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Mitochondrial fusion and maintenance of mitochondrial homeostasis in diabetic retinopathy

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ABSTRACT

Mitochondria are dynamic in structure, and undergo continuous fusion-fission to maintain their homeostasis. In diabetes, retinal mitochondria are swollen, their membrane is damaged and mitochondrial fusion protein, mitofusin 2 (Mfn2), is decreased. DNA methylation machinery is also activated and methylation status of genes implicated in mitochondrial damage and biogenesis is altered. This study aims to investigate the role of mitochondrial fusion in the development of diabetic retinopathy, and to illustrate the molecular mechanism responsible for Mfn2 suppression. Using human retinal endothelial cells, manipulated for *Mfn2*, we investigated the role of fusion in mitochondrial structural and functional damage in diabetes. The molecular mechanism of its suppression in diabetic milieu was determined by investigating *Mfn2* promoter DNA methylation, and confirmed using molecular and pharmacological inhibitors of DNA methylation. Similar studies were performed in the retinal microvasculature (prepared by hypotonic shock method) of diabetic rats, and human donors with documented diabetic retinopathy. Overexpression of *Mfn2* prevented glucose-induced increase in mitochondrial fragmentation, decrease in complex III activity and increase in membrane permeability, mtDNA damage and apoptosis. High glucose hypermethylated *Mfn2* promoter and decreased transcription factor (SP1) binding, and Dnmt inhibition protected *Mfn2* promoter from these changes. In streptozotocin-induced diabetic rats, intravitreal administration of *Dnmt1*-siRNA attenuated *Mfn2* promoter hypermethylation and restored its expression. Human donors with diabetic retinopathy confirmed *Mfn2* promoter DNA hypermethylation. Thus, regulating Mfn2 and its epigenetic modifications by molecular/pharmacological means will protect mitochondrial homeostasis in diabetes, and could attenuate the development of retinopathy in diabetic patients.

1. Introduction

Retinopathy is one of the most devastating complications of diabetes, and the molecular mechanism of this complex blinding disease remains obscure. Recent research has led to a greater appreciation of how mitochondrial dysfunction could contribute to diabetic complications including neuropathy, nephropathy and retinopathy [1–5]. Retinal mitochondria are damaged in diabetes, they become swollen and their membranes leak cytochrome *c* in the cytosol, which accelerates the apoptotic process. Furthermore, mitochondria copy numbers are decreased, and their DNA (mtDNA) is damaged. Due to impaired transcription of mtDNA-encoded proteins, the electron transport chain is compromised, and the vicious cycle of free radicals continues to self-propagate [6–8].

Mitochondria are small in size (0.75 to 3 μm), and have double membranes; while the outer membrane is porous with no membrane potential, the inner membrane has barriers to all ions and molecules and is the site of oxidative phosphorylation [9]. Structurally, mitochondria are highly dynamic, and depending on the energy demand and supply balance, mitochondria constantly fuse and change size and shape. Fragmentation of mitochondria is associated with a decline in ATP production, and fusion mixes the contents of two mitochondria and dilutes injured mitochondrial proteins and DNA [10–12]. Disruption in fusion results in mitochondria to fragment into short rods or spheres, and in accumulation of mtDNA point mutations and deletions. Mitochondrial fusion is a two-step process, which uses mitofusin 1 and 2 (Mfn1 and Mfn2) to control the outer mitochondrial membrane fusion and optic atrophy 1 (Opa1) for the inner mitochondrial membrane fusion [13]. Although Mfn1 and Mfn2 share some common functions, Mfn2 itself is necessary and sufficient to modulate mitochondrial metabolism by regulating mitochondrial fuel oxidation, membrane potential and oxidative phosphorylation [14,15]. In diabetes, expression of Mfn2 (gene and protein) is decreased in the retina [16,17]. However, the role of Mfn2 in diabetic retinopathy remains to be explored.

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Gene expression is also regulated by epigenetic modifications without altering the DNA sequence [18], and in diabetes, enzymes responsible for these epigenetic modifications are altered in the retina [3,19]. Enzyme machinery for DNA methylation, one of the epigenetic modifications which suppresses gene expression, is activated in the retina and its vasculature in diabetes, and mtDNA is hypermethylated, resulting in impaired transcription of mtDNA-encoded genes [3,20]. The mechanism responsible for decreased retinal Mfn2 expression in diabetes is, however, not clear.

The aim of this study was to investigate the importance of mitochondrial fusion in the development of diabetic retinopathy, and illustrate the molecular mechanism responsible for Mfn2 suppression. Using human retinal endothelial cells (HRECs), manipulated for *Mfn2* expression, we have investigated the role of mitochondrial fusion in increased mitochondrial free radical production and mitochondrial damage in diabetic retinopathy. To understand the mechanism responsible for Mfn2 suppression in diabetes, DNA methylation status of its promoter was evaluated. Key parameters were confirmed in the retinal microvessels from rat model of diabetic retinopathy, and also from human donors with documented diabetic retinopathy.

2. Methods

2.1. Retinal endothelial cells

HRECs were obtained from Cell Systems Corporation (Cat. No. ACBRI 181, Cell Systems Corp, Kirkland, WA, USA), and were cultured in an environment of 95% O_2 and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 20 μg/mL endothelial cell growth supplement, 12% heat-inactivated fetal bovine serum, and 1% each insulin- transferrin- selenium-Glutamax and antibiotic/antimycotic [21]. Cells from the 5th–8th passage were incubated in normal 5 mM or high 20 mM D-glucose (NG and HG respectively) for 96 h, in the presence or absence of a DNA methyltransferase (Dnmt) inhibitor, 5-Aza-2′-deoxycytidine, (Aza, 1 μM; Cat. No. A3656; Sigma-Aldrich, St. Louis, MO). Cells incubated in 20 mM L-glucose (L-Glu) were used as osmotic/metabolic control [22,23]. A group of cells were transfected with either *Mfn2* plasmid using Turbofectin reagent (Cat. No. RG202218 and TF81001; OriGene, Rockville, MD), or with *Dnmt1*-siRNA (Cat. No. SC-35204, Santa Cruz Biotechnology, Santa Cruz, CA, USA), employing transfection reagent SC-29528 (Santa Cruz Biotechnology). To alleviate any effect of cell passaging, each incubation condition was performed in HRECs from the same passage and the same batch. At the end of the incubation with the plasmids/siRNA, the cells were rinsed with DMEM, and incubated in either normal or high glucose for 96 h [21,24]. Parallel incubations with non-targeting scrambled RNA, or with reagent alone, were used as controls. The efficiency of transfection was determined by quantifying their gene transcripts (by SYBR Green-based quantitative real-time PCR, qPCR) and protein expression (western blot).

2.2. Rats

Wistar rats (male, ~200gBW), obtained from Harlan Labs (South Easton, MA, USA) were made diabetic by intraperitoneal injection of streptozotocin (55 mg/kg BW) [24]. Although downregulation of Mfn2 is an early event in the development of diabetic retinopathy, mitochondrial damage and histopathology, characteristic of diabetic retinopathy are seen after 6 to 8 months of diabetes in rodents [25,26]. To ensure downregulation of Mfn2 at a duration of diabetes when mitochondrial damage and histopathology are present, the retina from diabetic rats was collected after 8 months of diabetes. Age-matched nondiabetic rats were used as controls.

For *Dnmt1*-siRNA experiment, a group of rats was anesthetized with ketamine-xylazine (67 mg/kg ketamine and 10 mg/kg xylazine) soon after induction of diabetes, using a 32 gauge needle attached to a 5 μL glass Hamilton syringe, 10 μg *Dnmt1*-siRNA (Cat. No. ID: RSS331349, Thermo Fisher Scientific, Waltham, MA), mixed with 5 μL Invivofectamine (Cat. No. IVF 3001, Invitrogen, Carlsbad, CA) was administered intravitreally in the left eye under a dissecting microscope. The right eye received 5 μL of medium GC content siRNA negative control (Cat. No. 12935-300, Thermo Fisher Scientific). Four weeks after administration of *Dnmt1*-siRNA, the rats were sacrificed and their retina were collected. Dnmt1 expression (gene and protein) was decreased by ~50% in the retinal microvasculature obtained from the eye receiving *Dnmt1*-siRNA compared to the control eye. The treatment of the animals was in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, and our institutional guidelines.

Table 1
Primer sequences.

Primer	Sequence
Human	
Mfn2	Fwd- ATGCAGACGGAAAAGCACTT
	Rev- ACAACGCTCCATGTGCTGCC
Mfn2 promoter	Fwd- TGCCCGATGAGTCACTTCAC
	Rev- CAAGGGGCGAAAAACCAAGG
mtDNA-short	Fwd- CCCCACAAACCCCATTACTAAACCCA
	Rev- TTTCATCATGCGGAGATGTTGGATGG
mtDNA-long	Fwd- TCTAAGCCTCCTTATTCGAGCCGA
	Rev- TTTCATCATGCGGAGATGTTGGATGG
CytB	Fwd- TCACCAGACGCCTCAACCGC
	Rev- GCCTCGCCCGATGTGTAGGA
β-Actin	Fwd- AGCCTCGCCTTTGCCGATCCG
	Rev- TCTCTTGCTCTGGGCCTCGTCG
Rat	
Mfn2	Fwd- ACAAAGTTCTGCCATCTGGG
	Rev- TGCTCATCCTGATGGAGGGC
CytB	Fwd- CCCATTCATTATCGCCGCCC
	Rev- GGTCTCCTAGTAGGTCTGGG
mtDNA-short	Fwd- CCTCCCATTCATTATCGCCGCCCTTGC
	Rev- GTCTGGGTCTCCTAGTAGGTCTGGGAA
mtDNA-long	Fwd- AAAATCCCGCAAACAATGACCACCCC
	Rev- GGCAATTAAGAGTGGGATGGAGCCAA
Dnmt1	Fwd- ACCTACCACGCCGACAT
	Rev- AGGTCCTCTCCGTACTCCA
β-Actin	Fwd- CCTCTATGCCAACACAGTGC
	Rev- CATCGTACTCCTGCTTGCTG

Table 2
Rat *Mfn2* promoter.

	Sequence	CpG sites
Region 1, −393 to −297	Fwd- CCTCTCCTCTCCCTCTCCTG	5
	Rev- CAGCAACAACGGCGAATCA	
Region 2, 320 to −221	Fwd- GGCATTGATTCGCCGTTGTT	5
	Rev- GGGTGAAGTGACTCATCGG	
Region 3, −245 to −84	Fwd- GTCTGCCCGATGAGTCACTT	6
	Rev- AAACCAAGGGCGTGGAGTA	
Region 4, −93 to +7	Fwd- CCTTGGTTTTTCGCCCCTG	8
	Rev- GCATCATGGGGGCTGTAGTT	

2.3. Human donors

Eye globes (enucleated within 8 h of death) from human donors (40–75 years age) with clinically documented diabetic retinopathy were supplied by the Eversight Eye Bank, Ann Arbor, MI. Over 75% donors had type 2 diabetes, and the duration of diabetes ranged from 14 to 41 years. Age-matched non-diabetic donors served as their controls. Retina was isolated and a small portion (1/8th to 1/5th) was utilized to prepare microvessels [27].

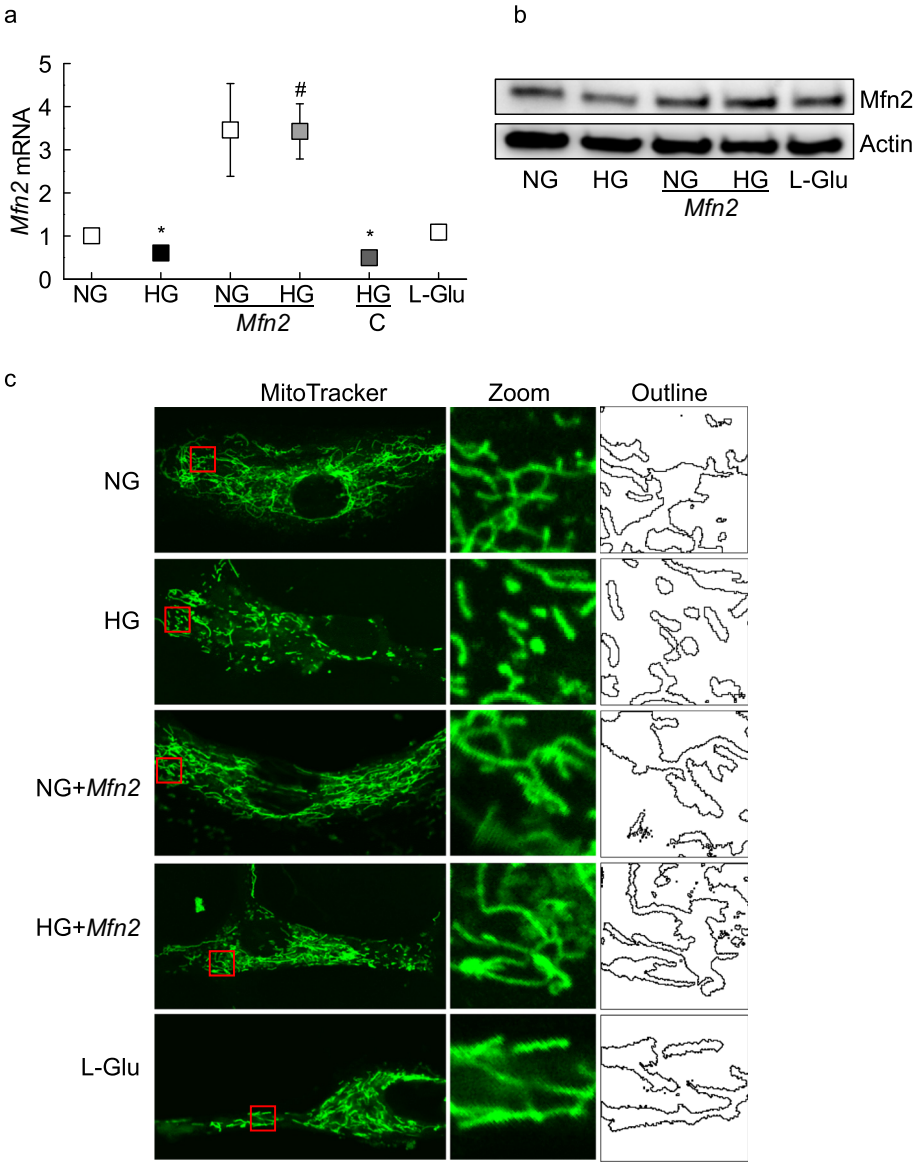


Fig. 1. Mfn2 expression and mitochondrial morphology of retinal endothelial cells in high glucose. HRECs, transfected with *Mfn2* plasmids, or untransfected, and incubated in high glucose, were analyzed for Mfn2 (a) gene expression by qPCR, and (b) protein expression by western blot technique. β-Actin was used as a housekeeping gene or loading protein respectively. (c) Live cell confocal microscopy (63×) was performed in 6–8 cells to examine mitochondrial morphology using MitoTracker Green dye. The middle panel shows the area inside the box enlarged. In the right panel, the area inside the box was analyzed in ImageJ for particles (size = 0- infinity; circularity = 0 to 1) after adjusting for color threshold. Bare outlines were drawn to illustrate the morphology. (d) Mitochondrial localization of Mfn2 was determined by immunofluorescence technique using Cox IV as a mitochondrial marker, and Alexa Fluor-488 (green)-conjugated secondary antibody for Cox IV and Texas red-conjugated for Mfn2. The image is representative of 3–4 different experiments, with each experiment performed in 5–7 cells. (e) Transfection efficiency of *Mfn2* plasmids was determined by quantifying its gene (qPCR) and protein (western blot technique) expressions using β-Actin as a housekeeping gene or loading protein respectively. Values obtained from untransfected cells in normal glucose are considered as 1, and are represented as mean ± SD from three different cell preparations, with each measurement performed in duplicate. NG and HG = normal and high glucose respectively; L-Glu = 20 mM L-Glucose; NG or HG + *Mfn2* = cells transfected with *Mfn2* plasmids and incubated in normal or high glucose for 96 h. C = cells with transfection reagent control. *$p < 0.05$ compared to NG and #$p < 0.05$ compared to HG.

2.4. Retinal microvessels

Retina (rat or human) was suspended in 15–20 mL deionized water, and incubated at 37 °C in a shaker water bath for 1 h. The non-capillary tissue was removed by repeated inspiration and ejection using a Pasteur pipette under a dissecting microscope [24,27]. They were then rinsed with sterile PBS, and were either crosslinked with 1% paraformaldehyde for chromatin immunoprecipitation (ChIP), or used for RNA/DNA isolation and western blotting. To maintain similar experimental conditions, each microvessel preparation included retina from both normal and diabetic rats, and each experiment was repeated in three or more different microvessel preparations. Although the hypotonic shock method lyses the cells, and the preparation is not ideal for mitochondrial functional studies, membranous enzymes, ChIP assays and DNA/RNA measurements can be performed reliably in these microvessel preparations [8,24,28].

2.5. Mitochondrial localization, morphology and function

Mitochondrial localization of Mfn2 was performed in HRECs by immunofluorescence technique on coverslips containing HRECs (transfected with *Mfn2* plasmids, or untransfected) exposed to normal

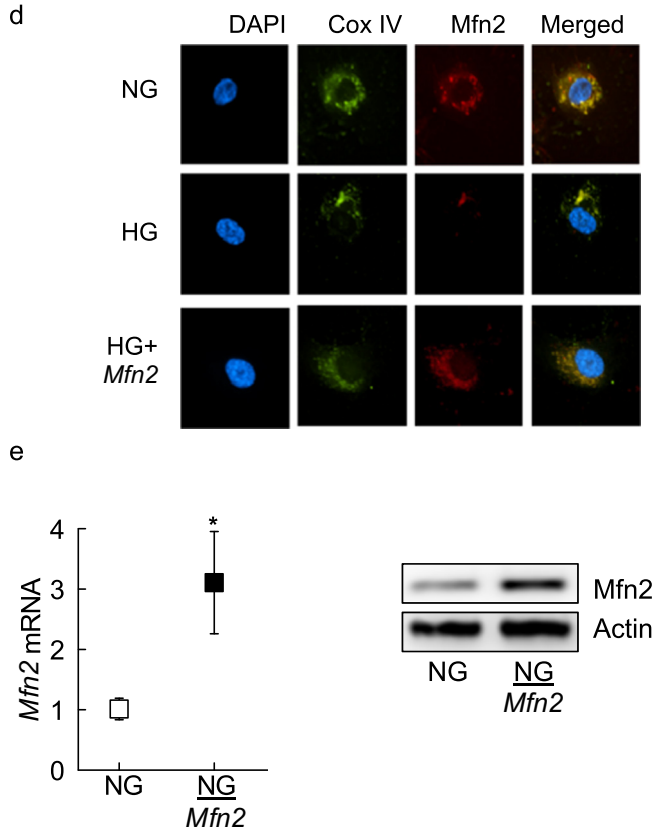


Fig. 1. (*continued*)

or high glucose using antibodies against Mfn2 (Cat. No. ab56889, Abcam; 1:250 dilution); CoxIV (Cat. No. ab153709, Abcam; 1:250 dilution) was used as a mitochondrial marker. Secondary antibodies included Alexa Fluor-488 (green) conjugated anti-rabbit (Cat. No. Molecular Probes-Life Technologies, Grand Island, NE, 1:500 dilution) and Texas red-conjugated anti-mouse (Cat. No. Vector Laboratories, Burlingame, CA; 1:500 dilution). Immuno-labelled cells were mounted using DAPI-containing (blue) Vectashield mounting medium (Vector Laboratories), and were examined under Eclipse 90i fluorescence microscope (Nikon, Melville, NY) at 40× magnification.

Coverslips containing HRECs (transfected with *Mfn2* plasmids, or untransfected) exposed to normal or high glucose, were incubated with 200 nM MitoTracker green FM (Cat. No. M7514, Thermo Fisher Scientific) for 10 min. After rinsing the coverslips with PBS, they were imaged using Leica SP5 confocal microscope at 63× magnification (Leica Microsystems, Wetzlar, Germany).

Reactive oxygen species (ROS) in the mitochondria were quantified using MitoSOX red (mitochondrial superoxide indicator). In brief, after experimental incubations, HRECs were incubated with 5 μM mitochondrial superoxide indicator, MitoSOX red (Cat. No. M36008, Thermo Fisher Scientific) and 200 nM MitoTracker green for 10 min at 37 °C [29]. Confocal images were obtained at 40× magnification. Six to eight cells were imaged in each group, and images are obtained from three different cell preparations. The intensity of the MitoSox was quantified using ImageJ and the data are expressed as percentage control.

Complex III activity was assayed in a 50 μL assay volume containing 0.25–0.5 μg mitochondrial protein, 40 μM reduced decylubiquinone and 2 mM KCN. Cytochrome c [50 μM] was added to initiate the reaction, and the reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm, as described previously [30].

Mitochondrial membrane permeability was determined by quantifying their calcium-induced swelling [30]. Briefly, in an assay volume of 50 μL, 2.5–10 μg mitochondrial protein was equilibrated with HEPES buffer (pH 7.4) containing 215 mM mannitol, 71 mM sucrose and 5 mM succinate for 30 s. The decrease in absorbance at 540 nm was monitored after addition of [400 μM] calcium chloride.

Apoptosis of retinal endothelial cells was measured using Cell Death Detection ELISAPLUS kit (Cat. No. 11774425001) from Roche Diagnostics (Indianapolis, IN) and 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt to detect absorbance (at 405 and 490 nm), as described previously [24].

Damage of mtDNA was quantified by extended length PCR using the method reported previously [6]. In brief, long (8.8 kb) and short (223 bp) mtDNA regions for human, and long (13.4 kb) and short (210 bp) for rats, were amplified using semiquantitative PCR, and the amplified products were resolved on 1.2% and 2% agarose gel respectively. Relative amplification was quantified by normalizing the intensity of the long product to the short product.

2.6. DNA methylation

5-Methylcytosine (5mC) was quantified in the immunoprecipitated genomic DNA using methylated DNA Immunoprecipitation (MeDIP) Kit (Cat. No. P-1015, EPIGENTEK, Farmingdale, NY) [8,31]. The enrichment of 5mC in HRECs and in human retinal microvessels was analyzed by qPCR using *Mfn2* promoter primers (Table 1). For rat microvessels, four regions of the rat *Mfn2* promoter (−393 to +7 region) were analyzed; region 1 (R1, −393 to −297) and region 2 (R2, −320 to −221) had 5 CpG sites each, region 3 (R3, −254 to −84) had 6 CpG sites and region 4 (R4, −93 to +7) had 8 sites (Table 2).

2.7. Chromatin immunoprecipitation (ChIP)

To analyze the binding of Dnmt1 or specificity protein 1 transcription factor (SP1) at the *Mfn2* promoter, ChIP was performed in the crosslinked samples. Protein-DNA complex (100 μg) was immunoprecipitated with the respective antibodies of interest (3 μg each, Dnmt1, Cat. No. D4567, Sigma Aldrich, MA or SP1, Cat. No. SC-17824, Santa Cruz Biotechnology). Normal rabbit IgG (3 μg; Cat. No. ab171870, Abcam, Cambridge, MA) was used as an antibody control. The immunoprecipitated complex was captured using Protein A Agarose/Salmon Sperm DNA (Cat. No. 16157, EMD Millipore, Temecula, CA), washed, and de-crosslinked at 65 °C for 6 h. This was followed by isolation of DNA with phenol: chloroform: isoamylalcohol, as reported previously [31]. Relative binding of Dnmt1 or SP1 was quantified by real- time qPCR using primers specific for *Mfn2* promoter. The target values were normalized to input controls respectively to obtain fold change.

2.8. Western blotting

Protein (30–40 μg) was separated on a 4–20% gradient acrylamide gel (BioRad, Hercules, CA), and transferred to a nitrocellulose membrane. Expression of Mfn2 was detected using anti-Mfn2 mouse monoclonal antibody (1:1000 dilution; Cat No. ab56889, Abcam). Mouse anti-β-Actin (1:2000 dilution; Cat No. A5441, Sigma Chemicals) was used as a loading protein.

2.9. Gene transcripts

Gene transcripts were quantified by real time qPCR using gene and species specific primers. 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 [8,31].

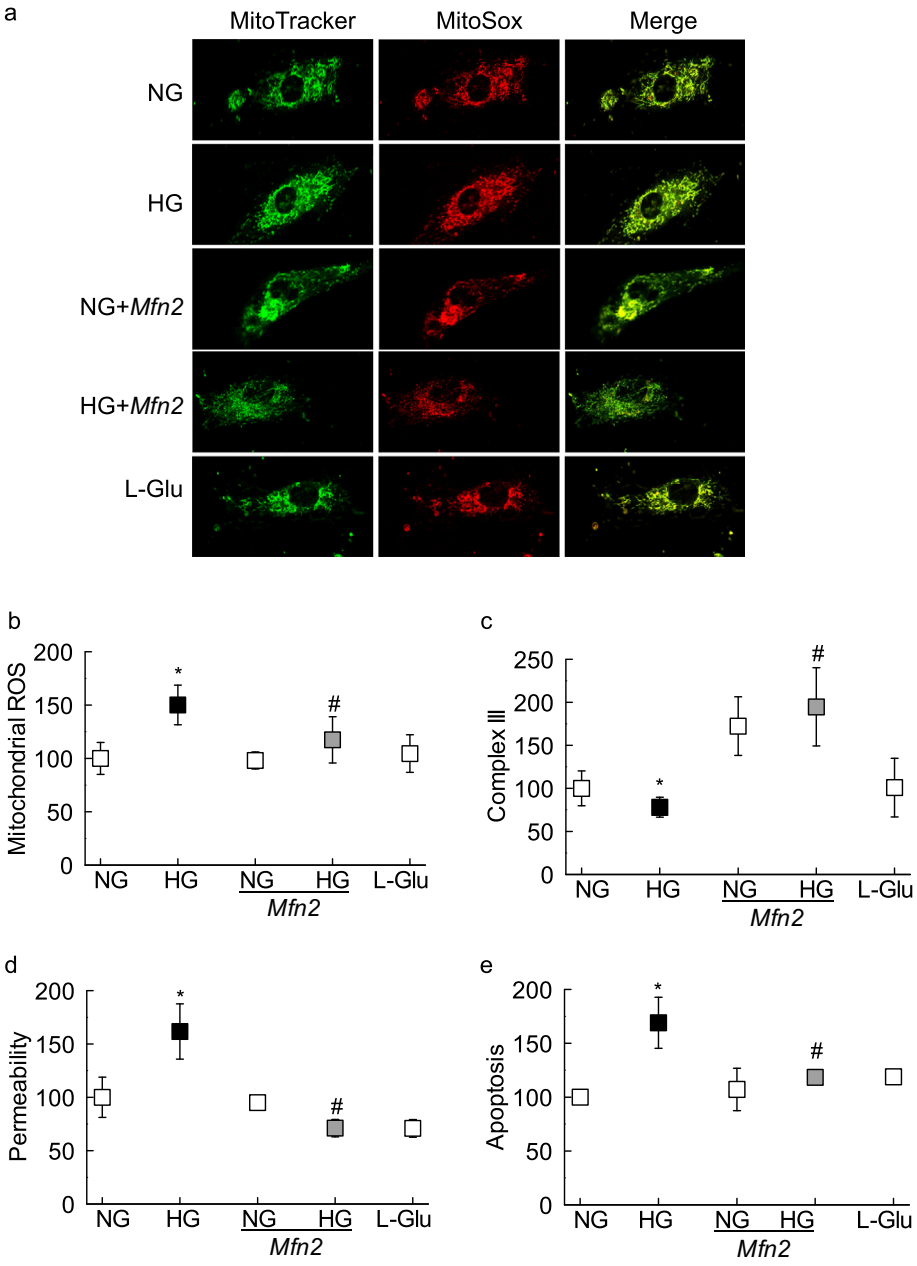


Fig. 2. Mfn2 overexpression and high glucose-induced mitochondrial dysfunction. (a) Mitochondrial ROS were measured by confocal imaging (40×) using MitoSOX Red, and MitoTracker Green was used to identify the mitochondria. (b) Shows the fluorescence intensity, quantified using ImageJ, from 5 to 6 images in each group. (c) Complex III activity and (d) mitochondrial membrane permeability in isolated mitochondria were measured spectrophotometrically by quantifying reduction of cytochrome *c* and calcium-induced swelling respectively. (e) Cell apoptosis was determined by quantifying nucleosomal DNA fragments using a Cell Death ELISA kit. Values are expressed as percentage compared to cells incubated in normal glucose and are represented as mean ± SD of three to four different cell preparations, with each measurement performed in duplicate. $*p < 0.05$ compared to NG, $\#p < 0.05$ compared to HG.

2.10. Statistical analysis

Data are presented as mean ± standard deviation. Comparison between groups was made using one way ANOVA followed by Dunn's *t*-test and a p value < 0.05 was considered significant.

3. Results

3.1. Retinal endothelial cells

Consistent with our previous work demonstrating decreased Mfn2 expression in the retina of diabetic rats and in human donors with diabetic retinopathy [16], Mfn2 expression (gene and protein) was decreased by 40–60% in the retinal endothelial cells exposed to high glucose for 96 h compared to the cells incubated in normal glucose (Fig. 1a & b). Confocal imaging of the same cell preparations showed fragmented mitochondria with disrupted morphology in high glucose conditions, compared to cells in normal glucose (Fig. 1c). Cells incubated in 20 mM L-glucose, instead of 20 mM D-glucose, had similar Mfn2 expression and elongated mitochondrial structure as observed in the cells incubated in normal glucose. Overexpression of *Mfn2* in HRECs ameliorated glucose-induced decrease in Mfn2 mRNA and protein expressions (Fig. 1a & b). Compared to untransfected cells, *Mfn2* overexpressing cells exposed to high glucose also had elongated mitochondria with less fragmentation (Fig. 1c), and increased levels of Mfn2 in the mitochondria (Fig. 1d). Fig. 1e shows *Mfn2* transfection efficiency in HRECs.

Mitochondrial function and dynamics are inter-connected [12,32], and in HRECs, mitochondrial ROS are elevated in high glucose conditions. Overexpression of *Mfn2* attenuated glucose-induced increased accumulation of mitochondrial ROS; *Mfn2* overexpressing HRECs, incubated in high glucose, had significantly lower Mitosox staining compared to the untransfected cells in high glucose (Fig. 2a & b). Consistent with this, glucose-induced decrease in complex III activity, and increase membrane permeability, were also ameliorated by *Mfn2* overexpression (Fig. 2c & d). In the same cell preparations, *Mfn2* overexpression also attenuated glucose-induced increase in apoptosis (Fig. 2e). The values obtained from cells in high glucose, transfected

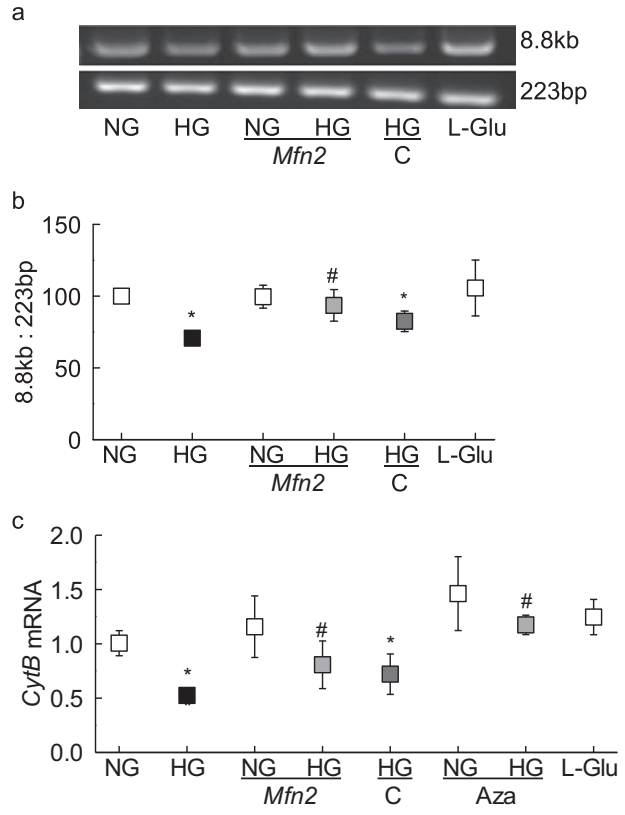


Fig. 3. *Mfn2* overexpression and mtDNA damage. High glucose exposed HRECs, transfected with *Mfn2* plasmids, or untransfected, were analyzed for (a) mtDNA damage by extended-length PCR using long mtDNA (8.8 kb) and short (223 bp) amplicons mtDNA. (b) Decrease in the ratio of 8.8 kb and 223 bp amplicon band intensities represents damaged mtDNA. (c) Transcripts of mtDNA-encoded *CytB* were measured by qPCR using *β-Actin* as a housekeeping gene. Each measurement was made in duplicate in three different cell preparations, and values are represented as mean ± SD. Values obtained from untransfected cells in normal glucose are considered as 100% (mtDNA damage) or 1 (*CytB* mRNA). NG and HG = normal and high glucose respectively; L-Glu = 20 mM L-Glucose; *Mfn2* and C = HRECs transfected with *Mfn2* plasmids or reagent control; Aza = cells incubated in the presence of azacytidine. *$p < 0.05$ and #$p < 0.05$ compared to NG and HG respectively.

with *Mfn2* were significantly different from those obtained from untransfected cells in high glucose. Incubation of cells overexpressing *Mfn2* in normal glucose had no effect on mitochondrial ROS levels and function, the values obtained from *Mfn2* overexpressing cells, or untransfected cells, were not different from each other.

Since mitochondrial membrane dynamic is intimately associated with alterations in mtDNA [33], and in diabetes mtDNA is damaged [6], the effect of *Mfn2* overexpression on mtDNA integrity was examined. Consistent with the protective effect of *Mfn2* overexpression on mitochondrial function, it also prevented mtDNA damage; similar ratios of short to long mtDNA amplicon were observed in *Mfn2* transfected cells incubated in high glucose and untransfected cells incubated in normal glucose (Fig. 3a & b). Damaged mtDNA results in impaired transcription of genes important in electron transport chain [34], and overexpression of *Mfn2* ameliorated glucose-induced decrease in mtDNA-encoded cytochrome B (*CytB*) gene transcripts (Fig. 3c).

Diabetes also activates DNA methyltransferases (Dnmts) [23], to understand the mechanism of suppression of Mfn2 in hyperglycemic conditions, DNA methylation status of its promoter was analyzed. As shown in Fig. 4a, 5mC levels at its promoter were increased by ~2.4 fold in the cells exposed to high glucose, compared to cells in normal glucose. Consistent with this, 2.5 fold increase in Dnmt1 binding at the same site of *Mfn2* promoter was also observed in the cells exposed to high glucose (Fig. 4b), and this was accompanied by ~40% decrease in the binding of transcription factor SP1 (Fig. 4c). Furthermore, regulation of DNA methylation by a pharmacological inhibitor Aza, or by its specific siRNA (*Dnmt1*-siRNA), attenuated glucose-induced increase in DNA methylation of *Mfn2* promoter and Dnmt1 binding. In the same cell preparations, regulation of Dnmts by either Aza or *Dnmt1*-siRNA also ameliorated glucose-induced decrease in SP1 binding (Fig. 4). Role of DNA methylation in *Mfn2* regulation was further confirmed by amelioration of glucose-induced decrease in *Mfn2* gene transcripts in cells transfected with *Dnmt1*-siRNA, or incubated with Aza (Fig. 4d). The values obtained from cells in high glucose, with and without Dnmt regulation were significantly different from each other (Fig. 4).

3.2. Rat retinal microvessels

In accordance with our results from *in vitro* model, microvessels from diabetic rat retina had over 50% decrease in Mfn2 expression compared to the values obtained from age-matched normal rats (Fig. 5a). Retinal microvessels from the same diabetic rats, as expected,

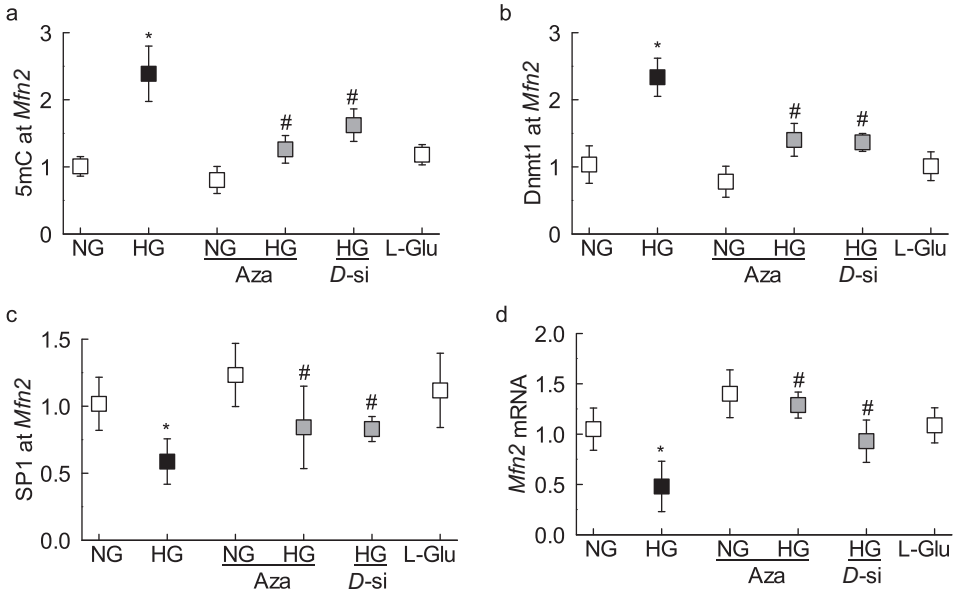


Fig. 4. High glucose and *Mfn2* promoter DNA methylation. The promoter of *Mfn2* was analyzed for (a) 5mC levels in the immunoprecipitated genomic DNA using MeDIP Kit, and (b & c) binding of Dnmt1 and SP1 in the crosslinked sample by ChIP technique using IgG as antibody control. (d) Gene transcripts of *Mfn2* were measured by qPCR and *β-Actin* was used as a housekeeping gene. Measurements were made in duplicate or triplicate in three different cell preparations. The values obtained from cells in normal glucose are considered as 1. NG = normal glucose; HG = high glucose; NG or HG + Aza = cells incubated in normal or high glucose in the presence of azacytidine; L-Glu = 20 mM L-Glucose; *D*-si = cells transfected with *Dnmt1*-siRNA. * and #$p < 0.05$ *vs* NG and HG respectively.

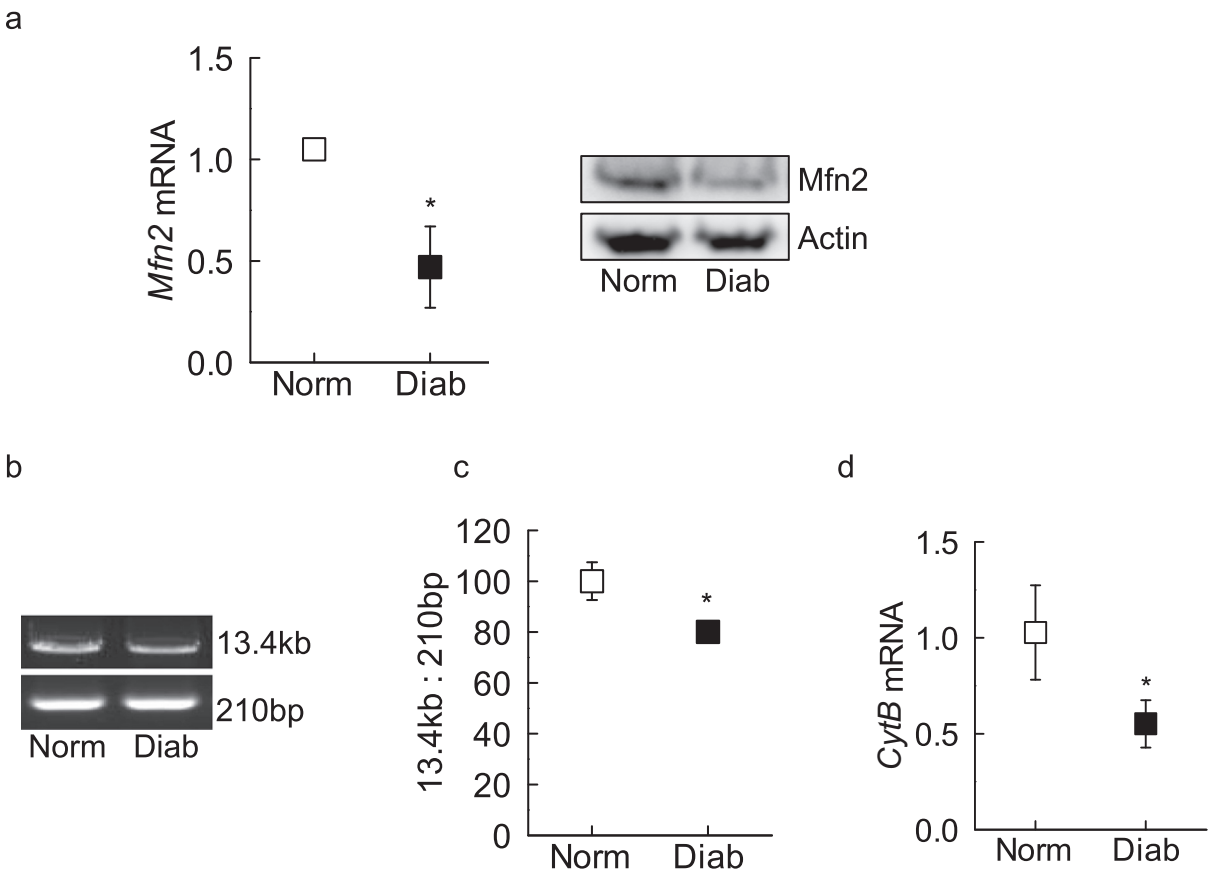


Fig. 5. Mfn2 in retinal microvasculature in diabetes. Microvasculature, prepared by hypotonic shock method, from retina of diabetic rats was analyzed for (a) Mfn2 gene and protein expression in by qPCR and western blot technique respectively, using β-Actin as housekeeping gene or loading control. (b) mtDNA damage was assessed by extended length PCR using its long (13.4 kb) and short (210 bp) amplicons. (c) Decrease in the ratio of band intensities of long and short amplicons represents mtDNA damage (d) *CytB* mRNA was quantified by qPCR using *β-Actin* as a housekeeping gene. Values are represented as mean ± SD from 5 to 6 rats in each group, and each measurement was made in duplicate. Norm and Diab = normal and diabetic rats respectively. *$p < 0.05$ compared to normal.

had increased mtDNA damage and decreased *CytB* gene transcript. Compared to normal rats, the ratio of long to short mtDNA amplicon (13.4 kb:210 bp) was ~25% lower (Fig. 5b & c) and *CytB* transcripts ~40% lower in diabetic rats (Fig. 5d).

Analysis of the four overlapping regions in the proximal (−393 to +7) region of *Mfn2* promoter (Fig. 6a) showed that, while retinal microvasculature from diabetic and normal rats had similar 5mC levels in R1 and R2, the two regions closest to the transcription start site (TSS), R3 and R4, had significantly higher 5mC levels in diabetic rats compared to normal rats. Among R3 and R4, R3 had over 9 fold increase in 5mC compared to ~3 fold in R4 (Fig. 6b). Furthermore, compared to normal rats, in diabetes, R3 of the *Mfn2* promoter had 4 fold higher Dnmt1 binding and 2 fold lower SP1 binding, R4 region of the promoter had < 2 fold increase in Dnmt1 binding and no decrease in SP1 binding (Fig. 6c & d).

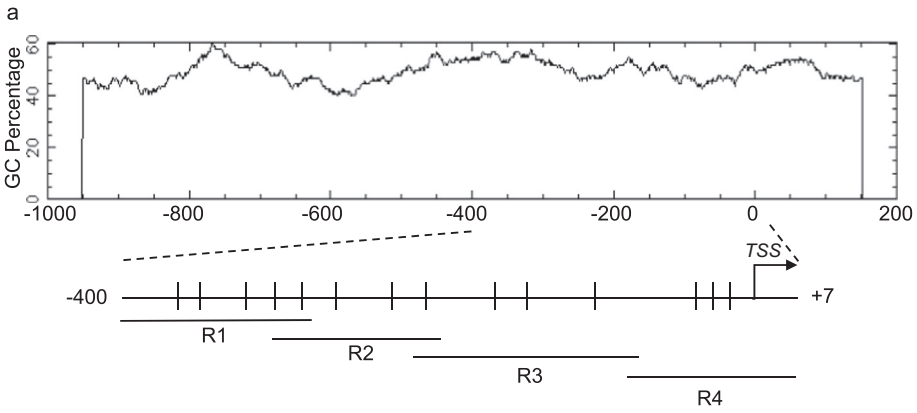
To further confirm the importance of DNA methylation in *Mfn2* transcription, retinal microvessels from diabetic rats receiving intravitreal administration of *Dnmt1*-siRNA were analyzed. Administration of *Dnmt1*-siRNA ameliorated diabetes-induced decrease in *Mfn2* transcription (Fig. 7a), and increase in 5mC at its promoter (Fig. 7b). The values obtained from the diabetic rat eye receiving *Dnmt1*-siRNA were significantly different from those obtained from the collateral eye receiving siRNA negative control.

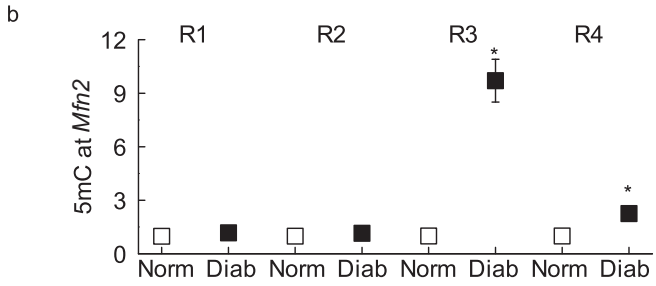
3.3. Human retinal microvessels

To transition from experimental models of diabetic retinopathy (*in vitro* and *in vivo*) to the human disease, retinal microvasculature from diabetic donors with established retinopathy were analyzed. Compared to age-matched non diabetic donors, retinal microvessels from donors with diabetic retinopathy had ~50% reduction in *MFN2* gene expression and ~2.5 fold increase in 5mC levels at its promoter (Fig. 8a & b), and reduction in transcription of mtDNA, as shown by 40% decrease in *CYTB* transcripts (Fig. 8c).

4. Discussion

Mitochondria, the primary source of cellular energy, are one of the most important subcellular organelle, and their homeostasis is critical for cell survival. They are considered to play central role in diabetic microvascular complications [3]. In diabetic retinopathy, retinal mitochondria are swollen, their membranes integrity is impaired, superoxide levels are elevated, mtDNA is damaged and the transcription of mtDNA is decreased, and compromised electron transport system continues to propagate a futile free radical cycle. Mitochondria are dynamic organelles that continuously undergo fission and fusion, and this process is critical for adaptation of a cell to changing energy demand and cell survival [10–12]. A dynamic regulatory fusion protein, Mfn2, is considered critical for the maintenance of normal mitochondrial function in mammalian cells [35]. Using *in vivo* and *in vitro* models of diabetic retinopathy, here we show that overexpression of Mfn2 in retinal endothelial cells prevents them from hyperglycemia-induced mitochondrial structural and functional damage, and ameliorates mtDNA damage and its transcription. In addition, our study has also identified a novel mechanism responsible for decreased Mfn2 expression in diabetes; due to DNA hypermethylation of *Mfn2* promoter in





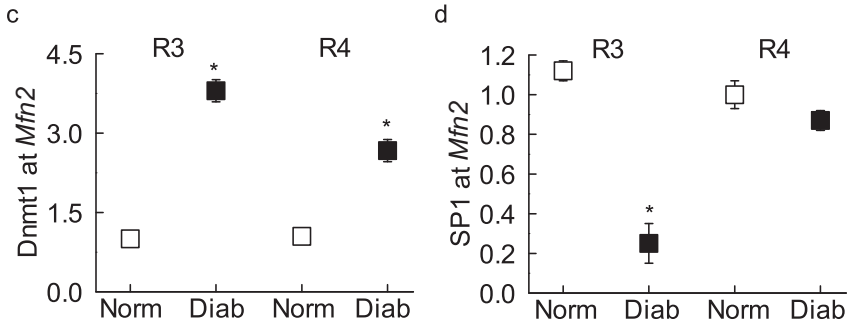


Fig. 6. DNA methylation of *Mfn2* promoter in diabetes. (a) GC percentage in the rat *Mfn2* promoter, and four (R1 to R4) regions between −400 bp to +7 bp used for analysis. Vertical lines indicate SP1 binding sites. Retinal microvessels from diabetic rats and age-matched normal rats were used for quantifying (b) 5mC levels in regions R1 to R4 by methylated DNA immunoprecipitation technique. The binding of (c) Dnmt1 and (d) SP1 in four regions of *Mfn2* promoter was determined in the crosslinked retinal microvessels using ChIP technique. Values are represented as mean ± SD from 5 to 6 rats in each group, with each measurement made in duplicate. $*p < 0.05$ compared to normal.

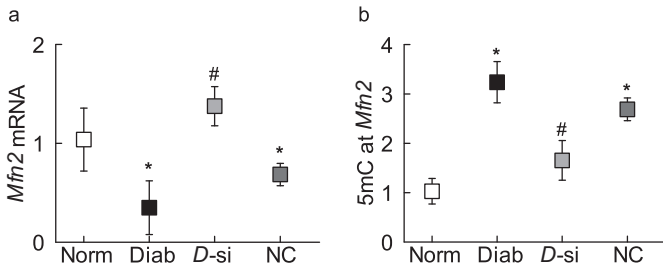


Fig. 7. Regulation of DNA methylation of *Mfn2* promoter. Retinal microvessels from the eye of diabetic rats receiving intravitreal administration of *Dnmt1*-siRNA (*D*-si) or negative control siRNA (NC) were analyzed for (a) *Mfn2* gene transcripts by qPCR and (b) 5mC levels at *Mfn2* promoter by methylated DNA immunoprecipitation method. Values are calculated as fold change as compared to normal and are represented as mean ± SD. *D*-si and NC = diabetic rats receiving intravitreal injection of *Dnmt1*-siRNA and GC content siRNA negative control respectively. $*p < 0.05$ compared to normal and $\#p < 0.05$ compared to diabetes or negative control.

hyperglycemic milieu, the binding of the transcription factors is decreased, suppressing its gene transcription. Results from the experimental models of diabetic retinopathy are further supported by significant reduction in Mfn2 and increase in its promoter DNA methylation in the retinal microvasculature from human donors with documented diabetic retinopathy. Taken together, this study shows a significant role of *Mfn2* in mitochondrial homeostasis in diabetic retinopathy, and epigenetic modification, especially DNA methylation, in its suppression.

Mitochondria are double membrane organelle, and they share proteins, membranes, solutes and metabolites through dynamic fusion-fission process. Fusion is a two-step process; the outer mitochondrial membrane fusion is controlled by Mfn1 and Mfn2, and the inner membrane by Opa1 1 [13]. *Mfn1* and *Mfn2* share approximately 80% similarity [36], and Mfn1-dependent mitochondrial dynamics is required for glucose sensing and insulin release in neurons [37], Mfn2 is a multifunctional protein and its expression is ubiquitous in mammalian tissues. Furthermore, tissues enriched in mitochondria including neuronal tissues have higher levels of Mfn2 compared to less metabolic tissues [35,38]. In hyperglycemic milieu, while Mfn2 is decreased in the retina and its mitochondria, Mfn1 levels remain unchanged [16]. Here, we show that Mfn2 is also decreased in the retinal microvessels in hyperglycemia, human donors with documented diabetic retinopathy also have decreased Mfn2 in their microvasculature compared to the age-matched nondiabetic donors. In addition, mitochondria are fragmented, and overexpression of *Mfn2* prevents mitochondrial fragmentation, confirming an integral role of Mfn2 in mitochondrial structural damage.

In diabetes, retinal mitochondria become swollen and their membrane potential is decreased, superoxide levels are elevated, and

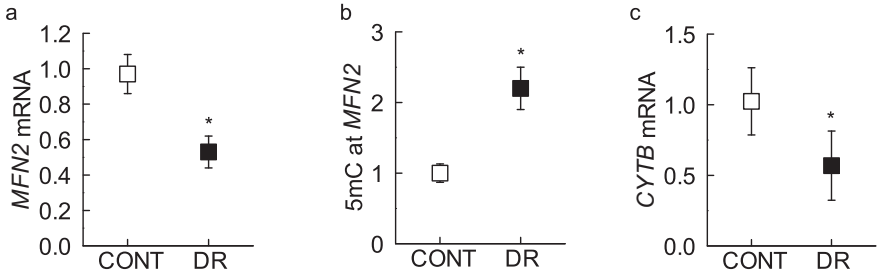


Fig. 8. MFN2 and human donors with diabetic retinopathy. Retinal microvessels from human donors with documented diabetic retinopathy (DR) and age-matched nondiabetic donors (CONT) were analyzed (a) for *MFN2* mRNA by qPCR using *β-ACTIN* as a housekeeping gene. (b) 5mC levels were quantified at *MFN2* promoter using MeDIP Kit. (c) Gene transcripts of mtDNA-encoded *CYTB* were quantified. Values are presented a fold change as compared to non-diabetic donors and are mean ± SD of at least six donors in each group, with each measurement was made in duplicate. *$p < 0.05$ compared to CONT.

complex III activity is decreased [30]. Here we show that overexpression of *Mfn2* alleviates diabetes-induced increase in superoxide radicals and mitochondrial dysfunction. It prevents alteration in mitochondrial membrane potential and complex III activity. In support, Mfn2 is also considered essential in maintaining mitochondrial energy metabolism and coenzyme Q levels, and Mfn2 deficiency is implicated with increased superoxide levels and impaired mitochondrial permeability and respiration and decrease ATP production [35,39,40].

Impairment in mitochondrial fusion is implicated in release of cytochrome *c* from mitochondria and accelerating the apoptotic machinery [41]. In the pathogenesis of diabetic retinopathy, mitochondrial dysfunction-accelerated capillary cell apoptosis precedes the development of histopathology characteristic of diabetic retinopathy [42,43]. Our results demonstrate that overexpression of *Mfn2* also attenuates increase in capillary cell apoptosis experienced by cells in hyperglycemic milieu, further supporting the role of Mfn2 in diabetic retinopathy.

Mitochondrial fusion, which allows the exchange of contents including DNA and metabolites between neighboring mitochondria, also preserves mtDNA function; abnormalities in mitochondrial fusion are implicated in mtDNA instability [32]. Loss in mitofusins is shown to cause mtDNA mutations and deletions in muscle atrophy [35]. *Mfn2* mutations are also shown to affect mtDNA replication, and alter mitochondrial oxidative phosphorylation [44]. Damaged mtDNA results in impaired transcription [34], and mitochondrial fusion helps maintain mtDNA [45], and a link between Mfn2 and mtDNA-encoded electron transport chain components is seen in neuropathy [46]. Our results demonstrate that overexpression of *Mfn2* prevents hyperglycemia-induced mtDNA damage, and also ameliorates decrease in expression of mtDNA-encoded genes including *CytB* of complex III of the electron transport chain.

We recognize that both Mfn1 and Mfn2 play role in outer mitochondrial membrane fusion, these two Mfns share many common functions and Mfn1 has higher GTPase activity [47]. However, while Mfn1 is implicated in increased susceptibility to certain pathogen infections, its mutations have not been associated with diseases [48]. In contrast, mutations in Mfn2 are associated with many diseases, and Mfn2 itself is necessary and sufficient to modulate mitochondrial metabolism by regulating mitochondrial fuel oxidation, membrane potential and oxidative phosphorylation [15,36]. Furthermore, our initial mitochondrial PCR array data in rat retina, and retina from human donors, have shown significant decrease in Mfn2 expression in diabetes [16]. However, in the same samples, decrease in Mfn1 was > 15% and was not significant compared to their age-matched nondiabetic controls, further supporting the role of Mfn2 in diabetic retinopathy.

Epigenetic modifications, the modifications that regulate gene expression without affecting DNA sequence, have been implicated in the development of diabetic retinopathy [19]. Methylation of cytosine in DNA, a repressive modification, by DNA methyltransferases, is a key component of the epigenetic machinery, and altered patterns of DNA methylation are observed in many diseases [49]. DNA methylation enzyme machinery is activated in the retina and its vasculature in diabetes, and while the levels of methylated cytosine are increased at the promoter of polymerase gamma (an enzyme responsible for replication of mtDNA), that of hydroxymethylated cytosine are increased at the promoters of *MMP-9* and *Rac1*, resulting in their gene suppression and activation respectively [5,31]. *Mfn2* promoter (human and rats) is rich in CpG islands [50], and here our results show it is hypermethylated in diabetes; 5mC levels are elevated at its promoter, and the binding of Dnmt1, the only isoform of the Dnmt family upregulated in hyperglycemic milieu [23], is increased. Regulation of Mfn2 expression-DNA methylation by pharmacological and molecular inhibitors of Dnmts, further supports the role of DNA methylation in Mfn2 expression. Binding of the transcription factor SP1 is considered essential for normal transcription of *Mfn2*, and this TATA-less human *MFN2* promoter has multiple SP1 binding sites [51]. We show that high glucose also decreases binding of SP1 at *Mfn2* promoter, and this can be regulated by both azacytidine and *Dnmt1*-siRNA, further supporting the role of DNA methylation in transcriptional activation of *Mfn2*. The role of DNA methylation in *Mfn2* regulation is further confirmed by increased 5mC levels and Dnmt1 binding in the retinal microvessels from diabetic rats, and also from human donors with diabetic retinopathy. *Mfn2* promoter has at least six transcription start sites [51], and our results show that in rat *Mfn2* promoter, −245 to −84 region had the highest level of 5mC and Dnmt1 binding (and the lowest SP1 binding) compared to other regions, suggesting that this ~150 bp promoter region (upstream of the transcription start site) is important in regulation of *Mfn2* expression in diabetes. Consistent with this, others have shown that the promoter activity in rat vascular smooth muscle cells is suppressed by deletion of the region between −229 and −54, suggesting the importance of this region of the core *Mfn2* promoter [51].

Our study is focused on the role of Mfn2 in mitochondrial homeostasis, and we recognize that both fusion and fission are critical in maintaining mitochondrial structural and functional homeostasis. In addition, many epigenetic modifications including histone modifications and DNA methylation can work in concordance to regulate a single gene expression [24,52]. The role of fission in mitochondrial homeostasis, and possibility of other epigenetic modifications and micro RNAs regulating *Mfn2* expression in diabetes, cannot be ruled out. Furthermore, this study focused on mitochondrial fission in retinal microvasculature, however, photoreceptors are very rich in mitochondria, and are considered to play a major source of ROS production in diabetic retinopathy [53]; similar mechanism operating in photoreceptors remains to be investigated.

In conclusion, this is the first report showing the role of mitofusin in diabetic retinopathy; decreased levels of Mfn2 in retinal vasculature in diabetes, in addition to affecting the mitochondrial morphology, also contribute to mitochondrial ROS accumulation, mtDNA damage and a dysfunctional electron transport chain. Regulation of Mfn2 protects mitochondrial structural and functional abnormalities, and prevents electron transport chain damage and accumulation of free radicals, experienced in hyperglycemic milieu. Our results also imply the role of epigenetics in transcriptional regulation of *Mfn2* in diabetes, and show DNA hypermethylation of its promoter. Mitochondrial dysfunction is intimately associated in the development of diabetic retinopathy; our study provides a new and 'untested' avenue to regulate mitochondrial homeostasis, and prevent possible loss of vision in diabetic patients.

Conflict of interest

No financial interests for Arul J. Duraisamy, Ghulam Mohammad or Renu A. Kowluru.

Transparency document

The Transparency document associated this article can be found, in online version.

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