MicroRNA-29a Promotion of Nephrin Acetylation Ameliorates Hyperglycemia-Induced Podocyte Dysfunction


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ABSTRACT
Podocyte dysfunction is a detrimental feature in diabetic nephropathy, with loss of nephrin integrity contributing to diabetic podocytopathy. MicroRNAs (miRs) reportedly modulate the hyperglycemia-induced perturbation of renal tissue homeostasis. This study investigated whether regulation of histone deacetylase (HDAC) actions and nephrin acetylation by miR-29 contributes to podocyte homeostasis and renal function in diabetic kidneys. Hyperglycemia accelerated podocyte injury and reduced nephrin, acetylated nephrin, and miR-29a levels in primary renal glomeruli from streptozotocin-induced diabetic mice. Diabetic miR-29a transgenic mice had better nephrin levels, podocyte viability, and renal function and less glomerular fibrosis and inflammation reaction compared with diabetic wild-type mice. Overexpression of miR-29a attenuated the promotion of HDAC4 signaling, nephrin ubiquitination, and urinary nephrin excretion associated with diabetes and restored nephrin acetylation. Knockdown of miR-29a by antisense oligonucleotides promoted HDAC4 action, nephrin loss, podocyte apoptosis, and proteinuria in nondiabetic mice. In vitro, interruption of HDAC4 signaling alleviated the high glucose–induced apoptosis and inhibition of nephrin acetylation in podocyte cultures. Furthermore, HDAC4 interference increased the acetylation status of histone H3 at lysine 9 (H3K9Ac), the enrichment of H3K9Ac in miR-29a proximal promoter, and miR-29a transcription in high glucose–stressed podocytes. In conclusion, hyperglycemia impairs miR-29a signaling to intensify HDAC4 actions that contribute to podocyte protein deacetylation and degradation as well as renal dysfunction. HDAC4, via epigenetic H3K9 hypoacetylation, reduces miR-29a transcription. The renoprotective effects of miR-29a in diabetes-induced loss of podocyte integrity and renal homeostasis highlights the importance of post-translational acetylation reactions in podocyte microenvironments. Increasing miR-29a action may protect against diabetic podocytopathy.


Podocytopathy in renal tissue is an important event of glomerular dysfunction that results in diabetes-mediated proteinuria and renal disorders.1,2 Glomerular damage in diabetic kidneys has been linked to hyperglycemia-induced promotion of podocyte dedifferentiation3 and apoptosis,4 as well as disturbance of angiogenic...
Nephrin acts as an important slit diaphragm protein that orchestrates interaction between cellular junctions and cytoskeleton dynamics in podocytes. On the other hand, lack of nephrin has been observed to result in glomerular deterioration and proteinuria. Defective nephrin integrity is a prominent feature in hyperglycemia-mediated podocyte injury that accelerates glomerular damage in vivo and reduces podocyte viability in vitro. Accumulating evidence demonstrates that post-translational modification of nephrin, including phosphorylation, ubiquitination, or sialylation, modulates podocyte functions in various physiologic or pathologic conditions. However, the biologic role of nephrin acetylation in diabetic podocytopathy has scarcely been addressed.

Histone deacetylase 4 (HDAC4), one of the class II HDACs, has been found to modify acetylation reactions in histones or nonhistone proteins and has been reported to regulate glucose homeostasis, neuron plasticity, degenerative neural disorders, and hepatic carcinoma. Administration of HDAC inhibitors ameliorates both adriamycin-induced renal injury and diabetes-induced glomerular hypertrophy. The contribution of HDAC4 signaling to nephrin stability in the progression of diabetes-induced podocyte injury is worthy of characterization.

MicroRNAs (miRs) are short and non-coding RNAs that have been observed to not only interrupt protein translation through the degradation of mRNA targets, but to also regulate homeostasis and deterioration of renal tissues. Loss of miR-192 signaling accelerates fibrotic matrix deposition in diabetic kidneys, whereas a decrease in miR-29 signaling accelerates TGF-β1–mediated renal fibrosis reactions. It has also been reported that miR-29s regulate HDAC actions in myogenic differentiation and acute myeloid leukemia. We previously demonstrated that nephrin destabilization reduced podocyte survival and accelerated diabetes-induced renal damage. We propose that miR-29a interacts with HDAC signaling to regulate the nephrin acetylation, podocyte integrity, and renal function in diabetic mice.

This study investigated the acetylation status of nephrin, podocyte integrity, and renal function in diabetic miR-29a transgenic and wild-type mice. We also verified whether HDAC4 was involved in hyperglycemia-induced perturbation of nephrin acetylation and miR-29a signaling in podocyte cultures.
RESULTS

Diabetes Reduced Nephrin Acetylation, Podocyte Survival, and miR-29a Expression

We utilized streptozotocin-induced hyperglycemic mice to test whether diabetes-mediated podocyte destruction was linked to the acetylation status of nephrin. Hyperglycemia significantly increased urinary protein excretion throughout the study period (Figure 1A). Podocytes in the diabetes group strongly displayed the podocyte injury marker desmin immunostain and the apoptotic cell marker terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) stain (Figure 1B). Few podocytes in diabetic kidneys weakly expressed cell markers nephrin and Wilms’s tumor-1 protein (WT-1) immunoreactions at 12 weeks after hyperglycemia compared with the normal control kidneys (Figure 1C). We harvested renal glomeruli to detect miR-29a expression and isolate nephrin immunoprecipitates; we also probed the immunocomplexes by acetylated lysine antibodies to identify acetylated nephrin. Diabetes significantly decreased the levels of nephrin, acetylated proteins, and acetylated nephrin throughout the study period (Figure 1D). Quantitative RT-PCR analyses revealed that diabetes significantly reduced the expression of miR-29a, but not miR-29b or miR-29c in renal glomeruli throughout the study period (Figure 1E). In situ hybridization observations showed that miR-29a transcripts were detectable in podocytes and tubular cells of the normal control kidneys, whereas few podocytes in diabetic kidneys expressed the miR-29a transcripts. However, podocytes and tubular cells in normal and diabetic kidneys ubiquitously expressed miR-29b and miR-29c transcripts (Figure 1F). Given that miR-29a reportedly regulates tissue fibrosis and protein acetylation,23,24 it was selected for the following experiments.

Gain of miR-29a Function Improved Renal Function and Podocyte Survival

We investigated whether miR-29a overexpression changed diabetes-induced podocytopathy and created miR-29a transgenic mice expressing a human phosphoglycerate kinase (PGK) promoter–coded miR-29a precursor. Agarose gel electrophoresis revealed that renal glomeruli constitutively expressed the miR-29 precursor construct corresponding to 506 bp (Figure 2A). Quantitative RT-PCR analyses confirmed that renal glomeruli in transgenic mice had higher miR-29a expression than that in wild-type mice (Figure 2A). Podocytes and tubular cells in transgenic mice with or without diabetes strongly expressed miR-29a transcripts compared with that in wild-type mice (Figure 2B). All animals gained body weight throughout the study period (Figure 2C). There was no significant difference in BUN, creatinine, cholesterol, creatine phosphokinase, glutamic pyruvic transaminase, Na, K, or Cl levels in sera between miR-29a transgenic mice and wild-type mice with or without diabetes (Supplemental Material). miR-29a overexpression did not significantly change the diabetes promotion of blood glucose levels in diabetic mice (Figure 2D). The HbA1c levels were significantly lower in miR-29a transgenic mice than in wild-type mice with diabetes (Figure 2E). Overexpression of miR-29a reduced diabetes-induced hyperfiltration (Figure 2F), kidney weight loss (Figure 2G), and urinary protein secretion (Figure 2H). Data are expressed as the mean ± SEM calculated from six to eight mice at each time point. *P<0.05, significant difference versus the NC group; #P<0.05, significant difference versus wild-type group. CCR, creatinine clearance; DM, diabetic mice; HbA1c, hemoglobin A1c; NC, normal control; PC, positive control; STZ, streptozotocin; Tg, transgenic mice; WT, wild-type mice.
glucose (Figure 2D) or hemoglobin A1c (Figure 2E) levels. Of note, miR-29a overexpression significantly reduced the diabetes-induced hyperfiltration as evidenced by increased creatinine clearance at 4 weeks after hyperglycemia (Figure 2F), and decreased kidney weight (Figure 2G) and urinary protein levels (Figure 2H) at 8 weeks after hyperglycemia compared with wild-type mice.

Glomeruli in diabetic kidneys underwent hypertrophy and strongly displayed periodic acid–Schiff stain. The miR-29a overexpression reduced diabetes-mediated glomerular hypertrophy and periodic acid–Schiff stain (Figure 3A). Few podocytes and tubular cells in the diabetic kidneys of the transgenic mice weakly expressed desmin immunoreactivity (Figure 3B) and TUNEL stain (Figure 3C) compared with the diabetic kidneys in wild-type mice. By contrast, podocytes in the transgenic mice with or without diabetes strongly expressed nephrin (Figure 3D) and WT-1 (Figure 3E) immunofluorescence reactions. Immunoblotting and quantitative RT-PCR analyses showed that miR-29a overexpression attenuated the diabetes-induced loss of nephrin and WT-1 (Figure 3F) and decreased the expression of fibrogenic factors TGF-β1, fibronectin, and IL-1β in diabetic renal glomeruli (Figure 3G).

miR-29a Modulated HDAC Expression and Nephrin Acetylation

We isolated nephrin immunoprecipitates from renal glomerular extracts and tested whether miR-29a signaling changed the acetylation status of nephrin. Electrophoreograms and liquid chromatography–tandem mass spectrometry analyses showed that a protein corresponding to 120 kD presented in the nephrin immunoprecipitates (Figure 4A) and the protein of interest showed high homology with HDAC4 (Figure 4A). Bioinformatics searches indicate that HDAC4, HDAC7a, and histone acetyltransferase p300/CREB-associated factor (PCAF) are predicated as putative miR-29a targets (http://www.microrna.org). Diabetes significantly increased HDAC4 (Figure 4B) and reduced acetylated nephrin levels (Figure 4C); miR-29a overexpression significantly reduced the promotion of HDAC4 levels (Figure 4B) and restored acetylated nephrin concentrations (Figure 4C). There was no significant difference in HDAC7A or PCAF levels between the miR-29a transgenic mice and wild-type mice with or without diabetes (data not shown).

We tested whether diabetes-induced nephrin loss was linked to ubiquitination. Nephrin immunoprecipitates were probed by ubiquitin mAbs to indicate ubiquitinated nephrin corresponding to 100 kD. In the wild-type mice, diabetes significantly increased ubiquitinated nephrin levels (Figure 4D) and urinary nephrin levels (Figure 4E) compared with normal control mice. These effects were significantly reduced in miR-29a transgenic mice and wild-type mice with or without diabetes (data not shown).

Histologic observation revealed that few podocytes in diabetic kidneys of wild-type mice weakly expressed immunoreactivity of nephrin (green fluorescence) and acetylated lysine (red fluorescence) compared...
with those of the normal control group (Figure 5). Podocytes in transgenic mice with or without diabetes strongly displayed nephrin and acetylated lysine immunofluorescence reactions (Figure 5).

**Knockdown of miR-29a Promoted Podocyte Apoptosis and Nephrin Deacetylation**

We tested whether knockdown of miR-29a affected nephrin stability or podocyte integrity. Exogenous miR-29a antisense oligonucleotides significantly reduced miR-29a expression (Figure 6A). Few podocytes and tubular cells in renal glomeruli expressed miR-29a transcripts (Figure 6B), whereas podocytes and tubular cells strongly displayed desmin immunostain and TUNEL stain (Figure 6C) at 8 weeks after treatment. The miR-29a interruption significantly reduced the levels of nephrin and acetylated nephrin, but increased the levels of HDAC4 and ubiquitinated nephrin (Figure 6D) and excretion of urinary nephrin and protein (Figure 6E) compared with those of the control group. Empty vector treatment did not significantly affect either the levels of HDAC4 or nephrin or secretion of urinary nephrin or protein.

**HDAC4 Interference Reduced High Glucose–Induced Nephrin Loss and Apoptosis in Podocyte Cultures**

We incubated primary mouse kidney podocytes and immortalized podocyte cultures in high glucose conditions to test whether miR-29a or HDAC4 regulated podocyte injury. High glucose treatment significantly reduced the levels of nephrin, WT-1, and acetylated nephrin and increased the levels of HDAC4 and ubiquitinated nephrin in primary podocytes (Figure 7A) and immortalized podocyte cultures (Figure 7B). Transfection of miR-29a precursor or HDAC4 RNA interference (RNAi) significantly reduced the high glucose promotion of HDAC4 and ubiquitinated nephrin levels and restored nephrin, WT-1, and acetylated nephrin levels in primary podocytes (Figure 7A, Supplemental Material) and immortalized podocyte cultures (Figure 7B, Supplemental Material). Protein ligation analyses revealed that podocytes positive for nephrin and HDAC4 ligation displayed red fluorescence in the cytoplasm. Cell cultures incubated in high glucose conditions strongly displayed fluorescence reactions (Figure 7C) and green fluorescence TUNEL staining (Figure 7D). These effects were reduced after miR-29a precursor or HDAC4 RNAi treatment. Neither scramble control nor osmolality control mannitol significantly changed the levels of nephrin, WT-1, or HDAC4 or apoptosis in podocytes.

**HDAC4 Regulated miR-29a Transcription**

We tested whether HDAC4 changed histone acetylation or miR-29a expression in podocyte cultures. High glucose significantly reduced the levels of acetylated histone H3 at the lysine 9 site (H3K9Ac), whereas the miR-29a precursor or HDAC4 RNAi transfection significantly restored H3K9Ac levels in primary podocytes (Figure 8A) and

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**Figure 4.** Effect miR-29a overexpression on HDAC level and nephrin acetylation in renal glomeruli. (A) Electrophoretography and liquid chromatography–tandem mass spectrometry spectrum of HDAC4. (B) miR-29a overexpression reduces the diabetes promotion of HDAC4 levels but not the HDAC7A or PCAF level. (C–E) Overexpression of miR-29a increased acetylated nephrin levels (C) and reduced ubiquitinated nephrin levels (D) in renal glomeruli and urinary nephrin excretion in diabetic kidneys (E). Data are expressed as the mean±SEM calculated from six to eight mice in each group. *P<0.05, significant difference versus the NC group; #P<0.05 indicates significant difference versus the wild-type group. Ac-lysine, acetylated lysine; Ac-nephrin, acetylated nephrin; DM, diabetic mice; IB, immunoblotting; IP, immunoprecipitates; MM, molecular mass; NC, normal control; PCAF, P300/CBP-associated factor; PI, isoelectric point; Tg, transgenic mice; Ub-nephrin, ubiquitinated nephrin; WT, wild-type mice.
immortalized podocyte cultures (Figure 8B). Chromatin immunoprecipitation-quantitative PCR analyses confirmed that the H3K9Ac antibodies were specific for detecting the H3K9Ac enrichment in the miR-29a proximal promoter (Figure 8C). High glucose significantly decreased the levels of H3K9Ac in the miR-29a proximal promoter region in primary podocytes (Figure 8C) and immortalized podocyte cultures (Figure 8D). The miR-29a precursor or HDAC4 RNAi significantly increased the enrichment of H3K9Ac in the miR-29a promoter. Moreover, the miR-29a precursor or HDAC4 RNAi significantly increased miR-29a transcription in both primary podocytes (Figure 8E) and immortalized podocyte cultures (Figure 8F) exposed to high glucose.

**DISCUSSION**

Podocyte loss and dysfunction in renal microenvironments are important features in the pathogenesis of diabetic nephropathy. Podocyte nephrin disintegration in the slit diaphragm has been observed to contribute to loss of protein filtration capacity resulting in excessive urinary protein excretion. However, post-translational acetylation events underlying diabetes-induced loss of podocyte integrity have not yet been elucidated. In this study, we demonstrated that hyperglycemia-induced deacetylation and ubiquitination reactions on the podocyte microstructure deteriorated podocyte homeostasis and renal function in diabetic mice. Interplay between miR-29a and HDAC4 signaling modulated the acetylation status of podocyte microenvironments and counteracted against the diabetes-induced podocyte damage, glomerular fibrosis and inflammation, and renal dysfunction. This study marks the first attempt to shed light on both nephrin acetylation in the stabilization of podocyte function and epigenetic actions on maintaining nephrin stability and renal podocyte integrity in diabetic kidneys.

Diabetic kidneys in the miR-29a transgenic mice had better podocyte and tubular cell viability and lower fibrogenic factor expression and urinary protein secretion than those of the wild-type mice, suggesting that protective effects triggered by miR-29a signaling occur in renal microenvironments. Exogenous miR-29a antisense oligonucleotide induction of podocyte injury and excessive urinary protein levels in non-diabetic mice also support the postulation that miR-29a signaling is beneficial for maintaining renal homeostasis. The results of our analysis agree with previous studies demonstrating that miR-29a is a potent regulator that inhibits fibrotic matrix expression in high glucose–stressed renal proximal tubule cells. However, the biologic roles of the miR-29 family in renal function remain unclear. Muscle wasting–mediated CKD is associated with the decreased expression of miR-29a and miR-29b. In addition, miR-29b has been found to ameliorate fibrosis reactions in tubular epithelial cells and obstructive nephropathy. TGF-β1 decreases the expression of miR-29b and miR-29c but not miR-29a in immortalized human podocyte cultures. By contrast, knockdown of miR-29c reduces high glucose–induced apoptosis in mouse podocytes and attenuates albuminuria and mesangial fibrosis in db/db mice. Urinary miR-29b and miR-29c further correlate with proteinuria in patients with IgG nephropathy. In this study, miR-29a, but neither miR-29b nor miR-29c, in renal glomerular microenvironments actively responded to hyperglycemic stress. Moreover, miR-29a signaling reduced diabetes-induced podocyte and tubular cell apoptosis, renal fibrosis, and proteinuria. We speculate that different kidney disease models may have varying expression of the miR-29 family in renal microenvironments. Each miR-29 family member may have its own distinct biologic action on renal tissue remodeling. These phenomena rationalize our hypotheses to focus on the molecular events underlying miR-29a protection against diabetes-mediated podocyte dysfunction.
Our experimental results revealed that diabetes led to a reduction in the nephrin level in conjunction with nephrin deacetylation. Whereas nephrin has been shown to act as a potent tyrosin kinase that triggers biologic reactions in maintaining podocyte function and renal filtration capacity in various renal disorders,9,14,33,34 podocyte nephrin acetylation in diabetic kidneys has seldom been delineated until now. This study suggests that miR-29a antisense oligonucleotide-induced nephrin deacetylation impedes podocyte integrity in nondiabetic kidneys. Conversely, restoration of acetylated nephrin by miR-29a overexpression alleviated the diabetes-mediated podocyte injury and renal function. We are the first group to report the biologic action of noncoding miR signaling on nephrin acetylation in podocyte microenvironments. Nephlin ubiquitination and urinary nephrin excretion also explain the phenomenon of diabetes-induced nephrin loss causing podocyte microarchitecture destabilization.

Immunoprecipitation and liquid chromatography–tandem mass spectrometry analyses revealed that acetylation regulators PCAF, HDAC7a, and HDAC4 presented in the nephrin immunocomplexes. HDAC4 actively responded to hyperglycemic stress and miR-29a signaling and contributed to hyperglycemia-mediated loss of nephrin acetylation reactions and viability in podocytes. HDAC4 was found to be essential for embryonic kidney development35 and the regulation of angiogenic reactions in kidney cancer cells.36 Protein ligation analyses also confirmed the interaction between HDAC4 and nephrin in high glucose-stressed podocyte cultures. This study highlights the new molecular insight that HDAC4-dependent nephrin loss augments hyperglycemia-mediated podocyte destruction.

This study demonstrates that high glucose reduced both levels of H3K9Ac as well as the enrichment of H3K9Ac in the miR-29a proximal promoter region. Emerging evidence has proven that HDAC4 signaling regulates miR-mediated somatic cell reprogramming37 and keratinocyte senescence,38 as well as represses the miR-29b action in chronic lymphocytic leukemia.39 It is noteworthy that this study revealed that HDAC4 interruption restored H3K9Ac enrichment in the miR-29a promoter region and increased miR-29a transcription in high glucose–stressed podocyte cultures. The experimental results provide a new perspective that HDAC4 via an epigenetic

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**Figure 6.** Effect of exogenous miR-29a antisense oligonucleotide on podocyte survival and renal function. (A) The miR-29a antisense oligonucleotide decreases miR-29a expression in renal glomeruli. (B and C) Podocytes (arrows) and tubular cells weakly express miR-29a transcripts (B) and strongly express desmin immunoreactivity and TUNEL stain (arrows) (C). (D) Knockdown of miR-29a reduces nephrin and acetylated nephrin and increases the levels of HDAC4 and ubiquitinated nephrin. (E) Knockdown of miR-29a increases the excretion of urinary nephrin and protein. Data are expressed as the mean±SEM calculated from six mice in each group. *P<0.05, significant difference versus empty vector group. Ac-nephrin, acetylated nephrin; AS, miR-29a antisense oligonucleotide; IB, immunoblotting; IP, immunoprecipitates; NC, normal control; Ub-nephrin, ubiquitinated nephrin; Vector, empty vector.
pathway represses miR-29a transcription in the progression of high glucose–induced podocyte injury. Disturbed orchestration between miR-29a and HDAC4 signaling accelerates glomerular podocyte and renal deterioration. We do not exclude the possibility that miR-29a signaling may change post-translational modification or survival of other bioactive proteins or renal cell populations. Upregulation of podocyte marker WT-1 and tubular cell viability and reduction of glomerular fibrosis and inflammation in diabetic kidneys of miR-29a transgenic mice also reflect the diverse functions of miR-29a signaling on renal glomerular homeostasis.

Taken together, miR-29a is an important regulator in the maintenance of podocyte ultrastructure integrity and renal homeostasis. By suppressing HDAC4 action, miR-29a restores the acetylation status of nephrin and protects against diabetes-induced podocyte injury and renal dysfunction. HDAC4-dependent H3K9 hypoacetylation counteracts miR-29a transcription in high glucose–stressed podocytes (Figure 9). This study highlights an emerging view of an epigenetic mechanism underlying nephrin acetylation in podocytes and suggests that the addition of the miR-29a function is beneficial for improving diabetic podocytopathy.

CONCISE METHODS

**Streptozotocin-Induced Diabetes**

Three-month-old male FVB mice were given 50 mg/kg streptozotocin to induce hyperglycemia, as previously described. At 2 weeks after streptozotocin treatment, animals with postfast blood glucose of 200–300 mg/dl were considered diabetic. Blood glucose in animals was equalized by insulin. Diabetic or normal animals were euthanized by an overdose of sodium pentobarbital at 4 weeks (n=8), 8 weeks (n=8), and 12 weeks (n=8) after diabetes. All protocols for animal use and experiment were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan.

**miR-29a Transgenic Mice**

The PGK promoter and miR-29a precursor full-length sequence were cloned from the cDNA library by PCR protocols. The cDNAs were inserted into the pUSE empty expression vector; and the linear human PGK-miR-29a-BGH poly-A cDNAs were cloned. The designed constructs were transferred into fertilized eggs from FVB/N mice. The eggs were further transferred into Crl:CD1 foster mothers, as previously described. Transgenic mice were...
bred in a specific pathogen-free condition and genotyped by PCR using specific primers (forward: 5'-GAGGATCCCCCTCAAGGATAC-CAAG-GGATGAAT-3' and reverse 5'-CTTCTAGAAGGAGTG-9CAGG-3').

**Lentivirus-Mediated miR-29a Inhibitor Treatment**

miR-29a antisense oligonucleotide constructs were inserted into pMIF-cGFP-zeo and pmiR-ZIP shRNA expression vectors and transfected with pPACKF1 vector into 293T cells, according to the manufacturer’s instructions (System Biosciences, Mountain View, CA). Titers of lentivirus were measured using the plaque-forming method. Anesthetized mice were given 10^9 pfu/kg miR-29a antisense oligonucleotide (n=8), empty vector (n=8), or vehicle (n=8) via tail vein. At 8 weeks after injection, animals were euthanized and renal tissues were dissected for studies.

**Urine and Serum Biochemistry**

Serum hemoglobin A1c and blood glucose levels were detected according to the manufacturer’s instructions (Primus Diagnostics, Kansas City, MO). Urine excretion of each animal was collected using a metabolic cage system. Total protein (Dade Behring, Inc., Newark, NJ), creatinine (Formosa Biomedical Technology Corp., Taipei, Taiwan), and nephrin (Life Science, Inc., Houston, TX) concentrations in urine were measured using the respective kits. In some experiments, BUN, creatinine, cholesterol, creatine phosphokinase, and glutamic pyruvic transaminase levels were detected using a FujiFilm Serum Biochemistry Analyzer specific for experimental animals.

**Isolation of Primary Renal Glomeruli and Podocyte Cultures**

Primary glomeruli were harvested using magnetic isolation protocols, as previously described. Briefly, animals receiving Dynabead M-450 perfusions were euthanized, and their kidneys were coarsely dissected and then filtered through a 100-mesh sterile stainless sieve. Dynabead-trapped glomeruli were subjected to magnetic isolation, collagenase digestion, washing with PBS, and further magnetic isolation. In some experiments, primary podocyte cultures were isolated according to previous protocols. Briefly, isolated glomeruli were incubated in flask containing RPMI 1640 for 7 days. Outgrowing epithelial cells were trypsinized, filtered through a 33-μm sieve, and then incubated in RPMI 1640 with 10% FBS at 37°C. Isolated cell cultures displayed podocyte marker WT-1 and nephrin.

**Quantitative RT-PCR**

Total miR in primary glomeruli or podocyte cultures were isolated (BioChain Institute, Inc., Hayward, CA) and reverse transcribed into cDNA (Ambion, Inc., Austin, TX). Aliquots of cDNA templates were mixed with a PCR mixture containing miR-29a, miR-29b, miR-29c, or U6 primers (Applied Biosystems, Foster City, CA) and a 2X TaqMan Universal PCR Master Mix and an ABI 7900 Detection System (Applied Biosystems). Fold change for the treatment was calculated as 2^(-ΔΔCt), where ΔΔCt=ΔCt_target-ΔCt_control and ΔCt=Ct_target-Ct_U6.

**Liquid Chromatography and Mass Spectrometry**

Protein bands of interest in SDS-PAGE gels were cut and digested with 20 ng/ml trypsin. Peptide sequences in the digestion products were separated by an Acclaim PepMap 100 column in liquid chromatography ( UltiMate 3000; Dionex, Sunnyvale, CA) and identified by electrospray ionization mass spectrometry ( Bruker Daltonik GmbH, Leipzig, Germany) according to the manufacturer’s instructions. The peptide mass data were matched via the National Center for Biotechnology Information or SwissPort bioinformatics stations. Each tandem mass spectrometry ion with molecular weight search scores ≥39 of the match peptide was considered to have high homology with the candidate proteins.

**Immunoprecipitation and Immunoblotting**

Tissue and cell lysates from glomeruli and podocyte cultures were extracted. Aliquots of lysates were immunoblotted and probed by
H3K9Ac and actin monoclonal antibodies (Millipore, Billerica, MA). In some experiments, lysates were immunoprecipitated using nephrin monoclonal antibodies (R&D Systems, Minneapolis, MN). Nephrin immunocomplexes were immunoblotted and probed by HADC4, HDAC7a, PACF, acetylated lysine, and ubiquitin mAbs and followed by horseradish peroxidase–conjugated IgG and visualization with chemiluminescence agents.

**In Situ Hybridization, Immunohistochemistry, and Immunofluorescence**

Specimens were fixed and sectioned in RNase-free conditions. *In situ* hybridization for miR-29a, miR-29b, and miR-29c transcripts was performed using IsHyb *In Situ* Hybridization kits (Biochain) and digoxigenin-labeled miR-29a probes (Exiquon A/S, Vedbaek, Denmark), as previously described. The miR-29a transcripts were visualized by digoxigenin antibody–conjugated horseradish peroxidase and counterstaining with hematoxylin. Desmin immunostaining in specimens were probed by desmin mAb (Cell Signaling Technology, Danvers, MA) and a horseradish peroxidase–3′-3′-diaminobenzidine kit (BioGenex, San Ramon, CA). Sections without primary antibodies were enrolled as negative controls for immunostaining. Apoptotic cell maker TUNEL stains in specimens were detected using *in situ* cell death detection kits (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. In some experiments, specimens were subjected to immunofluorescence staining probed by nephrin, acetylated lysine mAbs as primary antibody, and followed by fluorescence FITC and Rhodamin (Abcam, Cambridge, MA) as secondary antibodies and then counterstained with 4′,6-diamidino-2-phenylindole (DAKO, Carpinteria, CA).

**Histomorphometry**

Ten glomeruli in each section were randomly selected for microscopy under ×200 magnification (Carl Zeiss, Gottingen, Germany). Six regions within renal glomeruli from three sections obtained from six mice were detected. The positive immunolabeled and total cells per high-power field in each section were counted, and the percentage of positive labeled cells was calculated.

**In Vitro High Glucose–Treated Podocyte Cultures**

Primary mouse podocytes (5 × 10⁵ cells per well, six-well plate) were incubated in RPMI 1640 with 10% FBS in the presence or absence of 25 mM D-glucose or mannitol at 37°C for 48 hours. Immortalized mouse podocyte cell lines were grown as previously described and incubated in the presence or absence of 25 mM D-glucose at 37°C for 48 hours. In some experiments, subconfluent podocyte cultures were transfected with miR-29a precursor or empty vector by 10⁹ pfu/ml lentivirus-mediated constructs or HDAC4 siRNA or scrambled control by Lipofectamine 2000 according to the manufacturer’s instructions.

**Protein Ligation Assay**

Cell cultures were fixed by cold methanol and probed using Duolink II Fluorescence kits (Olink Bioscience, Uppsala, Sweden) with HDAC4 and nephrin antibodies and followed by Duolink proximity ligation assay. After ligation and amplification solution rinses, specimens were counterstained by 4′,6-diamidino-2-phenylindole. Specimens displaying red fluorescence reactions indicate intensive ligation between HDAC4 and nephrin.

**Chromatin Immunoprecipitation**

Chromatin in the nuclear extracts of cell cultures was sonicated and immunoprecipitated using H3K9Ac mAbs, IgG, and Megan ChIPA/G kits (Millipore), according to the manufacturer’s instructions. DNA in the immunoprecipitate were eluted by chromatin immunoprecipitation solution buffer and digested by proteinase K. DNA was subjected to purification and PCR amplification using Cy3-labeled probes (forward: 5′-ACGACAGATTGAAGGGG-3′; reverse: 5′-GGTGCTCTTTTCCCCAATCAT-3′) (Applied Biosystems) for the miR-29a proximal promoter. Enrichment of H3K9Ac in the miR-29a proximal promoter region of interest was calculated as the input percentage, as previously described.

![Figure 9. Scheme of miR-29a signaling protection against diabetes-induced podocyte injury and renal dysfunction. miR-29a signaling attenuates the promoting effects of hyperglycemia on HDAC4-dependent nephrin deacetylation and ubiquitination and WT-1 integrity loss that accelerates podocyte apoptosis, proteinuria, and renal fibrosis.](https://www.jasn.org/BASIC-RESEARCH/doi/10.1681/NKES-14-0189/Fig9.jpg)
Statistical Analyses
All values were expressed as means±SEs. In vitro experimental data were collected from at least three repeated experiments. Parametric ANOVA and a Bonferroni post hoc test were used to analyze the differences among various groups.

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DISCLOSURES
None.

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