEGF-LacZ mice were harvested under sterile conditions. Kidney capsules were removed and kidney cortices dissected, and cut into small pieces (around 1 mm). Kidney cortex pieces were cultured using a roller bottle incubator (Robbins Scientific model 1000, Sunnyvale, CA) at 37°C, 5% CO2, 20% O2, 75% N2 for 72 hrs in DMEM, containing 10% FBS, 1× penicillin and streptomycin, under either normal glucose (5mM D-glucose) or high glucose conditions (25mM D-glucose). miR-93 morpholino oligomers (10µM) was delivered into the cultured kidneys using 4mM of Endo-Porter delivery system (Gene Tools, Philomath, OR) (32). After 72 hrs, kidneys were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde. X-gal staining (Millipore, Phillipsburg, NJ) was performed at 37°C in 0.02% glutaraldehyde, 5mM K3Fe(CN)6, 5mM K4Fe(CN)6, and 2mM MgCl2 as described previously (33). Kidneys were then postfixed in 4% paraformaldehyde followed by paraffin embedding. Paraffin sections were dewaxed and mounted, and examined under a Nikon Eclipse 80i microscope.

Promoter Cloning and Luciferase Reporter Assay—Based on the reported human MCM7 promoter sequence (34), the mouse MCM7 promoter spanning 500bp upstream of the gene was PCR amplified from podocytes genomic DNA using HotMaster Taq DNA Polymerase (5PRIME, Gaithersburg, MD) with the following primer set: 5’-GTAGGTACCGCGGACTAGCCGAGTTGGA AAGATAG-3’(forward), and 5’-GCAGCTAGCT CTGGGGAAGCAGAAAAAC GCG-3’(reverse). PCR products were cloned into KpnI-NheI sites of pGL4.10 [luc2]. The MCM7 promoter construct or the empty pGL4.10 [luc2] vector was cotransfected with pSV-β-gal (Promega, Madison, WI) into podocytes. Luciferase activity was measured using Dual-Glo Luciferase Reporter Assay Kit (Promega, Madison, WI) on a FLUOstar Omega luminometer (BMG Labtech, Cary, NC) as previously reported (19), using β-gal as internal control.

Statistical Analysis—All data are shown as mean±SEM. Statistical significance was assessed by performing analysis of variance (ANOVA) followed by the Tukey–Kramer post-hoc analysis for multiple comparisons using an alpha value of 0.05 in Graphpad Prism software (San Diego, CA).

RESULTS

Hyperglycemia Elicits Downregulation of miR-93 Expression — Comparative miRNA arrays from high glucose-exposed (25 mM) podocytes and kidney microvascular endothelial cells, as well as in the kidney glomeruli obtained from diabetic db/db mice revealed that a number of miRNAs were differentially modulated in high glucose conditions (Fig.1 and Supplementary Tables 1-4). Since we were interested in the role of miRNAs on the expression of VEGF-A in the diabetic environment, we initially focused on miRNAs which were preferentially downregulated under hyperglycemic conditions. Among those individual miRNAs differentially expressed, we focused on miR-93 which was consistently decreased within all samples, and potentially had a highly conserved binding site in the 3’-UTR region of vegfa in several species (Fig.2A, upper panel). As shown in the Fig. 2A (lower panel), the 3’-UTR of the mouse vegfa gene contains a 7-mer that is perfectly complementary to the seed region of miR-93. The minimum free energy predicted for hybridization with the VEGF 3’-UTR and miR-93 at this site was ΔG = −27.4 kcalmol⁻¹, consistent with an authentic miRNA targeting (35). As determined by RNA Hybrid analysis, we also found that miR-93 and its binding site in vegfa could potentially form a very stable secondary structure (Fig. 2B). Thus, we hypothesized that VEGF-A might serve as a target for miR-93 in the diabetic milieu.

Validation of miR-93 Expression In Vitro and In Vivo— We initially examined miR-93 expression pattern by Northern blot analysis from
multiple tissues obtained from control db/m mice (Fig. 3A). Using a LNA-labeled miR-93 probe, we found that the expression level of miR-93 was high in spleen, lung, and kidney (Fig.3A). To further examine the localization of miR-93 expression in the kidney, we carried out in situ hybridization using DIG-labeled LNA-miR-93 probe (Fig.3B). Hybridization with the miR-93-specific probe revealed the presence of miR-93 predominantly in the cortical region of the kidneys. Generalized staining of miR-93 was detected throughout glomeruli and tubular epithelial cells.

To verify our array analysis and to address whether miR-93 expression is differentially expressed in DN, expression of miR-93 was validated by Northern blot analysis in the glomeruli of db/db and control db/m mice from independent experiments (Fig.4A). As shown in Fig.4A and Fig.4B, diabetic db/db mice had significantly lower expression levels of miR-93 compared to db/m control mice (p<0.05, Fig.4B). These results confirmed that the glomeruli expression of miR-93 is markedly downregulated in diabetic mice.

Next, we validated the expression profile of miR-93 in podocytes, the main source of VEGF production in the kidneys (4,18). Differentiated podocytes were cultured under normal glucose (5 mM) or high glucose (25 mM) medium for 24 hrs. Northern blot analysis in podocytes indicated that miR-93 expression was significantly downregulated in high glucose as compared to normal glucose condition (Fig.4C and Fig.4D). Using qPCR, we also found that the exposure of cultured podocytes to high glucose levels (25 mM), led to a near two-fold decrease in miR-93 expression levels (Fig.4E). Taken together, these findings validated our predicted profiling data on miR-93 expression both in vitro and in vivo.

miR-93 Targets vegfa 3’-UTR — To address whether binding of miR-93 to the vegfa 3’-UTR leads to translational suppression of VEGF, we cloned mouse vegfa 3’-UTR into luciferase reporter vector 3.1-luc (18) (Fig. 5A). We also generated a mutated miR-93 binding site (miR-93 mutant) in which the putative miR-93 binding site (GCACUUU) in the vegfa 3’-UTR was mutated into UGUAGCG (Fig. 5B).

Transient cotransfection of miR-93 mimics and luciferase expression plasmids in HeLa cells resulted in significant repression of the basal level of the vegfa transcript, whereas transfection of cells with miR-690 mimics, a control microRNA which was not predicted to target VEGF, did not have any effect on the expression of luciferase (Fig 5C). Importantly, suppression of the vegfa 3’-UTR by miR-93 mimics was abrogated when cells were transfected with miR-93 mutant (Fig. 5C), consistent with the conclusion that miR-93 acts as a negative regulator of vegfa by binding to the vegfa 3’-UTR.

A connection between miR-93 and VEGF-A as its target was further substantiated when we assessed the protein levels of VEGF-A in the luciferase lysates using a monoclonal VEGF-A antibody. Western blot analysis indicated that transfection of cells with miR-93 mimics significantly decreased VEGF protein levels compared to that in control miR-690 transfected cells (Fig. 5D). Taken together, these results indicate that miR-93 downregulates VEGF expression, through binding to the 3’-UTR of vegfa gene.

Effect of Gain of Function and Silencing of miR-93 on VEGF Protein Levels—To address whether miR-93 modulates VEGF-A protein levels in podocytes, we transfected miR-93 mimics into podocytes, and measured VEGF release in the medium under normal glucose (5 mM) or elevated glucose conditions (25 mM) by using ELISA assays. Consistent with several previous reports (36,37), we found that elevated glucose levels increased VEGF secretion (Fig. 6A). In contrast, transfection of podocytes with miR-93 mimics significantly reduced high glucose-induced VEGF release in the medium. Conversely, inhibition of miR-93 in podocytes with anti-miR-93 increased the secretion of VEGF in the medium, while inhibition of miR-690, as a control, did not have any effect (Fig. 6B). Thus, these findings suggest that overexpression or knock-down of miR-93 modulate VEGF production in podocytes.

To support the notion that miR-93 could directly regulate VEGF-A expression in podocytes, we generated a podocytes cell line with stable expression of mmu-miR-93 precursor using pEGP-miR-93 construct, which enabled us to monitor miR-93 expression by GFP fluorescence. As shown in Fig.6C, we observed significantly reduced fluorescent immunoreactivity of VEGF in miR-93-GFP-transfected podocytes compared with non-transfected control cells (Fig. 6C and Fig.6D).
Taken together, these results indicate that miR-93 directly downregulates VEGF expression.

**miR-93 Represses High Glucose-induced Downstream Targets of VEGF in Podocytes**—To investigate the biological significance of miR-93 as a regulator of VEGF in the kidneys, we analyzed the effect of miR-93 on the expression of two widely studied VEGF downstream targets, α3 collagen IV (COL4A3) and fibronectin (FN1) genes (3,38). To this end, we transfected podocytes with miR-93 mimics before culturing them in normal glucose or high glucose medium. As shown in Fig.7A, high glucose caused a significant increase in the expression of COL4A3 gene. Transfection of miR-93 mimics in podocytes abrogated high glucose-induced COL4A3 expression. The effect of miR-93 on COL4A3 expression was VEGF-dependent since forced expression of VEGF cDNA lacking 3'-UTR in podocytes rescued the inhibitory effect of miR-93 on COL4A3 expression. Similar results were obtained with miR-93 and FN1 expression in podocytes (Fig.7B).

**Inhibition of miR-93 Expression By Morpholino Oligos Mimics the Effect of Hyperglycemia on Glomerular VEGF Expression**—To assess the effect of miR-93 in normal and high glucose conditions on VEGF-A expression in the kidneys, we took advantage of a transgenic mouse line containing a VEGF-LacZ bicistronic transcript (22) (Fig. 8A). Use of these transgenic mice allowed visualization of β-galactosidase activity following increased VEGF-A expression in the kidneys (39). Kidney cortices from VEGF-LacZ mice were removed, and cultured under either normal glucose (5mM) or high glucose conditions (25mM). After 72 hrs, kidneys were fixed and whole mount Lac-Z staining of samples was performed. In normal glucose conditions, cultured glomeruli and tubular cells were very faintly stained, indicating very low expression of VEGF. In contrast, glomerular VEGF was strongly expressed when kidneys were cultured in high glucose conditions for 72hrs (Fig.8C). Importantly, miR-93 morpholino oligomers exposed to normal glucose conditions also induced β-galactosidase activity in cultured kidneys (Fig. 8C). These results strongly suggest that inhibition of miR-93 with morpholino oligomers leads to upregulation of VEGF-A expression, mimicking the effect of high glucose conditions on VEGF.

To provide further experimental evidence for the effect of miR-93 on the expression VEGF in vivo, we explored VEGF-A expression in miR-93 knock-out (miR-93−/-) mice (40). As shown in Fig. 8D, glomerular VEGF immunostaining in miR-93−/- mice was markedly increased compared to that in control mice. This result suggests that miR-93 plays a critical role in regulating VEGF expression in vivo.

**Transcriptional Regulation of MCM7 Promoter by High Glucose Is Responsible For miR-93 Repression**—To address the underlying mechanism by which high glucose represses expression of miR-93, we sought to assess the effect of high glucose on miR-93 promoter. miR-93 is encoded by intron 13 of the host MCM7 (mini-chromosome maintenance complex component 7) gene (41), and its abundance is linked to the expression of MCM7 promoter (42) (Fig.9A). The only reported human MCM7 promoter (34) has several transcription factor binding sites (E2F1, GC box and E-box); some of which have been reported to be glucose responsive (43-45). Thus, we amplified a 500bp upstream region of the mouse MCM7 promoter from podocytes genomic DNA by PCR, and subcloned it into pGL4 luciferase reporter to create pGL4-miR-93-Luc construct. We then tested the promoter activity of pGL4-miR-93-Luc under normal or high glucose exposure in podocytes. Luciferase assay showed that the cloned 500bp upstream region of MCM7 gene indeed had strong promoter activity, and hyperglycemia decreased luciferase activity reproducibly over its basal activity in normal glucose environment in podocytes (Fig. 9B), suggesting that the underlying regulatory mechanism by which miR93 is downregulated in hyperglycemic conditions is because of the regulatory effect of high glucose on a 500 bp upstream region of the transcriptional start of MCM7 promoter.

**DISCUSSION**

Previous reports have demonstrated the requirement of maintaining appropriate levels of VEGF-A for the proper development and function of kidney glomeruli (2-4). In the current study, we have identified miR-93 as a signature miRNA in the diabetic environment and a critical regulator of VEGF-A expression.

A major finding of this report is the
identification of VEGF as a target of miR-93 in hyperglycemic conditions. It has been well established that the regulation of VEGF is tightly controlled at transcription, post-transcription, translation, and differential cellular localization of various isoforms (3,46). Translational regulation of VEGF-A depends on the presence of internal ribosome entry sites in the 5′-UTR, whereas its transcriptional regulation involves a plethora of transcription factors (3). Identification of miR-93 in this report uncovers an additional layer of control by this class of regulatory molecules on VEGF expression in diabetic environment. Consistent with the conclusion that miR-93 regulates VEGF expression, forced expression of miR-93 repressed the transcription of vegfa 3′-UTR and prevented high glucose-induced increase in VEGF protein secretion. In cultured podocytes, the use of miR-93 mimics led to attenuated VEGF secretion, while the use of anti-miR-93 inhibitors caused a significant increase in VEGF production. We further showed that overexpression of miR-93 abrogated VEGF downstream targets such as collagen IV and fibronectin.

Another major finding of this study is that miR-93 can directly modulate VEGF expression in vivo. Indeed, by using a transgenic VEGF/LacZ mouse as our experimental model in vivo, we were able to 1) assess the effects of high glucose on VEGF in vivo, and 2) to investigate the effects of miR-93 inhibition on VEGF expression in kidneys ex vivo by taking advantage of morpholino oligomers. These findings indicate that VEGF is an important target of miR-93 in vivo in an animal model that recapitulates the regulatory events leading to modulation of VEGF expression.

The precise regulation of miRNAs expression is largely unknown. However, it is becoming increasingly evident that the integration of miRNAs into introns of protein coding genes represents a common and convenient mechanism for regulating the expression of miRNAs (47,48). miR-93 is encoded by intron 13 of the MCM7 gene (41), and while our results suggest the presence of a glucose-responsive element on MCM7 promoter as the underlying regulatory mechanism by which miR93 is downregulated in hyperglycemic conditions, a recent report has suggested that miR-93 might also have its own promoter (47). Further studies are needed to validate the presence of a specific miR-93 promoter, and examine the potential effect of high glucose on the putative miR-93 specific promoter.

VEGF plays myriad roles in microvascular complications of diabetes, including diabetic retinopathy and nephropathy. Recent publications have clearly established the roles of miR-192 and miR-377 in regulating a number of key genes involved in the pathogenesis of diabetic nephropathy in vitro (30,49). However, the role of miRNAs on VEGF expression in the diabetic milieu remained unknown. The identification of miR-93 in this study as a novel regulator of VEGF in the diabetic environment could have important implications in further understanding the biology and functions of VEGF as a key mediator of angiogenesis and microvascular complications of diabetes.

In summary, we show that miR-93 has a modulatory effect on VEGF expression and its downstream signaling which might play important roles in the pathogenesis of diabetic nephropathy (Fig. 9C). Future studies are underway in our laboratory to examine whether forced in vivo expression of miR-93 in an experimental murine model could override the stimulatory effect of hyperglycemia on VEGF expression. Dissecting molecular mechanisms by which miR-93 regulates VEGF both in normal and pathological conditions can lead to insights into preventing microvascular complications of diabetes. Although many obstacles remain to be addressed, miRNA mimics and miRNA antisense constructs could hold promise for the design of a new generation of drugs for the treatment of patients with diabetic kidney disease.

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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: VEGF, vascular endothelial growth factor; DN, diabetic nephropathy; NG, normal glucose; HG, high glucose; UTR, untranslated region; DIG, digoxigenin; LNA, locked nucleic acid; MCM, minichromosome maintenance complex; PCR, polymerase chain reaction; GFP, green fluorescence protein.

FIGURE LEGENDS

FIG. 1: Identification of miR-93 as a signature miRNA in high glucose conditions. Comparative microarray analysis indicating that 5 miRNAs were downregulated within all samples, whereas 45 miRNAs were downregulated in db/db glomeruli, 86 in high glucose (HG)-treated kidney microvascular endothelial cells, and 32 miRNAs in podocytes treated with HG (25 mM) for 24 hrs as compared to normal glucose conditions.
FIG. 2: **miR-93 is predicted to target vegfa gene.** A (upper panel), A miR-93 target site resides at nt162–168 (shown in the blue box) of the vegf-3′ UTR, and is highly conserved in several species. Lower panel, sequence alignment of miR-93 with the mouse vegfa 3′-UTR. B, miR-93 can potentially form a strong secondary structure with the target sequence of 3′-UTR of vegfa (predicted by RNA Hybrid).

FIG. 3: **Expression pattern of miR-93 in vivo.** A, Expression of miR-93 in different tissues as detected by Northern blot. U6 snRNA serves as a loading control. B, Expression pattern of miR-93 in a db/m kidney tissue by in situ hybridization. Inset: A higher power view. G: glomerulus, T: tubule. Figures are representative of three independent experiments.

FIG. 4: **miR-93 is downregulated in high glucose conditions.** A, Northern blot analysis shows representative results from glomerular miR-93 expression in two control db/m and two diabetic db/db mice. B, Quantitative analysis of miR-93 expression. Mean values for miR-93 expression were generated by measuring the pixel intensity in each band using NIH Image J ver. 1.42q. Measured transcript levels were normalized to U6 snRNA expression. Samples were run in triplicate. Data are shown as mean±SE. C, Northern blot analysis of miR-93 expression in high glucose (HG)-treated podocytes compared to normal glucose (NG). D, Quantitative analysis of miR-93 expression in podocytes. Measured transcript levels were normalized to U6 snRNA expression. Samples were run in triplicate. Data are shown as mean±SE. E, RT-qPCR analysis of miR-93 in podocytes and kidney microvascular endothelial cells (EC) following exposure to high glucose (HG) for 24 hrs. Measured transcript levels were normalized to U6 snRNA expression. Data represent three independent experiments with three replicates each. Data are shown as mean±SE.

FIG. 5: **miR-93 targets vegfa.** A, Schematic diagram of vegfa 3′-UTR reporter construct. B, Sequence alignment between miR-93 and mouse vegfa 3′-UTR wild type (wt) and miR-93 mutant (mut). Red color indicates the sequence of the mutated miR-93 binding site. C, HeLa cells were transfected with either luc-vegfa-wt or luc-vegfa-mutant, along with miR-93 mimics (30 nM). A non-related miRNA (miR-690) was used as control. Luciferase activities were normalized to β-gal activities. Results were obtained from three independent experiments. Data are shown as mean±SE. NS: non significant. D, Western blot analysis of VEGF-A protein levels in cells transfected with miR-93 mimics.

FIG. 6: **miR-93 represses VEGF-A production in podocytes.** A, VEGF released into the culture medium was measured following 24hrs of exposure to high glucose (HG) by ELISA. Similar experiments were carried out in cells cultured in normal glucose (NG) medium. B, Effect of miR-93 inhibitor on VEGF following 24hrs of exposure to high glucose (HG) was measured by ELISA. C, Podocytes were transfected with pEGP-miR-93 plasmid (green), and expression of VEGF (red) was assessed by deconvolution microscopy. Original magnificationn×400. D, Quantitative analysis based on fluorescence intensity of VEGF.

FIG. 7: **miR-93 inhibits high glucose-induced downstream target genes of VEGF.** A, Podocytes were transfected with miR-93 mimics with or without VEGF cDNA lacking 3′-UTR. Cells were serum starved and exposed to high glucose (HG) for 24hrs, and a3 collagen (IV) (COL4A3) or fibronectin (FN1) (B)
mRNAs were assessed by RT-qPCR. Expression levels of mRNAs were normalized as described in “Experimental Procedures”. Data are shown as mean±SE.

FIG. 8: **miR-93 regulates VEGF expression in kidneys.**  
**A,** Schematic diagram of the VEGF-LacZ targeting construct.  
**B,** Mechanism of Endo-Porter system delivery through endocytosis.  
**C,** Light microscopic images of whole mount X-gal staining demonstrating β-galactosidase activity (blue) in the glomeruli and tubular cells in the kidney sections from adult VEGF-lacZ mice. Kidney cortex pieces were cultured ex-vivo in normal glucose, high glucose, or normal glucose in the presence of miR-93 morpholino oligomers. Original magnifications are ×200 and ×400. G: glomerulus.  
**D,** Immunofluorescence staining of glomerular VEGF with anti-VEGF-A (red) and anti-nephrin (green) antibodies using confocal laser scanning microscopy. Original magnification ×600.

FIG. 9: **High glucose downregulates MCM7 promoter activity.**  
**A,** Diagram of the genomic organization of the mouse MCM7 gene. miR-93 is localized within the intron 13 of MCM7.  
**B,** Luciferase activity of the cloned mouse MCM7 promoter in podocytes. Podocytes were transfected with empty vector (Vec) or mouse MCM7 promoter constructs. Luciferase activity in normal glucose (NG) or high glucose (HG) medium were measured and normalized to β-gal internal control. Quantitative analysis of three independent experiments is shown as mean±SE.  
**C,** Proposed mechanism for the putative effects of high glucose on miR-93-mediated VEGF downstream signaling leading to diabetic nephropathy.
Figure 1
Gene:
Mouse vegfa NM_001025250 3'-UTR length: 1809

..130.......140.......150.......160.......170.......180......
Mmu AAGCCUGACUAAGAAGAGAAGGAGACUCUUCGAGGA GCACUUGUGGCGAGGCGAGA
Hsa AA-CCUGAAUGAAGAAGGAGACUCUUCGAGGA GCACUUGUGGCGAGGCGAGA
Rno AAGCCUGACUAAGAAGAGAAGGAGACUCUUCGAGGA GCACUUGUGGCGAGGCGAGA
Cfa AA-CCUGAAUGAAGAAGAGAAGGAGACUCUUCGAGGA GCACUUGUGGCGAGGCGAGA

Figure-2
Figure 3
miR-93
U6 snRNA

Figure-4

Relative miR-93 expression (fold of change)

p<0.05

p<0.01

p<0.05

p<0.05

podocytes   EC

NG HG   NG HG

NG HG   NG HG
A

\[5'\text{CMV} \rightarrow \text{luciferase} \rightarrow \text{vegfa 3'-UTR} \rightarrow 3'\]

B

\[
\begin{align*}
\text{wt} & \quad \text{mmu-miR-93} & \quad 3'\text{GAUGGAC-GUGCU} & \quad \ldots \ldots \ldots & \quad \ldots \ldots \ldots & \quad \text{UUCGAAAC} & \quad 5' \\
& \quad \text{vegfa 3'-UTR} & \quad 5'\text{-AGCCGACAGAAGGAAAGGAGACUCUUGCAGGGACACUUUG} & \quad 3'
\end{align*}
\]

\[
\begin{align*}
\text{mut} & \quad \text{mmu-miR-93} & \quad 3'\text{GAUGGAC-GUGCU} & \quad \ldots \ldots \ldots & \quad \ldots \ldots \ldots & \quad \text{UUCGAAAC} & \quad 5' \\
& \quad \text{vegfa 3'-UTR} & \quad 5'\text{-AGCCGACAGAAGGAAAGGAGACUCUUGCAGGGACACUUUG} & \quad 3'
\end{align*}
\]

C

- p<0.001
- p=NS

D

- VEGF-A
- \(\beta\)-actin

Figure-5
Figure 6
Figure-7

A

HG+miR-93 mimics
HGNG
Relative COL4A3 expression (fold of change)

* p<0.01 compared to NG
† p<0.01 compared to HG

HG+miR-93 mimics + VEGF

B

HG+miR-93 mimics
HGNG
Relative FN1 expression (fold of change)

* p<0.01 compared to NG
† p<0.01 compared to HG
Figure 9

Diagram showing the relationship between hyperglycemia, MCM7, miR-93, VEGF-A, and collagen & fibronectin, leading to diabetic nephropathy.
Identification of MicroRNA-93 as a novel regulator of vascular endothelial growth factor (VEGF) In hyperglycemic conditions
Jianyin Long, Yin Wang, Wenjian Wang, Benny H. J. Chang and Farhad R. Danesh

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