Aurora kinase B disruption inhibits angiogenesis by inducing cell cycle arrest and apoptosis.

Running title: AURKB disruption inhibits angiogenesis

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Abstract

AIM: Angiogenesis is a complex process, which involves the sprouting of new blood vessels from pre-existing vasculature. Pathological retinal angiogenesis can lead to vision loss and even blindness. Aurora kinase B (AURKB), a member of the Aurora family of serine/threonine protein kinases, is a key player in chromosome segregation and is reported to contribute to tumor disease. However, the role of AURKB in retinal angiogenesis is largely unknown. The purpose of the present study was to investigate the role of AURKB in retinal angiogenesis.

METHODS: Western blotting, RT-qPCR were used to evaluate the expression of AURKB in pathological angiogenesis using oxygen induced retinopathy (OIR) mouse model. Small interfering RNA sequences against AURKB were synthesized and incubated with retinal cells. The efficiency of knockdown was confirmed by western blot and RT-qPCR assays. The effect of AURKB siRNAs on angiogenesis *in vitro* was investigated using EdU cell proliferation, cell migration and tube formation assays. Subsequently, flow cytometry was used to assess the effects of AURKB siRNAs on cell cycle distribution and on the induction of apoptosis.

RESULTS: The expression levels of the cell cycle and apoptosis-related genes were detected using western blotting. AURKB was overexpressed in pathological retinal angiogenesis. AURKB siRNAs could inhibit human umbilical vein endothelial cells (HUVEC) proliferation, migration and tube formation. The possible mechanism was that AURKB siRNAs could induce cell cycle arrest via AURKB-p21 complexes and apoptosis.

CONCLUSION: In conclusion, the data indicated that AURKB was overexpressed in OIR model and silencing of AURKB could inhibit angiogenesis *in vitro*. AURKB could be a novel therapeutic target for pathological retinal angiogenesis.

KEYWORDS: angiogenesis, oxygen induced retinopathy, AURKB, cell cycle

Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vasculature [1]. It is a complex process that involves the degradation of the extracellular matrix, the proliferation and migration of endothelial cells and the sprouting of vessels [2]. Physiological angiogenesis is of considerable significance in growth, development, wound healing and the normal function of several organs. However, the pathological angiogenesis can lead to several diseases such as tumour formation, retinopathy and rheumatoid arthritis [1,2]. Angiogenic retinopathy, such as diabetic retinopathy, retinopathy of prematurity and retinal vein occlusion, can eventually lead to blindness [3]. The current methods of treatment for angiogenesis retinopathy are limited. They can improve vision and pathological conditions to some extent; however, they fail to achieve the desired effects [4,5]. Therefore, novel therapeutic targets are required for the treatment of retinal pathological angiogenesis and for the investigation of

the potential molecular mechanisms associated with this process.

Aurora kinases represent a family of serine/threonine kinases that play a key role in regulation of cell division and mitosis [6,7]. Aurora B was found to control several aspects for chromosome segregation in all eukaryotes [8,9]. Two additional members of the Aurora family named Aurora A and Aurora C exist in mammals [10,11]. AURKB expression level is greatly increased in many human cancers, which is linked to tumorigenesis and angiogenesis [12,13]. It contributes to a number of processes that impact fidelity to cell division, inducing kinetochore stabilization, kinetochore-microtubule attachment, and the regulation of a surveillance mechanism named the spindle assembly checkpoint [13]. Recently, researchers find that inhibition of AURKB resulted in the induction of apoptosis and autophagy in tumor disease [14]. Both cell mitosis and apoptosis are well known biological processes that involve the development of many diseases such as tumor and angiogenesis [15]. AURKB was aberrantly expressed in various malignancies, including breast and colon cancers [16,17]. However, the expression and the role of AURKB in angiogenesis are largely unknown up to now.

Therefore, in the present study, we investigated the expression of AURKB in pathological angiogenesis using oxygen induced retinopathy (OIR) mouse model. Furthermore, we studied the effect of AURKB disruption using siRNAs on angiogenesis *in vitro* and revealed the underlying molecular mechanism. The study highlights the role of AURKB in retinal angiogenesis and may provide novel insights for pathological neovascularization therapy.

Material and methods:

Reagents. The antibodies for GAPDH, cyclin A1, cyclin B1, cyclin D1, cyclin E1, CDK1, CDK2, CDK4and CDK6 were purchased from Proteintech Group, Inc. DMEM, fetal bovine serum (FBS), DAPI, TRIzol®, Super Signal West Pico chemiluminescent substrate, and HRP, FITC and Alexa Fluor555-conjugated secondary antibodies were obtained from Thermo Fisher Scientific, Inc. Propidium iodide (PI) was obtained from Beyotime Institute of Biotechnology. The PrimeScript RT kit and SYBR Premix were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The Annexin V-FITC apoptosis kit was purchased from eBioscience (San Diego, CA, USA). *Griffonia simplicifolia* isolectin B4 was purchased from Invitrogen (Carlsbad, CA). Matrigel Matrix was purchased from BD transduction laboratories (Shanghai, China). The EdU staining kit was obtained from Guagzhou RiboBio Co., Ltd. (Guangzhou, China).

Oxygen-induced retinopathy in Mice. C57BL/6 mice (SIPPR-BK Experimental Animal Co., China) were kept in alternate dark-light cycles of 12 h at room temperature (RT). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize the suffering of the animals. The oxygen-induced retinopathy model was used to assess retinal neovascularization as previously described. Newborn mice were kept in a hyperoxia (75% oxygen) chamber connected to an oxygen controller from the first postnatal week (P7 to P12). At P12, the mice were returned to normoxia. Following exposure to normoxic air for 5 days (P12-P17), the eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour at room temperature. The retinas were cut four times from the edge to the center, and then incubated overnight at 4 ℃ with iso-lectin B4 in PBS containing 1 mM $CaCl_2$. The images were obtained using an IX81 microscope (Olympus). All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee of The Second Military Medical University.

Cell culture. Human umbilical endothelial cells (HUVECs), obtained from Sciencell Research Laboratories (Carlsbad, CA, USA), were cultured in DMEM supplemented with 10% FBS. The cells that were authenticated by cell short tandem repeat (STR) genotyping were cultured at 37 ℃, in a 5% CO_2 cell incubator. The cells were

authenticated using immunofluorescence staining for cytoplasmic VWF, PECAM1 expression and for the uptake of Di-I-Ac-LDL. When the cells were grown to approximately 80% confluence, they were used for the different assays.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. TRIzol reagent was used to extract the total RNA from mouse retinal tissues and from HUVECs. Subsequently, PrimeScript RT reagent was used to reverse transcribe total RNA to cDNA. SYBR Premix Ex Taq was subsequently used to assess the quantity of cDNA. The reactions were performed using a Rotor Gene 3000A (Corbett Research) instrument and the mRNA levels were normalized to the expression of *GAPDH*. The primer sequences (5'→3') used were the following: CCCTGAGGAGGAAGACAATG (forward) and GCACCACAGATCCACCTTCT (reverse) for *AURKB* (*Homo*), TGGGCTACACTGAGCACCAG (forward) and AAGTGGTCGTTGAGGGCAAT (reverse) for *GAPDH* (*Homo*), GATCCCAGAACAAGCAGCCT (forward) and TCGATTTCGATCTCTCGGCG (reverse) for *AURKB* (*Mus*), CAATGAATAGGGCTACAGCA (forward) and AGGGAGATGCTCAGTGTTGG (reverse) for GAPDH (*Mus*).

RNA interference. The siRNAs were designed and synthesized by a commercial company (Shanghai GenePharma Co., Ltd., Shanghai, China). The target sequences against *AURKB-1* were the following: Sense, GGAGGAUCUACUUGAUUCUTT; and anti-sense, AGAAUCAAGUAGAUCCUCCTT. The target sequences for *AURKB-2* were as follows: Sense, CCAAACUGCUCAGGCAUAATT; and anti-sense, UUAUGCCUGAGCAGUUUGGTT. The sequences of scramble siRNA were the following: Sense, UUCUCCGAACGUGUCACGUdTdT; and anti-sense, ACGUGACACGUUCGGAGAAdTdT. Lipofectamine® 2000 was used to transfect siRNAs according to the manufacturer's instructions. The efficiency of transfection is assessed by the AURKB expression level in western blotting and RT-qPCR.

Cell proliferation assay. Cell proliferation was assessed using 5-ethynyl-2'-deoxyuridine (EdU). Following transfection of the cells with AURKB siRNAs, they were incubated for 72 h and their medium was removed. EdU (100 μM) was added to the cells and they were incubated for 2 h. Staining was achieved using previous method [18]. The images were visualized by IX81 microscope (Olympus, Hamburg, Germany).

Cell migration assay. A Transwell chamber containing a polycarbonate filter with an 8-μm pore size was used to evaluate cell migratory ability. A total of $2x10^4$ cells that were treated with AURKB siRNAs for 72 h were seeded in the upper chamber in 0.5% FBS medium. A total of 700 μl of DMEM containing 1% FBS was added to the 24-well plate. The cells were allowed to migrate for 12 h, and the chambers were then fixed with 4% paraformaldehyde for 20 min. Subsequently, a cotton swab was used to remove the cells from the upper surface. The chambers were subsequently stained with 0.1% crystal violet and the migrated cells were counted and analyzed using IX81 microscope (Olympus).

Tube formation assay. The 96-well plate was coated with Matrigel and incubated at 37 ℃ for 0.5 h. The cells were treated with AURKB siRNAs for 72 h and resuspended in fresh medium. A total of $1.5x10^4$ cells were mixed with 100 μl of DMEM and added to the Matrigel-coated plates. Following 4 h of incubation at 37 ℃, the capillary-like structures were imaged and analyzed using IX81 microscope (Olympus Corporation).

Cell cycle assay. HUVECs were seeded in a 6-well plate and starved for 16 h. Subsequently, the cells were treated with AURKB siRNAs for an additional 72 h, harvested and finally fixed in 70% ethanol at 4 ℃ overnight. The ethanol was removed and the fixed cells were washed with PBS twice. Propidium iodide was used to stain the cells for 30 min at room temperature. The stained cells were assessed using Cell Lab Quanta SC (Beckman Coulter, Inc.). The data were finally analyzed using Modfit software.

Cell apoptosis assay. HUVECs were seeded in a 6-well plate and starved for 16 h. Subsequently, the cells were treated with AURKB siRNAs for an additional 72 h. The cells were harvested and stained with an Annexin V-FITC apoptosis kit, according to the manufacturer's protocol. The stained cells were assessed using Cell Lab Quanta SC (Beckman Coulter, Inc.). The data were finally analyzed using Flowjo software.

Western blot analysis. Lysis buffer containing protease inhibitor cocktail was used to extract total protein from mouse retinas and HUVECs. Approximately 10 μg of protein was separated in 10% SDS/PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Nonfat milk was used at 5% to block the non-specific sites. Subsequently, the samples were incubated with different primary antibodies at 4℃ overnight and the following morning the membranes were washed three times with TBS-T and incubated with HRP conjugated secondary antibodies. GAPDH was used as loading control and the protein signals were finally detected using the Super Signal West Pico chemiluminescent substrate and semi-quantified by the GeneGnome HR Image Capture System

Statistical analysis. The data were analyzed and presented using Prism software (GraphPad5.0). All data are presented as the mean ± SD, and the differences between the groups were evaluated using the t-test or one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results:

AURKB is highly expressed in pathological retinas. The OIR mouse model is a well-recognized model for the evaluation of retinal angiogenesis. Newborn pups were exposed to hyperoxia from P7 to P12. On P12, the pups were grown under normoxic conditions. On P17, retinal neovascularization was determined in OIR mice by isolectin-B4 staining. In order to investigate the role of AURKB in retinal angiogenesis, the expression of AURKB was initially studied in OIR mouse retinas using RT-qPCR and western blot analysis. OIR mice exhibited high retinal neovascularization (Fig. 1A). RT-qPCR and western blot assays demonstrated that the expression levels of AURKB were significantly higher in the OIR mouse retinas compared with those in the control group (Fig. 1B and C). The data indicated that AURKB was overexpressed in pathological retinas and, notably, in pathological retinal angiogenesis.

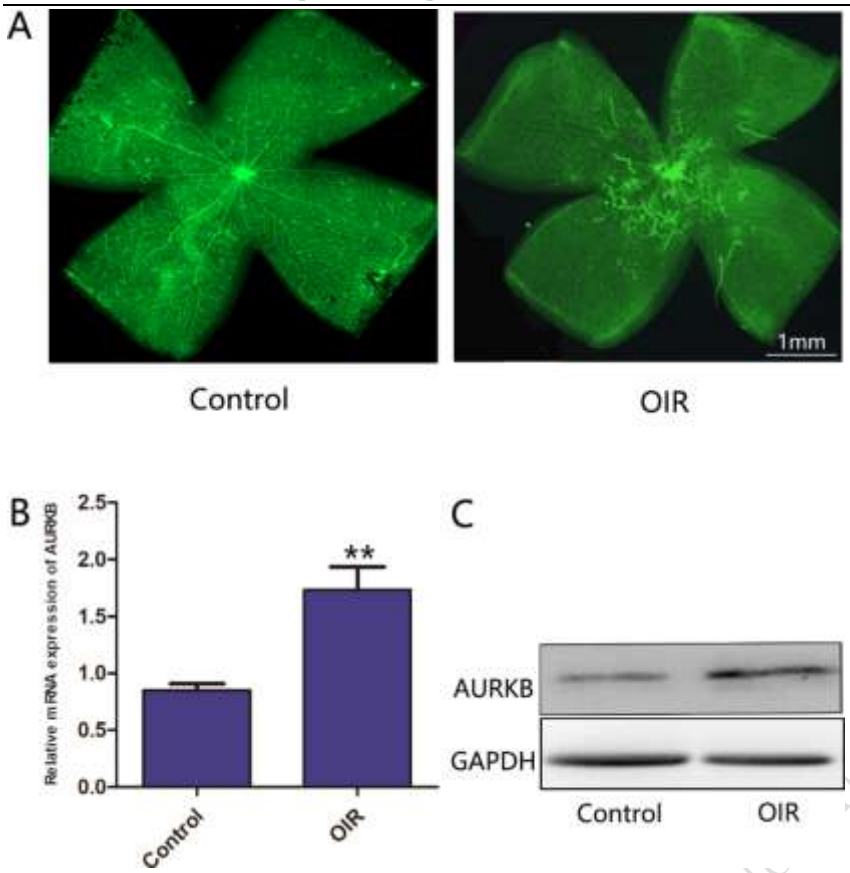

Figure 1. AURKB was overexpressed in the retina of the OIR mouse. (A) Representative images of isolectin-B4 staining of the retina in the OIR and normal groups. (B) mRNA levels of AURKB from OIR and control retinas. (C) Western blot analysis for AURKB in OIR and control retinas. GAPDH was used as a loading control. $^{***}P<0.001$. Each value represents the mean ± SD of three independent tests.

Silencing of AURKB inhibits HUVEC growth. To further explore the role of AURKB in angiogenesis, AURKB siRNAs were synthesized and their knockdown efficiency assessed. The results indicated that the AURKB siRNAs could significantly inhibit the expression of AURKB at the mRNA and protein level (Fig. 2A and B). The effect of AURKB siRNAs on cell proliferation was subsequently investigated by EdU. The data indicated that the cells treated with AURKB siRNAs proliferated more slowly than those of the control and scramble groups (Fig. 2C and D). The data indicated that the transfection was efficient and that the knockdown of AURKB may exert anti-angiogenic activity, partially through inhibition of the growth of vascular endothelial cells.

Silencing of AURKB inhibits the migration of HUVECs. The Transwell chamber assay was used to determine cell migration. The results indicated that AURKB siRNAs could inhibit the number of migrated cells significantly compared with the control and the scramble groups (Fig. 3A-D). No differences were observed between the control and the scramble groups (Fig. 3E). The number of migrated cells in the siAURKB-1 group was decreased to 62.3% compared with that of the control group, whereas the number of migrated cells in the siAURKB-2 group was decreased to 61.2% compared with that of the control group (Fig. 3E). The data suggested that silencing of AURKB could inhibit vascular endothelial cell migration, which is considered a vital process for angiogenesis.

Silencing of AURKB inhibits the tube formation of HUVECs. The tube formation assay is a well-known method to evaluate the angiogenic activity of endothelial cells *in vitro*. In the present study, tube formation assays were performed using Matrigel. The results indicated that AURKB siRNAs could significantly inhibit tube formation of

HUVECs compared with that noted in the control and scramble groups (Fig. 4A-D). No significant difference was noted with regard to tube formation between the control and scramble groups (Fig. 4E). The percentage of branching points in the siAURKB-1 group decreased to 61.2% compared with that of the control group, and the percentage of migrated cells in the siAURKB-2 group decreased to 64.1% compared with that of the control group (Fig. 4E). The data suggested that AURKB played an important role in angiogenesis, and that knockdown of AURKB could inhibit angiogenesis *in vitro*.

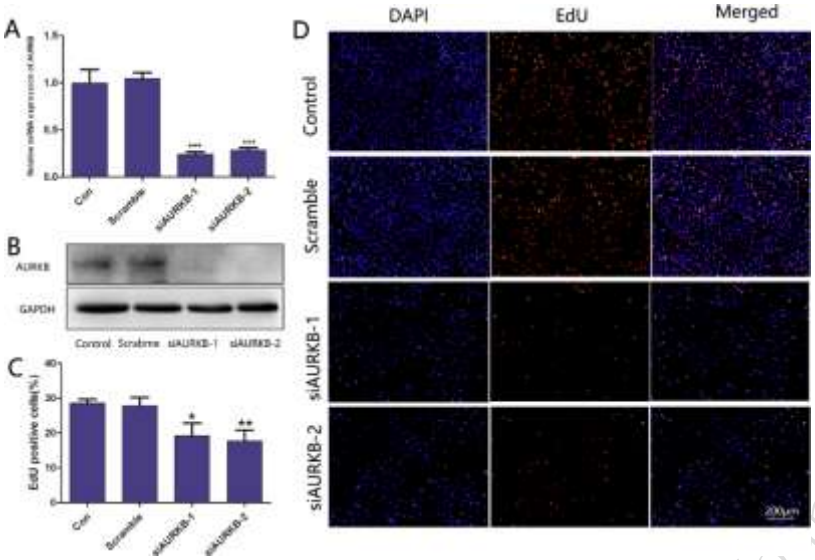

Figure 2. AURKB siRNAs could inhibit endothelial cell proliferation. (A) mRNA levels of AURKB from control, scramble and siAURKBs groups. (B) Western blot analysis of AURKB in the control, scramble and siAURKBs groups. GAPDH was used as a loading control. (C) Quantitative analysis of EdU-positive cells, which represented proliferating cells. (D) Representative images of cell proliferation assay using EdU. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Each value represents the mean ± SD of three independent tests.

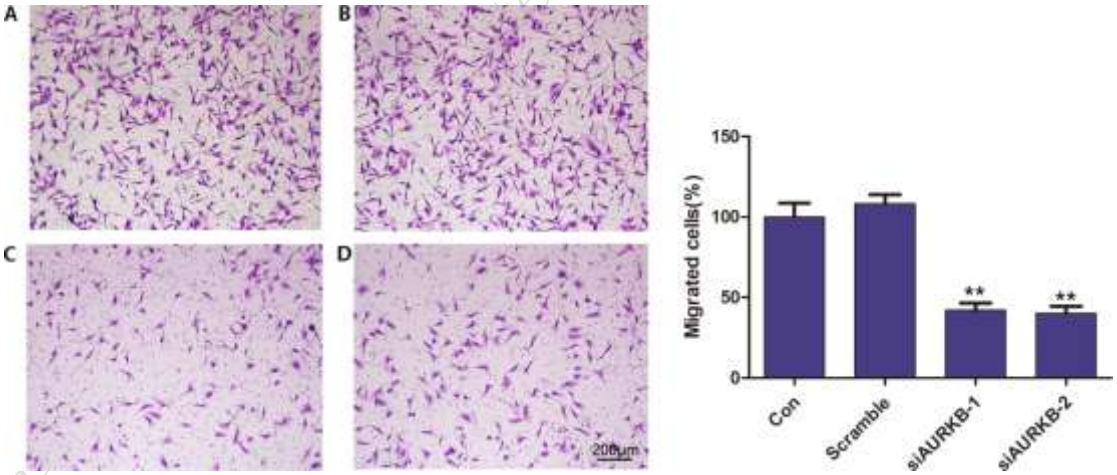

Figure 3. AURKB siRNAs inhibit endothelial cell migration. Representative images of migrated cells of the (A) control, (B) scramble, (C) siAURKB-1 and (D) siAURKB-2 groups using Transwell chamber assay. (E) Quantitative analysis of migrated cells. $^{**}P<0.01$. Each value represents the mean ± SD of three independent tests.

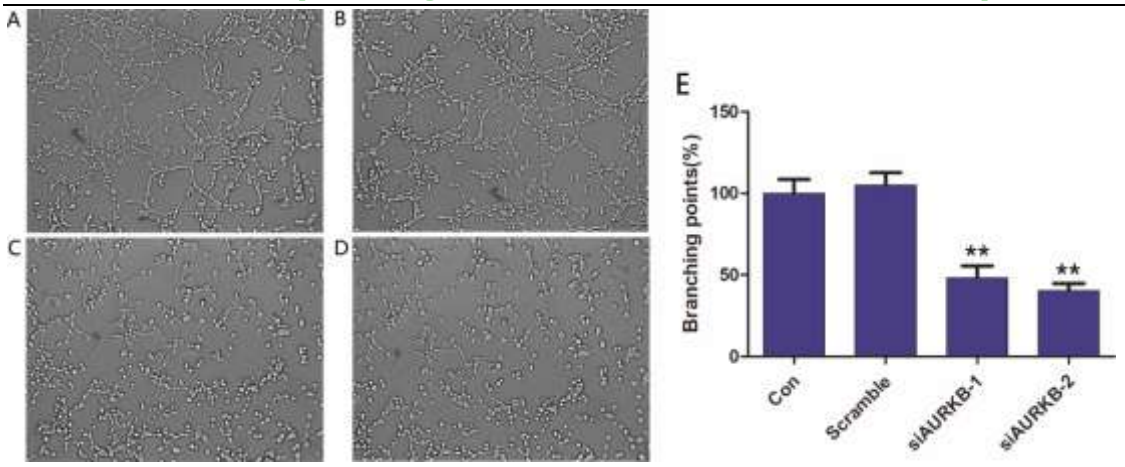

Figure 4. AURKB siRNAs inhibit endothelial cell tube formation. Representative images of tube formation of (A) control, (B) scramble, (C) siAURKB-1 and (D) siAURKB-2 groups. (E) Quantitative analysis of tube branching points. $^{**}P<0.01$. Each value represents the mean ± SD of three independent tests.

Silencing of AURKB disrupts the cell cycle and apoptosis process. The effects of AURKB knockdown on cell cycle progression and the induction of apoptosis were investigated. Knockdown of AURKB induced cell cycle arrest at the G2/M phase (Fig. 5A). The percentage of the cell population in the G2/M phase increased by ~12% in both the siAURKB-1 and siAURKB-2 groups compared with that noted in the control group (Fig. 5B). Furthermore, knockdown of AURKB induced apoptosis compared with the control and scramble groups (Fig. 5C). The percentage of early and late apoptotic cells in the AURKB knockdown groups increased by ~10% compared with that of the control groups (Fig. 5D). Subsequently, we investigated the expression levels of cell cycle-related proteins using western blot analysis. The results indicated that silencing of AURKB could inhibit the expression levels of CDK1, CDK2, CDK4, CDK6 and cyclin A1, cyclin B1, cyclin D1 and cyclin E1, and increase the expression levels of p21 (Fig. 6A and B).

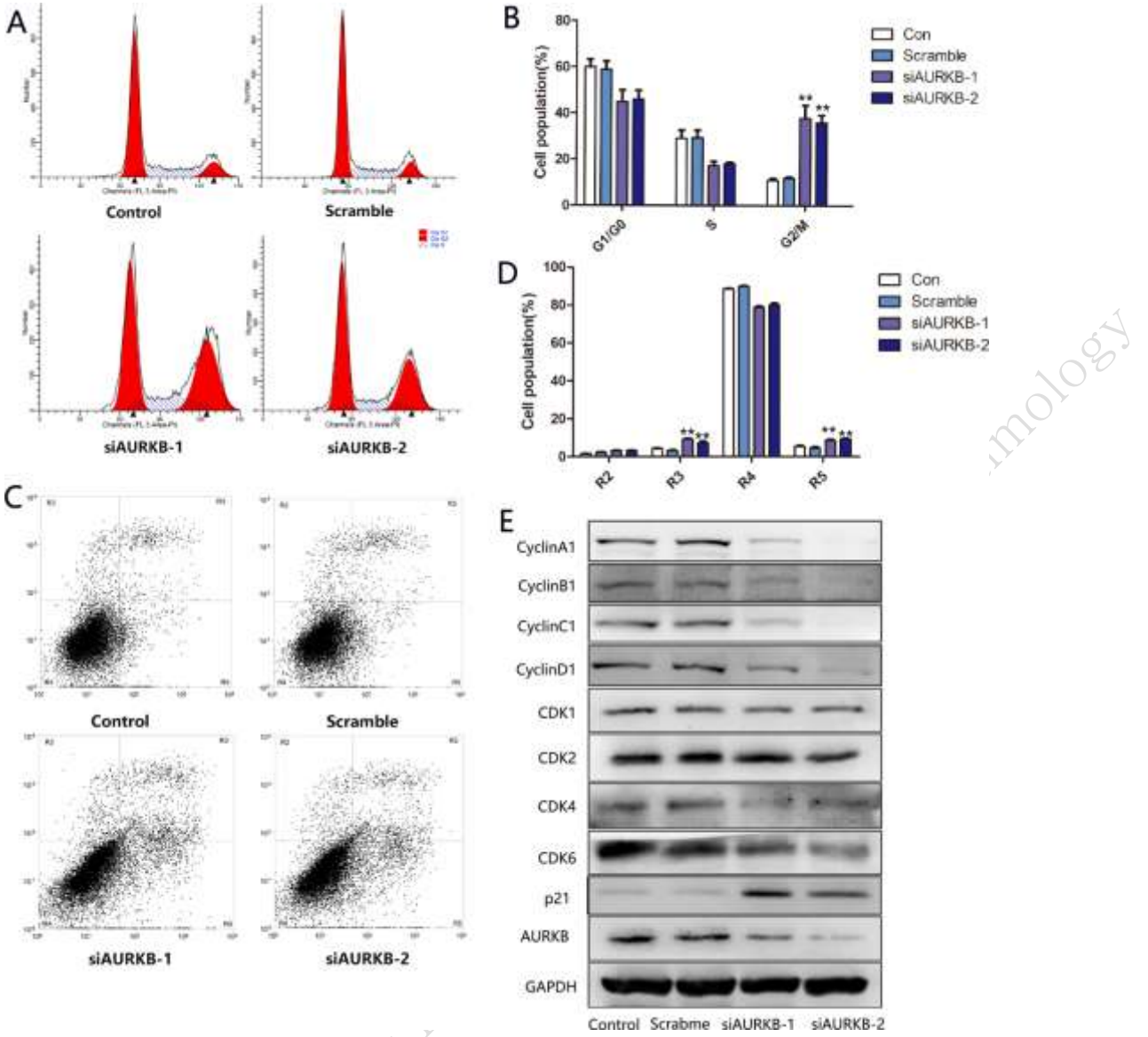

Figure 5. Possible mechanism of AURKB siRNAs inhibited angiogenesis. (A) Representative images of cell cycle analysis. (B) Quantitative analysis of the result of cell cycle assay. (C) Representative images of cell apoptosis assay. R2 represented dead cells, R3 represented late apoptotic cell, R4 represented live cells and R5 represented early apoptotic cells. (D) Quantitative analysis of the result of cell apoptosis assay. (E)The Western blot images of the effect of siAURKB on the expression of cyclin A1, cyclin B1, cyclin D1, cyclin E1, CDK1, CDK2, CDK4, CDK6 and p21. *p<0.05, **p<0.01. Each value represents the mean ± SD of three independent tests.

Discussion:

Pathological retinal angiogenesis is a severe disorder, which can result in retinal detachment, optic neuropathy and glaucoma [19,20]. These diseases can cause vision loss and even blindness. The vascular endothelial growth factor (VEGF) antibody has been clinically used as an inhibitor of retinal angiogenesis. However, subsequent studies have demonstrated that VEGF may cause cardiovascular adverse effects and that certain patients can develop drug resistance [21]. Therefore, the identification of novel therapeutic targets is required to treat retinopathy in combination with the VEGF antibody and/or independently.

Previous studies revealed AURKB was highly expressed in several tumor disease [12,13]. Deregulation of AURKB activity can result in mitotic abnormality and genetic instability, leading to defects in centrosome

function, spindle assembly, chromosome condensation, microtubule-kinetochore attachment, and cytokinesis [22]. And inhibition of AURKB can induce cell cycle arrest in G2/M phase and apoptosis via the mitochondria-dependent pathway in THP-1, HL-60 and K562 cells [14]. However, the role of AURKB in angiogenesis has not been fully investigated. The formation of pathological angiogenesis resembles the formation of tumor angiogenesis, and both of these phenomena are characterized by the uncontrolled growth of multiple cells [23]. These studies suggested that AURKB may participate in the formation of pathological angiogenesis.

The induction of AURKB during G2 and M-phase and the requirements for this kinase in microtubule-kinetochore attachment and biorientation of chromosomes have been widely described in recent studies [24,25]. In the absence of AURKB, CDK1 activity is diminished as a consequence of the induction of CDK inhibitor p21 and the presence of an increased pool of p21-CDK1 complexes [26]. The CDK1-cyclin B1 complex, also known as the M-phase promoting factor (MPF) is particularly relevant with regard to mitotic progression [27-29]. The data presented in the current report indicated that the expression of cyclin B1 was inhibited in the AURKB siRNA group. In addition, the expression levels of the cell cycle related genes, namely *cyclin A1*, *cyclin D1*, *cyclin E1*, *CDK2*, *CDK4* and *CDK6* were significantly altered. These might partially account for the cell cycle arrest induced by AURKB siRNAs.

In the present study, the expression of AURKB in OIR, its role in angiogenesis in vitro and the possible mechanism of this process were investigated for the first time. The mouse model of oxygen-induced retinopathy was triggered retinal neovascularization by hypoxic at P12, and the retinal neovascularization is maximal at P17. Angiogenesis spontaneously regresses between P17 and P25 [30]. So we detected the correlation of AURKB and retinal angiogenesis *in vivo* at P17. The data indicated that AURKB was highly expressed in pathological retinal angiogenesis and that AURKB siRNAs could inhibit endothelial cell proliferation, migration and tube formation. Furthermore, AURKB siRNAs could induce cell cycle arrest and apoptosis. The possible mechanism was associated with the inhibition of the expression of the cell cycle-related proteins. Taken collectively, the results highlight the role of AURKB in retinal angiogenesis and suggest its potential application for pathological neovascularization therapy.

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