ORIGINAL ARTICLE

Interleukin-2 induces extracellular matrix synthesis and TGF-β2 expression in retinal pigment epithelial cells

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Abstract

Macular fibrosis is a vital obstacle of vision acuity improvement of age-related macular degeneration patients. This study was to investigate the effects of interleukin 2 (IL-2) on epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) synthesis and transforming growth factor β2 (TGF-β2) expression in retinal pigment epithelial (RPE) cells. 10 μg/L IL-2 was used to induce fibrosis in RPE cells for various times. Western blot was used to detect the EMT marker α-smooth muscle actin (α-SMA), ECM markers fibronectin (Fn) and type 1 collagen (COL-1), TGF-β2, and the activation of the JAK/STAT3 and NF-κB signaling pathway. Furthermore, JAK/STAT3 and NF-κB signaling pathways were specifically blocked by WP1066 or BAY11-7082, respectively, and the expression of α-SMA, COL-1, Fn and TGF-β2 protein were detected. Wound healing and Transwell assays were used to measure cell migration ability of IL-2 with or without WP1066 or BAY11-7082. After induction of IL-2, the expressions of Fn, COL-1, TGF-β2 protein were significantly increased, and this effect was correlated with IL-2 treatment duration, while α-SMA protein expression did not change significantly. Both WP1066 and BAY11-7082 could effectively downregulate the expression of Fn, COL-1 and TGF-β2 induced by IL-2. What's more, both NF-κB and JAK/STAT3 inhibitors could suppress the activation of the other signaling pathway. Additionally, JAK/STAT3 inhibitor WP1066 and NF-κB inhibitor BAY 11-7082 could obviously decrease RPE cells migration capability induced by IL-2. IL-2 promotes cell migration, ECM synthesis and TGF-β2 expression in RPE cells via JAK/STAT3 and NF-κB signaling pathways, which may play an important role in proliferative vitreoretinopathy.

KEYWORDS
age-related macular degeneration, epithelial-mesenchymal transition, extracellular matrix synthesis, Interleukin 2, retinal pigment epithelial cells

1 | INTRODUCTION

As a major cause of severe loss of vision among the old, age-related macular degeneration (AMD) is a complex multifactorial disease clinically caused by the degeneration of the photoreceptors and retinal pigment epithelium (RPE) cells in the old (Yonekawa & Kim, 2014). AMD is broadly classified into the wet form (neovascular or exudative) and dry form. Both two types of AMD pathology start with the formation of insoluble aggregates and drusen, which forms in the extracellular matrix between Brush's membrane and RPE (Mitchell, Liew, Gopinath, & Wong, 2018). Nowadays the main therapeutic medication of neovascular age-related macular degeneration (nAMD)- anti-vascular endothelial growth factor (VEGF)—includes bevacizumab and ranibizumab (Solomon, Lindsley, Vedula, Krzystolik, & Hawkins, 2019). Delightedly, the treatment could prevent the decrease or even improve vision acuity. However, after frequently, costly and invasive intravitreal injections, up to one-third of patients could not benefit from the therapy due to the development of macular fibrosis or atrophy (Little, Ma, Yang, Chen, & Xu, 2018).

Considering TGF-β is a dominant factor in the development of AMD and macular fibrosis or atrophy (Wang et al., 2019), the mechanism of enhanced TGF-β activity after anti-VEGF by intravitreal injection may provide a new way to prevent the development of nAMD and macular fibrosis or atrophy. While inflammation is an inevitable response after intravitreal injection and it plays important roles in nAMD (Kauppinen, Paterno, Blasiak, Salminen, & Kaarniranta, 2016; Rezar-Dreindl et al., 2016; Roh et al., 2009). IL-2, a characteristic inflammatory factor, plays major roles in many eye diseases and is involved in fibrosis in many tissues (Jung, Woo, & Park, 2019; Wang, Wang, Zhu, Geng, & Yang, 2015). The expression level of IL-2 has been found to be highly expressed in the aqueous humour of polypoidal choroidal vasculopathy (PCV) and proliferative vitreoretinopathy (PVR) patients (Ricker et al., 2011; Roybal et al., 2018; Sakurada et al., 2015). In addition, the inflammation pathways associated to IL-2 were activated in AMD (Makarev et al., 2014; Newman et al., 2012). Various studies have confirmed that IL-2 could interact with TGF-β to enhance their activities (Battaglia et al., 2013; Chambers et al., 2014; Freudenberg et al., 2018; Tischner, Wiegers, Fiegl, Drach, & Villunger, 2012). It is speculated that persistent activation of IL-2 may be an important contributor to the increase of TGF-β effects. However, whether IL-2 could directly induce the occurrence of EMT and synthesis of ECM and whether there was a connection between TGF-β activity and IL-2 in RPE cells remain unknown.

Numerous studies have elucidated various functions of JAK/STAT3 signaling pathway related to ROS, inflammatory, immune and so on in AMD pathogenesis (Fasler-Kan, Wunderlich, Hildebrand, Flammer, & Meyer, 2005; Kutty et al., 2018; Lin et al., 2013; Yamamoto, Fara, Dasgupta, & Kemper, 2013). While JAK/STAT3 signaling pathway is validated to cause EMT or ECM synthesis in multiple tissue or diseases (Kim et al., 2019; Li et al., 2018). Meanwhile, NF-κB signaling pathway was also proved to work in AMD or EMT or ECM synthesis in other disease (Hseu et al., 2019; Shen, Xie, Gu, Li, & Tong, 2016; Zhang & Huang, 2018). Additionally, cytokines like interleukin 6 (IL-6) were found to activate JAK/STAT3 and NF-κB signaling pathways (Hendrayani, Al-Harbi, Al-Ansari, Silva, & Aboussekhra, 2016; Li, Ye, Huang, Zhang, & He, 2019). Whereas the relationship of IL-2 and JAK/STAT3 and NF-κB pathways is not clear in nAMD.

According to these effects of IL-2 and fibrosis in AMD, we hypothesized that IL-2 may serve as an inducer function by interacting with TGF-β and activating certain pathways. To validate, we simulated the inflammatory microenvironment of the AMD with IL-2 in this study, then used Western blot method to detect the EMT marker, the ECM markers, and the activation of the JAK/STAT3 and NF-κB signaling pathway. In addition, Wound healing and Transwell assays were enrolled to detect cell migration capability. We found that IL-2 could promote RPE cells migration, ECM markers fibronectin (Fn) and type 1 collagen (COL-1), TGF-β2 but not EMT marker α-smooth muscle actin (α-SMA) depending on the activation of JAK/STAT3 and NF-κB signaling pathways.

2 | MATERIALS AND METHODS

2.1 | Culture and treatment of retinal pigment epithelial cells

The ARPE19 cell line was purchased from ATCC. A total of 1×10^6 cells within 12 passages were seeded into a culture flask with DMEM/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The medium was renewed by serum-free DMEM/F12 when the cells get to 70% confluence, and then cells were treated with IL-2 for different durations (0, 12, 24, 36 and 48 hr) or at different concentrations (0, 0.5, 1, 2, 4, 6 and 8 μg/L). Cells in the control group were treated with an equal volume of medium.

2.2 | Western blot

Western blot was performed as described in our previous study (Ma, Yang, et al., 2018). Briefly, RPE cell lysis was loaded at a concentration of 20 μg per well to separate in SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked using 1% BSA (Beijing Biodee Biotechnology Co., Ltd) in tris-buffered saline containing 0.1% Tween-20 for 1 hr at room temperature and probed using antibodies shown in Table 1 overnight at 4°C. After incubation with appropriate secondary antibodies, protein signals were detected using enhanced chemiluminescence western blotting detection kit (Bio-Rad).

2.3 | Wound healing and Transwell® migration assay

Wound healing and Transwell® migration assay were performed as described in our previous study (Ma, Jing, et al., 2018). The cells were in serum-free medium containing 10 μg/L IL-2 with or without WP1066 (S2796; Selleck) or BAY 11-7082 (S2913; Selleck).

TABLE 1 List of various antibodies used for Western blot

Antibody	Dilution	Source
Mouse anti-α-SMA	1:1,000	ab5694, Abcam, Cambridge, MA
Rabbit anti-Fn	1:1,000	ab2413, Abcam, Cambridge, MA
Rabbit anti-COL-1	1:200	AB745, Millipore, Billerica, MA
Rabbit anti-TGF-β2	1:1,000	ab36495, Abcam, Cambridge, MA
Rabbit anti-p-STAT3	1:1,000	#9145, Cell Signaling, Danvers, MA
Rabbit anti-STAT3	1:1,000	#12640, Cell Signaling, Danvers, MA
Rabbit anti-IκBα	1:1,000	12045, Sino Biological, Beijing, China
Rabbit anti-p-IκBα	1:1,000	#9246, Cell Signaling, Danvers, MA
Rabbit anti-β-actin	1:1,000	#4970, Cell Signaling, Danvers, MA
Goat anti-rabbit IgG secondary antibody	1:3,000	401315, Millipore, Billerica, USA
Goat anti-mouse IgG secondary antibody	1:3,000	401215, Millipore, Billerica, USA

2.4 | Cell viability detection using CCK8 kit

Approximately 10,000 cells were seeded in a 96 well plate. The medium was replaced with serum-free medium containing IL-2 and renewed 24 hr later. Cells were treated with different time (0, 24 and 48 hr) and concentration (0, 1, 5, 10, 15 and 20 μg/L) grades. After induction, 100 μl/ml CCK8 solution in medium was added into the microplate. 4 hr later, absorbance at 450 nm and relevance at 650 were measured using a microplate reader.

2.5 | Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, Inc.) was employed to perform all of the statistical analyses. All of the data are reported as the mean ± *SEM* with at least three independent experiments. Statistical comparisons of the Western blot data were analyzed using one-way ANOVA, whereas differences between groups were compared using Tukey's honest significant difference (HSD) test. Differences of $p < .05$ were considered statistically significant.

3 | RESULTS

3.1 | IL-2 stimulates RPE cells migrate

Varies cytokines were verified to be involved in fibrosis disease among which IL-2 was also discovered to function in prolapsed lumbar intervertebral disc and hypertension after glaucoma surgery (Jung et al., 2019; Wang et al., 2015). Whether IL-2 had the analogous effect was unknown on RPE cells. We first investigated the impact of IL-2 on the migration ability of RPE cells using

Transwell and wound healing assays. The results showed that the relative wound area was much less in the IL-2 treated group than the control group ($p < .05$, Figure 1a,b). Similar results were also observed in the Transwell assay ($p < .01$, Figure 1c,d). The results indicated that IL-2 promoted the migration capability of RPE cells in vitro.

3.2 | IL-2 promotes ECM synthesis and TGF-β2 expression in RPE cells

Previous studies have shown that IL-2 could promote cell migration and ECM synthesis in nucleus pulposus cells (Wang et al., 2015). However, the ability of IL-2 to promote EMT and ECM synthesis in RPE cells was not clear. Our results showed that after treating RPE cells with IL-2 for various lengths of time (0, 12, 24, 36 and 48 hr), the protein expression levels of COL-1, Fn and TGF-β2 were significantly upregulated in a time-dependent manner (Figure 2a,c–e). However, the expression of α-SMA was unchanged after treatment with different concentrations of IL-2 for various durations of time (Figure 2b). Therefore, the results indicate that IL-2 could promote ECM synthesis but not EMT in RPE cells.

3.3 | Role of the JAK/STAT3 and NF-κB signaling pathway in ECM synthesis in RPE cells treated with IL-2

Previous studies have shown that IL-2 can activate the JAK/STAT3 and NF-κB signaling pathway in some cells (Fujii, 2007; Marzec et al., 2008). However, whether JAK/STAT3 and NF-κB signaling pathway play important roles in RPE cells was not clear. Our results confirmed that IL-2 could activate the JAK/STAT3 and NF-κB signaling pathways in RPE cells (Figure 3a–c). Then we wondered whether the JAK/STAT3 and NF-κB signaling pathways were involved in the IL-2 induced expression of ECM proteins in RPE cells. RPE cells were treated with the JAK/STAT3 inhibitor WP1066 (4 μM) to block the JAK/STAT3 signaling pathway prior to IL-2 for 2 hr. Consistent with prior observations, after treatment with 10 μg/L IL-2, the protein expression levels of Fn, COL-1 and TGF-β2 were significantly increased compared with those in the control group. Nonetheless, the induction of Fn, COL-1 and TGF-β2 protein expression by IL-2 was significantly inhibited by blocking the JAK/STAT3 signaling pathway with WP1066 or BAY (Figure 3d–i). Similarly, when BAY (10 μM) was used to block the NF-κB signaling pathway, the induction of Fn, COL-1 and TGF-β2 protein expression by IL-2 was significantly inhibited (Figure 3j–o). Intriguingly, either WP1066 or BAY were applied to block the specific pathway, the other pathway was blocked significantly (Figure 3i,n). These results might indicate that STAT3 and NK-κB signaling pathways might interact with each other and played important roles in fibrosis in RPE cells together.

3.4 | JAK/STAT3 and NF-κB signaling pathways were involved in migration capability regulation

Cell migration is a central process in fibrosis disease (Anderluh, Kocic, Tomovic, Kocic, & Smelcerovic, 2019; Shelef, Bennin, Mosher,
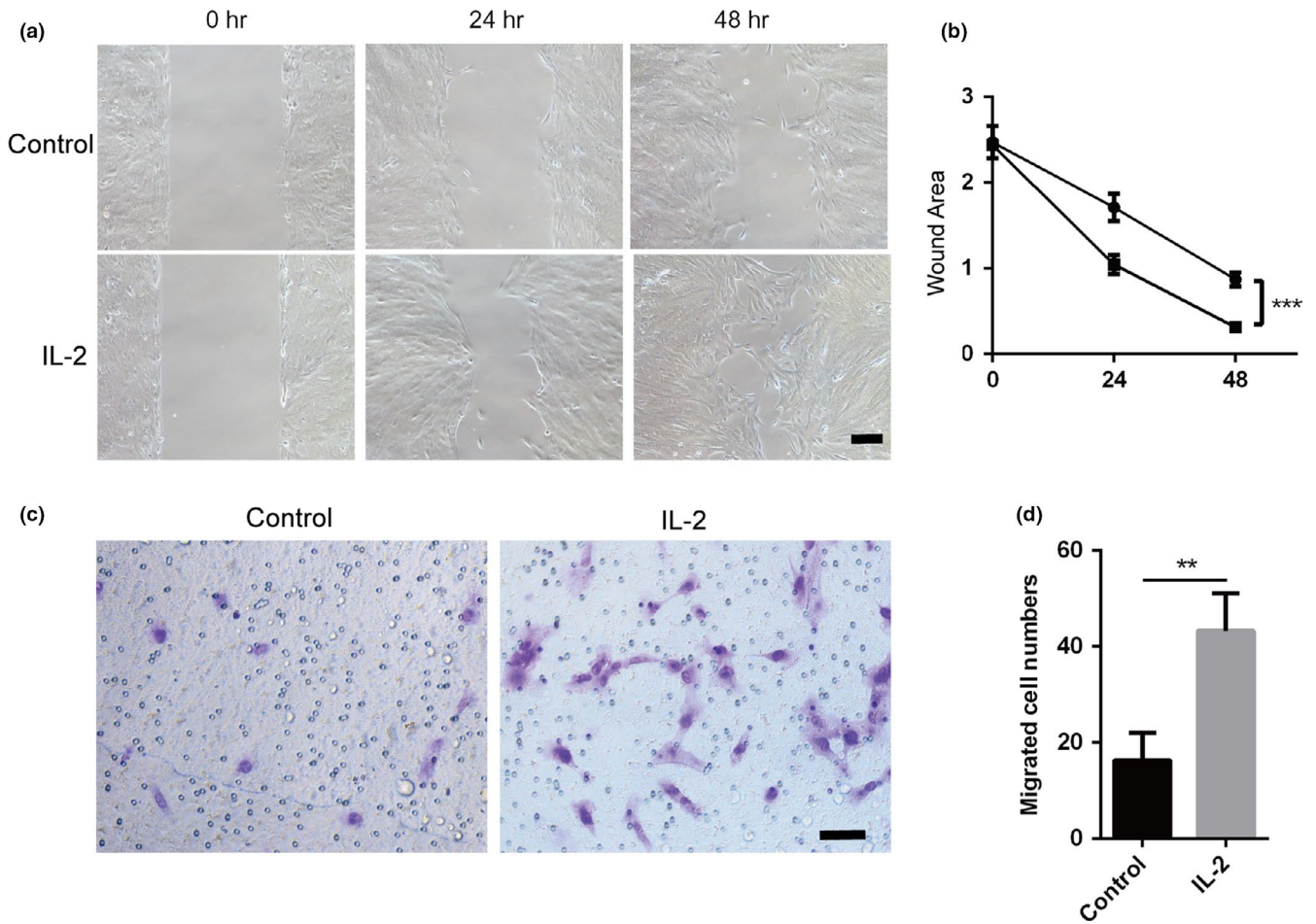
FIGURE 1 IL-2 promoted RPE cells migration. (a) RPE cells were treated by 10 μg/L IL-2 when cells were in 70% confluence after the insert was removed in serum-free medium. Wound healing at 0, 24 and 48 hr. (b) The wound area was measured and analyzed with ImageJ software and there was a significance in 48 hr between the control group and IL-2 treated group (━•━, Control; ━•━, IL-2). (c) Transwell assay between control group and 10 μg/L IL-2 treated group after cells were seeded in the Transwell chamber 24 hr later. (d) Migrated RPE cells in control group and IL-2 group, respectively. **$p < .01$, ***$p < .001$. Scar bar: 100 μm
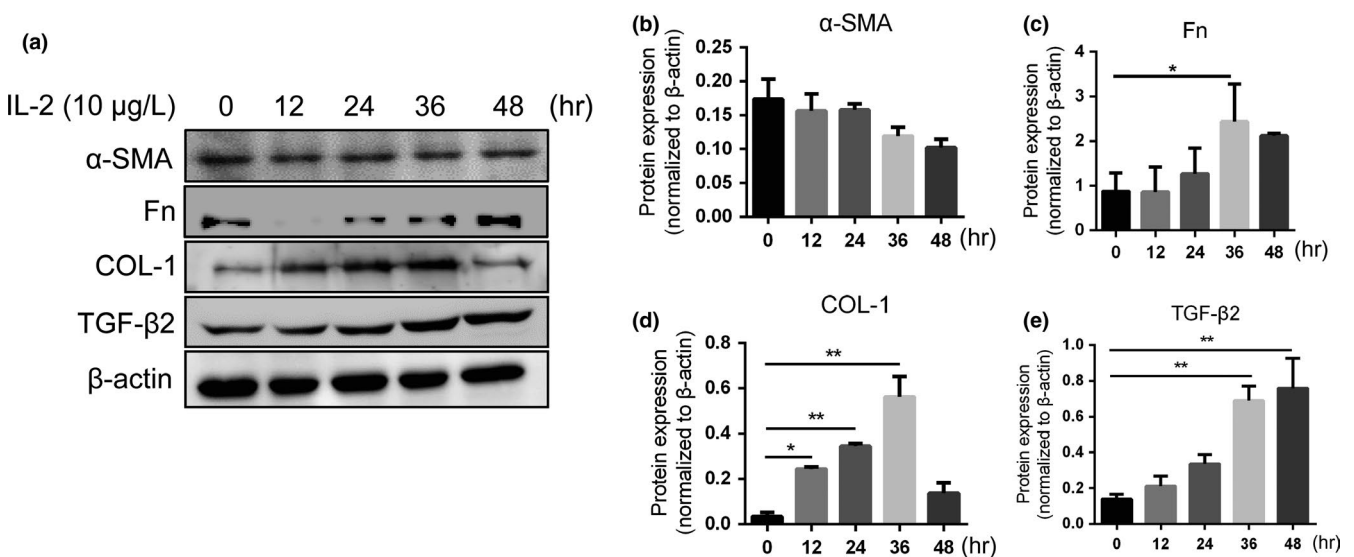

FIGURE 2 IL-2 promoted RPE cells ECM synthesis and TGF-β2 expression. (a) RPE cells were treated by 10 μg/L IL-2 for 0, 12, 24, 36 and 48 hr, α-SMA, COL-1, Fn and TGF-β2 protein expression were detected by Western blot. (b–e) Quantification of the Western blot analysis results. IL-2 treatment increased COL-1, Fn and TGF-β2 protein expression but not α-SMA time dependently. *$p < .05$, **$p < .01$
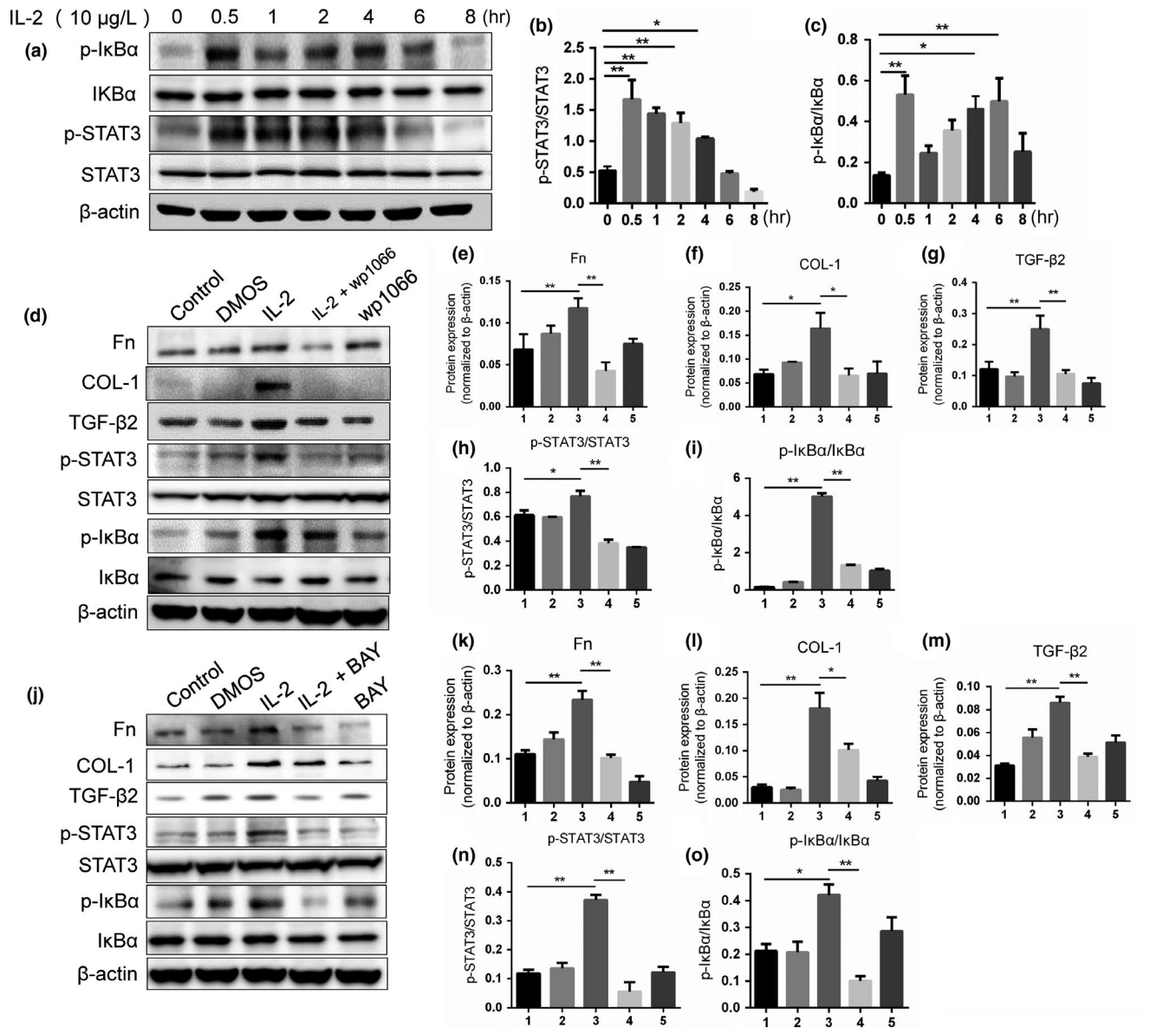
FIGURE 3 Both JAK/STAT3 and NF-κB signalling pathway involved in ECM synthesis and TGF-β protein expression. (a) RPE cells were treated by 10 μg/L IL-2 for different duration (0, 0.5, 1, 2, 4, 6 and 8 hr) and p-IκBα, IκBα, p-STAT3 and STAT3 expression were detected by Western blot. (b–c) Quantification of the Western blot analysis of p-STAT3/STAT3 and p-IκBα/IκBα. (d) RPE cells were preteated by 4 μM WP1066 for 1 hr and then 10 μg/L IL-2 was added in serum-free medium. (e–i) Quantification of the Western blot analysis (■, Control; ■, DMSO; ■, IL-2 10 μg/L; ■, IL-2 10 μg/L+WP1066; ■, WP1066). (j) RPE cells were preteated by 10 μM BAY for 1 hr and then 10 μg/L IL-2 was added in serum-free medium. (k–o) Quantification of the Western blot analysis (■, Control; ■, DMSO; ■, IL-2 10 μg/L; ■, IL-2 10 μg/L+BAY; ■, BAY). *$p < .05$, **$p < .01$

& Huttenlocher, 2012). Whether JAK/STAT3 and NF-κB signaling pathways were involved in migration in RPE cells remain unknown. IL-2 was identified to activate JAK/STAT3 and NF-κB signaling pathways and furthermore increased ECM synthesis. Consistent with our results, IL-2 could stimulate RPE cells migrate quickly. Our results indicated that with WP1066, the RPE cell migration rate is well below the IL-2 group ($p < .01$, Figure 4a,c) during 48 hr. Similarly, the migration speed of IL-2 with BAY was far lower ($p < .001$, Figure 4a,c) than that of IL-2 group. In parallel, similar outcomes emerged in Transwell assay. The migrated cell in IL-2 with WP1066 or BAY was far less

than IL-2 group (Figure 4b,d) after 24 hr. These results showed both JAK/STAT3 and NF-κB pathways were involved in RPE cell migration induced by IL-2.

4 | DISCUSSION

Based on our experimental results, we hypothesized that the inflammatory microenvironment in the nAMD induced cytokines like IL-2 expression. IL-2 promoted RPE cells migration, ECM synthesis and
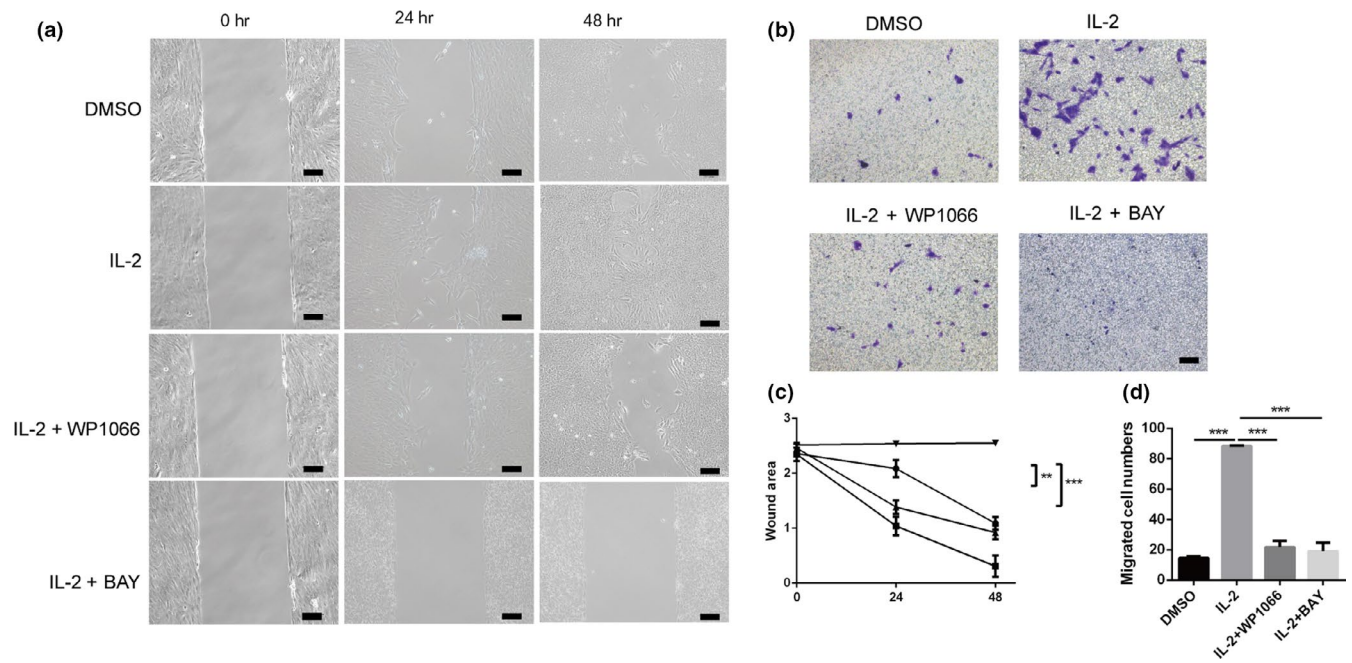
FIGURE 4 Both inhibition of JAK/STAT3 and NF-κB signalling pathway lower migration rate. (a) RPE cells were pre-seeded in the culture insert. When cells were 70% confluence, the insert was removed and serum-free medium was added. Before 10 µg/L IL-2 were loaded, WP1066 or BAY has been added into the medium for 1 hr. (b) Transwell assay of 10 µg/L IL-2 and IL-2 with 4 µM WP1066 or 10 µM BAY preteated for 1 hr. (c) The wound area was measured and analyzed with Image J software and there was a significance in 48 hr between the IL-2 and IL-2 with WP1066 or BAY group (-•-, DMSO; -•-, IL-2; -•-, IL-2+WP1066; -▼-, IL-2+BAY). (d) Migrated RPE cells in DMSO group and IL-2 with P1066 or BAY group respectively. **$p < .01$, ***$p < .001$. Scar bar: 100 µm

TGF-β2 expression by activating the STAT3 and NK-κB signaling pathways but did not induce the expression of EMT-specific proteins. Moreover, IL-2 interacted with TGF-β2 leading to ECM proteins expression excessively which aggravated macular fibrosis. In conclusion, IL-2 played an important role on the fibrosis of macular degeneration. Anti-inflammatory therapy might effectively prevent the fibrosis of macular in nAMD.

Fibrosis is a complicated pathophysiological process including cell migration, apoptosis, inflammation, EMT, ECM synthesis and so on (Chen et al., 2019; Li, Li, et al., 2019; Mack, 2018). As a pleiotropic inflammatory cytokine, IL-2 is involved in cell proliferation, differentiation, immunoregulation and autoimmune or inflammatory diseases through the activation of intracellular signal transduction pathways (Klatzmann & Abbas, 2015). Several studies showed that IL-2 could promote cell proliferation and migration (Llavero, Artaso, Lacerda, Parada, & Zugaza, 2016; Tauriainen et al., 2015). However, our results showed that IL-2 could promote RPE cells migration but not proliferation (Figure S1A,B) in vitro. Considering cell proliferation and migration are two separate independent process and there was no proliferation pathways activated by IL-2, IL-2 may specifically promote RPE cells migration in nAMD rather than induce cell proliferation according our in vitro results. Whether IL-2 could promote RPE cells proliferate directly or indirectly in vivo needs further research.

As a pleiotropic effects inflammatory factor, IL-2 also plays an important role in promoting fibrosis (Taylor et al., 2018; Wang et al., 2016). IL-2 plays important role in human nucleus pulposus cells

migration and ECM synthesis (Wang et al., 2015). Moreover, a previous study showed that IL-2 could mediate the EMT process through interacting with TGF-β1 (Qian et al., 2017). However, whether IL-2 could induce EMT and ECM synthesis in RPE cells and whether there is a relationship between this cytokine and TGF-β2 remain unknown. According to the results, IL-2 induced RPE cells ECM synthesis. Intriguingly, IL-2 could not induce the expression of the EMT-specific protein α-SMA, which indicated that in RPE cells, IL-2 might specifically promote the synthesis of ECM rather than induce EMT.

TGF-β was considered a key member in the development of fibrosis. Our previous and several other studies found that IL-1, IL-6 and IL-17A could enhance the effects of TGF-β on fibrosis (Fabre, Kared, Friedman, & Shoukry, 2014; Ma, Yang, et al., 2018; Qian et al., 2017; Van Den Akker et al., 2017). A previous study showed anti-VEGF could reduce TGF-β1 expression but not cytokines in the aqueous humor of nAMD patients (Rezar-Dreindl et al., 2016). Besides, our results showed IL-2 could induce the expression of TGF-β2 in RPE cells. These results indicated that cytokines could promote TGF-β work. Inflammation might link to fibrosis in macular and nAMD relapse. However, the mechanism of interaction between IL-2 and TGF-β2 requires further study.

IL-2 is involved in cell fibrosis, immunoregulation, development and other biological behaviors via activation of the STAT3 and NK-κB signaling pathways (Jung et al., 2018; Luo et al., 2018; Mitrokhin, Gorbacheva, Mladenov, & Kamkin, 2018; Park et al., 2017; Wu et al., 2018). In our study, IL-2 could activate STAT3 and NK-κB signaling pathways. Moreover, WP1066 and BAY, the specific inhibitor of

the STAT3 and NK-κB signaling pathways, respectively, effectively suppressed the ECM synthesis induced by IL-2 in RPE cells. These results indicate that the STAT3 and NK-κB signaling pathways played important roles in IL-2 induced ECM synthesis in RPE cells. Interestingly, Inhibition of STAT3 signaling pathway could suppress IκBα phosphorylation. Similarly, Inhibition of NK-κB signaling pathway could suppress STAT3 phosphorylation. Because WP1066 could also partially inhibit ERK signaling according to the data sheet, herein we thought there might be key factors downstream of both JAK/STAT3 and NK-κB function or other molecules regulated by signaling pathway inhibitors.

ECM is known to acquire substantial changes in composition, structure, and mechanics, which together exacerbate disease etiology through promotion of angiogenesis, cell differentiation, and immune activation (Shelef et al., 2012). Fn was verified to activate FAK consequently promote HLEC cell migration (Liu et al., 2018). Our results showed that with STAT3 and NF-κB pathways inhibitors, the migration rate of RPE cells induced by IL-2 dropped as well. So decreased ECM synthesis resulted from downregulated JAK/STAT3 and NF-κB signaling pathways might reduce focal adhesion and thus migration capability.

These results might indicate that STAT3 and NK-κB signaling pathways might interact with each other and played important roles in IL-2-induced fibrosis in RPE cells together. Our findings will offer new insights into our understanding of the molecular mechanisms underlying the pathogenesis of nAMD.

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CONFLICT OF INTEREST

All of the authors declare that there is no interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.