Mitofusin 2 attenuates the histone acetylation at collagen IV promoter in diabetic nephropathy

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Abstract

Extracellular matrix (ECM) increase in diabetic nephropathy (DN) is closely related to mitochondrial dysfunction. The mechanism of protective function of mitofusin 2 (*Mfn2*) for mitochondria remains largely unknown. In this study, the molecular mechanisms for the effect of *Mfn2* on mitochondria and subsequent collagen IV expression in DN were investigated. Ras-binding-deficient mitofusin 2 (*Mfn2*-Ras(Δ)) were overexpressed in rat glomerular mesangial cells, and then the cells were detected for mitochondrial morphology, cellular reactive oxygen species (ROS), mRNA and protein expression of collagen IV with advanced glycation end-product (AGE) stimulation. Preliminary results reveal that the mitochondrial dysfunction and the increased synthesis of collagen IV after AGE stimulation were reverted by *Mfn2*-Ras(Δ) overexpression. Bioinformatical computations were performed to search transcriptional factor motifs in the promoter region of collagen IV. Three specific regions for TFAP2A binding were identified, followed by validation with chromatin immunoprecipitation experiments. Knocking down TFAP2A significantly decreased the TF binding in the first two regions and the gene expression of collagen IV. Furthermore, results reveal that *Mfn2*-Ras(Δ) overexpression significantly mitigated TFAP2A binding and also reverted the histone acetylation at Regions 1 and 2 after AGE stimulation. In streptozotocin-induced diabetic rats, *Mfn2*-Ras(Δ) overexpression also ameliorated glomerular mesangial lesions with decreased collagen IV expression, accompanied by decreased acetylation and TFAP2A binding at Region 1. In conclusion, this study highlights the pathway by which mitochondria affect the histone acetylation of gene promoter and provides a new potential therapy approach for DN.

Key Words

- ▶ Mitofusin 2
- ▶ histone
- ▶ acetylation
- ▶ collagen
- ▶ diabetic nephropathy

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Introduction

More than 347 million people suffer from diabetes worldwide (Danaei *et al.* 2011). Diabetic nephropathy (DN) is a serious complication of diabetes with poor prognosis and the most common cause of end-stage renal disease (Atkins & Zimmet 2010). However, the mechanism by which DN arises is yet to be elucidated even though there is an urgent need to identify an

effective early intervention for DN. One of the hallmarks of DN is the expansion of glomerular mesangial area for increased synthesis of extracellular matrix (ECM), which is mainly composed of collagen IV and V, fibronectin and laminin, among others (Mene *et al.* 1989, Haneda *et al.* 1991). Several recent studies have shown substantial increase in the mesangial content of collagen IV induced

by high glucose levels in mesangial cells (Whiteside *et al.* 2009, Li *et al.* 2013).

Emerging evidence indicates that oxidative stress serves as a critical pathogenic process in the development of DN (Onozato *et al.* 2002, Koya *et al.* 2003). Reactive oxygen species (ROS), which is mainly produced by mitochondria, is a significant factor causing oxidative stress in numerous pathological processes. Mitochondrial dysfunction in diabetes leads to overproduction of ROS, which has a major effect on disease progression (Brownlee 2001, 2005, Dugan *et al.* 2013). In addition, increasing evidence indicates that ECM accumulation in DN is closely associated with oxidative stress and mitochondrial dysfunction (Pozzi *et al.* 2009, Ribaldo *et al.* 2009). However, the underlying mechanism that explains the relationship remains unclear.

Mitofusin 2 (*Mfn2*), a dynamic regulatory protein found in the mitochondria, is critical for the maintenance of normal mitochondrial function in mammalian cells (Bach *et al.* 2003). *Mfn2* is a multifunctional protein that promotes mitochondrial fusion (Anton *et al.* 2013), activates Ras–MAPK signaling pathway via its Ras-binding site (77th to 91th amino acid of *Mfn2*, N-DVKGYSKVRGISEV-C) (de Brito & Scorrano 2009), regulates mitochondrial transport (Misko *et al.* 2010) and maintains the stability of mitochondrial DNA (Vielhaber *et al.* 2013). Both basic and clinical studies suggest that *Mfn2* is involved in mitochondrial biogenesis and metabolism in diabetes (Hernandez-Alvarez *et al.* 2010, Zorzano *et al.* 2010). In our previous study, overexpression of wild-type *Mfn2* attenuated numerous pathological changes in kidney structure and function associated with diabetes in rats, including repression of collagen IV synthesis (Tang *et al.* 2012). However, the mechanism by which overexpression of *Mfn2* affected the expression of pathogenic genes in DN remained unclear.

Abnormal gene transcription is a response to extracellular stimulation signals and is regulated by *cis*-regulatory elements, such as promoters, enhancers, silencers and insulators (Smith & Shilatifard 2014). Among these elements, promoters perform a central function in activating gene transcription. The initial step in gene expression involves chromatin remodeling in the promoter region, which is mainly regulated by histone acetylation or methylation (Jenuwein & Allis 2001, Kouzarides 2007). Important clues for the involvement of histone modifications in the regulation of pathologic genes that are associated with DN have emerged gradually in recent years. Previously, in endothelial cells cultured in high glucose,

the prolonged upregulation of p65 with sustained changes in H3 lysine-4 monomethylation was implicated (El-Osta *et al.* 2008). In uninephrectomized db/db mice, a model of non-insulin-dependent diabetes mellitus, H3K9 and H3K23 acetylation and H3K4 dimethylation significantly increased with severe glomerulosclerosis (Sayyed *et al.* 2010). The epigenetic characteristic of the promoter is enriched with histone 3 lysine-4 trimethylation (H3K4me3), and the active form is enriched with histone 3 lysine-27 acetylation (H3K27ac) (Jin *et al.* 2011, Zhou *et al.* 2011). Findings of such epigenetic changes have offered a new framework for understanding the pathogenesis of DN.

Increasing evidence indicates that ROS can directly induce epigenetic modification, consequently affecting gene expression. For instance, insulin alters the acetylation of histone H3 by enhancing ROS production under hyperglycemic conditions (Kabra *et al.* 2009). The level of H4K12 histone acetylation is significantly increased by superoxide overproduction in porcine oocytes during aging (Cui *et al.* 2011). In rat hepatocytes, oxidative stress induced by ethanol significantly stimulates histone H3 acetylation (Choudhury *et al.* 2010). To our knowledge, how ROS regulates key genes in DN at transcriptional level has yet to be extensively studied.

Elucidation of molecular mechanisms underlying DN at the transcriptional level is critical for understanding the pathogenesis of DN. In this study, we report that overexpression of Ras-binding-deficient mitofusin 2 (*Mfn2*–Ras(Δ)) attenuates AGE-dependent mitochondrial fragmentation and ROS production under conditions of diabetes in rat mesangial cells. We also found that overexpression of *Mfn2*–Ras(Δ) has effects on histone acetylation and transcriptional factor (TF) binding at specific regions near the collagen IV transcriptional start site (TSS), which may be responsible for the increased expression of collagen IV in DN. In addition, epigenetic changes were also validated in rat DN models. Our results provide some new insights into the complex molecular pathogenic mechanisms of DN and identify potential avenues for early intervention of DN.

Methods

Cell culture

Rat mesangial cells were obtained from Texas Health Science Center and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 17% fetal bovine serum (FBS) (Gibco) at 37°C in 5% CO₂ in a

humidified incubator. The cells were grown in complete medium for further validation experiments at 80% confluence after synchronization.

Preparation of advanced glycation end-product (AGE) proteins

AGE proteins were synthesized as described previously (Oldfield *et al.* 2001). In brief, 10 mg/mL bovine serum albumin (BSA) was incubated with 0.5 M D-glucose in 0.4 M phosphate buffer at 37°C for 12 weeks under sterile conditions. Unincorporated sugars were then removed through dialysis against phosphate-buffered saline (PBS). Control non-glycated BSA was prepared by incubation without glucose. Glycation was assessed by characteristic fluorescence (excitation of 370 nm and emission of 440 nm) with an 8- to 10-fold increase in fluorescence of AGE-BSA in comparison with BSA.

Construction of Mfn2 gene vectors and transfection

The rat *Mfn2* mRNA sequence (NM_130894.4) was obtained from the NCBI website. The *Mfn2*-Ras(Δ) sequence was subjected to Ras-binding region deletion. The target gene sequences were then subcloned between BamHI and AgeI restriction sites of the GV287 vector (element sequence: Ubi-MCS-3FLAG-SV40-EGFP). After amplification, the positive clone was confirmed by sequencing (GeneChem, Shanghai, China). Afterward, the purified reconstructed vectors combined with lentivirus (GeneChem) were used for transfection. In brief, rat glomerular mesangial cells (GMCs) were seeded in six-well plates in entire DMEM medium (Gibco) under normal culture condition. When cell growth reached 30–50% confluency, both 1×10^8 TU/mL lentivirus containing 20 μ L of reconstructed vectors and 1 μ L of 10 mg/mL Polybrene were added. After 8 h, the culture liquid was replaced with fresh medium.

Figure 1

Downregulation of mitofusin 2 and increased production of cellular ROS and hydrogen peroxide induced by AGE in GMCs. GMCs were exposed to control BSA or AGE-BSA at the concentration of 40 μ M. (A) The expression of mfn2 protein over time in GMCs under control BSA or AGE stimulation by Western blotting. β -actin was used as an internal control. * $P < 0.05$ vs control group. (B) The relative production of cellular ROS over time under control BSA or AGE stimulation. * $P < 0.05$ vs control at 0 h. (C) The hydrogen peroxide concentration changed over time under AGE stimulation. * $P < 0.05$ vs control group. (D) ROS and hydrogen peroxide relative levels in the presence of NAC under AGE stimulation. * $P < 0.05$ vs AGE group at 48 h. (E) The expression of mfn2 protein under AGE stimulation or ROS blocking by Western blotting. The samples were pretreated with NAC (5 mM) for 2 h before the addition of AGE in AGE + NAC group. β -actin was used as internal control. * $P < 0.05$ by *t*-test vs control group; ** $P < 0.05$ by *t*-test vs AGE group. Data are mean $\pm$ S.E.M. of three separate experiments.

The transfected GMCs were assessed for transfection efficiency and harvested for sub-culture after 72 h.

Co-immunoprecipitation

GMC lysates from various groups were used for co-immunoprecipitation as described previously (Koshiba *et al.* 2004, Zhang *et al.* 2009). In brief, cell lysates were first cleared with 50% protein A-agarose (Santa Cruz Biotechnology) and were then incubated with rabbit polyclonal antibody against *mfn2* protein (Thermo Scientific) for 1 h at 4°C, followed by incubation with protein A-agarose overnight at 4°C. The antigen-antibody-protein A-agarose complex pellets were collected through centrifugation and washed with RIPA buffer. The bound proteins were separated using SDS-PAGE and were then transferred to PVDF membranes for Western blot analyses with anti-*mfn2* (Thermo Scientific), anti-Ras (Santa Cruz Biotechnology) or non-specific IgG antibodies (Santa Cruz Biotechnology).

Western blot analysis

Western blot was performed to determine the expression levels of *mfn2*, Ras, collagen IV and TFAP2A proteins in GMCs. In brief, cells were pelleted and directly lysed with 2% SDS lysis buffer, boiled for 10 min and quantified by NanoDrop 2000. About 10 µg of protein was loaded in 4–12% NuPAGE Bis-Tris gels (Invitrogen), subjected to electrophoresis and transferred to Immobilon-P PVDF Membranes (Millipore). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (Invitrogen) before incubation overnight at 4°C with primary antibodies against *mfn2* (Thermo Scientific), Ras (Santa Cruz), collagen IV (Santa Cruz) and TFAP2A (ABCAM). Protein bands were visualized using HRP (Bio-Rad), as well as secondary antibodies (Sigma) and SuperSignal West Pico (Thermo Scientific).

Laser confocal microscopy for mitochondrial morphology

Rat mesangial cells were grown on coverslips inside a Petri dish filled with DMEM culture medium. When cells reached 80% confluency, the medium was removed from the dish, and a prewarmed (37°C) staining solution containing diluted MitoTracker Red CMXRos M7512 (Invitrogen) was added to the final working concentration (300 nM) as described previously (Amiott *et al.* 2008). The mixture was incubated for 30 min under growth conditions. Afterward, the cells were stained,

washed with PBS and fixed with growth medium containing 3% formaldehyde at 37°C for 15 min. The cells were then subjected to permeabilization with 0.2% Triton X-100 for 10 min. Finally, the coverslips were removed and placed on a dry platform for laser confocal microscopy by using a Zeiss LSM700 system with excitation and emission wavelengths of 579 and 599 nm, respectively.

Cellular ROS detection

ROS production in rat mesangial cells was assessed by flow cytometry (Becton Dickinson, Mountain View, CA, USA) as described previously, with minor modifications (Park *et al.* 2011). In brief, GMCs from each group under different conditions were washed twice with PBS with 10% FBS containing 5 µmol/L fluorescent probe CM-H2DCF-DA (Invitrogen). After incubation at routine temperature (RT) for 30 min in the darkness, cells were washed and resuspended in PBS. DCF intensity was analyzed using flow cytometry with excitation and emission wavelengths of 488 and 530 nm, respectively.

Hydrogen peroxide measurement

Hydrogen peroxide was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) according to the manufacturer's instructions. In brief, after treating the cells, media supernatants were collected and centrifuged immediately before use. For tissues, the total protein content in each sample was quantified by the Bradford method. A lower hydrogen peroxide concentration range was used for a standard curve to quantify H₂O₂ in supernatants. Hydrogen peroxide levels in supernatants were measured by characteristic fluorescence (excitation of 530 nm and emission of 590 nm) (Moreira *et al.* 2015).

Gene knockdown

GMCs were seeded and transfected at 60% confluence. Lipofectamine 3000 Reagent (Invitrogen) and 10 nM TFAP2A siRNA (a total of five sequences are listed in Table 1) were diluted in Opti-MEM medium, mixed together and added to cells according to the manufacturer's protocols. After 48 h, cells were used for further experiments.

Quantitative real-time PCR

GMCs were lysed with TRIzol-LS immediately (Ambion), and total RNA was prepared with RNeasy Plus Mini

Table 1 siRNA sequences for TFAP2A knockdown.

1	S 5': CUGGGAGCAUUAACUUUUAU UU mRNA: ggctgggagcattaactttatta AS 3': UU GACCUCGUAAUUGAAUA
2	S 5': CCAGAUCAAACUGUAAUUA UU mRNA: tccagatcaactgtaattaag AS 3': UU GGUCUAGUUUGACAUUAAU
3	S 5': GAGCAGGGUAUCAUUUAGA UU mRNA: cagagcagggtatcatttagata AS 3': UU CUCGUCCCAUAGUAAUUCU
4	S 5': GGUACAACCACCAUUUGA UU mRNA: aaggtacaaccacccatttgaac AS 3': UU CCAUGUUGGUGGUAAACU
5	S 5': GUAGGUCAAUCUCCCUACA UU mRNA: ctgtaggtcaatctccctacacc AS 3': UU CAUCCAGUUAGAGGGAUGU

Kit (Qiagen 74134) according to the manufacturer's instructions. cDNA was obtained by reverse transcription by using iScript cDNA Synthesis Kit (Bio-Rad). Afterward, cDNA was amplified with specific primers and detected using a SYBR Green Supermix (Bio-Rad), with the BIO-RAD CFX-96 Real-Time PCR system (Bio-Rad) under the following conditions: 95°C for 3min, followed by 40 cycles of 95°C for 10s and 55°C for 30s. The relative expression amount of cDNA was calculated using the 2^{-ΔΔCt} method. Expression levels were normalized with glyceraldehyde-3-phosphate dehydrogenase(GAPDH) mRNA as an internal control. (The primers for collagen IV are listed in Table 2.)

Mfn2 overexpression in vivo

Male Sprague–Dawley rats (10 weeks old, weighing 200–250g) were purchased from the Experimental Animal Center of Sichuan University. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma) at a dose of 65mg/kg dissolved in 0.05M citrate monosodium (ACROS) buffer (Brosius *et al.* 2009). All animals injected with STZ developed diabetes, as indicated by plasma glucose levels (13.9 mmol/L at 48h following the injection using a ReliOn Ultima glucose reader (Solartek)) (Lin *et al.* 2013). The animals were fed under the same condition without insulin therapy, subjected to different treatments, and then killed at the 12th week. Subsequently, kidney tissues were harvested. For determining *Mfn2*

overexpression, the animals were first anesthetized with an intramuscular injection of ketamine (35mg/kg) and xylazine (5mg/kg). After median incision of the abdomen, the abdominal aorta below the renal vessels, inferior vena cava and hepatic portal vessel were clamped. Approximately 15μL of the lentiviral vector-containing solution (either lenti-GFP or lenti-GFP-*Mfn2*, titer at 1×10⁸ tuberculin units/mL) was injected into the renal artery. After blood flow was re-established, the animals were allowed to recover. All animal procedures were approved by the Institutional Animal Care and Use Committee of the West China Hospital of Sichuan University, China.

Renal histology

The kidney tissues of rats were harvested and placed in 4% PBS-buffered paraformaldehyde for 24h and then embedded in paraffin after killing for each group. The paraffin-embedded tissues were sectioned at 3–5μm and stained with periodic acid Schiff (PAS). Digital images were obtained from a Leica DM5000B microscope system. PAS staining was used for assessing ECM deposition in glomeruli. The scores of sections were investigated in a blinded manner according to a previous scoring system (range, 0–4: 0, no ECM deposition; 4, ECM deposition in all sections of the glomeruli) (Tak *et al.* 2013).

Isolation of glomeruli

Glomeruli were isolated from the kidneys of rats by sequential sieving, as described previously (Holdsworth *et al.* 1978). In brief, after the rats were killed, kidneys were removed and de-capsulated. The renal cortical tissues were cut into 1 mm³ pieces, ground gently on 250μ stainless mesh with a syringe handle and rinsed with 1× PBS. The filtered material was sequentially passed through 100 and 70μ nylon meshes (Biologix, Jinan, China). The glomeruli-enriched fraction was retained on top of the 70μ mesh and subjected to further experiments.

Chromatin immunoprecipitation (ChIP)

For each ChIP experiment, approximately 1×10⁶ cells were fixed with 1% formaldehyde at RT for 5 min, lysed,

Table 2 Primers for collagen IV in q-PCR.

Primer name	Forward 5'–3'	Reverse 5'–3'
Collagen IV-a1 cDNA	GAAAGGAGACCAGGGAGA	TCCTCGGGAACCTTTATC
GAPDH	GTTACCAGGGCTGCCTTCTC	GATGGTGATGGGTTCCCGT

prepared for sonication with TruChIP Chromatin Shearing Reagent kit (Thermo Scientific, Covaris, 520127) and sonicated using Covaris E220 system to shear chromatin DNA. TFAP2A and H3K27 acetylation-bound chromatin fragments were enriched by immunoprecipitation with rabbit pAbs (Abcam) raised against the two immunogens. The ChIP reactions were carried out with a ChIP-IT high-sensitivity kit (Active Motif, 53040). After reversal of cross-linking, enriched DNA fragments were purified using a MinElute PCR purification kit (Qiagen, 28004) (Fan *et al.* 2012). Enrichments for specific DNA binding or histone modifications were quantified by qPCR.

Quantitative real-time PCR for enriched DNA segments after ChIP reaction (ChIP-qPCR)

TFAP2A binding and H3K27 acetylation enrichments were assessed by ChIP-qPCR in GMC. After the ChIP step, enriched DNA segments from matched IP sample and input control sample (without ChIP reactions) were subjected to quantitative real-time PCR (qPCR) analysis using SYBR Green Supermix (Bio-Rad) and CFX96 Real-time PCR system (Bio-Rad). qPCR positive primers were designed to cover the core consensus of three motifs (All primers are listed in Table 3). qPCR negative primers were referenced to the known negative regions. Enrichment scores were computed as $2^{-\Delta\Delta C_t}$ using the following formula:

$$2^{-\Delta\Delta C_t} = 2^{-[(C_{t_{\text{positive}}^{\text{IP}}} - C_{t_{\text{negative}}^{\text{IP}}}) - (C_{t_{\text{positive}}^{\text{Input}}} - C_{t_{\text{negative}}^{\text{Input}}})]}$$

(IP: DNA after ChIP reaction; Input: DNA without ChIP reaction; Positive: positive region primer pair; Negative: negative primer pair) (All primers for ChIP-qPCR are listed in Table 3.)

Statistical analysis

All data are expressed as the mean $\pm$ S.E.M. The comparison between groups was performed using one-way ANOVA. $P < 0.05$ was considered statistically significant. SPSS statistical software for Mac (version 20.0) was used for analysis.

Results

Effects of AGE on expression of Mfn2 and production of ROS

In a previous study, we found that the decreased expression of *Mfn2* induced by high glucose is combined with mitochondrial dysfunction (Tang *et al.* 2012). In this study, we detected the expression of *Mfn2* protein in GMCs stimulated by AGE at 0, 24, 36, 48 and 60h. The results indicated that *Mfn2* expression did not change over time in the control group (Fig. 1A, line 1). By contrast, with AGE stimulation, *Mfn2* expression declined significantly at 36h, dropped to the lowest level at 48h and showed no further decline at 60h (Fig. 1A, line 3). Inversely, AGE increased cellular ROS production over time, peaking at 48h (Fig. 1B), which was 1.77 ± 0.09 -fold higher than that at 0h ($P < 0.05$). Furthermore, changes in the hydrogen peroxide (H_2O_2)

Table 3 Primers for ChIP q-PCR.

Primer name	Forward 5'–3'	Reverse 5'–3'
Region1-core	GGCAGGAAGGAGACAT	CCTGCTCTGCTGGGAT
Region1-(–400bp)	CAGATAGGTGAGGGAAAG	CCAGTGCCTAGACAGAAC
Region1-(–200bp)	TAAACAGCACTACCTATGAG	TAAATGTAGCCAATCCTT
Region1-(+200bp)	CTCAAGATTGGGACAGA	GAAACTCAGCGGTGGT
Region1-(+400bp)	TTGGCAGCAAAAGGTG	CGAGTGCCTGTAGGTGT
Region2-core	TGCATTGCACAGGACA	GGAAGACAGGCTCACC
Region2-(–400bp)	ATAGGAGAAGGAGGGAGG	AGGGGACTTTGAATGTATCT
Region2-(–200bp)	GACCAGAGGCTGAGAAC	GCTGCGATGATAATGC
Region2-(+200bp)	AGACTCTTCTCCGTAGGC	GCTGCATGGGCTGAAC
Region2-(+400bp)	CAAGGGGCTCGTGGAA	CGCACCAGCTCTTCC
Region3-core	AGGGGCTCGTGGAATC	GGCTCGGGACTTGGTG
Region3-(–400bp)	GCAGCGCCAACAAATT	GCGAGGTGCTCAGTCT
Region3-(–200bp)	CGCTGCCGAAGGTGAGT	CGCTGGACAAAGAGGC
Region3-(+200bp)	ATCGCACATCCATTACG	GCTTCAGTCCCTTCTTAA
Region3-(+400bp)	CCACTCAGAGTCCACA	CTCCAAACAACCAGAAAC
TFAP2A negative	AGAGGAAGGCTACTGATT	CCACAAAAGTTCCACCA
H3K27ac negative	AGCTGCTGGTTTTCTCA	AGAATGCTTACATAGTCCC

concentration over time under AGE stimulation showed a similar trend as ROS (Fig. 1C). These results indicate that AGE reduces *Mfn2* expression and increases ROS production simultaneously, and further suggest that *Mfn2* expression changes are closely correlated with ROS production in DN. Based on these trends, 48h of AGE stimulation is the optimal experimental condition for conducting further experiments *in vitro*. Beyond that, we found that pre-treatment with N-acetylcysteine (NAC) could also effectively reduce ROS or H₂O₂ production induced by AGE (Fig. 1D). In addition, blocking ROS with N-acetylcysteine (NAC) had a slight reverse effect

on the decreased *mfn2* expression under AGE stimulation (Fig. 1E). For these reasons, we chose NAC as the ROS blocker. Taken together, our results suggest that decreased expression of *Mfn2* after AGE stimulation may serve as a distinct feature of dysfunctional mitochondria associated with ROS overproduction.

Interaction between *Mfn2* and Ras *in vitro*

To further elucidate the unique direct effect of *Mfn2* on mitochondria, we deleted the Ras-binding site sequences (77th to 91st amino acids) while conserving the domain

mediating mitochondrial function. Sequences of both wild-type and *Mfn2*-Ras(Δ) mRNA are shown in Fig. 2A. Co-immunoprecipitation was used to detect the interaction between Ras and *Mfn2* proteins. After 48h of stimulation with AGE, we found that either *Mfn2* expression or Ras-*Mfn2* binding increased (Fig. 2B, lane 2). Overexpression of wild-type *Mfn2* significantly increased the amount of *Mfn2* protein and Ras-*Mfn2* binding at the same time (Fig. 2B, lane 3). *Mfn2*-Ras(Δ) overexpression also notably facilitated the expression of *Mfn2* protein but did not increase Ras-*Mfn2* binding (Fig. 2B, lane 4), demonstrating that *Mfn2*-Ras(Δ) cannot bind to Ras and only has the ability to interact with mitochondria.

In reciprocal immunoprecipitation experiments with RAS and immunoblot for *Mfn2* (Fig. 2C), AGE did not change Ras protein expression but slightly increased the amount of Ras-*Mfn2* binding (Fig. 2C, lane 2).

Moreover, wild-type *Mfn2* overexpression significantly increased the amount of Ras-*Mfn2* binding but not *Mfn2*-Ras(Δ) overexpression (Fig. 2C, lane 3,4). These results suggest that only *Mfn2* with the Ras-binding domain and RAS can co-immunoprecipitate with each other. *Mfn2*-Ras(Δ) eliminates the potential for a Ras signaling mechanism, thereby providing us with a tool to examine the unique mitochondria-protective function of this protein.

In our study, NAC treatment of GMC had a modest effect on *Mfn2* expression, and the decrease in *Mfn2* with AGE treatment of GMC was only modestly reversed by the ROS blocker NAC (Fig. 2D, first line, right lane). However, it had barely any effect on *Mfn2*-Ras binding (Fig. 2D, second line, right lane). These results suggest that the dysfunctional mitochondria overproduce ROS and that ROS has adverse effects on mitochondria for facilitating mitochondrial damage.

Figure 3

Overexpression of *Mfn2*-Ras(Δ) ameliorated the mitochondrial morphology and function, thus reducing collagen IV synthesis. (A) The mitochondrial morphology changes in different groups by laser scanning confocal microscopy. The white arrows indicate the fragmental mitochondria. Views with high magnification were shown at the low right box. The right part is quantitation result for % number of cells with fragmental mitochondria, * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. (B) The relative level of ROS after *Mfn2*-Ras(Δ) transfection. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. (C) The hydrogen peroxide concentration changed after *Mfn2*-Ras(Δ) transfection. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. (D) The expression of collagen IV in rat mesangial cells by Western blotting. β -actin was used as an internal control. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. Data are mean $\pm$ s.e.m. of three separate experiments.

Effects of *Mfn2*-Ras(Δ) overexpression on AGE-induced mitochondrial dysfunction and collagen IV synthesis

Optimal expression of *Mfn2* in mitochondria aids in maintaining normal mitochondrial morphology and function (Baumann 2010, Chen *et al.* 2010a). In this study, either total *mfn2* or mitochondrial *mfn2* expression decreased after AGE stimulation. By contrast, overexpression of *Mfn2* increased the amount of *Mfn2* on mitochondria even with AGE stimulation, indicating that *Mfn2* mitochondrial distribution also changed after transfection (Fig. 2F). Furthermore, mitochondrial morphology was examined using MitoTracker labeling and confocal microscopy. The results revealed prominent fragments of mitochondria in the AGE group. Fragmented mitochondria were found to contain small spheres or short rods with AGE stimulation, whereas normal mitochondria were found to shape an elongated network under normal conditions (Fig. 3A). Mitochondrial fragments are related to mitochondrial dysfunction (Detmer & Chan 2007). This mitochondrial morphological change was accompanied by increase in ROS, up to 1.79 ± 0.09 -fold compared with that of the control group (Fig. 3B). Beyond this point, ROS overproduction may lead to further damage to cells. Consistent with previous reports (Li *et al.* 2014, Peng *et al.* 2015, Yu *et al.* 2006), *Mfn2*-Ras(Δ) overexpression dramatically reversed all of the previously mentioned adverse indicators of mitochondrial health under AGE stimulation, including the increased *Mfn2* mitochondrial distribution, elongated mitochondrial morphology and decreased ROS and hydrogen peroxide production, which was 1.17 ± 0.11 -fold compared with that in the control group and significantly different from that in the AGE group ($P < 0.05$) (Figs 2F and 3A, B and C). These results collectively demonstrate that *Mfn2*-Ras(Δ) overexpression rescued AGE-induced mitochondrial dysfunction partially by, but not limited to, alleviating mitochondrial fragmentation. Eventually, expression of collagen IV, a major component of ECM, increased with ROS overexpression under AGE stimulation. The involvement of ROS in increase in collagen IV has previously been reported in numerous studies (Taye *et al.* 2013, Papadimitriou *et al.* 2014, Yan *et al.* 2015). We also found that *Mfn2*-Ras(Δ) overexpression mitigated ROS overproduction along with decreased collagen IV synthesis (Fig. 3D). Our results suggest that *Mfn2* performs a protective function in DN through the mitochondria-ROS pathway.

Motif search for proximal regions near the transcriptional start site (TSS) of collagen IV

A computer search of the DNA sequence from chr16:82985857 to chr16:82989857 covering 2000bp upstream and downstream of collagen IV TSS was performed to further investigate how the mitochondrial dysfunction-ROS pathway affects collagen IV expression. First, the sequence was calculated using FIMO software, a motif search tool (Grant *et al.* 2011), with the command line: 'fimo --oc . --verbosity 1 --thresh 1.0E-4 All-motif.meme sequences.fa.' The results revealed several consensus sequences for binding of the transcription factor TFAP2A (Fig. 4A) in this area. Second, accurate positions of TFAP2A motifs were calculated using the MAST (version 4.10.2) software (Bailey & Gribskov 1998) with the command line: 'mast motifs.meme sequences.fa -oc . -nostatus -remcorr -minsegs 1 -ev 10.0.'. Results indicate that these sequences lie in the upstream -1497 to -1474bp and -625 to -602bp and the downstream +288 to +309bp of TSS. A high degree of similarity was observed between each identified sequence and TFAP2A ($P < 1.0E-4$) (Fig. 4B).

Relationship between TFAP2A and collagen IV

On account of the interesting results of our bioinformatic analysis, we conducted additional experiments to probe the relationship between TFAP2A and collagen IV. First, we successfully knocked down TFAP2A expression using siRNA. Relative expression of the TFAP2A protein significantly decreased upon siRNA administration to $26.04 \pm 3.89\%$ of that in the control group without siRNA ($P < 0.05$) (Fig. 4C). Second, by Western blotting, the expression level of TFAP2A after AGE stimulation was found to have no significant difference compared with that in the control group (Fig. 4D). These results indicate that AGE stimulation did not change the expression level of TFAP2A protein in this study. Third, the effect of TFAP2A knockdown on the expression of collagen IV mRNA was investigated by qPCR. Our results show that inhibition of TFAP2A significantly decreased the relative level of collagen IV mRNA from 3.31 ± 0.21 to 1.49 ± 0.12 -fold higher compared with that in the control group ($P < 0.05$) (Fig. 4E). These results indicate that TFAP2A is involved in the regulation of collagen IV expression at the transcriptional level. Furthermore, we found that changes in the level of collagen IV protein were consistent with changes in mRNA levels upon TFAP2A inhibition (Fig. 4F). Taken together, our results

Figure 4

Motif search for the proximal regions near the TSS of collagen IV and validation for matched transcript factor. (A) The logo of transcript factor TFAP2A.

The logo was drawn using meme software according to the occurrence rate for each base of TFAP2A motif.

(B) The block diagram of three motifs for TFAP2A binding near the TSS of collagen IV. In each block, the '+' indicates the motif locates at forward chain, whereas '-' at reverse chain; P value is the possibility which means the core sequence is dissimilar to that of the given motif matrix. The '+' above each base sequence means it has high similarity to that of the given motif; the core sequence of each motif was marked with colors; the grey block indicates the sequence of TFAP2A; TSS is transcriptional start site; the number '0' on the scale at the bottom means the location of TSS, upstream locations are indicated in negative numbers, whereas downstream in positive numbers.

(C) Western blotting result for knocking down TFAP2A in rat GMCs using siRNA technology. β-actin was used as an internal control. * $P < 0.05$ vs control group.

(D) AGE stimulation did not change the expression of TFAP2A protein. β-actin was used as internal control.

(E) Knocking down of TFAP2A has effect on the mRNA expression of collagen IV under AGE stimulation. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group.

(F) Knocking down of TFAP2A has final effect on the expression of collagen IV protein. β-actin was used as an internal control. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. Data are mean $\pm$ s.e.m. of three separate experiments.

Figure 5

ChIP-qPCR results for TFAP2A DNA binding and the effects of Mfn2-Ras(Δ) overexpression on H3K27 acetylation. (A) TFAP2A enrichments at Region 1 and near regions within 400 bp distance with or without AGE stimulation. * $P < 0.05$ vs the same region without AGE stimulation. (B) TFAP2A enrichments at region 2 and near regions within 400 bp distance with or without AGE stimulation. * $P < 0.05$ vs the same region without AGE stimulation. (C) TFAP2A enrichments at Region 3 and near regions within 400 bp distance with or without AGE stimulation. (D) Mfn2-Ras(Δ) overexpression has an effect on TFAP2A enrichment at both Regions 1 and 2. * $P < 0.05$ vs vectors transfection under AGE stimulation. (E) The changes of H3K27 acetylation in Regions 1 and 2 after AGE stimulation with or without Mfn2-Ras(Δ) transfection. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. (F) ROS blocking has effects on H3K27 acetylation in both Regions 1 and 2. * $P < 0.05$ vs control group; # $P < 0.05$ vs AGE group. Data are mean $\pm$ s.e.m. of three separate experiments.

indicate that TFAP2A has a positive regulatory function in the synthesis of collagen IV in DN.

Effects of AGE on TFAP2A DNA binding

Subsequently, we performed TF-ChIP experiments to test the DNA binding of TFAP2A at the corresponding motif regions with or without AGE stimulation. As shown in Fig. 5A, no TFAP2A enrichment peaks were found at the motif center (0 bp), as well as -400, -200, +200 and +400 bp from the motif center of Region 1 without AGE

stimulation. On the contrary, with AGE stimulation, the signal of TFAP2A enrichment was found at the motif center (0 bp), which was significantly stronger than that without AGE stimulation at the same site ($P < 0.05$). Similar results were also found in Region 2, as shown in Fig. 5B. The results indicate that AGE stimulation observably increased the DNA binding of TFAP2A at the motif centers of Regions 1 and 2, consequently facilitating the regulatory function of TFAP2A. However, unlike Regions 1 and 2, TFAP2A-binding enrichment signals have not been detected at Region 3 (Fig. 5C), suggesting that not

all calculated motifs could bind with the corresponding TFs under certain conditions. Hence, Region 3 may not be involved in this pathological process and may instead participate in another unknown mechanism.

Effect of *Mfn2*-Ras(Δ) overexpression on TFAP2A DNA binding

As described previously, *Mfn2*-Ras(Δ) overexpression mitigates the synthesis of collagen IV in DN. Therefore, we conducted another TF-ChIP experiment to detect the effect of *Mfn2*-Ras(Δ) overexpression on TFAP2A DNA binding at Regions 1 and 2. Our results reveal that TFAP2A relative enrichment at Region 1 was significantly reduced by *Mfn2*-Ras(Δ) overexpression to 0.27 ± 0.04 -fold of that in the AGE group ($P < 0.05$). *Mfn2*-Ras(Δ) overexpression also decreased TFAP2A enrichment at Region 2 to

0.17 ± 0.05 -fold of that in the AGE group ($P < 0.05$) (Fig. 5D). Correspondingly, vector transfection did not manifest these effects. Taken together, these consequences demonstrate that *Mfn2*-Ras(Δ) overexpression attenuates abnormal TFAP2A DNA binding, contributing to inhibition of the mechanism driving collagen IV gene expression through the mitochondria-ROS-TF pathway in DN.

Epigenetic changes in TFAP2A-binding sites

Furthermore, considering that the transcription factor TFAP2A can bind at Regions 1 and 2 near the collagen IV TSS and enhance gene expression, whether these two regions possess epigenetic modifications remains unclear. Our results suggest H3K27 acetylation of both Regions 1 and 2 at low levels under normal conditions.

Figure 6

Extracellular matrix deposition, TF binding and H3K27 acetylation *in vivo*. (A) Extracellular matrix deposition as determined by PAS staining. Black arrows indicate the ECM deposition. In Fig. 6(Aa) Normal rats as control; (Ab) diabetic rats without *Mfn2*-Ras(Δ) transfection; (Ac) diabetic rats with vectors transfection *in vivo*; (Ad) diabetic rats with *Mfn2*-Ras(Δ) transfection *in vivo*. (B) Histological scores of PAS staining sections. The scores were assessed at least in ten glomeruli in sections of three different mice. * $P < 0.05$ vs normal group; # $P < 0.05$ vs DN group. (C) Relative mRNA expression level for collagen IV in isolated glomeruli of rats as determined by q-PCR. * $P < 0.05$ vs normal group; # $P < 0.05$ vs DN group. (D) The hydrogen peroxide concentration changed after *Mfn2*-Ras(Δ) transfection *in vivo*. * $P < 0.05$ vs normal group; # $P < 0.05$ vs DN group. (E) The changes of H3K27 acetylation enrichment in Regions 1 and 2 in isolated glomeruli of rats. * $P < 0.05$ vs normal group; # $P < 0.05$ vs DN group. (F) The changes of TFAP2A binding enrichment in Regions 1 and 2 in isolated glomeruli of rats. * $P < 0.05$ vs normal group; # $P < 0.05$ vs DN group. Quantitative data are reported as the mean $\pm$ s.d. (Six animals were used for 6 independent experiments in each group).

On the contrary, the levels of H3K27 acetylation of Regions 1 and 2 are notably increased under AGE stimulation and can be mitigated by *Mfn2*-Ras(Δ) overexpression. In comparison, vector transfection showed no effect (Fig. 5E). To investigate the effect of ROS on H3K27 acetylation, we used ChIP-qPCR for determining the H3K27 acetylation level. In our study, we found that AGE significantly increased H3K27 acetylation level in both Regions 1 and 2. Interestingly, ROS blocking with NAC significantly decreased H3K27 acetylation in the two regions (Fig. 5F). The results indicate that ROS blocking has negative effect on the H3K27 acetylation level in Regions 1 and 2 after AGE stimulation. *Mfn2*-Ras(Δ) overexpression, like ROS blocking, can mitigate the H3K27 acetylation level in Regions 1 and 2 after AGE stimulation. The protective role of *Mfn2*-Ras(Δ) overexpression may be responsible for its effects on decreasing ROS production (Fig. 3B). These novel results are consistent with changes in TFAP2A binding under different conditions. Thus, H3K27 acetylation at the two regions may facilitate subsequent TFAP2A binding.

***In vivo* investigation of the effects of *Mfn2*-Ras(Δ) overexpression on epigenetic changes at TFAP2A-binding sites**

To further validate the epigenetic changes at TFAP2A-binding regions *in vivo*, we established STZ-induced diabetic animal models and overexpressed *Mfn2*-Ras(Δ) in their kidneys through targeted transfection. Consistent with our previous results, *Mfn2*-Ras(Δ) overexpression *in vivo* notably attenuated glomerular mesangial lesions (including ECM deposition and mesangial area expansion) and significantly decreased mesangial pathological scores (Fig. 6A and B). To further verify whether *Mfn2*-Ras(Δ) overexpression affected collagen IV expression at the transcriptional level, we detected glomerular collagen IV gene expression by qPCR. In accordance with the results *in vitro*, an evident increase in collagen IV gene expression in diabetic animal can be reversed by *Mfn2*-Ras(Δ) overexpression at the transcriptional level (Fig. 6C). In DN models, hydrogen peroxide production increased significantly, whereas in the *Mfn2*-Ras(Δ) overexpression group, hydrogen peroxide production was significantly reversed (Fig. 6D). The results indicate that *Mfn2*-Ras(Δ) overexpression can mitigate hydrogen peroxide production in DN animals. Interestingly, H3K27 acetylation changes were detected at Region 1 with

notable increase in the glomerulus of diabetic animals. However, H3K27 acetylation signals were fairly weak at Region 2 both in diabetic and normal animals (Fig. 6E). Consequently, TFAP2A binding changed similarly at the two regions (Fig. 6F). Moreover, *Mfn2*-Ras(Δ) overexpression *in vivo* significantly decreased both H3K27 acetylation and TFAP2A binding at Region 1. These results suggest that expression of collagen IV in DN *in vivo* depends on a considerably more complex regulatory mechanism than that *in vitro*.

Discussion

AGEs, as well as hyperglycemia, are known to be critical factors in the progression of DN (Brownlee *et al.* 1988, Soulis-Liparota *et al.* 1995). Here, we found that AGE significantly increased ROS production in GMCs, which is consistent with previous reports. *In vivo* experiments strongly demonstrated that infusion of AGEs into mice leads to increased cytosolic ROS, followed by increased mitochondrial permeability and deficiency of the mitochondrial respiratory complex I. By contrast, use of AGE cross-link breaker agent reduces mitochondrial superoxide generation (Coughlan *et al.* 2009). Moreover, similar results were found in cultured renal mesangial cells (Ide *et al.* 2010) and interstitial fibroblasts (Chen *et al.* 2010b). In our study, we also revealed that decreased expression of *Mfn2* over time was accompanied by ROS overproduction under AGE stimulation. The downregulation of *Mfn2* has been proven previously in cultured renal tubular cells with high glucose (Zhan *et al.* 2014) and in DN rat models (Tang *et al.* 2012). Interestingly, AGE exerts a similar effect on *Mfn2* expression in our study, and our results indicate that the change in *Mfn2* expression after AGE stimulation is not ROS dependent. Generally, expression of *Mfn2* is known to be regulated by peroxisome proliferator-activated receptor-coactivator-1 α (PGC-1 α) (Zorzano *et al.* 2010). The regulatory mechanism is extremely complicated as it involves gene transcription, translation and post-translational modification. The precise mechanism by which AGE affects *Mfn2* expression needs to be investigated further. To the best of our knowledge, the relationship between AGE and *Mfn2* has not been investigated previously. Our study shows that binding of *Mfn2* to mitochondria, and not Ras binding, mediates the protective effects of AGE stimulation and associated changes in ROS production, collagen IV expression and TFAP2A promoter binding to DN.

Overproduction of collagen IV in DN was previously shown either under high glucose (Tahara *et al.* 2012) or AGE stimulation in cultured mesangial cells (Abe *et al.* 2004). In both our previous and present studies, *Mfn2* exhibited a protective effect against DN. For instance, overexpression of either wild-type *Mfn2* (Tang *et al.* 2012) or *Mfn2*-Ras(Δ) attenuated the synthesis of collagen IV. In a recent report by Peng and coworkers, without DN limitation, *Mfn2* overexpression attenuated hypoxia-induced mitochondrial dysfunction, and the restored mitochondrial morphology subsequently reduced apoptosis in cultured HT22 cells (Peng *et al.* 2015). *Mfn2* overexpression was also found to attenuate injury-induced astrocyte hyperplasia, activation-relevant protein synthesis and cellular proliferation (Liu *et al.* 2014). In addition, *Mfn2* overexpression has been shown to suppress the development of atherosclerosis in ApoE (–/–) mice (Guo *et al.* 2007). However, distinguishing the exact function of *Mfn2* from others in these reports is difficult. In this study, overexpression of *Mfn2*-Ras(Δ) to compensate for AGE-induced *Mfn2* decrease attenuated collagen IV synthesis and subsequently reduced ECM expansion through direct interaction with mitochondria but not in a Ras signal pathway-dependent manner. This strongly supports the idea that *Mfn2* plays protective roles against DN. Without limitation, the protective role for *Mfn2* with other functions in DN should be investigated in future studies.

TFs, which are DNA-binding proteins that usually target specific DNA sequences, perform a regulatory role in gene expression. In this study, we found three candidate regions with TFAP2A (transcription factor AP-2 alpha)-like motifs near the rat collagen IV gene TSS. TFAP2A is found in various cellular and viral regulatory complexes and can bind the consensus sequence 5'-GCCNNNGGC-3' to activate the transcription of genes involved in a variety of important biological functions, including proper development of the eye, face, body wall, limb and neural tube (Rada-Iglesias *et al.* 2012, Hallberg *et al.* 2014). A previous study demonstrated that various inflammatory cytokines and prostaglandins induce the expression of TFAP-2, which subsequently causes aberrant activation of genes associated with hyperproliferation of mesangial cells and nephrosclerosis, suggesting an important function for TFAP-2 in glomerular disorders (Suyama *et al.* 2001). In another report, increase in TFAP-2 DNA-binding activity induced by estradiol was found to be involved in collagen metabolism (Guccione *et al.* 2002).

Consistently, our results revealed that knockdown of TFAP2A by siRNA significantly decreased collagen IV synthesis at the transcriptional level *in vitro*, indicating that TFAP2A performs a key function in collagen IV gene expression under AGE stimulation. Interestingly, the DNA-binding activity of TFAP2A was suppressed by *Mfn2* overexpression, which may be responsible for the observed decrease in collagen IV expression after *Mfn2* transfection under AGE stimulation.

However, the mechanisms for the effect of *Mfn2* overexpression on TFAP2A binding remain unclear. In this study, we analyzed the epigenetic changes in TFAP2A DNA-binding regions. In general, active promoters are specifically marked by H3K27ac (Rada-Iglesias *et al.* 2011), which can recruit TFs and thereby activate gene transcription (Spitz & Furlong 2012). Therefore, we focused on H3K27 acetylation, which is a key step for promoter activation. We observed acetylation changes at the two TFAP2A-binding regions with AGE stimulation or *Mfn2* overexpression, which could explain the TFAP2A DNA-binding changes observed previously. Epigenetic modifications and gene expression changes can be induced by changes in metabolite levels because numerous cofactors of chromatin-modifying enzymes are intermediate metabolic products (DeBerardinis & Thompson 2012, Wellen & Thompson 2012). For example, acetyl groups for histone acetyltransferases (HAT) originate from fatty acid and glucose metabolism (El-Osta *et al.* 2008). Mitochondrial functions, including oxidative TCA cycle, membrane potential and ROS production, are closed to histone acetylation (Martinez-Reyes *et al.* 2015). ROS enhancement of histone acetylation in diabetes or other diseases has also been reported (Bartling & Drumm 2009, Kabra *et al.* 2009). Consistent with the results of previous studies, our findings show that ROS performs a key function in histone acetylation. More specifically, ROS affects the acetylation of regulatory elements near the collagen IV TSS. In addition, *Mfn2* transfection reduces mitochondrial ROS production, thereby inhibiting the acetylation of regulatory elements and gene expression. This study is the first to report these effects. However, in our further study in the renal glomeruli of diabetic animals, change in acetylation was only found in Region 1, but not in Region 2. This result can be explained as follows: (a) the glomerulus is a mixture of numerous types of cells, which results in higher background noise and lower signal intensity associated with acetylation; (b) acetylation at Regions 1 and 2 may be asynchronous

and (c) gene regulation *in vivo* is considerably more complex than that *in vitro*. Therefore, unraveling the complexity of gene expression regulation *in vivo* remains a major challenge for future research.

Several questions need to be addressed to fully elucidate the mechanisms underlying the observed results. First, inhibition of TF binding at regulatory elements partially reduces the expression of collagen IV. This suggests that regulation of collagen IV expression is not restricted to interactions between the regulatory elements and TSS. Second, the interaction between regulatory elements and collagen IV TSS is unclear for technical reasons. Whether direct or indirect interaction is involved requires further investigation. Third, the detailed mechanism by which ROS facilitates histone acetylation is unclear. This may involve an extremely complex unknown molecular network, including localization of HAT, the pioneer TF for acetylation and the recruitment of numerous other cofactors, among others (Smith & Shilatifard 2014). In conclusion, our results reveal that overexpression of *Mfn2*-Ras(Δ), which has mitochondrial protective function, inhibits the synthesis of collagen IV in DN. Overproduction of ROS by dysfunctional mitochondria affects histone acetylation at regulatory elements near the collagen IV TSS in DN in a cell-specific manner. Furthermore, TF TFAP2A is involved in the mitochondria-ROS-collagen IV pathway through binding to regulatory elements. Therefore, the pathway by which mitochondria affect the function of regulatory elements at promoter regions may be an important area of research for tackling DN. Taken together, our findings improve our understanding of DN and offer new insights into possible therapeutic targets in DN.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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