MicroRNA-665 inhibits the oncogenicity of retinoblastoma by directly targeting high-mobility group box 1 and inactivating the Wnt/β-catenin pathway

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Purpose: Previous studies have revealed that microRNA-665 (miR-665) is dysregulated in a variety of human cancers. However, little is known regarding its expression profiles and functions in retinoblastoma (RB). Therefore, the aims of our study were to evaluate miR-665 expression in RB and determine the precise roles of miR-665 in the progression of RB.

Patients and methods: Herein, RT-qPCR was used to determine miR-665 expression levels in RB tissues and cell lines, and a series of functional experiments were performed to explore the influence of miR-665 on RB cell proliferation, colony formation, apoptosis, migration, and invasion as well as tumor growth. The molecular mechanisms underlying the tumor-suppressive action of miR-665 in RB were also explored.

Results: We found that miR-665 was markedly reduced in RB tissues and cell lines and that lower miR-665 expression was strongly associated with tumor size, TNM stage, and differentiation in patients with RB. Exogenous expression of miR-665 suppressed cell proliferation, colony formation, migration, and invasion, and induced cell apoptosis in RB cells, while silencing miR-665 expression had the opposite effects. In addition, upregulation of miR-665 decreased the tumor growth of RB cells in vivo. High-mobility group box 1 (HMGB1) was identified as a direct target of miR-665 in RB cells, and decreasing the expression of HMGB1 simulated the regulatory effects of miR-665 overexpression in RB cells, while knockdown of HMGB1 expression counteracted the miR-665-mediated antitumor effects in RB cells. Moreover, miR-665 was shown to regulate the Wnt/β-catenin signaling pathway by targeting HMGB1 in vitro and in vivo.

Conclusion: Taken together, our in vitro and in vivo results suggest that miR-665 acts as a tumor-suppressive miRNA in RB by directly targeting HMGB1 and inactivating the Wnt/β-catenin pathway. Hence, this miRNA is a candidate prognostic biomarker and therapeutic target in patients with RB.

Keywords: microRNA-665, retinoblastoma, high-mobility group box 1, Wnt/β-catenin pathway, oncogenicity

Introduction

Retinoblastoma (RB) is an aggressive intraocular malignancy that arises from the primitive retinal layer. It accounts for 2–4% of all malignant tumors in children under 5 years of age. Approximately 9,000 cases of RB are diagnosed every year worldwide, and approximately 20% of these occur in China. In developing
countries, most patients are diagnosed with RB at an advanced stage; therefore, their clinical outcomes are poorer than those of patients in developed countries.\textsuperscript{5,6} The primary treatment options for patients with RB are enucleation, laser photocoagulation, chemotherapy, and focal therapy.\textsuperscript{7} Although tremendous progress has been made in the diagnosis and treatment of RB over the past decade,\textsuperscript{8} the prognosis is still unsatisfactory due to its rapid progression. The allelic inactivation of the RB1 gene along with other oncogenes or tumor suppressors has been shown to play a crucial role in the development and progression of RB;\textsuperscript{9,10} however, the exact molecular mechanisms are poorly understood. Therefore, delineating the molecular events involved in the pathogenesis of RB is of great and urgent significance for the identification of effective molecular therapeutic strategies for patients with RB.

In recent years, our knowledge of the importance of microRNAs (miRNAs) in cancer has greatly increased.\textsuperscript{11} miRNAs are a group of short (19–23 nt), noncoding RNA molecules that function as novel gene expression regulators.\textsuperscript{12} miRNAs pair imperfectly with the 3′-untranslated region (3′-UTR) of their target genes, which leads to mRNA degradation and/or transcription silencing.\textsuperscript{13} Because miRNAs modulate approximately 60% of all human protein-coding genes, they have been implicated in various physiological functions and pathological conditions, including carcinogenesis and cancer progression.\textsuperscript{14–16} Numerous studies have shown that the expression profiles of various miRNAs are altered in RB.\textsuperscript{17–19} For example, miR-101-3p,\textsuperscript{20} miR-506-3p,\textsuperscript{21} and miR-874\textsuperscript{22} are expressed at low levels in RB and function as tumor-suppressive miRNAs. In contrast, miR-10b,\textsuperscript{23} miR-198,\textsuperscript{24} and miR-498\textsuperscript{25} are upregulated in RB and have a tumor-promoting functions. Therapeutic techniques targeting the miRNAs that contribute to the initiation and progression of RB may have potential applications.

Abnormal miR-665 expression has been reported in multiple human cancers.\textsuperscript{26–29} However, the expression profile and biological function of miR-665 in RB and the underlying molecular mechanisms are largely unknown. Hence, in this study, we aimed to detect miR-665 expression in RB and evaluate its clinical value in patients with RB. Additionally, we investigated the function of miR-665 in RB progression and explored the molecular mechanisms underlying the tumor-suppressive action of miR-665 in RB.

### Material and methods

#### Patients and tissue samples

The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University, and the study was performed in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from the parents or guardians of all patients. A total of 34 RB tissues and 11 normal retinal tissues were collected at The First Affiliated Hospital of Zhengzhou University between March 2015 and October 2017. Normal retinal tissues were obtained from patients with ophthalmorrhexis who received enucleation. All enrolled patients were newly diagnosed and were being treated for the first time, and they had not been treated with laser photocoagulation, chemotherapy, or focal therapy before enucleation. All tissue specimens were quickly frozen in liquid nitrogen after enucleation and stored at −80 °C until use.

#### Cell lines and culture conditions

The normal retinal pigmented epithelial cell line ARPE-19 and three human RB cell lines, Y79, SO-RB50, and WERI-RB-1, were acquired from the American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO\textsubscript{2} and grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

#### Cell transfection

Human synthetic miR-665 mimics, a miR-665 inhibitor, negative control miRNA mimics (miR-NC), and a negative control miRNA inhibitor (NC inhibitor) were purchased from GenePharma (Shanghai, China). To knockdown HMGB1, an HMGB1-targeting small interfering RNA (siRNA; si-HMGB1) and a corresponding negative control (si-NC) were purchased from GeneCopoeia (Guangzhou, China). To increase HMGB1 expression, the full-length human HMGB1 cDNA was chemically synthesized by GenePharma and then inserted into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) to generate the plasmid pcDNA3.1-HMGB1 (pc-HMGB1). Cells were plated in 6-well plates 12 h prior to transfection, and Lipofectamine 2000 (Invitrogen) was utilized for cell transfection. Transfected cells were collected at different time points and then used for subsequent experiments.
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TRIzol (Invitrogen) was used to extract total RNA from prepared tissue specimens and cells. Then, the extracted total RNA was reverse transcribed using the miScript Reverse Transcription kit (Qiagen, Hilden, Germany). The obtained cDNA and the miScript SYBR Green PCR kit (Qiagen) were used to quantify miR-665 expression by using quantitative PCR (qPCR). To determine HMGB1 mRNA expression, the reverse transcription reaction was performed using the PrimeScript™ RT reagent Kit (Takara, for 24 h, and cells/mL. Then, the cells were stained with 5 µL of Annexin V-FITC and 5 µL of propidium iodide solution. After 15 min of incubation in the dark at 4 °C, the cells were evaluated with a flow cytometer (FACScan; BD Biosciences, San Jose, CA, USA). The data were analyzed with CellQuest software (BD Biosciences).

In vitro migration and invasion assays

The migration and invasion of RB cells were assessed using transwell inserts (8 µm pores; BD Biosciences). In the invasion assays, the upper side of the insert was precoated with Matrigel (BD Biosciences), while a non-Matrigel-coated insert was used for the migration assays. At 48 h post-transfection, cell suspensions were prepared using FBS-free DMEM. Then, 300 µL of a cell suspension containing 5×10⁴ transfected cells was placed into the upper compartment of the insert, and 700 µL of DMEM supplemented with 20% FBS was added to the lower compartment. The Transwells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h, and then the cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Finally, the number of migrated and invaded cells was counted under an inverted light microscope (magnification, 200×).

Tumor xenograft mouse model

All animal care and experimental protocols were approved by the Ethical Committee of The First Affiliated Hospital of Zhengzhou University, and the experiments were carried out in accordance with the Animal Protection Law of the People’s Republic of China-2009 for experimental animals. Seven-week-old BALB/c nude mice were obtained from the Chinese Academy of Sciences (Shanghai, China) and subcutaneously injected with miR-665 mimic- or miR-NC-transfected cells. Each group contained four mice. The tumors were monitored every 2 days, and tumor volume was calculated using the following formula: tumor volume = 0.5 × long diameter × short diameter². Four weeks after injection, the mice were sacrificed, and the weight of the tumor xenografts was measured.

Bioinformatics prediction

The putative targets of miR-665 were predicted using three miRNA target prediction programs, TargetScan (http://www.targetscan.org/vert_71/), miRDB (http://mirdb.org/), and microRNA (http://www.microrna.org/microrna/home.do).
Luciferase reporter assay

The wild-type (wt) and mutant (mut) 3′-UTR of HMGB1, containing putative and mutated miR-665 binding sites, respectively, were chemically synthesized by GenePharma and inserted into the pmiGLO luciferase reporter vector (Promega, Madison, WI, USA). The luciferase plasmids were then co-transfected with miR-665 mimics or miR-665 inhibitor into cells and plated in 24-well plates. At 48 h after transfection, the cells were harvested and lysed, and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Relative firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis

Total protein was extracted with the active protein extraction kit (KGP1050; Nanjing KeyGen Biotech, Nanjing, China), and protein levels were quantified with the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels, separated by electrophoresis, and transferred to polyvinylidene fluoride membranes (Beyotime Biotechnology). After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated overnight at 4 °C with primary antibodies against HMGB1 (ab77302; Abcam, Cambridge, MA, USA), β-catenin (sc-57534; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-β-catenin (sc-59737; Santa Cruz Biotechnology), cyclin D1 (ab40754; Abcam), and GAPDH (ab128915; Abcam), which were diluted to 1:1,000. The membranes were incubated with goat anti-rabbit (ab205718; Abcam) or goat anti-mouse (ab6789; Abcam) HRP-conjugated secondary antibodies at a dilution of 1:5,000 for detection. The bands were visualized using enhanced chemiluminescence solution (Pierce; Thermo Fisher Scientific, MA, USA). Equal amounts of protein were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels, separated by electrophoresis, and transferred to polyvinylidene fluoride membranes (Beyotime Biotechnology). After blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated overnight at 4 °C with primary antibodies against HMGB1 (ab77302; Abcam, Cambridge, MA, USA), β-catenin (sc-57534; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-β-catenin (sc-59737; Santa Cruz Biotechnology), cyclin D1 (ab40754; Abcam), and GAPDH (ab128915; Abcam), which were diluted to 1:1,000. The membranes were incubated with goat anti-rabbit (ab205718; Abcam) or goat anti-mouse (ab6789; Abcam) HRP-conjugated secondary antibodies at a dilution of 1:5,000 for detection. The bands were visualized using enhanced chemiluminescence solution (Pierce; Thermo Fisher Scientific, Inc.). GAPDH was used as a loading control.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Each assay was repeated at least three times, and all data were expressed as mean ± standard error. The chi-squared test was used to evaluate the correlation between miR-665 levels and clinicopathological parameters in patients with RB. Spearman’s correlation analysis was used for the correlation analysis between miR-665 and HMGB1 mRNA levels in tissues obtained from patients with RB. Two-tailed Student’s t-test and one-way analysis of variance with Student-Newman-Keuls tests were utilized for comparisons between two groups and multiple groups, respectively. P-values less than 0.05 were considered statistically significant.

Results

miR-665 expression is decreased in RB tissues and cell lines

To determine the expression profile of miR-665 in RB, we first detected its expression in 34 RB tissues and 11 normal retinal tissues. The results of the RT-qPCR analysis showed that miR-665 expression levels were lower in RB tissues than in normal retinal tissues (Figure 1A, P<0.05). We also measured miR-665 expression in the RB cell lines Y79, SO-RB50, and WERI-RB-1 and the normal retinal pigmented epithelial cell line ARPE-19. Consistently, lower miR-665 expression was observed in all three RB cell lines when compared with the level in ARPE-19 cells (Figure 1B, P<0.05).

We next attempted to evaluate the clinical role of miR-665 in patients with RB. All enrolled patients were divided into either the low or high miR-665 expression group using the median value as the cutoff. The statistical analysis showed that low miR-665 expression was significantly related to tumor size (P=0.015), TNM stage (P=0.004), and differentiation (P=0.032, Table 1) in patients with RB. These results indicate that downregulation of miR-665 might play a critical role in the malignant development of RB.

Figure 1 Decreased miR-665 expression in RB tissues and cell lines. (A) RT-qPCR was used to determine the expression profile of miR-665 in 34 RB tissues and 11 normal retina tissues. *P<0.05 compared with normal retina tissues. (B) The miR-665 expression level was assessed in three RB cell lines (Y79, SO-RB50, and WERI-RB-1) and a normal retinal pigmented epithelial cell line (ARPE-19). #P<0.05 compared with ARPE-19.
Table 1 The association between clinicopathological parameters and miR-665 expression in 34 patients with RB. RB patients were divided into a low miR-665 or high miR-665 expression group based on median value of miR-665 expression in RB tissues

| Parameters       | miR-665 expression | P     |
|------------------|--------------------|-------|
|                  | Low    | High  |       |
| Age (years)      |        |       |       |
| <5               | 14     | 11    | 0.438 |
| ≥5               | 3      | 6     |       |
| Gender           |        |       |       |
| Male             | 10     | 8     | 0.732 |
| Female           | 7      | 9     |       |
| Tumor size (mm)  |        |       |       |
| <15              | 5      | 13    | 0.015*|
| ≥15              | 12     | 4     |       |
| TNM stage        |        |       |       |
| I-II             | 6      | 15    | 0.004*|
| III-IV           | 11     | 2     |       |
| Differentiation  |        |       |       |
| Moderate and well| 7      | 14    | 0.032*|
| Poor             | 10     | 3     |       |

Note: *P<0.05.

miR-665 attenuates the malignant phenotypes of RB cells in vitro

To explore the biological functions of miR-665 in RB, gain- and loss-of-function assays were performed to determine the effect of altering miR-665 expression on the phenotypes of RB cells. Y79 cells, which exhibited the lowest miR-665 expression among the three tested RB cell lines, were transfected with miR-665 mimics, while WERI-RB-1 cells, which showed the highest miR-665 expression level, were transfected with a miR-665 inhibitor. Transfection efficiency was validated by RT-qPCR (Figure 2A, P<0.05). CCK-8 and clonogenic assays revealed that transfection of Y79 cells with the miR-665 mimics decreased cell proliferation and colony formation, respectively, while transfection of WERI-RB-1 cells with the miR-665 inhibitor led to enhancement of cell proliferation and colony formation (Figure 2B and C, P<0.05). Since miR-665 inhibited RB cell proliferation, we next examined whether miR-665 regulated apoptosis in RB cells. As shown in Figure 2D, ectopic miR-665 expression in Y79 cells led to an obvious increase in apoptosis, while silencing miR-665 expression in WERI-RB-1 cells led to a marked reduction in apoptosis (P<0.05). We also investigated the effects of miR-665 on the migration and invasion of RB cells in vitro. The results from the in vitro migration and invasion assays showed that restoration of miR-665 expression restricted the migration and invasion of Y79 cells. In contrast, downregulation of miR-665 promoted the migration and invasion of WERI-RB-1 cells (Figure 2E and F, P<0.05). Taken together, these results indicate that miR-665 may function as a tumor-suppressing miRNA in RB.

HMGB1 is a direct target of miR-665 in RB cells

Bioinformatics analysis was performed to search for putative targets of miR-665 and determine the mechanisms responsible for the effects of miR-665 in RB cells. The 3′-UTR of HMGB1 contained a highly conserved binding site for miR-665 (Figure 3A), suggesting that HMGB1 is a direct target of miR-665. Thus, a luciferase reporter assay was conducted to verify this prediction. The luciferase activity of a plasmid harboring the wild-type HMGB1 3′-UTR was decreased by upregulation of miR-665 expression in Y79 cells, whereas inhibition of miR-665 in WERI-RB-1 cells increased luciferase activity (Figure 3B, P<0.05). However, the luciferase activity of a plasmid containing a mutant HMGB1 3′-UTR was unaffected by a change in miR-665 expression.

RT-qPCR and Western blot analysis were employed to determine the effects of miR-665 on the endogenous expression levels of HMGB1 mRNA and protein. The results showed that the mRNA and protein levels of HMGB1 were suppressed by exogenous miR-665 expression in Y79 cells and were induced by silencing miR-665 expression in WERI-RB-1 cells (Figure 3C and D, P<0.05). Furthermore, we found that HMGB1 mRNA expression was notably upregulated in RB tissues relative to the level in normal retinal tissues (Figure 3E, P<0.05). RB tissues with high miR-665 expression showed lower HMGB1 mRNA levels than RB tissues with low miR-665 expression (Figure 3F, P<0.05). Moreover, Spearman’s correlation analysis indicated that the HMGB1 mRNA level was negatively correlated with the miR-665 level in RB tissues (Figure 3G; R²=0.3561, P=0.0002). These results suggest that HMGB1 is a direct target gene of miR-665 in RB.

HMGB1 knockdown can simulate the effect of miR-665 upregulation in RB cells

Loss-of-function assays were performed to investigate the biological functions of HMGB1 in RB progression. Western blot analysis was used to validate the knockdown efficiency of HMGB1 siRNA (si-HMGB1). Transfection of si-HMGB1 efficiently decreased HMGB1 protein levels in Y79 and WERI-RB-1 cells compared with the levels in cells transfected
with si-NC (Figure 4A, P<0.05). Cell proliferation and colony formation in the si-HMGB1-transfected cells were attenuated when compared with those in si-NC-transfected cells for both the Y79 and WERI-RB-1 cell lines (Figure 4B and C, P<0.05). In addition, transfection of both Y79 and WERI-RB-1 cells with si-HMGB1 promoted apoptosis (Figure 4D,
had expression affects the function of miR-665 in RB cells and the underlying mechanism, rescue experiments were performed by restoring HMGB1 expression in Y79 and WERI-RB-1 cells. To this end, miR-665-overexpressing Y79 cells were transfected with pc-HMGB1, and si-HMGB1 was introduced into WERI-RB-1 cells that were transfected with a miR-665 inhibitor.

Restoration of HMGB1 protein expression was corroborated by Western blot analysis (Figure 5A, P<0.05). Functional experiments revealed that cell growth (Figure 5B–D, P<0.05) and metastasis (Figure 6A and B, P<0.05) in Y79 cells were inhibited by miR-665 overexpression; however, these inhibitory effects were abolished by co-transfection with pc-HMGB1. Similarly, co-transfection with si-HMGB1 partially counteracted the miR-665 inhibitor-induced promotion of WERI-RB-1 cell growth and metastasis. These results suggest that miR-665 exerts its anticancer effects in RB, at least in part, through the negative regulation of HMGB1.

The functional roles of miR-665 in RB cells depend on HMGB1 expression

To investigate whether HMGB1 expression affects the functions of miR-665 in RB cells and the underlying mechanism, rescue experiments were performed by restoring HMGB1 expression in Y79 and WERI-RB-1 cells. To this end, miR-665-overexpressing Y79 cells were transfected with pc-HMGB1, and si-HMGB1 was introduced into WERI-RB-1 cells that were transfected with a miR-665 inhibitor.

miR-665 suppresses the Wnt/β-catenin signaling pathway in RB cells via regulation of HMGB1

The Wnt/β-catenin signaling pathway has previously been reported to be regulated by HMGB1.31,32 To investigate...
whether a change in miR-665 expression affects the Wnt/β-catenin pathway in RB cells, the expression levels of several important molecules in the pathway, including p-β-catenin, β-catenin, and cyclin D1, were measured in RB cells following upregulation or downregulation of miR-665. Transfection of Y79 cells with miR-665 mimics downregulated the protein levels of p-β-catenin and cyclin D1, while silencing miR-665 expression in WERI-RB-1 cells had the opposite effects (Figure 7). However, recovery of HMGBl expression partially alleviated the change in p-β-catenin and cyclin D1 levels caused by miR-665 upregulation or downregulation. These results indicate that miR-665 deactivates the Wnt/β-catenin pathway in RB cells via negative regulation of HMGBl.

miR-665 inhibits tumor growth in vivo via HMGBl and the Wnt/β-catenin pathway

To further illustrate the effects of miR-665 on RB cell growth, a tumor xenograft mouse model was established by inoculating Y79 cells transfected with the miR-665 mimics or miR-NC into the flanks of nude mice. The tumor xenografts generated from the miR-665 mimic-transfected cells were significantly smaller (Figure 8A and B, P<0.05) and lighter (Figure 8C, P<0.05) than the xenografts in mice of the miR-NC group. We then detected miR-665 expression in xenografts by RT-qPCR. The data showed that the expression level of miR-665 was significantly higher in xenografts originating from miR-665 mimic-
transfected Y79 cells (Figure 8D, P<0.05). Western blot analysis was performed to determine the levels of HMGB1, p-β-catenin, β-catenin, and cyclin D1 in the xenografts, and the results were consistent with those of the in vitro experiments. The expression levels of HMGB1, p-β-catenin, and cyclin D1 proteins were significantly downregulated in the tumor xenografts of mice in the miR-665 mimics group (Figure 8E). These
results show that miR-665 inhibits tumor growth by targeting HMGB1 via deactivation of the Wnt/β-catenin pathway.

**Discussion**

Dysregulation of miRNAs has been shown to be closely correlated with the malignant development of RB.\(^{17–19}\) Multiple miRNAs have been implicated in the aggressive behavior of RB by acting as tumor suppressors or oncogenes.\(^{33–35}\) Accordingly, further investigation of the specific miRNAs related to the development and progression of RB might facilitate the identification of promising therapeutic strategies for patients with RB. In the present study, we detected miR-665 expression in RB for the first time and subsequently investigated the roles of miR-665 in RB progression. More importantly, we explored the molecular mechanisms responsible for the tumor suppressor activity of miR-665 in RB cells.

miR-665 is upregulated in hepatocellular carcinoma, and its upregulation is significantly correlated with tumor size, vascular invasion, local invasion, Edmondson grading, and clinical stage.\(^{26,27}\) Hepatocellular carcinoma patients with high miR-665 levels show shorter survival times than patients with low miR-665 levels.\(^{26}\) In contrast, miR-665 is expressed at low levels in osteosarcoma tissues and cell lines, and osteosarcoma patients with low miR-665 expression have poorer prognoses than patients with high miR-665 expression.\(^{28}\) miR-665 expression is also downregulated in
ovarian cancer \textsuperscript{29} and pancreatic cancer.\textsuperscript{36} However, the expression profile of \textit{miR-665} in RB had not been investigated previously. Herein, we showed that \textit{miR-665} expression was decreased in RB tissues and cell lines and that decreased \textit{miR-665} expression was markedly correlated with tumor size, TNM stage, and differentiation in patients with RB. These observations suggest \textit{miR-665} might be a novel marker of prognosis in patients with RB.

Aberrantly expressed \textit{miR-665} is involved in the malignant phenotypes of multiple human cancers. For instance, \textit{miR-665} has been identified as a tumor-promoting miRNA in hepatocellular carcinoma, and upregulation of \textit{miR-665} promotes the growth, metastasis, and epithelial-mesenchymal transition of hepatocellular carcinoma cells in vitro and in vivo.\textsuperscript{27} In contrast, \textit{miR-665} plays an inhibitory role in the growth and invasion of ovarian cancer cells.\textsuperscript{29} A study by Dong et al revealed that upregulation of \textit{miR-665} expression attenuated the proliferation, invasion, and epithelial-mesenchymal transition of osteosarcoma cells.\textsuperscript{28} However, the functional roles of \textit{miR-665} in RB cells have remained largely unknown. In the present study, exogenous \textit{miR-665} expression inhibited RB cell proliferation and colony formation, promoted cell apoptosis, and decreased cell migration and invasion in vitro. Conversely, silencing \textit{miR-665} expression showed the opposite effects. Further, in vivo experiments showed that \textit{miR-665} overexpression was sufficient to impair tumor growth. These results suggest that \textit{miR-665} is a potential therapeutic target for patients with RB.

Multiple studies have indicated that miRNAs are tightly correlated with carcinogenesis and cancer progression and function by directly regulating the expression levels of target genes.\textsuperscript{37} Three genes, \textit{PTPRB}\textsuperscript{27} in hepatocellular carcinoma, \textit{RAB23}\textsuperscript{28} in osteosarcoma, and homeobox A10\textsuperscript{29} in ovarian cancer, have been identified as direct targets of \textit{miR-665}. Considering this, we attempted to identify the direct target gene involved in the antitumor roles of \textit{miR-665} in RB cells. HMGB1, a highly conserved DNA-binding protein, was found to be a direct and functional downstream target of \textit{miR-665} in RB. \textit{HMGB1} is located on chromosome 8q22 and has been found to be upregulated in various human malignancies, including endometrial carcinoma,\textsuperscript{38} gastric cancer,\textsuperscript{39} hepatocellular carcinoma,\textsuperscript{40} and renal cell carcinoma.\textsuperscript{41} It is also highly expressed in RB, and its overexpression is closely
associated with poor tumor differentiation and optic nerve invasion.\textsuperscript{42} HMGB1 increases oncogene activity in the genesis and development of RB, and it is involved in the regulation of RB cell proliferation, autophagy, apoptosis, the cell cycle, viability, metastasis, and chemotherapy sensitivity.\textsuperscript{43-46} In the present study, we demonstrated that miR-665 directly targets HMGB1 and inactivates the Wnt/\(\beta\)-catenin pathway to inhibit the various malignant behaviors of RB in vitro and in vivo. These findings suggest that miR-665-mediated silencing of HMGB1 and inactivation of the Wnt/\(\beta\)-catenin pathway may represent an effective therapeutic approach for patients with RB.

**Conclusion**

In summary, this is the first study to demonstrate that miR-665 is downregulated in RB tissues and cell lines and that low miR-665 expression is significantly associated with tumor size, TNM stage, and differentiation. Upregulation of miR-665 suppresses the development and progression of RB, likely by directly targeting HMGB1 and inhibiting the activation of the Wnt/\(\beta\)-catenin pathway. A better understanding of the relationship between miR-665, HMGB1, and the Wnt/\(\beta\)-catenin pathway in RB may elucidate the molecular pathogenesis of RB and provide a potential target for therapy.

**Disclosure**

The authors report no conflicts of interest for this work.

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