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MicroRNA-129-5p suppresses proliferation, migration and invasion of retinoblastoma cells through PI3K/AKT signaling pathway by targeting PAX6

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ABSTRACT

Background: Retinoblastoma (RB) is the most common primary intraocular malignancy in children. Accumulating evidences have clarified that microRNAs (miRNAs) modulated signaling molecules by acting as oncogenes or tumor-suppressor genes in RB. Thus, in our study, we aimed to investigate the function of miR-129-5p in RB cells through PI3K/AKT signaling pathway by targeting PAX6. Two RB cell lines, Y79 and WERI-Rb-1, were selected in our study, followed by transfection of miR-129-5p inhibitor or si-PAX6 to explore the regulatory role of miR-129-5p in RB cell proliferation, invasion and migration.

Material and methods: Dual-luciferase assay was used for the detection of targeting relationship between miR-129-5p and PAX6. Besides, western blot analysis was applied to detect expression of cell cycle-related factors (CDK2 and Cyclin E) and PI3K/AKT signaling pathway-related factors (p-AKT and AKT). Nude mice tumorigenesis experiment was used to evaluate the effect of miR-129a-5p on RB growth *in vivo*.

Results: miR-129-5p was down-regulated in RB cell lines. miR-129-5p directly targeted the 3′-untranslated region of PAX6. Artificial down-regulation of miR-129-5p promoted cell proliferation, migration and invasion in RB cell lines Y79 and WERI-Rb-1, and promoted RB growth *in vivo* via PI3K/AKT signaling pathway, which could be reversed by transfection with silencing PAX6.

Conclusion: This study provides evidences that RB progression was suppressed by overexpressed miR-129-5p via direct targeting of PAX6 through PI3K/AKT signaling pathway, which may provide a molecular basis for better treatment for RB.

1. Introduction

Retinoblastoma (RB), caused by the mutation of the RB1 gene, is the most common type of malignant intraocular cancer in children [1]. Statistics revealed that incidence of RB is relatively constant at one case for every 15,000–20,000 livebirths, which amounts to about 9000 new cases in the world each year [2]. To be specific, that kind of disease has greatest prevalence in Asia and Africa, which show high birth rates, and has the mortality about 40–70% [3]. There are many well-known clinical manifestations of RB, including leukocoria, red eye, strabismus, nystagmus, and loss of binocularity depending on tumor location [4,5]. Treatment methods for RB include laser photocoagulation, cryotherapy, thermotherapy, intra-arterial chemotherapy, intravenous chemoreduction, and external beam radiotherapy as well as enucleation [6,7]. However, previous evidence reported that delayed diagnosis and treatment may lead to the exacerbation and migration of RB [8]. Therefore, early treatment requires timely and accurate diagnosis, which may improve the cure rate and survival rate.

MicroRNAs (miRNAs), a class of mature non-coding single-strand RNAs with a length of 22 nucleotides, play crucial roles in various kinds of physiological and pathological processes, especially in tumor development and progression [8]. miR-129-1 and miR-129-2, located at chromosome 7q32 and 11p11.2, respectively, were reported to produce the same 5′ prime product, miR-129-5p [9]. Previous studies showed that miR-129-5p was lowly expressed in multiple kinds of cancers, including bladder cancer, gastric cancer, non-small cell lung cancer,

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Table 1
Quantitative polymerase chain reaction primer sequences.

Primer name	Primer sequence
miR-129a-5p - F	GGGGGCTTTTTGCGGTCTGG
miR-129a-5p - R	AGTGCGTGTCG TGGAGTC
U6 - F	CTCGCTTCGGCAGCACA
U6 - R	CTCGCTTCGGCAGCACA
PAX6 - F	GAATCAGAGAAGACAGGCCA
PAX6 - R	GTGTAGGTATCATAACTCCG
β-actin - F	TCCTTCCTGGGCATGGAGT
β-actin - R	AAAGCCATGCCAATCTCATC

glioma and hepatocellular carcinoma [10–14]. Besides, lowly expressed miR-129-5p was also found in breast cancer tissues, and down-regulation of miR-129-5p promoted epithelial-mesenchymal transition in breast cancer [15]. PAX6 encodes a 422 amino acid protein consisting of two highly conserved DNA-binding domains [16]. It is reported that the homeobox gene PAX6 is an evolutionarily conserved key regulator for eye development in metazoans, and PAX6 heterozygosity may lead to eye abnormalities [17]. The phosphoinositide 3-kinase/AKT (PI3K/AKT) signaling pathway is reported to be involved in cell survival, growth, as well as tumorigenesis [18]. Additionally, previous evidence proved that the development of RB was related to the activation of the PI3K/AKT signaling pathway [19]. Thus, we selected Y79 and WERI-Rb-1 cells for investigating the regulatory role of miR-129-5p in RB progression with the involvement of PAX6 and the PI3K/AKT signaling pathway.

2. Materials and methods

2.1. Cell culture and grouping

The human normal retinal vascular endothelial cell (AC-BRI-181) and the human RB cell lines (Y79 and WERI-RB1) (purchased from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured to 70% confluency in RPMI-1640 (Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C under normoxic conditions of 100% humidity, 95% air and 5% CO_2 or under hypoxic conditions of 2% O_2, 93% N_2 and 5% CO_2. Cells were then assigned into: inhibitor control group, miR-129-5p inhibitor group, and miR-129-5p inhibitor + si-PAX6 group.

2.2. Cell transfection

miR-129-5p inhibitor and inhibitor control were synthesized by GenePharma, Co., Ltd., (Shanghai, China). si-PAX6 was purchased from Santa Cruz Biotechnology (TX, USA). According to the manufacturers' instructions, Y79 and WERI-RB1 cells were transfected with miR-129-5p inhibitor (30 nM) using Lipofectamine® 3000 transfection reagent for 48 h and PAX6 siRNA (100 nM) using Metafectene reagent (Biontex, München, Germany) for 5 h, respectively.

2.3. RNA isolation and quantification

Total RNA from the cells was extracted using a miRNeasy Micro Kit according to the instructions (Y5-217084, QIAGEN, Valencia, CA, USA), and RNA was reverse transcribed using a miScript II RT Kit (218160, QIAGEN). The amplification was carried out in triplicate using a miScript SYBR Green PCR Kit (218076, QIAGEN) on a 7900 H T Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the instructions. The expression of miR-129-5p was normalized to U6, and PAX6 mRNA was normalized to β-actin. The primers were purchased from Sangon Biotech (Shanghai, China), and primer sequences are listed in Table 1. The reaction conditions were as follows: 95 °C for 10 min, and a total of 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative mRNA expression was calculated from three independent experiments using the $2^{-\Delta\Delta Ct}$ method [20].

2.4. Western blot analysis

The extracted protein from cells was separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes at 100 mA for 2 h. The membranes were blocked in skimmed milk for 1 h at room temperature and added with anti-PAK6 (ab37749, Abcam Inc., Cambridge, MA, USA), anti-CDK2 (ab32147, Abcam), anti-Cyclin E (ab33911, Abcam), anti-AKT (ab179463, Abcam) and anti-p-AKT (4058; 1:1,000; Cell Signaling Technology, Beverly, MA, USA) for incubation overnight at 4 °C. After that, the membranes were incubated with the secondary antibody, horseradish peroxidase-labeled goat anti-rabbit IgG (1:2,000; A0208; Beyotime Institute of Biotechnology, Shanghai, China). The protein bands were observed using Cano Scan (LiDE110, Tokyo, Japan).

2.5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cells were collected, and the concentration was adjusted to 5×10^3 cell/well before they were cultured in an incubator at 5% CO_2, 37 °C for 24, 48, 72 and 96 h respectively. After that, 20 μL of MTT solution (M2128, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added to the cells (5 mg/mL) for incubation at 5% CO_2, 37 °C for another 4 h before MTT was removed. Subsequently, optical density (OD) value was measured at 570 nm when cells were suspended in 150 μL of dimethyl sulfoxide.

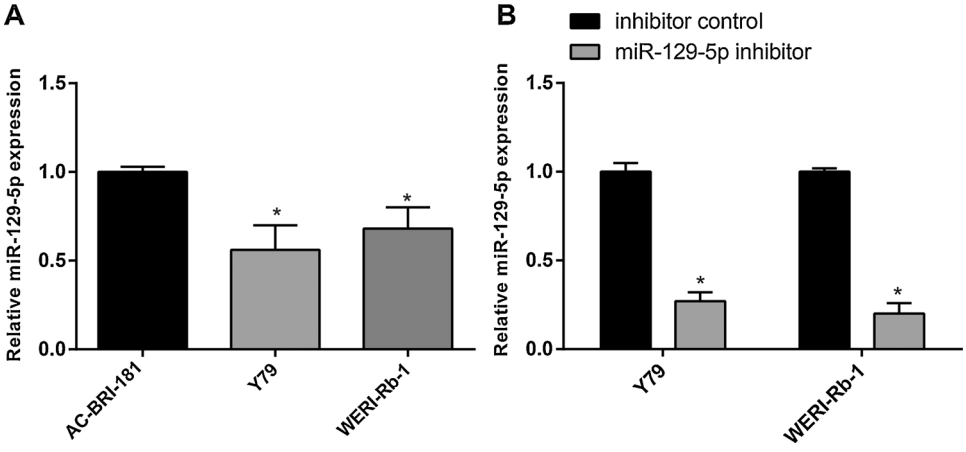

Fig. 1. miR-129-5p was significantly down-regulated in RB cell lines.
Note: A. Expression of miR-129-5p in RB cell lines (Y79 and WERI-RB1) and human normal retinal vascular endothelial cell (AC-BRI-181); * $P < 0.05$ *vs* AC-BRI-181 cell. B. Expression of miR-129-5p in transfected Y79 and WERI-Rb-1 cells; * $P < 0.05$ *vs* the inhibitor control group. Data are presented as the mean ± standard deviation. The experiments were repeated for three times. miR, microRNA; RB, retinoblastoma.

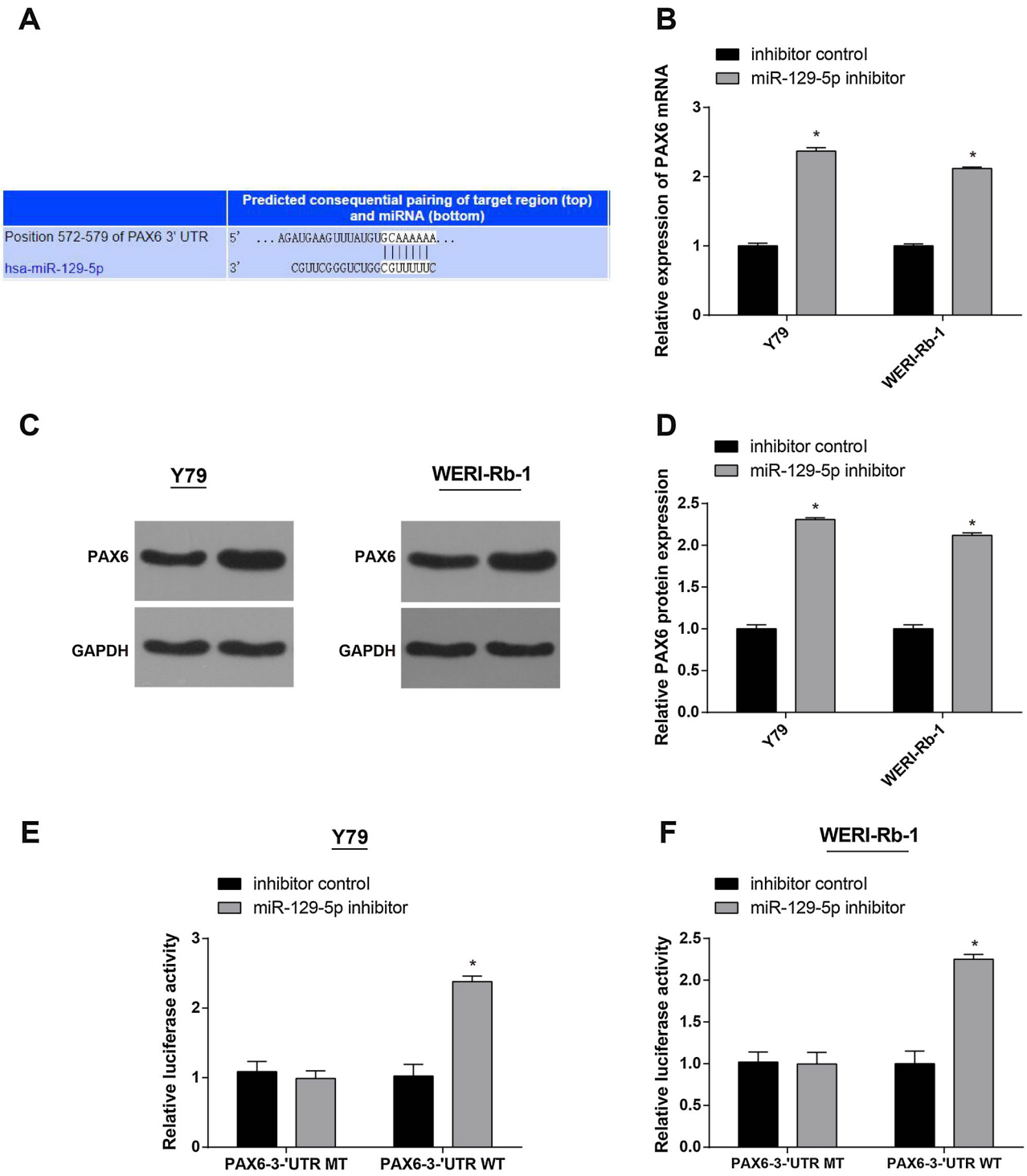

Fig. 2. PAX6 was one of the target genes of miR-129-5p.
Note: A, the binding site between the miR-129-5p and PAX6 3′-UTR; B–D, after inhibition of miR-129-5p, the mRNA and protein expression of PAX6 in RB cells; E–F, the fluorescence intensity after transfection of wild-type PAX6 3′-UTR and miR-129-5p inhibitor in Y79 and WERI-Rb-1 cells; Data are presented as the mean ± standard deviation. * $P < 0.05$ *vs* the inhibitor control group.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

Cell proliferation was also determined by BrdU incorporation assay (11647229001, Sigma-Aldrich). A total of 5 μM BrdU was added to the cells 4 h before PFA fixation. After that, cells were incubated with a rat anti-BrdU antibody (1:1,000; ab6326; Abcam) for incubation. The proliferating cells were visualized by addition of a goat anti-rat antibody labeled Alexa Fluor 594 (A-11007;1:1,000; Molecular Probes, Darmstadt, Germany).

2.7. Flow cytometry

After transfected for 48 h, transfected cells were washed by PBS two times and fixed with 75% ethanol at −20 °C overnight. After PBS washing again, cells were incubated with PBS containing 50 μg/ml propidium iodide (P4170, Sigma-Aldrich) and 100 μg/ml RNaseA

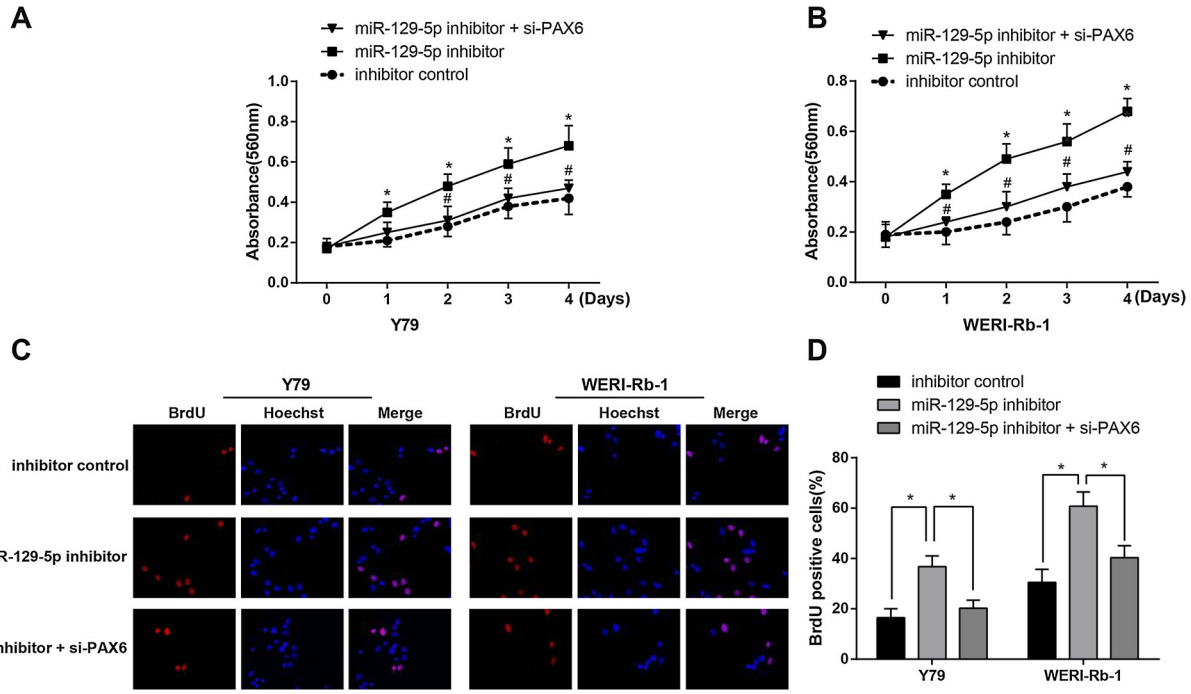

Fig. 3. Down-regulated miR-129-5p promoted RB cell proliferation through PAX6.
Note: A–B, MTT assay was applied to detect cell proliferation after transfection of si-PAX6 and/or miR-129-5p inhibitor in Y79 and WERI-Rb-1 cells; C–D, Detection of Brdu cell positive rate after transfection of si-PAX6 and/or miR-129-5p inhibitor in Y79 and WERI-Rb-1 cells; Data are presented as the mean ± standard deviation. * $P < 0.05$ *vs* the inhibitor control group; # $P < 0.05$ *vs* the miR-129-5p inhibitor group.

(R6148, Sigma-Aldrich) for 20 min at room temperature. The FACScan flow cytometer (BD Bioscience, NJ, USA) was adopted to analyze the cell cycle. Data was presented by the cell percentage in each phase of cell cycle.

2.8. Transwell assay

Matrigel (30 μl) (E1270, Sigma-Aldrich) was dissolved overnight, and diluted with FBS-free DMEM in triplicate volume. After that, Matrigel was added into each well in the apical chamber with a 15-min interval. Each well in the apical chamber was inoculated with 2×10^4 cells. Each well of the basolateral chamber was added with DMEM (0.5 ml) containing 10% FBS. After 24 h of incubation, the cells were fixed with paraformaldehyde and stained with 0.1% Crystal Violet for 0.5 h to remove the uninfected cells from the apical chamber. Subsequently, cells were washed with 0.1 M PBS. Fields were randomly selected for counting and photographing cells under a light microscope. The cells passing through the Matrigel was analyzed to determine the invasion ability. The experiment was repeated for three times.

2.9. Dual luciferase reporter gene assay

The potential binding site of PAX6 and miR-129-5p was predicted by TargetScan (http://www.targetscan.org/vert_71/). Wild-type and mutant 3′-UTR of PAX6 containing the target sequence were synthesized by Sangon Biotech Co., Ltd. and cloned into the pMIR-Report vector (Applied Biosystems). The Y79 and WERI-RB1 cells were transfected with inhibitor (50 and 100 nmol) and then co-transfected with wild-type and mutant 3′-UTR of PAX6 by Lipofectamine 2000. After 48 h, the cells were lysed using FastBreak™ Cell Lysis Reagent (V8572, Promega Corporation, Madison, WI, USA) and the cell lysates were incubated with dual-luciferase reagents (TM040, Promega Corporation). After that, the luciferase activities were assayed by Dual-Luciferase Reporter assay system (E1910, Promega Corporation).

2.10. Animal experiments

Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of College of Veterinary Medicine of Jilin University. Nude mice (BALB/C nu/nu) were fed with autoclaved water and laboratory rodent chow. A volume of 100 μl culture medium mixed with Matrigel (BD Biosciences) containing 3×10^6 Y79 cells with miR-129-5p inhibitor, miR-129a-5p inhibitor + si-PAX6 or inhibitor control was transplanted into the abdomen of the mice by subcutaneous injection. The tumor volume was monitored every 3 days for up to 35 days and calculated as follows: Volume (mm^3) = $(a \times b^2)/2$, where a indicates the largest diameter and b the perpendicular diameter. Once tumors had reached ~70 mm^3, the mice were randomly distributed into 3 groups (6 mice/group).

2.11. Immunohistochemical staining

Tumor sections at 5 μm from xenografts of RB nude mice were stained with anti-pAKT antibody (1:1,000; Cell Signaling Technology) and anti-Ki67 antibody (1:50, MIB-1, Immunotech, Marseille, France) at 4 °C overnight and then allowed to react with anti-IgG secondary antibody (1:1,000; ab6721; Abcam) for 30 min. Visualization was performed using 3,3-diaminobezidine (DAB, DA1010, Solarbio, Beijing, China). Five fields of view at 200 × magnification were randomly captured from each replicate using an inverted microscope (Nikon, Tokyo, Japan).

2.12. Statistical analysis

The data was analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The data are expressed as the mean ± standard deviation. The unpaired two-tailed Student's *t*-test or analysis of variance with Bonferroni t post-test was used to analyze the data depending on conditions. $P < 0.05$ was considered to be

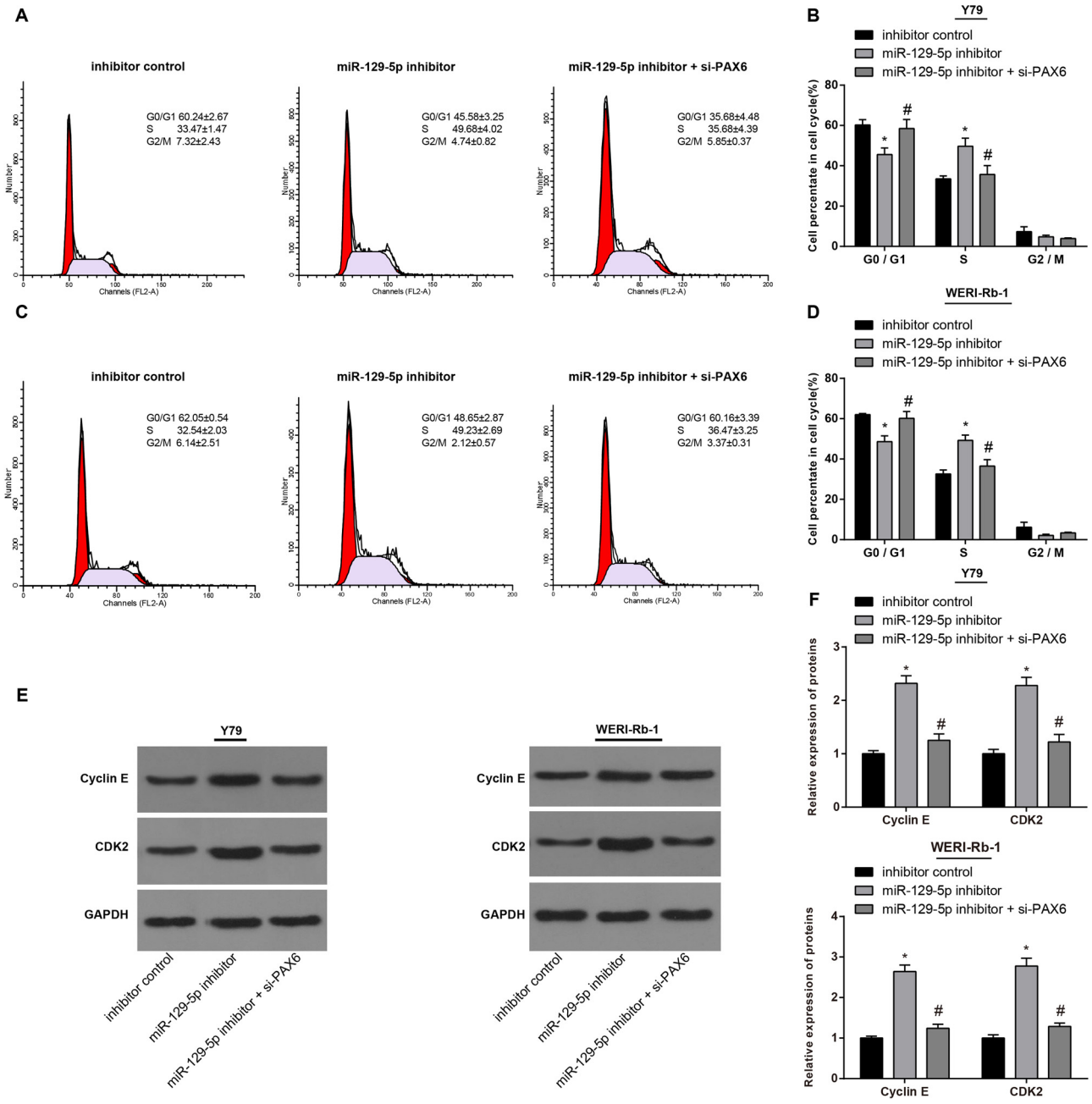

Fig. 4. Effect of miR-129-5p on RB cell cycle through PAX6.
Note: A–D, flow cytometry was applied to detect cell cycle after transfection of si-PAX6 and/or miR-129-5p inhibitor in Y79 and WERI-Rb-1 cells; E–F, detection of expression of cyclin E and CDK2 by western blot analysis; Data are presented as the mean ± standard deviation. * $P < 0.05$ *vs* the inhibitor control group; # $P < 0.05$ *vs* the miR-129-5p inhibitor group.

statistically significant.

3. Results

3.1. miR-129-5p was lowly expressed in RB cells

RT-qPCR was applied to detect expression of miR-129-5p in RB cell lines (Y79 and WERI-RB1) and human normal retinal vascular endothelial cell (AC-BRI-181), the result showed that miR-129-5p was lowly expressed in Y79 and WERI-RB1 cells when compared with the AC-BRI-181 cell ($P < 0.05$, Fig. 1A). miR-129-5p inhibitor and inhibitor control were successfully transfected into Y79 and WERI-RB-1 cells by using transfection reagent. The total RNA was extracted from transfected cells and the expression of miR-129-5p was tested by RT-qPCR. The results presented that compared with the inhibitor control group, Y79 and WERI-RB-1 cells in the miR-129-5p inhibitor group showed decreased expression of miR-129-5p (Fig. 1B).

3.2. PAX6 was one of the target genes of miR-129-5p

The results of bioinformatics prediction software showed that there was binding site between the 3′-UTR region of PAX6 and miR-129-5p (Fig. 2A). By detecting the expression of PAX6 in Y79 cells or WERI-RB-1 cells with overexpressed miR-129-5p, it was found that the expression of PAX6 was significantly lower than that of the control group, suggesting that miR-129-5p may be involved in regulating the expression of PAX6 (Fig. 2B-D). The luciferase reporter gene plasmids of PAX6 wild-type and mutant 3′-UTR were further constructed. The

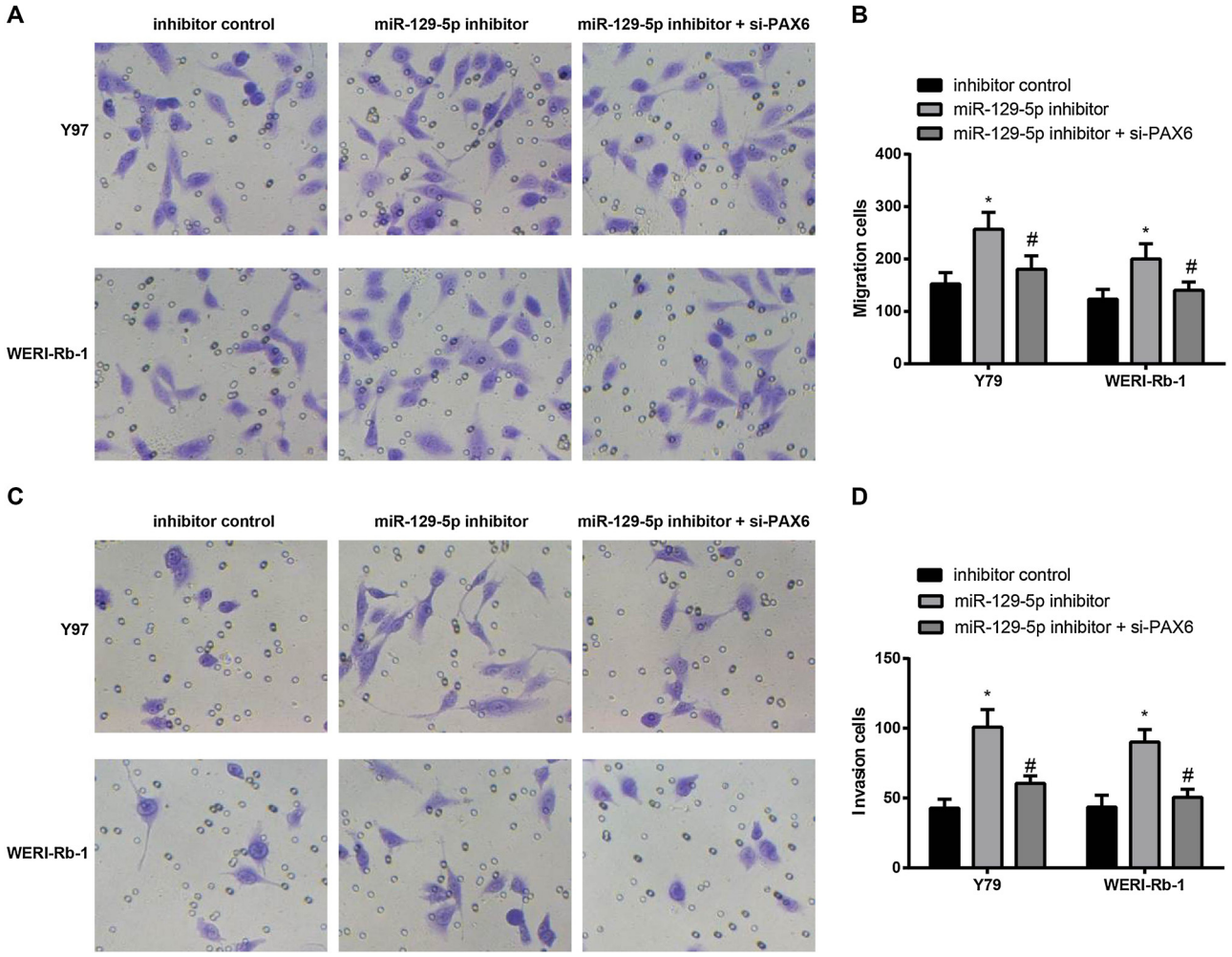

Fig. 5. Down-regulated miR-129-5p promoted RB cell invasion and migration through PAX6.
Note: A–B, cell passing through the membranes after transfection of si-PAX6 and/or miR-129-5p inhibitor was detected; C–D, cell invasion ability after transfection of si-PAX6 and/or miR-129-5p inhibitor was detected; Data are presented as the mean ± standard deviation. * $P < 0.05$ *vs* the inhibitor control group; # $P < 0.05$ *vs* the miR-129-5p inhibitor group.

fluorescence intensity after transfection of wild-type PAX6 3′-UTR and miR-129-5p inhibitor was significantly increased; while no significant difference was found in fluorescence intensity after transfection of wild-type PAX6 3′-UTR and miR-129-5p (Fig. 2E-F). These results suggested that PAX6 was the downstream target gene of miR-129-5p. miR-129-5p inhibited the transcription of PAX6 and negatively regulates the expression of PAX6 through binding to PAX6 3′-UTR.

3.3. Down-regulated miR-129-5p promoted RB cell proliferation through PAX6

In order to detect the effect of miR-129-5p on RB cells by targeting PAX6, miR-129-5p inhibitor was transfected into Y79 cells or WERI-RB-1 cells and PAX6 was silenced at the same time. The transfected Y79 cells and WERI-RB-1 cells were collected and the proliferation of RB cells was detected by MTT assay. The results (Fig. 3A-B) clarified that similar results were observed in Y79 and WERI-RB-1 cells. Compared with the inhibitor control group, the miR-129-5p inhibitor group showed increased cell proliferation ($P < 0.05$); while the cell proliferation ability was lower in the miR-129-5p inhibitor + si-PAX6 group when compared with the miR-129-5p inhibitor group ($P < 0.05$). Then, the proliferation of transfected Y79 cells and WERI-RB-1 cells was detected by BrdU labeling fluorescence staining, and the proliferation ability of the three groups was analyzed. The miR-129-5p inhibitor group showed increased Brdu cell positive rate compared with that in the inhibitor control group; while decreased Brdu cell positive rate was found in the miR-129-5p inhibitor + si-PAX6 group when compared with the miR-129-5p inhibitor group ($P < 0.05$) (Fig. 3C-D). The results suggested that miR-129-5p inhibited the proliferation of RB cells by inhibiting the expression of PAX6.

3.4. Effect of miR-129-5p on RB cell cycle through PAX6

Effect of transfection of miR-129-5p inhibitor/si-PAX6 on cell cycle was assessed by flow cytometry, the results revealed that (Fig. 4A-B) compared with the inhibitor control group, the miR-129-5p inhibitor group showed more cell at S phase and less cells at G0/G1 phase ($P < 0.05$); while the miR-129-5p inhibitor + si-PAX6 group showed decreased cell number at S phase and increased cell number at G0/G1 phase ($P < 0.05$). The results suggested that miR-129-5p regulated cell cycle by targeting PAX6. We further detected the relationship between miR-129-5p and cell cycle associated protein expression in RB cells by western blot analysis. The results presented that the miR-129-5p inhibitor group showed increased expression of cyclin E and CDK2; while the miR-129-5p inhibitor + si-PAX6 group showed decreased expression of cyclin E and CDK2 (Fig. 4C-D). In conclusion, miR-129-5p could inhibit the proliferation of RB cells by regulating the expression of cyclin E and CDK2 through PAX6.

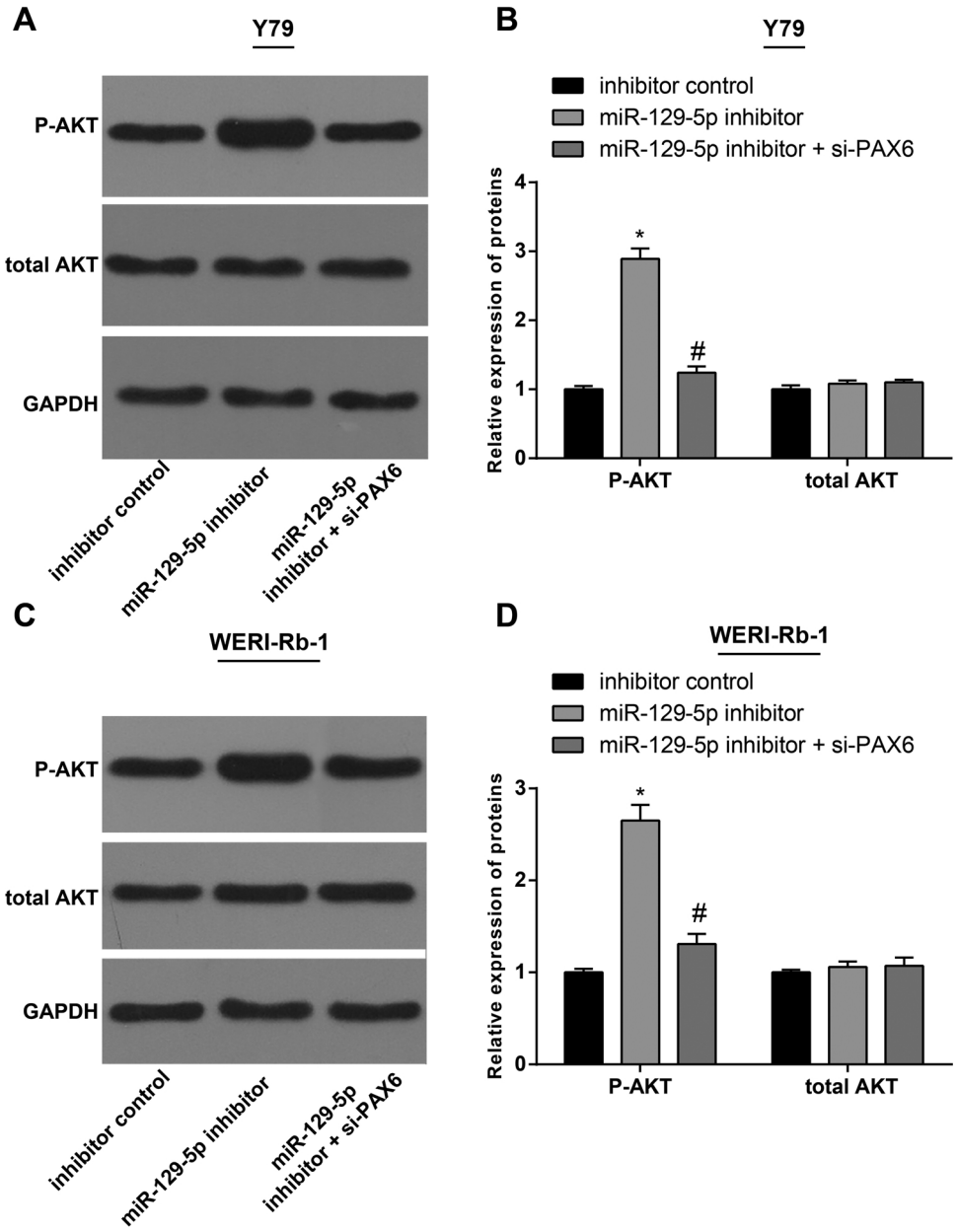

Fig. 6. miR-129-5p regulates PI3K/AKT signaling pathway through PAX6, which controls RB cell proliferation, migration and invasion.
Note: Expression of p-AKT and AKT after transfection of si-PAX6 and/or miR-129-5p inhibitor in Y79 and WERI-Rb-1 cells was detected; Data are presented as the mean ± standard deviation. * $P < 0.05$ *vs* the inhibitor control group; # $P < 0.05$ *vs* the miR-129-5p inhibitor group.

3.5. Down-regulated miR-129-5p promoted RB cell invasion and migration through PAX6

Effect of transfection of miR-129-5p inhibitor/si-PAX6 on cell invasion and migration was tested, the results revealed that (Fig. 5A-B) compared with the inhibitor control group, the miR-129-5p inhibitor group showed more cells in the basolateral of transwell chamber; while the miR-129-5p inhibitor + si-PAX6 group showed decreased cells in the basolateral of transwell chamber. Compared with the inhibitor control group, the miR-129-5p inhibitor group showed enhanced cell invasion ability; while the miR-129-5p inhibitor + si-PAX6 group showed decreased cell invasion ability (Fig. 5C-D). These results clarified that miR-129-5p affects the migration and invasion of RB cells by targeting PAX6.

3.6. miR-129-5p regulates PI3K/AKT signaling pathway through PAX6, which controls RB cell proliferation, migration and invasion

The results showed that PI3K/AKT signaling pathway was activated in RB cells in the miR-129-5p inhibitor group. However, in the miR-129-5p inhibitor + si-PAX6 group, the activity of PI3K/AKT signaling pathway was decreased to some extent (Fig. 6). These results suggested that miR-129-5p regulated the PI3K/AKT signaling pathway through PAX6, thereby controlling the proliferation, migration and invasion of RB cells.

3.7. Down-regulated miR-129-5p promoted RB tumor growth in vivo

By constructing the xenograft tumor model of Y79 nude mice, we found that interfering miR-129-5p expression could promote tumor growth, and further interfering PAX6 expression decreased the growth

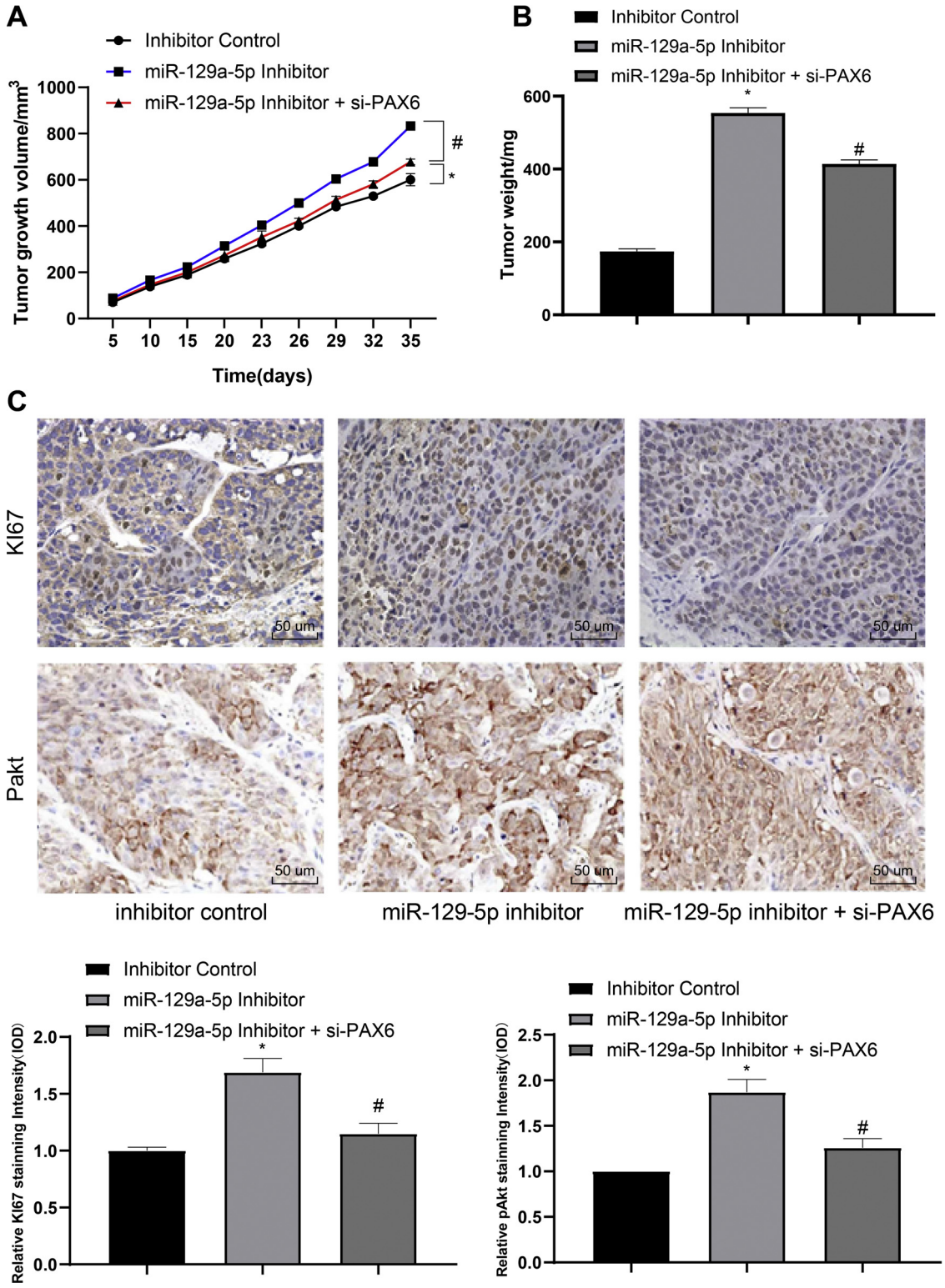

Fig. 7. Down-regulated miR-129-5p promoted RB tumor growth in vivo.
Note: Y79 cells with miR-129-5p inhibitor, miR-129a-5p inhibitor + si-PAX6 or inhibitor control were inoculated subcutaneously into BALB/c nude mice at a dose of 5×10^6 per mouse (n = 6 in each group). A, tumor size; B. tumor weight. Tumor sections were obtained and stained with anti-pAKT and anti-Ki67 antibodies. C, representative views of p-AKT and Ki67-positive tumor cells and quantification of immunostaining. In panel A two-way ANOVA was used to determine statistical significance of quantification of immunostaining, whereas in panel B one-way ANOVA was used. $*P < 0.05$ vs the inhibitor control group, $\#P < 0.05$ vs the miR-129a-5p inhibitor group.

rate of tumors significantly ($P < 0.05$) (Fig. 7A-B). Moreover, the results of Ki67 and p-AKT immunohistochemistry showed that the positive rates of Ki67 and p-AKT in the miR-129a-5p inhibitor group were significantly higher than those in the miR-129a-5p inhibitor/si-PAX6 group ($P < 0.05$) (Fig. 7C).

4. Discussion

RB was reported to affects infants and young children, although improvements have been made in treatment methods, the prognosis for patients with RB was still poor, with mortality rates range from 50 to 70% among children in underdeveloped countries [3]. Therefore, it is necessary to further explore the biological and molecular mechanisms of RB, and explore a new treatment to improve the prognosis of RB patients. Accumulating evidence demonstrated that many kinds of miRNAs were aberrantly expressed in RB and lead to RB development and progression [21,22]. However, the expression and roles of miR-129-5p in RB remain unclear. In this study, two RB cells, Y79 and WERI-Rb-1 cells, were selected and transfected with miR-129-5p inhibitor or si-PAX6 to explore the regulatory role of miR-129-5p in RB cell proliferation, migration and invasion through the PI3K/AKT signaling pathway by targeting PAX6. Importantly, we made conclusion that RB progression *in vivo* and *in vitro* was suppressed by overexpressed miR-129-5p via direct targeting of PAX6 through PI3K/AKT signaling pathway, which may provide a molecular basis for better treatment for RB.

Firstly, decreased expression of miR-129-5p was found in RB cells. miR-129-5p was frequently observed to be abnormally expressed in various kinds of human cancers. For example, miR-129-2 was down-regulated in glioma tissues and cell lines, and overexpression of miR-129-2 suppressed glioma cell proliferation, migration, and invasion and promoted cell apoptosis *in vitro* [12]. The miR-129-5p expression was decreased in hepatocellular carcinoma tissues and cells, and patients with low miR-129-5p expression had a higher ratio of vascular invasion, intrahepatic metastasis, and higher recurrence rate [14]. Furthermore, down-regulated miR-129-5p was also found in breast cancer tissues, and lowly expressed miR-129-5p could induce epithelial-mesenchymal transition in breast cancer [15]. These findings indicated that miR-129-5p expression may be a prognostic target for cancers.

The targeting relationship between PAX6 and miR-129-5p was verified in our study. Additionally, our study provided evidence that down-regulated miR-129-5p promoted RB cell proliferation, invasion and migration as well as arrested more cells at S phase (presented with increased cyclin E and CDK2) through PAX6. miR-129 is a miRNA family containing three well-known members, including miR-129-5p, miR-129-2-3p and miR-129-3p; among them, miR-129-5p is known as a tumor suppressor in multiple kinds of cancers, for example, hepatocellular malignancy and breast cancer [23,24]. Previous evidence demonstrated that up-regulated miR-129-5p could promote differentiation of retinal pigment epithelium [25]. Eye development has become an example of organogenesis, from lens induction through epidermal morphogenesis to the designation and differentiation of neurons; and PAX6 was proved to play a crucial role in these processes [26]. Cyclin E is important for cell cycle progression and is commonly overexpressed in cancer cells [27]. CDK6 is a key regulator of cell cycle, and over-expressed miR-129-5p was found to decrease CDK6 expression [28]. Lowly expressed miR-129-5p was found in glioma tissues and cell lines, and enforced miR-129-5p expression could inhibit human glioma cell proliferation and induces cell cycle arrest by targeting DNMT3A [29]. Taken together, it is reasonable to present that alterations in miR-129-5p expression regulate the proliferation, invasion and migration of RB cells via targeting PAX6.

Furthermore, the interaction between miR-129-5p and the PI3K/AKT signaling pathway was also investigated, and we found that miR-129-5p regulates the PI3K/AKT signaling pathway through PAX6, which controls RB cell proliferation, migration and invasion. Previous evidence revealed that AKT was activated in human RB, and once activated, AKT could phosphorylate many kinds of targets that regulate proliferation, apoptosis, and metabolism [30]. It is reported that the PI3K/AKT signaling pathway was involved in multiple kinds of cell processes, which was also important for tumorigenesis, including cell growth and mobility [31]. One recent study revealed that signaling pathway gene sets that primarily affected by miR-129-5p included PI3K-AKT signaling pathway, leading to the obvious changes in protein processing in the endoplasmic reticulum and proteoglycans process in cancer [32].

In conclusion, miR-129-5p was significantly downregulated in RB cells. Additionally, we demonstrated that overexpression of miR-129-5p works as a tumor suppressor in RB through direct targeting of PAX6 and indirect regulation of the PI3K/AKT signaling pathway. The above results contribute evidence that miR-129-5p plays a crucial role in the progression of tumor malignant, and miR-129-5p may present a potential gene-targeting approach for RB treatment.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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