• Basic Research •

Leptin activates the JAK/STAT pathway to promote angiogenesis in RF/6A cells *in vitro*

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Abstract

● **AIM:** To investigate the effect of leptin on the angiogenesis of RF/6A cells (monkey retinal choroidal endothelial cells) *in vitro* and test the cellular signaling in the mechanism.

● **METHODS:** RF/6A cells were cultured *in vitro* and randomly divided into four groups: normal control, with leptin at 50, 100, 200 ng/mL for cell counting kit-8 (CCK8). RF/6A cell proliferation and migration were examined by Transwell assays, while RF/6A cell tube formation by Matrigel assay. JAK2, p-JAK2, STAT3, and p-STAT3 protein expression was measured by Western blotting. Cells were then divided into the following treatment groups: control, 100 ng/mL leptin and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) for examinations of RF/6A cellular behaviour again. Analysis of differences was carried out using one-way ANOVA and least significant difference (LSD).

● **RESULTS:** RF/6A cell proliferation, migration and cell tube formation were promoted significantly by leptin in a dose-dependent manner (P<0.05). Western blotting showed that leptin up-regulated p-JAK2 and p-STAT3 expression levels. Treatment with the JAK/STAT pathway inhibitor, AG-490, decreased leptin-induced p-JAK2 and p-STAT3 expression, and inhibited cell proliferation, migration and cell tube formation induced by leptin (P<0.05).

● **CONCLUSION:** Leptin can promote RF/6A cell angiogenesis *in vitro via* activation of the JAK2/STAT3 signaling pathway.

● **KEYWORDS:** leptin; JAK/STAT; angiogenesis; RF/6A cells; proliferation; migration; tube formation

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INTRODUCTION

Diabetic retinopathy is the primary cause of adult blindness in the world. Obesity, which is due to weight gain caused by increased adipose tissue, is usually related to lifestyle-related cardiovascular and metabolic diseases, for instance, diabetes, hypertension, and hyperlipidemia which lead to a higher risk of developing vascular disease or atherosclerosis. Adipose tissue is a vital endocrine organ that secretes many bioactive substances, such as leptin, chemerin and apelin[1-3], collectively known as adipocytokines[4]. Evidence suggests that adipocytokines play a pathophysiological role in complications related to obesity and diabetes[5]. Leptin is an adipocytokine that acts directly on the hypothalamus, regulating energy intake and consumption[6-7]. Leptin receptors are expressed not only in the hypothalamus but also in various peripheral tissues[8-9]. Leptin concentrations in the peripheral blood of obese people is proportional to the degree of obesity, suggesting that leptin may play a pathophysiological role in obesity-related complications[10]. In addition, leptin is closely related to energy metabolism and insulin resistance[11]. Serum leptin concentrations were found to be significantly higher in

hyperplastic diabetic retinopathy patients than non-proliferative retinopathy patients[12]. In proliferative diabetic retinopathy patients, intravitreous leptin concentrations were higher[13-14], suggesting that leptin may play a role in proliferative diabetic retinopathy. The most crucial pathological change of proliferative diabetic retinopathy is the formation of retinal neovascularization[15-16]. However, it remains unclear whether leptin is involved in the process. Thus, this study aimed to clarify the role and mechanism of action of leptin on retinal neovascularization *in vitro*.

MATERIALS AND METHODS

Cell Grouping and Processing RF/6A cells were purchased from the Cell Bank of Typical Cell Culture Preservation Committee, Chinese Academy of Sciences. Cell culture consumables were purchased from Biyuntian Biotechnology Company (China). RF/6A cells growing in good condition were digested by 0.25% trypsin, collected and centrifuged at 1000 rpm for 5min, and then moistened with phosphate buffered saline (PBS) twice to remove the residual serum. According to different treatment, RF/6A cells were randomly divided into four groups: control, 50, 100, and 200 ng/mL leptin groups. In leptin treatment group, different concentrations of leptin (0, 50, 100, and 200 ng/mL) were added to the culture medium for culture. Recombinant human leptin was purchased from Peprotech (USA). In leptin+AG-490 treatment group, leptin (100 ng/mL) and AG-490 (10 μmol/L) were added to the cell culture medium. AG-490 inhibitor was purchased from AbMole (USA). RF/6A cells were treated for 24h, then assayed as outlined below.

CCK-8 Assay of Cell Proliferation RF/6A cells in the logarithmic growth phase were plated at a density of 5×10^4/mL in 96-well plates (100 μL cell suspension per well). Cells were treated as described above for 24h, then cultured at 37°C for a further 24h. Next, cells were cultured for 4h at 37°C with 10 μL CCK8 in each well. Plates were read at OD 450 using an Enzyme standard instrument (ThermoFisher Scientific, MULTISKAN MK3).

Transwell Assay to Determine Cell Migration Treated RF/6A cells were digested with 0.25% trypsin, collected and diluted (1×10^5 cells/mL). Medium containing 10% FBS (800 μL) was placed into a 24-well plate containing a Transwell chamber. One hour later, 200 μL of cell suspension was added to the upper section of the Transwell chamber and incubated at 37°C in an atmosphere of 5% CO_2 for 24h. Cells were washed with PBS and fixed in 10% methanol for 30min after the Transwell chamber was removed. The membrane was stained with 5% crystal violet at room temperature for 20min. After washing with PBS, samples were observed and photographed under the inverted microscope (Nikon, ECLIPSE Ts2).

Matrigel Cell Tube Formation Assay Matrigel was purchased from BD (USA). According to the manufacturer's instructions, the Matrigel was melted and 100 μL of liquid Matrigel was placed into 96 well plates. RF/6A cells were diluted to a concentration of 2×10^5 cells/mL and 50 μL of cell suspension was added to each well. The 50 μL of the various treatment groups [control (media alone), leptin (0-200 ng/mL), and leptin+10 μmol/L AG-490] were added to the wells. After 24h, cell tube formation in five randomly selected fields was photographed using a phase-contrast microscope at a magnification of 200× and counted using Image J software.

Western Blot Analysis Cells were lysed and then total protein was extracted. Samples were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Overnight at 4°C, membranes were incubated with the following primary antibodies: GAPDH (1:1000), JAK2 (1:1000), p-JAK2 (1:1000), STAT3 (1:1000), and p-STAT3 (1:2000). The JAK2 and p-STAT3 antibodies were purchased from Cell Signaling Technologies (USA), the p-JAK2 antibody was obtained from Abcam (UK), the STAT3 antibody was purchased from Wuhan Sanying Biotechnology Co., Ltd. (China) and the GAPDH antibody was purchased from Hangzhou Xianzhi Biological Co., Ltd. (China). After washing, membranes were incubated with the corresponding secondary HRP-conjugated antibodies at 37°C for 2h. Protein bands were analyzed by BandScan.

Statistical Analysis Data are analyzed using SPSS 20.0 software and given as the mean±standard deviation. Comparison of the means between groups was analyzed by single-factor ANOVA, while the LSD method was used to compare two groups. $P<0.05$ was considered to be statistically significant.

RESULTS

Leptin Promotes Proliferation of RF/6A Cells The effects of different concentrations of leptin on cell proliferation were examined using the CCK8 assay. As shown in Figure 1, varying concentrations of leptin (20, 50, 100, and 200 ng/mL) led to a significant dose-dependent increase in RF/6A proliferation after 24h ($P<0.05$).

Leptin Promotes Migration of RF/6A Cells Migration of endothelial cells is essential during early angiogenesis. To determine the effect of leptin on angiogenesis, we used the Transwell assay to measure the cell migration ability of RF/6A cells. We found that leptin led to an obvious increase in the migration of endothelial cells after 24h significantly. The number of migrating cells in the control, 50, 100, and 200 ng/mL leptin groups were 43±4.5, 65±6.5, 83.5±7.2, and 112.1±7.6, respectively ($F=145.8$, $P=0.001$; Figure 2).

Leptin Promotes Tube Formation in RF/6A Cells The Matrigel tube formation assay revealed that the number of tubular structures that developed in control, 50, 100, and 200 ng/mL leptin groups was 38.6±2, 50.1±3.5, 67.7±5.2, and 84±5.6, respectively ($F=68.7$, $P=0.001$). These findings suggest that leptin can promote RF/6A cell tube formation (Figure 3).

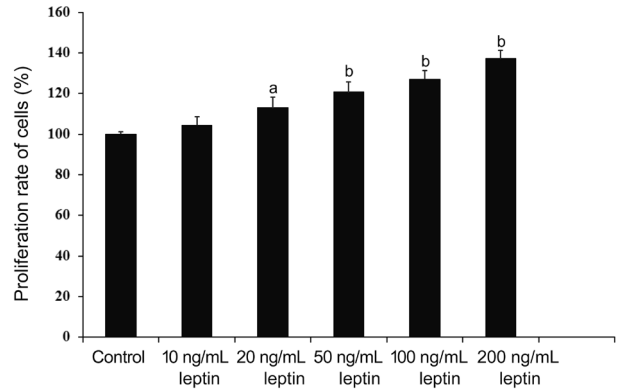

Figure 1 Effect of leptin on cell proliferation [a]*P*<0.05 *vs* control; [b]*P*<0.01 *vs* control.

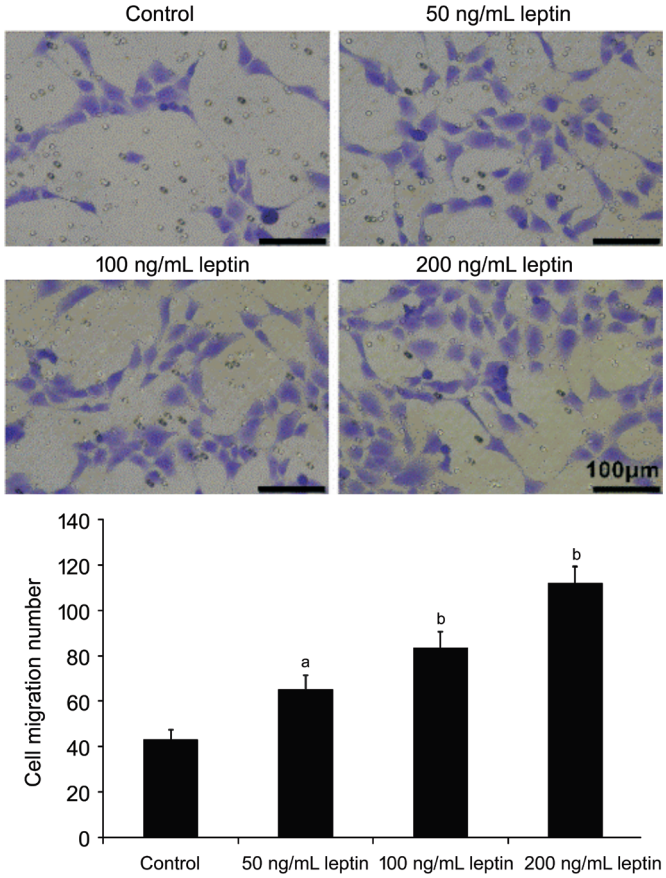

Figure 2 Effect of leptin on cell migration [a]*P*<0.05 *vs* control; [b]*P*<0.01 *vs* control.

Leptin Activates the JAK/STAT Signaling Pathway To determine the effect of leptin on the JAK/STAT signaling pathway, we used Western blot analyses to detect the protein expression levels of JAK2, p-JAK2, STAT3, and p-STAT3. It was found that incubation with leptin increased the phosphorylation levels of JAK2 and STAT3 were significantly increased in RF/6A cells significantly (P-JAK2: F=47.33, P=0.001; P-STAT3: F=111.86, P=0.001).

AG-490 is a tyrosine kinase inhibitor that inhibits STAT-3 activation by selectively blocking JAK2. Thus, JAK/STAT-3 activation is inhibited using AG-490 selectively. To verify the inhibitory effect of AG-490 on the JAK/STAT signaling

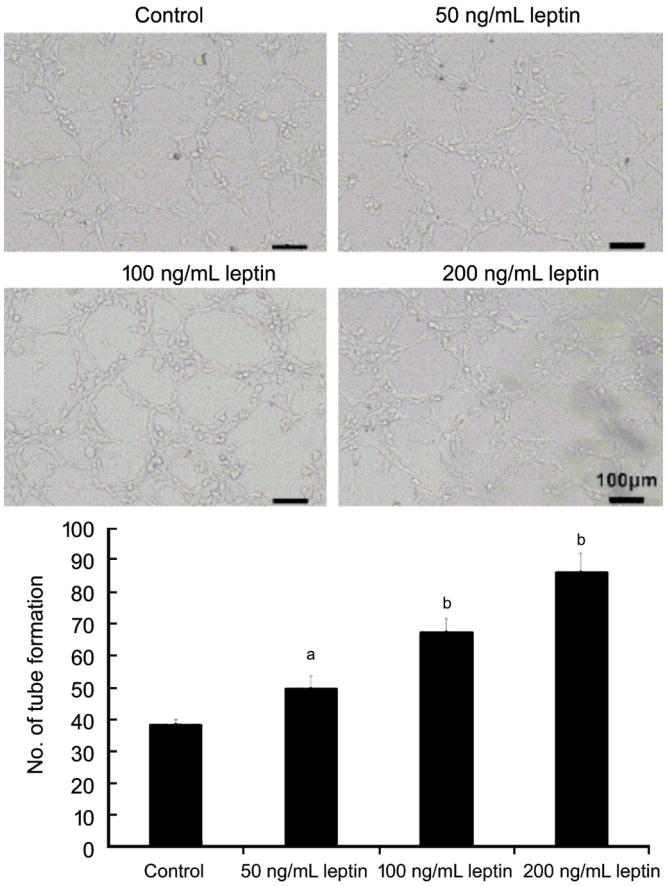

Figure 3 Effect of leptin on the construction of tubular structures in RF/6A cells [a]*P*<0.05 *vs* control; [b]*P*<0.01 *vs* control.

pathway, cells were separated into control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) groups. We found that AG-490 could inhibit leptin-induced JAK2 and STAT3 phosphorylation (P-JAK2: F=63.29, P=0.001; P-STAT3: F=70.68, P=0.001; Figure 4).

Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Proliferation The role of the JAK/STAT signaling pathway on leptin-induced cell proliferation was examined using AG-490, the selective JAK/STAT signaling pathway inhibitor. The effects of the following treatment groups: control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490) on cell proliferation were assessed using the CCK8 assay. We found that AG-490 could significantly inhibit the proliferation of RF/6A cells induced by 100 ng/mL leptin (F=131.99; P=0.001; Figure 5).

Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Migration To determine the role of the JAK/STAT signaling pathway on leptin-induced cell migration, the Transwell assay was used to assess the levels of migration in control, 100 ng/mL leptin, and AG-490 (100 ng/mL leptin+10 μmol/L AG-490)-treated cells. We found that inhibition of the JAK/STAT pathway by AG-490 significantly inhibited the migration of RF/6A cells induced by 100 ng/mL leptin (F=150.31; P=0.001; Figure 6).

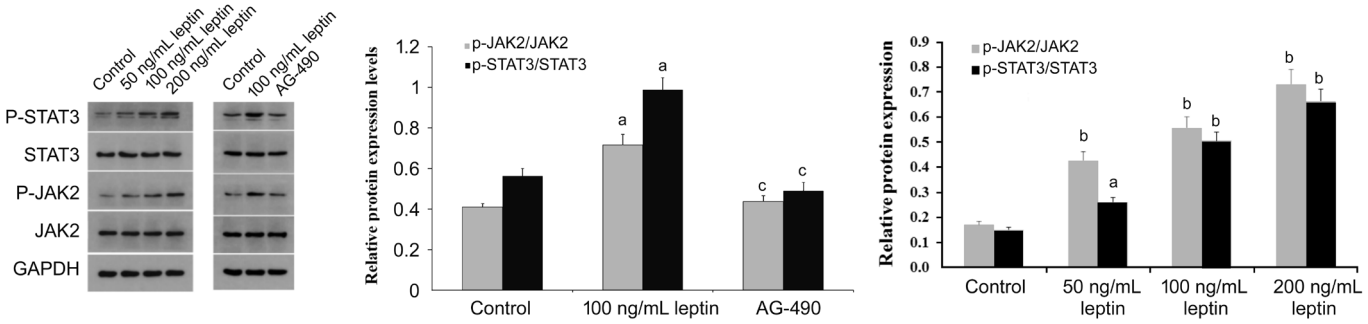

Figure 4 Effect of leptin on the JAK/STAT signaling pathway [a]*P*<0.05 *vs* control; [b]*P*<0.01 *vs* control; [c]*P*<0.05 *vs* 100 ng/mL leptin.

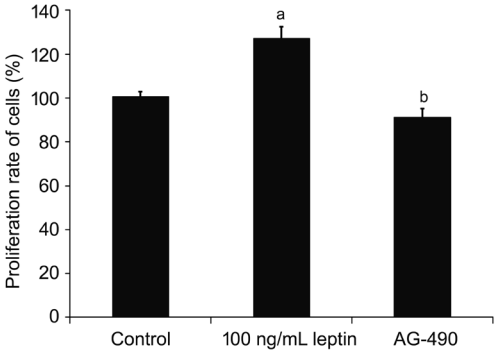

Figure 5 Effect of inhibition of the JAK/STAT signaling pathway on cell proliferation [a]*P*<0.05 *vs* control; [b]*P*<0.05 *vs* 100 ng/mL leptin.

Effect of Inhibition of the JAK/STAT Signaling Pathway on Cell Tube Formation The Matrigel tube formation experiment was used to detect the formation of tubular structures in control, 100 ng/mL leptin- and AG-490- (100 ng/mL leptin+10 μmol/L AG-490) treated cells. We found that inhibition of the JAK/STAT pathway by AG-490 inhibited the number of tube formations in RF/6A cells induced by 100 ng/mL leptin, significantly (F=80.50, P=0.001; Figure 7).

DISCUSSION

In the final stages of varieties of ocular diseases, including diabetic retinopathy and retinal vein occlusion, retinal neovascularization often leads to catastrophic vision loss. In this study, it was demonstrated that leptin can stimulate RF/6A cells' proliferation, migration, and tube formation *in vitro*. As an adipocytokine, leptin was found to be expressed nearly exclusively in adipose tissue in mammals[17]. It acts directly on the hypothalamus to regulates energy intake and expenditure. It has been shown that leptin expressed in retina, choroid, sclera, vitreous and tears[18-20]. Serum levels of leptin were closely related to obesity and obesity-associated microvascular complications[12]. Our findings are similar to previous studies[21-22]. Cell migration, proliferation, and tube formation are vital factors in the formation of new blood vessels[23-24]. Our data suggest that leptin could promote new blood vessels formation. Furthermore, our findings confirm that leptin could activate the JAK2/STAT3 signaling pathway in RF/6A cells, and that inhibition of the JAK2/STAT3 signaling pathway through AG-490 blocks leptin-induced endothelial cells' proliferation, migration, and tube formation.

The JAK/STAT pathway was initially regarded as a transmitter of interferon signaling on cells[25-26]. The JAK family is comprised of non-receptor tyrosine protein kinases and includes JAK1, JAK2, JAK3, and Tyk2. The JAK family has a C-terminal tyrosine kinase domain, while the N-terminus plays a regulatory role when JAK couples with the receptor protein. The JAK/STAT signaling pathway relays signals from the membrane to the nucleus, *via* tyrosine phosphorylation, leading to the activation and nuclear translocation of STATs[25]. Activation of the upstream JAK2 kinase by cytokines or growth factors results in tyrosine phosphorylation and activation of STAT3, which in turn regulates the transcription of target genes and mediates diverse biological effects including cell proliferation, differentiation, and apoptosis[27]. The activation of STATs is transient in normal cells, lasting only a few minutes or hours. In tumor cells, abnormal and persistent activation of STAT3 is closely associated with the biological behavior of the tumor and its pathogenesis[25].

In addition to cell proliferation, differentiation and immune regulation[28-29], the JAK2/STAT3 signaling pathway also plays a role in the process of neovascularization[30-31] and the regulation of VEGF expression. It has been shown that JAK2/STAT3 can activate VEGF expression in some studies[32], while inhibition of the JAK2/STAT3 pathway inhibits VEGF expression[33]. Abnormal activation of STAT3 regulates the STAT3/VEGF signaling pathway and promotes VEGF expression[34-35]. VEGF expression can be down-regulated by inhibiting the JAK2/STAT3 pathway[36]. The well-established JAK/STAT pathway inhibitor AG-490, also known as Tyrphostin AG-490, was used in our study to specifically block the phosphorylation and activation of JAK2 and STAT3[37-38]. Consistent with previous studies, leptin can activate the JAK/STAT signaling pathway[39-42]. Thus, leptin may increase the expression of VEGF in retinal endothelial cells by activating the JAK2/STAT3 pathway, and then promotes neovascularization.

Due to the shortage of human primary retinal vascular endothelial cells, RF/6A cells have been widely used in experiments

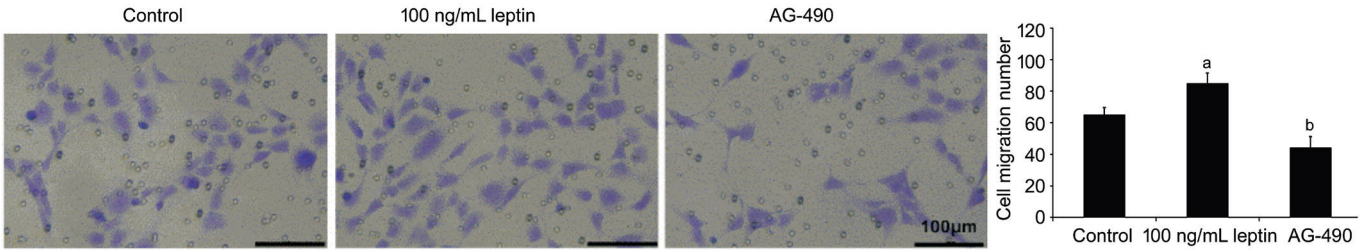

Figure 6 Effect of inhibition of the JAK/STAT signaling pathway on cell migration [a]*P*<0.05 *vs* control; [b]*P*<0.05 *vs* 100 ng/mL leptin.

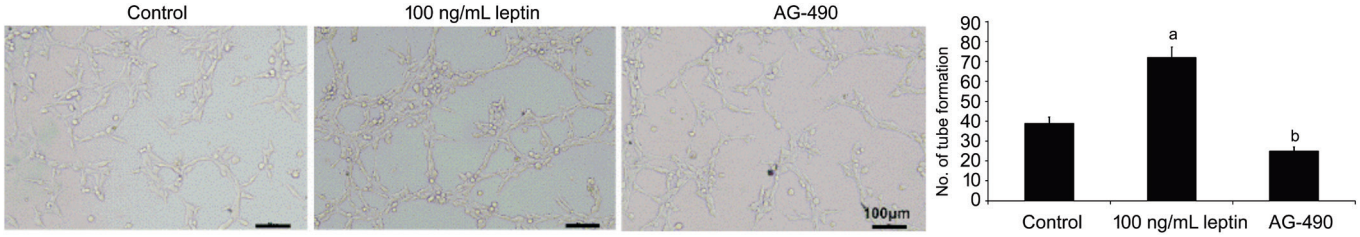

Figure 7 Effect of inhibition of the JAK/STAT signaling pathway on cell tube formation [a]*P*<0.05 *vs* control; [b]*P*<0.05 *vs* 100 ng/mL leptin.

of angiogenesis, cell differentiation and various drug and environmental treatments in the choroid and retina[43-44]. In this experiment, the RF/6A cells were selected because they were easy to obtain, stable and already widely used. Although RF/6A cells have been used for many years, they may also be different from real human retinal vascular endothelial cells. Further research is required to determine whether leptin has the same effect on human retinal microvascular endothelial cells and retinal neovascularization *in vivo*.

In conclusion, our study shows that leptin can activate the JAK2/STAT3 signaling pathway, resulting in increased RF/6A cell proliferation, migration, and tube formation, which may participate in neovascularization. If it can be proved by more studies that leptin promotes the angiogenesis of retinal endothelial cells *via* activation of the JAK2/STAT3 signaling pathway, the inhibitor or antagonist of this pathway could prevent the neovascularization of diabetic retinopathy. Further research is required to demonstrate whether leptin has the same effect on retinal neovascularization *in vivo*.

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