

# miR106b regulates retinoblastoma Y79 cells through Runx3

GE YANG<sup>1</sup>, YANG FU<sup>2</sup>, LUXI ZHANG<sup>1</sup>, XIAOYAN LU<sup>1</sup> and QIUMING LI<sup>1</sup>

Departments of <sup>1</sup>Ophthalmology and <sup>2</sup>General Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

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**Abstract.** MicroRNAs are increasingly recognized as important regulators of cancer. The aim of the present study was to investigate the role of miR-106b in the regulation of Y79 retinoblastoma. Y79 cells were transfected with antisense oligonucleotides (ASO) against miR-106b (ASO-miR-106b) or ASO-control. After transfection, the levels of miR-106b were monitored with real-time PCR (RT-PCR). The effects of ASO-miR-106b transfection on cell viability was evaluated by Cell Counting Kit-8 (CCK-8) analysis at 24, 48 and 72 h after transfection. Subsequently, the cells were stained with Annexin V-FITC and propidium iodide (PI) and subjected to flow cytometry to assess cell apoptosis. Transwell assay was used to analyze cell migration. Changes in Runt-related transcription factor 3 (*Runx3*) mRNA and proteins levels were also evaluated. miR-106b was downregulated by ASO-miR-106b at 48 and 72 h after transfection, accompanied by a decrease in cell viability and proliferation, as well as an increase in cell apoptosis. Transwell analysis indicated that cells treated with ASO-miR-106b exhibited significantly lower cell migratory abilities. The mRNA and protein level of *Runx3* were upregulated after transfection. These results demonstrated that suppression of miR-106b inhibited Y79 cell proliferation and migration. The upregulation of *Runx3* after miR-106b suppression ascertained that *Runx3* is a tumor-suppressor in retinoblastoma and is a target of miR-106b.

## Introduction

Retinoblastoma is an aggressive eye cancer and the most common intraocular cancer of infancy and childhood (1). Previous studies have indicated that various vulnerable groups, such as earthquake survivors (2) and rural-to-urban migrants (3), were in poor health, suffer from retinoblastoma. In addition, studies on retinoblastoma have

led to the discovery of the first tumor-suppressor gene, *RBI*, the loss of which accounts for aberrant cell cycle arrest and the tumorigenesis of retinoblastoma. Despite research into the *RB* family and cell cycle control, the specific pathways that contribute to the *RB* loss in tumorigenesis remain largely unknown. In addition, mice with *RB* deletion alone were not retinoblastoma-prone (4), suggesting the existence of other mechanisms that cooperatively contributes to the initiation of retinoblastoma.

It is increasingly recognized that microRNAs (miRNAs) are important regulators of cancer. They are short non-coding RNAs that post-transcriptionally modulate the expression of cancer-related genes (5). In retinoblastoma, a number of miRNAs have been identified to synergize with loss of the *RB* family to regulate cell cycles (6-8). Besides the *RB* family, the Runt-related transcription factor 3 (*Runx3*) gene, another tumor suppressor in retinoblastoma, was also found to be regulated by miRNAs. *Runx3* is located on the chromosome 1p36 and plays an important role in mammalian development (9). The loss of *Runx3* has been considered as a prognostic marker for bladder tumor (10), gastric cancer (11) and glioblastoma (12). Previously, the link between miR-106b and *Runx3* has been demonstrated in laryngeal carcinoma (13). By binding to the 3'-untranslated region (3'-UTR) of *Runx3*, miR-106b down-regulated *Runx3* expression, which consequently abolished the proliferation and invasion of laryngeal carcinoma cells (13). In retinoblastoma, although the downregulation of *Runx3* has been documented, the mechanism involved in the regulation of *Runx3* has not been investigated.

Surgery remains the primary treatment for retinoblastoma to date (14). However, being an invasive procedure, surgery exposes patients to substantial risk of losing vision (1). Chemotherapy has also been exploited for retinoblastoma, however chemotherapy is associated with potential toxicity and it also increases the risk of secondary cancers (15,16). Therefore, strategies capable of modulating retinoblastoma-causative genes are urgently needed to improve the clinical outcome of retinoblastoma therapy and minimize harm to the eye.

In the present study, we strived to elucidate the role of miR-106b in retinoblastoma and investigated the correlation between miR-106b and *Runx3*. We revealed that inhibition of miR-106b led to decreased cell proliferation and migration of human Y79 retinoblastoma cells. Concurrently, upregulation of *Runx3* was observed. The results shed light on the target of miR-106b in retinoblastoma, and provide a novel strategy for inhibiting retinoblastoma progression.

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*Correspondence to:* Dr Qiuming Li, Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou, Henan 450052, P.R. China  
E-mail: qiumingli111@126.com

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## Materials and methods

**Cell culture and transfection.** Human retinoblastoma Y79 cells were acquired from the American Type Culture Collection (ATCC; Rockville, MD, USA), and were maintained by Beina Co. Ltd. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 2.0 g/l sodium bicarbonate,  $1 \times 10^5$  IU/l penicillin and 100 mg/l streptomycin were used for cell culture. The cells were cultured in an incubator maintained at 37°C with saturated humidity and 5% CO<sub>2</sub>. ASO-miR-106b or ASO-control of 50 nM was used for transfection with Lipofectamine® 2000 (Invitrogen) according to the manufacturer's recommendations. Briefly, cells were seeded into 6-well plates at a density of  $10^5$  cells/well and cultured overnight. In each well, 100 pmol of ASO-control or ASO-miR-106b was added along with 5  $\mu$ l Lipofectamine® 2000. ASO-miR-106b and ASO-control were prepared by Jiman Pharmaceuticals (Shanghai, China).

**Cell viability assay.** For the cell viability assay, cells of  $2.5 \times 10^4$  cells/well were first seeded into 96-well plates, with each well containing 100  $\mu$ l medium, and treated with ASO-miR-106b or ASO-control. After culturing for 24, 48 and 72 h, 10  $\mu$ l Cell Counting Kit-8 (CCK-8) solution was added into the medium, and incubated for another 4-5 h. The absorbance of each well at 538 nm was assessed to calculate the cell viability using the following equation:

$$\text{Cell viability} = \frac{OD_{\text{untreated}} - OD_{\text{treated}}}{OD_{\text{untreated}}}$$

**Transwell cell migration assay.** The Transwell apparatus was coated with Matrigel (both from BD Biosciences, San Jose, CA, USA) of 20-30  $\mu$ l and allowed to gel overnight at 37°C. The other side of the membrane was coated with fibronectin (Invitrogen). Y79 cells ( $5 \times 10^5$  cells/ml) of 2,000  $\mu$ l were added to the chamber and cultured for 24 h before removing the cells on the membrane. The membrane on the lower chamber was collected and fixed with formalin for 30 min, and stained with hematoxylin. Dehydration was carried out using the standard procedure using ethanol. The membrane, which was dehydrated with ethanol and xylene, was mounted onto cover slips, followed by cell counting in 4 fields of view. The average cell number was calculated.

**Apoptosis analysis using flow cytometry.** Cells collected at 48 and 72 h after transfection were washed with 0.01 mol/l phosphate-buffered saline (PBS), and centrifuged at 1,500 rpm for 5 min. The supernatant was then discarded. Cell pellets were re-suspended to ensure a cell density of  $1 \times 10^6$  cells/ml. A cell suspension of 500  $\mu$ l was then dispensed into a microcentrifuge tube, followed by the addition of 5  $\mu$ l Annexin V-FITC and 10  $\mu$ l propidium iodide (PI) (Lianke Biology Co., Ltd., Hangzhou, China) for staining for 10 min at room temperature. Flow cytometry was used to detect cells with apoptotic activity. Experiments were performed in triplicate for each sample.

**RT-PCR.** Total RNA of the sample was extracted using a RNA Extract kit (Promega, Madison, WI, USA). Synthesis of cDNA was performed using 1  $\mu$ g purified RNA and a kit acquired from Takara (Shiga, Japan). RT-PCR was carried out using SYBR-Green mixture (Takara). Quantification was performed using the 2<sup>- $\Delta\Delta C_t$</sup>  method. Primers used in the present study were as follows: 5'-GGATTTGGTTCGTATTGGGCG-3' (sense) and 5'-TACTTCTCATGGTTCACAC-3' (antisense) for GAPDH; 5'-TGCGGCAACACCAGTCGATGG-3' (sense) and 5'-CCA GTGCAGGGTCCGAGGT-3' (antisense) for miR-106b; 5'-TGGCAGGCAATGACGA-3' (sense) and 5'-CAGGGAACG GCTTGGT-3' (antisense) for *Runx3*. Primers were synthesized by Sangon Biotech (Shanghai, China).

**Western blot analysis.** Cells transfected for 48 and 72 h were lysed using RIPA cell lysis buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein concentration was determined using the BCA assay (Pierce, Rockford, IL, USA). SDS-PAGE was performed using 40  $\mu$ g of protein. The protein was then transferred onto the polyvinylidene difluoride (PVDF) membrane, followed by blocking with 50 g/l non-fat milk for 1.5 h. The goat anti-human *Runx3* antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted (1:500) in 1% BSA and incubated with the membrane at 4°C overnight. After washing with TBST (1% Tween-20) 3 times (6 min each time), an HRP-conjugated anti-goat antibody (1:5,000 dilution; Boster Biotechnology Co. Ltd., Wuhan, China) was applied to the membrane and was incubated for 2 h. Visualization of the protein band was performed by adding ECL substrates in the dark. The band intensities of GAPDH were used to normalize the expression of the *Runx3* protein.

**Statistical analysis.** Statistical analysis was performed using SPSS 19.0 software package (IBM, Chicago, IL, USA). All data were presented as the mean  $\pm$  SD. Differences were considered significant when  $P < 0.05$ .

## Results

**Transfection of ASO-miR-106b downregulates miR-106b expression.** To validate the efficacy of ASO-miR-106b in downregulating miR-106b expression, RT-PCR was performed in Y79 cells transfected with ASO-miR-106b or ASO-control. As shown in Fig. 1, at 24 h after transfection, no clear difference in miR-106b levels was seen among all groups. After 48 and 72 h, a significant downregulation of miR-106b was observed in cells transfected with miR-106b ( $P < 0.01$ ), suggesting that ASO-miR-106 effectively inhibited the expression of miR-106. Conversely, no significant differences were observed in cells transfected with the ASO-control ( $P > 0.05$ ).

**Downregulation of miR-106b decreases cell viability.** Concomitant with the inhibition of miR-106b expression, the viability of Y79 was also decreased. As shown in Fig. 2, while no significant viability suppression was seen at 24 h after transfection, at 48 and 72 h after transfection, cell viability was significantly decreased, as revealed by CCK-8 assay ( $P < 0.05$ ).

**Downregulation of miR-106b decreases cell migration.** We next examined whether downregulation of miR-106b decreased

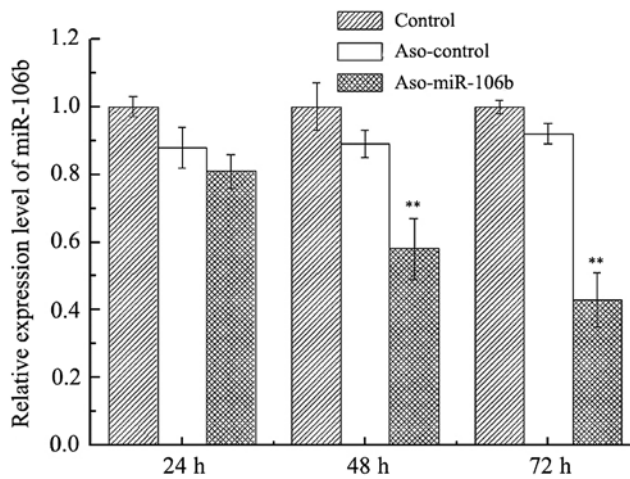


Figure 1. Relative expression level of miR-106b in Y79 cells transfected with ASO-control or ASO-miR-106b at 24, 48 and 72 h. The levels of miR-106b in Y79 cells without transfection were used for normalization. Compared with control group; \*\*P<0.01.

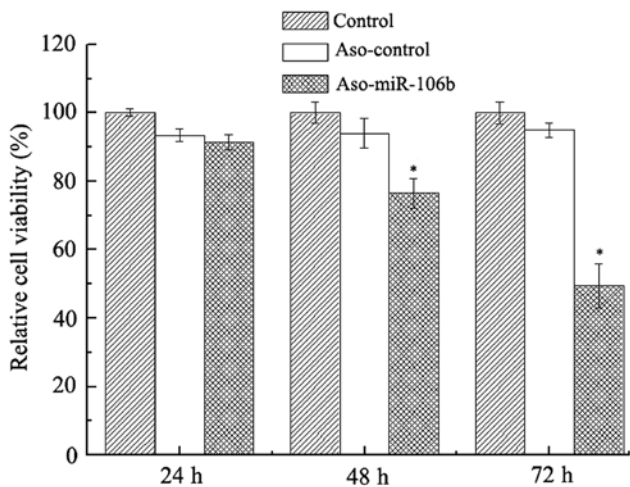


Figure 2. Cell viability analysis in cells transfected with ASO-control or ASO-miR106 at 24h, 48h, and 72h after transfection, as revealed by CCK-8 assay. Compared with control group; \*P<0.05.

the migration of Y79 cells. As expected, at 48 and 72 h after transfection, the migration of Y79 cells was significantly decreased as indicated by Transwell assay (P<0.05) (Fig. 3). In contrast, transfection with ASO-control did not suppress cell migration.

**Apoptosis is increased with miR-106 downregulation.** After transfection for 48 and 72 h, the apoptosis in Y79 cells was evaluated with Annexin-PI staining and flow cytometry. Consistent with the decreased cell viability and migration in Y79 cells after ASO-miR-106b transfection, a shift of cell population toward the Annex<sup>+</sup>-PI<sup>+</sup> population was recorded (Figs. 4 and 5), indicating an increase in apoptotic activity.

**Downregulation of miR-106b increases Runx3 expression.** To explore the target of miR-106b in retinoblastoma, we analyzed the expression level of *Runx3* mRNA and protein in Y79 cells after ASO-miR-106b transfection. The results revealed that both *Runx3* mRNA and protein expression

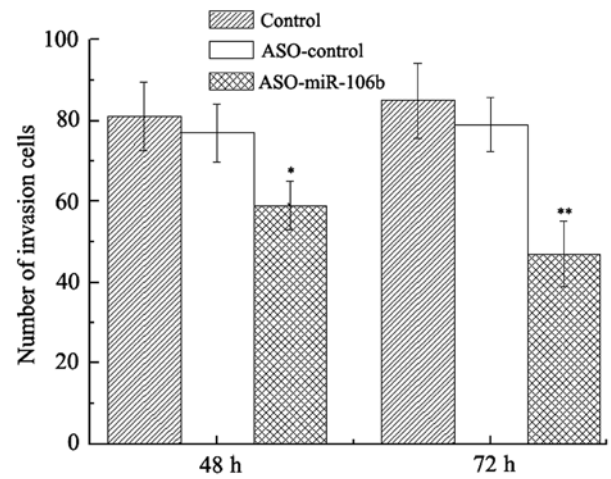


Figure 3. The number of invaded cells as determined by Transwell assay in Y79 cells transfected with ASO-control or ASO-miR-106b. Cells that received no transfection were used as a control. Compared with control group; \*P<0.05; \*\*P<0.01.

levels were upregulated after ASO-miR-106b transfection (P<0.05) (Figs. 6 and 7). Given the role of *Runx3* as a tumor suppressor, this *Runx3* upregulation after miR-106b inhibition is consistent with the diminished cell proliferation and migration. Therefore, *Runx3* acts as a target of miR-106b in the regulation of retinoblastoma.

## Discussion

A vast majority of people suffer from cancer (17,18). By fine-tuning the expression of multiple genes post-transcriptionally, miRNAs offer a new paradigm for re-modulating the gene network wired to the thriving cancer (19). Numerous miRNA-based strategies for correcting aberrant gene expression in cancer have emerged. These miRNAs may assume tumor-promoting or tumor-inhibiting roles in cancer. For example, given that the loss of *RBI* expression is a hallmark of cancer, miR-106a inhibition was employed to reverse the downregulation of *RBI* (20). In another study, miR-192 was ectopically overexpressed, which inhibited cell proliferation and induced cell apoptosis in lung cancer cells (21). For retinoblastoma, recent miRNA microarray analysis yielded a panel of miRNAs as essential effectors that modulate cancer progression, metastasis and resistance (22). Nevertheless, these miRNAs have yet to be utilized as therapeutic targets in retinoblastoma. Furthermore, despite extensive studies on the etiology of the disease and genetic diagnosis approaches (23), gene therapies for retinoblastoma are rarely studied. Therefore, in the present study, we set forth to explore the therapeutic value of miR-106b in retinoblastoma. The present study is preceded by much effort in correlating miR-106b overexpression to the malignant phenotype and resistance of cancer (24,25). We demonstrated that the inhibition of miR-106b induced a decrease in Y79 retinoblastoma cell viability and migration, and induced cell apoptosis. Instead of directly targeting oncogenes of retinoblastoma, our approach was to inhibit the expression of miR-106b with antisense oligonucleotides (ASO) to exert the antitumor effects. Our data potentiated the development of an *in vivo* gene therapy strategy that targets

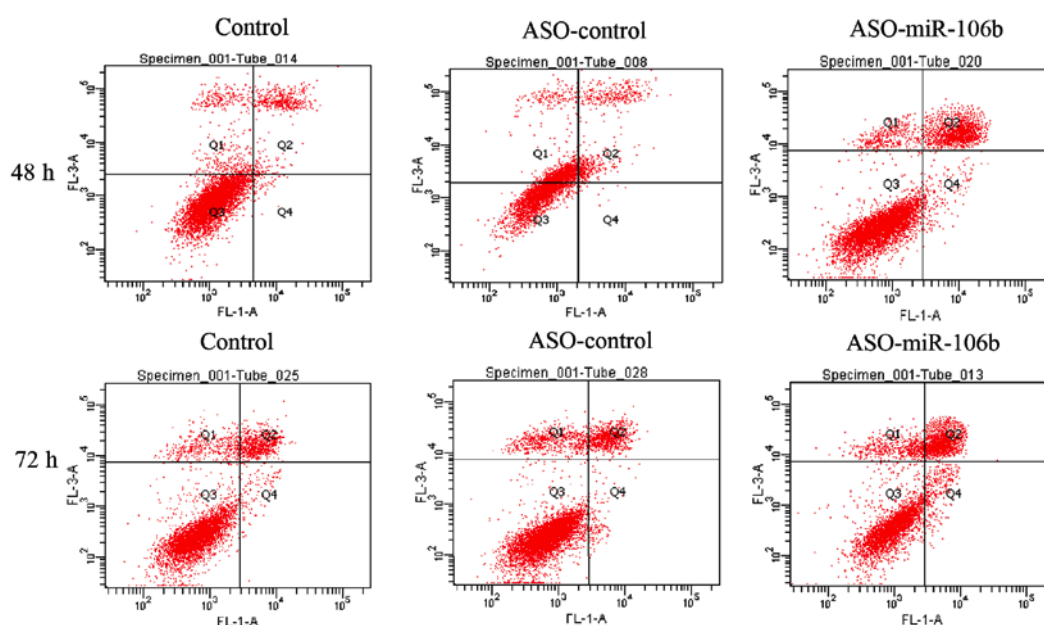


Figure 4. Apoptosis analysis with Annexin V/PI staining and flow cytometry in Y79 cells transfected with ASO-miR-106b or ASO-control. Y79 cells that received no transfection were used as a control.

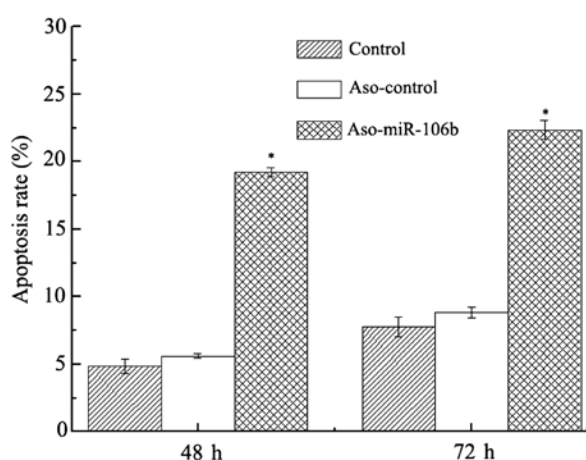


Figure 5. Apoptotic rates in Y79 cells. Y79 cells transfected with ASO-control, or Y79 cells transfected with ASO-miR-106b, as indicated by flow-cytometry as shown in Figure 4. Compared with the control group; \* $P < 0.05$ .

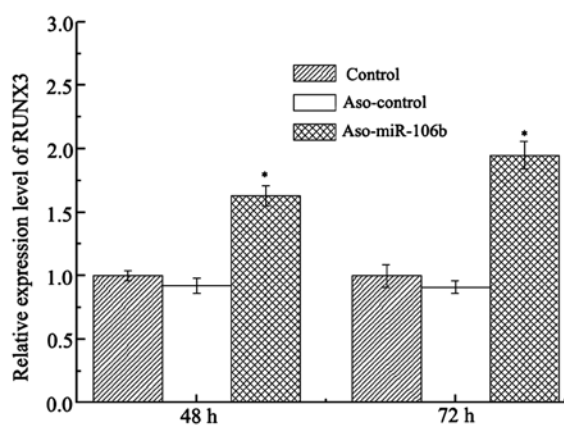


Figure 6. Runx3 mRNA levels in Y79 cells without transfection, or cells transfected with ASO-control or ASO-miR-106b. Compared with control group; \* $P < 0.05$ .

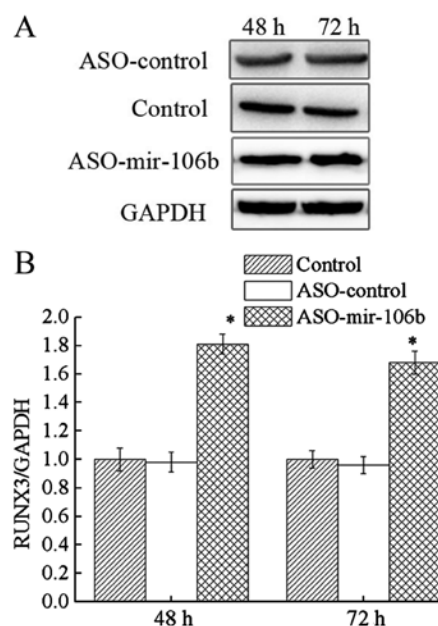


Figure 7. (A) Western blot analysis of Runx3 levels in Y79 cells that received no transfection (ASO-control transfection), or ASO-miR-106b transfection. GAPDH was used as a loading control. (B) Quantification of Runx3 protein levels in Y79 cells transfected with ASO-control or ASO-miR-106b. Compared with control group; \* $P < 0.05$ .

miR-106b; a strategy that lowers the non-specific toxicity commonly observed with chemotherapy drugs. With the advances in RNA-delivery systems, we could envision that retinoblastoma therapy based on miR-106b inhibition may improve the clinical outcome of patients and exempt them from painful and risky surgery.

Furthermore, we revealed that the antitumor effect of anti-miR-106b therapy was mediated by Runx3. Similar to *RBI*, Runx3 serves as a tumor suppressor and its methylation is pivotal to the transforming growth factor- $\beta$  (TGF- $\beta$ ) pathway.

Since its link to epithelial-to-mesenchymal transition and cancer metastasis, the loss of *Runx3* significantly affects the clinical outcome of cancer patients (26). In spite of efforts in unraveling the role of *Runx3* in cancer progression, very few *Runx3*-based therapeutic strategies have been devised. Previously, ectopic expression of *Runx3* was exploited as an antitumor strategy, whereby the restoration of *Runx3* was shown to drastically suppress tumor growth (26). The findings here revealed that inhibition of miR-106b is another viable avenue to upregulate *Runx3*. This mirrors recent evidence that revealed that miR-106b significantly promotes TGF- $\beta$  signaling and cancer metastasis (25,27). However, the fact that miR-106b is also an important player in the PTEN/PIK3/AKT pathway (27), WNT pathway (28) and RB family enhances its clinical value as a target in cancer.

In summary, we demonstrated that anti-miR-106b therapy effectively inhibited Y79 retinoblastoma cell viability, proliferation and induced cell apoptosis *in vitro*. *Runx3* was found to be a target of miR-106b, and the inhibition of miR-106b upregulated *Runx3*. Our data is significant for the development of novel strategies in gene therapy for retinoblastoma. Further *in vivo* studies are warranted to corroborate the role of miR-106b in retinoblastoma, and potentially apply this strategy to the clinic.

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