Diabetic retinopathy is the leading cause of vision loss in young adults, and despite ongoing research in the field, its etiology remains elusive. Animal models have clearly documented that apoptosis of retinal cells, including vascular and nonvascular cells, proceeds the development of histopathology characteristic of diabetic retinopathy,1–3 and mitochondrial damage is implicated in the accelerated apoptosis of capillary cells.4,5 Our previous work has shown that diabetes activates matrix metalloproteinase-9 (MMP-9), and MMP-9 via damaging retinal mitochondria, activates capillary cell apoptosis. MMP-9 promoter has binding sites for many transcription factors, and in diabetes its promoter undergoes epigenetic modifications, including histone modifications and DNA methylation. Enhancer of Zeste homolog 2 (Ezh2), which catalyzes dimethylation/trimethylation of histone 3 lysine 27 (H3K27me2 and me3), is also associated with DNA methylation. Our aim was to investigate link(s) between histone and DNA modifications in the regulation of MMP-9.

METHODS. Using human retinal endothelial cells, and also retinal microvessels from diabetic rats, effect of hyperglycemia on H3K27me3, and recruitment of Ezh2 at the MMP-9 promoter were quantified by chromatin-immunoprecipitation technique. Role of H3K27 trimethylation in regulating DNA methylation-transcription of MMP-9 was determined by regulating Ezh2 by its specific siRNA and also a pharmacologic inhibitor.

RESULTS. Hyperglycemia elevated H3K27me3 levels and the recruitment of Ezh2 at the MMP-9 promoter, and increased the enzyme activity of Ezh2. Inhibition of Ezh2 attenuated recruitment of both DNA methylating (Dnmt1) and hydroxymethylating (Tet2) enzymes and 5 hydroxymethyl cytosine at the same region of the MMP-9 promoter, and prevented increase in MMP-9 transcription and mitochondrial damage.

CONCLUSIONS. Activation of Ezh2 in diabetes, via trimethylation of H3K27, facilitates recruitment of the enzymes responsible for regulation of DNA methylation at the MMP-9 promoter, resulting in its transcriptional activation. Thus, a close crosstalk between H3K27 trimethylation and DNA methylation in diabetes plays a critical role in the maintenance of cellular epigenetic integrity of MMP-9.

Keywords: diabetic retinopathy, DNA methylation, epigenetics, histone methylation, matrix metalloproteinase-9
translocase 2 (Tet2), at the same site of the promoter, and increase in 5 hydroxymethyl cytosine (5hmC), in turn, activates MMP-9 transcription.20 However, the crosstalk between histone methylation and DNA methylation in the regulation of retinal MMP-9 in diabetes remains to be investigated.

This study aims to investigate link(s) between histone and DNA modifications in the regulation of MMP-9 expression in diabetes. Using both in vitro (human retinal endothelial cells; HRECs) and in vivo (retinal microvessels from diabetic rats) models of diabetic retinopathy, and retinal microvessels from human donors with documented diabetic retinopathy, we have investigated the effect of hyperglycemia on H3K27me3 and Ezh2 recruitment at the AP-1 region of the MMP-9 promoter. A crosstalk between H3K27 methylation and DNA methylation of MMP-9 promoter is investigated by pharmacologic and molecular regulation of Ezh2.

**METHODS**

HRECs, purchased from Cell Systems Corporation (Kirkland, WA, USA), were cultured in Dulbecco’s modified Eagle medium (DMEM)-F12 containing 10% heat-inactivated fetal bovine serum, endothelial cell growth supplement (15 g/mL), insulin transferrin selenium (1%), Glutamax (1%), and antibiotic/antimycotic (1%) in an environment of 95% O2 and 5% CO2 as described previously.21 Cells from the fifth to seventh passage were used. As reported previously, HRECs were either crosslinked with 1% paraformaldehyde for 30 minutes of diabetes, the rats were killed by CO2 asphyxiation and the retina was collected immediately.20 Age-matched normal rats served as their controls. The treatment of the animals was in accordance with the guidelines of the ARVO Resolution on the Use of Animals in Research.

Human donor retinas, isolated from eye globes obtained from donors between 54 and 76 years of age (supplied by the Eversight, Ann Arbor, MI, USA) with 10 to 43 years of diabetes and documented retinopathy, and their age-matched nondiabetic donors (Table 1), were used to isolate microvessels.

Retinal microvessels were prepared by osmotic shock method by incubating the retina (rats/human) in distilled water for 1 hour at 37°C with gentle shaking. The microvessels were isolated under microscope with repeated inspiration and ejection using Pasteur pipette. As reported previously, these microvessel preparations are largely devoid of nonvascular components.22,23 The microvessels rinsed with sterile PBS were either crosslinked with 1% paraformaldehyde for chromatin immunoprecipitation (ChIP), or used for RNA isolation with Trizol reagent.

ChIP was performed in the cross-linked microvessels, sonicated in ChIP lysis buffer. Protein-DNA complex (100 µg) was immunoprecipitated with either H3K27me3 or Ezh2 or Dnmt1 or Tet2 antibody (ab60002, ab191080, ab13537, and ab135087, respectively; Abcam, Cambridge, MA, USA). IgG (ab171870) was used as an antibody control. The antibody chromatin complex was precipitated using Protein A Agarose/Salmon Sperm DNA (EMD Millipore), washed and de-crosslinked at 65°C for 6 hours followed by DNA isolation with phenol:chloroform:isoamylalcohol using the methods reported previously.3,11 The DNA was resuspended in water and relative abundance of methylated H3K27 and enzyme binding at MMP-9 promoter was quantified by real-time quantitative (q)PCR using primers specific for MMP-9 promoter proximal AP-1 binding site (Table 2). The target values were normalized to input controls, respectively, to obtain fold change. The specificity of the assay was validated by resolving the PCR products in 2% agarose gel.

**Table 1. Human Donors**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Duration of Diabetes, y</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  71</td>
<td>-</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>2  34</td>
<td>-</td>
<td>Hydrocephalus with edema</td>
</tr>
<tr>
<td>3  70</td>
<td>-</td>
<td>Subarachnoid bleed</td>
</tr>
<tr>
<td>4  75</td>
<td>-</td>
<td>Subarachnoid hemorrhage</td>
</tr>
<tr>
<td>5  74</td>
<td>-</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>6  75</td>
<td>-</td>
<td>Acute renal failure</td>
</tr>
<tr>
<td>7  64</td>
<td>-</td>
<td>Pulmonary fibrosis</td>
</tr>
<tr>
<td>8  65</td>
<td>-</td>
<td>Acute myocardial infarction</td>
</tr>
</tbody>
</table>

**Table 2. Primer Sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 promoter</td>
<td>GAGTCAGCAGCTTTCCGTGTA</td>
</tr>
<tr>
<td>(−8 to −9)</td>
<td>CTGGTGTGTTGGGCGTTTTA</td>
</tr>
<tr>
<td>Ezh2</td>
<td>ACATCCCTTTTCTATGGAACACC</td>
</tr>
<tr>
<td>MMP-9</td>
<td>TGAGTGGGCTTATATTCCGG</td>
</tr>
<tr>
<td>CytB</td>
<td>GCCACTTGCCGTTGCCGATAGGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGCTCTGGCCCTTGCGGTGCCGTCGG</td>
</tr>
<tr>
<td>MMP-9 promoter</td>
<td>GAGCTTGGGCAAGGCGACATTAA</td>
</tr>
<tr>
<td>(−221 to −22)</td>
<td>GGTTGAGAAGCAGAAATTTGGCC</td>
</tr>
<tr>
<td>Ezh2</td>
<td>TGACTGCTCTCATACCTCTCT</td>
</tr>
<tr>
<td>β-actin</td>
<td>CTCCTATGCCAAACACAGGTGC</td>
</tr>
<tr>
<td>CD44</td>
<td>CATGCTACTCCCTTGTTGCG</td>
</tr>
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</table>
FIGURE 1. Effect of high glucose on trimethylation of H3K27 and its regulation by Ezh2. HRECs incubated in high glucose for 4 days in the presence or absence of DZNep (5 μM), or transfected with Ezh2siRNA, were analyzed for (a) H3K27me3 levels at the MMP-9 promoter by immunoprecipitating H3K27me3 in the cross-linked samples, followed by PCR using the primers for the AP-1 binding region of the MMP-9 promoter. IgG (▲) was used as an antibody control. Ct values were normalized with the values from input by delta delta Ct method. (b) Agarose gel picture showing the band intensity. Values are represented as mean ± SD from three different cell preparations; 5 and 20 mM glucose, respectively; 5 + D and 20 + D = cells in 5- and 20-mM glucose, respectively, with DZNep; 20+ESi and 20+SC = Ezh2-siRNA or scrambled RNA cells in 20-mM glucose; 5 + ESi = Ezh2-siRNA transfected cells in 5-mM glucose; Mann = 20-mM mannitol. ▲P < 0.05 compared with 5-mM glucose, #P < 0.05 compared with 20-mM glucose.

of methylated H3K27 was detected using specific antibody. The activity was represented as percentage controls.

Levels of 5hmC were quantified in sonicated DNA, which was immunoprecipitated for 5hmC using hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit (EPIGENTEK). The enriched 5hmC at the MMP-9 promoter was analyzed by qPCR using specific primers.10,20

Immunofluorescence technique was performed to confirm the effect of Ezh2 inhibition on MMP-9. HRECs grown on coverslips, exposed to 5- or 20-mM glucose, in the presence or absence of Ezh2 inhibitor DZNep, were fixed with paraformaldehyde and incubated with antibodies against MMP-9 and cytochrome oxidase IV (CoxIV, a mitochondrial marker). Secondary antibodies included a DyLight 488-labelled for MMP-9 and Texas red-conjugated for CoxIV mounting. The cells were washed and mounted with DAPI containing medium and the images were visualized using Zeiss ApoTome fluorescence microscope at ×40 magnification (Carl Zeiss, Inc., Chicago, IL, USA).

Gene expression of Ezh2, MMP-9, and cytochrome B (CytB) were quantified using specific primers using real time qPCR (Table 2). The specific products were confirmed by SYBR green single melt curve analysis. The results were normalized to the expression of the housekeeping gene β-actin, and relative fold change was calculated using delta delta Ct method.11,24

MMP-9 activity was quantified by fluorescence kit (Sensolyte Plus 520 MMP-9 Assay Kit; AnaSpec, Inc., Fremont, CA, USA) using approximately 30-μg protein. Cleavage of the fluorogenic peptide, induced by MMP-9, was measured at 490-nm excitation and 520-nm emission wavelengths.25

Cell apoptosis was determined in 20 μg of the cytoplasmic protein by the Cell Death Detection ELISAPLUS kit from Roche Diagnostics (Indianapolis, IN, USA) using monoclonal antibodies against DNA and histones, and peroxidase conjugated anti-DNA and biotin-labeled antihistone.9

Statistical Analysis

Data are presented as mean ± SD. Comparison between groups were made using one-way ANOVA followed by Dunn’s t-test and a P value less than 0.05 was considered significant.

RESULTS

Histone modification is a complex process and many repressive and activating modifications can regulate the expression of a gene.26 The effect of high glucose on methylation of H3K27 at the MMP-9 promoter was investigated by ChIP technique. High glucose increased methylation of H3K27, the levels of H3K27me3 were increased by approximately 4-fold in the AP-1 region of the MMP-9 promoter. At the same site, IgG control values were less than 1% of the values obtained from cell precipitated with H3K27me3 antibodies (Fig. 1a), accompanying Figure 1b shows the band intensity on a 2% agarose gel. However, addition of 20-mM mannitol, instead of 20-mM glucose, had no effect on H3K27 methylation.

Because Ezh2 specifically methylates H3K27,16 the effect of high glucose on Ezh2 was investigated. As shown in Figure 2a, its mRNA levels were elevated by approximately 2-fold in HRECs incubated in high glucose compared with cells in normal glucose. Increase in Ezh2 expression was accompanied by approximately 45% increase in its enzyme activity (Fig. 2b).

To investigate the role of Ezh2 in regulation of histone methylation, its binding at the MMP-9 promoter was determined, and as shown in Figure 3, high glucose increased Ezh2 recruitment by over 4-fold. The role of Ezh2 in regulation of
MMP-9 was confirmed using both pharmacologic (DZNep) and molecular (siRNA) inhibitors of Ezh2; addition of DZNep in high-glucose medium attenuated increase in H3K27me3 levels and Ezh2 recruitment at the MMP-9 promoter (Figs. 1, 3), however, inclusion of DZNep in cells incubated in 5-mM glucose had no effect on H3K27me3 levels and Ezh2 binding. In the same cells, DZNep also ameliorated glucose-induced increase in MMP-9 expression, the values in high glucose þ DZNep cells were reduced by approximately 2-fold compared with the cells in high glucose alone, but they remained significantly higher compared with the cells in normal glucose. Consistent with this, although MMP-9 expression in 5-mM glucose þ DZNep and 20-mM glucose þ DZNep were not different from each other, cells incubated in 5-mM glucose þ DZNep had an approximately 1.8-fold increase in MMP-9 compared with cells in 5-mM glucose alone (Fig. 4). However, transfection of cells with Ezh2-siRNA, but not with scrambled RNA, attenuated glucose-induced increase in H3K27me3 levels and Ezh2 recruitment at the MMP-9 promoter (Figs. 1, 2), and ameliorated increase in both MMP-9 expression and activity (Figs. 4a, 4b). Consistent with our previous results showing increased mitochondrial levels of MMP-9 in hyperglycemia, as shown in Figure 4c, Ezh2 inhibition also ameliorated glucose-induced increase in mitochondrial accumulation of MMP-9.

Ezh2, in addition to methylating H3K27, can also regulate DNA methylation by allosterically binding with Dnmt1. Among the DNA methylating-hydroxymethylating family of enzymes, our previous work has shown increased mRNA levels of Dnmt1 and Tet2 in the retinal capillary cells in diabetes, and a dynamic DNA methylation-hydroxymethylation is implicated in the regulation of MMP-9 transcription. Consistent with our previous results, despite increased Dnmt1 recruitment at the MMP-9 promoter, 5hmC levels and Tet2 binding were increased by 2.5-4 fold, however, regulation of Ezh2 by DZNep or its siRNA attenuated glucose-induced increase in the recruitment of both Dnmt1 and Tet2 at the promoter, and also ameliorated increase in 5hmC levels (Figs. 5a–d), suggesting a crosstalk between H3K27me3 and dynamic DNA methylation.

Because glucose-induced increase in MMP-9 is implicated in mitochondrial damage, the effect of regulation of Ezh2 on mitochondrial damage was investigated, and as shown in Figure 6a, glucose-induced decrease in CytoB was ameliorated by inhibition of Ezh2 by DZNep or its siRNA. Consistent with the amelioration of mitochondrial damage, Ezh2 inhibition also protected glucose-induced increase in capillary cell apoptosis (Fig. 6b). The values obtained from cells incubated in the presence of DZNep, or cells transfected with Ezh2-siRNA, and exposed to 20-mM glucose were not significantly different from those obtained from cells in 5-mM glucose.

To confirm the results in an in vivo model, retinal microvessels from rats diabetic for 2 months were analyzed. As with HRECs, diabetes increased H3K27me3 levels at the MMP-9 promoter by approximately 4-fold, and this was accompanied by an increase in Ezh2 recruitment at the same site of the promoter (Fig. 7a); accompanying gel picture (Fig. 7b) represents the band intensity on a 2% agarose gel. Figure 7c shows significant increase in Ezh2 mRNA in the same microvessel preparation.
donors (Fig. 8a). Figure 8b is included to show the band intensity. In the same donors with documented diabetic retinopathy, recruitment of Dnmt1 and Tet2 and 5hmC levels were also significantly higher and MMP-9 expression was elevated by approximately 4-fold (Figs. 8c, 8d).

**DISCUSSION**

In diabetes, activation of gelatinase MMPs (MMP-2 and MMP-9) in the retina is an early event, and activated MMPs, by damaging the mitochondria, activate the apoptotic machinery, culminating in the loss of capillary cells, a phenomenon seen before histopathology characteristic of diabetic retinopathy can be observed.25,26 Regulation of MMP activation is mediated via many different mechanisms including regulation of their tissue inhibitors, and their gene expressions by transcriptional factors and epigenetic modifications. Both, histone and DNA modifications in the MMP-9 promoter region, initiated by the diabetic milieu, are shown to regulate its activation in the retina.11,20 Here, we report that there is a crosstalk between histone modification and DNA methylation in regulating MMP-9 transcription. While the levels of histone repressor mark H3K27me3 are elevated and the enzyme methylating H3K27, Ezh2, is activated, due to increased recruitment of Dnmt1 by Ezh2 at the transcriptional factor binding site, the dynamic DNA methylation of MMP-9 promoter is initiated. The levels of 5hmC are increased at the promoter, and this ultimately results in the transcriptional activation of MMP-9.

Histone methylation and DNA methylation are considered to be tightly coordinated; methylation of lysine can initiate, target, or maintain DNA methylation, and this is true vice versa as well.29,30 Our recent work has shown that the methylation status of MMP-9 promoter is altered in diabetes, despite increased recruitment of Dnmt1 at the MMP-9 promoter, 5mC levels are decreased. However, due to simultaneous activation of the hydroxymethlating Tet2 of the Tet family, the MMP-9 promoter remains hypomethylated, suggesting an active cytosine methylation-demethylation process.20 Ezh2 can enhance DNA methylation by recruiting Dnmt1 at the promoter of a gene18; and the results presented here suggest a clear crosstalk between Ezh2 and DNA methylation. We show that the hyperglycemic environment favors binding of Ezh2 at the MMP-9 promoter, which facilitates the recruitments of Dnmt1 and Tet2, ultimately, leaving the promoter hydroxymethylated, and activating MMP-9 transcription. In support, recruitment of Dnmt1 by Ezh2 at the ABCA1 promoter is shown to transcriptionally silence ABCA1 expression, and accelerate progression of atherosclerosis.31

Unlike acetylation, which is generally associated with gene activation, methylation of histones can either activate or inhibit gene transcription depending upon the site of methylation, and the degree of methylation.32 Among different histone modification scenarios, modifications of lysine 4, 9, and 27 of histone 3 are considered to be the most important histone modifications in influencing gene expression.33,34 In diabetes, H3K9me2 levels are decreased at the retinal MMP-9 promoter...
Ezh2. In support, others have shown increased H3K27me3 and activates regulation of VEGF.\(^7\) However, high glucose–exposed human HRECs exposed to high glucose suggesting its role in maintenance of the integrity of the developing vasculature.\(^6\)

Values are represented as mean ± SD obtained from five to seven rats in each group. Norm and Diab = normal and diabetic rats, respectively. *\(P < 0.05\) compared with normal.

In the pathogenesis of diabetic retinopathy, MMP-9 promoter, in addition to histone modifications, also undergoes DNA methylation-hydroxymethylation.\(^{11,20,39}\) Although DNA methylation and histone modification are mediated by different sets of enzymes, these epigenetic modifications appear to be biologically interrelated, and the relationship can work in either direction. For example, histone methylation can direct DNA methylation patterns, and DNA methylation can serve as a template for some histone modifications after DNA replication.\(^{40}\) H3K27me3 itself is a repressive mark associated with gene repression,\(^{41}\) but Ezh2 can also control DNA methylation directly by regulating Dnmts.\(^{32,43}\) Here, we show that despite increased H3K27me3 at the MMP-9 promoter, its transcription is increased in diabetes. Regulation of Ezh2, along with inhibiting the binding of Dnmt1, also attenuates concomitant recruitment of Tet2 and 5hmC levels, which ultimately results in repression of MMP-9 transcription. In support, overexpression of Ezh2 is shown to increase recruitment of Dnmts at the TIMP2 promoter in ovarian cancer.\(^{44}\)

Consistent with the results obtained from retinal endothelial cells in culture, retinal microvessels from diabetic rats also have increased MMP-9 transcription.\(^{6,7,11}\) and we show that MMP-9 promoter has increased H3K27me3 and Ezh2 recruitment, confirming similar phenomenon in animal model of diabetic retinopathy. In addition, transitioning to the human disease, our exciting results show that similar increase in H3K27me3 and Ezh2 is also observed in the retinal microvessels from human donors with diabetic retinopathy. This is accompanied by increased recruitment of both Dnmt1 and Tet2, and elevated levels of 5hmC, keeping MMP-9 transcriptionally active. These results further confirm the role of Ezh2-H3K27me3-DNA methylation in the development of diabetic retinopathy.

Our study was focused on crosstalk between Ezh2-H3K27me3 and DNA methylation-demethylation of the MMP-9 promoter, however, we acknowledge the role of demethyl-
lases in regulating H3K27me3 levels. In addition, similar crosstalk between histone methylation-DNA methylation of the intracellular inhibitor of MMP-9, TIMP1, in regulating MMP-9 activity also cannot be ruled out; Ezh2-mediated transcriptional repression of TIMPs is considered to be one of the major mechanisms shifting the MMPs-TIMPs balance and MMPs activation in invasive prostate cancer, and as mentioned above, in ovarian cancer, overexpression of Ezh2 increases recruitment of Dnmts at the TIMP2 promoter.

In summary, using experimental models of diabetic retinopathy, and confirming results in the retinal microvessels from human donors with diabetic retinopathy, this study provides strong evidence of a crosstalk between histone and DNA modifications in the regulation of MMP-9 expression in diabetes. The results show that the recruitment of Ezh2 is increased at the MMP-9 promoter in diabetes. This facilitates the recruitment of Dnmt1/Tet2, ultimately resulting in transcriptional activation of MMP-9, and regulation of Ezh2 protects DNA methylation, attenuating MMP-9 transcription. Thus, in the pathogenies of diabetic retinopathy, MMP-9 has a major role in damaging the mitochondria and accelerating the apoptotic machinery, and Ezh2 appears to be critical in maintenance of cellular epigenetic integrity by regulating both histone modifications and DNA methylation.

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References


