Normal vitreous promotes angiogenesis via activation of Axl

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Abstract
Vitreous has been reported to prevent tumor angiogenesis, but our previous findings indicate that vitreous activate the signaling pathway of phosphoinositide 3-kinase (PI3K)/Akt, which plays a critical role in angiogenesis. The goal of this research is to determine which role of vitreous plays in angiogenesis-related cellular responses in vitro. We found that in human retinal microvascular endothelial cells (HRECs) vitreous activates a number of receptor tyrosine kinases including Anexelektro (Axl), which plays an important role in angiogenesis. Subsequently, we discovered that depletion of Axl using CRISPR/Cas9 and an Axl-specific inhibitor R428 suppress vitreous-induced Akt activation and cell proliferation, migration, and tuber formation of HRECs. Therefore, this line of research not only demonstrate that vitreous promotes angiogenesis in vitro, but also reveal that Axl is one of receptor tyrosine kinases to mediate vitreous-induced angiogenesis in vitro, thereby providing a molecular basis for removal of vitreous as cleanly as possible when vitrectomy is performed in treating patients with proliferative diabetic retinopathy.

KEYWORDS
Akt, Axl, angiogenesis, endothelial cell, vitreous

Abbreviations: Axl, anexelektro; EGM-2, endothelial growth medium-2; HEK 293T, human embryonic kidney 293T; HRECs, human retinal microvascular endothelial cells; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; sgRNA, single-guide RNA; VEP, visual evoked potential.

Huizuo Xu and Hetian Lei contributed equally to this work.

[Correction added on November 13, 2020, after first Online publication: Affiliation 5 ‘Jinan University’ added, text added in Funding and Acknowledgment section].

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1 | INTRODUCTION

Vitreous, which is a tissue that becomes avascular during development and appears to possess antiangiogenic properties. In 1976, Brem and Folkman observed that tumors planted in the vitreous fail to form vessels only those tumors directly attached to retina could elicit capillaries. Then Folkman who proposed the potential usefulness of antiangiogenic therapies made a hypothesis that avascular tissues, such as cartilage or vitreous may be logical candidates as endogenous sources of antiangiogenic factors. Lutty et al found the antiangiogenic activity of vitreous using chick choorioallantoic activity membrane assay. Afterwards, Glaser demonstrated that vitreous samples, obtained from diabetic patients, stimulate the proliferation of vascular endothelial cells (ECs) assessed by a thymidine incorporation assay whereas vitreous samples obtained from patients with non-vascular ocular disorders fail to show the same stimulatory activity. Recently, several papers have been published describing the angiogenic and inflammatory activity of vitreous obtained from proliferative diabetic retinopathy patients. However, our previous findings showed that vitreous from healthy animals enhances the signaling pathway of phosphoinositide 3-kinase (PI3K)/Akt, cell proliferation and migration of human pigment retinal epithelial cells.6,7 and the PI3K/Akt signaling pathway plays a critical role in angiogenesis. In addition, tandem mass spectrometry showed that there are some pro-angiogenic proteins in the vitreous, and the majority of vitreal proteins detected are intracellular proteins, some of which may originate from retina. Therefore, we hypothesize that vitreous can promote angiogenesis.

Herein, we report that vitreous activates a number of receptor tyrosine kinases including Axl, and that inactivation of Axl suppresses vitreous-induced Akt activation and proliferation, migration, and tube formation of human retinal microvascular endothelial cells (HRECs).

2 | METHODS AND REAGENTS

2.1 | Major reagents

Antibodies against p-Akt, Akt, p-Axl, and Axl were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody against RasGAP, a gift from Dr Andrius Kazlauska was characterized in the previous publications. Secondary antibodies of the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent substrate for detection of HRP was obtained from Thermo Fisher Scientific (Waltham, MA).

2.2 | Cell culture

Primary human retinal microvascular endothelial cells (HRECs) were purchased from Cell Systems (Kirkland, WA) and cultured in grown at 37°C with 5% CO2 with endothelial growth medium (EGM)-2 kit (Lonza, Walkersville, MD). Human embryonic kidney (HEK) 293T cells (HEK 293 containing SV40 T-antigen) from The Dana-Farber Cancer Institute/Harvard Medical School (Boston, MA) were cultured in high-glucose (4.5 g/L) DMEM supplemented with 10% FBS. The medium used to harvest the lentiviral supernatant from 293T cells was high-glucose DMEM supplemented with 30% FBS. All cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

2.3 | DNA constructs

The four 20nt target DNA sequences preceding a 5′-NGG PAM sequence in the genomic Axl locus (NC_000004.12) were selected for generating single-guide RNA (sgRNA) for SpCas9 targets using the CRISPR design website (https://chopchip.cb.uib.no). The three target sequences were 5′-AACGGTTCATCATGCAGG-3′ (A1), 5′-GGGAATGTACACGGC-3′ (A2) and 5′-CTTCTACCGGAA ACTGACT-3′ (A3). The control sgRNA sequence (5′-TGCC ATACCGCAGCAGTGGG-3′) was designed to target the lacZ gene from Escherichia coli. The lentiCRISPR v2 vector was purchased from Addgene (Cat. 52961) (Cambridge, MA). To express SpGuides in the targeted cells, the oligos of top oligos 5′-CACCG-20nt (target Axl DNA sequences A1, 2, 3, or the lacZ sgRNA sequence)-3′ and bottom oligos: 5′-AAAC-20nt (20nt: complimentary target Axl DNA sequences or lacZ sgRNA sequence)-C-3′ were annealed and cloned into the lentiCRISPR v2 vector by BsmB1 (New England Biolabs, Boston, MA), respectively. All clones were confirmed by DNA sequencing using a primer 5′-GGACTATCATATGCTTACCCG-3′ from the sequence of U6 promoter that drives expression of sgRNAs. Both synthesis of primers and oligos and sequencing of PCR products and clones were performed at the Massachusetts General Hospital (MGH) DNA Core Facility (Cambridge, MA).

2.4 | Preparation of lentivirus

The procedure for lentivirus production was described in detail in our previous publication. In brief, lentiviruses were produced by triple transfection of HEK 293T cells with pLentiCRISPRv2, psPAX2, and pVSV-G (From addgene: 52961, 12260, 8454) using Lipofectamine 3000. After harvest, viruses were concentrated by centrifuging in a JA17 rotor (Beckman Coulter) at 25 000 rpm for 90 minutes at 4°C.
The concentrated viral resuspended in 300 μL of sterile TNE (50 mM Tris pH 7.8, 130 mM NaCl, 1 mM EDTA) with gentle rotation overnight at 4 degree. Next these dissolved retroviruses were titered for infecting HRECs in combination with 8 μg/mL of polybrene (Sigma-Aldrich Corp., St. Louis, MO, USA) or kept at −80°C. The infected cells were selected in media using puromycin (Sigma) (0.5 μg/mL) and the resulting cells were examined by western blotting.

2.5 | Western Blot analysis

HRECs at 90% confluence in a 24-well plate were deprived from serum and growth factors for continuous incubation for 6-8 hours, and then some of these cells were pretreated with vitreous (100 μL/mL) for 15 minutes. Simultaneously, the serum-deprived cells were treated for 15 minutes with different inhibitors. After washing twice with ice-cold phosphate-buffered saline (PBS), cells were lysed in 1 x sample buffer, which was diluted with extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/mL aprotinin, 2 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) from the 5 x protein sample buffer (25 mM EDTA (pH = 7.0), 10% Sodium Dodecyl Sulfate (SDS) (Sigma), 500 mM dithiothreitol, 50% sucrose, 500 mM Tris-HCl (pH = 6.8), and 0.5% bromophenol blue. The samples were boiled for 5 minutes and then centrifuged for 5 minutes at 13 000 g. Proteins from the centrifuged and heated samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, and subjected to western blot analyses using the appropriate antibodies. Experiments were repeated at least three times. Signal intensity was determined by densitometry using NIH ImageJ software.

2.6 | Cell proliferation assay

Using a cell counter, HRECs at a density of 3 x 10^4 cells/well number in 24-well plates was estimated after 48 hours of continuous treatment with EGM-2 or bovine vitreous (1:3 dilution in EGM-2). At least three independent experiments were performed as described previously.

2.7 | Scratch-wound migration assay

Migration was assessed with the scratch-wound assay with minor modifications. Once cells reached 80% confluence in 24-well plates, they were starved for 8 hours. After the cell monolayer was scraped with a sterile pipette tip (200 μL), and washed twice to remove detached cells. One scratch was generated per well and imaged on an EVOS imaging system every 6 hours for 48 hours. Images were analyzed by measuring the number of pixels in the wound area using Adobe Photoshop (Adobe Systems, San Jose, CA) and analyzed using ImageJ software. For each assay, results were quantitated from three to five independent experiments, and values were normalized to those obtained for control cells.

2.8 | Tube formation assay

This assay was performed as previously described. Plating 15 000 HRECs onto wells precoated with basement membrane extract (Trevigen, Gaithersburg, MD) from the storage at −80°C was thawed overnight at ice. Cells were imaged 6 hours after plating or culture for change media every day for 7 days, and tube formation was quantified the total length using the Angiogenesis Analyzer plugin for ImageJ (National Institutes of Health). We performed for each of the three replicates in one experiment, and the values of three independent experiments were normalized to those obtained for the control group.

2.9 | RTK array

HRECs were grown in 10-cm dishes, treated with vitreous for 30 minutes after starving for 8 hours, then washed with cold Wash buffer, extracted, and lysed as described under “Western blotting.” Following protein quantitation, 500 μg of protein/sample was used to carry out the phospho-RTK array analysis according to the protocol described by the manufacturer (ARY001B, R&D Systems). Henceforth, all incubation and wash steps were accompanied by end-to-end rocking. Briefly, each array was incubated with 2 mL of Array Buffer 1 for 1 hours at room temperature. After removal of this blocking buffer, 500 μg of sample diluted in 1.5 mL of Array Buffer 1 was added to each array and incubated overnight at 4°C. Following washes with 1 x Wash Buffer, arrays were incubated with 2 mL of anti-phosphotyrosine-HRP antibody diluted in Array Buffer 2 for 2 hours at room temperature. Wash steps were repeated, and arrays were visualized by adding a 1:1 ratio of the SuperSignal West Dura Extended Duration Substrate and scanning them using the myECL imager. Phosphorylation signals obtained were mapped to their respective RTKs using the reference RTK coordinates included in the kit. The experiment was repeated twice, and the phosphorylation signals for each RTK were quantified using the dot-blot analyzer macro in the ImageJ 1.50i software. Each phospho-RTK signal had two representative spots on a given array. The GraphPad Prism 7.0 software was used to generate graphs and carry out statistical analysis.
2.10 | A mouse model of oxygen-induced retinopathy (OIR)

C57BL/6J litters on postnatal day (P) 7 were exposed to 75% oxygen and the procedure was described before. 21 Briefly, at P12, the pups were anesthetized by freezing on ice. Pupils were dilated using a drop of 1% tropicamide and the eyes were treated with topical proparacaine anesthesia. Intravitreal injections were performed under a microsurgical microscope using glass pipettes with a diameter of approximately 150 μm at the tip after the eye were punctured at the upper nasal limbus using a BD insulin syringe with the BD ultra-fine needle. One microliter of R428 (final concentration 400 nM) or its vehicle (0.1% DMSO) was injected. After the intravitreal injection, the eyes were treated with a triple antibiotic (Neo/Poly/Bac) ointment and kept in room air (21% oxygen). At P17, the mice were euthanized and retinas were carefully removed for western blot analysis or fixed in 3.7% paraformaldehyde (PFA). Mice under 6 g of total body weight were excluded from the experiments. Each experiment was at least repeated three times in this OIR model. Retinal whole mounts were stained overnight at 4°C with the murine-specific EC marker IB4-Alexa 594 (red). 12,22,23 The images were taken with Leica FL microscope.

2.11 | Examination of R428 toxicity in mouse eyes

At P12, five pups were anesthetized and underwent intravitreal injections as described above. A single dose of 1 μL of 2 μM R428 stock (dissolved in 0.1% DMSO) was injected. Control injections were performed with 1 μL of the 0.1% DMSO vehicle. Based on the notion that the mouse vitreous volume is about 5 μL, this is expected to give rise to an initial final vitreal concentration of 0.4 μM, which is expected to be further diluted over time.

Visual evoked potentials (VEPs) were obtained using a multifocal electroretinography recorder (GT-2008V-VI; Gotec, Chongqing, China) for functional evaluation. The stimuli intensity was set to 10.0 cd·s/m2, the flash frequency was 1 Hz, and the number of flashes was 64. After light adaptation for 15 minutes, anesthetized animals were fixed on a special holder with one silver-plate electrode inserted under the skin of the occipital bone (anode), anterior bregma (cathode), and ear (ground electrode), respectively. Then, the VEP of right and left eyes was recorded in order by a Ganzfeld electrodagnostic system (Gotec, Chongqing, China). The latency of the first positive wave (P1) and second positive wave (P2) of VEP was analyzed.

After VEP, euthanized the mice and eyeballs were carefully removed, fixed in 3.7% paraformaldehyde (PFA) and embedded in paraffin for histopathological analysis. Sections were prepared and stained with hematoxylin and eosin. The images were taken with Leica FL microscope.

All animal experiments followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Investigators who conducted analysis were masked as to the treatment groups. All the mice were cared for by following the ACUC protocol approved by the Institutional Animal Care and Use Committee at Xiangya School of Medicine (Changsha City, China).

2.12 | Bovine vitreous

The cattle eyeballs were obtained from euthanized healthy cattle in Research 87 Inc (Boston, MA) and frozen at −80°C. To prepare the bovine vitreous, the vitreous was dissected from the eyeball while it was still frozen, permitted to thaw, and then centrifuged at 4°C for 5 minutes at 13 000 g. The resulting supernatant was used for all analyses.

2.13 | Statistical analysis

Results are presented as mean ± SD. The Student t test was performed for comparisons between two groups, and one-way ANOVA (Kruskal-Wallis test) was used for comparisons between multiple groups. A P value < .05 was considered significant.

3 | RESULTS

3.1 | Vitreous stimulates Akt activation in vascular endothelial cells

Akt plays a critical role in angiogenesis, 24 therefore, we evaluated whether vitreous was able to stimulate Akt activation in HRECs. Since there is an ethical issue with obtaining normal human vitreous, we isolated vitreous from healthy bovines and the vitreous was clarified by centrifuge for 5 minutes at 13 000 g. The most relevant cell types that vitreous induces angiogenesis are retinal ECs, so in this research HRECs were selected used. At first, we examined that what was the minimal vitreous we could use without losing its bioactivity. Therefore, growth-factors-deprived HRECs were treated with different volume of vitreous fluid at different time point. Western blot analysis of their lysates indicated that vitreous at 1:4 dilution within 15 minutes did not reduce its bioactivity in stimulating Akt activation (Figure 1A), and that vitreous-induced Akt activation reached the maximum at 15 minutes lasting for at least additional 15 minutes and then gradually reduced with time (Figure 1B).
Vitreous promotes proliferation, migration, and tube formation of vascular endothelial cells

Vitreous-stimulated activation of Akt, which in turn triggers angiogenesis-related cellular responses including cell proliferation, migration, and tube formation.\(^{25-28}\) Next, we examined whether vitreous was able to enhance these responses on HRECs. As expected, the number of HRECs is increased with vitreous than those treated with media only (Figure 2A). Then, scratch-wound assay was subjected to observe the capability of HRECs stimulated by vitreous. These results (Figure 2B-C) showed that HRECs treated with vitreous migrated faster than those treated with control, demonstrating that vitreous enhances the migration of HRECs.

To investigate the impact of vitreous on angiogenesis in vitro, we employed matrigel tube formation assay, in which ECs were plated on the matrigel with diluted vitreous or their culture medium without growth factors. These results (Figure 2D-E) showed that vitreous-enhanced HRECs to form vessel-like tubes in this in vitro angiogenesis assay.

Vitreous enhances phosphorylation of receptor tyrosine kinases including Axl

Extracellular signals such as growth factors stimulate activation of RTKs, which in turn activate PI3K, triggering Akt activation. Therefore, we evaluated whether vitreous was able to induce phosphorylation RTKs using RTK array kit, which contained 49 RTKs. As shown in Figure 3A, vitreous induces a number of RTKs’ phosphorylation, including EGF, Eph R, and Axl. Axl is essential for VEGF-dependent activation of PI3K/Akt\(^{18}\) and plays an important role in angiogenesis.\(^{29}\) Next, Axl activation induced by vitreous was confirmed by western blot analysis (Figure 3B). Therefore, we hypothesized that Axl played an

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**FIGURE 1** Normal vitreous stimulated Akt activation. A, Bovine vitreous (BV) diluted in a ratio of 1:1, 2, 4, 8, 10, 20, and 40 was used to treat serum-deprived HRECs for 15 minutes, and their lysates were subjected to western blot analysis using indicated antibodies. pAkt: S473. This is representative of three independent experiments. B, BV diluted in a ratio of 1:3 was used to treat serum-starved HRECs for 0.25, 0.5, 1, and 2 hours, and their lysates were subjected to western blot analysis. This is representative of three independent experiments.

**FIGURE 2** Vitreous enhanced proliferation, migration, and tube formation of vascular endothelial cells. A, HRECs were plated into 24-well plates at a density of 30,000 cells/well. After attaching the plates, the cells were deprived of growth factors for 8 hours and then treated with or without BV. After 24 hours, the cells were counted in a hemocytometer under a light microscope. B, A wound was created within HRECs in 48-well plates and then treated with or without BV. At 24 hours post wounding, the wound was photographed under a microscope. Scale bar: 400 μm. C, After polymerization of the matrigel, HRECs were seeded in each well in 18,000 cells per well in 96-well plates with or without BV. 16 hours later, each well was photographed under a microscope. Scale bar: 400 μm. Each plot in A, C, and E indicates the mean ± SD of three independent experiments. *** and **** denote significant difference (\(P < .05\) and .01) using un-paired t test.
**FIGURE 3** Axl was one of RTKs that were activated in response to BV. A, HRECs were cultured until reaching 90% confluence and then starved in serum-free medium overnight. The cells were stimulated with vitreous from healthy bovine (BV) for 15 minutes. The lysates were subjected to a phospho-RTK array following the manufacturer's instructions. BV, bovine vitreous. B, Lysates from cells treated as described above for panel A were subjected to western blot analysis using the indicated antibodies.

**FIGURE 4** Depletion of Axl blunted vitreous-induced Akt activation, proliferation, migration, and tube formation. A, Lysates from HRECs expressing CRISPR/Cas9 targeting lacZ or Axl were subjected to western blot analysis using indicated antibodies. B, Lysates with HRECs characterized in A treated with or without BV were subjected to western blot analysis using indicated antibodies. C, HRECs characterized in A were plated into 24-well plates at a density of 30,000 cells/well. After attaching the plates, the cells were deprived of growth factors for 8 hours and then treated with or without BV. After 24 hours, the cells were counted in a hemocytometer under a light microscope. B, A wound was created within HRECs characterized in A in 48-well plates and then treated with or without BV. At 24 hours post wounding, the wound was photographed under a microscope. Scale bar: 400 μm. C, After polymerization of the matrigel, HRECs characterized in A were seeded in each well in 18,000 cells per well in 96-well plates with or without BV. 6 hours later, each well was photographed under a microscope. Scale bar: 400 μm.
essential role in vitreous-induced activation of Akt and angiogenesis-related cellular responses.

3.4 | Depletion of Axl in vascular endothelial cells attenuates vitreous-induced Akt activation, proliferation, migration as well as tube formation

To investigate role of Axl in vitreous-induced angiogenesis, expression of Axl was depleted in HRECs using a CRISPR approach as shown in Figure 4A. Notably, depletion of Axl decreased vitreous-induced phosphorylation of Akt at Serine 473 (Figure 4B).

Furthermore, vitreous-induced proliferation, migration, and tube formation decreased in the HRECs with depletion of Axl compared with those (Figure 4C-D). These results demonstrate that Axl plays a critical role in vitreous-stimulated activation of Akt and cellular responses in vascular ECs.

3.5 | Pharmacological inhibition of Axl abrogates vitreous-induced activation of Akt and cellular responses by vascular endothelial cells

Axl depletion suppressed vitreous-stimulated Akt activation and angiogenesis in vitro (Figure 4), suggesting that pharmacological inhibition of Axl could block vitreous-induced Akt activation so that a potential pharmacological inhibitor could be used for treating retinal pathological angiogenesis. To this end, we found that 4 μM R428, a specific inhibitor of Axl, completely blocked vitreous-induced Akt phosphorylation at S473 in HRECs, indicating that this drug at this concentration is able to abrogate vitreous-stimulated Akt activation and cellular responses. As expected, R428 inhibited vitreous-enhanced proliferation, migration, and tube formation of HRECs (Figure 5), suggesting that Axl is a potential mediator of abnormal retinal angiogenesis.
3.6 R428 prevents abnormal retinal angiogenesis in mouse model of oxygen-induced retinopathy

We next explore the impact of R428 on angiogenesis in mouse model of oxygen-induced retinopathy in the eye. We first tested the effects of an intravitreal injection of a single dose of R428 into postnatal day (P) 12 mice at a vitreal concentration of 0.4 μM (a dose which did not cause obvious damage to cultured HERE (Figure S1), followed by examination of the mouse eyes 60 days later. This was done VEP and histological analysis to evaluate retinal structure (Figure S2). These assays revealed no functional or structural damage to the retina 60 days after a single injection of R428. We next examined the impact of R428 on pathologic angiogenesis in the mouse model of OIR.23,30 At P17 the whole-mount retinas from the euthanized pups were stained with isoelectin B4 (IB4), a mouse endothelial marker. Results showed a dramatic decrease in the number of preretinal tufts after treatment with R428 compared to vehicle (Figure 6A-B), and the tufts stand for pathological angiogenesis were outline in Figure 6C-D. Taken together, these data show that inhibition of Axl prevents hypoxia-induced pathological angiogenesis in a mouse model of OIR.

4 DISCUSSION

We herein present evidence that vitreous enhances Akt activation and angiogenesis-related cellular responses by HRECs. It was initially demonstrated that tissue extracts stimulate cellular proliferation in explants.31 This presaged
the hypothesis in 1939 that biochemical factors including VEGF involve in tumor angiogenesis. Later another research has been postulated that the non-perfused retinal tissue liberates a diffusible angiogenic substance. However, the role of vitreous in the ocular pathology is still unclear. Based on our previous findings we hypothesize the vitreous contains some soluble factors that can induce pathological angiogenesis from retina.

It had been demonstrated that IL6-neutralizing antibody can inhibit endothelial tube formation induced in vitro using vitreous samples obtained from patients with PDR. Our results show that normal vitreous can positively regulate various steps of the angiogenic process in ECs including cell proliferation and migration. Our data herein showed that bovine vitreous activated Akt in a dose- and time-sensitive manner by activating Axl-mediated PI3K/Akt signaling, which promotes EC motility and proliferation. Therefore, we propose that vitreous stimulates Akt activation via Axl, and this proposal warrants further investigation.

Axl is a receptor tyrosine kinase and was first identified in 1991 in two patients with chronic myeloid leukemia. It has determined that Axl interacts with other molecules, including EGFR and HER3, and maintains survival of cells. Autophosphorylation of Axl stimulates cellular responses including cell migration and tube formation. Axl initiates its downstream signaling transduction (eg, PI3K/Akt). Akt also known as protein kinase B, is a serine/threonine kinase that plays a key role in multiple cellular responses such as cell proliferation and migration and angiogenesis. However, how which PI3K isoform transfers the signaling from Alx to Akt is still a mystery and requires further investigation.

Our findings herein demonstrate that vitreous provides an inducible environment that enhances pathological angiogenesis, supporting the notion that the vitreous may be a “reservoir” whose content of angiogenic modulators may recapitulate different events occurring during the progression of proliferative diabetic retinopathy. In contrast, a previous report shows that diabetes mellitus does not have any effect on the formation of neovessels except that high glucose induces a resistance to vessel regression induced by lysophosphatidic acid via a ROS/Src/Erk pathway. Therefore, we hypothesize that in diabetic condition vitreous induces two pathways. One is that vitreous activates the signaling pathway of PI3K/Akt for promoting angiogenesis; the other is that vitreous stimulates the Erk pathway for enhancing its resistance to vessel regression. As a consequence, diabetes induces proliferative diabetic retinopathy or diabetic adema, which can be VEGF-dependent and independent. Based on these findings antagonizing RTKs (eg, Axl) is a potential novel strategy to treat patients resistant to the anti-VEGF therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

W. Wu designed research, performed research, analyzed the data and wrote the manuscript. Fei Yao performed some animal experiment. X. Xia, L. Tang, H. Xu, and H. Lei analyzed the data and revised the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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