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Original Article

miR-145 regulates the proliferation and apoptosis of Y79 human retinoblastoma cells by targeting IGF-1R

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Abstract: Purpose: To investigate the effect of miR-145 on the proliferation and apoptosis of human retinoblastoma Y79 cells and to explore the underlying mechanism. Method: The Y79 cells were transfected by miR-145 mimics and IGF-1R siRNA with lipofection, respectively. The expression of miR-145 or IGF-1R was detected after transfection by real time-PCR. Cell proliferation inhibition was measured by Cell Counting Kit-8 (CCK-8) assay. Flow cytometry was used to examine cell cycles. Apoptosis was detected by Annexin/PI double immunofluorescence and flow cytometer. The interaction between miR-145 and IGF-1R was tested by luciferase activity measurement. Results: The expression of miR-145 in the miR-145 mimics group was significantly increased (P<0.05). The proliferation inhibition rate was higher in the miR-145 mimics group (P<0.01). The results of immunofluorescence and flow cytometry showed that ratios of Annexin or Annexin/PI double positive were increased in the miR-145 mimics group (P<0.05). The OD value of proliferation inhibition was lower in the IGF-1R siRNA group (P<0.05). The ratios of Annexin or Annexin/PI double positive were higher in the IGF-1R siRNA group (P<0.05). Luciferase activity was reduced in miR-145 mimics group (P<0.01). Conclusion: miR-145 inhibited proliferation and induced apoptosis in Y79 cells. Lower expression of IGF-1R suppressed the proliferation of Y79 cells. miR-145 restrained the proliferation of human retinoblastoma Y79 cells by down-regulating the expression of IGF-1R.

Keywords: miR-145, retinoblastoma, proliferation, apoptosis, IGF-1R, Y79 cell

Introduction

Retinoblastoma (RB), whose incidence is 1 in 17,000 in the world, is the most common intraocular malignancy in children [1], and is a rare life-threatening ophthalmic cancer [2]. There are approximately 1000 new cases occurring each year in our country, accounting for 20% of the world. Children with retinoblastoma are diagnosed before 3 years of age [3]. The diagnosis of retinoblastoma in older children is extremely rare. Children with RB are at risk for problems such as metastasis of RB and second primary tumors. Therefore, it is important to clarify the underlying mechanism and to search for an effective intervention target for RB.

MicroRNAs (miRNAs) are an abundant class of small, roughly 20-24 nucleotide (nt)-long, noncoding RNA molecules, and widely expressed in tissues and cells [4]. They control gene expression by binding to 3'-untranslated regions (UTR) of target messenger RNA inducing degradation or repressing translation, and regulate gene expression at the post-transcriptional level [5, 6]. miRNAs play the role in the process of differentiation, development, and apoptosis of normal cells. Emerging studies have showed that miRNAs are related to the pathogenesis of various human diseases such as metabolic disease [7, 8], cardiovascular disease [9, 10], and also particularly involved in the initiation and progression of cancer [11-13]. Matsushita reported that miR-145 inhibited bladder cancer cell aggressiveness [14], Karatas also showed that miR-145 carried crucial roles in laryngeal squamous cell carcinoma [15]. However, its role in the pathogenesis of RB is unknown.

The role of the insulin-like growth factor (IGF) system in cancer growth and metastasis has gotten more attention recently [16]. The receptor for the type 1 IGF (IGF-1R) is a regulator of cellular transformation and the acquisition of a tumorigenic phenotype [17]. In many human cancers, such as prostatic cancer [18],

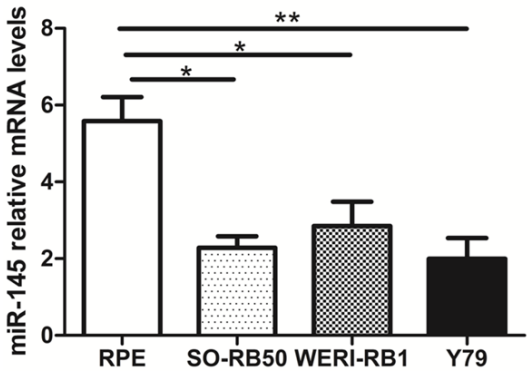

Figure 1. The mRNA expression of miR-145 in RB cell lines and RPE. Equivalent amounts of mRNA were analyzed by real time-PCR in three RB cell lines (WERI-RB1, SO-RB50, Y79) and in retinal pigment epithelium cells (RPE) as controls. The relative gene expression was normalized to U6. Data are presented as mean ± SEM and are representative of three independent experiments. *P<0.05, **P<0.01.

non-small cell lung cancer [19], and colorectal cancer [20], there is a strong association with dysregulated IGF signaling. Therefore, our study explored IGF-1R targeting for intervention in RB.

In the present study, we used liposome-mediated gene transfer technology, examined the effect of miRNA on retinoblastoma cell proliferation and apoptosis, explored the possibility of a new target treatment of IGF-1R on retinoblastoma. Furthermore, we studied the regulatory mechanism of miR-145 and IGF-1R on the human retinoblastoma cells.

Materials and methods

Cell culture

The human RB cell lines Y79, SO-RB50 and WERI-RB1 and retinal pigment epithelium (RPE) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco) containing 20% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The culture medium was replaced every 3 days.

Transfection of miR-145 mimic and IGF-1R siRNA

Human RB cell lines (Y79) were transfected with miR-145 mimic (miR09122105714), miR-NC (miR01101), IGF-1R siRNA (NM000875) and si-NC (RiboBio, Guangzhou, China) with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for western blotting or real time-PCR analysis.

Real-time PCR analysis

Total RNA was isolated from cell lines using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The concentration and quality of total RNA were assessed and then reverse-transcribed using RT Primer Mix and oligo dT primers (Takara, Dalian, China). Real-time PCR was carried out in the ABI 7500 system (Applied Biosystems, Foster City, CA, USA) using the specific primers. The PCR primer sequences were as follows: IGF-1R, sense: 5'-CTAAACCCGGGGAACTACACAG-3', antisense: 5'-TTCACAGAGGCATACA GC AC-3', 18S, sense: 5'-GGACACG GACAGGA TTGACA-3', antisense: 5'-ACCCACGGA ATCGAGAAAGA-3'. Primers of miR-145 mimic (cat. 4373133), miR-NC (cat. 4366596) and U6 (cat. 4373381) were purchased from RiboBio Co. Ltd., Guangzhou, China. PCR amplification was performed in a volume of 20 μl, using all-in-one quantitative PCR (qPCR) Mix (ABI, Carlsbad, CA, USA). Relative expression levels of miR-145 and IGF-1R were calculated by the $2^{-\Delta\Delta CT}$ method after normalization with reference to expression of U6 and 18S. Measurements were masked to group assignment.

Western blotting

Western blotting was performed as described previously [21]. Cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime, Shanghai, China) including 1% protease inhibitor (Roche). Fifty micrograms of protein extract were separated by 10% SDS-PAGE electrophoresis; the resolved proteins were electroblotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA) and incubated with specific antibodies against IGF-1R. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). The IGF-1R (ab39675) and β-actin (ab8227) antibody was purchased from Abcam.

Cell proliferation assay

For proliferation assay, 2 × 10^3 RB cells in 100 μl per well were cultured and transfected with

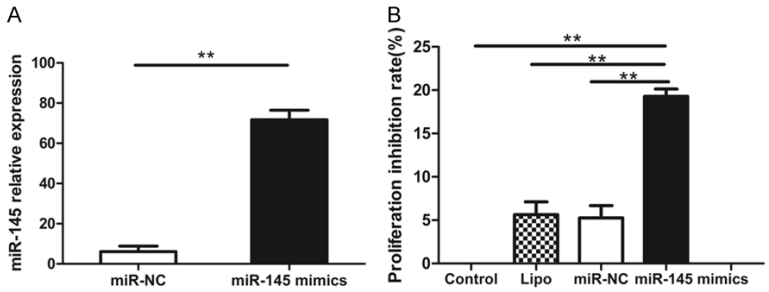

Figure 2. The expression of miR-145 in miR-145 mimics transfected Y79 cells and the effect of miR-145 on the proliferation of Y79 cells. A. The mRNA expression of miR-145 was detected in the miR-145 mimics or miR-NC group by real time-PCR. B. miR-145 inhibited Y79 cell proliferation. Data are presented as mean ± SEM and are representative of three independent experiments. ***P*<0.01.

miR-145 mimics or miR-NC. At 48 h after transfection, cell viability was detected by a modified MTT assay using a cell-counting kit (Cell Counting Kit-8; Sigma-Aldrich) as described previously [22]. After 60 minutes incubation under routine conditions, wavelength of 450 nm absorbance value (A) of each hole was detected by enzyme standard instrument (Bio-Tek, USA). The cell proliferation inhibition rate (%) = 1-(the average A value of experimental group/the average A value of control group) × 100%.

Cell apoptosis detection

Annexin V-FITC/PI Apoptosis Detection Kit (Invitrogen) was used to determine the percentage of apoptotic cells. Cell suspension at 48 hours after transfection, 100 μl cell solutions (3×10^5 cells/ml) were incubated with 5 μl Annexin V-FITC labeling solution and 5 μl propidium iodide (PI) as manufacturer's instructions. Following 10 min of incubation in dark, cells were resuspended in 400 μl incubation buffer. Annexin V-FITC/PI binding was analyzed by flow cytometry using FACS Canto II (BD Biosciences) and appropriate filters for FITC and PI fluorescence detection (Olympus IX 51 microscope; Olympus Optical Co., Hamburg, Germany). Data were analyzed using FACS Diva software.

Luciferase reporter assay

Y79 cells were cotransfected with 20 μM of either miR-145 mimics or the miR-NC and 500 ng of pMIR-REPORT-B7-H1 3'-UTR. Cells were collected 24 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System. Luciferase activity was detected using a fluorescence microplate reader (Bio-Tek FL × 800, USA). Renilla luciferase was cotransfected as an internal control. The ratio of firefly luciferase and renilla luciferase as the interaction between miR-145 and IGF-1R 3'-UTR.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Band intensity and relative mRNA expression experiments were analyzed with the unpaired Student's t-test. One-way analysis of variance (ANOVA) followed by a Bonferroni correction were applied for multiple comparisons. A *P*-value less than 0.05 was considered significant.

Results

miR-145 expression was decreased in human RB cell lines

First, we examined the relative expression levels of miR-145 in three RB cell lines (Y79, WERI-RB-1, and SO-RB50) by real time-PCR. As compared with the RPE cell, the mRNA expression level of miR-145 was significantly decreased in the three RB cell lines (**Figure 1**, *P*<0.05).

miR-145 inhibited the proliferation of Y79 cells

The Y79 cells were transfected by miR-145 successfully with lipofection. Real time-PCR analysis showed that the expression of miR-145 in the miR-145 mimics group was significantly increased as compared to the miR-NC group (79.06±3.45 *vs* 1.06±0.03, *P*<0.01) (**Figure 2A**). The proliferation inhibition rate was higher in the miR-145 mimics group (*P*<0.01) (**Figure 2B**).

miR-145 induced the apoptosis of Y79 cells

Representative results of immunofluorescence showed the Annexin or Annexin/PI double posi-

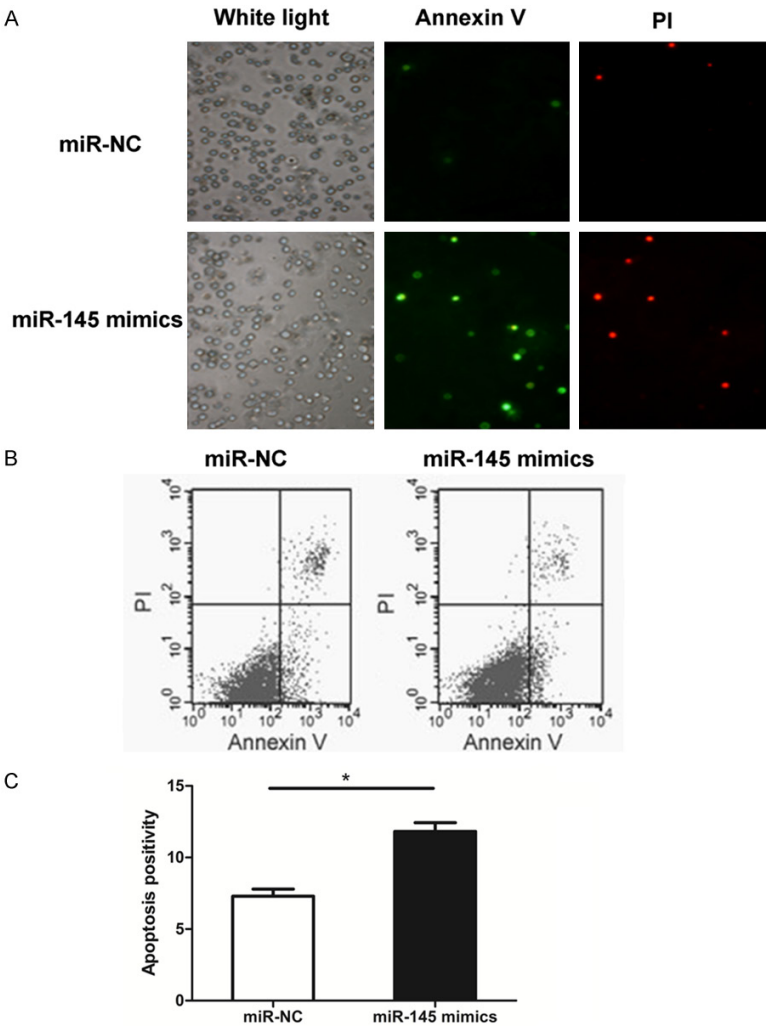

Figure 3. The effect of miR-145 on the apoptosis of Y79 cells. Representative immunofluorescence (A) and flow cytometry (B) pictures of Annexin or Annexin/PI double positive cells in the miR-145 mimics and the miR-NC groups. (C) Statistical results of Annexin or Annexin/PI double positive cells in the miR-145 mimics and the miR-NC groups. Data are presented as mean ± SEM and are representative of three independent experiments. *P<0.05.

tive cells in **Figure 3A**. Representative flow cytometer results are shown in **Figure 3B**. The statistical results showed that apoptosis positivity was obviously higher in the miR-145 mimics group than the miR-NC groups (P<0.05) (**Figure 3C**).

Depressed expression of IGF-1R inhibited the proliferation of Y79 cells

IGF-1R siRNA specially deters the expression of IGF-1R in IGF-1R siRNA transfected Y79 cells. Real time-PCR analysis showed that the expression of IGF-1R mRNA in three RB cell lines (Y79, WERI-RB-1, and SO-RB50) was increased (P< 0.05) (**Figure 4A**). Western blotting demonstrated the level of IGF-1R protein of siRNA group was 22.4% of the siR-NC group (**Figure 4B**). The result of the proliferation inhibition test demonstrated that the A value (OD) in the siRNA group (0.30) was lower than siR-NC group (0.40); the difference was significant (P< 0.05) (**Figure 4C**). The results of immunofluorescence and flow cytometer showed that ratios of Annexin were higher in the siRNA group (28.9%) than the siR-NC group (6.4%) (P<0.05) (**Figure 4D**).

miR-145 mimics inhibited the expression of IGF-1R in the Y79 cells

Western blotting demonstrated a reduced level of IGF-1R protein of the miR-145 mimics group (P<0.05) (**Figure 5**), which was 37.5% of the miR-NC group.

The interaction between miR-145 and IGF-1R in the proliferation of Y79 cells

In order to confirm the molecular mechanism by which miR-145 induces, we compared the sequences of 3'-UTR of IGF-1R with miR-145. The 3'-UTR of IGF-1R mRNA contained a complementary site for the seed region of miR-145 (**Figure 6A**).

To determine whether IGF-1R is a direct target gene for miR-145, a dual-luciferase reporter system was used. The luciferase reporter assay indicated that the luciferase activity of the reporter containing the IGF-1R gene's wild-type 3'-UTR was decreased in the miR-145 mimics group (**Figure 6B**). The result indicates that miR-145 most likely suppresses gene expression through miR-145 binding sequences at the 3'-UTR of IGF-1R.

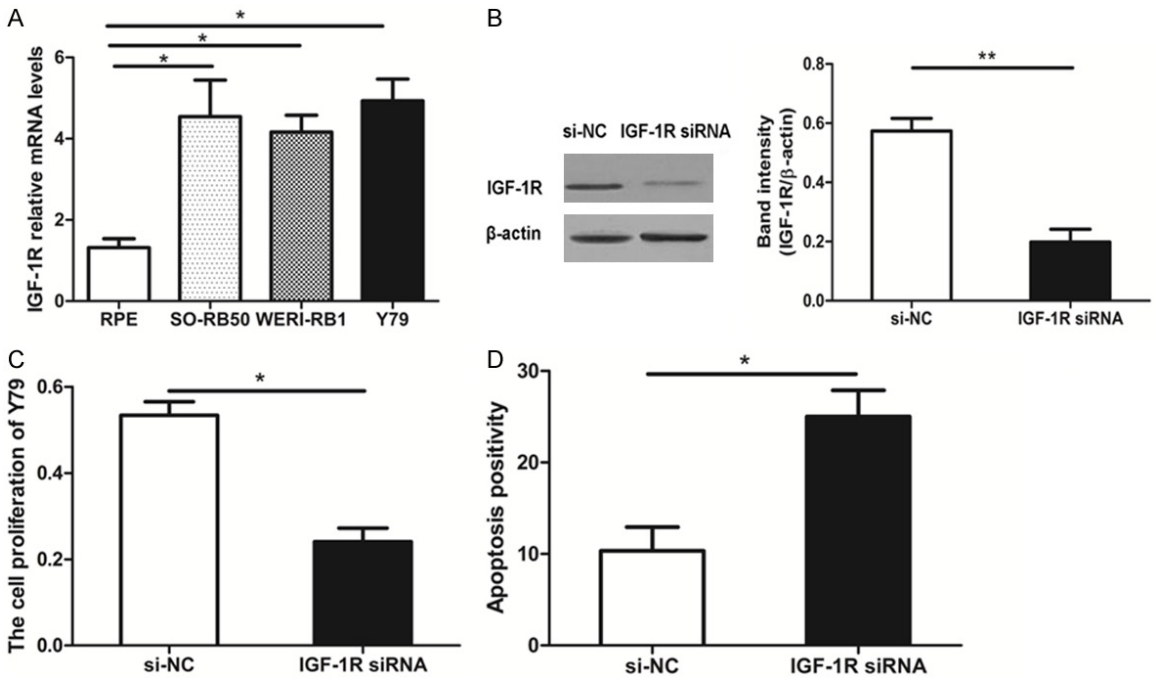

Figure 4. The expression of IGF-1R in RB cells and the effect of IGF-1R on the apoptosis of Y79 cells. A. The expression of IGF-1R was increased in RB cells. B. The expression of IGF-1R was depressed in the IGF-1R siRNA transfected Y79 cells. C. The A value (OD) in the siRNA group was lower than in the siR-NC group. D. Apoptosis positivity was higher in the siRNA group than the siR-NC group. Relative expression was normalized to 18S. Data are presented mean ± SEM and are representative of three independent experiments. **P*<0.05, ***P*<0.01.

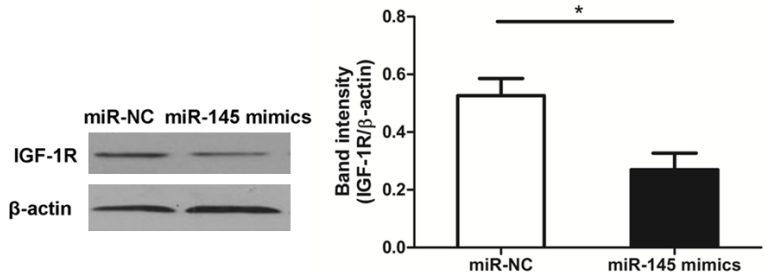

Figure 5. The effect of miR-145 mimics on the expression of IGF-1R in the Y79 cells. Western blotting analysis showed miR-145 mimics inhibited the expression of IGF-1R in the Y79 cells. Data are presented as mean ± SEM and are representative of three independent experiments. **P*<0.05.

Conclusion

Accumulating evidence shows that miR-145 is a tumor suppressor gene involved in cell suppression, invasion, and migration of cancer cells. It acted as a tumor suppressor in colorectal carcinoma cell lines, was a marker of undifferentiated progenitors in the colonic crypts, and was a key regulator of intestinal cell differentiation [23, 24]. miR-145 also suppressed tumor formation *in vivo* in nude mice and played an important role in tumorigenesis of prostate cancer [25]. Our study further expanded the tumor suppressive role of miR-145 in RB. Results showed that miR-145 was significantly decreased in RB cell lines; and overexpression of miR-145 could inhibit RB cell proliferation, and induce RB cell apoptosis.

IGF-1R, a heterotetrameric tyrosine kinase receptor, consists of two α-chains and two β-chains [26]. IGF-1R binds with IGF-1 and IGF-2, then it is phosphorylated and activates the downstream signaling pathway, promoting cell proliferation and inhibiting cell apoptosis [27]. There are clear data showing that IGF-1R signaling is involved in the oncogenesis and cancer progression [28, 29]. Both monoclonal antibodies to IGF1R and newer IGF-1R pathway targeting strategies (monoclonal antibodies to IGF1 and IGF2 and a small-molecule tyrosine kinase inhibitor of IGF1R) have been studied in human non-small cell lung cancer models [30-32]. In our study, we demonstrated that IGF-1R expres-

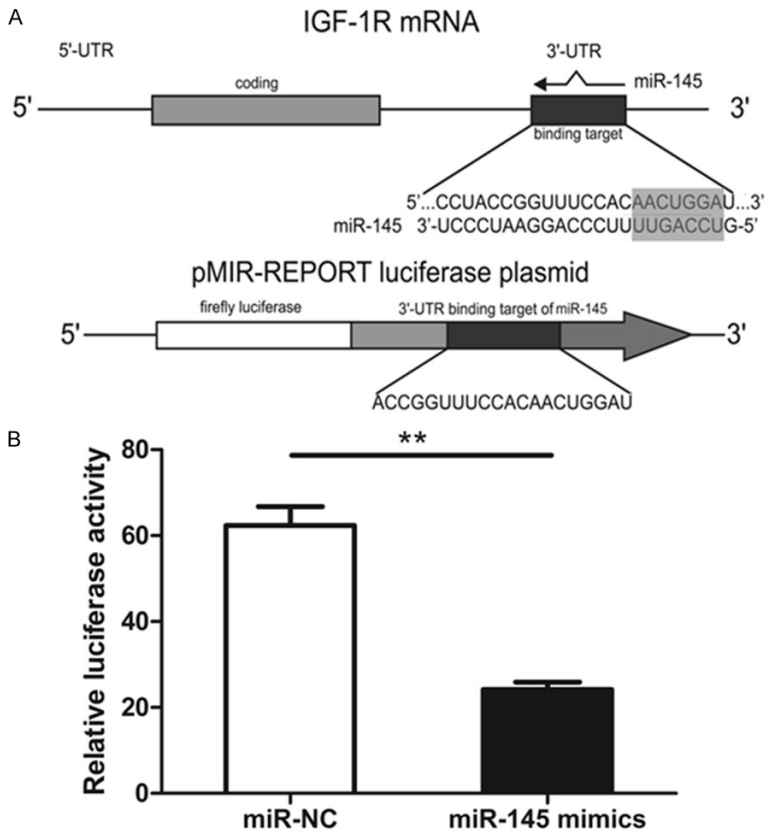

Figure 6. IGF-1R is a direct target of miR-145 in Y79 cells. A. Sequence alignment between miR-145 and the 3'-untranslated region (UTR) of IGF-1R mRNA seed-matching region or seed-mutated region. B. Relative luciferase activity was calculated by fluorescence microplate reader. Data are presented as mean ± SEM and are representative of three independent experiments. **P*<0.01.

sion was increased in RB, and depressed expression of IGF-1R inhibited the proliferation of Y79 cells. Last and most importantly, we showed over-expression of miR-145 depressed the level of IGF-1R in RB cells, and found oncogenic factor IGF-1R could be regulated by miR-145 in RB.

In summary, our study provided experimental evidence that miR-145 suppressed cell proliferation in RB by targeting IGF-1R. Therefore, these findings suggest that miR-145 may be a target for RB treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

RB, Retinoblastoma; miRNAs, MicroRNAs; IGF, Insulin-like growth factor; IGF-1R, The receptor for the type 1 insulin-like growth factor; RPE, Retinal pigment epithelium; UTR, Untranslated regions.

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