• Basic Research •

HMGB2 knockdown ameliorates retinal ganglion cell injury by inhibiting NLRP3 inflammasome activation after retinal ischemia

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Abstract

● **AIM:** To explore the neuroprotective effects of high mobility group box 2 (*HMGB2*) knockdown on retinal ganglion cells (RGCs) in the retinal ischemia-reperfusion injury (RIRI).

● **METHODS:** Oxygen-glucose deprivation (OGD)-injured RGCs from postnatal three-day C57BL/6 mice pups and high intraocular pressure (IOP)-induced RIRI mice were used as cellular and animal models of RIRI. The expression of HMGB2 in the retina of RIRI mice and OGD-injured RGCs was detected through reverse transcription-polymerase chain reaction (RT-qPCR) and Western blotting. The effects of *HMGB2* silencing on the morphological changes, RGCs survival, and cell apoptosis in mouse retinal tissues were observed through H&E staining, immunofluorescence staining with RNA-binding protein with multiple splicing (RBPMS) antibody, and TUNEL staining, respectively. RGC viability and apoptosis were examined by CCK-8 and flow cytometry assays. The levels of proteins associated with NOD-like receptor thermal protein domain associated protein 3 (NLRP3)-mediated pyroptosis [NLRP3, Caspase-1, GSDMD-N, interleukin (IL)-1β, IL-18] *in vivo* and *in vitro* were measured by Western blotting.

● **RESULTS:** HMGB2 protein and NLRP3 were upregulated in the retina of RIRI mice and OGD-injured RGCs (P<0.001). The retina was edematous, accompanied by disorganized cell arrangement and decreased thickness of all layers, and obvious vacuoles in ganglion cell layer. *HMGB2* silencing alleviated the reduction in total retinal thickness and the severity of retinal tissue damage as well as suppressed RGC loss and retinal cell apoptosis in RIRI mice. OGD-induced RGC apoptosis was ameliorated after downregulation of HMGB2 *in vitro*. Intravitreal injection of the AAV-sh-HMGB2 and si-HMGB2 resulted in significantly decrease of NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels in the retinal tissues of RIRI mice and OGD-injured RGCs, respectively (all P<0.001).

● **CONCLUSION:** *HMGB2* knockdown protects against RGC apoptosis and pyroptosis after RIRI through suppressing NLRP3 inflammasome activation.

● **KEYWORDS:** HMGB2; retinal ischemia-reperfusion injury; retinal ganglion cell; apoptosis; pyroptosis; NLRP3; mice
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INTRODUCTION

Retinal ischemia is characterized by an interruption of the blood supply to the retina, which leads to a lack of essential nutrients and oxygen to the retina, triggering a dangerous chain reaction and eventually resulting in cell death[1]. Subsequent restoration of circulation (called reperfusion) can further exacerbate the ischemic damage through a complex biochemical process[2]. Retinal ischemia-reperfusion injury (RIRI) is the common pathological basis of many retinal diseases, including acute angle-closure glaucoma, central retinal artery and vein occlusion, diabetic retinopathy, and ischemic optic neuropathy[3]. RIRI can lead to impairment of visual function and ultimately blindness[4]. Currently, various therapeutic measures have been applied to RIRI, including neurotrophic factors, antiapoptotic agents, calcium antagonists, and antioxidants[5]. Nevertheless, the clinical efficacy of these therapies is usually far from satisfactory. The management of RIRI has become an urgent medical issue, which prompted researchers to elucidate the pathogenesis of RIRI and develop effective treatment strategies.

Retinal ganglion cells (RGCs) are nerve cells located at the terminal end of the retina that are capable of transmitting information from the retina to the brain *via* their axons[6]. RIRI is hallmarked by the irreversible degeneration and loss of RGCs[7]. The pressure gradient of the lamina cribrosa elevates under conditions of high intraocular pressure (IOP), which results in a decline in blood supply and impairment in axonal transport in nerve fibers[8]. The loss of RGCs is more likely to occur because of the lack of neurotrophic support[9]. Studies have demonstrated that protecting RGCs from damage and loss after RIRI is critical to prevent visual impairment and improve neurological prognosis[10]. Apoptosis, autophagy, pyroptosis, necrosis, copper death, and iron death are all crucial pathways of RGC death, among which pyroptosis is a programmed mode of inflammatory cell death[11]. In classical pyroptosis, Caspase-1 is activated by different inflammasomes to cleave the substrate gasdermin D (GSDMD), forming pores in the cell membrane and releasing pro-inflammatory factors such as interleukin-1β (IL-1β) and interleukin-18 (IL-18)[12]. It has been reported that NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome-initiated pyroptosis pathway promotes the death of RGCs in retinal ischemia[13].

The high mobility group box (HMGB) protein family is composed of non-histone nuclear proteins that are widespread in mammalian cells and play vital roles in DNA recombination, replication, transcription, and repair[14]. There are four members in the HMGB family, that is HMGB1, HMGB2, HMGB3 and HMGB4[15]. Many studies have reported that downregulating HMGB1 contributes to alleviating retinal inflammation and hindering the progression of diabetic retinopathy[16]. Besides, HMGB1 can trigger pyroptotic cell death by activating NLRP3 and caspase-1-mediated pathways[17]. HMGB2 is highly homologous to HMGB1 and has indistinguishable biological characteristics[18]. Previously, it was discovered that HMGB2 knockdown suppressed retinal degeneration in light-induced retinal damage murine models and inhibited photoreceptor pyroptosis in mouse photoreceptor-derived cells through inactivating nuclear factor-kappa B (NF-κB)/NLRP3 pathway[19]. However, no study has clarified the function of HMGB2 in RIRI and its related mechanisms.

Therefore, our study employed both oxygen-glucose deprivation (OGD)-injured RGCs and high IOP-induced RIRI murine models to investigate the functional role of HMGB2 in the death of RGCs in RIRI.

MATERIALS AND METHODS

Ethical Approval All animal care and experimental procedures were approved by the Institutional Review Board of Wuhan Myhalic Biotechnology Co., Ltd (Approval number: HLK-20230314166; Approval date: 14/Mar/2023).

Animals Eighty six- to eight-week-old male C57BL/6J mice were bought from GemPharmatech Co., Ltd. (Jiangsu Province, China). The mice were housed in a pathogen-free facility with free access to food and water under a 12-h light/dark cycle.

Establishment of RIRI Mouse Models According to the previously described methods[20], the retinal ischemic-reperfusion (I/R) injury (RIRI) model was constructed after the induction of retinal ischemia in mice. After general anesthesia in mice by intraperitoneal injection of 50 mg/kg sodium pentobarbital, the right eye was topically dropped with 0.5% tetracaine hydrochloride (#T101909; Aladdin, Shanghai, China) for corneal anesthesia. The corneas were treated with 1% tropicamide (#LZR6328; Rayzbio, Shanghai, China) to dilate the pupils. Afterwards, a 30-gauge needle linked to a normal saline reservoir was inserted into the anterior chamber of the right eye. The IOP was increased to 110 mm Hg by adjusting the height (150 cm) of the saline reservoir (150 cm) and was maintained for 90min. The same operation was performed on the sham mice, without the elevation of the IOP. Corneal haze, conjunctival edema, and the disappearance of the fundus red-light reflex suggested retinal ischemia, while the recovery of the red-light reflex indicated the subsequent reperfusion. The IOP was allowed to return to the normal level after withdrawing the needle. The treated eyes were applied with levofloxacin eye drops to prevent infection. Eyes with cannulation-induced anterior chamber leakage, iris bleeding/injury, or cataracts were excluded from our study. Mice were sacrificed by cervical dislocation under anesthesia at different times during reperfusion, and retina tissues were isolated for subsequent experiments.

Intravitreal Injection of Adeno-Associated Virus The method described by Yang *et al*[21] was used to perform the accurate intravitreal injection under a dissecting microscope. Genbase Biotechnology (Shanghai, China) provided the adeno-associated virus (AAV), and the AAV vector carrying short-hairpin RNA targeting HMGB2 (AAV-sh-HMGB2) or its match control AAV-vehicle was constructed to interfere with HMGB2 expression in the retina. The viral vector suspension (5 μL; 5×10^{12} μg/mL final concentration) was slowly injected into the vitreous cavity using a Hamilton syringe fitted with a 30-gauge glass microneedle. Mice received AAV intravitreal injection 1d before RIRI induction.

Hematoxylin and Eosin Staining After mice were sacrificed at designated time points, their eyeballs were carefully removed and fixed overnight with 4% paraformaldehyde, followed by dehydration using an increasing ethanol gradient and paraffin embedding. Then, 5-μm thick sections across the optic nerve of each eye were prepared and stained using an hematoxylin and eosin (H&E) assay kit (#LM804004C;

LMAI Bio, Shanghai, China). Following dehydration using the gradient ethanol solution, transparency using xylene, and covering with a cover glass, the slides were observed under a microscope. The thickness of the retina (from the inner limiting membrane to the outer limiting membrane) was estimated in four adjacent areas with a 1 mm distance to the optic nerve center by using Image J software. A representative retinal thickness for each eye was obtained after the average of the measurements by two blinded investigators.

TUNEL Staining A one-step terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) apoptosis assay kit (#MA0223; Meilunbio, Shanghai, China) was employed to assess cell apoptosis in retinal tissues. The paraffin-embedded retinal slices were permeabilized for 5min at room temperature (RT) with 0.5% Triton X-100 (#S15022; Yuanye, Shanghai, China) in phosphate buffered saline (PBS; #AWR0213a; Abiowell, Changsha, China) and then incubated for 1h at 37℃ in darkness with TUNEL working solution. Thereafter, the sections were stained for five min in the dark with 4',6-diamidino-2'-phenylindole (DAPI) solution (#P4207; Warbio, Nanjing, China) after thrice rinse with PBS. Finally, the slices were examined under a fluorescent microscope (BX53, Olympus, Tokyo, Japan) to observe the TUNEL-positive cells with green fluorescence. After quantitating cell apoptosis using Image J software, TUNEL-positive cell numbers were expressed as the average per 1-mm length area.

Immunofluorescence Staining RNA binding protein with multiple splicing (RBPMS) is a specific marker for RGCs, and the antibody to RBPMS is a specific assay that specializes in the identification of RGCs in a wide range of mammals[22]. The survival rate of RGCs was assessed by observing RBPMS-positive cells in immunofluorescence staining. The paraffin retinal sections were dewaxed following the standard procedures, followed by the antigen retrieval through a heating method with 10 mmol/L sodium citrate solution (pH=6.0) at the sub-boiling temperature (95℃-98℃) for 30min. Subsequently, the sections were permeabilized and sealed by adding PBS containing 0.1% Triton X-100 and 5% donkey serum for 1h at RT. After overnight incubation at 4℃ with the primary antibody targeting rabbit polyclonal anti-RBPMS (#orb312900; 1:100; Biorbyt, Wuhan, China Abclonal) and 1h incubation at RT in the dark with the appropriate secondary antibody. Cell nuclei were counterstained with DAPI. After PBS wash, the retinal sections were observed under an Olympus fluorescence microscope. The counts of the RBPMS-positive cells were averaged.

Isolation and Culture of Primary RGCs The extraction of primary RGC cells was conducted as previously described with minor modifications[23]. In brief, retinas from postnatal three-day (P3) C57BL/6 mice pups were dissected and digested by trypsin-ethylene diamine tetraacetie acid (EDTA; #40127ES60; Yeason, Shanghai, China) for 30min, followed by incubation with anti-macrophage antiserum to clear endothelial cells and macrophages in the suspension. The suspension was processed through 3-step panning for RGC purification: 30min incubation in a lectin-coated negative panning plate (shaken every 15min), 10min incubation in another lectin-coated dish, and 45min incubation in an anti-mouse-Thy1.2 antibody-coated positive panning plate (shaken every 15min). Subsequently, cells attached to the plate were harvested and seeded on glass coverslips coated with Poly-D Lysine (#XK1022; Xuanke, Shanghai, China) and mouse Laminin (#23017015; SolelyBio, Shanghai, China). Purified RGCs were incubated in neurobasal medium (#21103049; Beinuo, Shanghai, China) supplemented with 1% GlutaMAX (#35050061; Reanta, Beijing, China), 2% B27 (#27010; Engreen, Beijing, China), and 1% penicillin-streptomycin (#60162ES76; Yeason) in a humidified atmosphere containing 5% CO_2 at 37℃.

Oxygen-Glucose Deprivation Model To simulate the ischemic injury *in vitro*, 5×10^5 RGCs were plated and grown to 80% confluence. After cells were washed thrice with PBS, the culture medium was replaced with glucose-free Neurobasal, and cells were incubated at 37℃ in an anoxic chamber (95% N_2 and 5% CO_2). Control cells were maintained under normoxic conditions (20% O_2, 1% CO_2, and 79% N_2) in the complete medium.

Cell Transfection Three small interfering RNAs (siRNAs) targeting HMGB2 (si-HMGB2#1/#2/#3) were designed to knock down Hmgb2 expression, with si-NC as the negative control. After the cultured RGCs entered the logarithmic growth phase, cell transfection was performed by using Lipofectamine 3000 (#L3000015; Reanta). After 48h, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to validate the interfering efficiency of Hmgb2, and the siRNA that resulted in the lowest expression of Hmgb2 in RGCs was selected for subsequent experiments.

Cell Counting Kit-8 Assay RGCs were seeded (1×10^4 cells/well) into 96-well plates and cultivated in 5% CO_2 at 37°C for 24h. After OGD stimulation for 30min, 1, 2, 4, and 6h, 10 μL of cell counting kit-8 (CCK-8) reagent (#FS-X9754; Fusheng Industrial; Shanghai, China) was added for 2h at 37℃. The optical density (OD) at 450 nm was measured using an enzyme-labeled analyzer (Mlbio, Shanghai, China). The percentage of cell viability was calculated as follows: cell viability (%)=$(OD_{experiment}-OD_{blank})/(OD_{control}-OD_{blank})\times100\%$.

Flow Cytometry Assay The apoptosis of RGCs was estimated using an Annexin V-FITC/Propidium Iodide (PI) apoptosis detection kit (#C9212; Warbio). After si-NC or si-Hmgb2#3 transfection, RGCs were exposed to OGD for 6h.

Then, about 1×10^5 cells per well were washed twice with cold PBS, trypsinized with EDTA-free trypsin, re-centrifugated, and resuspended in binding buffer. Next, 500 μL cell suspension was added with 5 μL Annexin V-FITC and 10 μL PI, followed by 15min incubation in the dark. At last, the apoptosis rate was evaluated by a flow cytometer (Mindray, Shenzhen, China).

RT-qPCR Total RNA from mouse retinal tissues or RGCs was extracted using TRIzol (#RY0871; Jisskang, Shangdong, China), whose concentration was evaluated by ScanDrop Ultra-micro spectrophotometer (Analytik Jena, Germany). To synthesize complementary DNA, total RNA (1 μg) was reverse transcribed by using a reverse transcription kit (#XY-PCR-1590; Xuanya, Shanghai, China). RT-qPCR was performed using ChamQ SYBR qPCR Master Mix (#Q711-02; Vazyme, Nanjing, China) on a LightCycler 480 II Real-time PCR Instrument (Roche, Swiss). The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was set as an internal control to normalize HMGB2 expression. The primer sequences were as follows: HMGB2, forward: 5'-GATGGAAGACCATGTCTGC-3', reverse: 5'-TCTCCCTGTCATAACGAGC-3'; GAPDH, forward: 5'-ACTCTTCCACCTTCGATGC-3', reverse: 5'-CCGTATTCATTGTCATACCAGG-3'.

Western Blotting Mouse retinal tissues or RGCs were homogenized in radio-immunoprecipitation assay lysis buffer (#YB25-191209; Yubo, Shanghai, China) containing protease and phosphatase inhibitors (#P1045; Beyotime, Shanghai, China). The lysates were then centrifuged (12 000 g, 4℃, 20min), and the resulting supernatant was harvested. A Bicinchoninic Acid Protein Assay Kit (ZY80815; Zeye, Shanghai, China) was employed to determine the protein concentration. A total of 40 μg protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes using a semi-dry method. The membranes were blocked with 5% bovine serum albumin (#abs9157; Absin, Shanghai, China) for 1h at RT to prevent non-specific binding, after which the primary antibodies (Affinity Biosciences) against HMGB2 (#DF3133; 1:1000), NLRP3 (#DF7438; 1:1000), Caspase-1 (#AF5418; 1:1000), GSDMD-N (#DF13758; 1:1000), IL-1β (#AF5103; 1:1000), and IL-18 (#DF6252; 1:1000) and GAPDH (#AF7021; 1:3000) were added to incubate the membranes at 4℃ overnight. The next day, the membranes were probed with the secondary antibody (#S0001; 1:3000; Affinity Biosciences, Hunan, China) for 1h at RT. In the end, the enhanced chemiluminescence detection reagent (#WP20005; Reanta) was used to develop the protein blots, followed by the quantification of the band intensity using ImageJ software.

Statistical Analysis All *in vitro* experiments were independently repeated three times. Statistical analysis was performed by utilizing SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) after checking the data for the normal distribution by the Shapiro-Wilk test. The experimental data are expressed in the form of mean±standard deviation (SD). Student's *t*-test was employed to compare two group means, while one-way analysis of variance with Bonferroni post hoc test was adopted to compare three or more group means. Statistical significance was considered at $P<0.05$.

RESULTS

HMGB2 is Upregulated in the Retina of RIRI Mouse Models and OGD-injured RGCs High IOP-induced RIRI murine models and OGD-injured RGCs were respectively used as animal and cellular models that simulated the pathological process of ischemic retinopathy. The typical morphological changes of retinal tissues were observed through H&E staining. After I/R injury, the mouse retina was markedly edematous, accompanied by disorganized cell arrangement and decreased thickness of all layers, and obvious vacuoles in the ganglion cell layer (GCL). These histopathologic changes were exacerbated with the prolongation of reperfusion time (Figure 1A-1B). As shown by CCK-8 assay, OGD induced a time-independent reduction in the viability of RGCs (Figure 1C). Western blotting revealed that HMGB2 protein levels were upregulated in both retinal tissues of mice after I/R injury and OGD-insulted RGCs compared with sham mice and control RGCs (Figure 1D-1G).

***HMGB2* Silencing Ameliorates Retinal Injury in Mice after I/R** To assess the function of HMGB2 in RIRI, mice received intravitreal injection of the AAV2-sh-HMGB2 virus and the control before the onset of I/R. First, the silencing of *HMGB2* gene was confirmed by RT-qPCR, which illustrated that HMGB2 mRNA expression in the retina of mice was remarkably reduced after injection with AAV2-sh-HMGB2 compared to the control AAV2-sh-NC (Figure 2A). Through H&E staining, we observed that HMGB2 silencing obviously mitigated retinal edema caused by I/R and restored retinal thickness with neatly arranged ganglion layers (Figure 2B-2C). Furthermore, immunofluorescence staining showed that I/R induced a notable decrease in the numbers of anti-RBPMS-labeled surviving RGCs, while such a decrease was reversed after the knockdown of HMGB2 (Figure 2D-2E). TUNEL-positive cell numbers were significantly higher in the retinal issues of mice after I/R than in those of sham mice, which however, were declined after silencing of HMGB2 (Figure 2F-2G), indicating that HMGB2 knockdown suppressed I/R-induced retinal cell apoptosis.

NLRP3-mediated Pyroptosis is Enhanced in RIRI Mouse Models and OGD-injured RGCs To evaluate whether

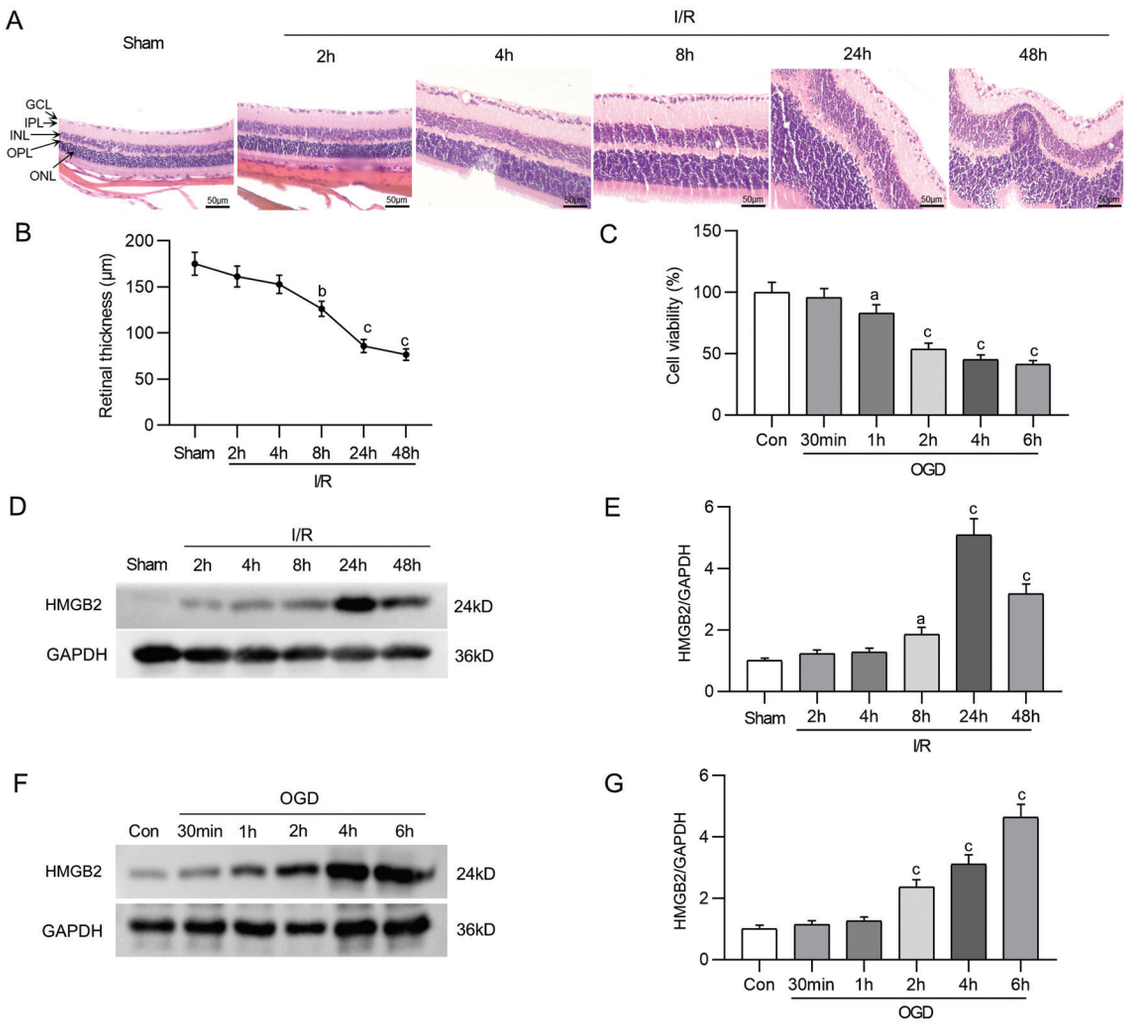

Figure 1 HMGB2 is upregulated in the retina of RIRI mouse models and OGD-injured RGCs A: Representative hematoxylin and eosin (H&E) staining images showing the morphological changes of mouse retinal tissues during I/R. Scale bar: 50 μm. B: The corresponding quantification of retinal thickness (from the internal to the outer limiting membrane). C: Detection of the viability of RGCs after OGD stimulation by CCK-8 assay. D-G: Measurement of HMGB2 protein levels in the retinal tissues of mice subjected to I/R for different times and in RGCs after OGD stimulation for different times by Western blotting. n=8. ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001. I/R: Ischemic-reperfusion; OGD: Oxygen-glucose deprivation; RGCs: Retinal ganglion cells; Con: Control; CCK-8: Cell counting kit-8; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer.

NLRP3-mediated pyroptosis participates in I/R-induced retinal injury and the time point of its occurrence, the levels of key related proteins (NLRP3/Caspase-1/GSDMD-N/IL-1β/IL-18) were examined at different time points following I/R. Western blotting manifested that the levels of these proteins in mouse retina increased and peaked 24h after I/R injury (Figure 3A-3F). In addition, the levels of the above proteins related to NLRP3-mediated pyroptosis were at a peak at 6h after OGD in RGCs compared with normal control RGCs (Figure 3G-3L). Taken together, both *in vivo* and *in vitro* evidence suggest that NLRP3-mediated pyroptosis was activated after RIRI.

Knockdown of HMGB2 Inhibits NLRP3-mediated Pyroptosis in RIRI Mouse Models The effects of HMGB2 on NLRP3-mediated pyroptosis were determined. As revealed in Figure 4, the intravitreal injection of the AAV virus vector carrying sh-HMGB2 resulted in a marked decrement in NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels in the retinal tissues of RIRI mouse models, indicating that Hmgb2 knockdown repressed the activation of NLRP3-mediated pyroptosis *in vivo*.

Knockdown of HMGB2 Suppresses Apoptosis and NLRP3-mediated Pyroptosis in OGD-injured RGCs Finally, whether downregulating HMGB2 exerts inhibitory effects on NLRP3-mediated pyroptosis was validated *in vitro*. RGCs were first transfected with Hmgb2-knockdown vectors si-HMGB2#1/#2/#3, which led to a substantial decline in HMGB2 mRNA levels (Figure 5A). Si-HMGB2#3 was used in the subsequent assays since the interfering efficiency of si-HMGB2#3 was better than that of si-HMGB2#1/#2. The apoptosis of RGCs after HMGB2 knockdown and

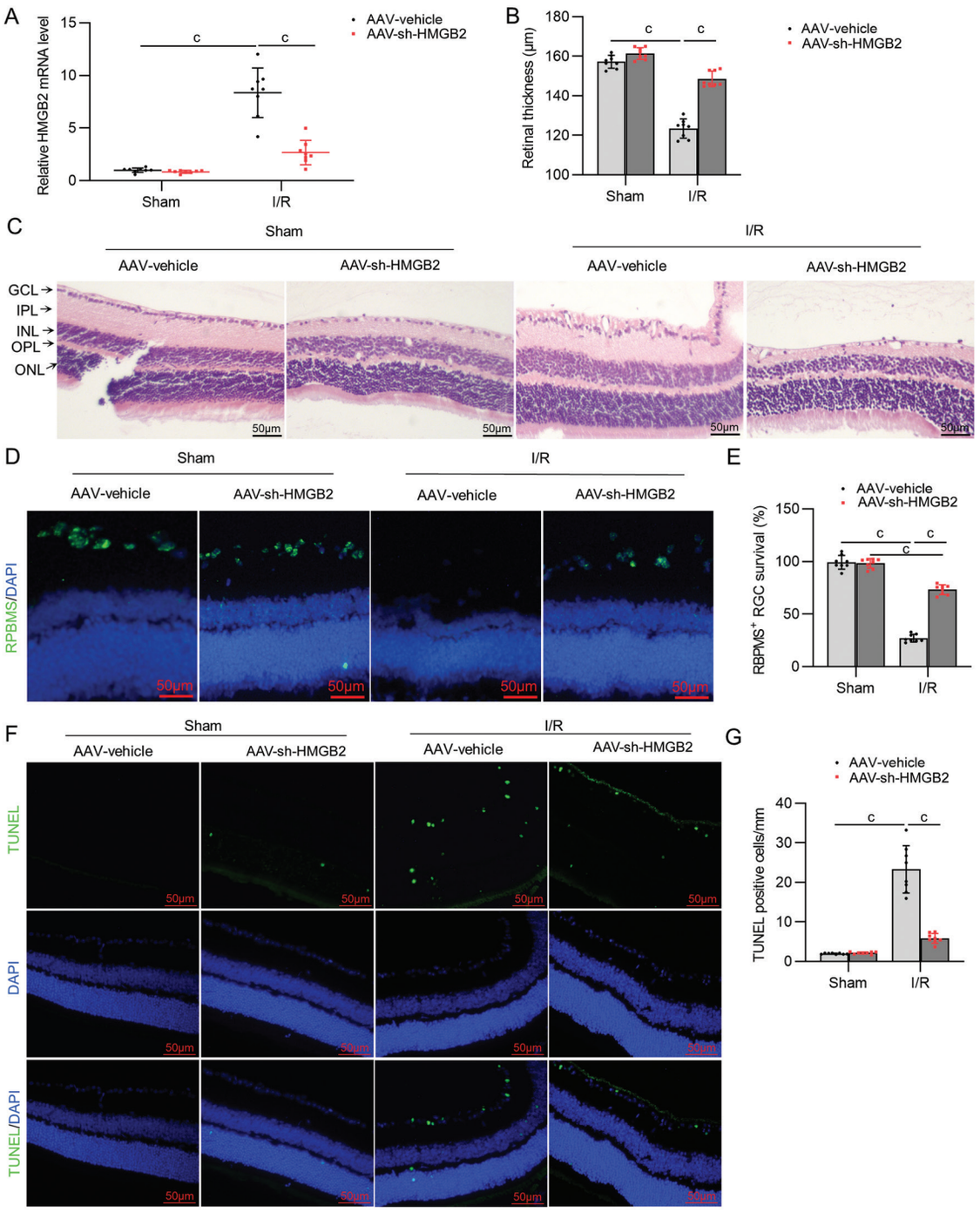

Figure 2 HMGB2 silencing ameliorates retinal injury in mice after I/R A: Detection of HMGB2 mRNA expression in retinal tissues of sham and I/R-injured mice after intravitreal injection of the AAV2-sh-HMGB2 virus and the control by RT-qPCR. B-C: Representative H&E staining images showing the morphological changes of mouse retinal tissues during I/R and the corresponding quantification of retinal thickness. Scale bar: 50 μm. D-E: Representative immunofluorescence images showing RBPMS-labeled RGCs in mouse retinal tissues and the quantification of RGC survival. Scale bar: 50 μm. F-G: Representative TUNEL staining images showing cell apoptosis in mouse retina and the quantification of TUNEL-positive apoptotic cells. Scale bar: 50 μm. n=8. ^{c}P<0.001. AAV: Adeno-associated virus; I/R: Ischemic-reperfusion; RGCs: Retinal ganglion cells; H&E: Hematoxylin and eosin; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; RBPMS: RNA binding protein with multiple splicing; DAPI: 4',6-diamidino-2'-phenylindole; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer.

OGD stimulation was analyzed using flow cytometry, which demonstrated that HMGB2 downregulation overturned OGD-induced elevation in the apoptosis of RGCs (Figure 5B-5C). Western blotting revealed that the elevation in NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels caused by OGD stimulation in RGCs was antagonized by HMGB2 knockdown (Figure 5D-5H), confirming that OGD-induced NLRP3-mediated pyroptosis in RGCs was inhibited by HMGB2 downregulation.

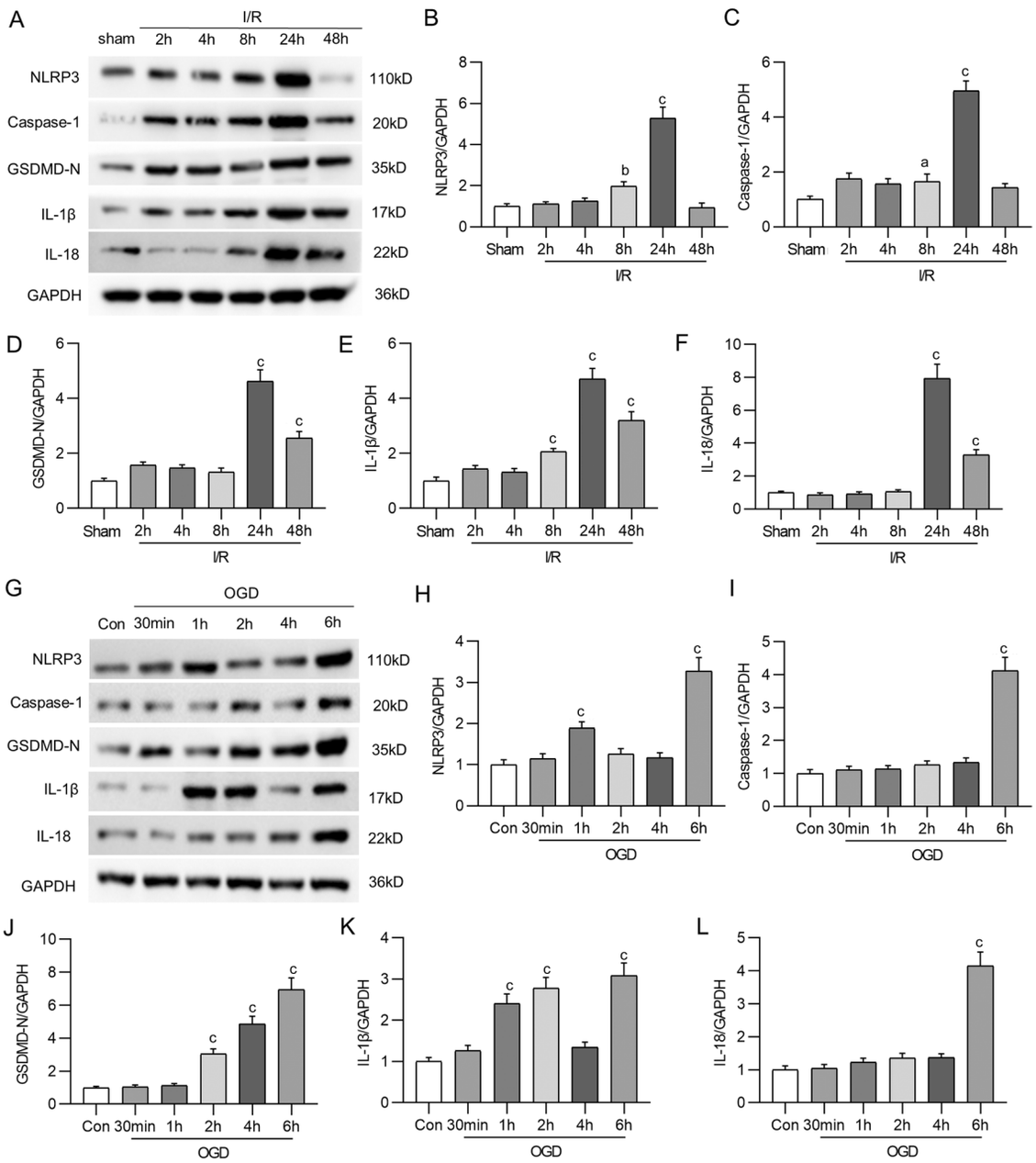

Figure 3 NLRP3-mediated pyroptosis is enhanced in RIRI mouse models and OGD-injured RGCs A-F: Determination of the levels of NLRP3-mediated pyroptosis-related proteins (NLRP3/Caspase-1/GSDMD-N/IL-1β/IL-18) in the retina of mice after I/R for different times through Western blotting. G-L: Measurement of NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels in RGCs after OGD stimulation for different times by Western blotting. n=8. [a]P<0.05, [b]P<0.01, [c]P<0.001. RIRI: Retinal ischemia-reperfusion injury; I/R: Ischemic-reperfusion; RGCs: Retinal ganglion cells; OGD: Oxygen-glucose deprivation; Con: Control; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GSDMD: Gasdermin D; IL: Interleukin; NLRP3: NOD-like receptor thermal protein domain associated protein 3.

DISCUSSION

RIRI is the main pathogenic process in a variety of retinal diseases, usually leading to irreversible visual impairment or even blindness, which severely impairs the life quality of patients[24]. The retina is metabolically active and is one of the most oxygen-dependent tissues in the body, which makes it particularly sensitive to ischemia and hypoxia. A series of inflammatory cascades occurs during the RIRI process, thereby destroying retinal neurons, especially RGCs. As are other neurons in the central nervous system, RGCs are difficult to regenerate once dead, which ultimately results in irreversible vision loss[25]. Hence, exploring the molecular mechanisms that induce RIRI-associated RGC death is of great value to develop therapeutic strategies to prevent vision loss. Our research provides the first evidence that HMGB2 is involved in regulating RIRI-associated RGC apoptosis and pyroptosis. We found that HMGB2 was upregulated in RIRI murine models and OGD-insulted RGCs. *HMGB2* silencing ameliorated retinal injury and RGCs apoptosis in RIRI mice as well as reduced apoptosis and pyroptosis in OGD-injured RGCs. Additionally, both *in*

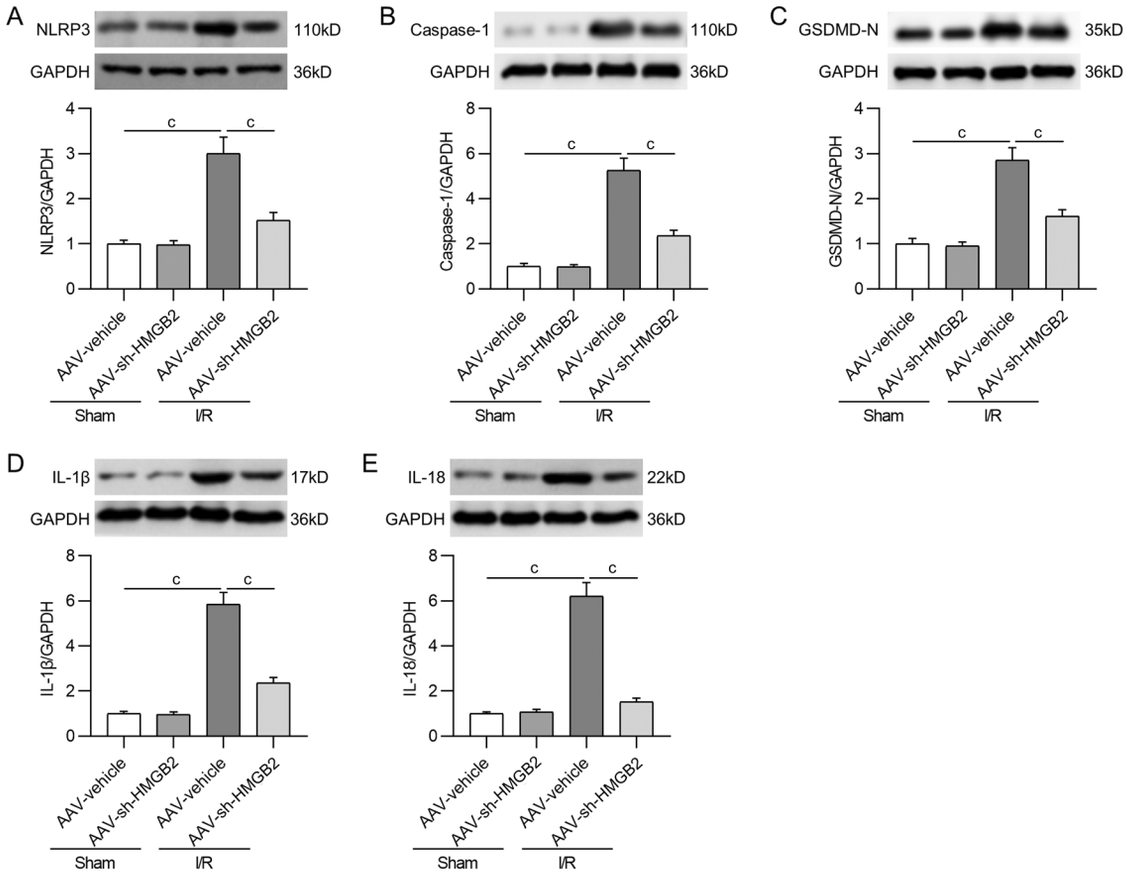

Figure 4 Knockdown of HMGB2 inhibits NLRP3-mediated pyroptosis in RIRI mouse models A-E: Examination of NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels in the retinal tissues of sham and I/R-injured mice after intravitreal injection of the AAV2-sh-HMGB2 virus and the control by western blotting. n=8. ^{c}P<0.001. RIRI: Retinal ischemia-reperfusion injury; AAV: Adeno-associated virus; I/R: Ischemic-reperfusion; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NLRP3: NOD-like receptor thermal protein domain associated protein 3; IL: Interleukin; GSDMD: Gasdermin D.

vivo and *in vitro* data suggested that HMGB2 knockdown suppressed NLRP3 inflammasome-mediated pyroptosis.

Progressive apoptosis of RGCs is an inevitable pathway in the progression of many retinopathies and optic nerve diseases, and is the underlying cause of blindness in many eye diseases[26]. RGCs are sensitive to external factors, such as trauma, high IOP, inflammation and neurotoxins, *etc*. These extracellular signals affect intracellular signaling through different pathways and regulate the expression of apoptosis-related genes, which ultimately leads to apoptosis of RGCs[27]. Overwhelming evidence indicates that apoptosis of RGCs is the primary mechanism in ischemic injury-induced retinal degeneration and irreversible blindness[28]. Earlier experimental studies found that apoptotic cells existed in the retinal GCL and inner nuclear layer in the RIRI model[29]. A growing body of studies have elucidated that suppressing the apoptotic pathway mitigates retinal ischemic injury[30-31]. Herein, our findings showed the activation of apoptosis in both retinal tissues of RIRI murine models and OGD-treated RGCs. Recent studies have revealed that RGCs also suffer from pyroptotic death in addition to RIRI-induced apoptosis[32]. Pyroptosis, an emerging form of pro-inflammatory programmed cell death, has also been implicated in diverse ischemic retina diseases[33]. The classical pathway of pyroptosis involves the activation of Caspase-1 by inflammasomes (*e.g.*, NLRP3), which cleaves the N-terminal sequence of GSDMD, allowing GSDMD proteins to bind to the cell membrane and create more pores, further contributing to the rupture of the cell membrane and the release of cellular contents, which induces an inflammatory response. At the same time, a series of inflammatory factors, such as IL-1β and IL-18 were released from the cells during the pyroptosis process, which recruit more inflammatory cells and amplify the inflammatory response[34]. It has been demonstrated that the inhibition of the NLRP3-mediated pyroptosis pathway contributes to alleviating RIRI[13]. As previously shown, the pyroptosis signaling upregulated and peaked 24h after RIRI in mice[35-36]. Consistently, the examination of pyroptosis pathway-related proteins (NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18) in the current study provided convincing evidence that pyroptosis occurred after RIRI in mice and peaked at 24h post I/R. The upregulation and activation of proteins associated with NLRP3-mediated

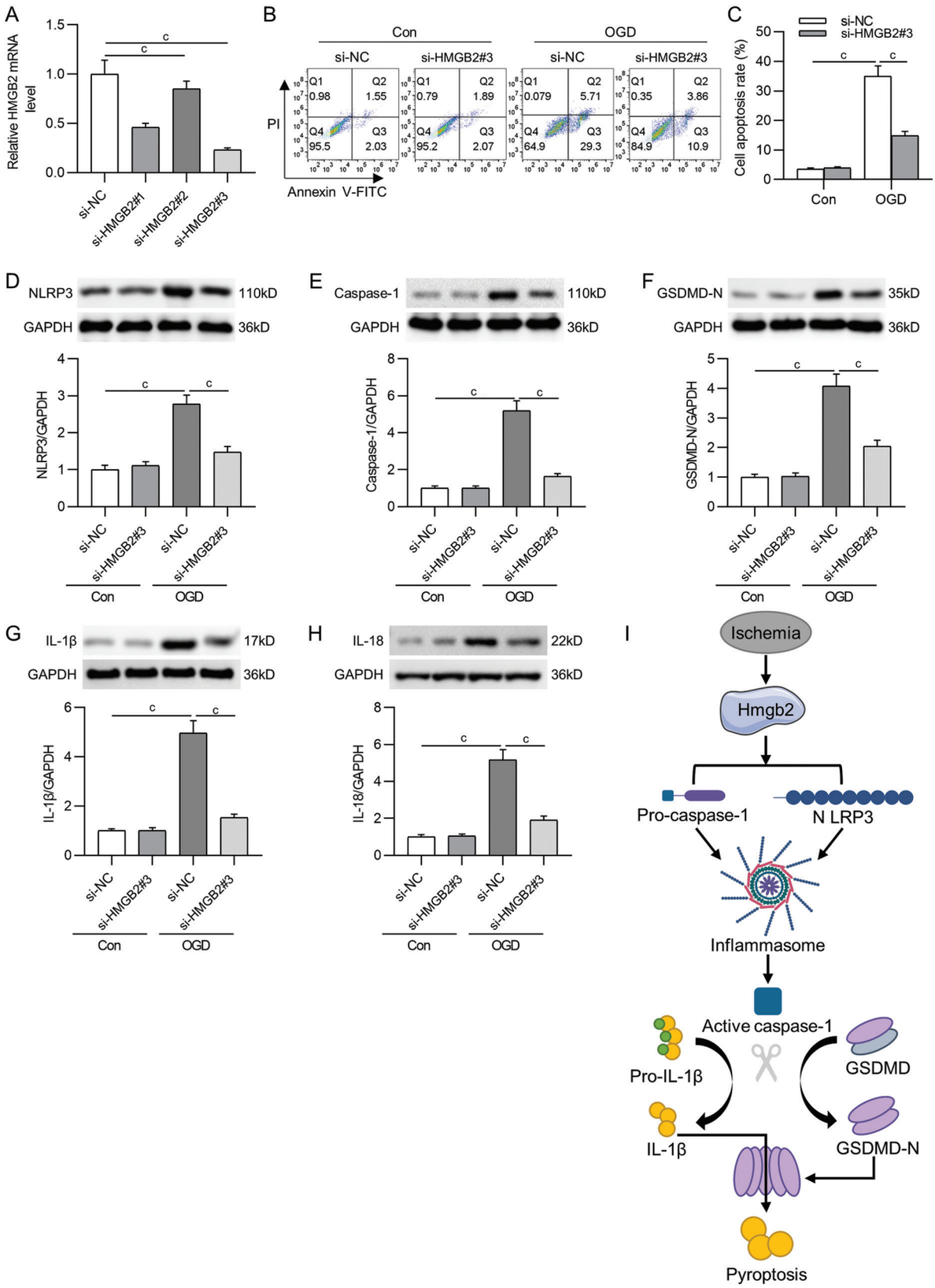

Figure 5 Knockdown of HMGB2 suppresses apoptosis and NLRP3-mediated pyroptosis in OGD-injured RGCs A: Analysis of HMGB2 mRNA levels in RGCs transfected with si-HMGB2#1/#2/#3 by RT-qPCR. B-C: Assessment of the apoptosis of RGCs after si-HMGB2#3 transfection and OGD treatment by flow cytometry assay. D-H: Determination of NLRP3, Caspase-1, GSDMD-N, IL-1β, and IL-18 protein levels in RGCs after si-HMGB2#3 transfection and OGD treatment by Western blotting. I: Schematic diagram showing the regulatory mechanism of HMGB2 in ischemia-induced pyroptosis. Ischemia upregulates HMGB2 expression to promote NLRP3 inflammasome activation, which further activates Caspase-1 that cleaves GSDMD and proinflammatory cytokine IL-1β, thereby inducing the onset of pyroptosis. $^{c}P<0.001$. OGD: Oxygen-glucose deprivation; RGCs: Retinal ganglion cells; Con: Control; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; GSDMD: Gasdermin D; IL: Interleukin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

pyroptosis were also observed in OGD-injured RGCs.

HMGB2, a member of HMGB proteins, is a highly conserved nuclear protein in mammals, which contains a long acidic carboxyl-terminal tail and two DNA-binding domains (HMG A-box and HMG B-box)[37]. In the nucleus, the non-specific binding between HMGB proteins and the minor groove of DNA induces the conformational changes of DNA and facilitates the assembly of transcription factors and other multiprotein complexes[38]. HMGB proteins serve as DNA structure factors and play essential roles in DNA replication, repair, transcription, recombination, stem cell differentiation, cell survival, and differentiation, inflammation, and cell signaling[39]. HMGB2 participates in chromatin binding and DNA repair by interacting with several proteins, including steroid receptor coactivator-1, steroid hormone receptors, tumor protein P53, and SET nuclear proto-oncogene[40]. Zhang *et al*[19] demonstrated the main distribution of HMGB2 in the outer nuclear layer and the inner and outer segments of retinal photoreceptor cells, and HMGB2 knockdown inhibited photoreceptor pyroptosis in light-induced retinal damage *via* restraining NF-κB/NLRP3 signaling pathway. HMGB1 is another member of the HMGB protein family and highly homologous to HMGB2, with 80% amino acid sequence identity. Previously, the neuroprotective role of HMGB1 silencing against RGC damage and loss has been confirmed in the experimental models of many retinal diseases. For example, Zhao *et al*[41]. suggested that N-acetylserotonin exhibited a neuroprotective effect against RIRI in rat models by decreasing HMGB1 and RAGE expression and inactivating the NF-κB/NLRP3 pathway. Peng *et al*[42] disclosed that the intravitreal injection of the HMGB1 inhibitor BoxA alleviated neuroinflammation-induced RGCs injury and death in experimental traumatic optic neuropathy mice by inactivating NLRP3 inflammasome. Tonner *et al*[43] clarified that the intravitreal injection of anti-HMGB1 monoclonal antibodies protected against RGC loss by repressing HMGB1-dependent inflammatory response in rat models of high IOP-induced glaucoma. Liu *et al*[44] reported that administration with glycyrrhizin, an HMGB1 inhibitor, attenuated proinflammatory cytokine levels, restored normal cell numbers in GCL, maintained normal retinal permeability, and curbed the retina thinning in diabetic mice, suggesting the effective role of HMGB1 inhibition in preventing diabetic retinal changes. Nevertheless, few studies have clarified the effects of HMGB2 on RGC damage and death in ischemic retinal diseases. Herein, our studies showed that HMGB2 expression was enhanced in both RIRI murine models and OGD-treated RGCs. HMGB2 downregulation mitigated retinal injury, restored RGC number, attenuated RGC apoptosis, and suppressed NLRP3-mediated pyroptosis in RIRI mice. Additionally, cellular experiments validated that knockdown of HMGB2 inhibited OGD-induced apoptosis and NLRP3-mediated pyroptosis in RGCs.

In conclusion, our study innovatively confirmed that downregulation of HMGB2 may become a potential therapy for RIRI by protecting against RGCs injury and death. Moreover, HMGB2 knockdown restrained NLRP3-mediated pyroptosis in both cellular and animal models of RIRI. More broadly, our work helps develop a promising therapeutic target that may promote the prophylaxis and treatment of retinal ischemic injury.

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