Down Syndrome Critical Region 1 Reduces Oxidative Stress–Induced Retinal Ganglion Cells Apoptosis via CREB–Bcl-2 Pathway

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Purpose. Irreversible retina ganglion cell (RGC) loss is a key process during glaucoma progression. Down syndrome critical region 1 (DSCR1) has been shown to have protective effects against neuronal death. In this study, we aimed to investigate the neuroprotective mechanisms of DSCR1 on RGCs.

Methods. DBA/2J mice and optic nerve crush (ONC) rat model were used for vivo assays. Oxidative stress model of primary RGCs was carried out with in vitro transduction. DSCR1 protein localization was assessed by immunofluorescence. Differential protein expression was validated by Western blot, and gene expression was detected by real-time PCR. TUNEL was used to identify cell apoptosis, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to analyze cell viability.

Results. Significant upregulation of DSCR1 was observed in DBA/2J mice, ONC rat model, and RGCs treated with H_2O_2, reaching peaks at the age of 6 months in DBA/2J mice, 5 days after ONC in rats, and 24 hours after H_2O_2 treatment in RGCs, respectively. DSCR1 was shown to be expressed in the ganglion cell layer. In vitro, overexpressed DSCR1 significantly promoted phosphorylation of cyclic AMP response element binding protein (CREB), B-cell lymphoma 2 (Bcl-2) expression, and RGC survival rate while reducing cleaved caspase 3 expression in H_2O_2-treated RGCs. On the other hand, the opposite effects were shown after knockdown of DSCR1. In addition, silencing of CREB inhibited expression of DSCR1.

Conclusions. Our results suggested that DSCR1 might protect the RGCs against oxidative stress via the CREB–Bcl-2 pathway, which may provide a theoretical basis for future treatments of glaucoma.

Keywords: DSCR1, retina ganglion cells, oxidative stress, CREB–Bcl-2 pathway, apoptosis

Glaucoma, the most prevalent neurodegenerative eye disease and a main causes of irreversible vision loss worldwide, is characterized by progressive apoptosis of retinal ganglion cells (RGCs).[1,2] Development of glaucoma is considered multifactorial, with aging, decreased blood flow, mitochondrial dysfunction, and oxidative stress all contributing to disease occurrence.[3] However, current treatments that act to lower intraocular pressure (IOP) do not always sufficiently prevent neuropathy in patients with glaucoma. Thus, further research is necessary to explore molecular mechanisms of glaucoma to develop alternative treatments to prevent vision deterioration.[4,5]

Down syndrome critical region 1 (DSCR1), a gene located on chromosome 21, has been shown to be involved in the pathogenesis of Down syndrome.[6] Previous studies have indicated that DSCR1 is widely expressed in the nervous system, with elevated DSCR1 levels preventing oxidative stress–induced neuronal apoptosis in the peri-infarct cortex after experimental stroke.[7–9] Furthermore, our previous study demonstrated that DSCR1 was expressed in the retina of a hypoxic-ischemic mouse model.[10] Moreover, the expression of DSCR1 has been shown to be upregulated by neuronal adaptation to oxidative stress.[11] As a major transcriptional regulator of gene expression, cyclic AMP response element binding protein (CREB) is necessary for the development and function of the nervous system. The activity of CREB was disturbed when mutations occur in *nebula*, the *Drosophila* homolog of human DSCR1.[12] However, the expression of DSCR1 in the retina exposed to oxidative stress remains unclear, and whether the regulation of DSCR1 on the pathogenesis of glaucoma through the activation of CREB also requires further study.

Therefore, in the present study, we aimed to investigate expression levels and distribution of DSCR1 in retinas of glaucomatous animal models at different time points. Additionally, we also explored the possible role and underlying mechanisms of DSCR1 in RGCs based on the H_2O_2-induced oxidative stress model.

Materials and Methods

Reagents

Fetal bovine serum (FBS) and neurobasal cell medium were purchased from Gibco BRL (Grand Island, NY, USA). Brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), papain, poly-L-lysine, forskolin, BSA, collagenase and ovomucoid were purchased from Sigma Chemical Company (St. Louis, MO, USA). B27 supplement was purchased from Invitrogen Life Technologies Company (Carlsbad, CA, USA). Anti-CREB (#9197) and anti-phospho-CREB (p-CREB, #9198) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti–B-cell lymphoma 2 (Bcl-2, #ab182858), anti–B-cell lymphoma extra large (Bcl-xl, #ab32370), anti–caspase 3 (#ab32351), OX-42 (#ab33827), and anti–cleaved caspase 3 (#ab2302) were purchased from Abcam (Cambridge, UK). Anti–tubulin $\beta 3$ (#801202) and Thy-1.1 (#202508) were purchased from BioLegend (San Diego, CA, USA). DSCR1 (#sc-377507) and Brn3a (#sc-31984) were purchased from Santa Cruz (Dallas, TX, USA). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Animals and Procedures

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Review Board of Zhongshan Ophthalmic Center (Guangzhou, China). DBA/2J mice, C57BL/6J mice, and Sprague-Dawley (SD) rats were purchased from the Animal Laboratory of Zhongshan Ophthalmic Center. C57BL/6J mice were used as the control group and source of primary RGCs. SD rats were used in optic nerve crush (ONC)–related experiments. All animals were kept in identical housing conditions with a controlled temperature of 20–26°C, a humidity of 40%–70%, and 14 hours of light per day. The ONC model was established in SD rats as previously described.[13,14] In brief, rats were sedated by intraperitoneal injection with 10% chloral hydrate. A 4-mm length of optic nerve was exposed and crushed for 10 seconds at 2 mm behind the optic nerve head. Funduscopy was used to examine the integrity of retinal blood vessels. Any rat with vascular damage was excluded from the study.

Purification and Identification of Primary RGCs

RGCs were isolated and purified as previously described.[10,15] In brief, retinas harvested from C57BL/6J mice at postnatal day 1 or day 2 were incubated in PBS containing papain and collagenase. A completely dissociated retinal suspension was prepared by trituration with an arrow-bore Pasteur pipette and centrifugated at 1500 rpm for 5 minutes. Cells were then rewashed and resuspended. The cell suspension was incubated in flasks coated with OX-42 (1:100) at 37°C for 30 minutes. The suspension was shaken gently every 20 minutes to remove all adherent cells. Nonadherent cells were placed in Thy-1.1 (1:100)–coated flasks and incubated for 50 minutes. The cells were washed gently with PBS for 5 times. Finally, the adherent cells in the Thy-1.1–coated flasks were washed with 10 mg/mL ovomucoid and 10 mg/mL BSA solution and centrifuged at 1500 rpm for 5 minutes before inoculation in poly-L-lysine–coated flasks.

Primary RGC Cultures

Purified primary RGCs were plated into 6-well plates precoated with poly-L-lysine and cultured in 1 mL neurobasal growth medium supplemented with penicillin, streptomycin, CNTF, BDNF, 10% FBS, forskolin, and B27 at 37°C in a humidified atmosphere with 5% CO_2. Previous studies have shown that the viability of cells significantly decreased when exposed to H_2O_2 at a concentration over 100 µM.[16–18] Thus, the concentration of H_2O_2 was maintained at 100 µM.

Lentiviral Transduction

Lentivirus particles, including DSCR1 short hairpin RNA (shRNA) lentivirus, DSCR1 lentivirus, CREB shRNA lentivirus, and empty Puro lentivirus, were designed and purchased from Genechem (Shanghai, China). The sequence of the mouse DSCR1 shRNA vector was GCTTCAAACGTGTCCGGATAACTCGAGTTATCCGGACACGTTTGAAGC. The mouse DSCR1 lentivirus vector was designed according to the opening reading frame of DSCR1 (NM_001081549.2). The sequence of the mouse CREB shRNA vector was GGAGTCTGTGGATAGTGTAACCTCGAGGTTACACTATCCACAGACTCC. Puromycin (2 µg/mL; Northern Marianas, Saipan, MP, USA) was used to identify the stable cells at day 3 after transduction. Western blot was used to confirm the efficiency of transduction. H_2O_2 was then introduced into transduced RGCs, which were incubated for another 12 hours.

TUNEL Staining

TUNEL staining was performed using a commercially available kit (In Situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. Apoptotic cells were visualized with fluorescence microscopy (Leica Microsystems, Mannheim, Germany). Four fluorescent nonoverlapping representative images per slide were captured and quantified using Image ProPlus software (Media Cybernetics, Rockville, MD, USA).

Immunohistochemistry

Paraffin-embedded sections were incubated with Bcl-2 (1:200) and Bcl-xl (1:200) antibodies at 4°C overnight, followed by secondary antibodies and diaminobenzidine at room temperature for visualization. Photography was obtained using the microscopy (Leica, Frankfurt, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MTT reagent from Cell Quaint-MTT Assay Kits (BioAssay Systems, Hayward, CA, USA) was used to evaluate viability of RGCs treated with H_2O_2. The cells were incubated with MTT for 4 hours at 37°C, followed by MTT solubilization solution for 1 hour at room temperature. The colorimetric analysis was performed with a wavelength of 570 nm. Cell viability was presented as the proportion of the control optical density.

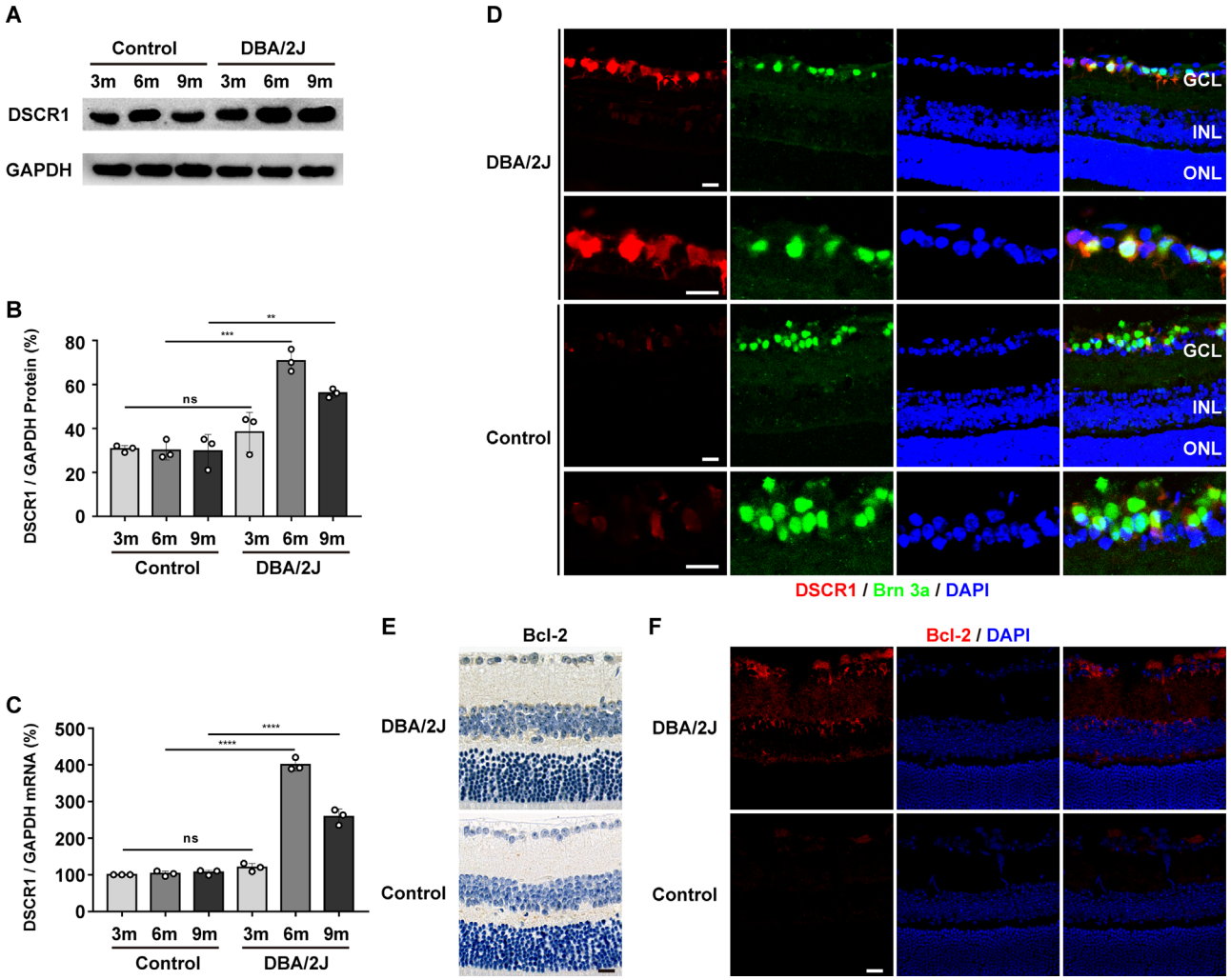

Figure 1. The expression of DSCR1 in the retina of DBA/2J mice. (**A–C**) The protein expression and mRNA level of DSCR1 were evaluated by Western blot ($n = 6$) and real-time PCR ($n = 6$) analysis at different time points in C57BL/6J and DBA/2J mice and then quantified. The expression of DSCR1 was significantly upregulated in DBA/2J mice at the age of 6 months and 9 months compared to the control group. The graphs are presented as mean ± SEM of three independent experiments. (**D**) Representative image of immunofluorescent staining with DSCR1 (red fluorescence) and retinal ganglion cell marker Brn3a (green fluorescence) in each group ($n = 3$). The colocalization of increased DSCR1 and Brn3a was detected in GCL at the age of 6 months in DBA/2J mice. (**E, F**) Representative immunohistochemistry and immunofluorescent images showed the expression of Bcl-2 in each group ($n = 3$). The increased expression of Bcl-2 was detected in GCL at the age of 6 months in DBA/2J mice. INL, inner nuclear layer; ONL, outer nuclear layer. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *Scale bar*: 10 µm.

Western Blot Analysis

The experimental procedure was performed as previously described.[19,20] In brief, retinas or RGCs were harvested and lysed with a radioimmunoprecipitation assay containing protease inhibitors. A total 20 µg of proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride filter membrane (Millipore, Temecula, CA, USA). Membranes were preincubated with 5% defatted milk for 1 hour at room temperature, then incubated with DSCR1 (1:400), caspase 3 (1:1000), cleaved caspase 3 (1:1000), CREB (1:1000), p-CREB (1:1000), Bcl-xl (1:1000), and Bcl-2 (1:2000) antibodies in defatted milk at 4°C overnight. Membranes were then incubated with secondary antibody (horseradish peroxidase antibody; 1:5000) for 2 hours at room temperature. Finally, an enhanced chemiluminescence system (Millipore) was used to visualize horseradish peroxidase (HRP) activity.

Real-Time PCR Analysis

Total RNA was extracted from retinas or RGCs using Trizol (Gibco BRL) and converted into cDNA with the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). SYBR Green Master mix (Qiagen, Redwood City, CA, USA) was used according to the manufacturer's instructions. Relative mRNA levels were quantified by the ΔΔCt method. The experiments were carried out in triplicate and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR primers were designed on the basis of the NCBI Gene Bank database, with primers of target genes as follows: rat DSCR1 (forward: 5′-CAGCGAAAGTGAGACCAGG-3′; reverse: 5′-GTAAAGTCTGGGCAAAGTACAGTT-3′), mouse DSCR1 (forward: 5′-CAAGACCGAGTTCCTGGGGA-3′; reverse: 5′-GTTCATACTTCTCTCCTGGCCCC-3′), mouse

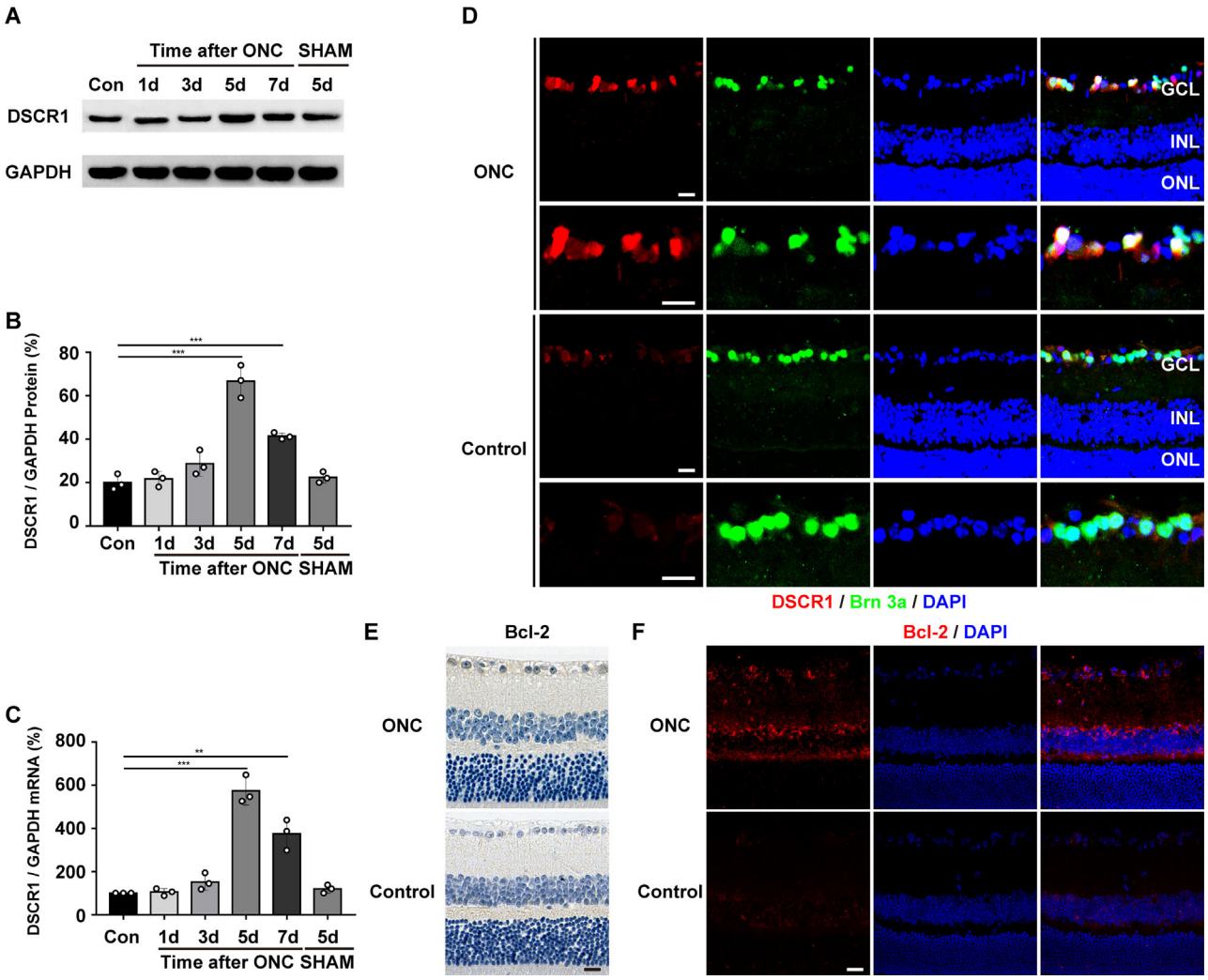

Figure 2. The expression of DSCR1 in the retina of SD rats after ONC. (**A–C**) The protein expression and mRNA level of DSCR1 were evaluated by Western blot ($n = 6$) and real-time PCR ($n = 6$) analysis at different time points after ONC and then quantified. The expression of DSCR1 was significantly upregulated at day 5 and day 7 after ONC. The graphs are presented as mean ± SEM of three independent experiments. (**D**) Representative image of immunofluorescent staining with DSCR1 (red fluorescence) and Brn3a (green fluorescence) in each group ($n = 3$). The colocalization of increased DSCR1 and Brn3a was detected in GCL on day 5 after ONC. (**E, F**) Representative immunohistochemistry and immunofluorescent images showed the expression of Bcl-2 in each group ($n = 3$). The increased expression of Bcl-2 was detected in GCL on day 5 after ONC. $^{**}P < 0.01$, $^{***}P < 0.001$. *Scale bar*: 10 µm.

TNF-α (forward: 5′-GTCCCCAAAGGGATGAGAAGT-3′; reverse: 5′-TTTGCTACGACGTGGGCTAC-3′), mouse IL-6 (forward: 5′-TGGAGTACCATAGCTACCTGGA-3′; reverse: 5′-GGAGAGCATTGGAAATTGGGG-3′), rat GAPDH (forward: 5′-GGGTGTGAACCACGAGAAT-3′; reverse: 5′-ACTGTGGTCATGAGCCTTC-3′), and mouse GAPDH (forward: 5′-AATGTGTCCGTCGTGGATCTGA-3′; reverse: 5′-GATGCCTGCTTCACCACCTTCT-3′).

Immunofluorescence

Cryosections and RGCs were incubated with the following primary antibodies: DSCR1 (1:50), Brn3a (1:50), and tubulin β3 (1:500) at 4°C overnight. Specimens were then treated with a mixture of fluorescein isothiocyanate- or CY3-conjugated secondary antibodies and DAPI at room temperature for 2 hours. Photography was obtained with confocal microscopy (Leica).

Statistical Analysis

All experimental data were analyzed using GraphPad Prism (v6.0; GraphPad Software, La Jolla, CA, USA) and are presented as mean ± SEM. Statistical significance was determined using independent samples *t* test or one-way ANOVA. *P* value less than 0.05 was regarded as statistically significant.

Results

Changes of the DSCR1 Expression in the Retina of DBA/2J Mice

It is widely known that DBA/2J mice are common animal models of glaucoma.[21–23] To investigate whether the expression of DSCR1 changed in the retina of DBA/2J mice at different time points, Western blot was performed to detect

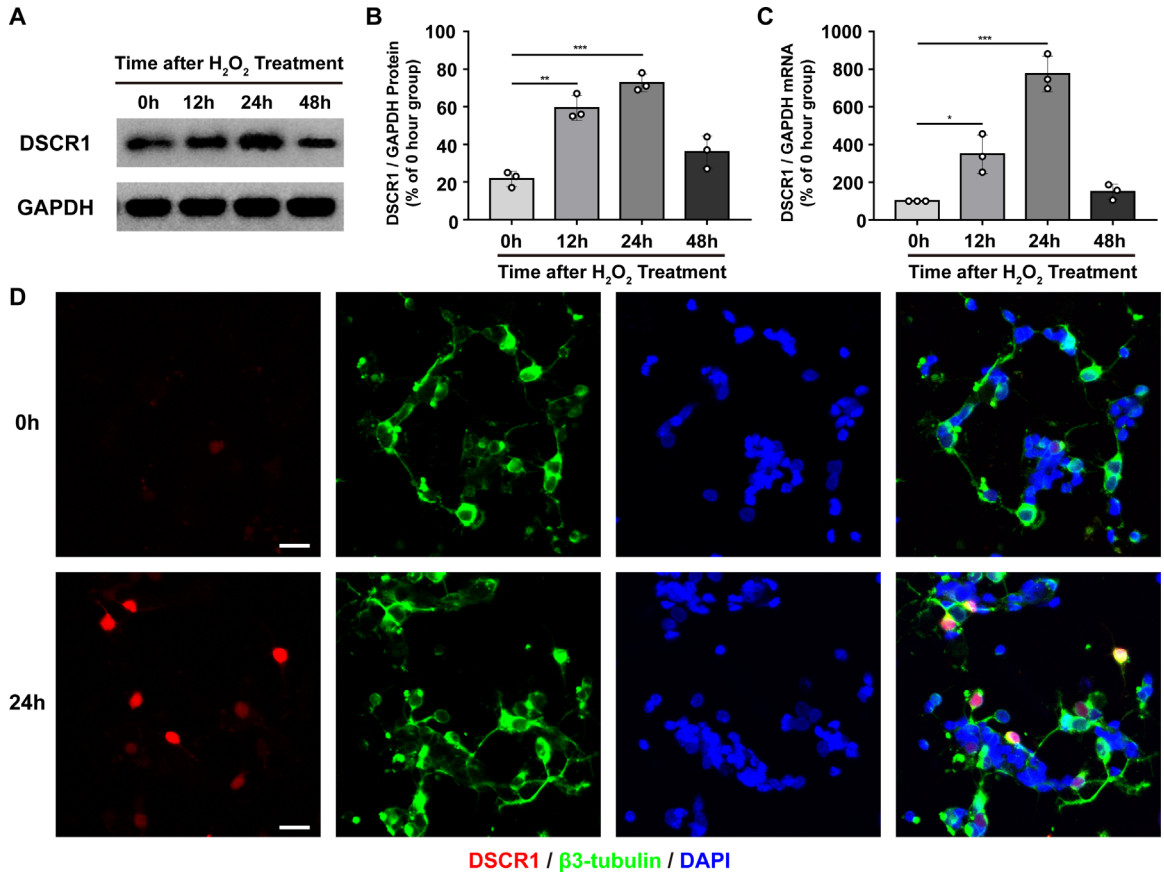

Figure 3. The expression of DSCR1 in the RGCs after the exposure of H_2O_2. (**A–C**) The protein expression and mRNA level of DSCR1 in the RGCs were evaluated by Western blot ($n = 12$) and real-time PCR ($n = 12$) analysis at different time points after H_2O_2 treatment and then quantified. The expression of DSCR1 significantly increased after exposing to H_2O_2. The graphs are presented as mean ± SEM of three independent experiments. (**D**) Representative image of immunofluorescent staining with DSCR1 (red fluorescence) and β3-tubulin (green fluorescence) in each group ($n = 6$). The colocalization of increased DSCR1 and β3-tubulin was detected in RGCs at 24 hours. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Scale bar*: 25 µm.

the DSCR1 expression in the retina of C57BL/6J mice and DBA/2J mice at 3, 6, and 9 months of age. DSCR1 is known to have two isoforms, DSCR1.1 (37 KD) and DSCR1.4 (28 KD). According to the DSCR1 antibody instructions and our results, the main isoform of DSCR1 in the retina was DSCR1.4. As shown in Figures 1A, 1B, the expression of DSCR1 was remarkably upregulated in DBA/2J mice at 6 and 9 months of age compared to the control group, while no significant alteration was observed at the age of 3 months between two groups. The Western blot results were consistent with those of PCR (Fig. 1C). To further address the location of DSCR1 in the retina, double immunofluorescent staining was performed. The colocalization of increased DSCR1 and the RGC marker, Brn3a, were detected in the ganglion cell layer (GCL) at the age of 6 months in DBA/2J mice (Fig. 1D).

Changes of the DSCR1 Expression in the Retina After ONC

The ONC animal model has been widely used to study oxidative stress–induced RGC apoptosis.[24,25] The role of DSCR1 in the retina after ONC was assessed in the current study. The protein level of DSCR1 in the retina was gradually upregulated starting on day 1, with peak levels reached at day 5, followed by declining levels (Figs. 2A, 2B). Meanwhile, the mRNA level of DSCR1 corresponded with the protein level (Fig. 2C). Additionally, double immunofluorescent staining showed colocalization of increased DSCR1 and Brn3a in the GCL on day 5 after ONC (Fig. 2D).

Changes of the DSCR1 Expression in RGCs After H_2O_2 Treatment

The expression of DSCR1 in RGCs exposed to H_2O_2-induced oxidative stress was further verified. As shown in Figures 3A, 3B, the protein expression of DSCR1 increased progressively over time, reaching a peak at 24 hours after H_2O_2 exposure, with a similar pattern for DSCR1 mRNA levels (Fig. 3C). Furthermore, significantly stronger immunofluorescent staining of DSCR1 was observed at 24 hours compared to 0 hours in the H_2O_2-treated group (Fig. 3D).

DSCR1 Promoted RGC Survival After H_2O_2 Treatment

HA-tagged or sh-tagged DSCR1 expression vectors were transduced into the primary RGCs with verification of

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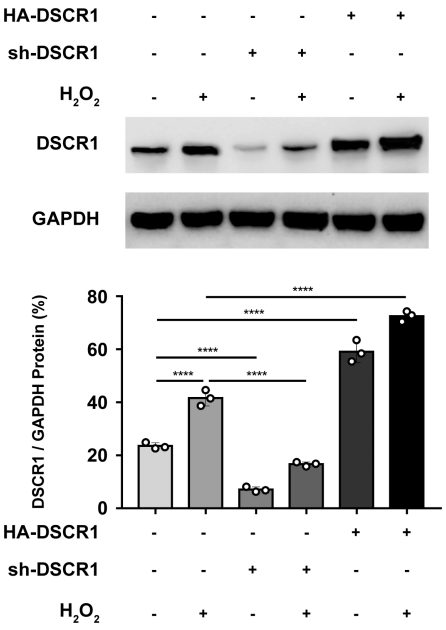

Figure 4. The expression of DSCR1 after knockdown or overexpression of DSCR1 under the H_2O_2 condition. RGCs were transduced with the sh-tagged DSCR1 or HA-tagged DSCR1 expression vector and were selected in neurobasal growth medium with H_2O_2 for 12 hours ($n = 12$). The stable DSCR1 expression was identified by Western blot and then quantified. In RGCs transduced with the sh-tagged DSCR1 vectors, the expression of DSCR1 decreased. In RGCs transduced with the HA-tagged DSCR1 vectors, the expression of DSCR1 increased. The graphs are presented as mean ± SEM of three independent experiments. ****$P < 0.0001$.

functionality using Western blot. As shown in Figure 4, decreased expression of DSCR1 was observed in RGCs after transduction of sh-tagged vector, with increased levels of DSCR1 after transduction of HA-tagged vector. Thus, confirmation of both a stable DSCR1 knockdown and DSCR1 overexpression model in RGCs was achieved.

The effect of DSCR1 on RGC survival was then evaluated using the MTT assay. In both DSCR1 knockdown and overexpression control groups, the viability of RGCs was stable (Figs. 5A, 5B). After H_2O_2 treatment, however, overexpression of DSCR1 in RGCs was shown to increase the RGC survival rate, and this effect lasted for at least 48 hours (Figs. 5C, 5D).

To further determine whether DSCR1 could promote RGC survival via inhibition of apoptosis, TUNEL staining was used. The apoptotic ratio of RGCs increased after stimulation by H_2O_2. RGCs treated with sh-tagged DSCR1 vectors showed a higher apoptotic ratio than the nontransduced group (Fig. 6A). On the contrary, a less apoptotic ratio was detected when RGCs were transduced with the HA-tagged DSCR1 vectors compared to the nontransduced group after H_2O_2 exposure (Fig. 6B).

DSCR1 Reduced the Expression of Cleaved Caspase 3 in RGCs After H_2O_2 Treatment

It has been confirmed that caspase 3 can elicit oxidative stress–induced apoptosis.[26–28] Thus, to assess whether the protective effect of DSCR1 on H_2O_2-mediated toxicity in RGCs was accompanied by inhibition of the caspase 3 pathway, stable DSCR1 knockdown and overexpression RGC models were used. As shown in Figure 7, cleaved caspase 3 expression in RGCs increased after exposure to H_2O_2. The RGCs transduced with sh-tagged DSCR1 had higher cleaved caspase 3 expression in response to H_2O_2 than the nontransduced group, while the expression of cleaved caspase 3 in RGCs exposed to oxidative stress decreased after being transduced with the HA-tagged DSCR1 vectors.

DSCR1 Enhanced the CREB Activity and Increased Bcl-2 Expression in RGCs After H_2O_2 Treatment

In many pathologic conditions, activation of CREB is essential for maintaining cell survival.[29] In order to further elucidate whether the protective role of DSCR1 in RGCs under oxidative stress depended on CREB phosphorylation, Western blot was performed in RGCs exposed to H_2O_2. As shown in Figure 8, p-CREB in RGCs was enhanced after exposure to H_2O_2. There was also an increase in the basal level of p-CREB in the stable DSCR1-overexpressed RGCs; however, the level of p-CREB was decreased in RGCs when DSCR1 was knocked down.

Since Bcl-2 contains a CREB-binding site and has a protective effect on cell injury,[30,31] we investigated whether transcription of Bcl-2 occurred after the activation of CREB by DSCR1. As shown in Figure 9, Bcl-2 expression in RGCs increased after exposure to H_2O_2. Moreover, expression of Bcl-2 was significantly enhanced in the stable DSCR1-overexpressed RGCs and decreased in RGCs after knockdown of DSCR1. Next, we validated whether upregulated expression of DSCR1 affected Bcl-2 expression in vivo. Immunohistochemistry and immunofluorescence staining showed that Bcl-2 expression was significantly increased in the retina of DBA/2J mice at the age of 6 months and on day 5 after ONC (Figs. 1E, 1F and Figs. 2E, 2F).

As another member of the Bcl-2 family, Bcl-xl has also been shown to be induced through phosphorylation of CREB, with subsequent promotion of cell survival.[32] Thus, we explored the involvement of Bcl-xl in this process. As shown in Supplementary Figures S1 and S2, the expression of Bcl-xl in RGCs increased after ONC and H_2O_2 treatment. In addition, the expression of Bcl-xl was increased in HA-tagged DSCR1-transduced RGCs and decreased in sh-tagged DSCR1-transduced RGCs.

Knockdown of CREB Suppressed the DSCR1 Activity in RGCs After H_2O_2 Treatment

To further clarify the relationship between CREB and DSCR1, we knocked down the CREB gene in RGCs with the sh-tagged CREB expression vectors. As shown in Figures 10A–C, the DSCR1 expression in RGCs increased after exposure to H_2O_2. The expression of DSCR1 in sh-CREB–transduced RGCs was significantly reduced in comparison to the nontransduced group. The results suggested the existence of a DSCR1-CREB loop in RGCs, which might transmit positive feedback signals to the downstream factors. Furthermore, Bcl-2 expression and viability of RGCs decreased in sh-tagged CREB-transduced RGCs. In addition, expression of Bcl-2 and viability of sh-tagged CREB-transduced RGCs did not change after transduction of HA-tagged DSCR1 vectors (Figs. 10D–F). Interestingly, although many studies have shown that DSCR1 exerts an anti-inflammatory effect on the nervous system,[6,9,33] the mRNA levels of TNFα and IL-6 did not change in H_2O_2-treated RGCs after interfering with

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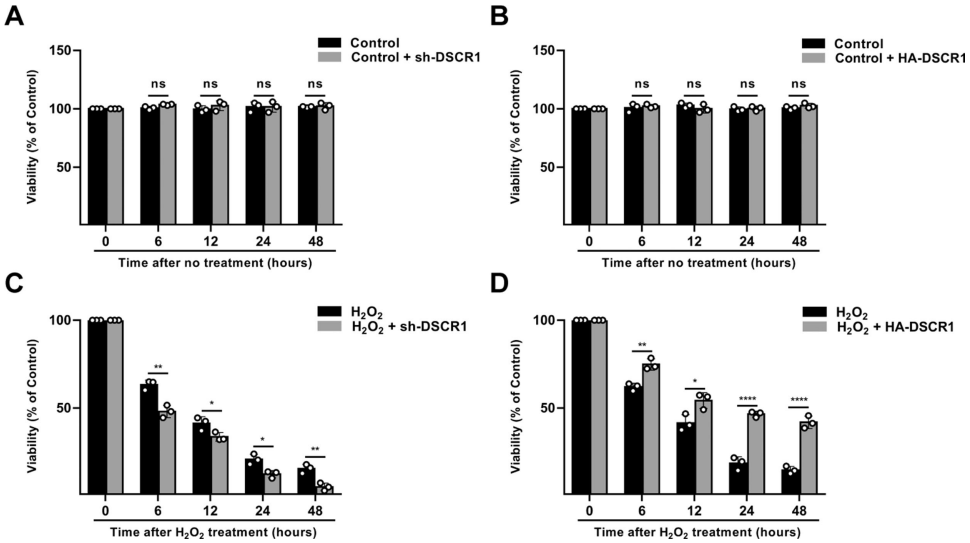

Figure 5. DSCR1 enhances the viability of RGCs after H_2O_2 treatment. The DSCR1 stable knockdown or overexpression RGCs were treated with H_2O_2 and the viability of RGCs was determined using MTT assay ($n = 12$). (**A, B**) Graph for RGC survival rate shows no differences in both DSCR1 knockdown and overexpression control groups. (**C, D**) Graph for RGC survival rate shows significant difference from 6 hours to 48 hours in both DSCR1 knockdown and overexpression RGCs treated with H_2O_2. The graphs are presented as mean ± SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

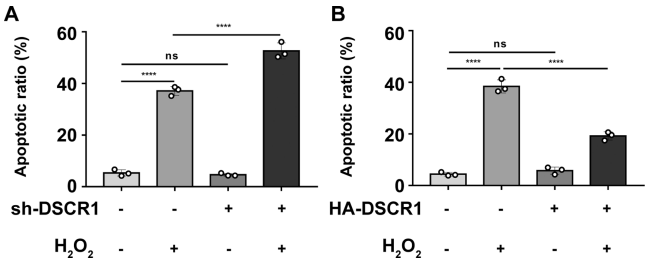

Figure 6. DSCR1 attenuates the apoptosis of RGCs after H_2O_2 treatment. The DSCR1 stable knockdown or overexpression RGCs were treated with H_2O_2 for 12 hours, and the apoptotic ratio of RGCs was determined using TUNEL staining ($n = 12$). (**A, B**) Graph for RGC apoptotic ratio shows significant difference in both DSCR1 knockdown and overexpression RGCs treated with H_2O_2. The apoptotic ratio of RGCs increased after exposure to H_2O_2. Higher apoptotic ratio was detected in RGCs treated with sh-tagged DSCR1 vectors when compared to the nontransduced group. Less apoptosis ratio was detected in RGCs treated with sh-tagged DSCR1 vectors when compared to the nontransduced group. The graphs are presented as mean ± SEM of three independent experiments. $^{****}P < 0.0001$.

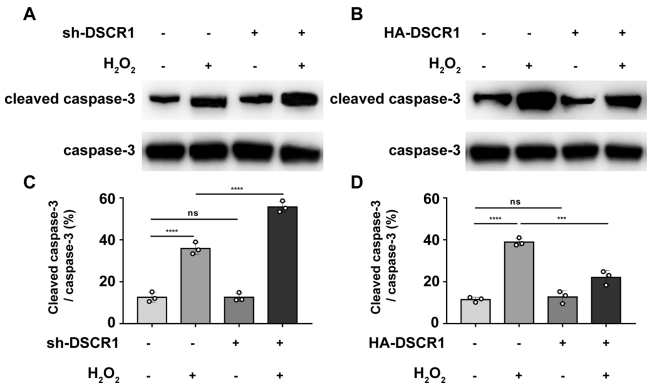

Figure 7. DSCR1 reduces the cleaved caspase 3 expression after H_2O_2 treatment. RGCs were transduced with the sh-tagged DSCR1 or HA-tagged DSCR1 expression vector and were cultured in neurobasal growth medium with H_2O_2 for 12 hours ($n = 12$). The caspase 3 and cleaved caspase 3 expression were identified by Western blot (**A, B**) and then quantified (**C, D**). The cleaved caspase 3 expression in RGCs increased after exposure to H_2O_2. RGCs transduced by sh-tagged DSCR1 had higher cleaved caspase 3 expression in response to H_2O_2 than the nontransduced group. RGCs transduced by HA-tagged DSCR1 had lower cleaved caspase 3 expression in response to H_2O_2 than the nontransduced group. The graphs are presented as mean ± SEM of three independent experiments. $^{***}P < 0.001$, $^{****}P < 0.0001$.

expression of DSCR1, suggesting that DSCR1 might play an antiapoptotic role in RGCs rather than an anti-inflammatory effect under oxidative stress (Supplementary Figure S3).

Discussion

In the present study, the DBA/2J mice, an ONC rat model, and an H_2O_2-induced RGC oxidative stress model were used to explore the potential role of DSCR1 in mediating oxidative stress–induced RGCs apoptosis. Our data revealed that DSCR1 was significantly upregulated in the retina in DBA/2J mice, post-ONC SD rats, and primary RGCs treated with H_2O_2. In addition, we found that DSCR1 increased RGC viability and reduced RGC apoptosis in response to oxidative stress, suggesting that DSCR1 may have neuroprotective effects on oxidative stress–induced RGC apoptosis. Meanwhile, DSCR1 produced by RGCs under oxidative stress enhanced phosphorylation of CREB and expression of Bcl-2. Additionally, expression of Bcl-2 and viability of RGCs did not change after transducing the HA-tagged DSCR1 vectors into sh-tagged CREB-transduced RGCs. These results indicated that the neuroprotective effect of DSCR1 was probably dependent on the CREB–Bcl-2 pathway.

DSCR1 has been shown to inhibit calcineurin, one of the characterized targets of which is the nuclear factor of

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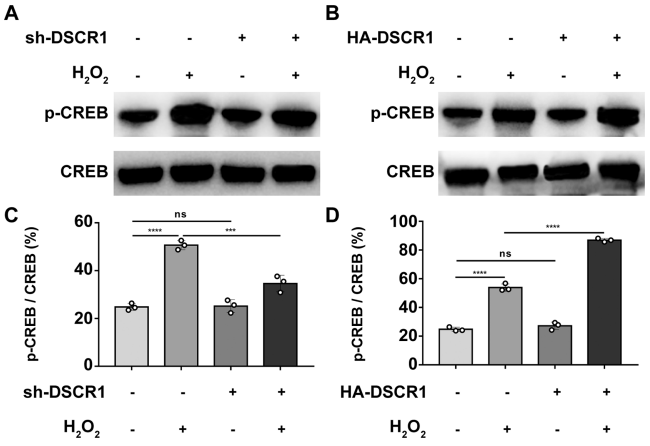

Figure 8. DSCR1 enhances CREB activity after H_2O_2 treatment. RGCs were transduced with the sh-tagged DSCR1 or HA-tagged DSCR1 expression vector and were cultured in neurobasal growth medium with H_2O_2 for 12 hours ($n = 12$). The CREB and p-CREB expression were identified by Western blot (**A, B**) and then quantified (**C, D**). The p-CREB expression in RGCs increased after exposure to H_2O_2. RGCs transduced by sh-tagged DSCR1 showed lower p-CREB expression in response to H_2O_2 than the nontransduced group. RGCs transduced by HA-tagged DSCR1 showed higher p-CREB expression in response to H_2O_2 than the nontransduced group. The graphs are presented as mean ± SEM of three independent experiments. ***$P < 0.001$, ****$P < 0.0001$.

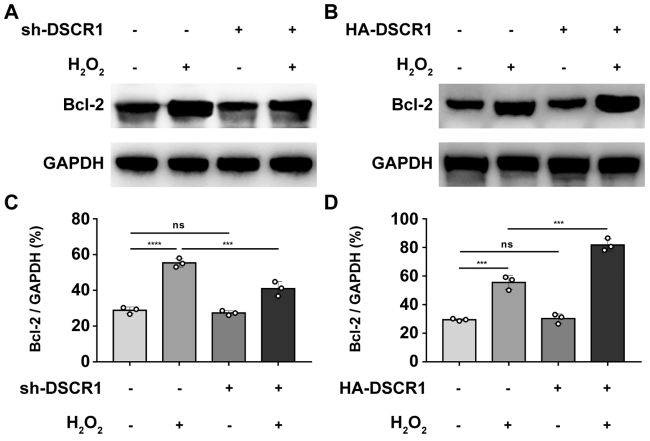

Figure 9. DSCR1 increases the Bcl-2 expression after H_2O_2 treatment. RGCs were transduced with the sh-tagged DSCR1 or HA-tagged DSCR1 expression vector and were cultured in neurobasal growth medium with H_2O_2 for 12 hours ($n = 12$). The Bcl-2 expression was identified by Western blot (**A, B**) and then quantified (**C, D**). The Bcl-2 expression of RGCs increased after exposure to H_2O_2. RGCs transduced by sh-tagged DSCR1 showed lower Bcl-2 expression in response to H_2O_2 than the nontransduced group. RGCs transduced by HA-tagged DSCR1 showed higher Bcl-2 expression in response to H_2O_2 than the nontransduced group. The graphs are presented as mean ± SEM of three independent experiments. ***$P < 0.001$, ****$P < 0.0001$.

activated T cells (NFAT). When calcineurin is activated, it dephosphorylates NFAT, which is then translocated into the nucleus and leads to transcription of proinflammatory and proapoptotic genes.[34] In addition, DSCR1 may also play an anti-inflammatory role by attenuating nuclear factor κB–mediated transcriptional activation.[35] It is known that DSCR1 is widely expressed in the brain, with functional impairments occurring secondary to DSCR1 defects.[36] Furthermore, DSCR1-null mice have been shown to exhibit deficits in learning and memory due to the increased activities of enzymatic calcineurin and protein phosphatase 1,[37] suggesting that DSCR1 had an effect on the process of neuroregulation. Previous studies have also revealed that DSCR1 contributes to the neuronal adaptations to oxidative stress and regulates neuronal apoptosis through the CREB-mediated Bcl-2 pathway.[38,39] Accordingly, there is a strong possibility that DSCR1 acts to protect neurons against oxidative stress–induced cell death via the antiapoptotic pathways. It is well known that oxidative stress plays a critical role in the pathogenesis of glaucoma, which is characterized by RGC apoptosis.[3] Our previous study has validated that DSCR1 is expressed in RGCs and upregulated in hypoxic RGCs.[10] Therefore, the aim of the current study was to discuss whether DSCR1 played an antiapoptotic role in RGCs under oxidative stress.

DBA/2J mice are the widely used models for the study of inherited glaucoma. Significant IOP elevations have been observed in DBA/2J mice at the age of 6 months,[40–42] and the presence of oxidative phosphorylation pathways has been confirmed in the DBA/2J mice.[43] The current study was the first to investigate expression of DSCR1 in this animal model. The increased expression of DSCR1 in DBA/2J mice was confirmed, and it reached a peak at the age of 6 months, suggesting that DSCR1 might be involved in the pathophysiology of glaucoma. And ONC SD rat model was also used to explore the role of DSCR1 in glaucoma. The ONC model is an established model that has been widely used in glaucoma-related research and has been shown to exhibit progressive RGC apoptosis.[25,44] The upregulation of DSCR1 in the GCL after ONC was found in our study. In addition, DSCR1 expression was enhanced in H_2O_2-treated RGCs. Furthermore, oxidative stress–induced RGC apoptosis was decreased after DSCR1 upregulation. These results agree with the previous findings demonstrating that increased expression of DSCR1 protected neuronal cells against oxidative stress.[45] Collectively, these findings suggest that DSCR1 has an important role in protecting RGCs exposed to oxidative stress.

Activation of the caspase signaling cascade is a critical event during apoptosis, which is hallmarked by caspase 8 and caspase 9 activation. As a main promoter of the Fas signaling pathway, caspase 8 is involved in the activation of Fas receptor and induction of cell apoptosis. Moreover, caspase 9 is an apoptotic effector in the mitochondrial channel, which initiates programmed cell death after activation. Caspase 3, which is downstream of caspase 8 and caspase 9 signaling, could be activated and finally results in cellular apoptosis.[46,47] In the current study, expression of cleaved caspase 3 was significantly decreased in RGCs overexpressing DSCR1 after H_2O_2 treatment. Fewer TUNEL-positive cells were also detected, likely due to the antiapoptotic effects of DSCR1. These results suggest that DSCR1 may promote RGC survival via inhibiting the caspase 3–dependent pathway.

CREB is known to play a vital role in various physiologic processes involving neuronal protection and cell survival.[48] In the mature central nervous system, CREB-mediated transcription is responsible for neuronal survival. Previous research has shown that CREB-null mice have enhanced levels of apoptosis and impaired sensory neuron growth in the peripheral nervous system.[49] Moreover, several studies have shown that the activation of CREB in the retina promotes the survival of RGCs upon oxidative stress.[50,51] On these bases, we hypothesized that DSCR1 may affect RGC

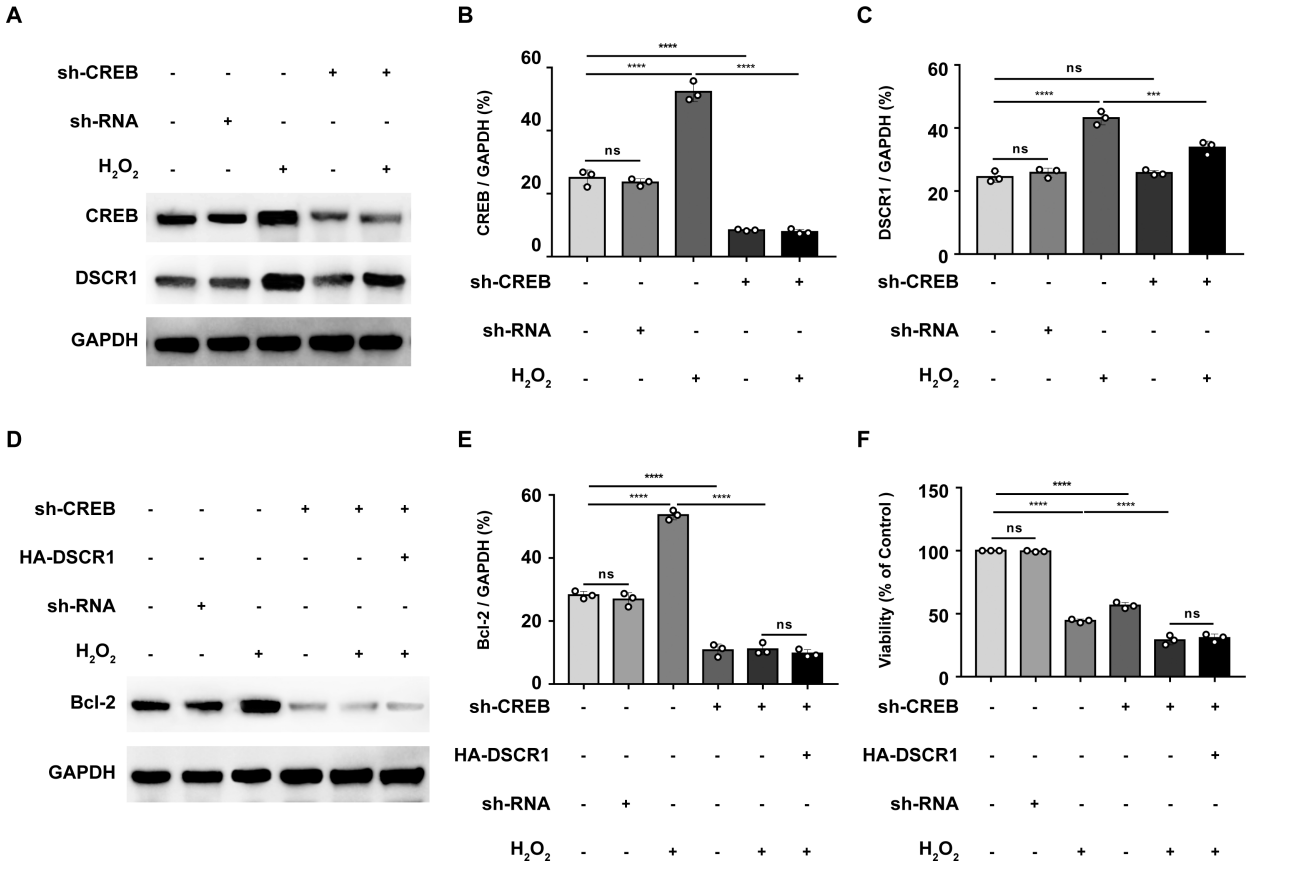

Figure 10. Knockdown of CREB reduces the expression of DSCR1 after H_2O_2 treatment. Sh-tagged CREB and HA-tagged DSCR1-transduced RGCs were cultured in neurobasal growth medium with H_2O_2 for 12 hours ($n = 12$). The DSCR1, CREB, and Bcl-2 expression were identified by Western blot (**A, D**) and then quantified (**B, C, E**). The expression of DSCR1, CREB, and Bcl-2 in RGCs increased after exposure to H_2O_2. RGCs transduced by sh-tagged CREB showed lower DSCR1, CREB, and Bcl-2 expression in response to H_2O_2 than the nontransduced group. The expression of Bcl-2 had no change in sh-tagged CREB-transduced RGCs after the transduction of HA-tagged DSCR1 vectors. (**F**) Graph for RGC survival rate shows that the viability of sh-tagged CREB-transduced RGCs did not change even if the HA-tagged DSCR1 vectors were transduced in RGCs ($n = 12$). The graphs are presented as mean ± SEM of three independent experiments. ***$P < 0.001$, ****$P < 0.0001$.

survival through activation of CREB. Our results showed that the activation of CREB by DSCR1 was correlated with the protection against oxidative stress–induced RGC apoptosis, which was in line with the previous finding that DSCR1 enhanced the phosphorylation of CREB in response to the cAMP intracellular pathway in neuronal cells.[39]

Moreover, the Bcl-2 gene, with a CREB-binding site in its upstream promoter region,[31] has been shown to be induced by activation of CREB during rescuing cells from apoptosis by B-cell activation,[52] indicating the antiapoptotic effects of the CREB–Bcl-2 pathway. In the current study, increased Bcl-2 expression was found in the DSCR1 overexpression group. Our results were consistent with previous findings in which PC-12 cells treated with H_2O_2 had stable expression of DSCR1 with increased expression of Bcl-2.[38]

It is noteworthy that increased basal level of p-CREB has been found in stably overexpressed DSCR1 cells in Down syndrome pathogenesis.[53] In accordance with this notion, we found that DSCR1 activity was suppressed in the CREB-downregulated group. CREB is a transcription factor responsible for cellular environmental homeostasis. Previous studies have shown that CREB activates E3 ubiquitin ligase,[54,55] which triggers the degradation of yeast Rcn1, a homologue of DSCR1.[56] These combined results imply that a DSCR1-CREB feedback loop might exist in RGCs under oxidative stress, with upregulation of DSCR1 activating phosphorylation of CREB and increasing Bcl-2 expression, which promotes RGC survival under the oxidative stress condition (Fig. 11).

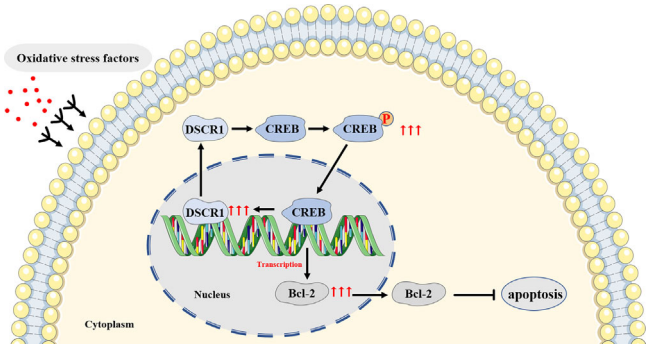

Figure 11. Schematic diagram showed that DSCR1 displayed anti-apoptosis properties in RGCs via CREB–Bcl-2 pathway.

Collectively, our results suggest that DSCR1 might promote RGC survival via activation of the CREB–Bcl-2 signaling pathway, implying that DSCR1 plays a protective role against oxidative stress–induced RGC apoptosis in

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glaucoma. Therefore, DSCR1 may be a potential new target for the treatment of glaucoma.

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