Annexin A2 Promotes Development of Retinal Neovascularization through PI3K/ AKT Pathway

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2 **Retinal Neovascularization through PI3K/**
3 **AKT Pathway**

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Chen-Yue Li, Zi-Chang Zhao, Shi-Hong Zhao*

Abstract

Background

RNV is a pathological characteristic of PDR and ANXA2 play an important role in the process of RNV while the mechanism remains unclear. We explore the role and molecular basis of ANXA2 in the formation of RNV and seek for new potential targets for the prevention and treatment of PDR.

Methods

Lentivirus containing plasmids which can interfere ANXA2 and overexpress ANXA2 were packaged and infected HRECs, dividing HRECs into 4 groups. Moreover, 1ul SC79 solution was added in shA2 group and 1ul LY294002 solution was added into lentiA2 group. Western Blot was used to detect expression of ANXA2 and changes in phosphorylation degree of major proteins in PI3K/AKT signaling pathway in each group of HRECs. HRECs of all groups were used to perform EDu cell proliferation assay, Transwell cell migration assay and Matrix tube formation assay. C57BL/6J mice were randomly divided into 3 groups. Mice of OIR group were injected intraperitoneally with 0.05ml PBS every day from 7th to 10th day while mice of LY294002 treatment group were injected with 0.05ml LY294002 solution and no

treatment in the control group. Lectin GS-IB4 fluorescence staining was used to observe RNV in mice in all groups. The expression of ANXA2 in mouse retinas was detected by Westen-blot.

Results

The proliferation, immigration and angiogenesis ability of HRECs is lower in shA2 group than shNC and SC79 treatment group while higher in lentiA2 group than lenti-EGFP and LY294002 treatment group. ANXA2 expression is significantly higher in retina of mice in OIR group and LY294002 treatment group. RNV is significantly less severe in LY294002 treatment group than that in OIR group.

Conclusions

ANXA2 can promote development of RNV through PI3K/ AKT Pathway.

Keywords: AnnexinA2, human retinal endothelial cell, retinal neovascularization, AKT, PI3K, phosphorylation

Background

Retinal neovascularization (RNV) is a pathological characteristic of proliferative diabetic retinopathy (PDR) ^[1, 2]. New vessels invade into the vitreous and neural retinal layers^[3]. Through retinal vascular leakage, lead to retinal edema, and fibrosis, ultimately causing visual impairment and blindness^[4].

Annexin A2 (ANXA2), a 39 kDa member of the annexin family of Ca²⁺- dependent, phospholipid-binding proteins, is expressed in the majority of cells and tissues and binds to numerous ligands^[5, 6]. ANXA2 is a pleiotropic protein and involved in diverse

cellular processes such as cell proliferation, differentiation, cell motility and also plays an important role in cytoskeletal organization, exocytosis, endocytosis, invasion, metastasis, fibrinolysis, ion channel formation and so on^[5-7].

Numerous papers have reported that upregulation of extracellular localization of ANXA2 has a profound impact on invasive and metastatic phenotype of cells and is directly correlated with neovascularization in cancer. ANXA2 can be phosphorylated and subsequent bind to S100A10, which acts as a premise for the localization of its translocation to the cell surface^[8, 9]. As a cell surface receptor for plasminogen (Plg) and tissue plasminogen activator, the ANXA2 complex can prominently accelerate the conversion of Plg to plasmin. As a result, a cascade of matrix metalloproteinases including MMP2 and MMP9 can be activated^[10] and ultimately lead to the degradation of fibrin, fibronectin, laminin, and several kinds of collagen^[11]. The degradation result in the liberation of not only vascular endothelial cells but also pro-angiogenic growth factors which is critical in the initiation of neovascularization^[12]. Playing a role in the process of neovascularization, ANXA2 may be regarded as a effective target for the development of neovascularization.

It has been well studied that PI3K/AKT signaling pathway regulates a wide variety of cellular processes such as cell proliferation, differentiation, survival, transformation and metastasis^[13]. The pathway also promotes neovascularization and recruitment of inflammatory cells^[14].

In this study, we assumed that ANXA2 played a role in the process of RNV and used

human retinal endothelial cells (HRECs) aiming at investigating the role and underlying mechanism of ANXA2 in the development of RNV and demonstrating that ANXA2 might regulate the formation of RNV through PI3K/AKT pathway.

Materials and methods

Animals

Wild type C57BL /6J mice were purchased from the animal experiment center of the Navy Medical University and housed at room temperature. We used the animals according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Materials

DMEM, FBS, Lipofectamine 2000 and Opti were purchased from Gibco. ECM was purchased from Sciencell. GS-IB4 and Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ANXA2, AKT, p-AKT(Ser473), phosphatidylinositol 3-Kinase (PI3K), p-PI3K(Tyr458), mTOR, p-mTOR(Ser2448), p70 S6 Kinase, p-p70 S6 Kinase(Thr389), 4E-BP1, p-4E-BP1(Thr37/46), Tsc2, p-Tsc2(Thr1462), LY294002, SC79 were purchased from Cell Signaling Technology. Gt anti-Rb IgG (H+L) Secondary Antibody was purchased from Invitrogen. Plasmids MDL, REV, VSVG, shNC, shA2, lenti-EGFP, lentiA2 were kindly provided by Microbiology office of The Navy Medical University. Transwell chambers were purchased from Corning Costar. Matrigel was purchased from BD biosciences.

Cell lines

94 HRECs were purchased from American ScienCell. Human embryonic kidney (293T)
95 cells were purchased from American Type Culture Collection (ATCC) and cultured
96 according to ATCC recommendations. 293T cells were cultured in DMEM and HRECs
97 were cultured in ECM at 37 °C in a humidified atmosphere of 5% CO₂.

98 **Lentivirus Packaging and Production**

99 293T cells were seeded in the 6-well culture plate in 2ml of DMEM then incubated at
100 37 °C with 5% CO₂ and DMEM was replaced by Opti when 293T grew to 80%
101 confluent. We mixed 40ul Lip2000, 3.2ml Opti, 1.554ug MDL, 1.166ug VSVG, 1.166
102 REV then added 3ug shNC, shA2, lenti-EGFP, lentiA2 respectively into the mixture.
103 Four kinds of mixture were added into different holes and cells were placed at 37 °C
104 with 5% CO₂ immediately. Transfection medium was replaced with 2ml DMEM after
105 6 hours of transfection and cells were incubated at 37 °C with 5% CO₂ for additional
106 48 hours. Finally, lentiviral supernatant was collected and filtered to remove cellular
107 debris.

108 **HREC Infection**

109 HRECs were seeded in the 24-well culture plate in 2ml of ECM then incubated at 37 °C
110 with 5% CO₂. 1.5ul lentivirus carrying lentiviral vectors shNC, shA2, lenti-EGFP,
111 lentiA2 was respectively added into four different wells when HRECs grew to 30%
112 confluent making HRECs into four groups: shNC, shA2, lenti-EGFP, lentiA2 group.
113 Cells were placed at 37 °C with 5% CO₂ immediately and then collected when they
114 grew to 90% confluent.

115 **Western Blot**

116 Total protein was extracted by RIPA lysis buffer and separated by sodium dodecyl
117 sulfate-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes.
118 Membranes were blocked with 5% skim milk at room temperature for 2.5h then
119 incubated overnight at 4°C with primary antibody against GAPDH (1:1000), AnnxA2
120 (1:1000), AKT(1:1000), p-AKT(Ser473) (1:1000), PI3K(1:1000), p-PI3K(Tyr458)
121 (1:1000), mTOR(1:1000), p-mTOR (Ser2448) (1:1000), p70 S6 Kinase(1:1000), p-p70
122 S6 Kinase(Thr389) (1:1000), 4E-BP1(1:1000), p-4E-BP1 (Thr37/46) (1:1000),
123 Tsc2(1:1000), p-Tsc2(Thr1462) (1:1000). PVDF membranes were incubated with the
124 corresponding secondary antibody at 37 °C for 2.5h. Protein bands were finally
125 visualized by imaging system.

126 **EDu Proliferation Assay**

127 3.0×10^4 HRECs were seeded in the 96-well culture plate in 200ul of ECM then
128 incubated at 37 °C with 5% CO₂. Cell nucleuses of all cells and cells in proliferative
129 stage were stained according to the protocol of EDu Kits and then counted under a
130 microscope.

131 **Cell Migration Assay**

132 Transwell assay was performed to assess the migration and invasive ability of HRECs.
133 100μl cell suspension containing 2.0×10^4 cells and ECM with 0.5% serum was added
134 into the upper chamber. Lower chamber was filled with 800μl ECM with 1% serum.
135 Cells were cultured at 37 °C with 5% CO₂ for next 12 hours and non-migrate ones were

gently removed with cotton swabs while the cells on the reverse surface of the membrane were fixed with 4% paraformaldehyde for 20 min and then stained with 0.5% crystal violet for 2-4h. Number of cells were finally counted under microscope.

Tube Formation Assay

Each well of the 96-well plates was coated with 60-70ul Matrigel. 2.0×10^4 HRECs were seeded onto the layer of Matrigel and then cultured at 37 °C with 5% CO₂ for 2-4h. Status of tube formation was observed under microscope and the ability of HRECs to form tubular structures was assessed.

Mice Model of Oxygen Induced Retinopathy and Treatment of LY294002

96 mice were randomly divided into normoxia group, oxygen-induced retinopathy (OIR) group and LY294002 treatment group (n=32/group). Mice of the normoxia group were raised in the normal air and mice in the OIR-control group were put in 75%±2% oxygen from P7 to P12 and returned to normal air on P12 in order to produce OIR model and injected intraperitoneally with 0.05 mL of phosphate-buffered solution (PBS) once a day from P7 to P10. While those of LY294002 treatment group were raised under the same phenomenon with OIR model but then injected intraperitoneally with 0.05 mL of LY294002 but not PBS once a day from P7 to P10. Three groups of mice were killed on P17. The retinas of left eyes of mice were stained with diluted GS-IB4 while those of the right eyes were used for protein extraction.

Quantitative Assessment of Retinal Neovascularization by GS-IB4 Staining

Retinas were stained with diluted GS-IB4 in room temperature for overnight and then

cut in four radial incisions with the optic disk as center under the microscope. The process of neovascularization was assessed using fluorescence microscope.

Statistical Analysis

As specified in figure legends, group of data are presented as mean $\pm$ SD error and were analyzed using t-test. Asterisks in each box and whisker plot indicate statistical significance (*=P < 0.05; **=P < 0.01; ***=P < 0.001).

Results

ANXA2 is closely related to PI3K/AKT signaling pathway in HRECs

We infected HRECs with lentivirus carrying lentiviral vectors shNC, shA2, lenti-EGFP, lentiA2 and made HRECs in four groups. Moreover, we also added AKT activator SC79 in shA2 group and PI3K inhibitor LY294002 in lentiA2 group. Western blot was performed to verify the relationship between ANXA2 and PI3K/AKT signaling pathway in HRECs. ShA2 group showed the degradation of phosphorylation of AKT and related proteins in the pathway and the best silencing efficiency on the PI3K/AKT signaling pathway while the changes were counteracted in the shA2+SC79 group. However, the level of phosphorylation of AKT and related proteins in the pathway was obviously enhanced in lentiA2 group and the up-regulation was dampened when LY294002 was added (Figure 1A, 1B, 1C, 1D, 1E).

Figure 1 The interference and overexpression of ANXA2 obviously influence the phosphorylation levels of important proteins in PI3K/ AKT signaling pathway in HREC cells of each group. Western blot assay for protein expression of AXNA2, p-PI3K, PI3K, p-AKT, AKT,

p-Tsc2, Tsc2, p-mTOR, mTOR, p-p70 S6K, p70 S6K, p-4E-BP1, 4E-BP1 in HRECs of each group

(A); Statistical analysis (B, C, D, E) * P <0.05, **P<0.01, *** P<0.001, n=3.

ANXA2 promotes proliferation of HRECs through PI3K/AKT signaling pathway

EDu assay was performed to investigate whether ANXA2 can influence the

proliferation on HRECs. The result shows number of HRECs in proliferative stage in

lentiA2 group was larger than that in lenti-EGFP group but the number decreased

when LY294002 was added. While the number of HRECs in proliferative stage in shA2

group was smaller compared to shNC group. However, proliferation of HRECs in shA2

group was strengthen when SC79 was Added. These results indicated that ANXA2

could promote proliferation of HRECs through PI3K/AKT signaling pathway (Figure

2A and 2B).

192

193 **Figure 2 The interference and overexpression of ANXA2 obviously influence the**

194 **proliferation of HREC cells and the influence is highly related with PI3K/AKT signaling**

195 **pathway.** EDu assay show the proliferation of HREC cells in each group(A). Statistical analysis (B, C)

196 ****P<0.01, *** P<0.001, n=3.**

197 **ANXA2 promotes migration and invasion of HREC cells through PI3K/AKT signaling**

198 **pathway**

199 To explore the influence of ANXA2 in HREC cells, Transwell assay was performed and

200 indicated that ANXA2 could significantly enhance the ability of migration and invasion

201 of HREC cells in lentiA2 group while the impact was counteracted by LY294002. On the

202 contrary, HREC cells in shA2 showed low migration and invasion but the ability was

203 restored by SC79, the result confirmed that ANXA2 may promote migration and

204 invasion of HREC cells through PI3K/AKT signaling pathway (Figure 3A and 3B).

205

206

207 **Figure 3 The interference and overexpression of ANXA2 obviously influence the migration**
 208 **and invasion of HRECs and the influence is highly related with PI3K/AKT signaling pathway.**

209 Transwell assay show the migration and invasion of HRECs in each group(A). Statistical
 210 analysis (B, C) * P <0.05, **P<0.01, *** P<0.001, n=3。

211 **ANXA2 promotes angiogenesis ability of HRECs through PI3K/AKT signaling**
 212 **pathway**

213 We performed tube formation assay to explore the potential relationship between
 214 ANXA2 and angiogenesis which plays a critical role in RNV. Tube formation assay
 215 revealed that HRECs in lentiA2 group had better angiogenesis compared with lenti-
 216 EGFP group while the angiogenesis ability of cells was dampened when LY294002 was
 217 added. While the HRECs in shA2 group had poorer angiogenesis compared to shNC
 218 group. However, the ability of HRECs in shA2 group was restored when SC79 was
 219 Added. These results indicated that ANXA2 could promote the expression of RNV and

220 facilitate angiogenesis ability of HREC's and the influence might be generated through
 221 PI3K/AKT signaling pathway (Figure 4A and 4B).

222

223

224 **Figure 4 The interference and overexpression of ANXA2 obviously influence the angiogenesis**
 225 **ability of HREC's and the influence is highly related with PI3K/AKT signaling pathway. Tube**
 226 **formation assay show the angiogenesis ability of HREC's in each group(A). Statistical analysis**
 227 **(B, C) **P<0.01, *** P<0.001, n=3.**

228 **Expression of ANXA2 is significantly increased in the retina of mice of OIR model**
 229 **and the inhibition of PI3K/AKT signaling pathway results in receding the process**
 230 **of neovascularization**

231 Western-blot was applied to measure the expression level of ANXA2 in the mice of
 232 normoxia, OIR and LY294002 treatment group. ANXA2 expression is significantly
 233 higher in the other two groups than normoxia group and that indicated high level of
 234 expression of ANXA2 is closely related to the development of retinal

neovascularization (Figure 5). Retinal vessels of the mice in normoxia group stretched out from the optic disc and distributed radially and we could hardly see the non-perfusion area. The diameter of vessels was thick and there were no abnormal branches. On the contrast, the retina of the OIR group showed a large amount of non-perfusion area in the center which was surrounded by bright and disorderly neovascular plexus. While the neovascular plexus, nonperfusion and abnormal branches were much less severe in the retina of mice in LY294002 treatment group (Figure 6).

Figure 5 ANXA2 expression is significantly higher in retina of mice in OIR group and LY294002 treatment group. Western blot assay for protein expression of AXNA2 in retina of mice of each group (A); Statistical analysis (B) *** $P < 0.001$, $n = 26$.

Figure 6 Inhibition of PI3K/AKT signaling pathway significantly recede the process of neovascularization. Images are typical retinal angiogram of the retina of mice in the normoxia group, OIR group and LY294002 treatment group. n=26.

Discussion

ANXA2 is a kind of Ca^{2+} - dependent protein which can bind to phospholipid. Previous studies pointed out a variety of biological functions of ANXA2 such as participating in cell signal transduction, cell cytoskeletal movement, cell proliferation, apoptosis and neovascularization ^[15]. Multiple pieces of evidence suggested that ANXA2 is highly expressed in the vascular system and overexpressed in angiogenesis, suggesting that it may play an important role in angiogenesis ^[16]. Immunohistochemical studies of human breast tumor tissues provided compelling evidence of a strong positive correlation between ANXA2 expression and neovascularization and suggested that ANXA2 was a potential target to delay or inhibit breast tumor growth by inhibiting neovascularization^[17]. The addition of an antibody against ANXA2 also produced a prominent inhibition of network formation of human umbilical vein endothelial cells in a co-culture system mimicking the process of neovascularization

[18]. However, the accurate function of ANXA2 in PDR has not been illustrated clearly.

PI3K/AKT signaling pathway regulates a wide variety of cellular processes, such as cell proliferation, differentiation, survival, cell transformation and metastasis of cells^[19].

It has been well documented that under the stimulation of Vascular endothelial growth factor (VEGF), AKT could promote proliferation and migration of vascular endothelial cell and participated in the process of vascular remodeling^[20]. The lack of AKT can

intensify the process of atherosclerosis in the mice model^[21]. The PI3K/AKT pathway has close relationship with tumor progression and abnormality of this pathway can be frequently observed in a variety of tumors^[22-24]. In both rabbit cornea model^[25] and

mouse ischemic retinal model^[26], PI3K/AKT pathway were confirmed to contain effective angiogenesis characteristics. Moreover, it was found the level of phosphorylation of AKT was significantly reduced while the total amount of AKT

remains when the gastric cancer cells were transfected with small interfering RNA containing shA2 plasmid which further indicated that ANXA2 may play a role by influencing the phosphorylation of AKT^[27]. When ANXA2 expression were inhibited,

the levels of phosphorylation of AKT and PI3K, the key molecular of AKT pathway, were distinctly lower and the same phenomenon also appeared when PI3K inhibitors were added in cells^[28]. The result suggested ANXA2 might accelerate cell proliferation

and migration by promoting the phosphorylation of AKT and related proteins in the pathway^[28].

Neovascularization, a complex process for new blood vessels formation in which

endothelial cells migration, adhesion and tubular formation play a very important role [29], is a pathological hallmark feature for PDR [30]. Edu assay, Transwell cell migration assay and tube formation assay are mature methods to simulate in vitro neovascularization [31-33]. We found that ANXA2 can promote the proliferation, migration and tube formation of HRECs. The results indicated that ANXA2 can be a positive regulator of human retinal neovascularization, which is consistent with the role of ANXA2 in endothelial cells [34] while the impact could be significantly weakened by the PI3K/AKT pathway inhibitor. These phenotypes had proved our hypothesis that ANXA2 promotes development of retinal neovascularization through PI3K/ AKT pathway.

To investigate its role in retinal neovascularization, we also constructed OIR mice model, the most mature and classic model of retinal neovascularization [35]. To ascertain whether we built the model effectively, we selected the P17 mice in all three groups because P17 is the peak of the formation of retinal neovascularization in OIR model and new blood vessels then begin to fade and is almost completely replaced by normal vascular system on P25 [36]. Compared with STZ-induced diabetic models, OIR mice model reveals not only the process of vaso-obliteration but also neovascularization phase [37], which is consistent with PDR and inspired our particularly strong focus. Therefore, we employed OIR mice model to illustrate the role of ANXA2 in PDR. Then we measured the protein expression level of ANXA2 in mice retinal, and the results showed the expression of ANXA2 in the OIR group was significantly increased in

307 comparison with negative controls which means ANXA2 was involved in the formation
308 of retinal neovascularization in mice and play an important role in retinal
309 neovascularization ^[16].

310 Furthermore, we hypothesized that the development of retinal neovascularization is
311 highly related to PI3K/AKT pathway and injected 0.05ml PI3K inhibitor LY294002
312 intraperitoneally once a day from P7 to P10. Finally, we found the retinas of mice which
313 was treated with LY294002 contained less severe neovascular and irregular expansion.

314 VEGF proved a key factor of hypoxia-inducible RNV and also an effective target to
315 inhibit the progress of it. What's more, intravitreal injection of anti-VEGF medicine
316 has led a significant breakthrough in the treatment of RNV ^[38, 39]. Inhibitors of VEGF
317 show great promise for treatment of RNV. However, a growing number of researches
318 suggest that the strategy may not address the underlying causes of pathology and have
319 some noteworthy deleterious side-effects including the acceleration of the formation of
320 proliferative epi-retinal membranes and aggravation of vitreoretinal traction^[40].

321 Increased incidence of systemic adverse events had scattered across many organ
322 systems induced by anti-VEGF treatment, the significance of which remains unclear
323 ^[41]. In addition, the effect of therapy can just last for about several months which means
324 the patients have to suffer repeat intravitreal injection of the medicine. Therefore, the
325 risk of intravitreal infection is much higher and some patients also respond poorly to
326 anti-VEGF treatments ^[42]. Thus, identification of novel targets that play important roles
327 in retinal neovascularization is urgently needed for those not responsible for anti-VEGF

therapy^[43]. Studies also demonstrated that celecoxib could inhibit the expression of VEGF and neovascularization growth in retinal pigment epithelial cells and the effect was mediated through a PI3K/AKT-dependent manner^[44] and abrogating of VEGF significantly reduced the risk of developing to PDR in type 2 diabetes patients^[45].

Conclusions

ANXA2 can promote development of RNV through PI3K/ AKT Pathway. We can make ANXA2 and PI3K/ AKT pathway as a potential target for the regulation of pathological neovascularization of retina, which also provides a novel idea for effective prevention and treatment of PDR in the future.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Abbreviations

ANXA2: Annexin A2

ATCC; American Type Culture Collection

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HRECs: human retinal endothelial cells

OIR: oxygen-induced retinopathy

PDR: proliferative diabetic retinopathy

Plg: plasminogen

RNV: Retinal neovascularization

Ethics declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration. Written informed consent was obtained from individual or guardian participants.

Consent for publication

All authors read and approved the final version of manuscript.

Competing interests

The authors declare that they have no competing interests.

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Contributions

Chen-Yue Li and Shi-Hong Zhao designed the study, Chen-Yue Li performed the tasks of study, Zi-Chang Zhao analyzed the data, Chen-Yue Li wrote the manuscript.

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Figures

Figure 2

The interference and overexpression of ANXA2 obviously influence the phosphorylation levels of important proteins in PI3K/ AKT signaling pathway in HREC cells of each group. Western blot assay for protein expression of AXNA2, p-PI3K, PI3K, p-AKT, AKT, p-Tsc2, Tsc2, p-mTOR, mTOR, p-p70 S6K, p70 S6K,

p-4E-BP1, 4E-BP1 in HRECs of each group (A); Statistical analysis (B, C, D, E) * P <0.05 **P<0.01 *** P<0.001 n=3.

Figure 4

The interference and overexpression of ANXA2 obviously influence the proliferation of HRECs and the influence is highly related with PI3K/AKT signaling pathway. EDu assay show the proliferation of HRECs in each group(A). Statistical analysis (B, C) **P<0.01 *** P<0.001 n=3.

Figure 6

The interference and overexpression of ANXA2 obviously influence the migration and invasion of HRECs and the influence is highly related with PI3K/AKT signaling pathway. Transwell assay show the migration and invasion of HRECs in each group(A). Statistical analysis (B, C) * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ $n = 3$

Figure 7

The interference and overexpression of ANXA2 obviously influence the angiogenesis ability of HRECs and the influence is highly related with PI3K/AKT signaling pathway. Tube formation assay show the angiogenesis ability of HRECs in each group(A). Statistical analysis (B, C) **P<0.01*** P<0.001n=3

Figure 9

ANXA2 expression is significantly higher in retina of mice in OIR group and LY294002 treatment group. Western blot assay for protein expression of AXNA2 in retina of mice of each group (A); Statistical analysis (B) *** P<0.001n=26.

Figure 12

Inhibition of PI3K/AKT signaling pathway significantly recede the process of neovascularization. Images are typical retinal angiogram of the retina of mice in the normoxia group, OIR group and LY294002 treatment group. n=26.