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

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Abstract
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 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. Effect of high glucose on 5-methylcytosine (5mC), 5-hydroxymethylcytosine (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.

Keywords
Diabetic retinopathy; DNA methylation; epigenetic modifications; matrix metalloproteinase

Introduction
Diabetic retinopathy remains one of the major causes of blindness in working adults. Number of 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 which 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-\(\kappa\)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-\(\kappa\)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 gamma 1 (POLG1) 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 \textit{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 mechanisms; while passive DNA demethylation is mostly found in mitotic cells, active demethylation, in which oxidation of 5mC forms 5 hydroxymethyl cytosine (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 20mM D-glucose on 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.

**Methods**

**Retinal endothelial cells**

Retinal endothelial cells, prepared from bovine eyes, from 4–7th passage were incubated in normal (5mM) or high (20mM) D-glucose for 4 days, and parallel osmotic controls included
cells incubated in 20mM mannitol or 20mM L-glucose. 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, 500μM; Cayman, Ann Arbor, MI). The cells received fresh media, including 2-HG, every 24 hours. Inhibition of Dnmts/Tets was further confirmed by transfecting the cells with their specific siRNAs (Dnmt1-siRNA, Santa Cruz Biotechnology, Santa Cruz, CA, and Tet2-siRNA, Integrative DNA Technology, Coralville, IA) using Lipofectamine® RNAiMAX transfection reagent (Thermo Fisher Scientific, IL), as routinely performed in our laboratory. Parallel incubations with non-targeting scrambled RNA were used as transfection controls. After transfection, the cells were rinsed and incubated in either 5mM or 20mM 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 weeks old C57BL/6J mice were randomly assigned to normal and diabetes group. Diabetes was induced by streptozotocin (i.p.; 55mg/kg BW) for 4 consecutive days, and 2 days after the last injection, animals presenting blood glucose over 250mg/dL were considered as diabetic. Animals in diabetic group received daily insulin injection (0.1~0.3 IU, NPH) to prevent weight loss and dehydration. The mice were sacrificed 6 months after induction of diabetes by carbon dioxide euthanasia, and retina were collected immediately. Age-matched non-diabetic, 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.

Chromatin Immunoprecipitation (ChIP)

To analyze potential interactions between DNA and epigenetic machinery, ChIP assay was performed using the methods routinely performed in our laboratory. Briefly, paraformaldehyde cross-linked samples were sonicated 6 times for 10 seconds 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) for 1 hour at 4°C. This was followed by overnight incubation of 100–120μg sample with Dnmt1 or Tet2 antibodies (Dnmt1, Cat # ab13537 and Tet2, Cat # ab135087, Abcam, Cambridge, MA). The antibody-chromatin complexes were pulled down with Protein A/G agarose beads and washed extensively before eluting with 1% SDS, 0.1M NaHCO₃. Protein-associated DNA was recovered by incubation at 65°C overnight, and then purified by phenol extraction. Purified DNA was precipitated with ethanol, re-suspended in water, and utilized for q-PCR using primers of the regions of interest. Normal rabbit IgG (Cat # 2729S, Cell Signaling, Danvers, MA) was used as negative antibody control, and DNA from the input (40μg 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). The enriched 5mC/5hmC fractions were analyzed by qPCR using species-specific MMP-9 primers (Table I), as recently reported by us \(^{20}\). The products were confirmed by semi-quantitative PCR on a 2% agarose gel.

Western Blot

Protein (30–40μg) was separated on a 4–20% gradient acrylamide gel (Bio-Rad, Hercules, CA). The proteins transferred onto nitrocellulose membranes were detected using antibodies against the proteins of interest (Dnmt1 and Tet2 from Abcam, and β-actin Cat # A-5316 from Sigma-Aldrich, St. Louis, MO). Images were quantified by Carestream MI software (Carestream, Rochester, NY), 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 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute, and 72°C for 5 minutes. Reaction specificity was validated by a single peak in the melting curve. For MMP-9, TaqMan real-time quantitative PCR 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 5mM 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 (3–6μg for Dnmt or 10–20μg 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), and the data are presented as mean ± standard deviations. Statistical test between two groups was done 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 20mM D-glucose resulted in ~50% reduction in 5mC levels in MMP-9 promoter (−720 to −547) compared to the cells incubated in normal glucose or 20mM mannitol (Figure 1a). In contrast, in the intragenic (1487 to 1701) CpG island region of 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\(^\text{20}\), 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&c are included to show the transfection efficiency of Dnmt1-siRNA; cells transfected with Dnmt1-siRNA had less than 50% Dnmt1 gene and protein expressions compared to the untransfected cells. Dnmt1-siRNA also inhibited significantly increase in Dnmt enzyme activity, observed in high D-glucose condition (Figure 2d).

Due to dynamic nature of DNA methylation, methylated cytosine can be converted to 5hmC\(^\text{23, 24}\); 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 3 fold in the cells incubated in high D-glucose compared to the cells in 5mM D-glucose or 20mM mannitol (Figure 3a). The role of demethylation in MMP-9 transcription was confirmed by supplementing 20mM 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 3 major members, Tet1, Tet2 Tet3\(^\text{24, 30}\); to determine the specific isoform(s) responsible for hypomethylation of MMP-9 promoter, effect of 20mM D-glucose on their expressions was quantified. Although 20mM D-glucose had no effect on Tet1 and Tet3, Tet2 expression (protein and gene) was significantly increased compared to 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 4 fold in cells incubated in 20mM D-glucose. In the same samples, IgG controls were less than 1% compared to the values obtained using Tet2 antibody (Figures 4b). Regulation of Tet2 by its specific siRNA prevented 20mM 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. The protective effect of inhibition of hydroxymethylation was confirmed by quantifying transcription of mtDNA. As expected, incubation of cells in 20mM D-glucose, but not in 20mM L-glucose, significantly decreased mtDNA 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&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 to the values from non-diabetic normal mice. However, 5mC levels in the intragenic CpG island region (1,361 to 1,600) remained unchanged in the retina from normal and diabetic mice (Figure 6a). As expected, in the same diabetic animals, retinal MMP-9 transcripts were significantly higher compared to those from normal mice (Figure 6b).

Dnmt is activated in the retina in diabetes, 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 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 2 fold higher compared to the non-diabetic 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 to that from age-matched normal mice. In the same retina samples, global Tet activity was also elevated by ~75% (Figure 7b), and among the 3 members of Tet family, only Tet2 expression (mRNA and protein) showed significant increase in the retina from diabetic mice compared to the retina from nondiabetic mice (Figure 7c). Diabetes also elevated Tet2 binding at the MMP-9 promoter by over 3 fold (Figure 7d), further confirming the role of Tet-mediated hypomethylation in MMP-9 transcriptional activation in diabetes.

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**Discussion**

Diabetes alters expressions of genes implicated in the metabolic abnormalities including genes encoding proteins for mitochondrial biogenesis and pro-inflammatory 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-κB 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 plays 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-κB 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-κB, and regulate its active transcription\(^27,28\), and in diabetes, the binding of NF-κB 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 \(\text{MMP-9}\) promoter, to our surprise, the binding of \(\text{Dnmt1}\) is significantly elevated at the same region, and regulation of \(\text{Dnmt1}\) by its siRNA further decreases 5mC levels at the \(\text{MMP-9}\) 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 \(\text{MMP-9}\) promoter, and amelioration of glucose-induced increase in \(\text{MMP-9}\) transcription by Tet inhibitor. Thus, though activation of \(\text{Dnmt}\) increases its binding, concurrent activation of the hydroxymethylating machinery keeps \(\text{MMP-9}\) promoter hypomethylated, facilitating the transcription process. In support, others have shown an inverse correlation between DNA methylation and \(\text{MMP-9}\) transcription in mouse thymic lymphoma cell line \(^{27}\). Although Tet family has 3 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 \(\text{MMP-9}\) 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 \(\text{MMP-9}\). 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 \(\text{MMP-9}\) promoter seen here in the retinal endothelial cells. Our \textit{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 \(\text{MMP-9}\) regulation in diabetes by a dynamic DNA methylation process.

Present study is focused on DNA methylation-demethylation of the \(\text{MMP-9}\) 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 \(\text{NF-\kappaB}\) \(^{9}\). Consistent with multiple epigenetic modifications seen in the retinal \(\text{MMP-9}\) promoter in diabetes, others have shown correlation among broad changes in H3K9 acetylation by high glucose, DNA hypomethylation and gene induction \(^{36}\). Furthermore, \(\text{MMP-9}\) is also regulated by its intracellular tissue inhibitor, \(\text{TIMP-1}\), whose levels are decreased in the retina in diabetes \(^{5}\); thus its role in regulating retinal \(\text{MMP-9}\) 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 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 Tet \(^{20}\) and induces a genome-wide demethylation and aberrant gene expression \(^{45}\). Site-specific demethylation of \(\text{MMP-9}\) promoter is shown to play an important role in the
pathogenesis of diabetic refractory ulcers\textsuperscript{28}, and here, we show that the regulation of hypomethylation of retinal \textit{MMP-9} promoter regulates its transcription and prevents mitochondrial damage. \textit{MMP-9} has important role in the pathogenesis of diabetic retinopathy, and mice with \textit{MMP-9} gene regulated are protected from the development of diabetic retinopathy\textsuperscript{4, 33, 34}, thus suggesting that the regulation of methylation of \textit{MMP-9} could serve as an important target in maintaining mitochondrial homeostasis, and the development of diabetic retinopathy.

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Figure 1.
High D-glucose hypomethylates MMP-9 promoter in retinal endothelial cells, and increases Dnmt1 binding at the same region of the promoter. The levels of 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 to 1701) of MMP-9 gene. The total genomic DNA from cells in 5mM 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 q-PCR. 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 ± SD from 4–5 samples in each group. 5mM and 20mM= 5mM or 20mM D-glucose, Mann= 20mM mannitol. *p< 0.05 compared to 5mM D-glucose.
Regulation of Dnmt1 has no effect on D-glucose-induced decrease in 5mC at the 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 5mM glucose or 20mM D-glucose. The transfection efficiency of Dnmt1-siRNA was measured by quantifying Dnmt1 (b) gene transcripts (q-PCR) and (c) protein expression (western blot). (d) The activity of Dnmt was assayed in 3–6μg sample using DNA Methyltransferase Activity/Inhibition assay kit, and the values obtained from cells in 5mM glucose are considered as 100%. 5mM and 20mM=D-glucose, 5+/D-si and 5+/SC= cells transfected with Dnmt1-siRNA or scrambled RNA respectively, followed by incubation in 5mM D-glucose; 20+/D-si and 20+/SC= cells transfected with Dnmt1-siRNA or scrambled RNA respectively and incubated in 20mM D-glucose; Mann= 20mM mannitol; L-Gl=20mM L-glucose. Data are represented as mean ± SD from each measurement made in duplicate in 3–5 cell preparations. *p< 0.05 compared to 5mM D-glucose.
Figure 3.
High D-glucose increases hydroxymethylation of MMP-9 promoter and Tet activity. (a) The levels of 5hmC were quantified in retinal endothelial cells using hMeDIP immunoprecipitation kit. (b) The enzyme activity of Tet was determined in 10–20μg sample using TET Activity/Inhibition Assay Kit, and the values obtained from cells in 5mM glucose are considered as 100%. (c) MMP-9 mRNA was quantified by q-PCR. Values are represented as mean ± SD from 4–5 cell preparations, with each measurement made in duplicate. 5mM and 20mM= 5mM or 20mM D-glucose; 5+/2-HG and 20+/2-HG= cells incubated in 5mM or 20mM D-glucose in the presence of 500μM 2-HG; Mann= 20mM mannitol; L-Gl=cells in 20mM L-glucose. *p< 0.05 compared to 5mM D-glucose.
Figure 4.
Tet2 expression and its binding at the *MMP-9* promoter are increased by high glucose. (a) The gene transcripts of *Tet1*, *Tet2* and *Tet3* were quantified by q-PCR using gene specific primers, and the protein expression of Tet2 was determined by western blot technique using β-actin as a house keeping gene (q-PCR)/loading control (western blot). (b) Tet2 binding at the *MMP-9* promoter was quantified in the Tet2 immunoprecipitated crosslinked cells using 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 ± SD from 3–5 samples in each group. 5mM and 20mM= 5mM 20mM D-glucose; Mann= 20mM mannitol. *p< 0.05 compared to 5mM D-glucose.
Figure 5.
Inhibition of Tet2 by its siRNA ameliorates D-glucose-induced increase in 5hmC and 5mC levels at the 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 mtDNA-encoded Cytb and ND6 by q-PCR using β-actin as the loading gene. Transfection efficiency of Tet2-siRNA was determined by quantifying its (c) gene transcripts and (d) protein expression. 5mM and 20mM= 5mM or 20mM D-glucose; 5+/T-si and 5+/SC= cells transfected with Tet2-siRNA or scrambled RNA respectively, followed by incubation in 5mM D-glucose; 20+/T-si and 20+/SC= Tet2-siRNA or scrambled RNA transfected cells incubated in 20mM D-glucose; 5+/2-HG and 20+/2-HG= cells incubated in the presence of 500μM 2-HG in 5mM or 20mM D-glucose respectively; L-Gl=20mM L-glucose. Data are represented as mean ± SD from 3–5 measurement made in duplicate. *p<0.05 compared to 5mM D-glucose.
Figure 6.
Diabetes hypomethylates *MMP-9* promoter, but the binding of Dnmt1 in the same region is increased. (a) The levels of 5mC were quantified in the promoter (−640 to −356) and the intragenic CpG island (1,361 to 1,600) 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 q-PCR 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 q-PCR. 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 ± SD from 5–7 mice in each group. Norm and Diab= retina from nondiabetic normal mouse or from diabetic mouse respectively. *p< 0.05 compared to normal.
Figure 7.
The levels of 5hmC are increased at the retinal **MMP-9** promoter in diabetes, and
dehydroxymethylation 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 10–20μg 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 q-PCR, and
the protein expression of Tet2 was determined by western blot technique. β-actin was used
as a house keeping gene (q-PCR)/loading control (western blot). (d) Binding of Tet2 at the
**MMP-9** promoter was determined in the Tet2 immunoprecipitated crosslinked retina using
IgG as a negative antibody control (indicated as ^). Data are represented as mean ± SD from
6–7 mice in each group, with each measurement made in duplicate. *p< 0.05 compared to
normal.
Table I

Primer sequences

| Gene          | Sequence (5′-3′)          | Amplicon length (bp) |
|---------------|--------------------------|----------------------|
| **Bovine**    |                          |                      |
| MMP-9 promoter (−720 to −547) | CAGACGCCACAAACACTCCCA TCCTCTCCCTGCCTCCACCTG | 174 |
| MMP-9 CGI (1,487 to 1,701) | TCTCTTGCTGCTTGCTGAA CATCTCCGTGCTTGAAAG | 215 |
| Dnmt1         | ACCATGTGACTACTCCCTGA GCGCTCATGTCCTTGCAAT | 149 |
| Tet1          | ACACATGACAGATGGCTTCTT ACCTCTCTCTCTCTACTACAA | 196 |
| Tet2          | GTGAGGGTGTGATACACCAGTG TCTCTTGCTGAGAAGGC | 89 |
| Tet3          | TCAAGTCGTGTCGTGAGAG GAGGTGGCATGAGAAGGC | 265 |
| Cytb          | CGATACATACACGCAACCGG AGATCAGGTAAAGGGCG | 298 |
| ND6           | CGTGATAGGTTTTGTGGGTT GCCAGTAAACAAATGGCCTA | 221 |
| β-actin       | CGCCATGGATGATGATATTG CTCTATGCCAACACAGTGC | 66 |
| **Mouse**     |                          |                      |
| MMP-9 promoter (−640 to −356) | CAGACGCCACAAACACTCCCA TCCTCTCCCTGCCTCCACCTG | 285 |
| MMP-9 CGI (1,361 to 1,600) | GTCGAGTCACCTTCTCTCA ACCAACAATGTCGTGTC | 240 |
| Dnmt1         | CCAAAGTCCCTGCTCTGGTA CTCAGATACACCAGGAAAGGC | 137 |
| Dnmt3a        | GCCGAATTGTGTCTTGGTGGATGA GCGATGAATGTCGCACTGGCAGAGAAG | 147 |
| Dnmt3b        | GCTGATACACCAGGAGAG GCATGATACACCAGGAAAGGC | 140 |
| Tet1          | GAGGCTGTTCGGCTCGATG TCTCTCTCCTCGTCATTCAC | 256 |
| Tet2          | TGTGTTGGTCAAGGTGAGAA CTCTCTCCTCGTCATTCAC | 103 |
| Tet3          | CGGGAATGGAGAAGATCTAC AAGATACACCATCGGCGATT | 162 |
| β-actin       | CCTCTATGCCAACACAGTC CCAAGACTTCCTACAGCTAC | 215 |
| Gene       | Sequence (5′-3′)          | Ampicon length (bp) |
|------------|---------------------------|---------------------|
|            | CATCGTACTCCTGCTTGCTG      |                     |