Dynamic DNA methylation of matrix metalloproteinase-9 in the development of diabetic retinopathy

Renu A Kowluru, Yang Shan and Manish Mishra

Diabetes elevates matrix metalloproteinase-9 (MMP-9) in the retina and its capillary cells, and activated MMP-9 damages mitochondria, accelerating retinal capillary cell apoptosis, a phenomenon which precedes the development of retinopathy. Diabetes also favors epigenetic modifications regulating the expression of many genes. DNA methylation is maintained by methylating–hydroxymethylating enzymes, and retinal DNA methyltransferase (Dnmt) is activated in diabetes. Our aim is to investigate the role of DNA methylation in *MMP-9* regulation. The effect of high glucose on 5-methylcytosine (5mC) and 5-hydroxymethyl cytosine (5hmC), and binding of Dnmt1 and hydroxymethylating enzyme (Tet2) on *MMP-9* promoter were quantified in retinal endothelial cells. Specific role of Tet2 in *MMP-9* activation was validated using *Tet2*-siRNA. The results were confirmed in the retina from streptozotocin-induced diabetic mouse. Although glucose increased Dnmt1 binding at *MMP-9* promoter, it decreased 5mC levels. At the same promoter site, Tet2 binding and 5hmC levels were elevated. *Tet2*-siRNA ameliorated increase in 5hmC and *MMP-9* transcription, and protected mitochondrial damage. Diabetic mice also presented similar dynamic DNA methylation changes in the retinal *MMP-9* promoter. Thus, in diabetes transcription of retinal *MMP-9* is maintained, in part, by an active DNA methylation–hydroxymethylation process, and regulation of this machinery should help maintain mitochondrial homeostasis and inhibit the development/progression of diabetic retinopathy.

Laboratory Investigation (2016) 96, 1040–1049; doi:10.1038/labinvest.2016.78; published online 25 July 2016

Diabetic retinopathy remains one of the major causes of blindness in working adults. Many diabetes-mediated retinal metabolic abnormalities are implicated in its development, but the pathogenesis of this progressing disease remains complex. Our previous work has documented that diabetes activates retinal gelatinase matrix metalloproteinases (MMPs), and activated MMPs damage the mitochondria. The damaged mitochondria allow cytochrome C to leak into the cytosol, initiating apoptosis of capillary cells,^{1–6} a phenomenon that is followed by the histopathology associated with diabetic retinopathy.⁷

Transcription of *MMP-9* gene is regulated by upstream sequences, which contain motifs with binding sites for transcriptional factors including NF- κ B and AP-1.⁸ In diabetes, *MMP-9* transcription is upregulated in the retina and its capillary cells, and the binding of transcriptional factors NF- κ B and AP-1 is increased at its promoter.⁹ In addition to transcription factors, gene expression is also regulated by epigenetic modifications, and diabetes induces many such epigenetic modifications including DNA methylation and histone methylation/acetylation.^{3,10,11} Methylation of

the fifth carbon of cytosine (5-methylcytosine, 5mC) in the promoter or other upstream regulatory regions of a gene is generally associated with impaired binding of the transcription factors and repressed gene expression.^{12,13} Although DNA methylation is necessary for establishment and maturation of cell fates,¹⁴ and hypermethylation of retinal neurons onsets their programmed cell death,¹⁵ aberrant DNA methylation is associated with many chronic diseases including cancer, diabetes, age-related macular degeneration and diabetic retinopathy.^{12,13,16–18} In diabetes, retinal mitochondrial DNA (mtDNA) and the gene encoding the enzyme responsible for mtDNA biogenesis, polymerase γ 1, are hypermethylated compromising the electron transport chain machinery and mtDNA biogenesis.^{19,20}

DNA methylation is catalyzed by DNA methyltransferases (Dnmts); Dnmt3a and 3b are *de novo* enzymes, and Dnmt1 is a maintenance enzyme important in regulating tissue-specific patterns of the methylated cytosine.^{21,22} In diabetes, Dnmt activity is increased and the expression of Dnmt1 is elevated in the retina and its capillary cells.²⁰ Methylation of DNA is dynamically regulated both through passive and active

Department of Ophthalmology, Kresge Eye Institute, Wayne State University, Detroit, MI, USA

Correspondence: Dr RA Kowluru, PhD, Department of Ophthalmology, Kresge Eye Institute, Wayne State University, 4717 St Antoine, Detroit, MI 48201, USA.

E-mail: rkowluru@med.wayne.edu

Received 22 April 2016; revised 13 June 2016; accepted 19 June 2016

Table 1 Primer sequences

Gene	Sequence (5'–3')	Ampicon length (bp)
<i>Bovine</i>		
MMP-9 promoter (–720 to –547)	CAGACGCCACAACACTCCCA TCCTCTCCCTGCTCCACCTG	174
MMP-9 CGI (1487–1701)	TCTCTGCCTGTCTCTGAA CATCTCCGTGCTCTGAAG	215
<i>Dnmt1</i>	ACCATGTCTGACCTCCCTGA GCGCTCATGTCCTTGCAAAT	149
<i>Tet1</i>	ACACAATGACAGATGGCTTCTT ACCTCTTCTCCTCTACACAA	196
<i>Tet2</i>	GTGAGGGTGCATACCACTGT GTCTGGGTCTGAAGGAAGGC	89
<i>Tet3</i>	TCAAGTCTGTGGCTGGAG GAGGTGGCGTGAGAAGGTT	265
<i>Cytb</i>	CGATACATACACGCAACCGG AGAATCGGGTAAGGGTTGCT	298
<i>ND6</i>	CGTGATAGGTTTTGTGGGGT GCCAGTAACAATGCCCTTA	221
<i>β-actin</i>	CGCCATGGATGATGATATTGC AAGCCGGCCTTGACAT	66
<i>Mouse</i>		
MMP-9 promoter (–640 to –356)	CTAGCCTGAGAAGGATGAAG TTCCTTGGGCTTCTGAGA	285
MMP-9 CGI (1361–1600)	GTCGCAGTCACCTCTTCTCA ACCACAACCTCGTCGTCGTC	240
<i>Dnmt1</i>	CCTAGTTCGGTGCTACGAGGAGAA TCTCTCTCTCTGCAGCCGACTCA	137
<i>Dnmt3a</i>	GCCGAATTGTGCTTGGTGGATGACA CCTGGTGAATGCACTGCAGAAGGA	147
<i>Dnmt3b</i>	GTCTGCACACCAGAGACCAGAG TCAGAGCCATTCCCATCATCTAC	140
<i>Tet1</i>	GAGCCTGTTCTCGATGTGG CAAACCCACCTGAGGCTGTT	256
<i>Tet2</i>	TGTTGTTGTCAGGGTGAGAATC TCTTGCTTCTGGCAAACCTACA	103
<i>Tet3</i>	CCGGATTGAGAAGGTCATCTAC AAGATAACAATCACGGCGTTCT	162
<i>β-actin</i>	CCTCTATGCCAACACAGTGC CATCGTACTCTGCTTGCTG	215

mechanisms; while passive DNA demethylation is mostly found in mitotic cells, active demethylation, in which oxidation of 5mC forms 5-hydroxymethylcytosine (5hmC),

is mediated by ten-eleven translocation enzymes (Tets).^{23–25} Hyperglycemic milieu activates Tets in zebrafish inducing a genome-wide demethylation and aberrant gene expression.²⁶ However, how altered DNA methylation machinery contributes to the development of diabetic retinopathy is unclear.

Although the promoter region of *MMP-9* has relatively few CpG sites, DNA methylation is shown to modulate its transcription.^{27,28} The aim of this study is to investigate the dynamic DNA methylation of *MMP-9* promoter in the development of diabetic retinopathy. Using retinal endothelial cells, one of the target cells of retinal histopathology characteristic of diabetic retinopathy, we investigated the effect of 20 mM D-glucose on the methylation–hydroxymethylation status of *MMP-9* promoter. The specific role of the DNA methylation–hydroxymethylation was determined by regulating these enzymes by their specific siRNA, and the *in vitro* results were confirmed in the retina from diabetic mice.

MATERIALS AND METHODS

Retinal Endothelial Cells

Retinal endothelial cells, prepared from bovine eyes, from 4th to 7th passage were incubated in normal (5 mM) or high (20 mM) D-glucose for 4 days, and parallel osmotic controls included cells incubated in 20 mM mannitol or 20 mM L-glucose.^{9,20} To investigate the effect of inhibition of Tet in demethylation of *MMP-9* promoter, a batch of cells were incubated with a cell-permeable inhibitor (2S)-octyl- α -hydroxyglutarate (2-HG, 500 μ M; Cayman, Ann Arbor, MI, USA).²⁹ The cells received fresh media, including 2-HG, every 24 h. Inhibition of Dnmts/Tets was further confirmed by transfecting the cells with their specific siRNAs (*Dnmt1*-siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and *Tet2*-siRNA (Integrative DNA Technology, Coralville, IA, USA)) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Rockford, IL, USA), as routinely performed in our laboratory.^{9,20} Parallel incubations with non-targeting scrambled RNA were used as transfection controls. After transfection, the cells were rinsed and incubated in either 5 or 20 mM D-glucose media for 4 days. The efficiency of transfection was determined by quantifying their protein (western blot) and gene (SYBR Green-based quantitative real-time PCR (qPCR)) expressions.

Animal Models

Eight-week-old C57BL/6J mice were randomly assigned to normal and diabetes group. Diabetes was induced by streptozotocin (intraperitoneally; 55 mg/kg BW) for 4 consecutive days, and 2 days after the last injection, animals presenting blood glucose over 250 mg/dl were considered as diabetic. Animals in the diabetic group received daily insulin injection (0.1–0.3 IU, NPH) to prevent weight loss and dehydration. The mice were killed 6 months after induction of diabetes by using carbon dioxide, and retina were collected

Figure 1 High D-glucose hypomethylates matrix metalloproteinase-9 (MMP-9) promoter in retinal endothelial cells, and increases DNA methyltransferase 1 (Dnmt1) binding at the same region of the promoter. The levels of 5-methylcytosine (5mC) were quantified in retinal endothelial cells by methylated DNA Immunoprecipitation Kit from EpiGentek, and primers for (a) promoter region (−720 to −547), and (b) intragenic CpG island region (1487–1701) of *MMP-9* gene. The total genomic DNA from cells in 5 mM glucose served as an input DNA control. (c) Dnmt1 binding at the *MMP-9* promoter was quantified using Dnmt1 monoclonal antibody, followed by amplification of the promoter region by quantitative real-time PCR (qPCR). Immunoglobulin G (IgG) was used as a negative antibody control (indicated as ^), and Ct values were normalized with the values from input by ddCt method. Product size was confirmed on an agarose gel. Values are represented as mean ± s.d. from four to five samples in each group. Five and 20 mM = 5 or 20 mM D-glucose, Mann = 20 mM mannitol. * $P < 0.05$ compared with 5 mM D-glucose.

immediately. Age-matched, nondiabetic mice served as controls. These procedures conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and are approved by our Institutional Animal Care and Use Committee, and are routinely used in our laboratory.^{2,4}

Chromatin Immunoprecipitation

To analyze potential interactions between DNA and epigenetic machinery, chromatin immunoprecipitation (ChIP) assay was performed using the methods routinely performed in our laboratory.^{9,20} Briefly, paraformaldehyde crosslinked samples were sonicated six times for 10 s each using a Sonic Dismembrator (Model 100; Thermo Fisher Scientific) at a setting 6 for retina and 4 for endothelial cells. The samples were then diluted in ChIP dilution buffer and precleared with protein A agarose/salmon sperm DNA (EMD Millipore,

Billerica, MA, USA) for 1 h at 4 °C. This was followed by overnight incubation of 100–120 µg sample with Dnmt1 or Tet2 antibodies (Dnmt1, cat. no. ab13537 and Tet2, cat. no. ab135087; Abcam, Cambridge, MA, USA). The antibody–chromatin complexes were pulled down with protein A/G agarose beads and washed extensively before eluting with 1% SDS and 0.1 M NaHCO₃. Protein-associated DNA was recovered by incubation at 65 °C overnight, and then purified by phenol extraction. Purified DNA was precipitated with ethanol, resuspended in water, and used for qPCR using primers of the regions of interest. Normal rabbit IgG (cat. no. 2729S; Cell Signaling, Danvers, MA, USA) was used as a negative antibody control, and DNA from the input (40 µg protein–DNA complex) as an internal control. The specificity of ChIP assay was confirmed by analyzing the products on a 2% agarose gel, and representative gel images are included in the accompanying figures.

Quantification of 5mC and 5hmC

Sonicated DNA was immunoprecipitated for 5mC/5hmC using methylated/hydroxymethylated DNA Immunoprecipitation (MeDIP/hMeDIP) Kits (EPIGENTEK, Farmingdale, NY, USA). The enriched 5mC/5hmC fractions were analyzed by qPCR using species-specific *MMP-9* primers (Table 1), as recently reported by us.²⁰ The products were confirmed by semiquantitative PCR on a 2% agarose gel.

Western Blot

Protein (30–40 µg) was separated on a 4–20% gradient acrylamide gel (Bio-Rad, Hercules, CA, USA). The proteins transferred onto nitrocellulose membranes were detected using antibodies against the proteins of interest (Dnmt1 and Tet2 from Abcam, and β-actin cat. no. A-5316 from Sigma-Aldrich, St Louis, MO, USA). Images were quantified by the Carestream MI software (Carestream, Rochester, NY, USA), and the intensities of the target bands were normalized to those of the loading control, β-actin.

Gene Transcripts

Gene transcripts were quantified by qPCR using target-specific primers. Amplification program included the holding stage at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and 72 °C for 5 min. Reaction specificity was validated by a single peak in the melting curve. For *MMP-9*, TaqMan qPCR was performed. Values of the products were normalized to the cycle threshold (Ct) value from the input sample, and those in cDNA were normalized to the Ct values from β-actin in the same sample. Relative fold changes were calculated by considering the values obtained from cells in 5 mM glucose or normal mice as one.^{4,9,20}

Enzyme Activities of Dnmt and Tet

The global activities of Dnmt and Tet were determined in nuclear fractions using EpiQuik DNA Methyltransferase

Activity/Inhibition and Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kits (EpiGentek), respectively. Briefly, nuclear extracts (3–6 μ g for Dnmt or 10–20 μ g for Tet activity) was added to the microplates coated with enzyme substrates, and the reaction products were detected by following the manufacturer's instructions.

Mitochondrial Damage

Mitochondrial damage was evaluated by quantifying the gene transcripts of mtDNA-encoded cytochrome b (*Cytb*) of complex III and *ND6* of complex I, as reported previously.^{4,9,20}

Statistical Analysis

Results were analyzed using SigmaPlot (Systat, Chicago, IL, USA), and the data are presented as mean $\pm$ standard deviations. Statistical test between two groups was carried out by *t*-test or Mann–Whitney rank-sum test. For multiple comparisons for the data with normal distribution, one-way ANOVA followed by Student–Newman–Keuls test was performed, and for the data that did not qualify the normal distribution pattern, one-way analysis on ranks followed by Dunn's test were used. A *P*-value <0.05 was considered as statistically significant.

RESULTS

Retinal Endothelial Cells

Incubation of retinal endothelial cells in 20 mM D-glucose resulted in ~50% reduction in 5mC levels in *MMP-9* promoter (–720 to –547) compared with the cells incubated in normal glucose or 20 mM mannitol (Figure 1a). In contrast, in the intragenic (1487–1701) CpG island region of the *MMP-9* gene, 5mC values were similar in the cells incubated in normal or high D-glucose (Figure 1b).

Since Dnmt plays a major role in the formation of 5mC, and among its 3 major isoforms, hyperglycemia increases Dnmt1 transcripts in retinal endothelial cells,²⁰ the binding of Dnmt1 at *MMP-9* promoter was determined by ChIP technique. Contrary to a significant decrease in 5mC levels, high D-glucose increased Dnmt1 binding by over 2.5-fold, suggesting an active methylation–demethylation processes. Values obtained from IgG controls were less than 1% compared to the values obtained using Dnmt1 antibody (Figure 1c).

To further confirm the role of Dnmt1 in the methylation status of *MMP-9* promoter, cells transfected with *Dnmt1*-siRNA were utilized. Transfection of cells with *Dnmt1*-siRNA significantly decreased 5mC levels, and the values obtained from *Dnmt1*-siRNA-transfected cells incubated in normal or high D-glucose were not significantly different from the untransfected cells in high D-glucose (Figure 2a). Figures 2b and c are included to show the transfection efficiency of *Dnmt1*-siRNA; cells transfected with *Dnmt1*-siRNA had <50% *Dnmt1* gene and protein expressions compared with the untransfected cells. *Dnmt1*-siRNA also inhibited

Figure 2 Regulation of DNA methyltransferase (Dnmt1) has no effect on D-glucose-induced decrease in 5-methylcytosine (5mC) at the matrix metalloproteinase-9 (*MMP-9*) promoter. (a) 5mC levels at the *MMP-9* promoter were quantified in the cells transfected with *Dnmt1*-siRNA or scrambled RNA, and incubated in either 5 mM glucose or 20 mM D-glucose. The transfection efficiency of *Dnmt1*-siRNA was measured by quantifying *Dnmt1* (b) gene transcripts (quantitative real-time PCR (qPCR)) and (c) protein expression (western blot). (d) The activity of Dnmt was assayed in 3–6 μ g sample using DNA Methyltransferase Activity/Inhibition Assay Kit, and the values obtained from cells in 5 mM glucose are considered as 100%. Five and 20 mM = 5 mM or 20 mM D-glucose, 5+/D-si and 5+/SC = cells transfected with *Dnmt1*-siRNA or scrambled RNA, respectively, followed by incubation in 5 mM D-glucose; 20+/D-si and 20+/SC = cells transfected with *Dnmt1*-siRNA or scrambled RNA, respectively, and incubated in 20 mM D-glucose; Mann = 20 mM mannitol; L-Gl = 20 mM L-glucose. Data are represented as mean $\pm$ s.d. from each measurement made in duplicate in three to five cell preparations. **P* < 0.05 compared with 5 mM D-glucose. siRNA, small interfering RNA.

significantly increase in Dnmt enzyme activity, observed in high D-glucose condition (Figure 2d).

Due to the dynamic nature of DNA methylation, methylated cytosine can be converted to 5hmC;^{23,24} to understand the mechanism for decreased 5mC levels at the *MMP-9* promoter, in spite of increased Dnmt1 binding, 5hmC levels were quantified. 5hmC levels at the *MMP-9* promoter were increased by three fold in the cells incubated in high D-glucose compared with the cells in 5 mM D-glucose or 20 mM mannitol (Figure 3a). The role of demethylation in *MMP-9* transcription was confirmed by supplementing 20 mM D-glucose medium with an inhibitor of the hydroxymethylation enzymes; addition of 2-HG, in addition to preventing glucose-induced increase in Tet activity (Figure 3b), also ameliorated increase in *MMP-9* transcripts (Figure 3c).

Tet family of enzymes has three major members, Tet1, Tet2, and Tet3;^{24,30} to determine the specific isoform(s) responsible for hypomethylation of *MMP-9* promoter, the effect of 20 mM D-glucose on their expressions was

Figure 3 High D-glucose increases hydroxymethylation of matrix metalloproteinase-9 (MMP-9) promoter and Tet activity. (a) The levels of 5-hydroxymethylcytosine (5hmC) were quantified in retinal endothelial cells using hMeDIP Immunoprecipitation Kit. (b) The enzyme activity of Tet was determined in 10–20 μ g sample using TET Activity/Inhibition Assay Kit, and the values obtained from cells in 5 mM glucose are considered as 100%. (c) MMP-9 mRNA was quantified by quantitative real-time PCR (qPCR). Values are represented as mean $\pm$ s.d. from four to five cell preparations, with each measurement made in duplicate. Five and 20 mM = 5 mM or 20 mM D-glucose; 5+/2-HG and 20+/2-HG = cells incubated in 5 or 20 mM D-glucose in the presence of 500 μ M 2-HG; Mann = 20 mM mannitol; L-Gl = cells in 20 mM L-glucose. * P < 0.05 compared with 5 mM D-glucose. 2-HG, (2S)-octyl- α -hydroxyglutarate.

quantified. Although 20 mM D-glucose had no effect on *Tet1* and *Tet3*, *Tet2* expression (protein and gene) was significantly increased compared with the values obtained from cells in normal glucose (Figure 4a). The role of *Tet2* in hydroxymethylation of 5mC was confirmed by its binding at the MMP-9 promoter using ChIP technique; consistent with increase in 5hmC levels, *Tet2* binding was also elevated by over fourfold in cells incubated in 20 mM D-glucose. In the same samples, IgG controls were <1% compared with the values obtained using *Tet2* antibody (Figure 4b). Regulation of *Tet2* by its specific siRNA prevented 20 mM D-glucose-induced increase in 5hmC levels, and, surprisingly, ameliorated decrease in 5mC levels at the MMP-9 promoter. In the same cell preparation, transcriptional activation of MMP-9 was also protected (Figure 5a).

Activated MMP-9 damages retinal mitochondria initiating capillary cell apoptosis, which precedes the development of degenerative capillaries in the retinal microvasculature.⁴ The protective effect of inhibition of hydroxymethylation was confirmed by quantifying transcription of mtDNA. As expected, incubation of cells in 20 mM D-glucose, but not in 20 mM L-glucose, significantly decreased mtDNA

Figure 4 Tet2 expression and its binding at the matrix metalloproteinase-9 (MMP-9) promoter are increased by high glucose. (a) The gene transcripts of *Tet1*, *Tet2*, and *Tet3* were quantified by quantitative real-time PCR (qPCR) using gene-specific primers, and the protein expression of *Tet2* was determined by western blot technique using β -actin as a housekeeping gene (qPCR)/loading control (western blot). (b) *Tet2* binding at the MMP-9 promoter was quantified in the *Tet2*-immunoprecipitated crosslinked cells using immunoglobulin G (IgG) as a negative antibody control (indicated as ^), and Ct values were normalized with the values from input by ddCt method. Values are represented as mean $\pm$ s.d. from three to five samples in each group. Five and 20 mM = 5 mM and 20 mM D-glucose; Mann = 20 mM mannitol. * P < 0.05 compared with 5 mM D-glucose.

transcription, as depicted by 40–60% decrease in mtDNA-encoded *Cytb* and *ND6*. However, regulation of hydroxymethylation ameliorated glucose-mediated decrease in transcription of both *Cytb* and *ND6* (Figure 5b). Figures 5c and d are included to show the ~50% decrease in mRNA and protein expression of *Tet2* in the cells transfected with *Tet2*-siRNA.

Diabetic Animal Model

Consistent with the results from isolated retinal endothelial cells in high D-glucose, diabetes decreased 5mC levels in the promoter region (–640 to –356) of the retinal MMP-9, compared with the values from nondiabetic normal mice. However, 5mC levels in the intragenic CpG island region (1361–1600) remained unchanged in the retina from normal and diabetic mice (Figure 6a). As expected,^{4,9} in the same diabetic animals, retinal MMP-9 transcripts were significantly higher compared with those from normal mice (Figure 6b).

Dnmt is activated in the retina in diabetes,²⁰ and consistent with the results from diabetic rats and retinal endothelial cells in high D-glucose, among the members of the Dnmt family, the expression of Dnmt1 was significantly elevated (Figure 6c). To investigate the role of Dnmt1 in the regulation

Figure 5 Inhibition of *Tet2* by its small interfering RNA (siRNA) ameliorates D-glucose-induced increase in 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) levels at the matrix metalloproteinase-9 (*MMP-9*) promoter, and protects mitochondrial damage. **(a)** The levels of 5hmC and 5mC, and *MMP-9* mRNA were quantified in the cells transfected with *Tet2*-siRNA or scrambled RNA. **(b)** Mitochondrial DNA damage was assessed by quantifying gene transcripts of mitochondrial DNA (mtDNA)-encoded cytochrome b (*Cytb*) and *ND6* by quantitative real-time PCR (qPCR) using β -actin as the housekeeping gene. Transfection efficiency of *Tet2*-siRNA was determined by quantifying its **(c)** gene transcripts and **(d)** protein expression. Five and 20 mM = 5 mM or 20 mM D-glucose; 5+/T-si and 5+/SC = cells transfected with *Tet2*-siRNA or scrambled RNA, respectively, followed by incubation in 5 mM D-glucose; 20+/T-si and 20+/SC = *Tet2*-siRNA or scrambled RNA transfected cells incubated in 20 mM D-glucose; 5+/2-HG and 20+/2-HG = cells incubated in the presence of 500 μ M 2-HG in 5 mM or 20 mM D-glucose respectively; L-Gl = 20 mM L-glucose. Data are represented as mean $\pm$ s.d. from three to five measurement made in duplicate. * P < 0.05 compared with 5 mM D-glucose. 2-HG, (2S)-octyl- α -hydroxyglutarate.

of retinal *MMP-9* promoter methylation status, its binding at the *MMP-9* promoter was determined. Figure 6d shows a significant increase in Dnmt1 binding at the *MMP-9* promoter; the values obtained from diabetic mice were over twofold higher compared with the nondiabetic mice. Similar discrepancies between increase in Dnmt1 binding but decrease in 5mC levels at the *MMP-9* promoter by high glucose in retinal endothelial cells and in the retina from diabetic mice support an active demethylation of the *MMP-9* promoter.

To further confirm the role of active hypomethylation in transcriptional activation of *MMP-9* in diabetes, 5hmC levels were quantified; as shown in Figure 7a, 5hmC levels at the *MMP-9* promoter were increased by over 2.5-fold in the retina from diabetic mice compared with that from age-matched normal mice. In the same retina samples, global Tet activity was also elevated by ~75% (Figure 7b), and among the three members of Tet family, only Tet2 expression (mRNA and protein) showed significant increase in the retina from diabetic mice compared with the retina from

Figure 6 Diabetes hypomethylates matrix metalloproteinase-9 (*MMP-9*) promoter, but the binding of DNA methyltransferase (*Dnmt1*) in the same region is increased. (a) The levels of 5-methylcytosine (5mC) were quantified in the promoter (–640 to –356) and the intragenic CpG island (1361–1600) regions of the *MMP-9* gene by methylated DNA Immunoprecipitation Kit. Total genomic DNA obtained from normal mouse retina served as input DNA control. Gene transcripts of (b) *MMP-9*, and (c) *Dnmts* were quantified by SYBR green-based quantitative real-time PCR (qPCR) using β -actin as the housekeeping gene. (d) The binding of *Dnmt1* at the *MMP-9* promoter region was measured in the crosslinked retina by immunoprecipitating the samples with *Dnmt1* antibody, followed by amplification of the promoter region by qPCR. Immunoglobulin G (IgG) was used as a negative antibody control (indicated as ^), and Ct values were normalized with the values from input by ddCt method. Values are represented as mean $\pm$ s.d. from five to seven mice in each group. Norm and Diab = retina from nondiabetic normal mouse or from diabetic mouse respectively. * $P < 0.05$ compared with normal.

nondiabetic mice (Figure 7c). Diabetes also elevated Tet2 binding at the *MMP-9* promoter by over threefold (Figure 7d), further confirming the role of Tet-mediated hypomethylation in *MMP-9* transcriptional activation in diabetes.

DISCUSSION

Diabetes alters expressions of genes implicated in the metabolic abnormalities including genes encoding proteins

Figure 7 The levels of 5-hydroxymethylcytosine (5hmC) are increased at the retinal matrix metalloproteinase-9 (*MMP-9*) promoter in diabetes, and hydroxymethylation enzyme machinery is activated. (a) 5hmC levels were quantified at the promoter region in sonicated DNA using hMeDIP Immunoprecipitation Kit. The total genomic DNA obtained from normal mouse retina was used as an input DNA control. (b) The enzyme activity of Tet was measured in 10–20 μ g retina using TET Activity/Inhibition Assay Kit and the values obtained from the normal mouse retina are considered as 100%. (c) The gene transcripts of *Tet1*, *Tet2*, and *Tet3* were quantified SYBR green-based quantitative real-time PCR (qPCR), and the protein expression of Tet2 was determined by western blot technique. β -Actin was used as a housekeeping gene (qPCR)/loading control (western blot). (d) Binding of Tet2 at the *MMP-9* promoter was determined in the Tet2-immunoprecipitated crosslinked retina using immunoglobulin G (IgG) as a negative antibody control (indicated as ^). Data are represented as mean $\pm$ s.d. from six to seven mice in each group, with each measurement made in duplicate. * $P < 0.05$ compared with normal.

for mitochondrial biogenesis and proinflammatory cytokines.^{3,31,32} *MMP-9* expression and mitochondrial accumulation are elevated in the retina and its capillary cells in diabetes, and increased *MMP-9* damages mitochondrial membrane, activating the apoptotic machinery, a phenomenon that precedes the development of diabetic retinopathy.^{4,7,9,33,34} Although gene expression is mainly

modulated by the transcriptional factors, it can also be regulated by metabolite fluctuations, and these epigenetic modifications alter gene expression without altering the DNA sequence.^{11,35} We have shown that in the pathogenesis of diabetic retinopathy, histones in the retinal *MMP-9* promoter are epigenetically modified, increasing the binding of NF- κ B and AP-1.^{6,9,34} Here, our exciting data obtained from both *in vitro* (retinal capillary cells that are the target of histopathology characteristic of diabetic retinopathy) and *in vivo* (retina from diabetic rodents) models show that the hyperglycemic milieu also alters the methylation status of *MMP-9* promoter. Despite decreased 5mC in the promoter region, the binding of the Dnmt1 is increased, but a concomitant activation of the demethylation machinery keeps the *MMP-9* promoter hypomethylated, resulting in its increased transcription. Silencing of the cytosine hydroxymethylation enzyme *Tet2* prevents hyperglycemia-induced increase in 5hmC and *MMP-9* transcription, and maintains mitochondrial homeostasis. These results clearly suggest that in diabetes active cytosine methylation–demethylation process of the *MMP-9* promoter has a significant role in its transcriptional activation, and regulation of the cytosine methylation–demethylation machinery could inhibit the development/progression of diabetic retinopathy by preventing *MMP-9*-mediated mitochondrial damage.

Aberrant DNA methylation is considered as an important mechanism of gene regulation. In normal cells, DNA methylation is associated with organization of chromatin into active and inactive state, regulation of gene expression, and tissue-specific expression and genomic imprinting.^{36,37} DNA methylation often represses gene transcription by interfering with the binding of the transcription factor, and/or recruitment of other factors to form repression complex.³⁸ The process acts as an interplay between external factors and genome, and is affected by many external factors including drugs and disease severity.^{10,24} *MMP-9* promoter has relatively few CpG islands, but it contains many CpG sites;^{27,28} the results presented here show that diabetes decreases 5mC levels in the promoter region of retinal *MMP-9*. Although methylation of the intragenic CpG islands can also regulate the gene expression,³⁹ we show that hyperglycemia has no effect on the intragenic region of the *MMP-9* gene, suggesting that DNA methylation of the promoter is critical in regulating its transcription. The promoter regions used in our study includes the binding sites of the transcription factors essential for *MMP-9* transcription, such as NF- κ B and Sp1, and reduced 5mC levels in this region indicate that hypomethylation of this region could be favoring the recruitment of these transcriptional factors. Consistent with this, hypomethylation of *MMP-9* promoter in other tissues is shown to influence the binding of NF- κ B, and regulate its active transcription,^{27,28} and in diabetes, the binding of NF- κ B and AP-1 is increased at the retinal *MMP-9* promoter.³⁴ The possibility that hypomethylation of *MMP-9* promoter could be repressing the

binding of the repressor proteins methyl-CpG-binding protein-2, which can interfere with the transcription machinery by binding to methyl-CpG pair,⁴⁰ however, cannot be ruled out.

Despite hypomethylation of *MMP-9* promoter, to our surprise, the binding of Dnmt1 is significantly elevated at the same region, and regulation of *Dnmt1* by its siRNA further decreases 5mC levels at the *MMP-9* promoter. These results clearly support concomitant activation of a demethylation process. Activation of demethylation is further supported by our data showing increased activity of the hydroxymethylating enzyme in the retina, elevated 5hmC levels in the same region of the *MMP-9* promoter, and amelioration of glucose-induced increase in *MMP-9* transcription by Tet inhibitor. Thus, although activation of Dnmt increases its binding, concurrent activation of the hydroxymethylation machinery keeps *MMP-9* promoter hypomethylated, facilitating the transcription process. In support, others have shown an inverse correlation between DNA methylation and *MMP-9* transcription in mouse thymic lymphoma cell line.²⁷ Although Tet family has three major members,^{24,30} we show that in diabetes *Tet2* is the most affected member in the retina and its capillary cells. The role of *Tet2* in regulating active demethylation of the *MMP-9* promoter is further confirmed by its increased binding at the same promoter region, and also by its siRNA, which, in addition to preventing glucose-induced increase in 5hmC, also prevents decrease in 5mC and protects increase in *MMP-9*. Amelioration of diabetes-induced increase in mtDNA damage and decrease in its transcription further confirms the role of Tet in diabetic retinopathy. We recognize that although demethylation could be initiated and processed by multiple pathways including passive dilution and deamination,^{23,24} capillary cells do not actively proliferate after birth, abating the possibility of a replication-dependent passive dilution assisting in the loss of methylation of the *MMP-9* promoter seen here in the retinal endothelial cells. Our *in vivo* experiments were performed in the retina, a complex tissue with multiple layers and cell type; however, similar results from retinal endothelial cells in culture and retina from diabetic rodents support retinal *MMP-9* regulation in diabetes by a dynamic DNA methylation process.

The present study is focused on DNA methylation–demethylation of the *MMP-9* promoter; however, hypomethylation of H3K9 is also observed in diabetes, and this frees up that lysine 9 of H3K9 for acetylation, facilitating the recruitment of NF- κ B.⁹ Consistent with multiple epigenetic modifications seen in the retinal *MMP-9* promoter in diabetes, others have shown correlation among broad changes in H3K9 acetylation by high glucose, DNA hypomethylation and gene induction.³⁶ Furthermore, *MMP-9* is also regulated by its intracellular tissue inhibitor, TIMP-1, whose levels are decreased in the retina in diabetes;⁵ thus, its role in regulating retinal *MMP-9* activity cannot be ruled out.

Recent studies have suggested that 5hmC is critical in various important normal processes, such as ion transport, transcription, cell adhesion, and cell death,^{23,41} and it is now considered not only as an intermediate in demethylation but also as an independent epigenetic marker whose altered levels are observed in many chronic diseases including cancer and Rett syndrome.^{41,42} Tet activation is being implicated in many diseases, including diabetic complications, Alzheimer's, and liver cancer.^{24,26,38,43,44} Hyperglycemia in zebrafish activates Tet²⁶ and induces a genome-wide demethylation and aberrant gene expression.⁴⁵ Site-specific demethylation of *MMP-9* promoter is shown to have an important role in the pathogenesis of diabetic refractory ulcers,²⁸ and here, we show that the regulation of hypomethylation of retinal *MMP-9* promoter regulates its transcription and prevents mitochondrial damage. *MMP-9* has important role in the pathogenesis of diabetic retinopathy, and mice with *MMP-9* gene regulated are protected from the development of diabetic retinopathy,^{4,33,34} thus suggesting that the regulation of methylation of *MMP-9* could serve as an important target in maintaining mitochondrial homeostasis, and the development of diabetic retinopathy.

ACKNOWLEDGMENTS

We thank Mangayarkarasi Thandampallayam Ajjea for her help with the maintenance of animal colony. This study was supported, in part, by grants from the National Institutes of Health (EY014370, EY017313, and EY022230) and from the Thomas Foundation (to RAK), and an unrestricted grant to the Ophthalmology Department from Research to Prevent Blindness.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

- Kowluru RA, Abbas SN. Diabetes-induced mitochondrial dysfunction in the retina. *Invest Ophthalmol Vis Sci* 2003;44:5327–5334.
- Kanwar M, Chan PS, Kern TS, *et al*. Oxidative damage in the retinal mitochondria of diabetic mice: possible protection by superoxide dismutase. *Invest Ophthalmol Vis Sci* 2007;48:3805–3811.
- Kowluru RA, Kowluru A, Mishra M, *et al*. Oxidative stress and epigenetic modifications in the pathogenesis of diabetic retinopathy. *Prog Retin Eye Res* 2015;48:40–61.
- Kowluru RA, Mohammad G, dos Santos JM, *et al*. Abrogation of *MMP-9* gene protects against the development of retinopathy in diabetic mice by preventing mitochondrial damage. *Diabetes* 2011;60:3023–3033.
- Santos JM, Tewari S, Lin JY, *et al*. Interrelationship between activation of matrix metalloproteinases and mitochondrial dysfunction in the development of diabetic retinopathy. *Biochem Biophys Res Commun* 2013;438:760–764.
- Kowluru RA, Santos JM, Zhong Q. Sirt1, a negative regulator of matrix metalloproteinase-9 in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2014;55:5653–5660.
- Kern TS, Tang J, Mizutani M, *et al*. Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci* 2000;41:3972–3978.
- Hsieh MJ, Lin CW, Yang SF, *et al*. Glabridin inhibits migration and invasion by transcriptional inhibition of matrix metalloproteinase 9 through modulation of NF- κ B and AP-1 activity in human liver cancer cells. *Br J Pharmacol* 2014;171:3037–3050.
- Zhong Q, Kowluru RA. Regulation of matrix metalloproteinase-9 by epigenetic modifications and the development of diabetic retinopathy. *Diabetes* 2013;62:2559–2568.
- Ling C, Groop L. Epigenetics: a molecular link between environmental factors and type 2 diabetes. *Diabetes* 2009;58:2718–2725.
- Villeneuve LM, Reddy MA, Natarajan R. Epigenetics: deciphering its role in diabetes and its chronic complications. *Clin Exp Pharmacol Physiol* 2011;38:401–409.
- Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484–492.
- Robertson KD, Wolffe AP. DNA methylation in health and disease. *Nat Rev Genet* 2000;1:11–19.
- Nasonkin IO, Lazo K, Hambright D, *et al*. Distinct nuclear localization patterns of DNA methyltransferases in developing and mature mammalian retina. *J Comp Neurol* 2011;519:1914–1930.
- Wahlin KJ, Enke RA, Fuller JA, *et al*. Epigenetics and cell death: DNA hypermethylation in programmed retinal cell death. *PLoS One* 2013;8:e79140.
- Yan B, Yao J, Tao ZF, *et al*. Epigenetics and ocular diseases: from basic biology to clinical study. *J Cell Physiol* 2013;229:825–833.
- Kowluru RA, Santos JM, Mishra M. Epigenetic modifications and diabetic retinopathy. *Biomed Res Int* 2013;2013:635284.
- He S, Li X, Chan N, *et al*. Review: epigenetic mechanisms in ocular disease. *Mol Vis* 2013;19:665–674.
- Tewari S, Zhong Q, Santos JM, *et al*. Mitochondria DNA replication and DNA methylation in the metabolic memory associated with continued progression of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2012;53:4881–4888.
- Mishra M, Kowluru RA. Epigenetic modification of mitochondrial DNA in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2015;56:5133–5142.
- Guibert S, Forne T, Weber M. Dynamic regulation of DNA methylation during mammalian development. *Epigenomics* 2009;1:81–98.
- Majumdar S, Buckles E, Estrada J, *et al*. Aberrant DNA methylation and prostate cancer. *Curr Genomics* 2011;12:486–505.
- Tahiliani M, Koh KP, Shen Y, *et al*. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by mll partner tet1. *Science* 2009;324:930–935.
- Kohli RM, Zhang Y. Tet enzymes, tdk and the dynamics of DNA demethylation. *Nature* 2013;502:472–479.
- Wu H, Zhang Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell* 2014;156:45–68.
- Dhaliwayo N, Sarra JR MP, Luczkowski E, *et al*. PARP inhibition prevents ten-eleven translocase enzyme activation and hyperglycemia-induced DNA demethylation. *Diabetes* 2014;63:3069–3076.
- Chicoine E, Esteve PO, Robledo O, *et al*. Evidence for the role of promoter methylation in the regulation of *MMP-9* gene expression. *Biochem Biophys Res Commun* 2002;297:765–772.
- Ling L, Ren M, Yang C, *et al*. Role of site-specific DNA demethylation in Tnf α -induced *MMP-9* expression in keratinocytes. *J Mol Endocrinol* 2013;50:279–290.
- Xu W, Yang H, Liu Y, *et al*. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α -ketoglutarate-dependent dioxygenases. *Cancer Cell* 2011;19:17–30.
- Williams K, Christensen J, Pedersen MT, *et al*. Tet1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 2011;473:343–348.
- Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. *Exp Diabetes Res* 2007;2007:95103.
- Kowluru RA. Mitochondria damage in the pathogenesis of diabetic retinopathy and in the metabolic memory associated with its continued progression. *Curr Med Chem* 2013;20:3226–3233.
- Kowluru RA, Zhong Q, Santos JM. Matrix metalloproteinases in diabetic retinopathy: potential role of mmp-9. *Expert Opin Investig Drugs* 2012;21:797–805.
- Mishra M, Flaga J, Kowluru RA. Molecular mechanism of transcriptional regulation of matrix metalloproteinase-9 in diabetic retinopathy. *J Cell Physiol* 2016;231:1709–1718.
- Deaton AM, Bird A. CpG islands and the regulation of transcription. *Genes Dev* 2011;25:1010–1022.
- Pirola L, Balcerzyk A, Okabe J, *et al*. Epigenetic phenomena linked to diabetic complications. *Nat Rev Endocrinol* 2010;6:665–675.
- Gemenetz M, Lotery AJ. The role of epigenetics in age-related macular degeneration. *Eye (Lond)* 2014;28:1407–1417.

38. Kato M, Natarajan R. Diabetic nephropathy—emerging epigenetic mechanisms. *Nat Rev Nephrol* 2014;10:517–530.
39. Medvedeva YA, Fridman MV, Oparina NJ, *et al*. Intergenic, gene terminal, and intragenic cpg islands in the human genome. *BMC Genomics*. 2010;11:48.
40. Nan X, Ng HH, Johnson CA, *et al*. Transcriptional repression by the methyl-cpg-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 1998;393:386–389.
41. Yang H, Liu Y, Bai F, *et al*. Tumor development is associated with decrease of tet gene expression and 5-methylcytosine hydroxylation. *Oncogene* 2013;32:663–669.
42. Wang J, Tang J, Lai M, *et al*. 5-Hydroxymethylcytosine and disease. *Mutat Res Rev Mutat Res* 2014;762:167–175.
43. Liu ZZ, Zhao XZ, Zhang XS, *et al*. Promoter DNA demethylation of *keap1* gene in diabetic cardiomyopathy. *Int J Clin Exp Pathol* 2014;7: 8756–8762.
44. Li D, Guo B, Wu H, *et al*. Tet family of dioxygenases: crucial roles and underlying mechanisms. *Cytogenet Genome Res* 2015;146:171–180.
45. Olsen AS, Sarras MP, Leontovich A, *et al*. Heritable transmission of diabetic metabolic memory in zebrafish correlates with DNA hypomethylation and aberrant gene expression. *Diabetes* 2012;61: 485–491.