

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 *Mlb1* 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, *Mlb1* 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 *Mlb1* 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 *Mlb1* promoter hypermethylation and a decrease in its gene transcripts. Similar decreases in *Mlb1* gene transcripts and its promoter DNA hypermethylation were observed in human donors.

CONCLUSIONS. Thus, as a result of the epigenetic modifications of the *Mlb1* 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

Diabetic retinopathy, a sight-threatening progressing disease, has a multifactorial etiology, and many metabolic, functional, and structural abnormalities have been implicated in its development. However, the exact molecular mechanism of this major complication of diabetes still remains unclear. Diabetic environment increases oxidative stress and inflammatory mediators in the retina and its vasculature and damages the mitochondria, resulting in a vicious cycle of free radicals.^{1,2}

Mitochondria are equipped with their own small circular DNA (mtDNA), and depending on cellular needs, each cell can have 100 to 10,000 copies of mtDNA.³ This circular mtDNA contains a noncoding regulatory region, the displacement loop (D-loop), which is the site of transcription and replication. Although mtDNA encodes genes for only 13 proteins, these proteins are essential for the functioning of the electron transport chain.⁴ Retinal mtDNA is damaged in diabetes, and compared to the other regions, the damage is more extensive in the D-loop. In addition, mtDNA copy numbers are decreased, and the electron transport chain system is compromised.^{5,6}

Mitochondrial DNA resides in close proximity to the reactive oxygen species (ROS)-producing electron transport chain, and

unlike nuclear DNA, it is devoid of protecting histones, making it very mutagenic; the mutation rate of mtDNA is several folds higher than that of nuclear DNA.⁷ Healthy mitochondria can harbor both mutant and nonmutant DNA within the same cell; however, if mutated DNA is increased, the cells exhibit reduced energy capacity.^{8,9} Increase in mtDNA sequence variants have been associated with many chronic diseases, including open-angle glaucoma.^{10,11} Experimental models of diabetic retinopathy have shown increased sequence variants in retinal mtDNA, especially in its D-loop, affecting the mitochondrial function.¹² Similar to other cellular organelle, to repair uncomplimentary base pairs incorporated into the DNA sequences and the insertion and/or deletion loops that are formed during DNA replication, mitochondria also uses a base mismatch repair (MMR) system to recognize mismatches and cut them, namely, mutS homolog 2 (Msh2) and mutL homolog 1 (Mlh1) enzymes, respectively.¹³ While the number of sequence variants is increased in the retinal mtDNA in diabetes, MMR enzymes Mlh1 and Msh2 are decreased. However, the overexpression of *Mlb1* ameliorates increases in mismatches, that of *Msb2* fails to have any effect.¹²



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

Mlb1 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 *Mlb1* 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 *Mlb1* 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 *Mlb1* 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. A3656, Sigma-Aldrich Corp., St. Louis, MO, USA). L-glucose (20 mM), instead of 20 mM D-glucose, was used as the osmotic/metabolic control.^{17,26,27}

A group of cells from the fifth to sixth passage were transfected with *Dnmt1*-siRNA (cat. no. SC-35204, Santa Cruz Biotechnology, Santa Cruz, CA, USA) using transfection reagent (cat. no. SC-29528, Santa Cruz Biotechnology). As a control, parallel incubations were run using nontargeting scrambled RNA or reagent alone.²⁶ Transfection efficiency was evaluated by quantifying *Dnmt1* gene transcripts and protein expression and, as reported earlier, was about 40%.²⁸ Each incubation condition had HRECs from the same batch and the same passage.

Mice

Two days after establishment of streptozotocin-induced diabetes in C57BL/6 mice (7–8 weeks old, either sex),²⁸ 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. ab33985, 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 40× (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 fraction³¹ using Mlh1 and CoxIV antibodies (ab92312 and ab33985; Abcam) at 1:1000 dilution each.

Plasmid and Constructs

Mlb1 promoter (–2077 to –617) was amplified and cloned into the promoter-less pGL3-Basic-IRES plasmid (plasmid no. 64784,

TABLE 1. Human *MLH1* Promoter Primers for Cloning

| Restriction Endonuclease | Position | Primers With 5' RE-Recognition Sites |
|--------------------------|-----------|--------------------------------------|
| HindIII | 617-636 | 5'-TCTAAGCTTTGGACTGTTGTTGCCCTAC-3' |
| BamHI | 2077-2058 | 5'-CAGGGATCCCCTTCAGCGGCAGCTATTGA-3' |
| HindIII | 1137-1156 | 5'-CAGAAGCTTCACCGCGGATAAAGACCAGG-3' |
| BamHI | 2070-2050 | 5'-ACCGGATCCCAGCTATTGATTGGACAG-3' |
| HindIII | 1235-1254 | 5'-CGGAAGCTTAGCTGGTTGCGTAGATTCCT-3' |
| BamHI | 2077-2057 | 5'-CAGGGATCCCCTTCAGCGGCAGCTATTGAT-3' |

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 BamHI 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 µM Aza.

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 performed in triplicate, and all transfection experiments were 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)³¹ using *Mlh1* promoter primers (Table 2).

TABLE 2. Primers for ChIP Analysis of Human *MLH1*

| Region | Position | Primer | Number of CpG |
|--------|--------------|--|---------------|
| R1 | −659 to −522 | Forward: CCTGGCTCGGTTAAAAAGC Reverse: GGAGGTGTTGCTGAGAGAGG | 7 |
| R2 | −383 to −261 | Forward: AGTATTCGTGCTCAGCCTCG Reverse: GCGTTATTTGGTGGTGGAGC | 16 |
| R3 | −246 to −122 | Forward: GTCATCCACATTCTGCGGGA Reverse: CTCTGCTGAGGTGATCTGGC | 8 |
| R4 | −13 to +72 | Forward: CCCCAGCTCCTAAAAACGAA Reverse: CTGCCCGCTACCTAGAAGGA | 8 |

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 13537, 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-

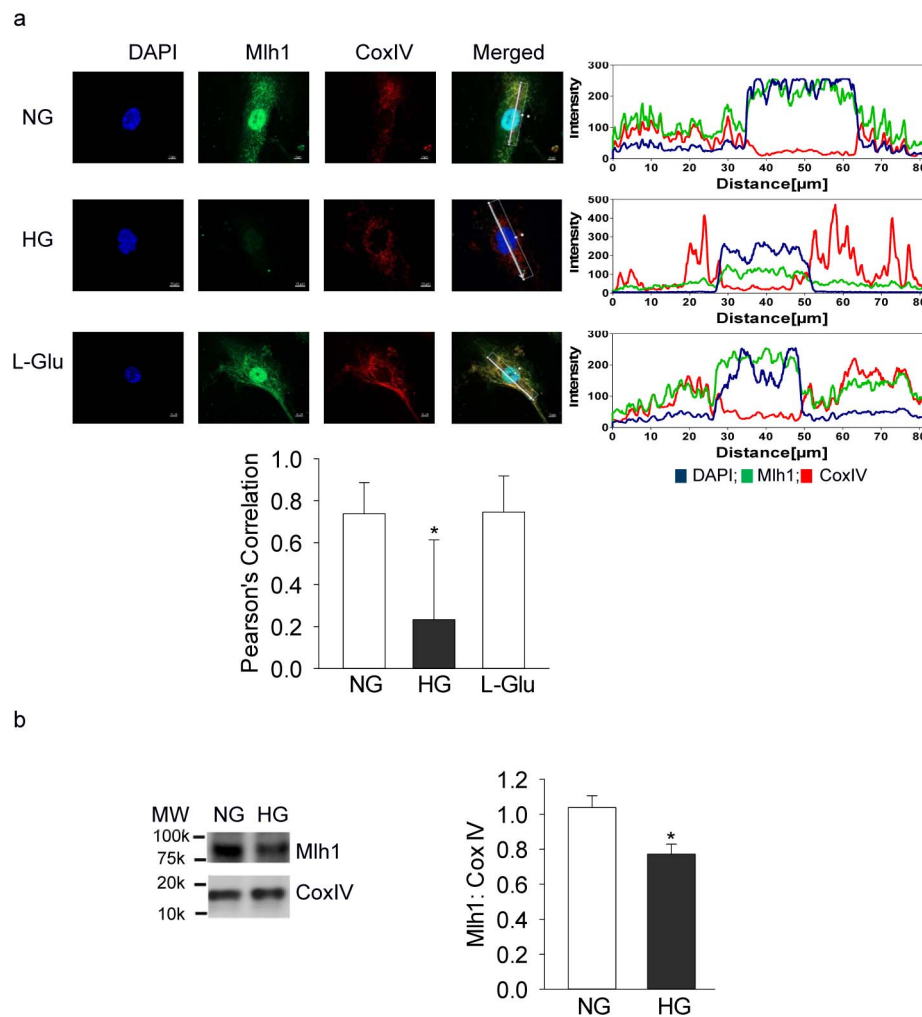


FIGURE 1. Effect of high glucose on mitochondrial localization of Mlh1 in retinal endothelial cells. Mitochondrial localization of Mlh1 was (a) determined by immunofluorescence technique using AlexaFluor 488 (green)-conjugated and Texas Red (red)-conjugated secondary antibodies for Mlh1 and CoxIV, respectively. After capturing the images in ZEISS 40X objective magnification with the Apotome module, the images were calibrated with ZEISS pro-inbuilt software package and modules, and the random regions of interest were made in the outer nuclear regions. A colocalization software module was used to determine the Pearson's correlation coefficient, and the line region of interest (white line in the merged column) was used to quantify the intensity profiles of Mlh1 and CoxIV across the cell. (b) Isolated mitochondrial fraction was western blotted for Mlh1, and CoxIV was used as the loading protein. Data in the histograms are represented as mean \pm SD of the values obtained from three or more cell preparations. NG and HG = HRECs in 5 mM and 20 mM D-glucose, respectively; L-Glu = 20 mM L-glucose. * $P < 0.05$ compared to NG.

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 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

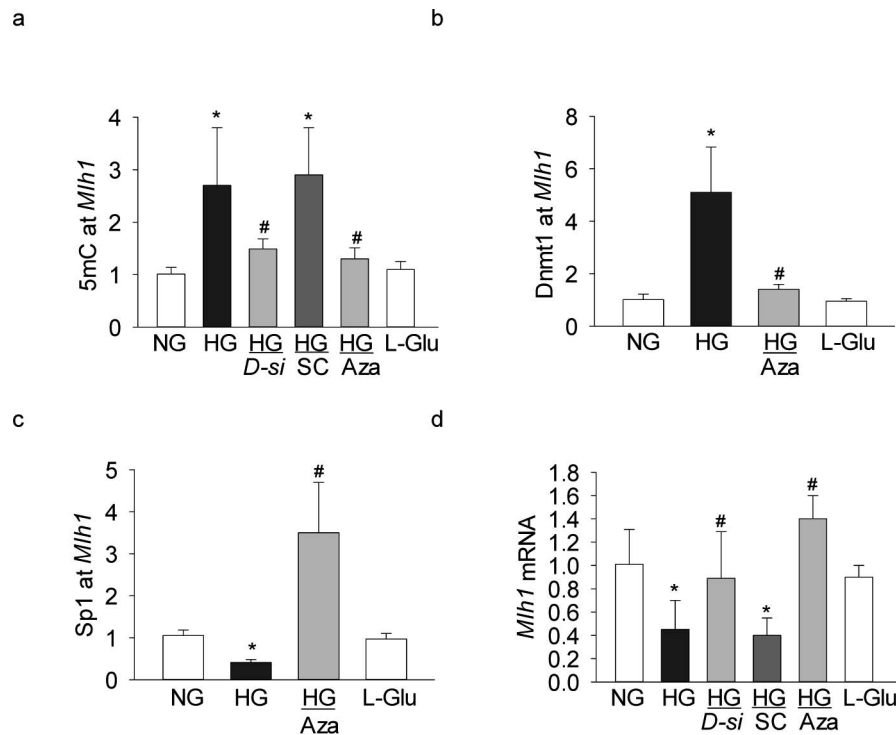


FIGURE 2. Effect of high glucose on DNA methylation of *Mlb1* promoter and its transcriptional regulation. HRECs, incubated in high glucose, were analyzed for (a) 5mC levels at *Mlb1* promoter by methylated DNA immunoprecipitation method, (b, c) Dnmt1 and Sp1 binding by ChIP technique, and (d) *Mlb1* gene transcripts by qPCR. Each measurement was made in duplicate in three to four samples per group. The values obtained from cells in NG are considered as 1. NG and HG = 5 mM and 20 mM glucose, respectively; *D-si* and SC = cells transfected with *Dnmt1*-siRNA and control negative RNA, respectively, and incubated in HG; Aza = 1 μ M Aza-2'-deoxyxytidine. * $P < 0.05$ versus NG and # $P < 0.05$ versus HG.

increase in 5mC levels and Dnmt1 binding and a decrease in Sp1 binding at the *Mlb1* promoter and ameliorated a decrease in *Mlb1* gene transcripts. Similarly, *Dnmt1*-siRNA transfected cells, but not scrambled RNA transfected cells, were also protected from a glucose-induced increase in methylation of the *Mlb1* promoter DNA and a decrease in *Mlb1* gene transcripts (Figs. 2a–d).

In the same cell preparations, the inhibition of Dnmts by either *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 *Mlb1* transcriptional regulation (Fig. 3).

To further investigate the role of DNA methylation, the 5' region of the human *Mlb1* 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 pGL-MLH1Pro1.65 as compared to the cells transfected with an empty plasmid vector pGL3-basic in normal glucose conditions. High glucose downregulated *Mlb1* promoter activity, and the inhibition of Dnmts significantly ameliorated ($P < 0.05$) glucose-induced downregulation of the luciferase activity (Fig. 4), confirming the role of DNA methylation in the repression of its gene transcripts in high-glucose conditions.

The *Mlb1* promoter is a CpG-rich promoter, and to identify the methylated sites, four regions (R) of its proximal promoter (–659 to 72; R1–R4) with 8 to 16 CpG counts were analyzed (Table 1). Regions R3 and R4 had significantly higher 5mC levels in the cells exposed to high glucose when compared with the cells in normal glucose, and R1 had slightly higher 5mC levels in high glucose, but the values were not significantly different from those in normal glucose (Fig. 5a). Differential distribution of 5mC in these four CpG-rich regions

was further confirmed by quantifying the binding of Dnmt1; as shown in Figure 5b, in high-glucose conditions, R3 had a more than fourfold increase in Dnmt1 binding compared to about threefold in R4 and less than twofold in R1, further confirming the importance of the R3 region. In the same samples, the values obtained from the control IgG were almost negligible. Regulation of *Dnmt1* by siRNA or by Aza in a high-glucose medium, in addition to preventing a decrease in *Mlb1* gene transcripts, also attenuated an increase in 5mC and Dnmt1 binding at the *Mlb1* promoter and prevented a decrease in Sp1 binding, further confirming the role of DNA methylation in the transcriptional regulation of *Mlb1* (Fig. 2).

Consistent with the in vitro model, *Mlb1* 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 *Mlb1* promoter (Fig. 6b). To further confirm the role of DNA methylation in *Mlb1* transcriptional regulation, retinas from mice receiving intraperitoneal administration of Aza or intravitreal administration of *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 *Mlb1* and protected the hypermethylation of its promoter. Compared to *Dnmt1*-siRNA, Aza administration had a better effect in ameliorating diabetes-induced *Mlb1* promoter DNA methylation, but these two groups were not significantly different from each other.

To transition from experimental models to the human disease, *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, *MLH1* expression was decreased by ~50% and that of *DNMT1* was increased by

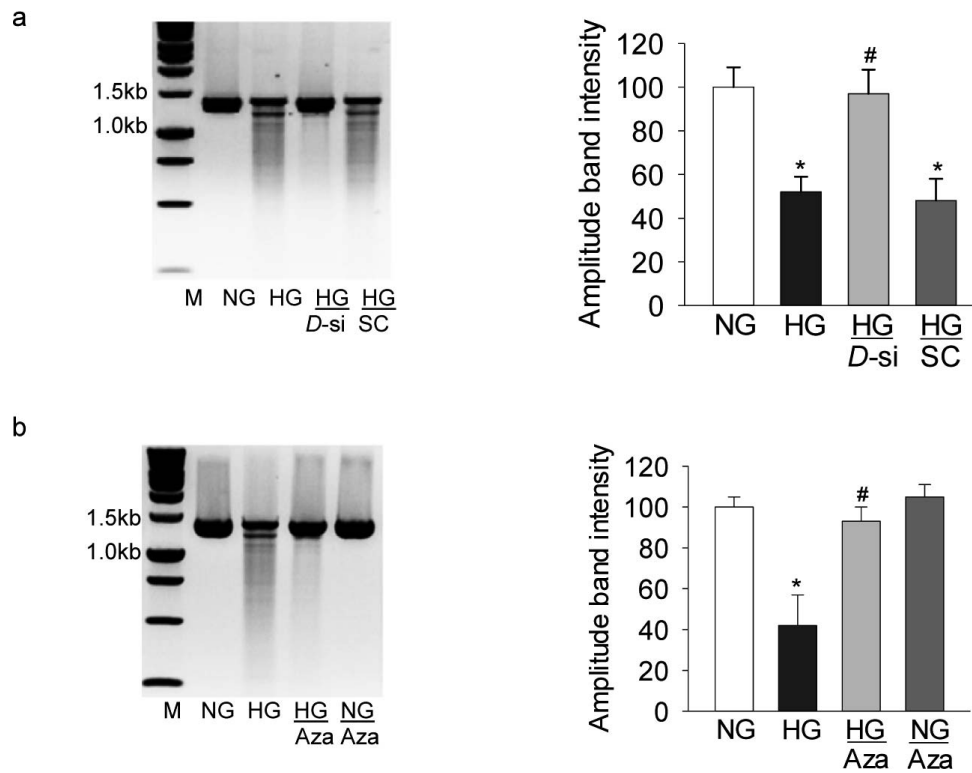


FIGURE 3. Regulation of DNA methylation and base mismatches. DNA from HRECs was amplified using semi-qPCR for the D-loop and digested with mismatch-specific surveyor endonuclease. The samples were analyzed on a 2% agarose gel. The parent amplicon band intensity was quantified by densitometry, and the values obtained from cells in NG was considered as 100%. Data are represented as mean \pm SD, and each measurement was made in duplicate in three to five samples in each group. Mismatches and the parent amplicon band intensity from (a) *Dnmt1*-siRNA transfected HRECs and (b) Aza-treated HRECs. NG and HG = HRECs in 5 mM and 20 mM glucose, respectively; D-si and SC = cells transfected with *Dnmt1*-siRNA and negative control RNA, respectively; Aza = 1 μ M 5-Aza-2'-deoxythymine. * P < 0.05 compared with NG; # P < 0.05 compared with HG.

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.^{15,33-35} Mitochondria are the only other subcellular structures with their own DNA; each cell has multiple mitochondria, and each mitochondria has several copies of DNA.³⁶ 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.^{37,38} 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.¹⁵ 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.¹² Furthermore, mtDNA is hypermethylated, and the inhibition of Dnmts also ameliorates sequence variants in the mtDNA.³¹ 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

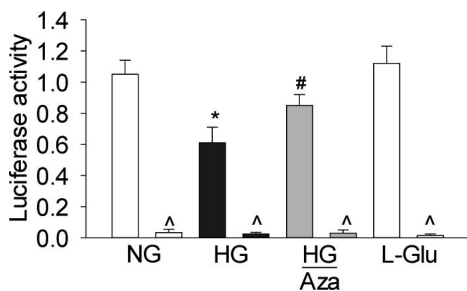


FIGURE 4. Effect of inhibition of Dnmts on *Mlb1* promoter activity. *Mlb1* promoter was amplified and cloned into the promoter less pGL3-Basic-IRES plasmid, and deletion promoter fragments generated were used for the transfection of HRECs. The promoter activity was determined by a Pierce Firefly Luciferase 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 \pm SD of three or more assays, each performed in duplicate. NG and HG = HRECs in 5 mM and 20 mM glucose, respectively; Aza = 1 μ M Aza-2'-deoxythymine; L-Glu = 20 mM L-Glucose. * P < 0.05 compared to NG and # P < 0.05 compared to HG.

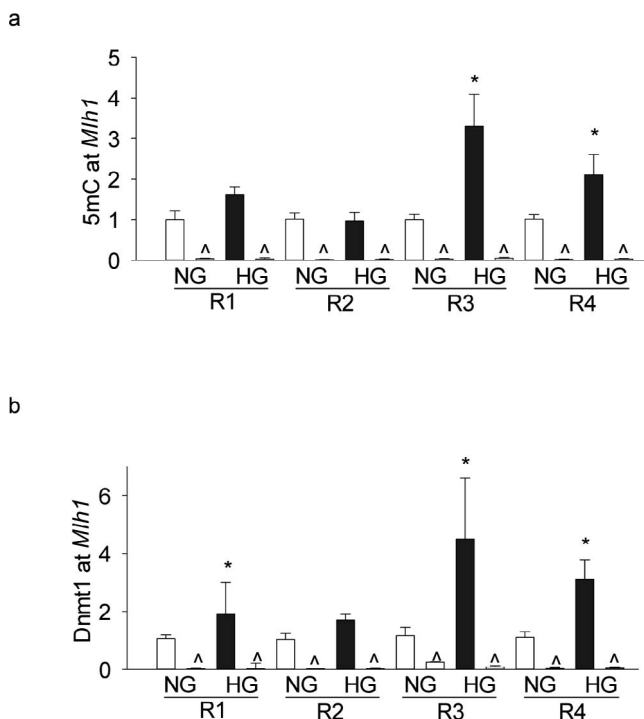


FIGURE 5. Differential DNA methylation of the *Mlb1* promoter in HRECs incubated in high glucose. Four CpG-rich regions of the proximal promoter of *Mlb1* (–659 to 72; R1–R4) were analyzed for (a) 5mC levels by methylated DNA immunoprecipitation method and (b) Dnmt1 binding by ChIP using IgG (represented 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 = 1 μM Aza-2'-deoxycytidine. **P* < 0.05 compared to NG.

the promoter is decreased. Hyperglycemia produces differential methylation in different regions of the *Mlb1* 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 *Mlb1* promoter, ameliorates a decrease in its promoter activity, transcriptional factor binding, and gene transcripts. Consistent with our in vitro results, *Mlb1* promoter hypermethylation is also observed in the retinal microvessels from diabetic mice, and the inhibition of Dnmts prevents the promoter hypermethylation and

decrease in *Mlb1* 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 *Mlb1*.

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,³⁹ an increase in heteroplasmy decreases the energy production.⁴⁰ Our previous work has shown that although both Mlh1 and Msh2 are decreased in the retina in diabetes, the overexpression of *Mlb1* ameliorates a diabetes-induced increase in mtDNA mismatches, but that of *Msb2* fails to provide any benefit.¹² 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.^{41,42} The results presented here clearly demonstrate that the localization of *Mlb1* 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 of a gene is generally associated with impaired transcriptional factor binding.¹⁹ The *Mlb1* promoter is a TATA-less promoter with multiple CpG and transcription factor-binding sites^{22,23} and is frequently hypermethylated in many tumor types.⁴⁴ Here, the data show that diabetes hypermethylates the *Mlb1* promoter and decreases its promoter activity. This is accompanied by decreased binding of the transcriptional factor Sp1. Furthermore, pharmacological or molecular inhibition of Dnmts reduces a glucose-induced increase in 5mC levels at the *Mlb1* promoter, ameliorates a decrease in Sp1 binding, and maintains its promoter activity and gene transcription, suggesting an important role of DNA methylation in the transcriptional suppression of *Mlb1* in diabetes.

Mlb1 promoter methylation also shows differential distribution of 5mC in four regions of the CpG-rich proximal promoter region (–659 to 72). Among the four regions, R3, a short region of ~100 bp just upstream of the start site, has the highest levels of 5mC and increased Dnmt1 binding, suggesting an irregular methylation pattern in the *Mlb1* promoter region.

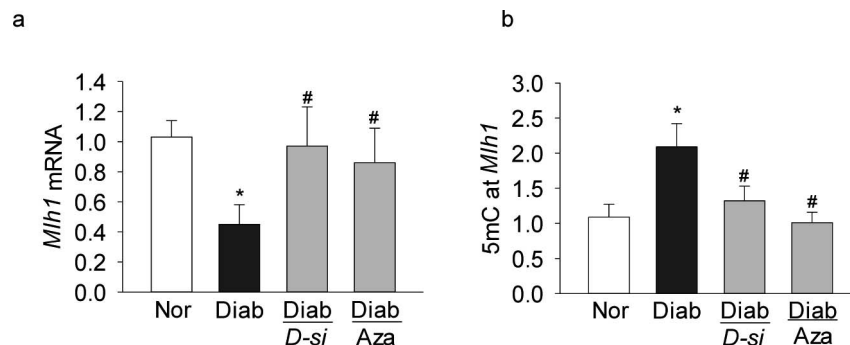


FIGURE 6. DNA methylation of the *Mlb1* promoter in diabetes and its regulation by Dnmt inhibitors. Retinal microvessels from diabetic mice receiving intravitreal administration of *Dnmt1*-siRNA (D-si) or intraperitoneal injection of Aza-2'-deoxyxytidine (Aza) were analyzed for (a) *Mlb1* mRNA levels by qPCR and (b) 5mC levels at the promoter region of *Mlb1* 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.

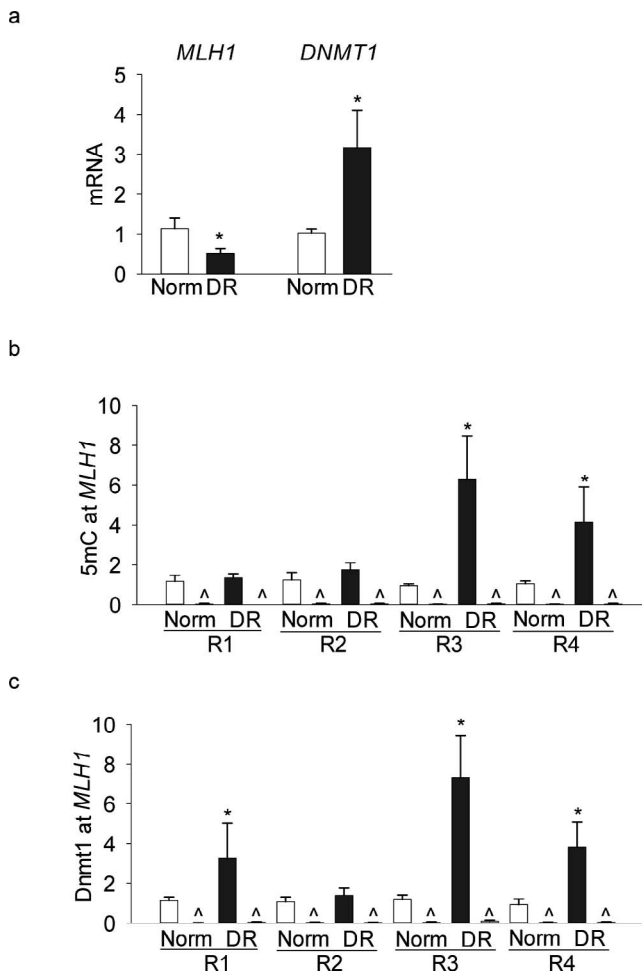


FIGURE 7. Methylation of *MLH1* in human donors with diabetic retinopathy. Retinal microvessels from human donors with documented diabetic retinopathy (DR) and age-matched nondiabetic donors (Norm) were analyzed for *MLH1* and *DNMT1* gene transcripts by qPCR using β -actin as a housekeeping gene (a). The *MLH1* promoter was analyzed for (b) 5mC levels using a methylated DNA immunoprecipitation method and (c) Dnmt1 binding by ChIP technique. Values are mean \pm SD of at least six donors in each group, with each measurement made in duplicate, and are presented as fold change compared to nondiabetic donors. Δ IgG antibody control. * $P < 0.05$ compared to nondiabetic control.

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 in 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.²³

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

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.^{31,49} 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

- Kowluru RA, Mishra M. Oxidative stress, mitochondrial damage and diabetic retinopathy. *Biochim Biophys Acta*. 2015;1852:2474–2483.
- Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. *Exp Diabetes Res*. 2007;2007:95103.
- Burr SP, Pezet M, Chinnery PF. Mitochondrial DNA heteroplasmy and purifying selection in the mammalian female germ line. *Dev Growth Diff*. 2018;60:21–32.
- Rebelo AP, Dillon LM, Moraes CT. Mitochondrial DNA transcription regulation and nucleoid organization. *J Inherit Metab Dis*. 2011;34:941–951.
- Mishra M, Lillis J, Seyoum B, Kowluru RA. Peripheral blood mitochondrial DNA damage as a potential noninvasive biomarker of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2016;57:4035–4044.
- Tewari S, Santos JM, Kowluru RA. Damaged mitochondrial DNA replication system and the development of diabetic retinopathy. *Antiox Redox Signal*. 2012;17:492–504.
- Lagouge M, Larsson NG. The role of mitochondrial DNA mutations and free radicals in disease and ageing. *J Int Med*. 2013;273:529–543.
- Wallace DC. Mitochondrial diseases in man and mouse. *Science* 1999;283:1482–1488.
- DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. *New Eng J Med* 2003;348:2656–2668.
- Bahreini F, Houshmand M, Modaresi MH, et al. Mitochondrial copy number and D-loop variants in pompe patients. *Cell J*. 2016;18:405–415.
- Collins DW, Gudiseva HV, Trachtman B, et al. Association of primary open-angle glaucoma with mitochondrial variants and haplogroups common in African Americans. *Mol Vis*. 2016;22:454–471.
- Mishra M, Kowluru RA. Retinal mitochondrial DNA mismatch repair in the development of diabetic retinopathy, and its continued progression after termination of hyperglycemia. *Invest Ophthalmol Vis Sci*. 2014;55:6960–6967.

13. Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech Ageing Dev.* 2008;129:391-407.
14. Handy DE, Castro R, Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. *Circulation.* 2011;123:2145-2156.
15. Kowluru RA. Mitochondrial stability in diabetic retinopathy: lessons learned from epigenetics. *Diabetes.* 2019;68:241-247.
16. Reddy MA, Zhang E, Natarajan R. Epigenetic mechanisms in diabetic complications and metabolic memory. *Diabetologia.* 2015;58:443-455.
17. Kowluru RA, Shan Y, Mishra M. Dynamic DNA methylation of matrix metalloproteinase-9 in the development of diabetic retinopathy. *Lab Invest.* 2016;96:1040-1049.
18. Zhang X, Zhao L, Hambly B, Bao S, Wang K. Diabetic retinopathy: reversibility of epigenetic modifications and new therapeutic targets. *Cell Biosci.* 2017;7:42.
19. Li L, He S, Sun JM, Davie JR. Gene regulation by Sp1 and Sp3. *Biochem Cell Biol.* 2004;82:460-471.
20. Bansal A, Pinney SE. DNA methylation and its role in the pathogenesis of diabetes. *Pediatr Diabetes.* 2017;18:167-177.
21. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A.* 1998;95:6870-6875.
22. Rodriguez-Jimenez FJ, Moreno-Manzano V, Lucas-Dominguez R, Sanchez-Puelles JM. Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. *Stem Cell.* 2008;26:2052-2062.
23. Deng G, Chen A, Hong J, Chae HS, Kim YS. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res.* 1999;59:2029-2033.
24. Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.* 1997;57:808-811.
25. Mishra M, Kowluru RA. Role of PARP-1 as a novel transcriptional regulator of MMP-9 in diabetic retinopathy. *Biochim Biophys Acta Mol Basis Dis.* 2017;1863:1761-1769.
26. Duraisamy AJ, Mohammad G, Kowluru RA. Mitochondrial fusion and maintenance of mitochondrial homeostasis in diabetic retinopathy. *Biochim Biophys Acta Mol Basis Dis.* 2019;1865:1617-1626.
27. Mishra M, Kowluru RA. The role of DNA methylation in the metabolic memory phenomenon associated with the continued progression of diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2016;57:5748-5757.
28. Duraisamy AJ, Mishra M, Kowluru A, Kowluru RA. Epigenetics and regulation of oxidative stress in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2018;59:4831-4840.
29. 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.
30. Haokip DT, Goel I, Arya V, et al. Transcriptional regulation of Atp-dependent chromatin remodeling factors: Smarcat1 and Brg1 mutually co-regulate each other. *Sci Rep.* 2016;6:20532.
31. Mishra M, Kowluru RA. Epigenetic modification of mitochondrial DNA in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2015;56:5133-5142.
32. Mishra M, Kowluru RA. DNA methylation-a potential source of mitochondrial DNA base mismatch in the development of diabetic retinopathy. *Mol Neurobiol.* 2019;56:88-101.
33. Kowluru RA, Kowluru A, Mishra M, Kumar B. Oxidative stress and epigenetic modifications in the pathogenesis of diabetic retinopathy. *Prog Retin Eye Res.* 2015;48:40-61.
34. Kowluru RA. Diabetic retinopathy: mitochondrial dysfunction and retinal capillary cell death. *Antioxid Redox Signal.* 2005;7:1581-1587.
35. Roy S, Trudeau K, Roy S, Tien T, Barrette KF. Mitochondrial dysfunction and endoplasmic reticulum stress in diabetic retinopathy: mechanistic insights into high glucose-induced retinal cell death. *Curr Clin Pharmacol.* 2013;8:278-284.
36. Clayton DA. Transcription and replication of mitochondrial DNA. *Hum Reprod.* 2000;15:11-17.
37. Ramos A, Santos C, Mateiu L, et al. Frequency and pattern of heteroplasmy in the complete human mitochondrial genome. *PLoS One.* 2013;8:e74636.
38. Santos C, Sierra B, Alvarez L, et al. Frequency and pattern of heteroplasmy in the control region of human mitochondrial DNA. *J Mol Evol.* 2008;67:191-200.
39. Wallace DC, Chalkia D. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. *Cold Spring Harbor Pres Biol.* 2013;5:a021220.
40. Bris C, Goudenege D, Desquiret-Dumas V, et al. Bioinformatics tools and databases to assess the pathogenicity of mitochondrial DNA variants in the field of next generation sequencing. *Front Genetics.* 2018;9:632.
41. Mason PA, Matheson EC, Hall AG, Lightowlers RN. Mismatch repair activity in mammalian mitochondria. *Nucl Acid Res.* 2003;31:1052-1058.
42. Modrich P. Mechanisms in eukaryotic mismatch repair. *J Biol Chem.* 2006;281:30305-30309.
43. Hunter A, Spechler PA, Cwanger A, et al. DNA methylation is associated with altered gene expression in AMD. *Invest Ophthalmol Vis Sci.* 2012;53:2089-2105.
44. Kanaya T, Kyo S, Maida Y, et al. Frequent hypermethylation of MLH1 promoter in normal endometrium of patients with endometrial cancers. *Oncogene.* 2003;22:2352-2360.
45. Branham MT, Pellicer M, Campoy E, Palma M, Correa A, Roque M. Epigenetic alterations in a gastric leiomyoma. *Case Report Gastro Med.* 2014;2014:371638.
46. Holliday R, Grigg GW. DNA methylation and mutation. *Mutat Res.* 1993;285:61-67.
47. Poulos RC, Olivier J, Wong JWH. The interaction between cytosine methylation and processes of DNA replication and repair shape the mutational landscape of cancer genomes. *Nucl Acid Res.* 2017;45:7786-7795.
48. Scarpulla RC. Nucleus-encoded regulators of mitochondrial function: integration of respiratory chain expression, nutrient sensing and metabolic stress. *Biochim Biophys Acta.* 2012;1819:1088-1097.
49. Ehrlich M, Norris KF, Wang RY, Kuo KC, Gehrke CW. DNA cytosine methylation and heat-induced deamination. *Biosci Rep.* 1986;6:387-393.