Regulation of Matrix Metalloproteinase-9 by Epigenetic Modifications and the Development of Diabetic Retinopathy

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Diabetes activates retinal matrix metalloproteinase-9 (MMP-9), and MMP-9 damages the mitochondria and augments capillary cell apoptosis. Our aim is to elucidate the mechanism responsible for MMP-9 activation. Histone modifications and recruitment of the nuclear transcriptional factor κB (p65 subunit) at the MMP-9 promoter and the activity of lysine-specific demethylase 1 (LSD1) were measured in the retina from streptozotocin-induced diabetic rats. The role of LSD1 in MMP-9 activation was investigated in isolated retinal endothelial cells transfected with LSD1 small interfering RNA (siRNA). The results were confirmed in the retina from human donors with diabetic retinopathy. Diabetes decreased histone H3 dimethyl lysine 9 (H3K9me2) and increased acetyl H3K9 (Ac-H3K9) and p65 at the retinal MMP-9 promoter. LSD1 enzyme activity and its transcripts were elevated. LSD1 siRNA ameliorated the glucose-induced decrease in H3K9me2 and increase in p65 at the MMP-9 promoter, and prevented MMP-9 activation, mitochondrial damage, and cell apoptosis. Human donors with diabetic retinopathy had similar epigenetic changes at the MMP-9 promoter. Thus, activated LSD1 hypomethylates H3K9 at the MMP-9 promoter and this frees up that lysine 9 for acetylation. Increased Ac-H3K9 facilitates the recruitment of p65, resulting in MMP-9 activation and mitochondrial damage. Thus, the regulation of LSD1 by molecular or pharmacological means has the potential to retard the development of diabetic retinopathy. Diabetes 62:2559–2568, 2013

Mitochondrial dysfunction is considered as one of the mechanisms responsible for the development of diabetic retinopathy, possibly by initiating the loss of retinal capillary cells, a phenomenon that precedes the development of histopathology (1–3). Increased matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are observed in the retina of patients with diabetic retinopathy and in diabetic rodents (4–6), and activated MMPs (2 and 9) damage retinal mitochondria and accelerate cell apoptosis (5–8). MMP-9 gene transcripts are increased in the retina in diabetes (5,6,9,10), and the retinal vasculature of diabetic mice lacking the MMP-9 gene shows normal mitochondrial ultrastructure. The vasculature is also protected from the increased apoptosis and histopathology associated with diabetic retinopathy (5). However, the mechanism responsible for the regulation of retinal MMP-9 transcripts is not clear.

Transcription of genes is controlled by protein complexes, including chromatin-remodeling and transcription factors. Acetylation of histone lysine activates gene transcription (11), and a decrease in general histone acetylation in liver fibrosis and human sarcoma cells is implicated in subnormal MMP-9 promoter activity (12,13). Unlike acetylation, lysine residues can be mono-, di-, and trimethylated (me1, me2, and me3, respectively). Methylation of lysine 9 of histone 3 (H3K9) is associated with repressive chromatin conformation, but that of lysine 4 of H3 (H3K4) is associated with an open chromatin structure and active transcription (11,14). How histone modifications activate retinal MMP-9 in diabetes needs further investigation.

Demethylation of histones is regulated by a dynamic enzyme, lysine-specific demethylase 1 (LSD1), and demethylation of methyl H3K4 results in gene repression and that of methyl H3K9 in gene activation (15–17). In diabetes, LSD1 reduces methylation of H3K9 at the promoter of the p65 subunit of NF-κB (p65), and this is associated with the persistent activation of nuclear transcriptional κB, NF-κB (14). The effect of diabetes on retinal LSD1 and the role of LSD1-mediated demethylation in the activation of retinal MMP-9 remain to be explored.

Transcription of MMP-9 is controlled by 670 bases of upstream sequence that includes binding sites for transcriptional factors including AP-1, NF-κB, and SP1, and deletion or mutation in any of these binding sites decreases MMP-9 promoter activity (18,19). Induction of MMP-9 through activation of NF-κB is considered key for many signaling pathways, and the classic pathway of p65/p50 and alternative pathway p52/RelB can bind to the NF-κB binding site to activate the MMP-9 promoter (20,21). In the development of diabetic retinopathy, activation of retinal NF-κB is considered proapoptotic (22,23), and the binding of p65 is increased at the promoter and enhancer regions of Sod2, the gene that encodes MnSOD (24). The effect of diabetes on the regulation of the MMP-9 promoter by NF-κB remains unclear.

The aim of this study is to elucidate the mechanism responsible for the regulation of the retinal MMP-9 promoter in diabetes. Using retina from diabetic rats, we investigated histone methylation at the retinal MMP-9 promoter and the role of LSD1 in regulating methylation. In addition, the recruitment of p65 at the MMP-9 promoter was evaluated. The results were confirmed in retinal endothelial cells, the cells that are the target of the histopathology characteristic of diabetic retinopathy, by genetically modulating LSD1 or NF-κB. To transit from the animal models and in vitro studies to the human disease, the key findings were investigated in the retina from human donors with diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Rats. Six months after streptozotocin-induced diabetes in male Wistar rats (glycated hemoglobin 12.3 ± 2.0%), the retina was removed immediately. One retina was cross-linked with 1% paraformaldehyde for chromatin
immunoprecipitation (ChIP), and the other was stored in liquid nitrogen for future analysis. Age-matched normal rats (glycated hemoglobin 5.9 ± 0.9%) served as control.

**Mice.** Mice overexpressing Sod2 (MnSOD-Tg) and their wild-type (WT) littermates (−8 weeks of age) or C57BL/6 (control) or MMP-9 gene knockout (MMP-KO, B6.FVB [Cg]-MMP-9tm1Tvu/J; The Jackson Laboratory, Bar Harbor, ME) were made diabetic by streptozotocin (2,3,5,7,8,25). MnSOD-Tg mice were originally generated in the C3H background, and the hemizygous mice were then bred with WT B6C3 F1 mice to generate MnSOD-Tg mice (26). To avoid any m1-related retinal complications coming from the C3H strain, mice were bred for seven to eight generations with C57BL6 mice, and the absence of m1 was confirmed by genotyping for m1. As previously reported by us, nondiabetic WT and MnSOD-Tg mice had a similar number of degenerative retinal capillaries (2), and nondiabetic and diabetic MMP-KO mice had a similar retinal MMP-9 activity (5,6). The mice were killed after 7–12 months of diabetes by an overdose of pentobarbital, and the retina was isolated under a dissecting microscope.

Treatment of animals followed the National Institutes of Health principles of laboratory animal care and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research guidelines.

**Retinal endothelial cells.** Bovine retinal endothelial cells (BRECs) from fourth to fifth passage were transfected with LSD1 small interfering RNA (siRNA) or p65 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) as routinely performed in our laboratory (5,24). Parallel incubations were carried out using nontargeting scrambled RNA. At the end of the transfection, the cells were rinsed with PBS and incubated in 5 or 20 mmol/L glucose for 4 additional days. BRECs incubated in 20 mmol/L mannitol served as an osmotic control. Transfection efficiency was determined by quantifying the gene transcripts of LSD1 or p65 by semiquantitative PCR and confirmed by real-time quantitative PCR (qPCR).

**Human retina.** The retina was isolated from human postmortem eyes (Midwest Eye Banks, Ann Arbor, MI). Diabetic donors with an average duration of diabetes of 10–30 years had clinically documented proliferative retinopathy. Age-matched nondiabetic donors served as controls (Table 1). The eyes were enucleated between 6 and 9 h after death and transported on ice. One-third of the retina was selected randomly and fixed in paraformaldehyde, and the rest of the retina was frozen for further analysis. Our previous work has shown that the retina prepared from such postmortem eyes present similar diabetes-induced alterations in the gene expressions as observed in the fresh retina from diabetic rodents (3,6). Furthermore, others have shown stable transcripts from diabetic rodents (3,6). Furthermore, others have shown stable transcripts in the postmortem samples that are not influenced by the delay (7–24 h) in processing the tissue (27,28).

Gene expression was quantified by SYBR green-based qPCR using the 7500 Real-Time PCR System (Applied Biosystem). Amplification was performed in ~5 ng of ChIP-purified DNA or cDNA using the primers provided in Table 2. The PCR conditions included denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 60 s. This was followed with dissociation at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. The specific products were confirmed by SYBR green single melting curve and a single correct-size product on a 1.2% agarose gel. Each sample was measured in duplicate. Values obtained from the immunoprecipitate were normalized to the cycle threshold (Ct) value from the input sample, and those from cDNA were normalized to the Ct value from β-actin in the same sample using the delta-delta cycle threshold (ddCt) method. Relative fold changes were calculated by setting the mean fraction of normal rats or cells incubated in 5 mmol/L glucose as 1 (6,24). Taqman primers were used to quantify MMP-9 gene expression in the rat retina and LSD1 in BRECs with β-actin or 18S rRNA, respectively, as their housekeeping genes (24). LSD1 expression was also confirmed by semiquantitative PCR.

**Western blotting.** Proteins were separated on a 4–20% gradient polyacrylamide gel using primary antibody against the protein of interest and β-actin as the loading control. The band intensity was quantified using UnScan-It Gel digitizing software (Silk Scientific, Orem, UT).

ChIP was performed using a ChiP assay kit (Millipore Corp., Temecula, CA) according to the methods used in our laboratory (24,25). Protein-DNA complex (100–120 μg) was immunoprecipitated with the antibody against H3K4me1, H3K4me2, H3K9me2, LSD1, acetyl H3K3, p65, or general transcription factor IIIB (TFIIB). DNA fragments were recovered by phenol:chloroform:isoamyl alcohol extraction, followed by ethanol precipitation, and resuspended in 14 μL water for PCR. MMP-9 promoter was quantified in the extracted DNA (~5 ng) by SYBR green-based qPCR. Normal rabbit IgG was used as a negative antibody control and DNA from the input (20–40 μg protein-DNA complex) as an internal control. Each ChIP measurement was made with five to six samples per group. MMP-9 promoter precipitated by H3K4me2, p65, or normal rabbit IgG was confirmed by semiquantitative PCR, and the conditions included denaturation at 95°C for 2 min, 30 cycles of denaturation at 92°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 45 s. Specificity of the ChIP assay was verified by semiquantitative PCR; the off-target region of rat MMP-9 (nonpromoter region, +1859 to +2232) occupied by H3K4me2 was amplified for negative target control, and rat β-actin promoter bound by TFIIB served as a positive control. Whereas the promoter sequence of the MMP-9 gene (~650 to +1 bp upstream) is ~70% homologous among human, rat, cow, and mouse, the NF-κB binding site sequence is ~90–100% conserved among these species (18).

LSD1 activity was quantified in the retina using the LSD1 Fluorescent Assay kit (BPS Bioscience, San Diego, CA) according to the manufacturer’s instructions. Nuclear protein (5 μg), isolated by the Nuclear Extract kit (Active Motif, Carlsbad, CA), was incubated at room temperature for 30 min with 2× assay buffer and methyl peptide. The reaction mixture was then incubated for 5 min with Amplex red and peroxidase, and H2O2 production was quantified at 540-nm excitation and 600-nm emission wavelengths.

### TABLE 1

| Human donors | Age (years) | Duration of diabetes (years) | Cause of death | Laser therapy |
|--------------|-------------|------------------------------|----------------|---------------|
| Nondiabetic donors | 1 | 44 | — | Intracranial hemorrhage |
| | 2 | 37 | — | Asthma |
| | 3 | 70 | — | Myocardial infarction |
| | 4 | 72 | — | Myocardial infarction |
| | 5 | 55 | — | Subarachnoid hemorrhage |
| | 6 | 77 | — | Myocardial infarction |
| | 7 | 57 | — | Myocardial infarction |
| | 8 | 70 | — | Cerebrovascular accident |
| Donors with diabetic retinopathy | 1 | 75 | 25 | Pulmonary edema |
| | 2 | 66 | 30 | Acute myocardial infarction |
| | 3 | 54 | 10 | Unknown |
| | 4 | 61 | 10 | Acute myocardial infarction |
| | 5 | 68 | 10 | Congestive heart failure |
| | 6 | 59 | 25 | Pneumonia |
| | 7 | 47 | 27 | Acute myocardial infarction |
| | 8 | 75 | 30 | Acute myocardial infarction |
| | 9 | 68 | 10 | Congestive heart failure |

+, Patients who received laser therapy.
LSD1 activity in BRECs was quantified by chemiluminescent assay (BPS Bioscience). Nuclear protein (5–10 μg) was incubated for 1 h with 1× assay buffer on a methylated histone H3–coated plate. The formation of demethylated H3 was captured by a specific antibody and developed using horseradish peroxidase–labeled secondary antibody.

MMP-9 activity was measured by in situ zymography (5,6). In brief, conditioned medium (15–30 μL) was mixed with 4× zymograph sample buffer and electrophoresed on 8% SDS-polyacrylamide gel containing 1 mg/mL gelatin. The gel was washed with 2.5% Triton X-100, developed using 1× developing buffer (Bio-Rad, Hercules, CA), and stained with 2% (vol/vol) Coomassie blue G-250 (Sigma-Aldrich, St. Louis, MO). The intensity of the active band of MMP-9 (82–86 kDa) was quantified using Un-Scan-It Gel digitizing software.

Caspase-3 activity was measured using a kit from Enzo Life Sciences (Farmingdale, NY) as reported previously (29). In brief, 20–25 μg of retinal homogenate was incubated with Ac-DEVD-p-nitroaniline, and the formation of p-nitroaniline was quantified spectrophotometrically at 405 nm.

Apoptosis of retinal endothelial cells was determined by ELISA using Cell Death Detection ELISAPLUS as routinely performed in our laboratory (1,29).

Reactive oxygen species. The cells were incubated for 45 min with 10 μmol/L of 2′,7′-dichlorofluorescein diacetate (DCFH; Enzo Life Sciences), washed with PBS, and resuspended in PBS with protease inhibitors. Fluorescence was measured at 485-nm excitation and 516-nm emission wavelengths (30,31).

Mitochondrial morphology was determined by electron microscopy. After incubation of cells (transfected with LSD1 siRNA or untransfected cells) with

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**Table 2**

| Gene        | Primer sequence   | Position   |
|-------------|-------------------|------------|
| Rat         |                   |            |
| *MMP-9* promoter |                 |            |
| Forward     | TGCCCCTGAGGCTCCCACA | −644 to −625 |
| Reverse     | TGGAGCCCTCCTCCCACACTG | −466 to −485 |
| β-actin promoter |              |            |
| Forward     | ACGGAACCAGCACCACCAGTGC | −267 to −248 |
| Reverse     | CGCAGCTCTGCCAGGTGCTG | +12 to −9 |
| *MMP-9* off-target |       |            |
| Forward     | ATGGTGCCCCATGGTCCTTT | 1859 to 1878 |
| Reverse     | GAAATGGAAATACGCAGGGTTT | 2232 to 2211 |
| *LSD1*     |                   |            |
| Forward     | ACAGCAGCACAACACTCCA | −719 to −700 |
| Reverse     | TCCTCTCCTCCTCCCACACTCCTG | −546 to −565 |
| *MMP-9*     |                   |            |
| Forward     | CAGGCCACAGGCGGAAGACAGG | 1917 to 1937 |
| Reverse     | TGGGTGACTGACACCATATGTT | 2117 to 2095 |
| β-actin     |                   |            |
| Forward     | CATTATGGCAACACAGTGTCG | 89 to 109 |
| Reverse     | AAGCCGCGCTTGGCCACAT | 154 to 138 |
| Bovine      |                   |            |
| *MMP-9* promoter |                 |            |
| Forward     | CAGAGCCACAGGCGGAAGACAGG | −638 to −616 |
| Reverse     | CCAAACCCCCCACACTCCA | −471 to −492 |
| *MMP-9*     |                   |            |
| Forward     | CACTGTCCACACCCTACAGAGC | 1438 to 1457 |
| Reverse     | GCCACCTGTCGGAAGATGAGG | 1700 to 1681 |
| *LSD1*     |                   |            |
| Forward     | AGCTCGCCTTGGGCGATTCATG | 2117 to 2136 |
| Reverse     | TCACCCCCATGCTGGCACTG | 2290 to 2271 |
| β-actin     |                   |            |
| Forward     | ACGCTCGCCTTGGGCGATTCATG | 34 to 54 |
| Reverse     | TCCTCTGTCGGCTGGCTGCG | 270 to 249 |
| Human       |                   |            |
| *MMP-9* promoter |                 |            |
| Forward     | GATTCAACCCCTCGGGAGACAGGG | −638 to −616 |
| Reverse     | CCAAACCCCCACACTCCA | −471 to −492 |
| *MMP-9*     |                   |            |
| Forward     | CACTGTCCACACCCTACAGAGC | 1438 to 1457 |
| Reverse     | GCCACCTGTCGGAAGATGAGG | 1700 to 1681 |
| *LSD1*     |                   |            |
| Forward     | AGCTCGCCTTGGGCGATTCATG | 2117 to 2136 |
| Reverse     | TCACCCCCATGCTGGCACTG | 2290 to 2271 |
| β-actin     |                   |            |
| Forward     | ACGCTCGCCTTGGGCGATTCATG | 34 to 54 |
| Reverse     | TCCTCTGTCGGCTGGCTGCG | 270 to 249 |
| Mouse       |                   |            |
| *MMP-9*     |                   |            |
| Forward     | CAGAGCCTCTGCGGGTGGA | 1993 to 2012 |
| Reverse     | AAGGGCAGCTCAGAGGTCGTA | 2197 to 2176 |
| *LSD1*     |                   |            |
| Forward     | CGAAGCGGCGGCCAAGGGTAG | 478 to 497 |
| Reverse     | ACCAGACGGCTTCTCGGCTG | 660 to 641 |
| β-actin     |                   |            |
| Forward     | CTTCTGCTCCTGGCCTGCG | 955 to 974 |
| Reverse     | CATCGATCTCTCGCTGCTG | 1169 to 1150 |
high glucose, they were fixed in 2% glutaraldehyde in 100 mmol/L cacodylate, washed with 7% sucrose, embedded in 2% agar, and incubated in 1% osmium oxide (3,5). They were then dehydrated using graded ethanol solutions and embedded in Epon 812 resin (Electron Microscope Sciences, Hatfield, PA). Ultrathin transverse sections (70–80 nm) were stained with uranyl acetate–lead citrate mixture and imaged under a transmission electron microscope (model 400; Phillips, Eindhoven, the Netherlands).

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was carried out using SigmaStat software. The Kolmogorov-Smirnov test was used to test for normal distribution of the data. One-way ANOVA followed by Dun’s test was used for data that did not present normal distribution. The Kolmogorov-Smirnov test was used for data that obtained after normal distribution. P < 0.05 was considered statistically significant.

RESULTS
Diabetes epigenetically modifies retinal MMP-9 promoter. Since demethylation of H3K4 is associated with gene repression and that of H3K9 with activation (11,14–17), we investigated the effect of diabetes on the methylation status of H3K4 and H3K9 at the MMP-9 promoter. Diabetes increased methylation of H3K4 at the MMP-9 promoter (Fig. 1A), and the levels of H3K4me1 and -me2 were elevated by twofold compared with the values obtained from age-matched normal rat retina. Normal rabbit IgG, used as a negative antibody control, precipitated <10% of the product from ChIP with H3K4me1, -me2, and H3K9me2 antibodies. Increased H3K4me2 at the MMP-9 promoter was confirmed by semiquantitative PCR, and Fig. 1B shows a significant increase in H3K4me2 at the MMP-9 promoter. In contrast, as a control, IgG antibody produced almost undetectable PCR products at the MMP-9 promoter. To verify the specific binding at the MMP-9 promoter and validate the ChIP assay, the binding of H3K4me2 at the off-target region of MMP-9 was also quantified, and Fig. 1B shows almost negligible H3K4me2 at the off-target region of the MMP-9 promoter. Contrary to this, β-actin promoters occupied by TFIIB were strong in both normal and diabetic rat retina (Fig. 1B). In the same animals, however, H3K9me2 was decreased by ~45% (Fig. 1A).

To investigate the mechanism responsible for decreased methylation of H3K9, the demethylation enzyme LSD1 was quantified. Figure 2A shows that diabetes increased the activity of LSD1 by ~50% and its gene and protein expressions by over twofold compared with the values from age-matched normal rats (Fig. 2B and C). Consistent with a decrease in H3K9me2 (Fig. 1A), an increase in LSD1 was accompanied by its increased recruitment at the MMP-9 promoter (Fig. 2D). To determine if the decrease in H3K9me2 was sufficient to open up the chromatin and facilitate binding of MMP-9 activators, gene activation markers acetyl H3K9 and p65 were quantified. Contrary to a decrease in H3K9me2 at the MMP-9 promoter, acetyl H3K9 was increased by almost 18-fold and the recruitment of p65 was elevated by 30-fold (Fig. 2E).

Since MMP-9 is regulated by oxidative stress and activated MMP-9 in diabetes damages retinal mitochondria (5,32), to further corroborate the role of increased superoxide in the regulation of MMP-9, the effect of overexpression of MnSOD on LSD1 and MMP-9 was determined. Regulation of mitochondrial superoxide in mice ameliorated the diabetes-induced increase in LSD1 and MMP-9 gene expression of retinal MMP-9 in diabetes diabetessjournals.org

![FIG. 1. Methylation of H3K4 and H3K9 at the MMP-9 promoter is altered in the retina of diabetic rats. A: H3K4me1, H3K4me2, and H3K9me2 at the MMP-9 promoter were determined using ChIP technique followed by SYBR green–based qPCR. Retinal chromatin was immunoprecipitated with antibodies against H3K4me1, H3K4me2, and H3K9me2, and the MMP-9 promoter region was amplified. The qPCR value in each immunoprecipitate was normalized to the Ct value from the input sample using the ddCt method. Rabbit IgG served as a negative antibody control (indicated as *). B: The specificity of the ChIP assay was verified by semiquantitative PCR. Cross-linked retina was immunoprecipitated with H3K4me2 or p65, and the MMP-9 promoter occupied by H3K4me2 or p65, respectively, was amplified. DNA from the input (protein-DNA complex) was used as an internal control. For the ChIP assay, the positive controls were β-actin promoter occupied by TFIIB, and the negative controls included the MMP-9 promoter pulled down by normal rabbit IgG as negative antibody control and off-target region of MMP-9 (+1859 to +2232 bp region) occupied by H3K4me2 as off-target control. C: Gene expression of MMP-9 was quantified by Taqman-based qPCR and analyzed by ddCt method using β-actin as the housekeeping gene. Fold changes were calculated by setting the mean fraction of normal rats as 1. Values are represented as mean ± SD of five to seven rats in each group. *P < 0.05 compared with normal. Diab, diabetes; Nor, normal.](diabetes.diabetesjournals.org)
transcripts; the values obtained from the retina of MnSOD-Tg diabetic mice were significantly lower compared with those obtained from WT diabetic mice (Fig. 3A and B). In the same MnSOD-Tg diabetic mice, an increase in retinal MMP-9 protein was also prevented (Fig. 3C).

To investigate the effect of MMP-9 deletion on retinal apoptosis, caspase-3 activity was measured. As expected, caspase-3 activity was increased in the retina from control diabetic mice, which was prevented by the deletion of MMP-9; caspase-3 activity in MMP-KO diabetic mice was significantly lower compared with that in control diabetic mice (Fig. 3D).

**High glucose epigenetically modifies MMP-9 in retinal endothelial cells.** To confirm the role of epigenetic modifications of MMP-9 in diabetic retinopathy, the effect of high glucose on the methylation status of H3K9 was quantified in BRECs. Glucose decreased H3K9me2 at the MMP-9 promoter by >70% and increased the recruitment of LSD1 by 1.6-fold compared with the cells exposed to normal glucose (Fig. 4A and B). Acetyl H3K9 and the recruitment of p65 at the MMP-9 promoter were also increased by more than twofold (Fig. 4B). The specificity of the ChIP assay was validated using IgG antibody control, and the values obtained from the IgG antibody were <10% of the ChIP antibodies. Incubation of BRECs in 20 mmol/L mannitol did not affect H3K9me2 at the MMP-9 promoter, suggesting that the observed effects of high glucose on histone modifications were not due to an increase in the osmolarity. High glucose also increased LSD1 enzyme activity by 75% and its protein and gene expressions by over twofold compared with the cells incubated in normal glucose (Fig. 5A–C).

**Effect of regulation of LSD1 in retinal endothelial cells on glucose-induced epigenetic changes at the promoter of MMP-9.** To investigate the effect of inhibition of LSD1 on histone modifications at the MMP-9 promoter, the BRECs were transfected with LSD1 siRNA; Fig. 5D is included to show the efficiency of transfection. LSD1 siRNA alleviated the glucose-induced decrease in H3K9me2 and prevented increased recruitment of LSD1 at the MMP-9 promoter (Fig. 4A and B). Furthermore, p65 recruitment was also significantly decreased (Fig. 4B). In the same LSD1 siRNA–transfected cells, the glucose-induced increase in LSD1 protein and mRNA was also prevented (Fig. 5B and C). The values obtained from LSD1 siRNA–transfected cells in high glucose were significantly different from those obtained from untransfected cells or scramble RNA–transfected cells in high glucose.

To confirm if glucose-induced activation of MMP-9 is via LSD1, the effect of LSD1 siRNA on MMP-9 was determined. Transfection of cells with LSD1 siRNA prevented the glucose-induced increase in MMP-9 transcripts (Fig. 6A) and the activation of MMP-9, as determined by decreased formation of the active form of MMP-9 in the media (Fig. 6B). The values obtained from the cells transfected with scramble RNA or untransfected cells incubated in high glucose were significantly higher compared with LSD1 siRNA–transfected cells. Consistent with our previous results showing similar high glucose–induced activation of MMP-9 in the medium and mitochondria (5), these results strongly suggest that the regulation of LSD1 may also prevent activation of mitochondrial MMP-9.

To further confirm that LSD1-mediated regulation of MMP-9 is via p65, the effect of LSD1 siRNA on p65 was investigated. LSD1 siRNA prevented the glucose-induced...
increase in p65 binding at the MMP-9 promoter (Fig. 4B) and attenuated the increase in p65 gene and protein expressions (Fig. 6C). Furthermore, direct regulation of p65 by its siRNA also prevented the glucose-induced increase in MMP-9 transcripts (Fig. 6A). The transfection efficiency of p65 siRNA was 40–50%, and these values were similar to those reported by us previously (24).

The role of LSD1-mediated regulation of MMP-9 in the loss of retinal capillary cells was confirmed by measuring the effect of LSD1 siRNA on glucose-induced apoptosis, inhibition of LSD1, in addition to ameliorating the increase in MMP-9 and NF-κB, also prevented endothelial cell apoptosis (Fig. 6D). High glucose–induced values obtained from the untransfected cells or the cells transfected with scramble RNA were about twofold higher than those obtained from cells transfected with LSD1 siRNA.

To determine if LSD1-mediated regulation of MMP-9 is via mitochondrial superoxide, mitochondrial damage and reactive oxygen species (ROS) levels were quantified. High glucose–induced mitochondrial swelling, partial cristolysis, and increase in ROS were prevented in LSD1 siRNA–transfected cells (Fig. 6E and F).

Epigenetic modifications of MMP-9 in the retina from human donors with diabetic retinopathy. As with the retina from diabetic rats and mice (5,10), MMP-9 gene transcripts were elevated by ∼50% in the donors with diabetic retinopathy, and this was accompanied by similar increases in LSD1 gene transcripts compared with non-diabetic donors (Fig. 7A and B). Although H3K9me2 at the MMP-9 promoter was decreased in diabetic retinopathy donors, the values did not achieve statistical significance (Fig. 7C). However, consistent with the results from the rat retina and BRECs, acetyl H3K9 and the recruitment of p65 at the MMP-9 promoter were elevated by almost twofold compared with age-matched nondiabetic donors, and as a control, normal rabbit IgG yielded <10% of the ChIP antibodies (Fig. 7C).

DISCUSSION

In the pathogenesis of diabetic retinopathy, MMP-9 is activated in the retina, and the activation of MMP-9 is considered one of the key events that damages mitochondrial function and structure and activates the apoptotic machinery (5,10,29,33,34). Here we have provided the mechanistic details to show how diabetes activates retinal MMP-9; this is the first report showing that diabetes epigenetically modifies MMP-9; H3K9me2 is decreased and acetyl H3K9 and the recruitment of LSD1 and transcription factor NF-κB are increased at the MMP-9 promoter. LSD1 silencing in BRECs ameliorates the glucose-induced decrease in H3K9me2 and increase in p65 at the MMP-9 promoter, prevents the activation of MMP-9 (transcripts and enzymatic activity), and inhibits cell apoptosis. Furthermore, damage to the mitochondrial ultrastructure and an increase in ROS are also prevented by LSD1 siRNA, suggesting a close association between LSD1-mediated MMP-9 epigenetic modifications and oxidative stress. Together, these results strongly imply that the histone modifications of the MMP-9 promoter, mediated via
LSD1 are important in the activation of retinal MMP-9 in diabetes.

Histone modifications regulate gene expression by affecting the accessibility of chromatin to the transcription factors. Depending on the site of methylation, histone lysine methylation can signal either activation or repression of the gene. For example, although hypermethylated H3K4 is generally related to gene activation, hypermethylated H3K9 is related to gene repression (11,14). The results presented here show that hyperglycemia elevates H3K4me1 and H3K4me2 at the MMP-9 promoter but decreases H3K9me2. These results are consistent with those reported by others showing an association between hypermethylation of H3K4 and hypomethylation of H3K9 and the transcriptional activation of MMP-9 in a human cancer cell line (19). Furthermore, in support of our results, hyperglycemia produces similar dynamic cooperativity of histone methylase and demethylases in aortic endothelial cells where hypermethylation of H3K4 and hypomethylation of H3K9 are considered to participate in the activation of NF-κB (14).

Methyl status of histones is regulated by histone-modifying enzymes; histone methyltransferases catalyze the transfer of one, two, or three methyl groups to lysine or arginine residues of histones, and histone demethylases remove methyl groups (35). LSD1 specifically removes the methyl group from mono- and dimethylated H3K4 and H3K9 via a redox process (15–17,36). Removal of methyl groups from H3K4me2 by LSD1 induces transcriptional repression, whereas removal from H3K9me2 results in activation (11,37). Here we show that this demethylating enzyme is activated in hyperglycemic conditions, and increase in LSD1 activity is accompanied by its increased expression, both gene and protein. Furthermore, the recruitment of LSD1 at the MMP-9 promoter is also increased, suggesting that in addition to demethylation of H3K9me2, this increased recruitment of LSD1 at the promoter region could be contributing to the maintenance of active MMP-9 that the retina experiences in diabetes. The role of LSD1 in MMP-9 activation is further confirmed by our results showing that the regulation of LSD1 by its siRNA prevents a decrease in H3K9me2 and an increase in the recruitment of LSD1.

Gene expression of MMPs is largely regulated at the transcriptional level and MMP-9 promoter contains cis-regulatory elements for binding with the transcription factors, including AP-1 (−533 and −79 bp) and NF-κB (−600 bp). Regulation of MMP-9 protein levels has been largely ascribed to transcriptional activation of the gene via binding with these transcription factors (38–40). In accordance with decreased H3K9me2 at retinal MMP-9 promoter in diabetes, our results also show that acetyl H3K9 and the recruitment of p65 are also increased. Since hypomethylation and hyperacetylation of H3K9 are associated with the loosening of chromatin, and acetylation of histones alters the folding properties of the chromatin to facilitate the binding surface for the recruitment of other elements (41), these results clearly demonstrate that diabetes-induced modifications at H3K9 help recruit p65 at the MMP-9 promoter, resulting in the activation of MMP-9. To further confirm this, we show that the inhibition of LSD1 by its siRNA prevents increased recruitment of p65 at the MMP-9 promoter. In support, others have shown LSD1-mediated demethylation of H3K9 at the promoter of
FIG. 6. LSD1 siRNA of retinal endothelial cells prevents glucose-induced increase in MMP-9, p65, cell apoptosis, and ROS and protects mitochondrial damage. Cells transfected with LSD1 or p65 siRNA or scramble RNA were exposed to high glucose for 4 days. A: MMP-9 gene expression was quantified by SYBR green-based qPCR. B: MMP-9 activity was measured in the incubation medium by in situ zymography using pro-MMP-9 (human recombinant) as a marker. The active form of MMP-9 (82–86 kDa) relative to the latent form (92 kDa) was quantified and is presented in the accompanying histogram. C: Gene expression and protein level of the p65 subunit of NF-κB were quantified by qPCR and Western blot techniques, respectively, using β-actin as an internal control. D: Cell apoptosis was measured using an assay kit from Roche Diagnostics. ELISA for cytoplasmic histone-associated DNA fragments was performed. E: Morphology of the mitochondria was determined by electron microscopy using ultrathin sections of the cells embedded in 812 resin. Mitochondria were analyzed by ImageJ software in 8–10 images per sample and three to five
induced activation of both LSD1 and MMP-9, and silencing capillary cells (5,6,10). Our results clearly show that the oxidative stress (32), and the activated MMP-9 dam-
doxx-sensitive enzyme, which is also activated by increased and its capillary cells (25,44). In addition, MMP-9 is a re-
alterations in histone methylation at the strenghten a close association between LSD1-mediated and accelerated cell apoptosis. Thus, these results further
with the results from the retina of diabetic rats and retinal endothelial cells exposed to high glucose and further confirm the role of epigenetic modifications of the MMP-9 promoter in the development of diabetic retinopathy.

The current study is focused on the regulation of MMP-9 by epigenetic modifications; however, MMP-9 activity is also regulated by TIMP-1, the tissue inhibitor selective for MMP-9, and diabetes decreases retinal TIMP-1 levels (10). Thus, the regulation of MMP-9 in diabetes by TIMP-1 cannot be ruled out. Furthermore, as mentioned earlier, diabetes also alters other MMPs, especially MMP-2, and activated MMP-2 damages mitochondria and increases pericyte death (4,7–9,45). We cannot rule out the possibility that a similar mechanism could be regulating retinal MMP-2 in diabetes

In conclusion, we provide a novel mechanism of MMP-9 activation in diabetes; the results imply that the activated LSD1 in diabetes hypomethylates H3K9 at the MMP-9 promoter and this frees up that lysine (K9) for acetylation. Acetyl H3K9 opens up the chromatin, increasing the accessibility to recruit the transcriptional factor NF-κB, and this culminates in the activation of MMP-9 and mitochondri
damage. Thus, the regulation of LSD1 by molecular or pharmacological means could have the potential to pre-
vent/retard the development and progression of retinopa-
thy by regulating MMP-9–mediated mitochondrial damage and accelerated capillary cell apoptosis in diabetic patients. Fortunately, LSD1 inhibitors have shown promising results for other chronic diseases, such as carcinoma and leukemia (46,47), and our results could support their potential benefit for diabetic retinopathy.

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FIG. 7. Retinal MMP-9 is epigenetically modified in human donors with diabetic retinopathy. Retina from human donors with clinically document-
diabetic retinopathy (Diab) and age-matched nondiabetic (Nor) donors were analyzed to quantify gene transcripts of MMP-9 (A) and LSD1 (B) by qPCR. C: Cross-linked retina was immunoprecipitated with anti-H3K9me2, acetyl (Ac)-H3K9, p65, or normal rabbit IgG, and the MMP-9 promoter region was amplified in the precipitated DNA by SYBR green–based qPCR. IgG served as a negative antibody control (indicated as *), and Ct values were normalized with the values from input by ddCt method. Values from the retina of nondiabetic donors are adjusted to 1 and are represented as mean ± SD from four to six samples in each group. *P < 0.05, compared with the values in the retina from nondiabetic donors.

NF-κB, resulting in its glucose-induced persistent activation in aortic endothelial cells (14). Furthermore, we have also demonstrated alterations in retinal global histone acetylation in diabetes and epigenetic modification of Sod2 with increased binding of acetyl H3K9 and p65 at its pro-
moter and enhancer (24,42).

The demethylation reaction catalyzed by LSD1 is via a redox process that produces H2O2 and also results in local DNA oxidation producing 8-oxo-2′-deoxyguanosine (8-OHDG) (43), and in the development of diabetic retinopathy, H2O2 and 8-OHDG levels are elevated in the retina and its capillary cells (25,44). In addition, MMP-9 is a re-
doxx-sensitive enzyme, which is also activated by increased oxidative stress (32), and the activated MMP-9 dam-
gages mitochondria, accelerating the apoptosis of retinal capillary cells (5,6,10). Our results clearly show that the regulation of mitochondrial superoxide prevents diabetes-induced activation of both LSD1 and MMP-9, and silencing of LSD1 ameliorates glucose-induced mitochondrial damage (as evidenced by a decrease in the swelling and protection from partial cristolysis), increase in ROS levels, and accelerated cell apoptosis. Thus, these results further strengthen a close association between LSD1-mediated alterations in histone methylation at the MMP-9 promoter and mitochondrial damage in the development of diabetic retinopathy.

We have shown increased MMP-9 (activity and mRNA) in the retina and its microvessels from human donors with diabetic retinopathy (5,6), and here we show that MMP-9 is epigenetically modified with decreased H3K9me2 and increased binding of p65 at its promoter. In addition, these donors have increased LSD1 compared with their age-
matched nondiabetic donors. These results are consistent with the results from the retina of diabetic rats and retinal endothelial cells exposed to high glucose and further confirm the role of epigenetic modifications of the MMP-9 promoter in the development of diabetic retinopathy.

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Q.Z. planned the experiments, performed the literature search, researched data, and wrote the draft of the manuscript. R.A.K. planned the experiments, performed the literature search, and wrote and edited the manuscript. R.A.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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