Epigenetic Modifications Compromise Mitochondrial DNA Quality Control in the Development of Diabetic Retinopathy

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PURPOSE. Diabetes causes dysfunction in the retinal mitochondria and increases base mismatches in their DNA (mtDNA). The enzyme responsible for repairing the base mismatches, MutL homolog 1 (Mlh1), is compromised. Diabetes also favors many epigenetic modifications and activates DNA methylation machinery, and Mlh1 has a CpG-rich promoter. Our aim is to identify the molecular mechanism responsible for impaired mtDNA mismatch repair in the pathogenesis of diabetic retinopathy.

METHODS. Human retinal endothelial cells, incubated in 20 mM glucose, were analyzed for mitochondrial localization of Mlh1 by an immunofluorescence technique, Mlh1 promoter DNA methylation by the methylated DNA capture method, and the binding of Dnmt1 and transcriptional factor Sp1 by chromatin immunoprecipitation. The results were confirmed in retinal microvessels from streptozotocin-induced diabetic mice, with or without Dnmt inhibitors, and from human donors with diabetic retinopathy.

RESULTS. Compared with cells in 5 mM glucose, high glucose decreased Mlh1 mitochondrial localization, and its promoter DNA was hypermethylated with increased Dnmt-1 binding and decreased Sp1 binding. Dnmt inhibitors attenuated Mlh1 promoter hypermethylation and prevented a decrease in its gene transcripts and an increase in mtDNA mismatches. The administration of Dnmt inhibitors in mice ameliorated a diabetes-induced increase in Mlh1 promoter hypermethylation and a decrease in its gene transcripts. Similar decreases in Mlh1 gene transcripts and its promoter DNA hypermethylation were observed in human donors.

CONCLUSIONS. Thus, as a result of the epigenetic modifications of the Mlh1 promoter, its transcription is decreased, and decreased mitochondrial accumulation fails to repair mtDNA mismatches. Therapies targeted to halt DNA methylation have the potential to prevent/halt mtDNA damage and the development of diabetic retinopathy.

Keywords: diabetic retinopathy, DNA methylation, epigenetics, mitochondria, mismatch repair, mutL homolog 1
The expression of a gene can also be regulated by epigenetic modifications, and these modifications are now implicated in many chronic diseases, including cancer and diabetes.14–16 Diabetes alters the epigenetic machinery in the retina and its vasculature, and several genes implicated in the development of diabetic retinopathy are epigenetically modified.15,17,18 Methylation of cytosine in the cytosine-guanosine rich region (CpG island) of a gene promoter is generally associated with decreased transcriptional factor binding and suppression of its expression,19,20 and in diabetes, the machinery critical in maintaining the DNA methylation status is activated.17

**Mlh1** promoter is a TATA-less promoter with multiple CpG islands,21 and Sp1 is considered as one of the transcription factors important in its transcription.22 Increased **Mlh1** promoter DNA methylation and its transcriptional suppression is implicated in many chronic diseases associated with mtDNA heteroplasmy.21,23,24 How diabetes decreases Mlh1 in the retinal microvasculature is not clear. The goal of this study is to investigate the molecular mechanism responsible for the impaired mismatch repair mechanism in the pathogenesis of diabetic retinopathy. DNA methylation status of the **Mlh1** promoter was evaluated in human retinal endothelial cells (HRECs) using the pharmacological and molecular inhibitors of DNA methyltransferases (Dnmts). In vitro results were confirmed in an in vivo model using retinal microvessels from streptozotocin-induced diabetic mice, with or without the administration of Dnmt inhibitors. To transition into the human diseases, the key parameters of **Mlh1** transcription were confirmed in the retinal microvessels from human donors with documented diabetic retinopathy.

**METHODS**

**Retinal Endothelial Cells**

Human retinal endothelial cells, obtained from Cell Systems Corporation (cat. no. ACBRI 181, Cell Systems Corp., Kirkland, WA, USA), were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 12% heat-inactivated fetal bovine serum, 20 µg/ml endothelial cell growth supplement, and 1% each insulin, transferrin, selenium, glutamax, and antibiotic/antimitotic.25 Cells from the fifth to eighth passage were incubated in normal D-glucose (5 mM [NG]) or high D-glucose (20 mM [HG]) for 96 hours in the presence or absence of a Dnmt inhibitor, 5-Aza-2'-deoxyxytidine (Aza, 1 µM; cat. no. A5656, Sigma-Aldrich Corp., St. Louis, MO, USA). L-glucose (20 mM), instead of 20 mM D-glucose, was used as the osmotic/antimitotic.26 Cells from the fifth to eighth passage were incubated in normal D-glucose (5 mM [NG]) or high D-glucose (20 mM [HG]) for 96 hours in the presence or absence of a Dnmt inhibitor, 5-Aza-2'-deoxyxytidine (Aza, 1 µM; cat. no. A5656, Sigma-Aldrich Corp., St. Louis, MO, USA). L-glucose (20 mM), instead of 20 mM D-glucose, was used as the osmotic/antimitotic.25

**Mice**

Two days after establishment of streptozotocin-induced diabetes in C57BL/6 mice (7–8 weeks old, either sex), they were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg ketanest and 12 mg/kg xylazine).

**Dnmt1-siRNA** (ID: MSS203624, Thermo Fisher Scientific, Waltham, MA, USA), 2 µg in 2 µL nuclease-free water and mixed with Invivofectamine (cat. no. IVF 3001, Invitrogen, Carlsbad, CA, USA), was injected intravitreally in the right eye under a dissecting microscope. The left eye was injected with a negative control siRNA (SC, cat. no. 12935-300, Invitrogen).28 Five weeks after the administration of siRNA, the mice were killed, and their retinas were quickly isolated. Controls included age-matched normal mice and diabetic mice without any siRNA administration. Intravitreal administration of **Dnmt1-siRNA** reduced the **Dnmt1** gene transcripts in the retinal microvessels by 35% to 45%. The treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Wayne State University’s Institutional Animal Care and Use Committee.

**Human Donors**

Eye globes from human donors with documented diabetic retinopathy (40–75 years age, 14–41 years of diabetes) and age-matched nondiabetic donors enucleated within 6 to 8 hours of death were obtained from the Eversight Eye Bank (Ann Arbor, MI, USA). The retinas were isolated, and the microvessels were prepared by hypotonic shock method.29

**Mitochondrial Localization**

Mitochondrial localization of Mlh1 was performed by immunofluorescence technique using an Mlh1 antibody (cat. no. ab92312, Abcam, Cambridge, MA, USA; 1:100 dilution). CoxIV (cat. no. ab533985, Abcam; 1:100 dilution) was used as a mitochondrial marker. Secondary antibodies against Mlh1 and CoxIV included AlexaFluor 488 (green)-conjugated antirabbit (cat. no. A11008, Molecular Probes-Life Technologies, Grand Island, NE, USA; 1:500 dilution) and Texas red (red)-conjugated antimouse (cat. no. TI-2000, Vector Laboratories, Burlingame, CA, USA; 1:500 dilution), respectively. After mounting the coverslips in 4',6-diamidino-2-phenylindole (DAPI)-containing (blue) Vectashield (Vector Laboratories) mounting medium, they were captured in ZEISS 40X (Carl Zeiss, Inc., Chicago, IL, USA) objective magnification with the Apotome module, using 408 nm, 488 nm, and 562 nm wavelengths. The captured images were calibrated with the ZEISS proinbuilt software package and modules. The random regions of interest were made in the outer nuclear regions, and using the colocalization software module, the Pearson’s correlation coefficient was determined.30 The intensity profile was determined by line region of interest across the cell, and the fluorescence intensity of blue (DAPI), green (Mlh1), and red (CoxIV) channels were plotted.

The mitochondria were isolated using a mitochondria isolation kit from Thermo Fischer (Wilmington, DE, USA; cat. no. 89874).31 After digesting the homogenate with the kit reagents, the mitochondrial rich fraction was obtained by differential centrifugation at 700g for 10 minutes followed by centrifugation at 3000g for 15 minutes. The mitochondrial pellet, after rinsing, was suspended in PBS.

**Western Blotting**

Western blotting was performed in the isolated mitochondrial fraction32 using Mlh1 and CoxIV antibodies (ab92312 and ab533985; Abcam) at 1:1000 dilution each.

**Plasmid and Constructs**

**Mlh1** promoter (−2077 to −617) was amplified and cloned into the promoter-less pGL3-Basic-IREs plasmid (plasmid no. 64784,
Addgene, Watertown, MA, USA). Deletion promoter fragments containing 934bp (−1137 to −2050) and 843bp (−1235 to −2077) of the Mlh1 promoter were generated by PCR. The primers used to clone these promoter fragments containing BamH1 and HindIII are listed in Table 1. The pGL3-control vector (cat. no. E1741, Promega, Madison, WI, USA) was used as a control vector.

Transient transfection was performed in the HRECs using TurboFect transfection reagent (cat. no. R0534, Thermo Fisher) according to the manufacturer’s instructions. Briefly, 1 μg of DNA in 100 μL of serum-free Dulbecco’s modified Eagle’s medium was mixed with 2 μL of transfection reagent and incubated at room temperature for 20 minutes. After rinsing and incubating the cells in Dulbecco’s modified Eagle’s medium overnight, they were incubated for 96 hours in normal or high glucose media in the presence or absence of 1 mM 2-deoxy-d-glucose.

Luciferase Activity

Luciferase activity was determined using Pierce Firefly Luciferase Glow Assay Kit (cat. no. 16176, Thermo Fisher). Briefly, after washing the cells with 1X Dulbecco’s phosphate-buffered saline buffer and lysing with 1X cell lysis buffer for 15 minutes, 10 to 20 μL cell lysate was transferred to a black opaque 96-well plate containing 50 μL working solution. The samples were incubated for 10 minutes at room temperature, and the light output was measured at 613 nm. The results were normalized with the value obtained from the cell lysate of pGL3-control vector transfected cells. Each luciferase assay was normalized with the value obtained from the cell lysate of untransfected cells. Each experiment was repeated three or more times.

Quantification of Methylated Cytosin

The levels of 5-methyl cytosine (5mC) were quantified in the total genomic DNA by a MethyLamp Methylated DNA capture kit (cat. no. P-1015, EPIGENTEK, Farmingdale, NY, USA). Base mismatches were identified using the Surveyor Mutation Detection kit from Transgenomics (Omaha, NE, USA). Using 50 ng DNA and a high-fidelity Elongase enzyme mix (Invitrogen), mitochondrial D-loop was amplified. Amplified mtDNA products were digested with a surveyor nuclease, a mismatch-specific endonuclease with high specificity for the sites of base substitution mismatch, using the method previously reported by us.12,32 The digested products were run on a 2% agarose gel, and fragmentation was detected under a UV transilluminator. A no-template negative control was always run simultaneously.

Table 1. Human MLI1 Promoter Primers for Cloning

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Position</th>
<th>Primers With 5’ RE-Recognition Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>HindIII</td>
<td>617-636</td>
<td>5’-TCTAAGCTTTTGACTTGGTGGCCCCTAC-3’</td>
</tr>
<tr>
<td>BamH1</td>
<td>2077-2058</td>
<td>5’-CAGGGATCCCTCTTCCGGCAGCTATTGA-3’</td>
</tr>
<tr>
<td>HindIII</td>
<td>1137-1156</td>
<td>5’-CAGAGGTCACGCGGTAAAGACCCAGG-3’</td>
</tr>
<tr>
<td>BamH1</td>
<td>2070-2050</td>
<td>5’-ACCGGATCCGGCGGCTATTGATTGGACAG-3’</td>
</tr>
<tr>
<td>HindIII</td>
<td>1235-1254</td>
<td>5’-CGGAGGATAGCTTGCGTAGATATCTT-3’</td>
</tr>
<tr>
<td>BamH1</td>
<td>2077-2057</td>
<td>5’-CAGGGATCCCCTTCAGGGGACAGCTATTGA-3’</td>
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</tbody>
</table>

Gene Expression

Gene transcripts were quantified by SYBR green-based, real-time quantitative PCR (qPCR) using the ABI 7500 Cycler detection system (Applied Biosystems, Foster City, CA, USA) and β-actin as the housekeeping gene.31

Chromatin Immunoprecipitation (ChIP)

A protein–DNA complex (100 μg) was immunoprecipitated with antibodies against either Dnmt1 (cat. no. Ab 15537, Abcam) or Sp1 (cat. no. sc14027, Santa Cruz Biotechnology). Normal rabbit immunoglobulin G (IgG) (cat. no. Ab 171870, Abcam) was used as an antibody control. The antibody–chromatin complex was precipitated using Protein A Agarose/Salmon Sperm DNA (cat. no. 16-157, Sigma-Aldrich Corp.), and the DNA were isolated after de-cross-linking at 65°C for 6 hours. Dnmt1/Sp1 binding at the Mlh1 promoter was quantified by qPCR using primers specific for the proximal promoter regions. The specificity of the assay was validated by resolving the PCR products on a 2% agarose gel. The target values were normalized to the input controls.26

Base Mismatches

Base mismatches were identified using the Surveyor Mutation Detection kit from Transgenomics (Omaha, NE, USA). Using 50 ng DNA and a high-fidelity Elongase enzyme mix (Invitrogen), mitochondrial D-loop was amplified. Amplified mtDNA products were digested with a surveyor nuclease, a mismatch-specific endonuclease with high specificity for the sites of base substitution mismatch, using the method previously reported by us.12,32 The digested products were run on a 2% agarose gel, and fragmentation was detected under a UV transilluminator. A no-template negative control was always run simultaneously.

Statistical Analysis

Sigma Stat software (San Jose, CA, USA) was used to perform statistical analysis. The results are expressed as mean ± standard deviation. Significance of variance among experimen-

Table 2. Primers for ChIP Analysis of Human MLI1

<table>
<thead>
<tr>
<th>Region</th>
<th>Position</th>
<th>Primer</th>
<th>Number of CpG</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>−659 to −522</td>
<td>Forward: CTCGGCTCGGTGTTAAAAGGC</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GAGACGCTGTGCTGAGAG</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>−383 to −261</td>
<td>Forward: AGTATTGAGGTCGTCGAGCTC</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTCGATGAGTCGTCGAGCTC</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>−246 to −122</td>
<td>Forward: GTCATGACAGTCTGCTGAGG</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTGAGTGACAGTCTGCTGAGG</td>
<td></td>
</tr>
<tr>
<td>R4</td>
<td>−13 to +72</td>
<td>Forward: CCCGAGCTCCTAAAAACGGA</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTGAGTGACAGTCTGCTGAGG</td>
<td></td>
</tr>
</tbody>
</table>
tal groups was analyzed using one-way ANOVA. A P value < 0.05 was considered as statistically significant.

RESULTS

The accumulation of Mlh1 in the mitochondria was significantly lower, and the overlap of Mlh1 and CoxIV intensity peaks was also compromised in the cells exposed to high glucose when compared to the cells in normal glucose. The accompanying figure shows lower Pearson’s correlation in the cells exposed to high glucose when compared with the cells in normal glucose, further confirming decreased Mlh1-CoxIV interactions in high glucose. However, the use of 20 mM L-glucose, instead of 20 mM D-glucose, had no effect on Mlh1 accumulation in the mitochondria (Fig. 1a). Glucose-induced decrease in mitochondrial localization of Mlh1 was further confirmed by western blotting of Mlh1 in the isolated mitochondria, and as shown in Figure 1b, compared to cells in normal glucose, the mitochondria isolated from the cells in high glucose had significantly lower Mlh1 expression.

DNA methylation suppresses gene expression, and in diabetes the machinery responsible for maintaining DNA methylation status is activated.17,33 Because Mlh1 has a CpG-rich promoter,21 to investigate the role of epigenetics in Mlh1 suppression, 5mC levels were quantified at its promoter. Figure 2a shows about a threefold increase in 5mC levels at the Mlh1 promoter in the cells exposed to high glucose when compared with the cells in normal glucose. This increase in 5mC was accompanied by a fivefold increase in the binding of Dnmt1, the only member of the Dnmt family elevated in the retinal capillaries in hyperglycemic milieu17 (Fig. 2b). In the same cell preparations, the binding of the transcriptional factor Sp1 and Mlh1 gene transcripts were decreased by more than 50% (Figs. 2c, 2d). The incubation of cells in 20 mM L-glucose, instead of D-glucose, had no effect on either 5mC or the binding of Dnmt1 or Sp1 at the Mlh1 promoter.

To confirm the role of DNA hypermethylation in the decreased expression of Mlh1 in hyperglycemic milieu, the cells manipulated for Dnmt function were analyzed. The inhibition of Dnmts by Aza attenuated a glucose-induced
increase in 5mC levels and Dnmt1 binding and a decrease in Sp1 binding at the \textit{Mlh1} promoter and ameliorated a decrease in \textit{Mlh1} gene transcripts. Similarly, \textit{Dnmt1}-siRNA transfected cells, but not scrambled RNA transfected cells, were also protected from a glucose-induced increase in methylation of the \textit{Mlh1} promoter DNA and a decrease in \textit{Mlh1} gene transcripts (Figs. 2a–d).

In the same cell preparations, the inhibition of Dnmts by either \textit{Dnmt1}-siRNA or by Aza attenuated an increase in base mismatches in the D-loop region experienced by the cells in high-glucose conditions, further supporting the role DNA methylation in \textit{Mlh1} transcriptional regulation (Fig. 3).

To further investigate the role of DNA methylation, the 5’ region of the human \textit{Mlh1} promoter (from –2077 to –617) was subcloned into a luciferase reporter plasmid, pGL3-basic vector. Transient transfection of HRECs with this luciferase reporter plasmid had a strong promoter activity in pGL3promoter and its transcriptional regulation. HRECs, incubated in high glucose, were analyzed. The inhibition of Dnmt by either its siRNA or by Aza in a high-glucose medium, in addition to preventing a decrease in \textit{Mlh1} gene transcripts, also attenuated an increase in 5mC and Dnmt1 binding at the \textit{Mlh1} promoter and prevented a decrease in Sp1 binding, further confirming the role of DNA methylation in the transcriptional regulation of \textit{Mlh1} (Fig. 2).

Consistent with the in vitro model, \textit{Mlh1} gene transcripts were significantly decreased in retinal microvessels from diabetic mice when compared with normal mice (Fig. 6a). Furthermore, diabetic mice had a twofold increase in 5mC levels at the \textit{Mlh1} promoter (Fig. 6b). To further confirm the role of DNA methylation in \textit{Mlh1} transcriptional regulation, retinas from mice receiving intraperitoneal administration of Aza or intravitreal administration of \textit{Dnmt1}-siRNA were analyzed. The inhibition of Dnmt by either its siRNA or by its pharmacologic inhibitor significantly attenuated diabetes-induced alterations in the gene transcripts of \textit{Mlh1} and protected the hypermethylation of its promoter. Compared to \textit{Dnmt1}-siRNA, Aza administration had a better effect in ameliorating diabetes-induced \textit{Mlh1} promoter DNA methylation, but these two groups were not significantly different from each other.

To transition from experimental models to the human disease, \textit{MLH1} gene expression and its promoter DNA methylation were determined in the retinal microvessels from human donors with diabetic retinopathy. Consistent with our results from the experimental models, \textit{MLH1} expression was decreased by ~50% and that of \textit{DNMT1} was increased by...
threefold in the retinal microvessels from donors with diabetic retinopathy when compared with age-matched nondiabetic donors (Fig. 7a). As seen in our in vitro model, the methylation analysis of $MLH1$ promoter also showed differential DNA methylation in R1 to R4; although R1 and R2 had similar 5mC levels in diabetic retinopathy and nondiabetic donors, the 5mC levels were more than sixfold higher in R3 and about fourfold higher in the R4 regions in diabetic retinopathy donors when compared with nondiabetic donors. Similarly, in R3 and R4 regions, Dnmt1 binding was also increased by about sevenfold and threefold, respectively. However, although R1 had similar 5mC levels in diabetic retinopathy and nondiabetic donors, Dnmt1 binding was threefold higher in diabetic retinopathy donors. However, normal rabbit IgG values obtained from the same samples were <1% compared to those obtained from antibodies against 5mC or Dnmt1 (Figs. 7b, 7c).

**DISCUSSION**

Recent studies have shown that mitochondrial dysfunction plays a major role in the development of diabetic retinopathy. Mitochondria are the only other subcellular structures with their own DNA; each cell has multiple mitochondria, and each mitochondria has several copies of DNA. The mutation and substitution rate of mtDNA is significantly higher than that of nuclear genes, but each cell can also have a population of distinct mtDNA genomes; mismatches in mtDNA, without apparent functional consequences, are routinely observed in normal subjects. The MMR system corrects the postreplication errors and maintains genetic stability, and an efficient MMR system also recognizes sequence variants in mtDNA and cuts them. Our previous study has shown that in diabetic retinopathy, the number of sequence variants is increased in the mtDNA, and the expression of $MLH1$ is also decreased. Furthermore, mtDNA is hypermethylated, and the inhibition of Dnmts also ameliorates sequence variants in the mtDNA. Here we show that the mitochondrial accumulation of $MLH1$, which helps fix the mismatches made during DNA replication, is decreased in diabetes. The promoter is hypermethylated, and the activity of

![Figure 3](image-url)  
**Figure 3.** Regulation of DNA methylation and base mismatches. DNA from HRECs was amplified using semi-qPCR for the D-loop and digested with mismatch-specific surveyor endonuclease. The samples were analyzed on a 2% agarose gel. The parent amplicon band intensity was quantified by densitometry, and the values obtained from cells in NG was considered as 100%. Data are represented as mean ± SD. (a) $Dnmt1$-siRNA transfected HRECs and (b) Aza-treated HRECs. NG and HG = HRECs in 5 mM and 20 mM glucose, respectively; Dsi and SC = cells transfected with $Dnmt1$-siRNA and negative control RNA, respectively. Aza = 1 μM 5-Aza-2-deoxyxytidine. *P < 0.05 compared with NG; #P < 0.05 compared with HG.

![Figure 4](image-url)  
**Figure 4.** Effect of inhibition of Dnmts on $MLH1$ promoter activity. $MLH1$ promoter was amplified and cloned into the promoter less pGL3-Basic-IRES plasmid, and deletion promoter fragments generated were used for the transfection of HRECs. The promoter activity was determined by a Pierce Firefly Luciferase Glow Assay Kit, and intensity was measured at 613 nm. pGL3-control vectors transfected cells were used as control. The values are represented as mean ± SD of three or more assays, each performed in duplicate. NG and HG = HRECs in 5 mM and 20 mM glucose, respectively; Aza = 1 μM Aza-2-deoxyxytidine; L-Glu = 20 mM L-Glucose. *P < 0.05 compared to NG and #P < 0.05 compared to HG.
the promoter is decreased. Hyperglycemia produces differential methylation in different regions of the *Mlh1* promoter; two of the four regions examined are hypermethylated, and the region proximal to the transcription start site presents the highest 5mC levels. Regulation of Dnmt, in addition to inhibiting DNA methylation of the *Mlh1* promoter, ameliorates a decrease in its promoter activity, transcriptional factor binding, and gene transcripts. Consistent with our in vitro results, *Mlh1* promoter hypermethylation is also observed in the retinal microvessels from diabetic mice, and the inhibition of Dnmts prevents the promoter hypermethylation and decrease in *Mlh1* expression. A similar differential DNA methylation pattern is also observed in the retinal microvessels from human donors with documented diabetic retinopathy and further confirms the role of DNA methylation in the transcriptional regulation of *Mlh1*.

Mitochondria are the major source of ROS production, and ROS leaked during oxidative phosphorylation can react with cellular components including DNA. The mutation rate of mtDNA is 10-fold to 50-fold higher than that of nuclear DNA. Although both the mutant and wildtype copies can coexist in mtDNA, an increase in heteroplasmy decreases the energy production. Our previous work has shown that although both Mlh1 and Msh2 are decreased in the retina in diabetes, the overexpression of *Mlh1* ameliorates a diabetes-induced increase in mtDNA mismatches, but that of *Msh2* fails to provide any benefit. To recognize and correct the biosynthetic errors formed during DNA replication or the mispaired bases generated in DNA recombination, the localization of MMR enzymes in the mitochondria is essential. The results presented here clearly demonstrate that the localization of *Mlh1* in the mitochondria is also decreased in hyperglycemic milieu, further supporting its role in mtDNA mismatch repair.

Diabetes activates DNA methylation machinery in the retina and its vasculature, and along with the mtDNA, DNA methylation status of many genes associated with mitochondrial homeostasis is also altered.27,32,43 Our previous work has shown a positive correlation between mtDNA methylation and an increase in base mismatches.32 The methylation of CpG in the promoter region of *Mlh1* by a methylated DNA immunoprecipitation kit. Values are calculated as fold change, and the values obtained from the cells in NG are considered as one. NG and HG = 5 mM and 20 mM glucose, respectively; Aza = 1μM Aza-2'-deoxycytidine. *P < 0.05 compared to NG.

![Figure 5](image-url)

**Figure 5.** Differential DNA methylation of the *Mlh1* promoter in HRECs incubated in high glucose. Four CpG-rich regions of the proximal promoter of *Mlh1* (~659 to 72; R1-R4) were analyzed for (a) 5mC levels by methylated DNA immunoprecipitation method and (b) Dnmt1 binding by ChIP using IgG (represented as ^) as an antibody control. The values are represented as fold change, and the values obtained from the cells in NG are considered as one. NG and HG = 5 mM and 20 mM glucose, respectively; Aza = 1μM Aza-2'-deoxycytidine. *P < 0.05 compared to NG.

DNA methylation of the *Mlh1* promoter in diabetes and its regulation by Dnmt inhibitors. Retinal microvessels from diabetic mice receiving intravitreal administration of Dnmt1-siRNA (D-si) or intraperitoneal injection of Aza-2'-deoxycytidine (Aza) were analyzed for (a) *Mlh1* mRNA levels by qPCR and (b) 5mC levels at the promoter region of *Mlh1* by a methylated DNA immunoprecipitation kit. Values are calculated as fold change as compared to normal and are represented as mean ± SD. Nor and Diab = nondiabetic control and streptozotocin-induced diabetic mice, respectively. *P < 0.05 compared to normal and #P < 0.05 compared to diabetes.

![Figure 6](image-url)

**Figure 6.** DNA methylation of the *Mlh1* promoter in diabetes and its regulation by Dnmt inhibitors. Retinal microvessels from diabetic mice receiving intravitreal administration of Dnmt1-siRNA (D-si) or intraperitoneal injection of Aza-2'-deoxycytidine (Aza) were analyzed for (a) *Mlh1* mRNA levels by qPCR and (b) 5mC levels at the promoter region of *Mlh1* by a methylated DNA immunoprecipitation kit. Values are calculated as fold change as compared to normal and represented as mean ± SD. Nor and Diab = nondiabetic control and streptozotocin-induced diabetic mice, respectively. *P < 0.05 compared to normal and #P < 0.05 compared to diabetes.
result of the conversion of cytosine to thymine, and the deamination rate of 5mC is considerably higher than that of cytosine or guanosine to adenine.46-47 A lack of protective histones in mtDNA, and its close proximity to the ROS-producing electron transport chain, also make mtDNA prone to mutations.48 Hypermethylation of mtDNA in diabetes further makes it more susceptible to mutation.31,32 A decreased translocation of Mlh1, the main enzyme responsible for fixing up the mismatches, inside the mitochondria in diabetes and the hypermethylation of its promoter further adds insult to the already damaged mtDNA, compromising mitochondrial homeostasis. Thus, strategies to regulate of DNA methylation (or its deamination) have the potential to protect mitochondrial homeostasis by preventing base mismatches and impeding the development of diabetic retinopathy.

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References


Similar differential DNA methylation patterns in the retinal microvessels from human donors with diabetic retinopathy further support the importance of CpG methylation in the regulation of Mlh1 in diabetic retinopathy. However, the significance of such differential methylation of the Mlh1 promoter in retinal microvasculature is the development of diabetic retinopathy remains to be investigated. In support, irregular methylation of the Mlh1 promoter is considered as a useful molecular marker to screen or diagnose precancerous and early endometrial malignancies,45 and the methylation of a small proximal region in the Mlh1 promoter is shown to correlate with the lack of its expression in colorectal cell lines.25

In conclusion, we have provided the first report showing the role of DNA methylation in the regulation of a mismatch repair enzyme in diabetic retinopathy; hypermethylation of the Mlh1 promoter in a hyperglycemic medium impedes the binding of the transcription factor and transcriptionally suppresses its expression. A majority of the mutations are the
mtDNA Quality Control in Diabetic Retinopathy


