Epigenetics and Regulation of Oxidative Stress in Diabetic Retinopathy

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PURPOSE. Oxidative stress plays a central role in the development of diabetic retinopathy, and in the pathogenesis of this blinding disease, activation of NADPH oxidase 2 (Nox2)-mediated cytosolic reactive oxygen species (ROS) production precedes mitochondrial damage. The multicomponent cytosolic Nox2 has an obligatory component, Ras-related C3 botulinum toxin substrate 1 (Rac1); in diabetes, Rac1 is functionally and transcriptionally active. Diabetes also facilitates many epigenetic modifications, and activates both DNA methylating (Dnmts) and hydroxymethylating (Tets) enzymes. Our aim was to investigate the role of epigenetics in Rac1 regulation in diabetes.

METHODS. Using human retinal endothelial cells, exposed to high glucose, 5-methyl cytosine (5mC) and 5-hydroxy methyl cytosine (5hmC) levels, and binding of Dnmt and Tets were quantified at the Rac1 promoter. The effect of inhibition of Dnmts/Tets (pharmacological inhibitors or short interfering RNA [siRNA]) on glucose-induced activation of Rac1-ROS production was evaluated. Results were confirmed in retinal microvessels from streptozocin-induced diabetic mice receiving intravitreally Dnmt1-siRNA.

RESULTS. Despite high glucose-induced increased binding of Dnmt1, 5mC levels remained subnormal at Rac1 promoter. But, at the same site, 5hmC levels and transcription factor nuclear factor (NF)-κB binding were increased. Inhibition of Dnmts/Tets prevented increase in 5hmC and NF-κB binding, and attenuated Rac1 activation. Similarly, in mouse retinal microvessels, Dnmt1-siRNA ameliorated diabetes-induced increase in Rac1 transcripts and activity, and decreased ROS levels.

CONCLUSIONS. Thus, despite Dnmts activation, concomitant increase in Tets rapidly hydroxymethylates 5mC, allowing NF-κB to bind and activate Rac1. These results imply a critical role of an active DNA methylation in cytosolic ROS regulation in the development of diabetic retinopathy.

Keywords: diabetic retinopathy, DNA methylation, epigenetics, oxidative stress, ras-related C3 botulinum toxin substrate 1 (Rac1)

Diabetic retinopathy, a slow progressing complication, is the leading cause of blindness in working age adults. The pathology of the disease is complex and involves many interrelated metabolic abnormalities initiated by persistent high circulating glucose.² In the cytosol, ROS are mainly generated by NADPH oxides (Nox), and among this family of enzymes, Nox2 is one of the isomers activated in the retina in diabetes.³ Nox2 is a multicomponent enzyme, and for its stabilization, a small molecular weight GTPase, Ras-related C3 botulinum toxin substrate 1 (Rac1),⁴ plays an important role. Experimental models have shown that the activation of Rac1-Nox2-ROS precedes mitochondrial damage, and the development of diabetic retinopathy.³

Functional activation of Rac1 requires specific guanine exchange factors, including Tiam1 and Sos1, and in diabetes, Tiam1-mediated activation of Rac1 is implicated in retinal Nox2-ROS production.²,⁵ Hyperglycemic milieu also increases Rac1 transcription, and our recent work has shown that hyperglycemia promotes this transcriptional activation by increasing the binding of nuclear transcriptional factor-κB (NF-κB, p65 subunit) at the Rac1 promoter.⁶ However, the putative mechanism underlying increased transcription factor binding remains to be explored.

Epigenetic modifications, the modifications that can alter gene expression without affecting the DNA sequence, play an important role in regulating the binding of the transcription factors in many disease conditions.⁷ Many epigenetic modifications, including DNA methylation and histone acetylation, are shown to play a role in the development of diabetic complications, including retinopathy.⁸⁻¹¹ Addition of a methyl group to the cytosine by DNA methyl transferases (Dnmts) forms 5-methylcytosine (5mC), and methylated cytosine impedes transcriptional factor binding, resulting in gene repression. However, 5mC can be rapidly oxidized by Ten-Eleven Translocation dioxygenases (Tets), and the resulting formation of 5-hydroxymethylcytosine (5hmC) stimulates gene expres-
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In diabetes, both Dnmts and Tets are activated in the retina and its vasculature, and among these two families of enzymes, Dnmt1 and Tet2 are the only respective isoforms that are upregulated. How these opposing enzymes regulate DNA methylation status of Rac1 is not clear.

The aim of this study was to investigate the role of epigenetic modifications in the transcriptional regulation of Rac1. Using human retinal endothelial cells (HRECs), we investigated the effect of high glucose on DNA methylation status of the Rac1 promoter. The role of DNA methylation in Rac1 transcriptional activation was further established using pharmacological inhibitors and specific small interfering RNAs (siRNAs) of Dnmt1 and Tet2. Key parameters were confirmed in an in vivo model using retinal microvessels from streptozotocin-induced diabetic mice, receiving intravitreal administration of Dnmt1-siRNA or negative control siRNA.

Methods

Retinal Endothelial Cells

HRECs, obtained from Cell Systems Corporation (catalog no. AGBRI 181; Cell Systems Corp., Kirkland, WA, USA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-F12 containing 10% heat-inactivated fetal bovine serum, endothelial cell growth supplement (15 μg/mL), insulin transferrin selenium (1%), Glutamax (1%) and antibiotic/antimycotic (1%) in an environment of 95% O2 and 5% CO2, as described previously. Cells from the sixth to eighth passage were incubated in 5-mM or 20-mM D-glucose (glucose) for 6 to 96 hours in the presence or absence of inhibitors of Dnmts (5-deoxy-2-azacytidine, 5-Aza, 1 μM; catalog no. A3650; Sigma-Aldrich Corp., St. Louis, MO, USA) or Tets (2-hydroxyglutarate, 2-HG, 500 μM; catalog no. 16574; Cayman, Ann Arbor, MI, USA). The role of p65 in Rac1 transcriptional activation was confirmed in the cells incubated with a cell-permeable inhibitor of NF-κ B activation, SN 50 (50 μg/mL; Biomol Research Laboratories, Plymouth Meeting, PA, USA). A group of cells was transfected with a single siRNA pool of Dnmt1 or Tet2 (Cat. Nos. sc-35204 and sc-88954, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using siRNA transfection reagent (catalog no. sc-29528; Santa Cruz Biotechnology), and the transfected cells were incubated in 5-mM or 20-mM glucose for 96 hours. The transfection efficiency was evaluated by quantifying mRNA levels of the respective genes (Dnmt1 or Tet2) by SYBR green-based real-time quantitative reverse transcription PCR (qRT-PCR) and by protein expression (western blotting), and it was ~40% for Dnmt1-siRNA and >50% for Tet2-siRNA. Untransfected cells and cells transfected with Dnmt1-siRNA or Tet2-siRNA had similar Dnmt3α or Tet4 expression, confirming the specificity of the Dnmt1- and Tet2-siRNA. (Fig. 1). Parallel controls included HRECs incubated in 20-mM L-glucose (osmotic/metabolic), and cells transfected with scrambled RNA (transfection).

Mice

Seven- to 8-week-old C57BL/6J mice (either sex) were made diabetic by streptozotocin injection (55 mg/kg body weight for 4 consecutive days). Mice with blood glucose more than 250 mg/dL 2 days after the last injection were considered diabetic. Soon after establishment of diabetes, a group of diabetic mice received intravitreal administration of 2-μg Dnmt1-siRNA (ID: MSS203624; Thermo Fisher Scientific, Waltham, MA, USA); this stealth Dnmt1-siRNA is a combination of sequences targeting three positions at exon 4. Dnmt1-siRNA was suspended in 2 μL nuclease-free water and mixed with Invivofectamine (catalog no. IVF 3001; Invitrogen, Carlsbad, CA, USA). The right eye received Dnmt1-siRNA and the left eye received a medium GC content negative control siRNA (catalog no. 12935-300, Thermo Fisher Scientific). Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100 mg/kg ketanest and 12 mg/kg xylazine), and using a 32-gauge needle attached to a 5-μL glass syringe (Hamilton), intravitreal injections were performed under a dissecting microscope. Care was taken to position the needle 1 mm posterior to the limbus, and siRNA mixture was slowly injected into the vitreous chamber. Four weeks after administration of siRNA, the mice were killed, and the retina was quickly isolated. Age-matched normal mice and diabetic mice, without receiving any siRNA, served as controls. 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.

Freshly isolated retina was used to prepare microvessels by incubating it in 5-mL de-ionized water for 60 minutes in a shaking water bath at 37°C. The nonvascular tissue was gently removed under the microscope.

Quantification of 5mC and 5hmC

Genomic DNA was isolated using Qiagen DNA isolation kit (Qiagen, Valencia, CA, USA), and was immunoprecipitated with 5mC or 5hmC antibodies. The levels of 5mC and 5hmC were quantified using methylated or hydroxymethylated DNA Immunoprecipitation (MeDIP)/(hMeDIP) kits, respectively (Cat. Nos. P-1015 and P-1038, respectively; EPIGENTEK, Farmingdale, NY, USA). The enrichment of 5mC or 5hmC at the Rac1 promoter was analyzed by qRT-PCR using specific primers.

Chromatin Immunoprecipitation (ChIP)

The binding of DNA modifying enzymes, or of the transcription factor NF-κB (p65 subunit), at the Rac1 promoter was determined by ChIP assay using cross-linked samples sonicated in ChIP lysis buffer. Protein-DNA complex (100 μg) was immunoprecipitated with Dnmt1, Tet2, or p65 antibody (Cat. Nos. ab13537, ab135087, and ab7970, respectively; Abcam, Cambridge, MA, USA). Each experiment included antibody control, where the samples were immunoprecipitated with IgG (catalog no. ab171870, Abcam). The immunoprecipitated complex was captured using Protein A Agarose/Salmon Sperm DNA (catalog no. 16157; EMD Millipore, Temecula, CA, USA), washed and de-crosslinked at 65°C for 6 hours, followed by DNA isolation with phenol:chloroform:isoamylalcohol, using the methods reported previously. Relative binding of Dnmt1/Tet2/p65 at the promoter was quantified by qRT-PCR using Rac1 promoter specific primer (Table). The target values were normalized to the input controls respectively to obtain fold change.

Gene Transcripts

Gene expression was quantified using gene/species-specific primers by qRT-PCR (Table), and 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 (human) or 18S (mice), and the relative fold change was calculated using delta delta Ct method.

Rac1 Activation

Active Rac1 was measured by G-LISA colorimetric assay kit (catalog no. BK-128; Cytoskeleton, Denver, CO, USA) in 20- to 25-μg protein, as described previously. Fold change was calculated considering the values obtained from cells in normal glucose as one.
**Reactive Oxygen Species**

Total ROS levels were quantified using 2',7'-dichlorofluorescein diacetate (DCFH-DA; catalog no. D6883; Sigma-Aldrich Corp.). Briefly, 5-µg protein was incubated with 4-µM DCFH-DA for 20 minutes, and the fluorescence was measured at 485-nm excitation wavelength and 535-nm emission wavelengths. Fold change was calculated as percent change with respect to values obtained from cells in normal glucose.

**Statistical Analysis**

Data are presented as mean ± SD. Comparison between groups was made using 1-way ANOVA followed by Dunn’s t-test; $P < 0.05$ was considered significant.

**RESULTS**

Rac1 is activated in hyperglycemic milieu in the retina and its vasculature and its gene transcripts are increased, to investigate the role of DNA methylation in Rac1 transcriptional regulation, 5mC levels at the Rac1 promoter were quantified in the cells exposed to high glucose for 96 hours, a duration when cell apoptosis is detected. Figure 1a shows that high glucose decreased 5mC levels by 50%. Because 5mC is associated with transcriptional repression, and hyperglycemic milieu activates both Dnmts and Tets, the effect of high glucose on hydroxymethylation of 5mC was investigated; compared with cells in normal glucose, cells incubated in high glucose had 2-fold higher 5hmC levels at Rac1 promoter (Fig. 1b).
To investigate the mechanism responsible for decrease in 5mC at Rac1 promoter, despite activation of Dnmts, in hyperglycemic milieu, the binding of Dnmt1 was investigated. Compared with the cells in normal glucose, high glucose exposure for 96 hours increased Dnmt1 binding by more than 1.5-fold, and in the same samples, Tet2 binding at Rac1 promoter was also elevated by more than 2-fold (Figs. 2a, 2b).

To confirm the role of active DNA methylation-hydroxymethylation in the regulation of Rac1 promoter methylation status, the effect of inhibition of Dnmt by its chemical (5-Aza) or molecular (Dnmt1-siRNA) inhibitors was analyzed; both 5-Aza and Dnmt1-siRNA prevented glucose-induced increase in Dnmt1 binding at Rac1 promoter (Fig. 2a). However, these inhibitors had no effect on Dnmt1 binding in the normal glucose conditions. To further understand the role of 5mC hydroxymethylation in Rac1 transcriptional regulation, the effect of Tet regulation on its binding at Rac1 promoter was investigated. Figure 2b shows that Tet2 inhibition either by 2-HG or Tet2-siRNA significantly attenuated glucose-induced increase in Tet2 binding. In the same cell preparations, inhibition of Dnmts or Tets also ameliorated glucose-induced increase in 5hmC levels at Rac1 promoter (Fig. 1b). Untransfected cells or scrambled RNA transfected cells, incubated in high glucose for 96 hours, had similar values, and also values from cells in 20-mM L-glucose or in normal glucose were not different from each other, confirming the use of 20-mM L-glucose as a valid osmotic/metabolic control.

Inhibition of DNA methylation-hydroxymethylation machinery also attenuated glucose-induced increase in p65 binding at Rac1 promoter, and the values obtained from cells in high glucose with Dnmt or Tet inhibitors were not significantly different from the cells in normal glucose (Fig. 3a). Consistent with the p65 binding, these inhibitors ameliorated glucose-induced increase in Rac1 gene transcripts and activity, and ROS levels (Figs. 3b–d). The role of NF-κB in Rac1 transcriptional activation was confirmed by inhibiting glucose-induced NF-κB activation by SN50, and as shown in Figure 3b, SN50 ameliorated glucose-induced increase in Rac1 transcription.

Rac1-Nox2-ROS signaling is one of the early events in the pathogenesis of diabetic retinopathy; in retinal endothelial cells it can be seen within 6 hours of high glucose insult. To investigate if DNA-methylation-hydroxymethylation is playing any role in the early activation of Rac1, DNA methylation status of Rac1 promoter was analyzed in HRECs exposed to high glucose for 6 to 48 hours. Compared with cells in normal glucose, while at 6 hours of high glucose both 5mC levels and Dnmt1 binding were decreased, at 24 hours and beyond, despite increased Dnmt1 binding, 5mC levels remained subnormal (Fig. 4a). Consistent with this, although Dnmt1 inhibitors had no effect on Dnmt1 binding in the normal glucose conditions. To further understand the role of 5mC hydroxymethylation in Rac1 transcriptional regulation, the effect of Tet regulation on its binding at Rac1 promoter was investigated. Figure 2b shows that Tet2 inhibition either by 2-HG or Tet2-siRNA significantly attenuated glucose-induced increase in Tet2 binding. In the same cell preparations, inhibition of Dnmts or Tets also ameliorated glucose-induced increase in 5hmC levels at Rac1 promoter (Fig. 1b). Untransfected cells or scrambled RNA transfected cells, incubated in high glucose for 96 hours, had similar values, and also values from cells in 20-mM L-glucose or in normal glucose were not different from each other, confirming the use of 20-mM L-glucose as a valid osmotic/metabolic control.

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transcripts were elevated by approximately 2-fold at 24 hours (and beyond) of exposure of high glucose, at 6 hours Dnmt1 expression was significantly decreased (Fig. 4b). In the same samples, 5hmC levels at Rac1 promoter were, however, not affected, but within 24 hours, >50% increase in 5hmC was observed. In accordance, 6 hours of high glucose also failed to affect Tet2 binding at Rac1 promoter, and the gene transcripts of Tet2 were also not different from those obtained from cells in normal glucose. But, at 24 hours (and beyond) of high glucose incubation, 5hmC levels, Tet2 binding, and its gene transcripts were significantly elevated, and they remained elevated at 48 to 96 hours of high glucose exposure (Figs. 4c, 4d). Cells in high glucose for 6 hours and beyond, as expected, had an approximately 2-fold increase in Rac1 gene transcripts (Fig. 4e). Values obtained from cells in 20-mM L-glucose were not different from those obtained from cells in 5-mM glucose.

To confirm the key parameters in an in vivo model, DNA methylation status of the Rac1 promoter was investigated in retinal microvessels obtained from diabetic mice. Compared with normal mice, although diabetic mice had decreased 5mC levels at the Rac1 promoter, 5hmC levels were significantly elevated (Figs. 5a, 5b). The role of DNA methylation in Rac1 transcriptional regulation was further confirmed in the mice receiving intravitreal administration of Dnmt1-siRNA. Although due to some technical issues, Dnmt1 silencing could not be
confirmed in the target cells, and off-target effects of the
Dnmt1-siRNA could not be investigated, administration of
siRNA ameliorated diabetes-induced increase in gene tran-
scripts and activity of Rac1 and ROS levels; the values obtained
from the retinal microvessels prepared from the diabetic
mouse eye receiving Dnmt1-siRNA were significantly different
from those obtained from the eye receiving siRNA negative
control (Figs. 5c, 5d).

**Figure 4.** Temporal relationship between promoter DNA methylation and transcription of Rac1. Cells incubated in 5-mM or 20-mM glucose for 6 to 96 hours were analyzed for (a) 5mC levels and Dnmt1 binding and (b) 5hmC levels and Tet2 binding. Gene transcripts of (c) Dnmt1, (d) Tet2, and (e) Rac1 were measured by qRT-PCR. Values obtained from cells in 5-mM glucose are considered as 1, and are represented as mean ± SD from three different cell preparations, each measurement made in duplicate; 5 mM and 20 mM indicate 5-mM and 20-mM glucose, respectively; L-Glu indicates 20-mM L-Glucose. *P < 0.05 compared with 5-mM glucose.
DISCUSSION

Diabetic retinopathy has a complex underlying pathophysiology, and in spite of cutting edge research in the field, molecular events contributing to its development remain unclear. Among many abnormalities implicated in the development of diabetic retinopathy, experimental models have documented oxidative stress to be at a central place. Although mitochondria are the major source of ROS, ROS are also produced in the cytosol by Nox enzymes, and our previous study has shown that in the pathogenesis of diabetic retinopathy, activation of cytosolic Nox2 precedes mitochondrial damage. Rac1, an obligatory component of the multicomponent Nox2, is activated in hyperglycemic milieu in the retina and its vasculature, and its gene transcripts are elevated. The data provided in the current report suggest that the role of epigenetic modifications in Rac1 transcriptional regulation. DNA at the Rac1 promoter undergoes active methylation-hydroxymethylation; in the initial stages of hyperglycemic insult (6 hours of high glucose), due to inhibition of Dnmts, the promoter is hypomethylated. However, at 24 hours, the binding of Dnmt1 is increased, concomitant activation of Tets hydroxymethylates 5mC to 5hmC, resulting in Rac1 activation. Role of active DNA methylation-hydroxymethylation in regulation of Rac1 transcription is further confirmed by our results showing increased 5hmC levels at the Rac1 promoter in the retinal microvessels from mice diabetic for 2 weeks, and amelioration of diabetes-induced activation of Rac1 (transcription and activity) by Dnmt1-siRNA. These results have significant implications for diabetic retinopathy because, although the appearance of retinal histopathology, characteristic of diabetic retinopathy, in rodent models is visible only after 6 to 8 months of diabetes, Rac1-Nox2-ROS signaling is an early event, which can be seen within 2 weeks after induction of diabetes.

Addition of a methyl group to carbon-5 of a cytosine base, forming 5mC, is considered as a fundamental epigenetic mediator, and is an important component in many cellular processes, including genomic imprinting and chromosome stability. DNA methylation is mainly associated with repression of gene expression by changing the chromatin structure and restricting access of the transcription factors to the gene promoter. However, errors in DNA methylation are observed with many genes in chronic diseases including cancer and diabetes. Human Rac1 promoter has four CpG islands within ~1200 bases upstream of transcription start site, and the binding site for the transcription factors lies in the CpG island close to the transcription start site between ~317 to 147 bp. Our previous work has shown that in diabetes, the binding of transcriptional factor NF-κB at Rac1 promoter is increased in the retinal vasculature, and here we show that at a duration of high glucose insult when mitochondrial damage and apoptosis are also observed (96 hours), 5mC levels are also decreased. As mentioned above, Dnmts catalyze the transfer of a methylation residue from S-adenosyl-L-methionine to cytosine, and among these, Dnmt3a and Dnmt3b are the de novo methyltransferases, Dnmt1 is considered as the maintenance methyltransferase and recognizes hemimethylated sites restoring symmetric methylation during replication. In diabetes, Dnmts are activated, and among this family of enzymes, only Dnmt1 is upregulated in the retina and its vasculature. At 96 hours, despite decrease in 5mC levels, binding of Dnmt1 at Rac1 promoter is increased, suggesting that the 5mC formed by Dnmt1 could be demethylated. The results from the in vivo model further

![Figure 5](https://example.com/figure5.png)
support the role of active DNA methylation in Rac1 activation, as administration of Dnmt1-siRNA ameliorates both Rac1 transcription and activation, and decreases ROS levels, seen in the retinal microvasculature of diabetic rodents.

DNA is a dynamic structure, and the methylated cytosine can be rapidly demethylated. Demethylation of cytosine is considered necessary for epigenetic reprogramming of genes, and is directly involved in many diseases including tumor progression. Demethylation of DNA can either be passive or active, or a combination of both, and the enzyme-based oxidation of 5mC to 5hmC promotes DNA demethylation by binding to CpG-rich regions to prevent unwanted Dnmt activity. Retinal Tets are activated in diabetes, and among this family of enzymes, Tet2 is the only member with upregulated gene transcripts. The results presented here clearly show that, although Dnmt1 binding at Rac1 promoter is increased in diabetes, due to increased Tet2 binding, 5hmC levels are elevated, suggesting a rapid hydroxymethylation of 5mC formed by Dnmt1. This increase in 5hmC allows binding of the transcription factor, increasing Rac1 expression. Further, we show that inhibition of Tets, in addition to regulating DNA methylation status of Rac1 promoter, also inhibits NF-kB binding and Rac1 transcription, further confirming the role of active DNA methylation in Rac1 activation. In support, diabetes increases transcription of matrix metalloproteinase MMP-9, an enzyme implicated in mitochondrial damage, via dynamic DNA methylation-hydroxymethylation of its promoter. Moreover, hyperglycemia has a direct impact on changes in the epigenome, and high DNA methylation and hydroxymethylation levels are observed in poorly controlled diabetic patients compared with healthy individuals. We recognize that Tets can further oxidize 5hmC to 5-formylcytosine, and 5-formylcytosine to 5-carboxylcytosine; their role in transcription factor binding at their recognition site, and regulation of Rac1 transcription and activation, and decreases ROS ultimately activating the receptor; imbalance in genomic 5mC/5hmC levels are associated with aberrant gene activation and oncogenic transformation. Thus, despite increased 5mC formed at the Rac1 promoter due to activation of Dnmt1, concomitant increase in Tet2 hydroxymethylate it to 5hmC. This allows the transcription factor to bind, and activate Rac1 transcription. Rac1, via Nox2, elevates cytosolic ROS levels, and ROS damage mitochondria and increase capillary cell apoptosis, which, ultimately, results in diabetic retinopathy (Fig. 6). These data clearly imply a critical role of DNA methylation in diabetic retinopathy, and demonstrate the importance of DNA methylation/hydroxymethylation machinery in regulation of Rac1-mediated oxidative stress. This opens up the possibility of

There are some limitations of our study, including the following:

- Retinal endothelial cells are not the only cells that are lost in the early stages of diabetic retinopathy; instead, pericytes of the vasculature also undergo accelerated apoptosis before any pathology, characteristic of diabetic retinopathy, can be observed, and in diabetes, photoreceptors are also a major source of ROS. The possibility of a similar mechanism(s) operating in retinal pericytes, and in other nonvascular cells, cannot be ruled out.

- Diabetic retinopathy is a slow progressing disease, mitochondrial damage and capillary cell apoptosis precedes the development of microvascular histopathology, and the histopathology not visible until 6 to 8 months of diabetes in rodents, raising a possibility that the in vitro model might not fully reproduce the in vivo model. However, most of the mechanism-based data are comparable between the in vitro and in vivo models.

- As mentioned above, high glucose-induced increase in Rac1-ROS can be seen within 6 hours in retinal endothelial cells and within 15 days of diabetes in rodents, and increase in mitochondrial damage and capillary cell apoptosis within 96 hours in cells and approximately 6 months of diabetes in mice. However, our retinal microvessel Rac1-ROS data, presented here, are from diabetic mice receiving Dnmt1-siRNA for only 4 weeks. In support of a possible link between early changes in DNA methylation and the onset of diabetic retinopathy, our previous work has shown that inhibition of the enzymes responsible for maintaining DNA methylation ameliorates mitochondrial damage and capillary cell apoptosis, the events that precede retinal microvascular pathology in the in vivo models of diabetic retinopathy.

- Because epigenetic modifications are influenced by external factors, and in vitro culture of endothelial cells may introduce additional epigenetic modifications, this may interfere with the true recapitulation of the disease state. To alleviate the effect of cell passaging, the present study compared Rac1 promoter DNA methylation in high glucose and normal glucose conditions using HRECs from the same passages (and the same batch). Moreover, experiments in in vitro models for many chronic diseases, including diabetic retinopathy, using appropriate controls, have provided consistent results as seen in the in vivo disease models.

In summary, despite the limitations stated above, our convincing data clearly demonstrate a role of active DNA methylation-demethylation in the regulation of Rac1 in the development of diabetic retinopathy. Although DNA methylation is an epigenetic repressive mark, hydroxymethylation of 5mC is also now considered as an independent epigenetic marker; imbalance in genomic 5mC/5hmC levels are associated with aberrant gene activation and oncogenic transformation. Thus, despite increased 5mC formed at the Rac1 promoter due to activation of Dnmt1, concomitant increase in Tet2 hydroxymethylate it to 5hmC. This allows the transcription factor to bind, and activate Rac1 transcription. Rac1, via Nox2, elevates cytosolic ROS levels, and ROS damage mitochondria and increase capillary cell apoptosis, which, ultimately, results in diabetic retinopathy (Fig. 6). These data clearly imply a critical role of DNA methylation in diabetic retinopathy, and demonstrate the importance of DNA methylation/hydroxymethylation machinery in regulation of Rac1-mediated oxidative stress. This opens up the possibility of
using this machinery as a target to regulate ROS production during early stages of the disease, which could help diabetic patients from facing the devastating consequences of this progressing disease, and protect them from losing their vision.

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