• Basic Research •

CCR7/p-ERK1/2/VEGF signaling promotes retinal neovascularization in a mouse model of oxygen-induced retinopathy

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Received: 2016-10-24 Accepted: 2017-02-14

Abstract

• AIM: To investigate the role of CCR7/p-ERK1/2/VEGF signaling in the mouse model of oxygen-induced retinopathy (OIR).

• METHODS: Neonatal C57BL/6J mice were evenly randomized into four groups: normoxia, OIR, OIR control (treated with scramble siRNA), and OIR treated (treated with CCR7 siRNA). Normoxia group was not specially handled. Postnatal day 7 (P7) mice in the OIR group were exposed to 75%±5% oxygen for 5d (P7-P12) and then maintained under normoxic conditions for 5d (P12-P17). Mice in the OIR control and OIR treated groups were given injections of scramble or CCR7 siRNA plasmid on P12 before returning to normoxic conditions for 5d (P12-P17). Retina samples were collected from all mice on P17, stained with adenosine diphosphatase (ADPase), and retinal neovascularization (RNV) was assessed. Retinas were also stained with hematoxylin and eosin (H&E) for RNV quantitation. The distribution and expression of CCR7, p-ERK1/2 and vascular endothelial growth factor (VEGF) were assessed *via* immunohistochemistry, Western blot, and quantitative real-time polymerase chain reaction (qRT-PCR).

• RESULTS: High oxygen promoted retinal neovascularization (*P*<0.05) and increased the number of endothelial nuclei in new vessels extending from the retina to the vitreous body; CCR7 promoted this process (*P*<0.05). CCR7 and VEGF mRNA were expressed at higher levels in the OIR and OIR control groups than in the normoxia and OIR treated groups. CCR7, p-ERK1/2, and VEGF protein were expressed in the retinas of mice in the OIR and OIR control groups. Intravitreal injection of CCR7 siRNA significantly reduced CCR7, p-ERK1/2, and VEGF expression in the OIR mouse model (all *P*<0.05). CCR7 significantly enhanced the neovascularization and non-perfusion areas in the OIR group (*P*<0.05). CCR7 siRNA significantly reduced levels of p-ERK1/2 and VEGF as compared to OIR controls (*P*<0.05).

• CONCLUSION: These results suggest that CCR7/p-ERK 1/2/VEGF signaling plays an important role in OIR. CCR7 may be a potential target for the prevention and treatment of retinopathy of prematurity.

• KEYWORDS: chemokine receptor type 7; vascular endothelial growth factor; extracellular signal-regulated kinase; retinal neovascularization; retinopathy of prematurity

DOI:10.18240/ijo.2017.06.06

Yuan LH, Chen XL, Di Y, Liu ML. CCR7/p-ERK1/2/VEGF signaling promotes retinal neovascularization in a mouse model of oxygen-induced retinopathy. *Int J Ophthalmol* 2017;10(6):862-869

INTRODUCTION

C-C chemokine receptor type 7 (CCR7) is mainly expressed in immunocytes such as dendritic cells, naive T cells, B cells, regulatory T cells, memory T cells, and natural killer (NK) cells. CCR7 participates in many physiological and pathological processes *in vivo*[1-2] and mediate cell migration and angiogenesis in many diseases[3].

The extracellular signal-regulated kinase (ERK) signaling pathway participates in the proliferation and angiogenesis of endothelial cells *in vitro*. The ERK signaling pathway is involved in the release of vascular endothelial growth factor (VEGF) in the retinas of diabetic rats[4]. VEGF is known as the most powerful angiogenesis promoting factor. However, the relationship between CCR7, ERK, and VEGF in retinopathy of prematurity (ROP) has not been illustrated, and the action of CCR7 in retinal neovascularization (RNV) during ROP remains unclear. Therefore, we measured the expression of CCR7, p-ERK1/2, and VEGF in RNV to investigate the role of CCR7/p-ERK1/2/VEGF signaling in the mouse model of oxygen-induced retinopathy (OIR).

MATERIALS AND METHODS

Animals All experiments were performed in accordance with guidelines set by the Animal Experiment Committee of the Shengjing Hospital of China Medical University, and the study was approved by Shengjing Hospital of China Medical

Table 1 Primer sequences for qRT-PCR

Gene		Primer sequences (5′-3′)	Product length (bp)	Tm (℃)
β-actin	Forward	CCTCCTCCTGAGCGCAAGTA	117	55
	Reverse	GATGGAGGGGCCGGACT		
CCR7	Forward	AACGGGCTGGTGATACTGAC	139	55
	Reverse	AGGACTTGGCTTCGCTGTAG		
VEGF	Forward	CAACTTCTGGGCTCTTCTCG	144	55
	Reverse	CCTCTCCTCTTCCTTCTCTTCC		

qRT-PCR: Quantitative real-time reverse transcriptional polymerase chain reaction; CCR7: C-C chemokine receptor type 7; VEGF: Vascular endothelial growth factor; Tm: Temperature; bp: Base pair.

University's Ethics Committee. Mice were housed in a barrier facility with free access to normal food and tap water. They were maintained under standard conditions for lighting (a 12h/12h light/dark cycle), temperature (23℃-25℃), and humidity (50%-60%).

Animal Model Specific pathogen-free healthy C57BL/6J neonatal mice (China Medical University, Shenyang, China) were evenly randomized into four groups: normoxia, OIR, OIR control (treated with scramble siRNA), and OIR treated (treated with CCR7 siRNA). Mice in the OIR group were handled based on Smith's method[5]. On postnatal day 7 (P7), mice in the the last 3 groups as well as their dams were transferred into a specially made glass case then exposed to 75%±5% oxygen for 5d (P7-P12). The mice in OIR group were returned to room air (normoxic conditions) for 5d (P12-P17). Mice in the last two groups were administered injections of 1 μL scramble or CCR7 siRNA plasmid with a 33-gauge needle attached to a Hamilton syringe on P11, and then returned to normoxic conditionsfor 5d (P12-P17). Mice in the normoxia group were maintained under normoxic conditions for 17d (P0-P17). Mice were humanely euthanized by cervical dislocation on P17, and then their eyeballs were harvested.

Small Interfering RNA CCR7 and scrambled small interfering RNA (siRNA) was purchased from GenePharma Co. Ltd. (Shanghai, China). The sequence of CCR7 siRNA used is 5'-GAAGUGCAUACACCGAGAC-3', and its efficacy has been previously demonstrated[6].

Observation of Retinal Neovascularization Eyeballs were fixed with 4% paraformaldehyde for 3h. The cornea and lens were removed, then the entire retina was dissected and radial cut into four quadrants. Retinas were stained with magnesium-activated adenosine diphosphatase (ADPase). ADPase stained retinal flat mounts were carefully imaged with an optical microscope (Olympus Corporation, Tokyo, Japan). Images were carefully examined to assess the severity of neovascularization with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA).

Quantification of Retinal Neovascularization To quantify preretinal neovascular cells, retinal structures were analyzed on 6-μm H&E stained sections. Eyeballs were fixed with 4% paraformaldehyde for 24h, then embedded in paraffin. Whole eyes were sagittally cut into serial sections (6-μm thick) through the cornea and parallel to the optic nerve, and then stained with H&E, dehydrated, vitrified, mounted, observed, and imaged with a light microscope (Olympus B201, Olympus Corp., Tokyo, Japan). Three blinded researchers counted the cells.

Immunohistochemistry Eyeballs were fixed with 4% paraformaldehyde then dehydrated with ethanol and xylene. Deparaffinized the eye tissue sections. For heat-induced antigen retrieval, antigen retrieval buffers were prepared with citric acid and sodium citrate. Sections were incubated with rabbit anti-CCR7 polyclonal antibody (1:200; Abcam, Cambridge, UK), rabbit anti-p-ERK1/2 (1:150; Cell Signaling Technology, Boston, MA, USA), and rabbit anti-VEGF polyclonal antibody (1:100; Proteintech, Chicago, IL, USA) overnight at 4℃. Sections were then incubated with biotinylated secondary antibody (1:1000; Zhongshan Jinqiao Biotechnology Co. Ltd., China) and the avidin-biotinylated peroxidase complex was activated. Primary antibody was replaced with PBS for negative controls. The peroxidase reaction was developed with horseradish peroxidase labeled avidin/streptomycin, and sections were colored with dimethyl amino-azo-benzene (DAB), dehydrated with alcohol, and sealed with neutral gum. Images were digitally captured using an Olympus B201 optical microscope.

Quantitative Real-time Reverse Transcriptional Polymerase Chain Reaction RNA was extracted from retinal samples with Trizol (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription into cDNA was performed with the reverse transcriptase kit TAKARA 047A (PrimeScript RT Reagent kit-Perfect Real-Time; Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Primers were designed and purchased from Sangon Biotech Co. Ltd. (Shanghai, China); β-actin served as a normalizing control. The sequences of primers used are shown in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to determine relative quantification of gene expression[7].

Western Blot We added 500 μmol/L RIPA Lysis Buffer (Sigma-Aldrich Co. St Louis, MO, USA) and 5 μmol/L phenylmethanesulfonyl fluoride (Sigma-Aldrich Co. St

Louis, MO, USA) to the retinas. For retinal samples in which p-ERK1/2 was to be tested, we also added 5 μmol/L protein phosphatase inhibitor. We then determined protein concentration using the BCA method. Membranes were then incubated with horse radish peroxidase-conjugated secondary antibody (1:2000; Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) for 2h. Signals were detected with enhanced chemiluminescence Azure c300 chemiluminescent Western blot imaging system (Azure Biosystems, Inc., USA). The ratio between the optical densities of the protein of interest and GAPDH in each same sample was calculated to determine relative protein content.

Statistical Analysis Statistical analysis of all data was performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are represented as mean±standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with the least significant difference post hoc analysis. $P<0.05$ was considered statistically significant.

RESULTS

Quantitation of Retinal Neovascularization We examined the retinal vasculature in the normoxia, OIR, OIR control, and OIR treated groups using ADPase in retinal flat mounts at P17 (Figure 1). No abnormal blood vessels were observed in the retinas of the normoxia group. Two layers of retinal vessels were evenly distributed in the retina, the superficial blood vessels were well formed, and the deep blood vessel formed a polygon mesh pattern. The blood vessels in OIR and OIR control groups showed non-perfusion areas and neovascularization. The ratio of new blood vessel area to total retinal area was higher in the OIR treated (0.34±0.04), OIR (0.62±0.08), and OIR control groups (0.60±0.05) than in the normoxia group (0.25±0.01; all $P<0.05$). In contrast, retinas in the OIR treated group (0.34±0.04) developed less severe neovascular tufts and regions of non-perfusion as compared to the OIR and OIR control groups (both $P<0.05$), which demonstrates a strong inhibitory effect of CCR7 siRNA on RNV in the OIR treated group. No significant difference was detected between the OIR and OIR control groups ($P>0.05$).

Qualitative of Retinal Neovascularization H&E stained retina sections are shown in Figure 2. Preretinal neovascular cells were nearly absent from retinas in the normoxia group (Figure 2A) but more abundant in the OIR (Figure 2D) and OIR control (Figure 2C) groups than in the OIR treated (Figure 2B) group, demonstrating an inhibitory effect of CCR7 siRNA on RNV in the OIR treated group.

CCR7/p-ERK1/2/VEGF Signaling in the Oxygen-induced Retinopathy Mouse Model Immunohistochemistry was analyzed with Nis Elements BR3.0 (Nikon Instruments Inc., Japan) (Figure 3). The mean density of CCR7, p-ERK1/2, and VEGF was low in the normoxia and OIR treated groups, but high in the OIR and OIR control groups (all $P<0.05$). These

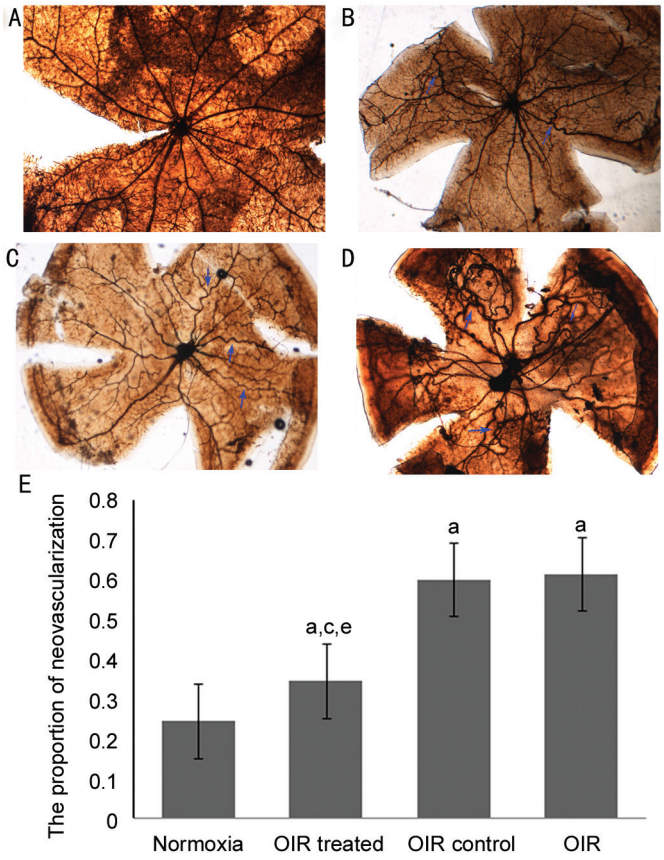

Figure 1 Inhibitory effect of CCR7 siRNA on RNV in the OIR model Representative retinal angiographs from the eyes of mice in the normoxia (A), OIR (B), OIR control (C), and OIR treated groups (D). The results of statistical analysis are illustrated in (E). The blue arrows indicate neovascularization (magnification: ×100). Data are shown as mean±SD (n=15). [a]$P<0.05$ *vs* normoxia group, [c]$P<0.05$ *vs* OIR group, and [e]$P<0.05$ *vs* OIR control group.

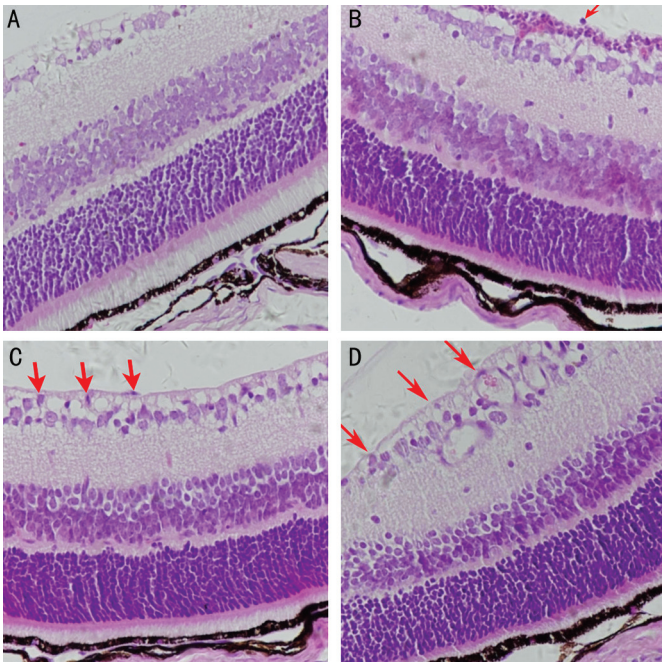

Figure 2 Effect of CCR7 siRNA on pre-RNV in mice with OIR Images shown are of representative retinal sections from the normoxia (A), OIR treated (B), OIR control (C), and OIR (D) groups. The red arrows indicate preretinal neovascular cells (magnification: ×400).

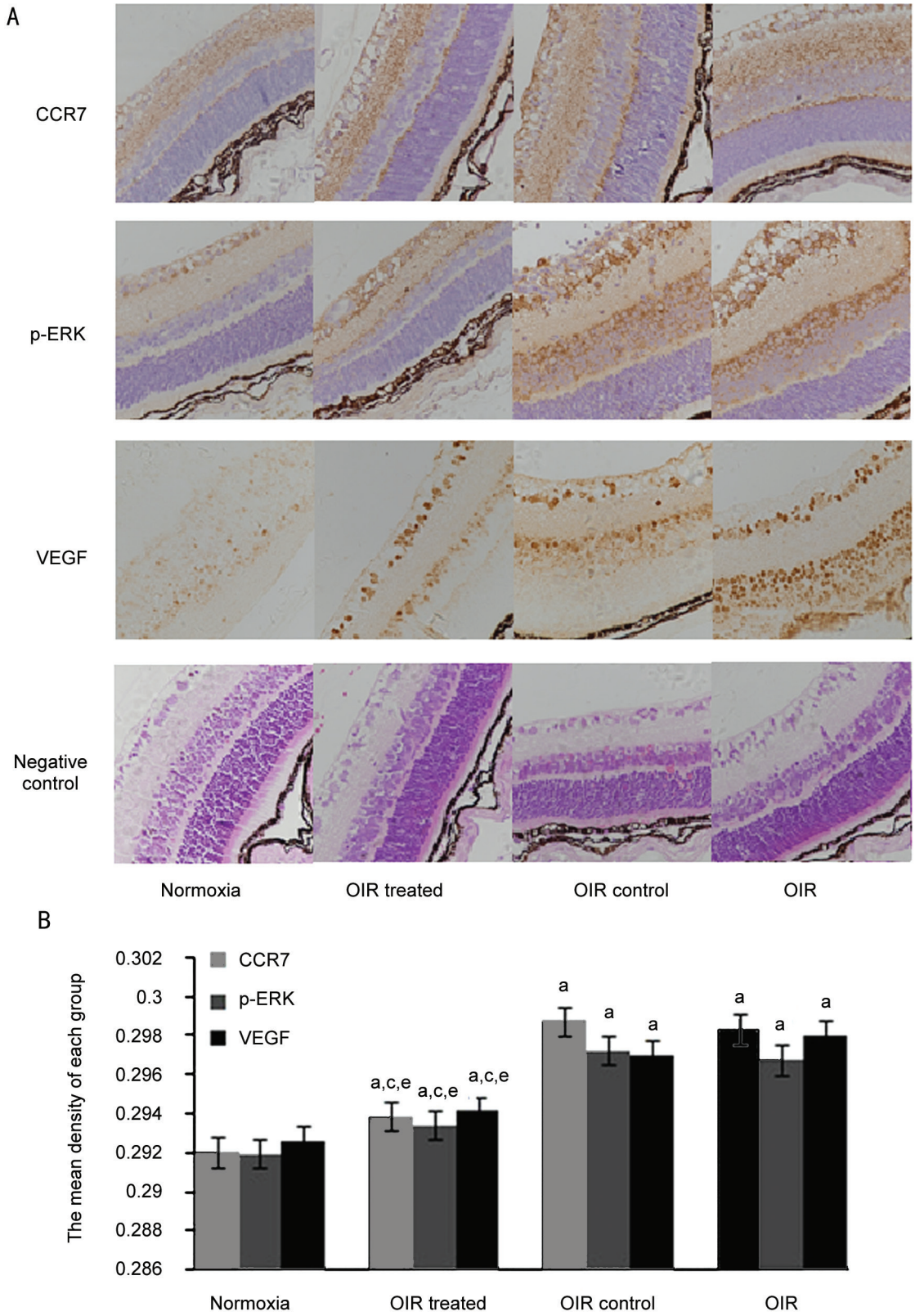

Figure 3 Hypoxia-induced CCR7 expression is mediated through the p-ERK1/2-VEGF pathway in the OIR mouse model A: Protein expression of CCR7, p-ERK1/2, and VEGF was determined by immunohistochemistry (magnification: ×400); B: The mean density of CCR7, p-ERK1/2, and VEGF in each group. [a]*P*<0.05 *vs* normoxia group, [c]*P*<0.05 *vs* OIR group, and [e]*P*<0.05 *vs* OIR control group.

results indicate that hypoxia induced the expression of CCR7, p-ERK1/2, and VEGF, and that reducing CCR7 could inhibit the expression of p-ERK1/2, and VEGF.

Expression of C-C Chemokine Receptor Type 7 and Vascular Endothelial Growth Factor The relative expression of CCR7 mRNA as compared to the normoxia group was 2.51±0.04 (OIR treated), 6.95±0.75 (OIR control), and 6.72±0.77 (OIR) and the expression of VEGF was 1.97±0.04 (OIR treated), 3.78±0.29 (OIR control), and 4.04±0.16 (OIR). CCR7 and VEGF mRNA levels in the OIR treated group were

decreased as compared to the OIR and OIR control groups (all $P<0.05$). Additionally, the expression of these mRNAs in the OIR and OIR control groups was increased as compared to the normoxia group (all $P<0.05$). No significant difference was detected between the OIR and OIR control groups (all $P>0.05$) (Figure 4).

CCR7/p-ERK1/2/VEGF Signaling Activity in Oxygen-induced Retinopathy Mice The relative protein quantity of CCR7 was 0.51±0.01, 0.68±0.02, 1.16±0.01, and 1.16±0.03 in the normoxia, OIR treated, OIR control, and OIR groups; p-ERK1/2 was 0.77±0.05, 1.00±0.08, 1.53±0.07, and 1.57±0.05; VEGF was 1.30±0.04, 1.85±0.03, 2.41±0.05, and 2.44±0.04, respectively. As compared to the OIR control group, CCR7, p-ERK1/2, and VEGF protein levels were decreased in the OIR treated group (all $P<0.05$). Additionally, the expression of each of these proteins was significantly increased in the OIR and OIR control groups as compared to the normoxia group (all $P<0.05$), whereas no significant difference was detected between the OIR and OIR control groups (all $P>0.05$) (Figure 5).

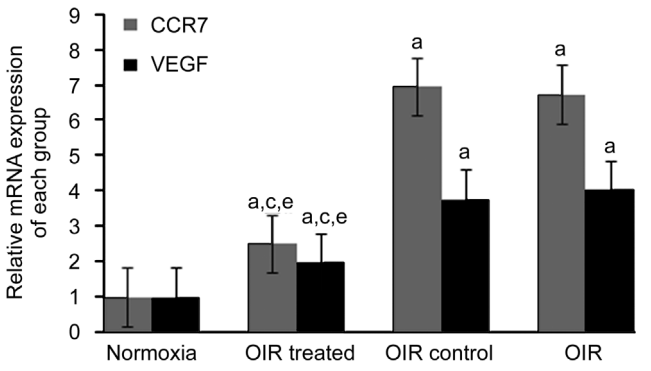

Figure 4 CCR7 siRNA inhibited RNV through inhibition of the expression of VEGF in the OIR mouse model The mRNA expression of CCR7 and VEGF was determined by qRT-PCR. Data are shown as mean±SD (n=10). [a]$P<0.05$ *vs* normoxia group, [c]$P<0.05$ *vs* OIR group, and [e]$P<0.05$ *vs* OIR control group.

DISCUSSION

Chemokines are chemical induction factors for specific G-protein coupled receptors (GPCRs). Chemokines belong to a family of cytokines, are secreted by different cell types, and play a role in chemotaxis. They play an important role in both normal and abnormal physiological processes. CCR7 is a member of the chemokine receptor family. CCR7 expression on the cell surface combined with activation by its high affinity ligands, CCL19 and CCL21, can promote integrin aggregation, thereby activating the coupled G-protein in the cytoplasm. This activation quickly induces Ca^{2+} mobilization and stimulates the mitogen activated protein kinase (MAPK) and focal adhesion kinase (FAK) pathways, thereby activating protein kinase C and downstream guanosine triphosphatase (GTP)-binding tyrosine kinases. This stimulates signal transduction in a variety of ways, recombinants the bone scaffold protein in cells, cause target cell migration and produce efficient chemotaxis. A variety of stimulating factors such as growth factors, cytokines, viruses, GPCR ligands, and oncogenic proteins can activate this pathway[8].

CCL19/21-CCR7 signaling plays an important role in the initiation, development, and metastasis of many tumor types[9-11]. CCR7 mediates angiogenesis in different tumor microenvironments as well as metastasis-mediated angiogenesis[12]. Pathological angiogenesis that occurs during thymic hyperplasia in myasthenia gravis may be related abnormal recruitment of vascular endothelial cells by CCL21[13].

CCL19[14] and CCL21[15] expression was detected in vascular endothelial cells in the synovial tissue of patients with rheumatoid arthritis (RA). CCL19 and CCL21 induce the formation of potent angiogenic factors in macrophages and RA fibroblasts[16]. In RA, CCR7 and its ligands regulate the formation of new blood vessels through different signaling pathways[17]. Furthermore, CCR7 and its ligands mediate cell migration and angiogenesis in many additional diseases[3]. Our data show that CCR7 promotes retinal neovascularization in OIR, and that CCR7 inhibition reduced this retinal neovascularization. We thus conclude that CCR7 promotes RNV in OIR.

ERK is a serine/threonine protein that was isolated and identified by Boulton *et al*[18]. The ERK signaling pathway plays a key role in transducing mitogenic cell signals[18]. ERK signals is the downstream of the three stages of MAPK signaling reactions, that is, the Ras-Raf-MEK cascade. The Ras-Raf-MEK-ERK pathway is one of the most important signal transduction pathways involved in cell growth, development, division, migration, metabolism, apoptosis, and other physiological processes *in vivo*[19-20]. Phosphorylated ERK can convert extracellular stimuli into intracellular reactions, promoting the phosphorylation and activation of multiple transcription factors in the nucleus, thereby enhancing transcriptional activity. ERK promotes tumorigenesis by mediating extracellular matrix degradation, adhesion, and migration of tumor cells as well as angiogenesis[19-21]. Various growth factors, ions, and hydrogen peroxide can activate the ERK pathway through phosphorylation. The ERK signaling pathway has been demonstrated to promote proliferation and angiogenesis of endothelial cells *in vitro*. In RNV diseases, the present study indicates that the ERK signaling pathway may be involved in the development and progression of diabetic retinopathy by regulating angiogenesis-related growth factors. The results of our study show that in the group with high RNV, p-ERK1/2 expression increased; in the normoxiaand OIR treated groups, p-ERK1/2 expression and RNV decreased synchronously. It can thus be inferred that p-ERK1/2 promotes RNV in OIR.

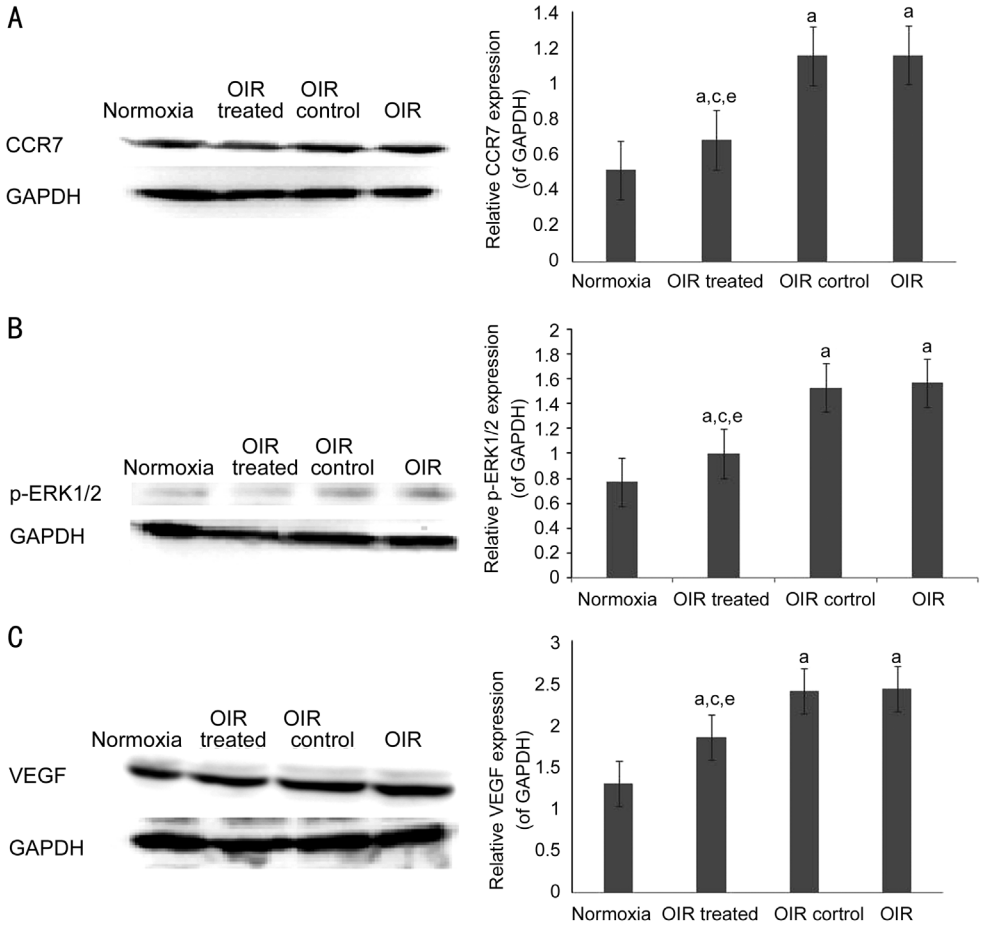

Figure 5 CCR7 siRNA decreases the protein expression of p-ERK1/2 and VEGF CCR7 (A), p-ERK1/2 (B), and VEGF (C) protein expression was determined by Western blot. Data are shown as mean±SD (n=10). [a]P<0.05 *vs* normoxia group, [c]P<0.05 *vs* OIR group, [e]P<0.05 *vs* OIR control group.

VEGF participates in many physiological and pathological processes in the body, strongly stimulates vascular endothelial proliferation and migration as well as maintenance of vascular integrity[22], increases vascular permeability[23], and promotes angiogenesis[24-25]. VEGF acts through both autocrine and paracrine signaling, promoting endothelial cell growth in arteries, veins, and lymphatic vessels by activating specific receptors on vascular endothelial cells[26]. VEGF expression promotes normal physiological vascular growth[27-29]. Abnormal VEGF expression can occur under pathological conditions. In ischemic disease, hypoxia can stimulate VEGF expression. The resulting strong induction of endothelial cell mitosis promotes the formation of new blood vessels and improves tissue blood supply. VEGF is a key factor for ocular neovascularization, directly promoting the formation and development of new blood vessels; its expression is closely related to disease severity. In this study, VEGF also played a role in promoting RNV.

CCR7 signal can through the ERK pathway[30]. CCR7 can activate MAPK family proteins including p38, JNK, and ERK1/2 *via* G(i)-dependent mechanisms[31]. In monocytes, the phosphorylation of ERK1/2, p38, and JNK can be induced by the combination of CCR7 and CCL19, thus playing an important role in cell migration[32]. The expression of CCR7 was positively correlated to p-ERK1/2 in this study. CCR7 inhibition-mediated suppression of p-ERK1/2 signaling has also been shown to be important for the regulation of angiogenesis *via* VEGF and other pathways. In bovine retinal microvascular endothelial cells *in vitro*, VEGF stimulated the phosphorylation of ERK1/2 in a dose-dependent manner to promote cell proliferation and endothelial cell formation[33]. In a rat model of ROP, the ERK-VEGF signaling axis has been demonstrated to play a key role in endothelial cell proliferation. VEGF and other growth factors affect cellular functions through the ERK pathway, thereby promoting the transcription and expression of select genes, and in so initiating cell proliferation and differentiation. This signaling pathway plays an important role in cell growth, development, and proliferation[34]. In this study, p-ERK1/2 and VEGF expression was high in the group with high levels of-RNV; in the group with less RNV, p-ERK1/2 and VEGF expression was reduced accordingly. Thus, we concluded that p-ERK1/2/VEGF plays a prominent role in the OIR model of RNV.

It has been reported that CCR7 increases VEGF expression in fibroblasts such as synovial cells in RA and osteoarthritis through the p-ERK1/2 signaling pathway, and thereby promotes angiogenesis[17]. CCR7 can also increase VEGF expression in non-small cell lung cancer cells through the p-ERK1/2 signaling pathway, thus promoting tumor angiogenesis[32]. Taken together, all of the above studies have shown that the CCR7/p-ERK1/2/VEGF pathway promotes the production of new blood vessels. In addition, the CCR7-VEGF pathway has been shown to promote RNV[35]. The present study showed that the expression of CCR7 and VEGF in the OIR model was correlated with high expression of CCR7/VEGF, and the expression of CCR7 and VEGF decreased after CCR7 was inhibited.

Considering our data and those of previous reports, the CCR7/p-ERK1/2/VEGF pathway may be important in promoting neovascularization in OIR. We have found a positive correlation between CCR7, p-ERK1/2, and VEGF in the present study. Expression of CCR7 lead to p-ERK1/2 and VEGF expression as well as neovascularization, and CCR7 inhibition suppressed p-ERK1/2 and VEGF expression and neovascularization. Thus, we speculate that CCR7/p-ERK1/2/VEGF signaling plays an important role in ROP.

Vitreous cavity injection is a definitive and effective way to treat RNV; however, siRNA injection has not been applied in RNV treatments. Our research shows that CCR7 siRNA can be an effective part of anti-VEGF therapy. Unfortunately, the delivery of siRNA, toxicity, and off-target effects have not been successfully resolved; these challenges must be addressed in order to use siRNA for gene therapy.

In conclusion, CCR7/p-ERK1/2/VEGF signaling promotes RNV in a mouse model of OIR. CCR7-targeting intervention *via* siRNA represents a potential anti-angiogenic therapy for RNV of ROP, and may contribute to future therapeutic strategies.

ACKNOWLEDGEMENTS

Foundation: Supported by the Science and Technology Planning Foundation of Liaoning Province (No.2010225034).

Conflicts of Interest: Yuan LH, None; **Chen XL,** None; **Di Y,** None; **Liu ML,** None.

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