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Mini-peptide RPL41 attenuated retinal neovascularization by inducing degradation of ATF4 in oxygen-induced retinopathy mice

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ABSTRACT

Endoplasmic reticulum (ER) stress signaling is activated in retinal degeneration disease. Activating transcription factor 4 (ATF4), an important mediator of the unfolded protein response (UPR), is a key element that maintains cell survival and proliferation in hypoxic conditions. Our previous studies showed that a small ribosomal protein L41 (RPL41) inhibits ATF4 by inducing its phosphorylation and degradation. In the present study, the effects of mini-peptide RPL41 on retinal neovascularization (RNV) in oxygen-induced retinopathy (OIR) mice was investigated. We induced OIR in C57BL/6 mice and obtained retinas from normoxia, OIR, OIR control (treated with PBS), and OIR treated (treated with RPL41) mice. Our results showed that ER stress signaling was activated and ATF4 was overexpressed in the retinas of OIR mice. After intravitreal injection of RPL41, the size of RNV and vaso-obliteration, and the number of preretinal neovascular cell nuclei in the retinas of OIR mice were significantly decreased. Western blot analysis and quantitative real-time polymerase chain reaction (qPCR) showed ATF4 and VEGF expression decreased after intravitreal injection of RPL41. Furthermore, the expression levels of inflammatory genes including TNF-α, IL-1β, and IL-6 were significantly decreased compared with the OIR control mice. In conclusion, RPL41 prevented pathologic neovascularization and exerted anti-inflammatory effects by degrading the important ER stress factor ATF4, thus, RPL41 could be a promising therapeutic agent for the treatment of neovascular eye diseases, especially retinopathy of prematurity (ROP).

1. Introduction

Aberrant retinal neovascularization (RNV) occurs in many neovascular eye diseases such as diabetic retinopathy (DM), retinopathy of prematurity (ROP), and age-related macular degeneration (AMD), and is the leading cause of visual impairment and blindness [12,14]. Emerging evidence indicates endoplasmic reticulum (ER) stress and unfolded protein response (UPR) are activated and play important roles in the retina of diabetes and oxygen-induced retinopathy (OIR) animal models [16,20]. The role of ER stress in RNV is mediated by three major signal transducers including protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6; [18]), which are associated with neovascularization and inflammatory reactions [11,17,41]. Among the three arms of the UPR pathway, the PERK/eIF2α/ATF4 regulatory axis is especially important in response to hypoxia [37]. Moreover, ATF4 mediates many other cell signaling pathways such as hypoxia-inducible factor (HIF)-1α pathway [43] and c-Jun NH_2-terminal kinase (JNK) pathway [42], as well as several inflammatory genes such as TNF-α, IL-1β, IL-6, and ICAM-1. ATF4 plays an important role in the development of neovascular eye diseases and has become a potential target for their treatment [28,35,4].

Ribosomal protein L41 (RPL41) is a small basic peptide with 25 amino acids, originally purified from the ribosomes of *Saccharomyces cerevisiae* and identified as a very small, basic protein that appeared to have orthologues in human and *Schizosaccharomyces pombe* ribosomes [39]. RPL41 is not only the smallest but also the most basic eukaryotic protein. We previously demonstrated that RPL41 is commonly deleted in many tumors [34]. Our recent findings showed that RPL41 has potent anti-ATF4 effects by targeting ATF4 to the cytoplasmic proteasome for degradation [33]. In the present study, whether a synthetic RPL41 as an anti-ATF4 agent is effective in treating retinal angiogenesis and alleviating ER stress was investigated in the animal model of OIR.

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2. Material and methods

2.1. Preparation of RPL41

The RPL41 peptide (NH2-MRAKWRKKRMRRLKRKRRKMRQ RSK-OH) was synthesized by GenScript (Nanjing, China). The peptide was HPLC-purified to more than 95% and analyzed using mass analysis and analytical HPLC. Peptides were reconstituted in double-distilled water with a final concentration of 100 μg/μL.

2.2. Animal treatment and OIR model

C57BL/6 J mice were purchased from the Laboratory Animal Center of China Medical University. All animal experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act 1986. The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (approval ID 2016PS259K).

OIR was induced in C57BL/6 J mice as described previously [31]. Neonatal mice with nursing mothers were placed on postnatal day 7 (P7) in a hyperoxia chamber with 75% oxygen for five days; oxygen concentration, and room temperature were monitored and recorded 3 times a day. On P12, the mice were randomly divided into the following four groups: normoxia, OIR, OIR control (PBS), and OIR treated (RPL41). The OIR control or OIR treated mice received 1 μL intravitreal injection of PBS or RPL41, respectively, using a 33-gauge needle attached to a Hamilton syringe, and were maintained in room air for the following five days. On P17, pups were sacrificed by cervical dislocation. The eyes were removed for morphological and pathological studies or for mRNA and protein collection.

2.3. Retinal vascular morphology using lectin staining and hematoxylin and eosin (H&E) staining

The eyes were enucleated; cornea, lens, and vitreous were surgically removed and the neural retinas were dissected. Isolectin B4–594 staining (Alexa Fluor 594-I21413, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) was performed according to a previously described method [9]. Briefly, PBS was removed from the cell culture plate well using a disposable fine-tip pipette and 500 μL lectin solution was immediately added. Then, the plate was covered with aluminum foil and shaken at room temperature overnight. After washing the retinas 3–4 times for 15 min in 1 × PBS, the retinas were divided into four quadrants by four incisions under dissecting microscope. A drop of SlowFade anti-fade reagent (mounting media) was placed on a coverslip and dropped over the retina. Finally, the results were analyzed and photographed using Imaging Software NIS-Elements V3.0, fluorescent microscopy Eclipse NI (Nikon, Chiyoda, Japan).

For hematoxylin and eosin (H&E) staining, whole eyes were fixed in 4% paraformaldehyde in 0.1 M PBS for 24 h and embedded in paraffin. Serial 3.5-mm sections of whole eyes were cut sagittally through the cornea parallel to the optic nerve. FFPE slides were stained with H&E. Images were obtained and analyzed using a light microscope [8].

2.4. Quantification of RNV and Vaso-obliteration

Quantification of RNV is difficult to standardize among different investigators. Several approaches for measuring the extent of RNV coexist: (i) scoring retinal whole mounts using a grading system [15,24]; (ii) counting preretinal nuclei in retinal cross-sections [2,38]; (iii) manually measuring the area of neovascular tufts in retinal whole mounts [25,5]. In the current study, the last two approaches were utilized.

For the retinal cross-sections, 10 intact sections of equal length per eye were analyzed. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section. The 10 counted sections were evaluated by three independent reviewers. For retinal whole mounts, retinal images were imported into Adobe Photoshop CS6. As Connor et al. described, the Lasso tool was used to select the avascular area and the whole retinal area. To determine the amount of vaso-obliteration, the number of pixels in the avascular area was divided by the number of pixels in the total retinal area. Magic wand tool was used to select the RNV area. The amount of RNV was obtained by dividing the number of pixels in the total retinal area.

2.5. Western blot analysis

Fresh retinas were mixed with RIPA lysis buffer containing protease and phosphatase inhibitor cocktails. The homogenates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and supernatant was collected. Total protein concentration was evaluated using BCA protein assay. Briefly, 50 μg protein lysate was heated to 95–100 °C for 5 min with loading buffer. Proteins were separated on 10% SDS polyacrylamide gels and transferred onto a PVDF membrane. The membrane was blocked with 1 × TBST containing 5% nonfat dry milk with constant rotating for 1 h. Primary antibodies were added and membranes incubated overnight at 4 °C. The primary antibodies used were GRP78/BIP (1:1000), eIF2α (1:2000), CHOP (1:500), HIF-1α (1:600), ATF4 (1:2000), VEGF (1:1000), JNK (1:2000), TNF-α (1:500), IL-1β (1:500), IL-6 (1:500), ICAM-1 (1:2000) (Proteintech, Rosemont, IL, USA), rabbit anti-phospho-eIF2 alpha (Ser51) antibody (1:1000) (Bioss, Beijing, China), anti-phospho-JNK1/2/3 (T183 + T183 + T221) antibody (1:200) (Wuhan Boster Biological Technology, Ltd., Wuhan, China), and β-actin (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed with TBST three times for 10 min and then incubated with a suitable HRP-conjugated secondary antibody (1:20000) (Thermo Fisher Scientific) for 1 h at room temperature. Membranes were incubated with chemiluminescent HRP substrates. Images were scanned using a densitometer (Bio-Rad) and analyzed quantitatively with ImageJ® Software.

2.6. Quantitative real-time PCR (qPCR)

Total RNA was extracted using RNAiso Plus (Takara Bio, Otsu, Japan). Quantitative real-time PCR (qPCR) was performed using the GoTaq®qPCR Master Mix (Thermo Fisher Scientific) in a total volume of 20 μL in a GoScrip Reverse Transcription System (Thermo Fisher Scientific). PCR reactions were incubated at 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. Results were normalized against GAPDH according to the $2^{-\Delta\Delta Ct}$ method. The following primers were used: ATF4, forward: 5'-CATGGCGTATTAGAGGCAGC-3' and reverse: 5'-ACACTGCTGCTGGATTTCGT-3'; eIF-2α, forward: 5'-TGGAGA AAGTGCTGTGGTACAAT-3' and reverse: 5'-GTCCATGAGGGCTATAGA AGGC-3'; VEGF, forward: 5'-TCATGCGATCAAACCTCACC-3' and reverse: 5'-CTGGCTTTGTTCTGTCTTTCTTTG-3'; GAPDH, forward: 5'-ACCACCATGGAGAAGGCCGG-3' and reverse: 5'-CTCAGTGTAGCCCAA GATGC-3'.

2.7. Statistical analyses

Data are presented as the means ± standard deviation (SD). The Mann-Whitney *U* test was used to compare the differences between two groups. The Kruskal-Wallis test was used to compare the differences among three or more groups. Statistical analyses were performed using GraphPad Prism 7 Software (San Diego, CA, USA). Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Activation of ER stress and ATF4 in the retinas of OIR mouse model

The UPR is a complex cellular stress response associated with ER stress. Three proximal sensors IRE1, PERK, and ATF6 regulate the UPR

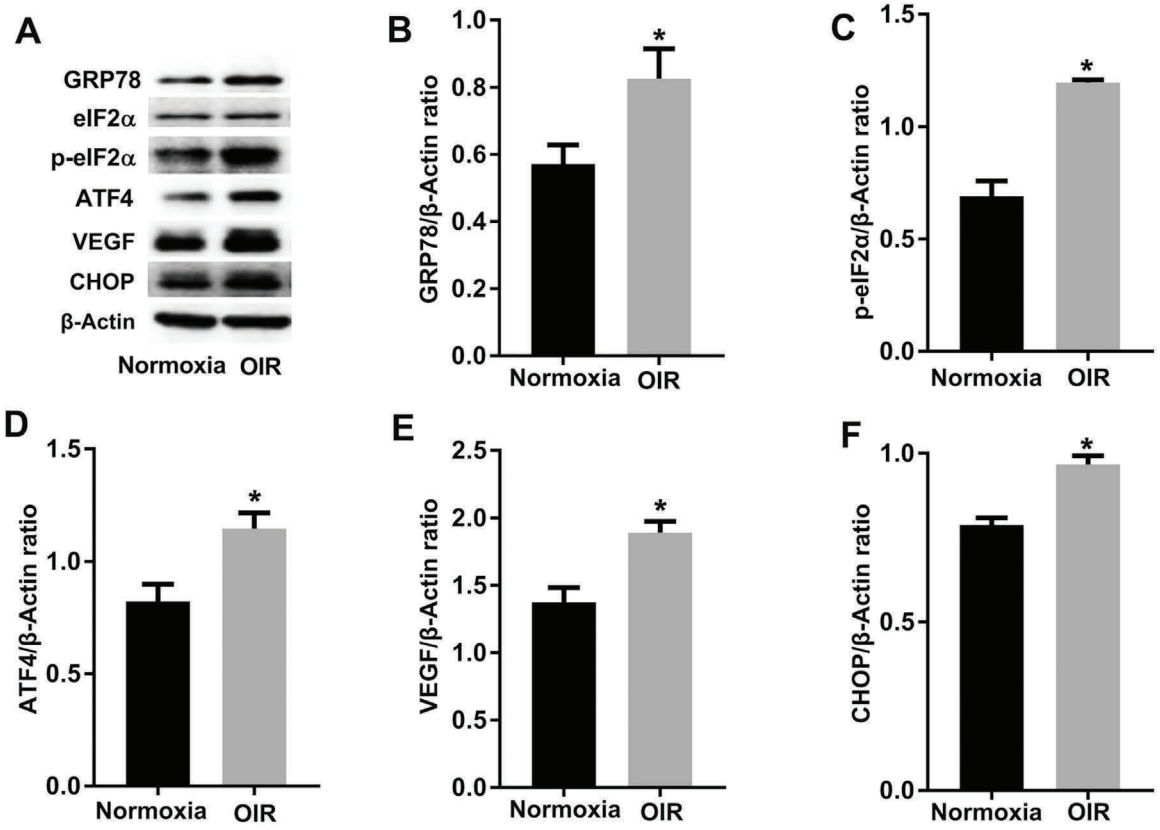

Fig. 1. Activation of ER stress and ATF4 in the retinas of OIR mice. (A) Western blot analysis of GRP78, eIF2α, p-eIF2α, ATF4, CHOP, and VEGF. (B-F) Relative protein levels of GRP78, eIF2α, p-eIF2α, ATF4, CHOP, and VEGF were quantified using densitometry (mean ± SD, n = 5). *$p < 0.05$ *vs.* normoxia group.

via their respective signaling cascades. In resting cells, these three transducers are maintained in an inactive state by interaction with the chaperone glucose-regulated protein 78 (GRP78, also known as immunoglobulin binding protein, BiP). Upon ER stress, GRP78 is sequestered from the stress sensors and subsequently activates the UPR signaling pathway. We examined retinal expression of ER stress markers in the retinas of OIR mice. As shown in Fig. 1, the expression of GRP78, p-eIF2α, ATF4, and CHOP were significantly increased in the retinas of OIR mice when compared with mice in the normoxia group (Fig. 1A–D and F), confirming that ER stress was significantly increased in the retinas of OIR mice. Moreover, an inflammatory cytokine, VEGF, was also significantly upregulated in the retinas of OIR mice (Fig. 1A and E).

3.2. RPL41 reduced the OIR neovascularization and vaso-obliteration in retinal whole mount staining images

The retinal vascular structures were examined using isolectin staining in normoxia, OIR, OIR control, and OIR treated mice on P17 (Fig. 2). As expected, the mice in the normoxia group developed a complete and clear retinal vasculature, with regular blood vessels extending from the optic nerve to the periphery. The retinas of mice in the OIR and OIR control groups were characterized by decreased central perfusion, and the neovascular response occurred in mid-periphery at the junction between the perfused and nonperfused retinas (Fig. 2A). The retinas of mice treated with RPL41 (OIR treated group) were significantly protected from pathologic neovascularization (3.9% ± 1.0%) compared with the retinas of mice in the OIR control group (14.3% ± 1.4%, $p \leq 0.05$). Moreover, the retinas of mice in the OIR control group had a vaso-obliterated/total retinal area of 13.1% ± 2.7% compared with 5.6% ± 0.8% in the retinas of OIR treated mice ($p \leq 0.05$; Fig. 2B and C), indicating RPL41 protected the retinas of postnatal mice from hyperoxia-induced vaso-obliteration.

3.3. RPL41 reduced the number of preretinal neovascular cells in the OIR mouse model

To further confirm the protective effects of RPL41 on RNV, we quantified preretinal neovascular cells from 10 non-continuous cross-sections on P17, an established method for evaluating the OIR mouse model [30]. The degree of oxygen-induced neovascularization was quantified by counting the number of vascular cell nuclei on the vitreal side of the internal limiting membrane. In our study, an average of 2.42 ± 1.7 nuclei extending into the internal limiting membrane per retinal cross-section was observed in the normoxia group compared with 70 ± 8.7 nuclei per cross-section in the OIR group (Fig. 3A, B, and E), confirming the successful establishment of the OIR mouse model. After intravitreal injection of RPL41, the number of preretinal neovascular cells in the retinas of mice in the OIR treated group (20 ± 7.3) was significantly decreased compared with the retinas of mice in the OIR control group (68 ± 8.3, $p < 0.05$, n = 7); this decreased number of preretinal neovascular cells result is consistent with the decreased OIR neovascularization in retinal whole mount, showing the protective effects of RPL41 on neovascularization.

3.4. RPL41 reduced the protein and mRNA levels of important ER stress factors p-eIF2α, ATF4, and VEGF in the OIR mouse model

Western blot analysis and qPCR assays were used to estimate the change of p-eIF2α, ATF4, and VEGF in the OIR mouse model. The results showed that both protein and mRNA levels of p-eIF2α, ATF4, and VEGF were substantially higher in the retinas of mice in the OIR group than in the retinas of mice in the normoxia group, which further confirmed the activation of ER stress in the OIR mouse model (Fig. 4). More importantly, the results demonstrated the protein and mRNA levels of p-eIF2α, ATF4, and VEGF were significantly decreased in the OIR

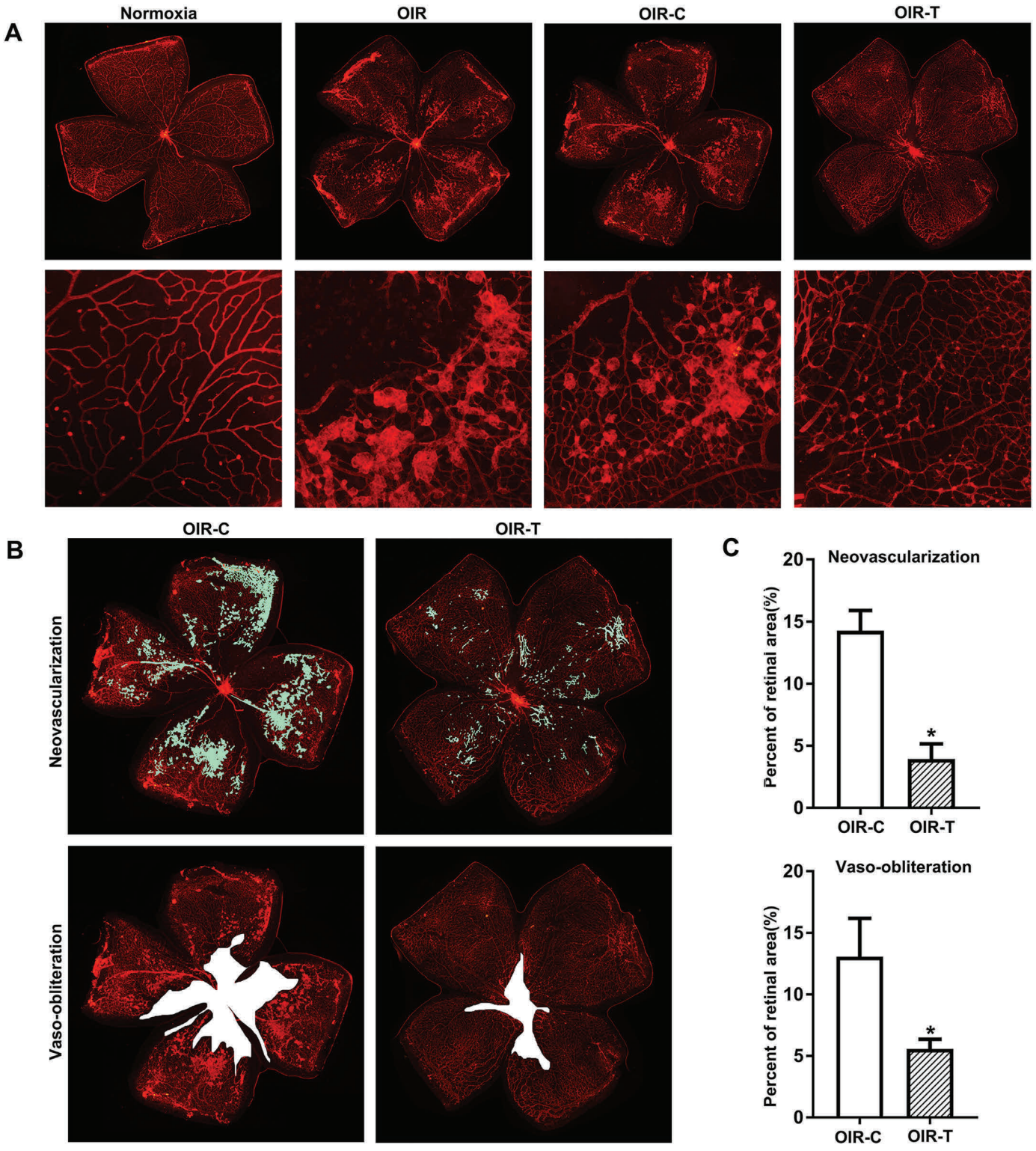

Fig. 2. RPL41 reduces the OIR neovascularization and vaso-obliteration in retinal whole mount staining images. (A) Representative images of retinal flat mount in the normoxia, OIR, OIR control, and OIR treated mice on P17. The upper panels show whole mount staining images from the four groups (magnification: 40×) and the lower panels show enlarged local areas of normal vessels and abnormal pathologic neovascularization in the four groups (magnification: 100×). (B) Representative images of retinal vasculature in the OIR control mice and OIR treated mice on P17. Areas of pathologic neovascularization or vaso-obliteration are highlighted in color. (C) Quantification of pathologic neovascularization and retinal vaso-obliteration in OIR control and OIR treated mice retinas. n = 15–18/group. *$p < 0.05$ *vs.* OIR control group.

treated group compared with the OIR control group. In brief, the results indicated the RPL41 administered in the OIR mouse model may ameliorate RNV by downregulating the protein and mRNA levels of p-eIF2α, ATF4, and VEGF.

3.5. RPL41 decreased the inflammatory response in the OIR mouse model

To investigate the effects of RPL41 on the inflammatory response, we examined several inflammatory cytokines using western blot

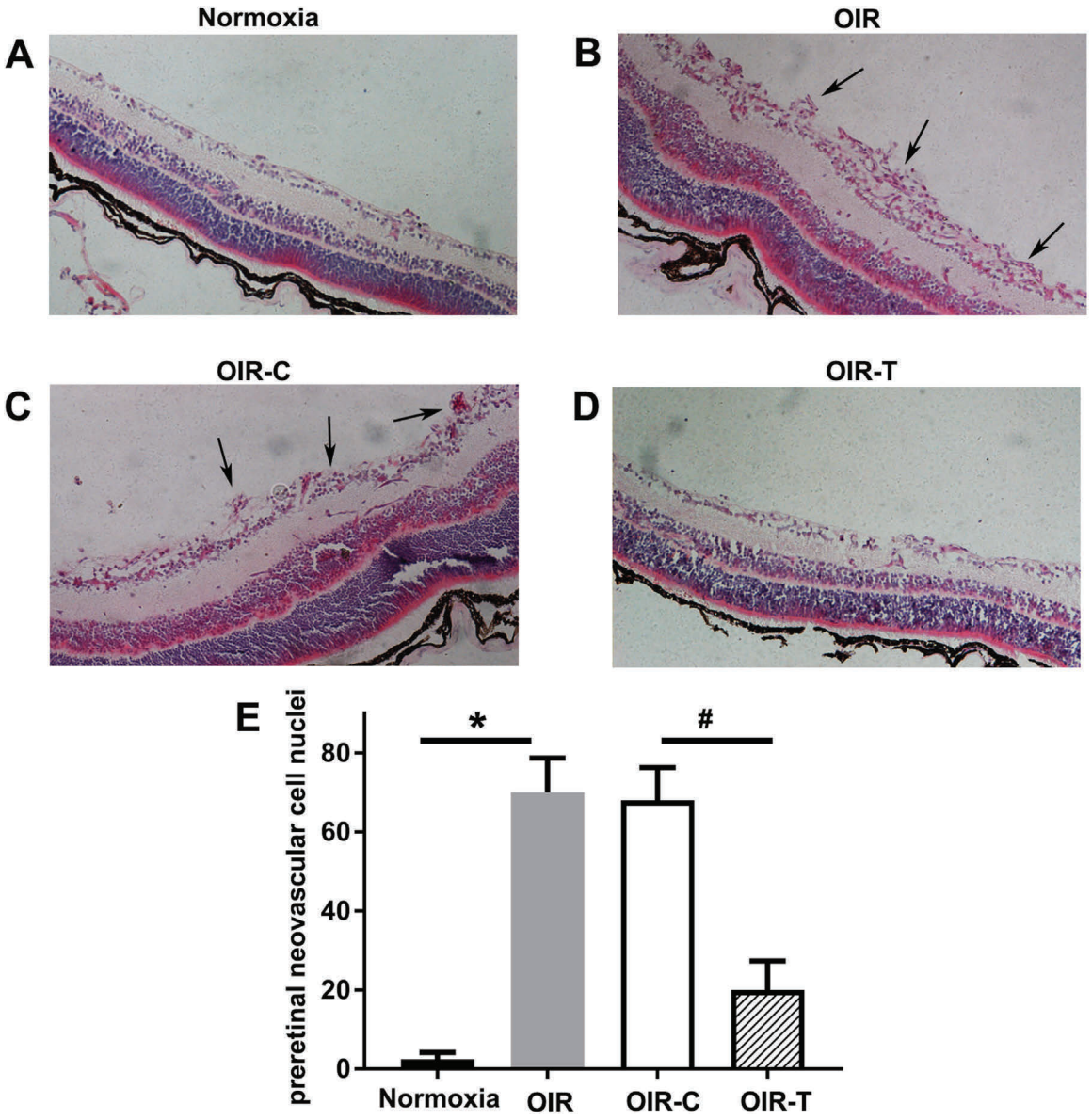

Fig. 3. RPL41 reduced the number of preretinal neovascular cells. (A) Normoxia group (B) OIR group (C) OIR control group (D) OIR treated group. Representative images of retinal cross-sections on P17 using H&E. Black arrows indicate the preretinal neovascular cells on the vitreous side of internal limiting membrane. Magnification 200×. (E) Assessment of preretinal neovascular cell nuclei on the vitreous side. Normoxia group *vs.* OIR group, $^{*}p < 0.05$; OIR control group *vs.* OIR treated group, $\#p < 0.05$; (n = 10).

analysis. As expected, ICAM-1, IL-1β, IL-6, and TNF-α were over-expressed in the retinas of mice in the OIR group compared with the normoxia group (Fig. 5A). Moreover, we demonstrated that two classical inflammatory signaling pathways mediated by HIF-1α and JNK were activated in the OIR mouse model (Fig. 5B and C). However, intravitreal injection of RPL41 significantly decreased protein levels of IL-1β, IL-6, and TNF-α in the OIR treated group compared with the OIR control group, but not ICAM-1 ($p = 0.3813$), indicating RPL41 could also decrease the inflammatory response (Fig. 5A).

4. Discussion

The activation of the ER stress and increased ATF4 levels have been previously reported in ischemic retinopathies [26,27,41]. ATF4 is a key response gene for tumor cells to survive stress. Under stress conditions, the accumulation of unfolded proteins in the ER lumen causes the release of PERK which dimerizes with BIP. Then, PERK is auto-phosphorylated and phosphorylates eIF2a on Ser51. Phosphorylated eIF2a induces translation of ATF4 mRNA [36]. In the OIR mouse model, neonatal mice were exposed to 75% oxygen from P7 to P12; vessel regression and cessation of normal radial vessel growth occurred in the first phase of OIR. Upon returning to room air, the non-perfused portion of the retina was oxygen-deficient, thereby inducing the expression of angiogenic factors and resulting in RNV [6]. We hypothesized the change of oxygen concentration could be a stress signal to the retinal cells and may induce the activation of ER stress signaling. In the present study, the expression of ER stress makers GRP78, p-eIF2α, ATF4, and CHOP were significantly increased in mice subjected to decreased oxygen concentration. VEGF was shown a critical factor contributing to RNV [1]. Roybal et al. [28] showed ATF4 could upregulate VEGF expression by binding to four amino acid response elements found in the *VEGF* promoter. Oskolkova et al., [21] demonstrated UPR, and more specifically its ATF4 branch, is an important mechanism mediating the upregulation of VEGF by oxidized phospholipids, and hypothesized the UPR cascade might play a role in pathologic angiogenesis in atherosclerotic plaques. In our study, protein and mRNA levels of ATF4 and

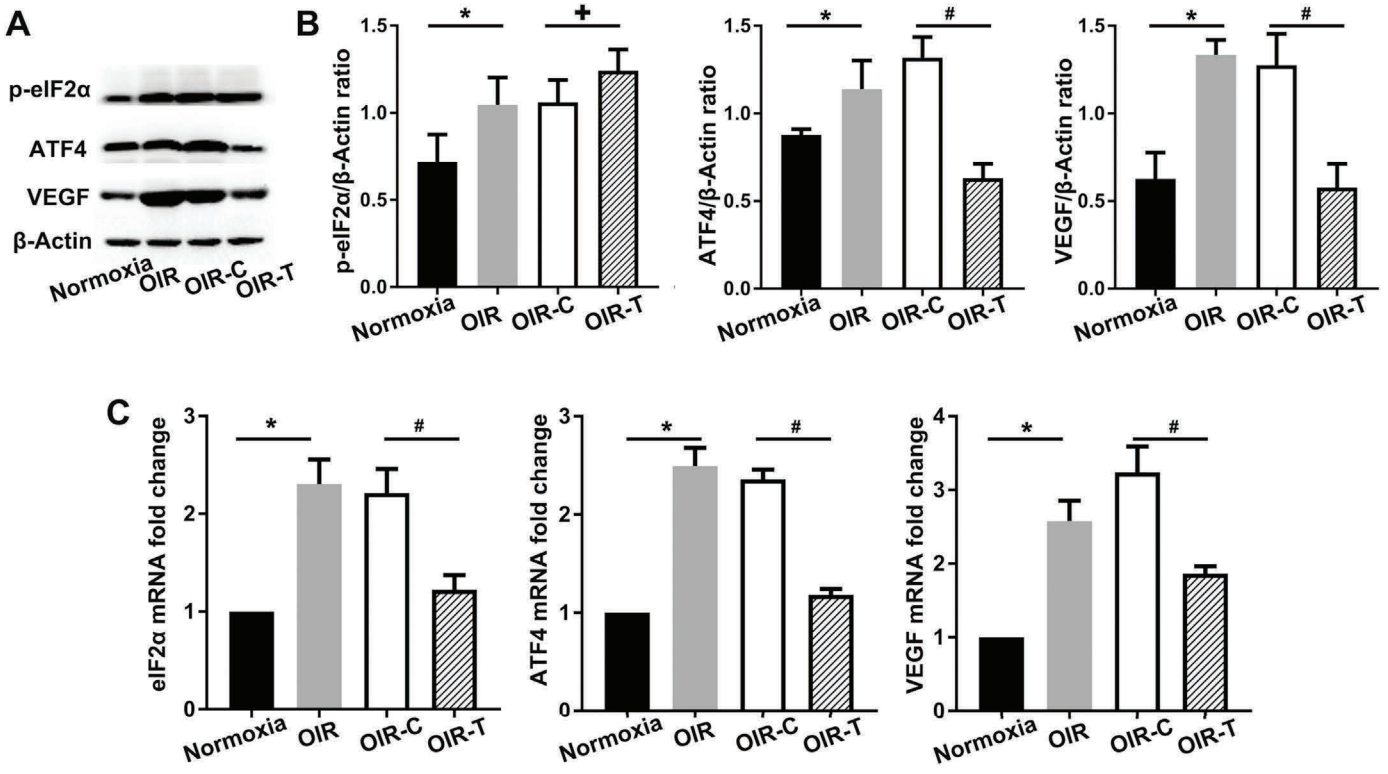

Fig. 4. RPL41inhibited RNV by reducing the protein and mRNA levels of important ER stress factors p-eIF2α, ATF4, and VEGF in the OIR mouse model. (A) Western blot analysis for protein expression. (B) Quantification of protein levels. Normoxia group *vs*. OIR group, $*p < 0.05$; Normoxia group *vs*. OIR group, $+ p > 0.05$; OIR control group *vs*. OIR treated group, $\#p < 0.05$; (n = 8). (C) mRNA expression of p-eIF2α, ATF4, and VEGF determined using qPCR. Normoxia group *vs*. OIR group, $*p < 0.05$; OIR control group *vs*. OIR treated group, $\#p < 0.05$; (n = 8).

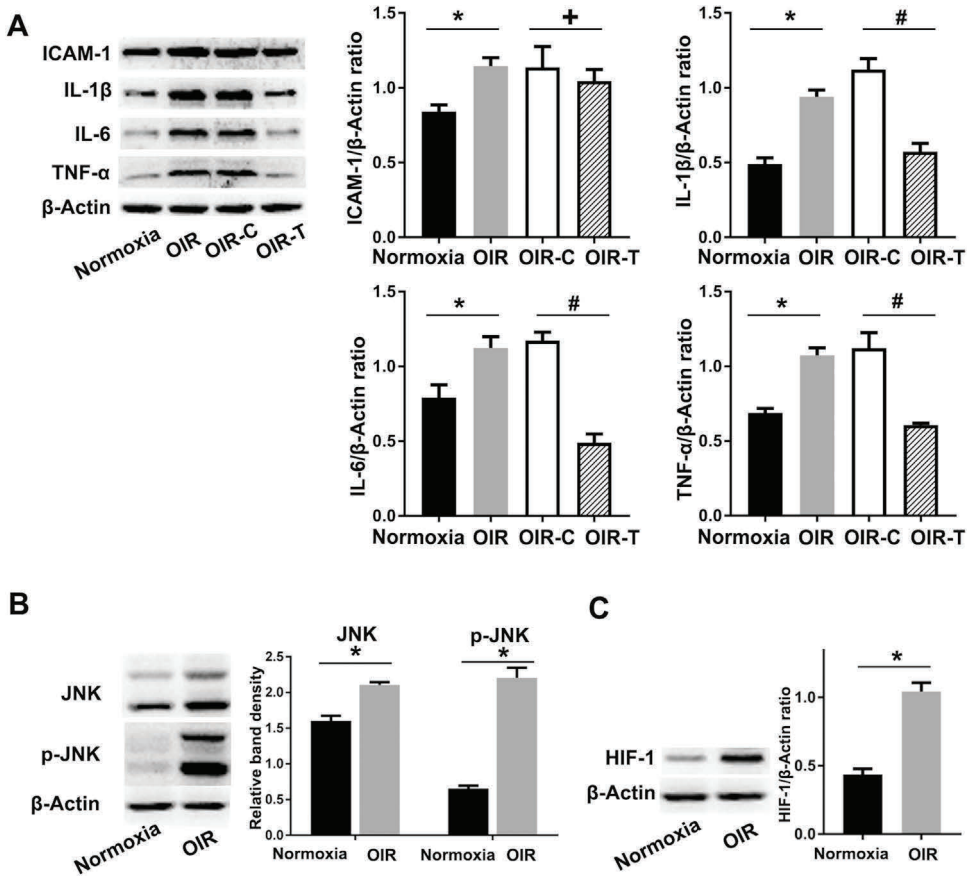

Fig. 5. RPL41 decreases the inflammatory cytokine expression and activation of JNK and HIF-1 pathways on P17 in the OIR mouse model. (A) Protein expression of ICAM-1, IL-1β, IL-6, and TNF-α. Normoxia group *vs*. OIR group, $*p < 0.05$; OIR control group *vs*. OIR treated group, $\#p < 0.05$, $+ p > 0.05$; (n = 8, respectively). (B) Protein expression of JNK. Normoxia group *vs*. OIR group, $*p < 0.05$, n = 6. (C) Protein expression of HIF-1. Normoxia group *vs*. OIR group, $*p < 0.05$; (n = 8).

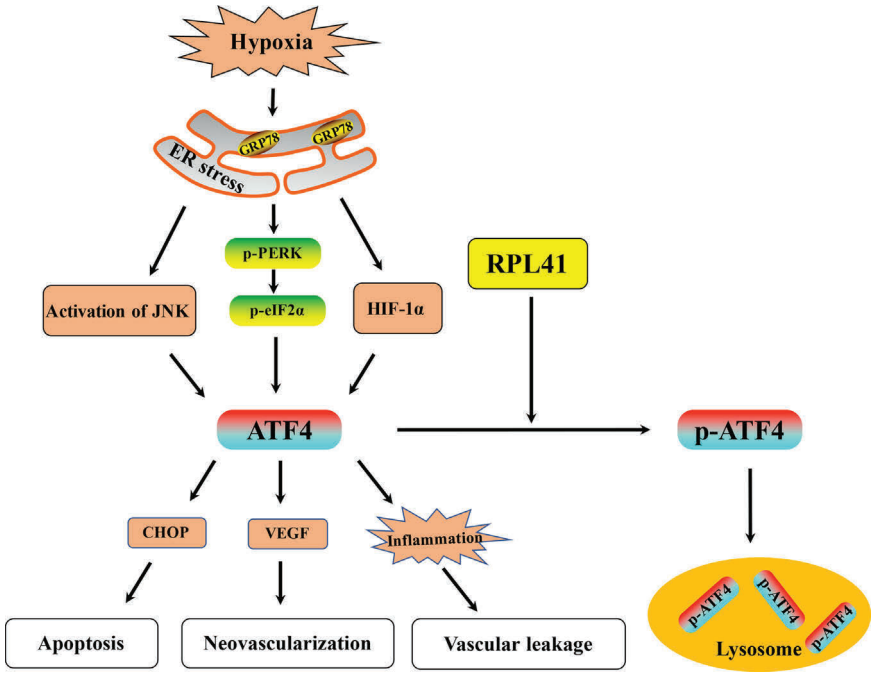

Fig. 6. Activation of the ER stress and function of RPL41 in the OIR model. Stress such as hypoxia induce ER stress resulting in activation of the PERK-UPR branch in retinal cells. Unrelieved ER stress upregulates ATF4, which directly binds to promoters of inflammatory genes (e.g., VEGF), and interacts with HIF-1α and JNK, resulting in exaggerated and sustained expression of inflammatory and apoptosis genes. Mini-peptide RPL41 induced rapid ATF4 degradation by inducing ATF4 phosphorylation and relocation from nuclei to cytoplasm.

VEGF were increased in the retinas of OIR mice, consistent with the conclusion stated by Roybal et al. [29]. In addition, the enhancement of ER stress or overexpression of ATF4 was sufficient to induce inflammation in cultured retinal endothelial cells [7]. Genetic manipulation with ATF4 can modulate the level of neovascularization and reprogram the UPR at both the mRNA and protein levels in proliferative retinopathy [35]. The results from the present study together with previous studies indicate that ATF4 shows great promise as a potential therapeutic target for treating ocular neovascular disorders.(Fig. 6)

The mini-peptide RPL41, a small molecular synthesized peptide rich in arginine and lysine capable of penetrating cell membranes, is analogous to the product of tumor suppressor gene RPL41 that is lost in many types of tumors. RPL41 is associated with several cytoskeleton components including tubulin β, γ, and myosin IIA, and a synthetic RPL41 could induce cellular α-tubulin acetylation and G2/M cell cycle arrest [34]. Our previous results showed the mini-peptide RPL41 induced rapid ATF4 degradation by inducing ATF4 phosphorylation and relocation from nuclei to cytoplasm, where ATF4 co-stained with a proteasome marker [33].

The mouse model of OIR is a widely used animal model of oxygen-induced ischemic retinopathy [31,9]. In the present study, we successfully built an OIR mouse model. Before the ER stress occurred, several OIR mice were administered an intravitreal injection of RPL41 on P12. The results showed the mini-peptide RPL41 blocked the formation of neovascular tufts and reduced the number of preretinal neovascular cells in the retina of the OIR mice. ATF4 and VEGF levels were markedly decreased in RPL41-treated mice compared with the OIR control group. At the protein and mRNA levels, results indicated RPL41 degraded the important ER stress factor ATF4, contributing to the attenuation of aberrant neovascularization in the OIR mouse model. In addition, we observed the OIR mice treated with RPL41 developed smaller areas of vaso-obliteration than the mice in the OIR control group. In retinal whole mount staining images with higher magnification, the size of neovascular tufts was much smaller but the number of neovascularization buds was significantly greater than in the OIR control group (data not shown). These results indicated RPL41 accelerated revascularization into the avascular area and the mini-peptide RPL41 plays an essential role in not only pathological angiogenesis but also the regrowth of normal vasculature in OIR; however, the mechanism remains unclear.

Several factors contribute to the pathogenesis of ischemic retinopathies; however, oxidative stress and inflammatory processes are major factors [26,27]. Cytokines such as ICAM-1, IL-1β, TNF-α, and IL-6 act as primary initiators of inflammation following infection or tissue damage [10]. Previous studies showed that JNK and HIF-1α are critical for hypoxia-induced retinal VEGF production and inflammatory processes, which closely interact with ATF4 [3]. In the present study, RPL41-induced degradation of ATF4 downregulated inflammatory gene expression, or indirectly inactivated other inflammatory pathways such as JNK and HIF-1α, thus preventing retinal inflammation and vascular leakage.

It has been well known that small peptides could function as biomolecules with a huge diversity of important roles in vivo. Meanwhile, small bioactive peptides also displayed multi-advantages as compared with chemical agents and proteins, like lower accumulation in tissues and less organ damage [19]. It has been identified that the different functional sequences in small bioactive peptides determine the mode multiplicity of their interactions with cellular molecules, of which arginine- and lysine-rich sequences possess the highly cationic nature that is important for binding to and penetrating though the cell membrane [13]. According to this property, various analogous peptides were analyzed and designed to function as cell-penetrating peptides (CPPs) for the agent delivery system [13,22], while the non-selective electrostatic binding lead to the absence of specific cellular delivery, which became the bottleneck of its clinical application [23,40]. Much efforts have been made to overcome these limitations, including local injection, conditional release induced by local microenvironment and the combining of local delivery device, so does our present study using intravitreal injection [32]. Additionally, increasing works have focused on the unique function of cell-targeting peptides (CTPs) with a cell-recognition peptide motif, of which assembling with CPPs are likely to be employed in the near future to associate both targeting and delivery properties [32].

In conclusion, mini-peptide RPL41 reduced RNV in OIR via ATF4 degradation and subsequent attenuation of VEGF expression. Because RPL41 is the only known anti-ATF4 agent, we expect this mini-peptide can be used not only for the treatment of ocular neovascular disorders but also other health conditions associated with neovascularization.

Acknowledgments

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Conflict of interest

None.

Disclosure

Wen Geng and Aiyuan Wang performed the research design. Wen Geng, Jiaxu Ren and Feng Qin conducted the experiments. Sheng Xiao designed and provided the RPL41. Wen Geng and Aiyuan Wang wrote the manuscript.

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