Epigenetic Modifications of Keap1 Regulate Its Interaction With the Protective Factor Nrf2 in the Development of Diabetic Retinopathy

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Purpose. Diabetes induces oxidative imbalance in the retina and impairs Nrf2-mediated antioxidant response, and elevates Keap1, the cytoplasmic repressor of Nrf2. The goal of this study was to understand the role of epigenetic modifications at *Keap1* promoter in regulation of Nrf2 function.

Methods. The effect of high glucose on the binding of transcriptional factor Sp1 at *Keap1* promoter and histone methylation status of the promoter was investigated in retinal endothelial cells. Role of histone methylation was confirmed in cells transfected with siRNA of methyltransferase enzyme Set7/9 (SetD7). In vitro results were confirmed in the retina from streptozotocin-induced diabetic rats. The role of epigenetic modifications of *Keap1* promoter in the metabolic memory was examined in rats maintained in poor control for 3 months followed by good control for 3 months.

Results. Hyperglycemia increased the binding of Sp1 at *Keap1* promoter, and enriched H3K4me1 and activated SetD7. *SetD7*-siRNA prevented increase in Sp1 binding at *Keap1* promoter and *Keap1* expression, and ameliorated decrease in Nrf2-regulated antioxidant genes. Cessation of hyperglycemia failed to attenuate increased binding of Sp1 at *Keap1*, and the promoter continued to be methylated with increased expression of *Keap1* and decreased expression of Nrf2-regulated genes.

Conclusions. Epigenetic modifications at *Keap1* promoter by SetD7 facilitate its binding with Sp1, increasing its expression. Keap1 restrains Nrf2 in the cytosol, impairing its transcriptional activity. Reversal of hyperglycemia fails to provide any benefit to epigenetic modifications of *Keap1* promoter, suggesting their role in both the development of diabetic retinopathy and the metabolic memory phenomenon.

Keywords: diabetic retinopathy, epigenetic modifications, Keap1, Nrf2, metabolic memory

The diabetic environment creates an imbalance between the oxidant production and their removal in the retina and its capillary cells, and increased reactive oxygen species are implicated in the development of diabetic retinopathy.[1–4] In the pathogenesis of diabetic retinopathy, the antioxidant defense mechanism is impaired, antioxidant defense enzymes, including superoxide dismutase is decreased, intracellular antioxidant glutathione (GSH) levels become subnormal, and the transcriptional activity of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), the primary cellular defense against oxidative stress, is decreased.[5–7]

Under normal conditions, Nrf2 remains latent in the cytosol, but once activated, it migrates into the nucleus and binds to the DNA at the location of the antioxidant response element (ARE) to control the expression of cytoprotective genes.[8–11] Kelch-like ECH-associated protein 1 (Keap1), an intracellular inhibitor of Nrf2, is a substrate adaptor protein for Cullin3/Rbx1 ubiquitin ligase. Keap1 tethers Nrf2 in the cytosol by continuously targeting its degradation by cullin-3–dependent proteasome, and this degradation ability is lost under stress conditions, releasing Nrf2 to move into the nucleus.[8] Our recent work has shown that in diabetes, although the overall cellular expression of Nrf2 is increased, due to its increased binding with Keap1, Nrf2 accumulation in the nucleus is decreased. This is accompanied by decreased binding of Nrf2 with ARE4, and subnormal levels of the downstream target gene glutamate cysteine ligase (Gcl), the enzyme important in GSH biosynthesis.[7] Others have shown that Nrf2-knockout diabetic mice have decreased retinal GSH levels, and the neuronal dysfunction is exacerbated.[12] Keap1 is rich in cysteine residues, and these residues sense the electrophilic or oxidative stress.[13] The promoter of *Keap1* has a stimulating protein-1 (Sp1) element site (−160/−153), and the binding of Sp1 with *Keap1* is considered essential for its transcriptional activation (e.g., in lung cancer cells), decreased binding of Sp1 with Keap1 is associated with its suppression.[14] How diabetes affects the binding of Sp1 with *Keap1* in the retina is not clear.

In addition to transcription factors, gene expression also is regulated by epigenetic modifications, and, depending on the modifications and their locations, the gene transcription is repressed or stimulated.[15,16] We have shown that in diabetes, histones are modified at the promoter regions of the retinal genes important in regulating oxidative stress, including the gene encoding mitochondrial superoxide dismutase (*Sod2*) and GSH biosynthesis-catalytic subunit of Gcl (*Gclc*).[7,17–20] Whether

epigenetic modifications play any role in regulating retinal *Keap1* expression remains elusive.

Clinical and experimental studies have demonstrated that the progression of diabetic retinopathy does not halt after termination of hyperglycemic insult, and the disease continues to progress beyond the point when good control is achieved, suggesting a "metabolic memory" phenomenon.[21,22] Recent studies have shown the role of epigenetic modifications in the metabolic memory associated with microvascular complications of diabetes, including retinopathy and nephropathy.[7,18–20,23,24] Histones at retinal *Gclc-ARE4* continue to be modified and Nrf2 transcriptional activity compromised even after normal glycemia is maintained for 3 months after a period of 3 months of hyperglycemia. The role of epigenetic modifications of *Keap1* promoter in the continued inhibition of Nrf2 activity remains to be determined.

The goal of this study was to understand the role of epigenetic modifications in the regulation of Nrf2 in diabetic retinopathy. Using retinal endothelial cells, the effect of high glucose on the regulation of *Keap1* transcription was investigated by evaluating the binding of Sp1 at its promoter, and histone methylation status of the promoter. To understand the mechanism responsible for altered histone methylation in *Keap1* transcription, the effect of genetic regulation of the histone methyltransferase enzyme Set7/9 (SetD7) on Sp1 binding and methylation status of lysine 4 of histone 3 (H3K4) at *Keap1* promoter was investigated. To transition in vitro results to an in vivo setting, modifications at *Keap1* promoter were also quantified in the retina from diabetic rats, and effect of reversal of glycemic insult on Sp1 binding and histone methylation was evaluated, and the results were confirmed in the retina from human donors with documented diabetic retinopathy.

Methods

Retinal endothelial cells, prepared from bovine retina (BRECs), were incubated in normal (5 mM) or high (20 mM) glucose for 4 days or in high glucose for 4 days, followed by normal glucose for 4 additional days (20-5). For transfection, the transfection complex containing *SetD7*-siRNA, transfection reagent, and transfection medium (Santa Cruz Biotechnology, Paso Robles, CA, USA) were used. After transfection, the cells were rinsed twice with PBS, and incubated in normal or high glucose media for 4 days. The transfection efficiency, as determined by quantifying the protein and gene expressions of SetD7, was approximately 50% (Figs. 1a, 1b). Cells incubated with scramble RNA served as transfection control, and incubated in 20 mM mannitol as osmotic control. These procedures are routinely performed in our laboratory.[7,25,26]

Male Wistar rats, obtained from Harlan Laboratories (South Easton, MA, USA), were made diabetic by streptozotocin, and soon after establishment of diabetes (blood glucose > 250 mg/dL, approximately 3 days after induction of diabetes) were either maintained in poor glycemic control (PC, glycated hemoglobin GHb approximately 12%) for 6 months, or maintained in good glycemic control (GC, GHb approximately 6%) for 6 months, or maintained in poor control for 3 months followed by good control for 3 additional months (Rev group), as reported by us.[17,18,27,28] These procedures are in compliance with the Declaration of Helsinki, and conformed to the ARVO Resolution on Treatment of Animals.

Human retina was isolated from the eye globes enucleated within 6 to 8 hours after death (obtained from Midwest Eye Banks, Ann Arbor, MI, USA) from donors (45–75 years of age) with diabetes for 10 to 20 years and established retinopathy.

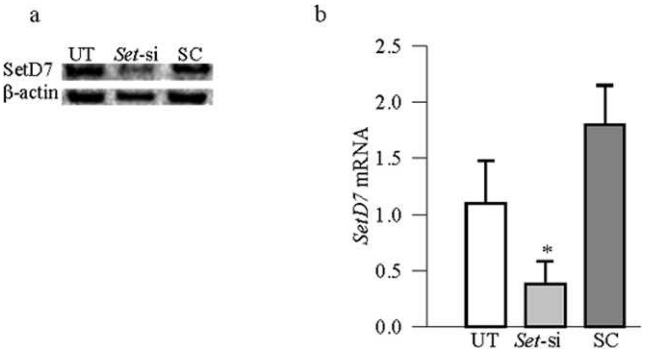

Figure 1. Transfection of retinal endothelial cells with *SetD7*-siRNA. The transfection efficiency was determined in cells transfected with *SetD7*-siRNA or scramble RNA by quantifying the (**a**) protein (Western blot) and (**b**) gene (q-PCR) expressions of SetD7. Transfection experiments were repeated in three to four different cell preparations, and the mRNA values are represented as mean ± SD. UT, untransfected cells, *Set*-si and SC, cells transfected with *SetD7*-siRNA or scramble RNA, respectively. $^*P < 0.05$ versus UT.

For controls, nondiabetic donors (45–75 years of age) were used. Retina from these donors has been in use in our laboratory.[7,20]

Chromatin immunoprecipitation (ChIP) was performed in the protein-DNA complex isolated from cross-linked retina or cells by immunoprecipitating Sp1 (sc-56774; Santa Cruz Biotechnology) or H3K4me1 or Nrf2 using their specific antibodies (ab8895 or ab89443 respectively; Abcam, Cambridge, MA, USA). DNA fragments recovered by phenol-chloroform-isoamyl alcohol extraction were ethanol precipitated, and resuspended in nuclease free water.

The Sp1 binding region of the *Keap1* promoter was quantified by SYBR green-based real-time quantitative PCR (q-PCR) using the 7500 PCR System (Applied Biosystems, Foster City, CA, USA). In human retina, semiquantitative PCR was performed to quantify the binding of Sp1 or level of H3K4me1 at *Keap1* promoter. Controls included rabbit anti-IgG (ab46540; Abcam) and DNA from the input.[18–20,29]

Gene expression was quantified by SYBR green–based q-PCR and the specific products were confirmed by SYBR green single melting curve using species-specific primers (Table). Relative fold changes were calculated by setting the mean values from normal rat retina or cells incubated in 5 mM glucose as one.[18–20,29,30]

Protein expression was determined by Western blot technique using protein-specific antibodies (SetD7, sc-56774 and Keap1, sc-365626; Santa Cruz Biotechnology), and β-actin (A5441; Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a loading control.

Activity of SetD7 methyltransferase, which methylates H3K4, was quantified using the SetD7 Fluorescent Assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. Nuclear protein (15–25 μg) was incubated with the assay mixture for 20 minutes, and H_2O_2 production was measured at 540 nm excitation and 590 nm emission wavelengths.

Statistical analysis was performed using SigmaStat (San Jose, CA, USA), and the results are presented as mean ± SD. The data presenting normal distribution were analyzed by ANOVA followed by Bonferroni's test and, for the data that did not present normal distribution, Kruskal-Wallis one-way analysis followed by Dunn's was performed; $P < 0.05$ was considered statistically significant.

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Table. Primer Sequence

Gene	Primer Sequence, 3′–5′	Product Length, bp
Bovine		
Sp1 at *Keap1* promoter	TGCGTAGCCTCCGATACTCT AATAGCGAAAGTCAGGCGGG	155
Keap1	ATGAGCGTACCCCGCAACCG AGCCACTCCCACCCCGATCC	174
SetD7	TCCCTCCAGGGTGCTGCCAT TGGAGGAGGCGGTGGAAGGG	161
Gclc	CCAGCACGTGGCGCATCTCT CAGCTGAACCTCCATGGGCCG	196
HO1	TGCTGTCTCCCTGTATCCCT AGGAACAGGAAGGGGTGAGT	146
β-actin	CGCCATGGATGATGATATTGC AAGCCGGCCTTGCACAT	66
Rat		
Sp1 at *Keap1* promoter	TGGCAGGGTCTGGCCTAATC TTTGACCACAGGGGCAGGAA	159
Keap1	TGGGCGTGGCAGTGCTCAAC GCCCATCGTAGCCTCCTGCG	195
SetD7	GGGCCAGCCCAGGAGTACGA TTTGACCACAGGGGCAGGAA	120
β-actin	CCTCTATGCCAACACAGTGC CATCGTACTCCTGCTTGCTG	215
Human		
SP1 at *KEAP1* promoter	TGTTAAAAGGAGAATAGCAG GAGATTCCTGCCTTACC	347
KEAP1	TGGCCAAGCAAGAGGAGTTC GGCTGATGAGGGTCACCAGTT	60
SETD7	CGCCATCAACCGCCACCCTT GCCCCTTGCCAGATGCTCCC	168
β-ACTIN	AGCCTCGCCTTTGCCGATCCG TCTCTTGCTCTGGGCCTCGTCG	237

Results

Retinal Endothelial Cells

Hyperglycemia decreases the transcriptional activity of Nrf2 and increases Keap1 expression in the retina and its endothelial cells.[7] *Keap1* transcription is considered to be regulated by the transcriptional factor Sp1.[14] To examine the role of Sp1 in increased *Keap1* expression in diabetes, the binding of Sp1 at *Keap1* promoter was evaluated. Figure 2a shows that high glucose increased the binding of Sp1 at *Keap1* by more than approximately 2-fold compared with the cells incubated in normal glucose; however, normal rabbit IgG control had less than 2% binding. In the same cell preparations, as expected, *Keap1* gene expression was also increased by more than 2-fold (Fig. 2b).

Because methylation of H3K4 favors binding of the transcription factor,[31] to understand the mechanism responsible for increased binding of Sp1 at *Keap1*, histone methylation at Sp1 binding region of the *Keap1* promoter was investigated. The *Keap1* promoter showed increased H3K4me1 by high glucose compared with cells in normal glucose. But, in contrast, 20 mM mannitol did not alter H3K4me1 levels, and the values obtained from cells in normal glucose were not different from those obtained from cells in mannitol (Fig. 3a). As a control, normal rabbit IgG yielded values that were less than 1% compared with those obtained from H3K4me1 antibody (Fig. 3b).

Because SetD7 mediates monomethylation of H3K4,[32] we quantified the effect of glucose on SetD7 in endothelial cells. Gene transcripts and methyltransferase activity of SetD7 were elevated by approximately 2-fold in high glucose compared with normal glucose (Figs. 4a, 4b).

Regulation of SetD7

To specifically investigate the role of histone methylation in *Keap1* transcription, *SetD7*-siRNA transfected cells were used. Regulation of *SetD7* prevented glucose-induced increase in Sp1 binding at *Keap1*, but transfection with scramble RNA had no beneficial effect on Sp1 binding (Fig. 2a). In the same cell

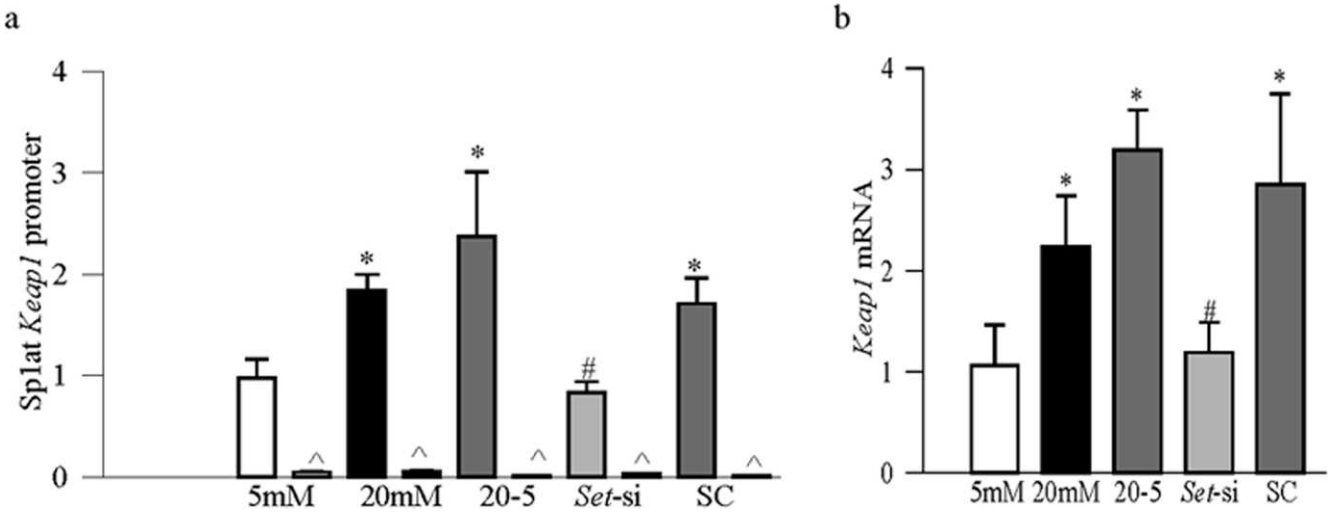

Figure 2. High glucose increases the binding of Sp1 at *Keap1* promoter and elevates its expression. Untransfected cells, or cells transfected with *SetD7*-siRNA or scramble RNA, were incubated in 20 mM glucose for 4 days, or untransfected cells incubated in 20 mM glucose for 4 days followed by 5 mM glucose for 4 additional days (20-5) were analyzed for (**a**) Sp1 binding at *Keap1* by ChIP technique. The q-PCR value in each immunoprecipitate was normalized to the Ct value from the input sample by using the delta-delta-Ct (ddCt) method. Rabbit IgG (^) was used as a negative antibody control. (**b**) *Keap1* mRNA was quantified by q-PCR using β-actin as a housekeeping gene. *Set*-si and SC, cells transfected with *SetD7*-siRNA or scramble RNA, respectively, and incubated in 20 mM glucose for 4 days. Data are represented as mean ± SD from four to six preparations in each group. $^*P < 0.05$ compared with 5 mM glucose and $\#P < 0.05$ versus high glucose.

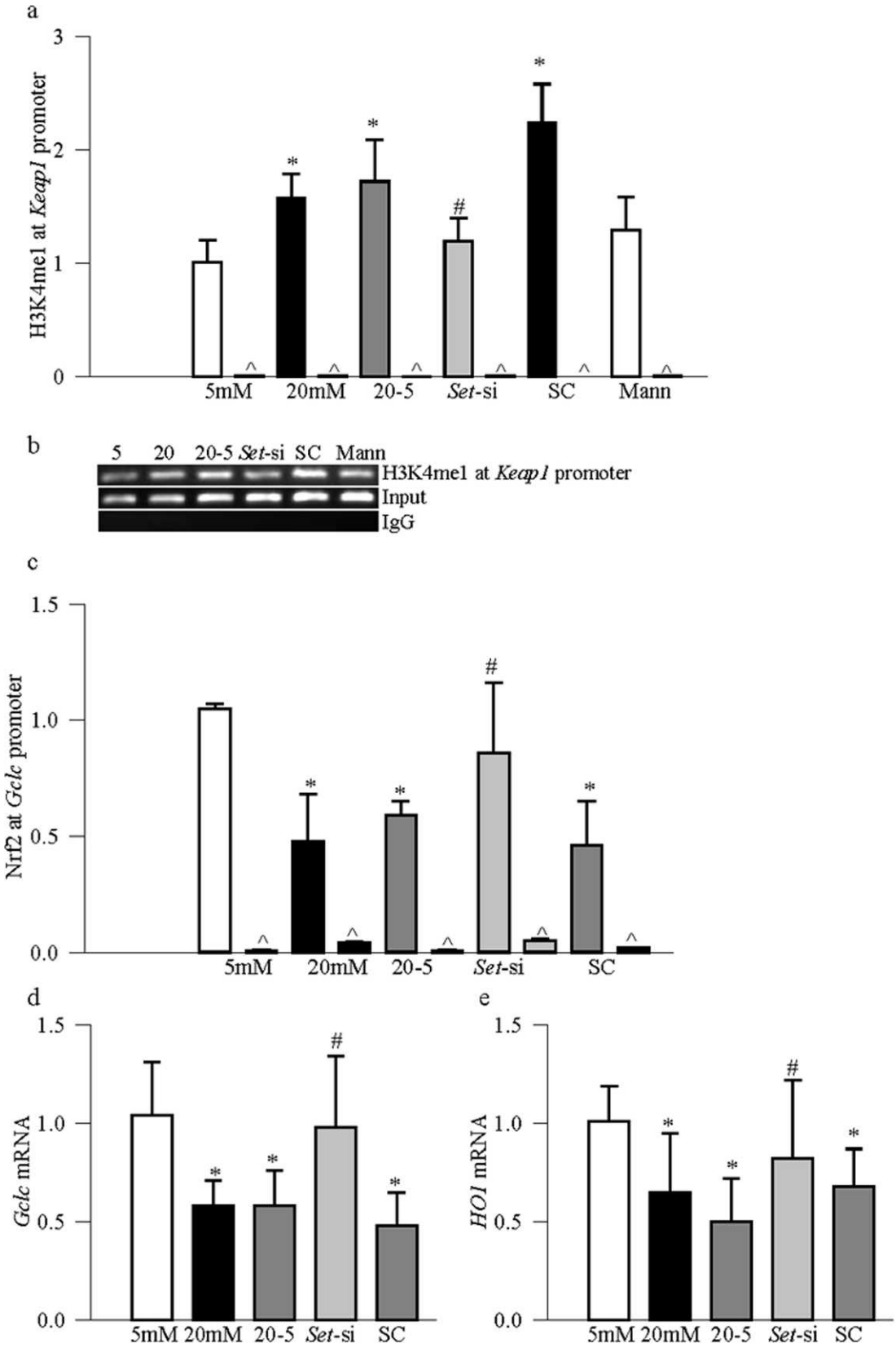

Figure 3. Enrichment of H3K4me1 at *Keap1* promoter impairs Nrf2 signaling. (**a**) H3K4me1 at *Keap1* was quantified by ChIP technique using rabbit IgG as negative antibody control (^), and (**b**) the products were confirmed by agarose gel electrophoresis. (**c**) Nrf2 binding at *Gclc* promoter was determined by ChIP technique, and mRNA levels of (**d**) *Gclc* and (**e**) *HO1* were quantified by q-PCR. Data are mean ± SD from three to four preparations in each group. $^{*}P < 0.05$ and $\#P < 0.05$ compared with 5 mM glucose and 20 mM glucose, respectively.

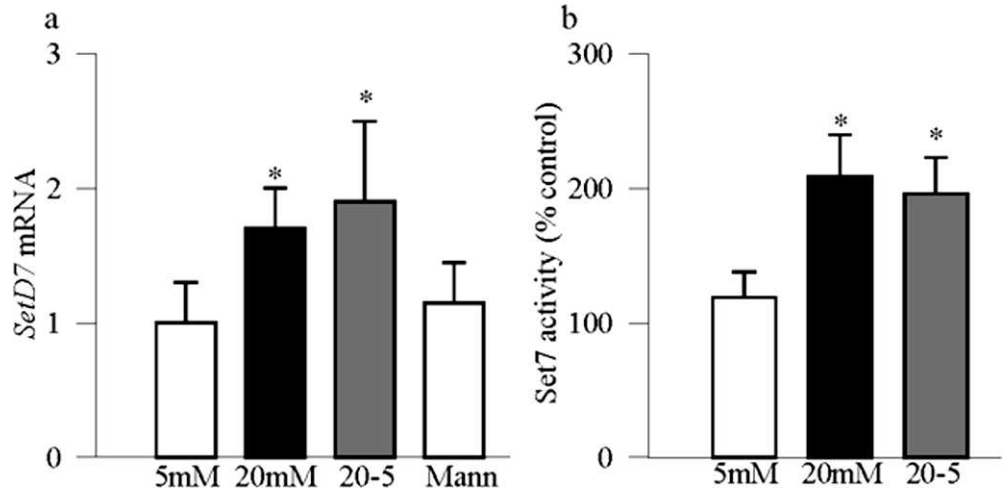

FIGURE 4. High glucose increases SetD7 in retinal endothelial cells. (**a**) *SetD7* mRNA was quantified by q-PCR using β-actin as housekeeping gene. (**b**) Activity was assayed by an ELISA-based methyltransferase activity assay. Values from the 5-mM glucose are considered as 1 for mRNA or 100% for activity. Values are mean ± SD from three to four experiments, and each experiment was performed in duplicate. $^*P < 0.05$ vs. 5 mM glucose.

preparations, increase in *Keap1* mRNA also was significantly ameliorated (Fig. 2b), and the values obtained from *SetD7*-siRNA–transfected cells were significantly different from the untransfected cells in high glucose, but were similar to those obtained from untransfected cells in normal glucose. The *SetD7*-siRNA–transfected cells showed significantly lower H3K4me1 at *Keap1* in high glucose conditions compared with the cells transfected with scramble RNA (Figs. 3a, 3b). This was accompanied by amelioration of glucose-induced decreased binding of Nrf2 at *Gclc* promoter and decreased expressions of Nrf2-regulated *Gclc* and *heme oxygenase1* (*HO1*). The values obtained from the cells transfected with *SetD7* or untransfected cells, incubated in high glucose medium, were significantly different from each other (Figs. 3c–e).

Rat Retina

Consistent with the results from retinal endothelial cells, Sp1 binding at retinal *Keap1* promoter was increased by more than 2-fold in diabetic rat retina compared with normal rats (Fig. 5a). In the same samples, mRNA and protein expressions of Keap1 were also increased by more than 1.5-fold (Figs. 5b, 5c). Similarly, *Keap1* promoter had increased H3K4me1 levels, and the ChIP antibody negative control (normal rabbit IgG) yielded values that were less than 1% compared with those obtained from Keap1 antibody (Figs. 6a, 6b). In the same samples, *SetD7* expression and its methyltransferase activity were also significantly elevated in diabetes (Figs. 7a, 7b).

Human Retina

To validate these findings in diabetic patients with retinopathy, SP1 binding at *KEAP1* promoter was determined in the retina from human donors. Figure 8a shows increased SP1 binding at *KEAP1* promoter in the diabetic donors compared with their age-matched nondiabetic donors, and, as expected, there was no change in the input samples. This was accompanied by increased expressions of *KEAP1* and *SETD7* in diabetic retinopathy donors (Figs. 8b, 8c).

Metabolic Memory

Retina continues to experience oxidative stress even after hyperglycemic insult is terminated.[18,33,34] To understand the role of epigenetic modifications of *Keap1* promoter in the metabolic memory phenomenon, Sp1 binding and H3K4me1 at the *Keap1* promoter were quantified in the retina of rats maintained in poor glycemic control for 3 months followed by good glycemic control for 3 additional months. Figures 5a–c show that even after termination of hyperglycemic insult, the binding of Sp1 at *Keap1* promoter remained high with increased mRNA and protein expressions of Keap1, and this was accompanied by continued increased H3K4me1 at *Keap1* promoter (Fig. 6). In the same rats, reversal of hyperglycemia also failed to ameliorate diabetes-induced increase in SetD7 expression and methyltransferase activity (Fig. 7). The values obtained from rats in the PC-Rev group were not different from the rats in the PC group, but were significantly higher than those obtained from ones that remained normal throughout the experiment ($P < 0.05$). Maintenance of good glycemia, soon after induction of diabetes in rats (GC group), prevented diabetes-induced increase in Keap1 expression and H3K4me1 at its promoter (Figs. 5b, 6). The values obtained from the GC group were significantly different from those in PC or PC-Rev groups ($P < 0.05$), but were not different from the normal group ($P > 0.05$).

Consistent with the results from rat retina, glucose-induced increase in Sp1 binding and H3K4me1 at *Keap1* remained elevated with increased *Keap1* and *SetD7* in the endothelial cells exposed to normal glucose for 4 days, which had followed 4 days of high glucose (20-5 group). The binding of Nrf2 at *Gclc* promoter remained compromised with decreased levels of the cytoprotective genes that are under the control of Nrf2, *Gclc*, and *HO1* (Figs. 2–4). The values from the cells in continuous high glucose were not different from the cells in the 20-5 group.

DISCUSSION

The transcriptional activity of the protective factor Nrf2 and its binding at the *ARE4* are decreased in the retina in diabetes, and the expression of its downstream target gene, *Gclc*, becomes subnormal.[7,35] Here, we show that the binding of the transcriptional factor Sp1 at *Keap1* promoter is significantly increased in diabetes, and the reason for this appears to be the methylation of H3K4 due to activation of SetD7. However, regulation of increase in *SetD7* by its siRNA prevents increase in Sp1 binding at *Keap1* promoter and *Keap1* expression, and ameliorates decrease in the Nrf2-regulated genes, *Gclc* and

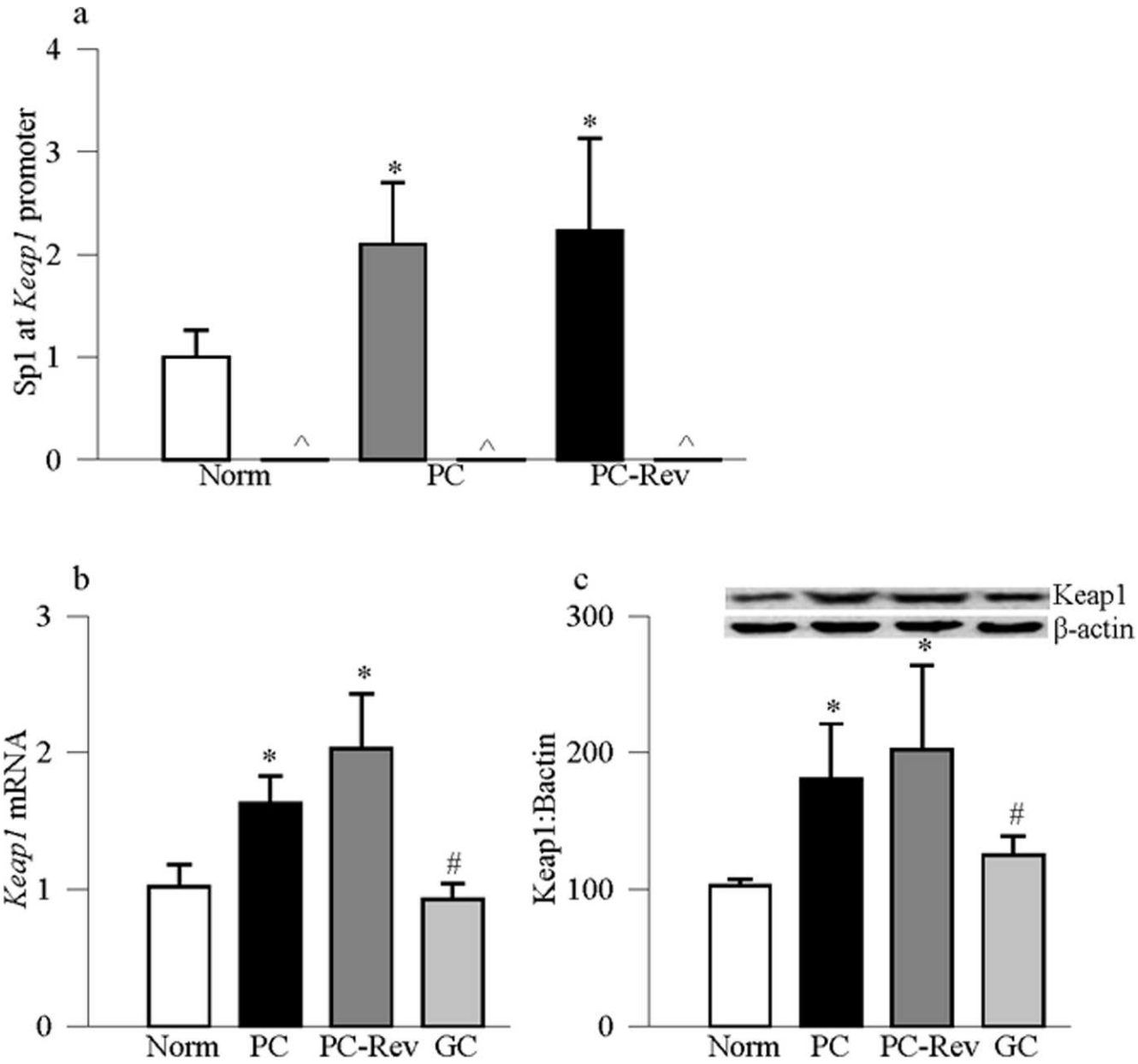

Figure 5. Diabetes increases the binding of Sp1 at *Keap1* promoter. Retina from rats in poor glycemic control for 3 months followed by good glycemic control for 3 months (PC-Rev), or continuous PC or GC for 6 months was analyzed for (**a**) Sp1 binding at *Keap1* using ChIP assay. Keap1 (**b**) mRNA was quantified by q-PCR using β-actin as a housekeeping gene, and (**c**) its protein expression by Western blotting technique and β-actin was used as a loading control. Results are represented as mean ± SD from five to six rats in each group, each analysis performed in duplicate. $^{*}P < 0.05$ versus normal and $\#P < 0.05$ versus PC rats.

HO1. In addition to the epigenetic modifications of the *Keap1* promoter in the development of diabetic retinopathy, the results also show the role of these modifications in the metabolic memory phenomenon. Cessation of high glucose insult fails to attenuate increased binding of Sp1 at *Keap1*, and the promoter continues to be methylated with increased expression of *Keap1* and decreased expression of Nrf2-regulated defense genes. The results clearly suggest the role of epigenetic modification in the regulation of retinal antioxidant status in the development of retinopathy, and in the metabolic memory phenomenon associated with its progression.

Keap1, an intracellular inhibitor of Nrf2, is one of the primary regulators of Nrf2, and its cysteine-rich Kelch/DGR domain maintains its interaction with Nrf2.[8] Under stress conditions, critical cysteine residues within Keap1, especially Cys151, is covalently modified and loses its ability to ubiquitinate Nrf2, and Nrf2 becomes free to move into the nucleus.[9] Sp1, a ubiquitously expressed transcription factor, acts as an essential transcriptional activator of *Keap1* by binding at its promoter (–160/–153).[14] We have shown that the expression of *Keap1* is increased in diabetes,[7] and here we show that the possible mechanism of increased *Keap1* expression could be the increased binding of SP1 at its promoter. In addition, we also present similar increase in the retina of human donors with diabetic retinopathy, further strengthening the role of increased Keap1-Nrf2 in the regulation of retinal Nrf2 activity.

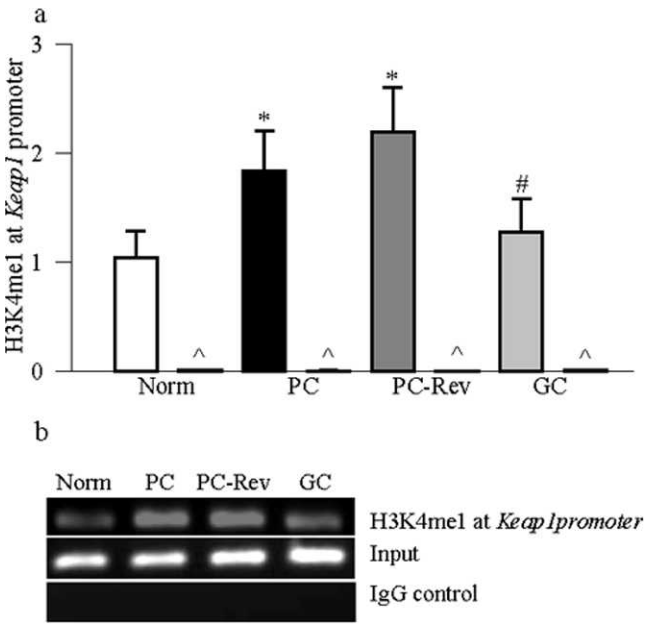

Figure 6. H3K4me1 is increased at retinal *Keap1* promoter in diabetes. (**a**) H3K4me1 at *Keap1* was quantified by ChIP technique, and (**b**) confirmed by agarose gel electrophoresis. Norm, normal; IgG control, immunoprecipitation with IgG (^); Input, total genomic DNA without ChIP. Data are represented as mean ± SD from five to seven rats in each group. $^{*}P < 0.05$ and $^{\#}P < 0.05$ versus normal and PC rats, respectively.

Because the binding of the transcription factor at the promoter of a gene is regulated by epigenetic modifications, including histones methylation/acetylation and DNA methylation,[15,16,31] and diabetes epigenetically modifies the transcriptional binding sites of a number of retinal genes,[7,18–20] the role of histone methylation in the increased binding of Sp1 at *Keap1* promoter was evaluated. H3K4me1 is significantly enriched at the *Keap1* promoter in the retina. In support, H3K4me1 enrichment at the promoter regions is generally associated with transcriptional activation,[16,36] and decreased H3K4me1 levels at retinal *Sod2* are implicated with the suppression of its expression in diabetes.[18]

Lysine 4 of histone 3 can be mono-, di-, or trimethylated, and depending on the methylation status, it can act as a transcriptional activator or repressor.[37] Our recent study has shown that although H3K4me2 is increased at *Gclc-ARE4* in the retina in diabetes, H3K4me3 and H3K4me1 are decreased, resulting in impaired binding of Nrf2 at *Gclc-ARE4*.[38] Here we show that the activity of SetD7 is increased in diabetes, and regulation of *SetD7* by its specific siRNA, in addition to ameliorating increase in H3K4me1 at *Keap1* promoter, also prevents increased binding of Sp1 at the *Keap1* promoter and *Keap1* expression. Histone methylation is carried out by methyltransferases, and SetD7, a monomethyltransferase, methylates H3K4 and other proteins.[36,39] Consistent with our results showing increased SetD7, this enzyme has been shown to regulate NF-κB-dependent inflammatory genes in diabetic nephropathy,[32] and NF-κB is activated in the retina in diabetes.[40] As Nrf2 binding with Keap1 regulates the movement of Nrf2 to the nucleus and controls the transcriptional activity of Nrf2, to further validate the role of histone methylation in Nrf2 signaling, we show that *SetD7*-siRNA also regulates the expressions of *Gclc* and *HO1*, the genes that are under the control of transcriptional activity of Nrf2. These results clearly suggest that epigenetic modifications at *Keap1* promoter play important role in the regulating Nrf2-mediated ARE genes.

Intensive glycemic control, after a period of hyperglycemia, does not halt the progression of diabetic retinopathy.[21,22,41] Metabolic memory phenomenon is also observed in animal models and in in vitro models of diabetic retinopathy, and persistent hyperglycemia-driven changes in many genes associated with diabetic complications, including retinopathy, have been identified.[18,20,42–45] As epigenetic modifications can persist in a system, recent comprehensive epigenomic profiling on the cells from subsets of The Diabetes Control and Complications Trial and Follow-up Epidemiology of Diabetes Interventions and Complications Study participants with varied complications have shown that epigenetics could be playing an important role in the further progression of complications during EDIC.[46] In animal models, good control, after a period of poor control, fails to provide any benefit to the histopathology associated with diabetic retinopathy,[33,34] and

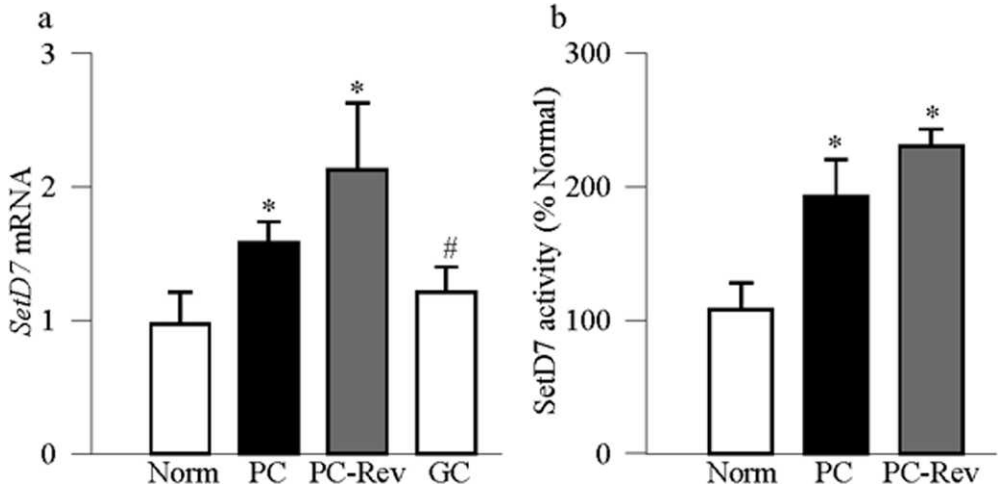

Figure 7. Diabetes increases retinal SetD7. (**a**) *SetD7* mRNA was quantified by q-PCR using β-actin as housekeeping gene. Fold change was normalized to the values of normal control by ddCt method. (**b**) SetD7 enzyme activity was measured by an ELISA-based activity assay kit. Values obtained from normal rat are considered as 1 for mRNA and 100% for activity. Values are mean ± SD from five or more rats in each group. $^{*}P < 0.05$ versus normal rats.

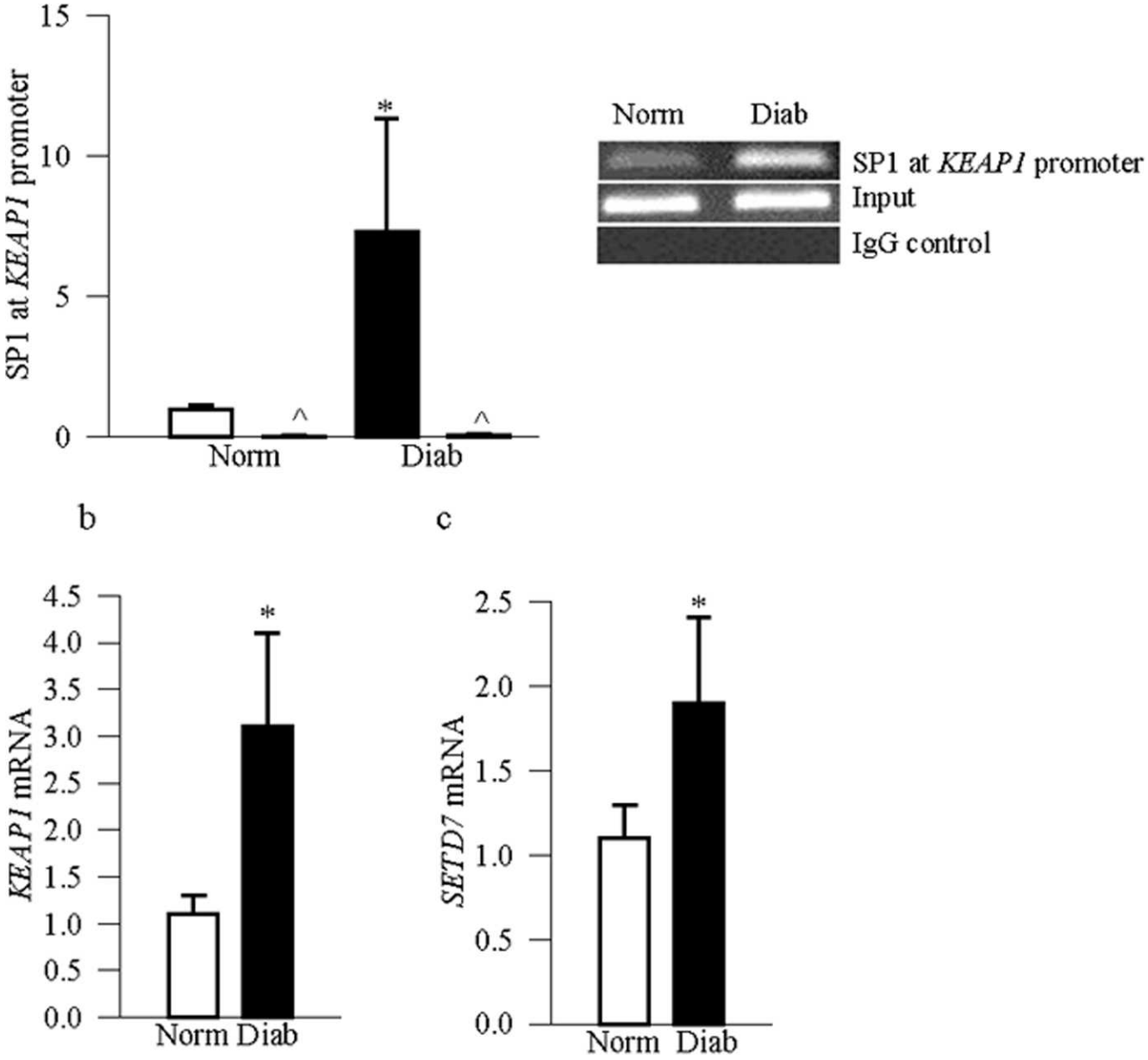

Figure 8. Retinal *KEAP1* promoter is epigenetically modified in the human donors with diabetic retinopathy. (**a**) SP1 binding at *KEAP1* was measured by ChIP technique by amplifying for the SP1 binding region at *KEAP1* promoter in protein-DNA complex using normal rabbit IgG (^) as antibody control. (**b**) *KEAP1* and (**c**) *SETD7* mRNA levels were quantified by qPCR using β-actin as a housekeeping gene. The values obtained from nondiabetic donors were considered as 1. Diab, donors with documented diabetic retinopathy; Norm, age-matched nondiabetic donors. Data are presented as mean ± SD from four donors in each group. $^*P < 0.05$ versus nondiabetic donors.

does not remove epigenetic modifications in the retinal genes.[18,20,29,47] H3K4me2 continues to be increased and H3K4me3 and H3K4me1 decreased at *Gclc-ARE4*, and Nrf2 activity compromised after hyperglycemic insult is terminated. Here, we show that H3K4me1 enrichment and Sp1 binding remain increased, and Keap1 continues to be elevated. Furthermore, SetD7 continues to be active even after hyperglycemia is replaced by normal glycemia, confirming the role of epigenetic modifications in continuing increased oxidative stress that the retina experience after hyperglycemia is terminated.[18,20,27,43] In support, transient glucose-induced SetD7-mediated H3K4me1 enrichment is associated with the persistent transcriptional activation inflammatory genes in human and bovine vascular endothelial cells.[32,48,49] However, if good glycemic control is initiated soon after induction of diabetes, consistent with the retina escaping epigenetic modifications of *Sod2*, and accelerated apoptosis and histopathology,[18,20,29,47,50] the binding of Sp1 and H3K4me1 enrichment at *Keap1* promoter also do not increase.

In conclusion, we have presented results showing that epigenetic modifications at *Keap1* promoter by SetD7 facilitate Sp1 binding with *Keap1*, increasing its expression, and increased Keap1 restrains Nrf2 in the cytosol, impairing the transcriptional activity of Nrf2. Reversal of hyperglycemia provides no benefit to Keap1 and its histone modifications, suggesting their role in both the development of diabetic retinopathy and in the metabolic memory phenomenon.

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References

1. Baynes JW, Thrope SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes*. 1999;48:1–9.
2. Kowluru RA, Engerman RL, Kern TS. Abnormalities of retinal metabolism in diabetes or experimental galactosemia VI. Comparison of retinal and cerebral cortex metabolism, and effects of antioxidant therapy. *Free Radic Biol Med*. 1999;26: 371–378.
3. Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. *Diabetes*. 2001;50:1938–1942.
4. Kowluru RA. Diabetic retinopathy, oxidative stress and antioxidants. *Curr Topics Nutraceutical Res*. 2005;3:209–218.
5. Kern TS, Kowluru R, Engerman RL. Abnormalities of retinal metabolism in diabetes or galactosemia. ATPases and glutathione. *Invest Ophthalmol Vis Sci*. 1994;35:2962–2967.
6. Kowluru RA, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system. *Free Radic Biol Med*. 1997;22: 587–592.
7. Zhong Q, Mishra M, Kowluru RA. Transcription factor Nrf2-mediated antioxidant defense system in the development of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2013;54: 3941–3948.
8. Villeneuve NF, Lau A, Zhang DD. Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. *Antiox Redox Signal*. 2010;13:1699–1712.
9. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. *Genes Dev*. 2013;27:2179–2191.
10. Shelton P, Jaiswal AK. The transcription factor NF-E2-related factor 2 (Nrf2): a protooncogene? *FASEB J*. 2013;27:414–423.
11. Taguchi K, Motohashi H, Yamamoto M. Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution. *Genes Cells*. 2011;16:123–140.
12. Xu Z, Wei Y, Gong J, et al. NRF2 plays a protective role in diabetic retinopathy in mice. *Diabetologia*. 2014;57:204–213.
13. Holland R, Fishbein JC. Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. *Antioxid Redox Signal*. 2010;13:1749–1761.
14. Guo D, Wu B, Yan J, Li X, Sun H, Zhou D. A possible gene silencing mechanism: hypermethylation of the Keap1 promoter abrogates binding of the transcription factor Sp1 in lung cancer cells. *Biochem Biophys Res Comm*. 2012;428:80–85.
15. Song SH, Han SW, Bang YJ. Epigenetic-based therapies in cancer: progress to date. *Drugs*. 2011;71:2391–2403.
16. Musselman CA, Lalonde ME, Cote J, Kutateladze TG. Perceiving the epigenetic landscape through histone readers. *Nat Struct Mol Biol*. 2012;19:1218–1227.
17. Tewari S, Zhong Q, Santos JM, Kowluru RA. Mitochondria DNA replication and DNA methylation in the metabolic memory associated with continued progression of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2012;53:4881–4888.
18. Zhong Q, Kowluru RA. Epigenetic changes in mitochondrial superoxide dismutase in the retina and the development of diabetic retinopathy. *Diabetes*. 2011;60:1304–1313.
19. Zhong Q, Kowluru RA. Regulation of matrix metalloproteinase-9 by epigenetic modifications and the development of diabetic retinopathy. *Diabetes*. 2013;62:2559–2568.
20. Zhong Q, Kowluru RA. Epigenetic modification of Sod2 in the development of diabetic retinopathy and in the metabolic memory: role of histone methylation. *Invest Ophthalmol Vis Sci*. 2013;54:244–250.
21. Aiello LP. Diabetic retinopathy and other ocular findings in the diabetes control and complications trial/epidemiology of diabetes interventions and complications study. *Diabetes Care*. 2014;37:17–23.
22. Diabetes Control and Complications Trial Research Group. Early worsening of diabetic retinopathy in the diabetes control and complication trial. *Arch Ophthalmol*. 1998;116:874–886.
23. El-Osta A, Brasacchio D, Yao D, et al. Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia. *J Exp Med*. 2008;205:2409–2417.
24. Reddy MA, Natarajan R. Epigenetic mechanisms in diabetic vascular complications. *Cardiovasc Res*. 2011;90:421–429.
25. Mohammad G, Kowluru RA. Diabetic retinopathy and signaling mechanism for activation of matrix metalloproteinase-9. *J Cell Physiol*. 2012;227:1052–1061.
26. Santos JM, Tewari S, Goldberg AFX, Kowluru RA. Mitochondria biogenesis and the development of diabetic retinopathy. *Free Radic Biol Med*. 2011;51:1849–1860.
27. Kowluru RA, Abbas SN, Odenbach S. Effect of re-institution of good metabolic control on oxidative stress in the kidney of diabetic rats. *J Diabetes Complications*. 2004;18:282–288.
28. Madsen-Bouterse SA, Mohammad G, Kanwar M, Kowluru RA. Role of mitochondrial DNA damage in the development of diabetic retinopathy, and the metabolic memory phenomenon associated with its progression. *Antiox Redox Signal*. 2010;13: 797–805.
29. Tewari S, Santos JM, Kowluru RA. Damaged mitochondrial DNA replication system and the development of diabetic retinopathy. *Antiox Redox Signal*. 2012;17:492–504.
30. Mohammad G, Kowluru RA. Matrix metalloproteinase-2 in the development of diabetic retinopathy and mitochondrial dysfunction. *Lab Invest*. 2010;90:1365–1372.
31. Ruthenburg AJ, Allis CD, Wysocka J. Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell*. 2007;25:15–30.
32. Sun G, Reddy MA, Yuan H, Lanting L, Kato M, Natarajan R. Epigenetic histone methylation modulates fibrotic gene expression. *J Am Soc Nephrol*. 2010;21:2069–2080.
33. Kowluru RA, Chakrabarti S, Chen S. Re-institution of good metabolic control in diabetic rats on the activation of caspase-3 and nuclear transcriptional factor (NF-kB) in the retina. *Acta Diabetologica*. 2004;44:194–199.
34. Kowluru RA, Kanwar M, Kennedy A. Metabolic memory phenomenon and accumulation of peroxynitrite in retinal capillaries. *Exp Diabetes Res*. 2007;2007:2196.
35. Zhong Q, Kowluru RA. Diabetic retinopathy and damage to mitochondrial structure and transport machinery. *Invest Ophthalmol Vis Sci*. 2011;52:8739–8746.
36. Keating S, El-Osta A. Transcriptional regulation by the Set7 lysine methyltransferases. *Epigenetics*. 2013;11:8.
37. Zhang Y, Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev*. 2001;15:2343–2360.

38. Mishra M, Zhong Q, Kowluru RA. Epigenetic modifications of nrf2-mediated glutamate-cysteine ligase: implications for the development of diabetic retinopathy and the metabolic memory phenomenon associated with its continued progression. *Free Radic Biol Med*. 2014;75:129–139.
39. Guo HB, Guo H. Mechanism of histone methylation catalyzed by protein lysine methyltransferase SET7/9 and origin of product specificity. *Proc Natl Acad Sci U S A*. 2007;104:8797–8802.
40. Kowluru RA, Koppolu P, Chakrabarti S, Chen S. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. *Free Radic Research*. 2003;37:1169–1180.
41. White NH, Sun W, Cleary PA, et al. Prolonged effect of intensive therapy on the risk of retinopathy complications in patients with type 1 diabetes mellitus: 10 years after the Diabetes Control and Complications Trial. *Arch Ophthalmol*. 2008;126:1707–1715.
42. Engerman RL, Kern TS. Progression of incipient diabetic retinopathy during good glycemic control. *Diabetes*. 1987;36:808–812.
43. Kowluru RA. Effect of re-institution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. *Diabetes*. 2003;52:818–823.
44. Kowluru RA, Zhong Q, Kanwar M. Metabolic memory and diabetic retinopathy: role of inflammatory mediators in retinal pericytes. *Exp Eye Res*. 2010;90:617–623.
45. Roy S, Sala R, Cagliero E, Lorenzi M. Overexpression of fibronectin induced by diabetes or high glucose: phenomenon with a memory. *Proc Natl Acad Sci U S A*. 1990;87:404–408.
46. Miao F, Chen Z, Genuth S, et al. Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. *Diabetes*. 2014;63:1748–1762.
47. Kowluru RA, Chan PS. Metabolic memory in diabetes—from in vitro oddity to in vivo problem: role of apoptosis. *Brain Res Bull*. 2010;87:297–302.
48. Okabe J, Orlowski C, Balcerczyk A, et al. Distinguishing hyperglycemic changes by Set7 in vascular endothelial cells. *Circ Res*. 2012;110:1067–1076.
49. Siebel AL, Fernandez AZ, El-Osta A. Glycemic memory associated with epigenetic changes. *Biochem Pharmacol*. 2010;80:1853–1859.
50. Zhong Q, Kowluru RA. Role of histone acetylation in the development of diabetic retinopathy and the metabolic memory phenomenon. *J Cell Biochem*. 2010;110:1306–1313.

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