

# MicroRNA-532 inhibits cell growth and metastasis in retinoblastoma by targeting MDM4

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## Research

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# Abstract

## Background

Now, numerous microRNAs (miRNAs) are found to exert effect in retinoblastoma (RB). This research mainly focused on the function of miR-532 in RB, which has not been investigated.

## Methods

RT-qPCR and Western blot analysis were used to measure expressions of miR-532 and genes. Transwell, CCK-8 and luciferase reporter assays were applied to explore functions of miR-532 and MDM4 in RB.

## Results

The expression of miR-532 was reduced in RB. Furthermore, overexpression of miR-532 restrained RB cell survival and metastasis and induced apoptosis. In addition, miR-532 directly targets MDM4. Moreover, downregulation of MDM4 blocked the progression of RB. And upregulation of MDM4 reversed the anti-tumor effect of miR-532 in RB.

## Conclusion

MiR-532 inhibited cell viability and metastasis in RB by targeting MDM4, indicating that miR-532 may be a novel therapeutic target for RB patients.

## Introduction

Retinoblastoma (RB) is the most common intraocular malignancy in infants and young children. Two-thirds of RB patients are under 3 years of age, and less than 5% of those over 5 years old [1]. As a genetic abnormal disease, RB has a certain genetic predisposition. Approximately 60% of RB patients are monogenic non-genetic, and 40% are caused by genetic inheritance of a parent who is sick or carrying a disease gene [2]. Nowadays, RB is one of the most cured cancers. 95–98% of children with RB can recover, and more than 90% of patients can survive to adulthood. Although the survive rate of China's patients is slightly lower, the current 5-year survival rate can reach 80–85% [3]. However, RB is prone to intracranial and distant metastasis, often endangering the lives of children [4]. Therefore, early detection, diagnosis and treatment are the key to keeping your child's eyes and vision and improving the cure rate.

MicroRNAs (miRNAs) inhibits expressions of target genes mainly by binding to the target mRNA, or by causing the mRNA to degrade or hinder its translation [5]. In the meantime, the dysregulation of miRNAs was also identified in RB. For example, miR-101 and miR-497 were downregulated in RB, while miR-221 and miR-222 were upregulated in RB [6–8]. Functionally, miR-138-5p acted as a tumor suppressor via

targeting PDK1 in human RB [9]. Inversely, miR-498 promoted cell proliferation and inhibited cell apoptosis in RB by directly targeting CCPG1 [10]. Now, the different roles of miR-532 in human cancers aroused our attention. First, upregulation of miR-532 was observed in colorectal cancer [11]. Moreover, miR-532-3p promoted hepatocellular carcinoma progression by targeting PTPRT [12]. However, downregulating of miR-532 was detected in epithelial ovarian cancer [13]. Furthermore, miR-532 inhibited cell proliferation and invasion by acting as direct regulators of hTERT in ovarian cancer [14]. In addition, Venkatesan et al. found that miR-532 was downregulated in RB [15]. But the function of miR-532 has not been illustrated in RB and need to be explored.

MDM4 (Mdm4 p53 binding protein homolog) is well-known to be involved in p53 pathway [16]. The tumor suppressor p53 has been widely identified in human cancers [17]. Furthermore, MDM4 promoter attenuated the p53 tumor suppressor pathway and accelerated tumor formation in humans [18]. Moreover, it was found that MDM4 in combination with p53 contributed to breast cancer susceptibility [19]. Zhou et al. reported that MDM4 accelerated the tumorigenesis of esophageal squamous cell carcinoma [20]. In addition, MDM4 expression was regulated by multiple microRNAs in prostate cancer, including miR-191-5p and miR-887 [21]. Mandke et al. suggested that miR-34a modulated MDM4 expression via a target site in the open reading frame [22]. Moreover, miR-1307 regulated cisplatin resistance by targeting MDM4 in breast cancer [23]. However, the interaction between MDM4 and miR-532 was not investigated in RB. Therefore, their relationship was verified in this study. Furthermore, the potential functions of miR-532 and MDM4 was explored in RB. This research will provide a potential therapeutic target against RB.

## Materials And Methods

### Clinical tissues

Twenty-two RB patients with informed consents in Weifang People's Hospital were participated in this study. Moreover, patients with RB did not receive any treatment except for surgery. The permission of this research was acquired from the Institutional Ethics Committee of Weifang People's Hospital.

### Cell culture

Normal retinal pigmented epithelium cell line ARPE-19 and RB cell line WERI-Rb-1 were purchased from ATCC (Manassas, VA, USA). These cells were incubated in RPMI-1640 medium (10% FBS, 5% CO<sub>2</sub>, 37°C) for further experiment.

### Cell transfection

MiR-532 mimics, miR-532 inhibitor, MDM4 siRNA or MDM4 overexpression plasmid was obtained from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, CA, USA) was applied to severally transfer them into WERI-Rb-1 cells. Untreated cells were used as controls.

## RNA isolation and RT-qPCR

Total RNA isolation was performed using TRIZOL reagent (Invitrogen, USA). And cDNA solution was obtained using microRNA reverse transcription kit (Takara, Dalian, China). RT-qPCR assay was performing using SYBR miRNA detection assays (Takara) based on the manufacturer's instruction. U6 or GAPDH was used as the control of miR-532 or MDM4, which were quantified with the  $2^{-\Delta\Delta Cq}$  method.

## Western blot analysis

RIPA lysis buffer (Beyotime) was used to obtain protein samples. Next, 10% SDS-PAGE was used to separate protein. Protein samples were transferred to PVDF membranes. Blocked with 5% non-fat milk, protein samples were incubated overnight at 4°C with E-cadherin, N-cadherin, vimentin, MDM4 and GAPDH primary antibodies (Abcam, Shanghai, China). Afterwards, secondary antibodies (Abcam, USA) were added to incubate protein samples for 1 hour. ECL kit (Beyotime) was used to assess protein bands.

## Dual luciferase reporter assay

First, WT-MDM4-3'UTR or MUT-MDM4-3'UTR was inserted into pmirGLO luciferase reporter vector (Promega, USA). Next, WERI-Rb-1 cells with the luciferase vector and miR-532 mimics were incubated for 48 h. Finally, the luciferase activity was observed by dual-luciferase reporter assay system (Promega, USA).

## Transwell assay

First, diluted Matrigel was added to the upper chamber for cell invasion. Cell migration assay was performed without Matrigel. After 30 min, WERI-Rb-1 cell suspension ( $3 \times 10^3$  cells/well) was added to the Transwell upper chamber, and RPMI-1640 medium (10% FBS) was added to 24-well plates in lower chamber. After 24 h, the moved cells were stained with 0.1% crystal violet. Observation and photographing were performed using a light microscope.

## CCK-8 assay

The prepared WERI-Rb-1 cells were incubated in a 96-well plate for 24 hours (at 37 °C, 5% CO<sub>2</sub>). Next, WERI-Rb-1 ( $3 \times 10^3$ /well) cells were incubated for 24, 48, 72 and 96 h. After that, 10 ml of CCK-8 (Dojindo, Kumamoto, Japan) solution was added to incubate the cells for 4 hours. The absorbance at 450 nm was observed with a microplate reader (Molecular Devices).

## Statistical analysis

Data was shown as mean  $\pm$  SD and analyzed by SPSS 17.0 or Graphpad Prism 6. Differences between groups were analyzed using one-way analysis of variance with Tukey's post hoc test. Differences were considered as significant at  $P < 0.05$ .

# Results

## **The expression of miR-532 was reduced in RB.**

To explore the dysregulation of miR-532, its expression was firstly detected in RB. We found that the expression of miR-532 was reduced in RB tissues compared with adjacent tissues ( $P < 0.01$ , Fig. 1A). Consistently, miR-532 was also downregulated in WERI-Rb-1 cells contrast to ARPE-19 cells ( $P < 0.01$ , Fig. 1B). The above results showed that miR-532 was indeed abnormally expressed in RB. Next, WERI-Rb-1 cells with miR-532 mimics or inhibitor were prepared to explore its role in RB. RT-qPCR indicated that miR-532 expression was elevated by its mimics and declined by its inhibitor ( $P < 0.01$ , Fig. 1C). Then, gain-loss functional experiment was performed to explore the function of miR-532 in RB.

## **Overexpression of miR-532 restrained the development of RB.**

Functionally, CCK-8 showed that cell proliferation was restrained by miR-532 overexpression, and was accelerated by downregulation of miR-532 in WERI-Rb-1 cells ( $P < 0.01$ , Fig. 2A). Then, apoptosis-associated proteins (Bcl-2/Bax) regulated by miR-532 were examined in WERI-Rb-1 cells. MiR-532 mimics were found to promote Bax expression and suppress survival gene Bcl-2 expression (Fig. 2B). Furthermore, miR-532 inhibitor suppressed Bax expression and promoted Bcl-2 expression (Fig. 2B). In addition, miR-532 mimics were found to restrain cell migration, whereas miR-532 inhibitor facilitated WERI-Rb-1 cell migration ( $P < 0.01$ , Fig. 2C). Furthermore, overexpression of miR-532 repressed cell invasion, when downregulation of miR-532 accelerated cell invasion in WERI-Rb-1 cells ( $P < 0.01$ , Fig. 2D). Collectively, overexpression of miR-532 restrained RB cell survival and metastasis and induced apoptosis.

## **MiR-532 directly targets MDM4.**

Further, miR-532 was found to have a site binding to 3'-UTR of MDM4 in TargetScan database (<http://www.targetscan.org/>, Fig. 3A). Luciferase reporter assay was then performed to verify this prediction. The results suggested that miR-532 mimics decreased Wt-MDM4 luciferase activity. But Mut-MDM4 luciferase activity was not affected by miR-532 mimics ( $P < 0.01$ , Fig. 3B). In addition, we also found that MDM4 expression was declined by miR-532 mimics, and was increased by its inhibitor in WERI-Rb-1 cells ( $P < 0.01$ , Fig. 3C). Besides, MDM4 was upregulated in RB tissues compared to normal tissues ( $P < 0.01$ , Fig. 3D). Furthermore, MDM4 expression had a negative association with miR-532 in RB tissues ( $P < 0.01$ ,  $R^2 = 0.7637$ ; Fig. 3E). This result revealed that miR-532 directly targets MDM4 and was negatively correlated with MDM4 expression in RB.

## **Downregulation of MDM4 blocked the progression of RB.**

Next, upregulation of MDM4 was identified in WERI-Rb-1 cells compared with ARPE-19 cells ( $P < 0.01$ , Fig. 4A). Then, MDM4 siRNA was transfected into WERI-Rb-1 cells to investigate its function in RB. RT-qPCR showed that MDM4 expression was reduced by its siRNA ( $P < 0.01$ , Fig. 4B). Functionally, downregulation of MDM4 suppressed WERI-Rb-1 cell proliferation ( $P < 0.01$ , Fig. 4C). In the meantime,

knockdown of MDM4 promoted Bax expression and suppressed Bcl-2 expression in WERI-Rb-1 cells ( $P < 0.01$ , Fig. 4D). Besides that, MDM4 silencing restrained WERI-Rb-1 cell migration and invasion ( $P < 0.01$ , Fig. 4E, 4F). These findings implied that MDM4 functioned as an oncogene in RB.

### **The interaction between miR-532 and MDM4 was involved in RB progression.**

Finally, MDM4 vector was transfected into WERI-Rb-1 cells with miR-532 mimics. The decreased expression of MDM4 induced by miR-532 mimics was restored by MDM4 vector (Fig. 5A). Functionally, upregulation of MDM4 impaired inhibition of cell proliferation mediated by miR-532 mimics (Fig. 5B). Furthermore, the reversal effect of MDM4 on Bcl-2/Bax expression was detected in WERI-Rb-1 cells with miR-532 mimics (Fig. 5C). In addition, the inhibitory effect of miR-532 on cell migration and invasion was also weakened by MDM4 vector (Fig. 5D, 5E). Based on these results, we considered that upregulation of MDM4 reversed the anti-tumor effect of miR-532 in RB.

## **Discussion**

Many literatures now indicate that the dysregulation of many miRNAs is associated with the formation and development of RB tumors. Many of these miRNAs have been reported to exert an inhibitory effect in RB, providing a potential therapeutic method for RB [24, 25]. In this study, miR-532 expression was reduced in RB. Furthermore, overexpression of miR-532 restrained RB cell viability and metastasis and induced apoptosis. In addition, MDM4 was confirmed to be a direct target gene of miR-532. Moreover, downregulation of MDM4 blocked the progression of RB. And upregulation of MDM4 reversed the anti-tumor effect of miR-532 in RB. Therefore, we considered that miR-532 acted as a tumor inhibitor in RB via binding to MDM4.

Consistent with our results, decreased expression of miR-532 was also detected in ovarian cancer and hepatocellular carcinoma [26, 27]. In addition, downregulation of miR-532 was found to promote the proliferation and invasion of bladder cancer cells [28]. Song et al. also reported that loss of miR-532 promoted cell proliferation and metastasis by in hepatocellular carcinoma [29]. Here, cell proliferation, migration and invasion were found to be inhibited by overexpression of miR-532 in RB. However, upregulation of miR-532 was identified in breast cancer and gastric cancer [30, 31]. Furthermore, miR-532 functioned as an oncogenic microRNA in human gastric cancer [32]. These findings are contrary to our results. Contradictory findings suggest that miR-532 expression patterns show tissue specificity in human cancers.

Previous studies revealed that miR-532 was involved in tumorigenesis through regulating target genes, such as ETS1 and Rab3IP [33, 34]. In current research, miR-532 was found to directly target MDM4 and inversely regulated its expression in RB. Upregulation of MDM4 has been identified in leukemia [35]. The p53 inhibitor MDM4 has been reported to be involved in tumorigenesis [36]. Moreover, it has been reported that MDM4 is an oncogene and cancer therapy [37]. Here, MDM4 was upregulated in RB and repressed cell viability and metastasis in RB, which was the same as previous studies. And upregulation of MDM4 reversed the anti-tumor effect of miR-532 in RB. The interaction between MDM4 and other

miRNAs has been identified in some human cancers. For example, miR-128 induced pancreas cancer cell apoptosis by targeting MDM4 [38]. Yan et al. also showed that miR-1205 functioned as a tumor suppressor by disconnecting MDM4 in non-small cell lung cancer [39]. Consistent with the above studies, miR-532 restrained cell proliferation and metastasis in RB by suppressing MDM4 expression.

## **Conclusion**

Briefly, this study proposed that miR-532 was an anti-tumor miRNA in RB through inhibiting survival and metastasis and inducing apoptosis. Moreover, MDM4 was confirmed as a direct target of miR-532. This research highlights the critical role of miR-532/MDM4 axis in RB progression.

## **Declarations**

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Not applicable.

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### **Availability of data and materials**

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

### **Authors' contributions**

CL designed the study and drafted the manuscript. YN and CW were responsible for the collection and analysis of the experimental data. TJ, QR and JX revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

### **Ethical approval**

The study was approved by Ethical Committee of Qilu Hospital of Shandong University (Qingdao) and conducted in accordance with the ethical standards.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

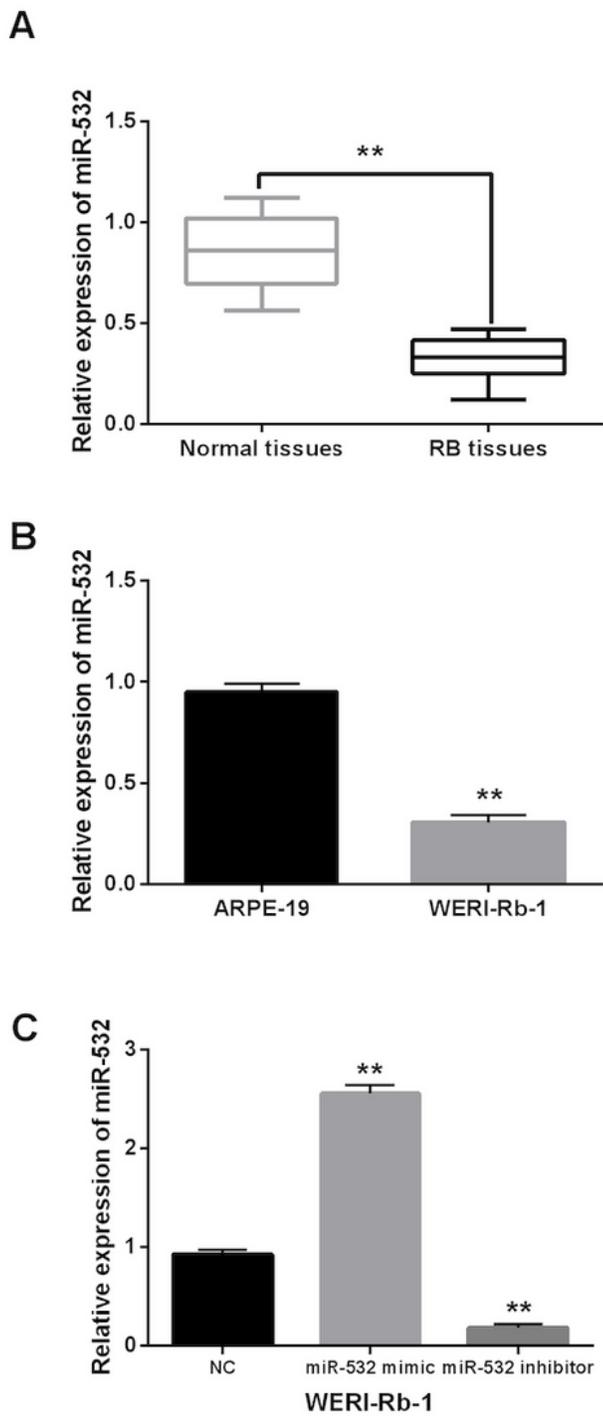
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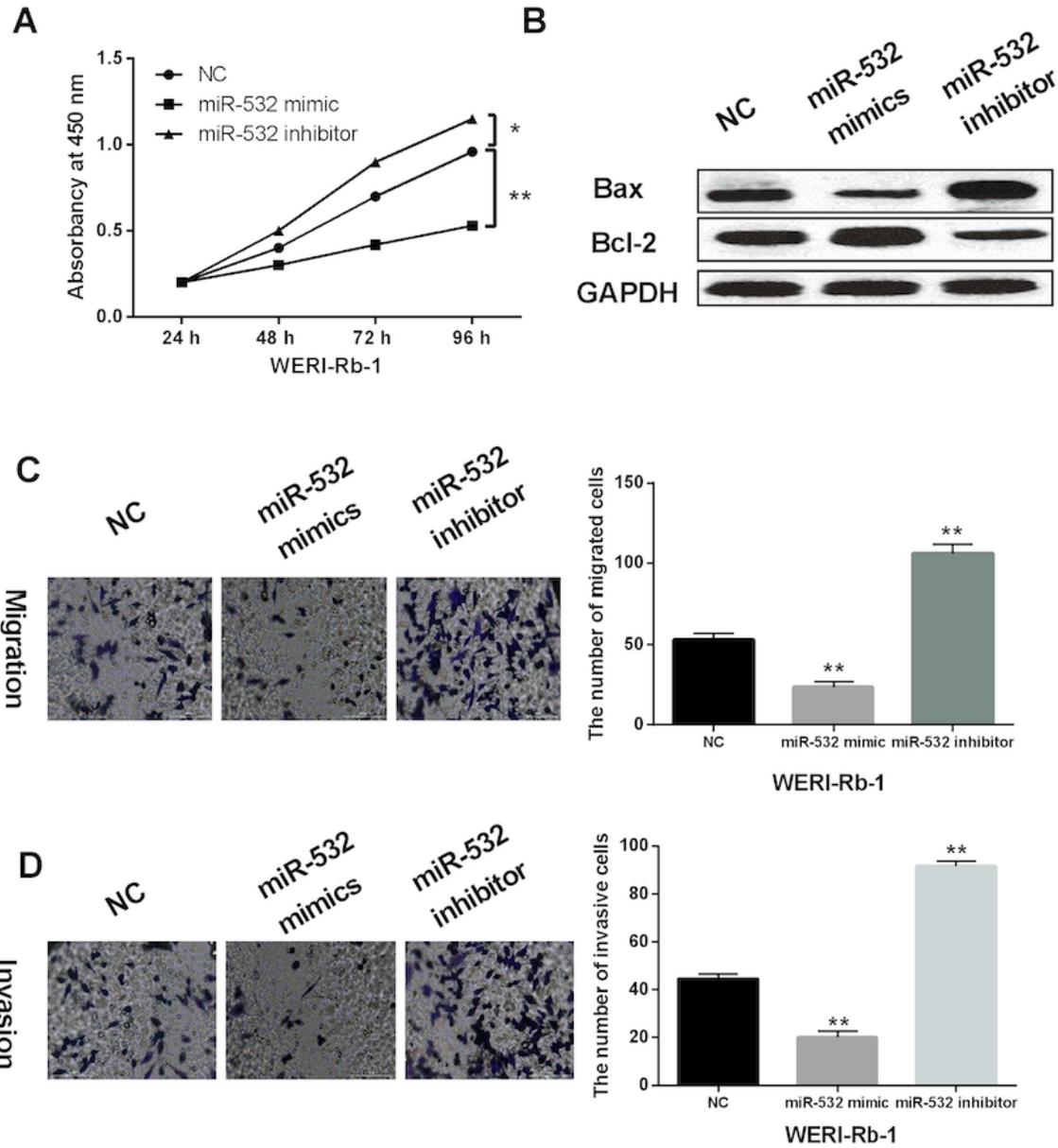
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## Figures



**Figure 1**

The expression of miR-532 was reduced in RB. (A) The mRNA miR-532 expression in RB tissues. (B) The miR-532 expression in WERI-Rb-1 and ARPE-19 cells. (C) MiR-532 expression was examined in WERI-Rb-1 cells with its mimics or inhibitor. \*\*P < 0.01.



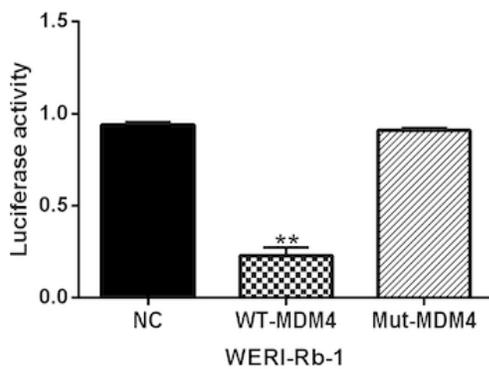
**Figure 2**

Overexpression of miR-532 restrained the development of RB. (A) Cell proliferation was measured in WERI-Rb-1 cells with miR-532 mimics or inhibitor (B) MiR-532 regulated protein expressions of Bax and Bcl-2 in WERI-Rb-1 cells. (C, D) Cell migration and invasion were measured in WERI-Rb-1 cells with miR-532 mimics or inhibitor. \*P < 0.05, \*\* P < 0.01

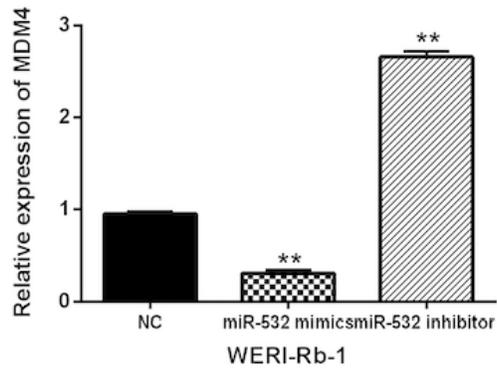
A

Predicted consequential pairing of target region (top) and miRNA (bottom)	
Position 738-744 of MDM4 3' UTR	5' ... AGACCAUCUCAAGCAUGGGAGAA ...
hsa-miR-532-3p	3' ACGUUCGGAACCCACACOCUCC

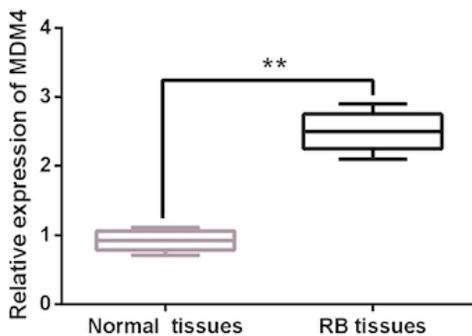
B



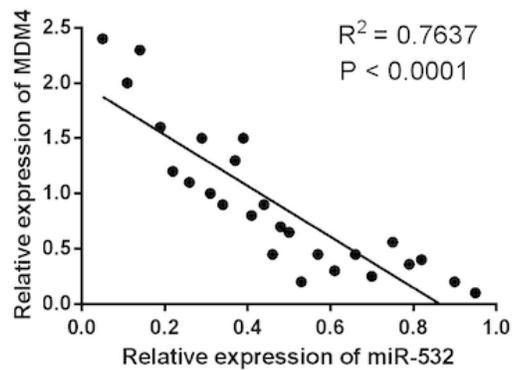
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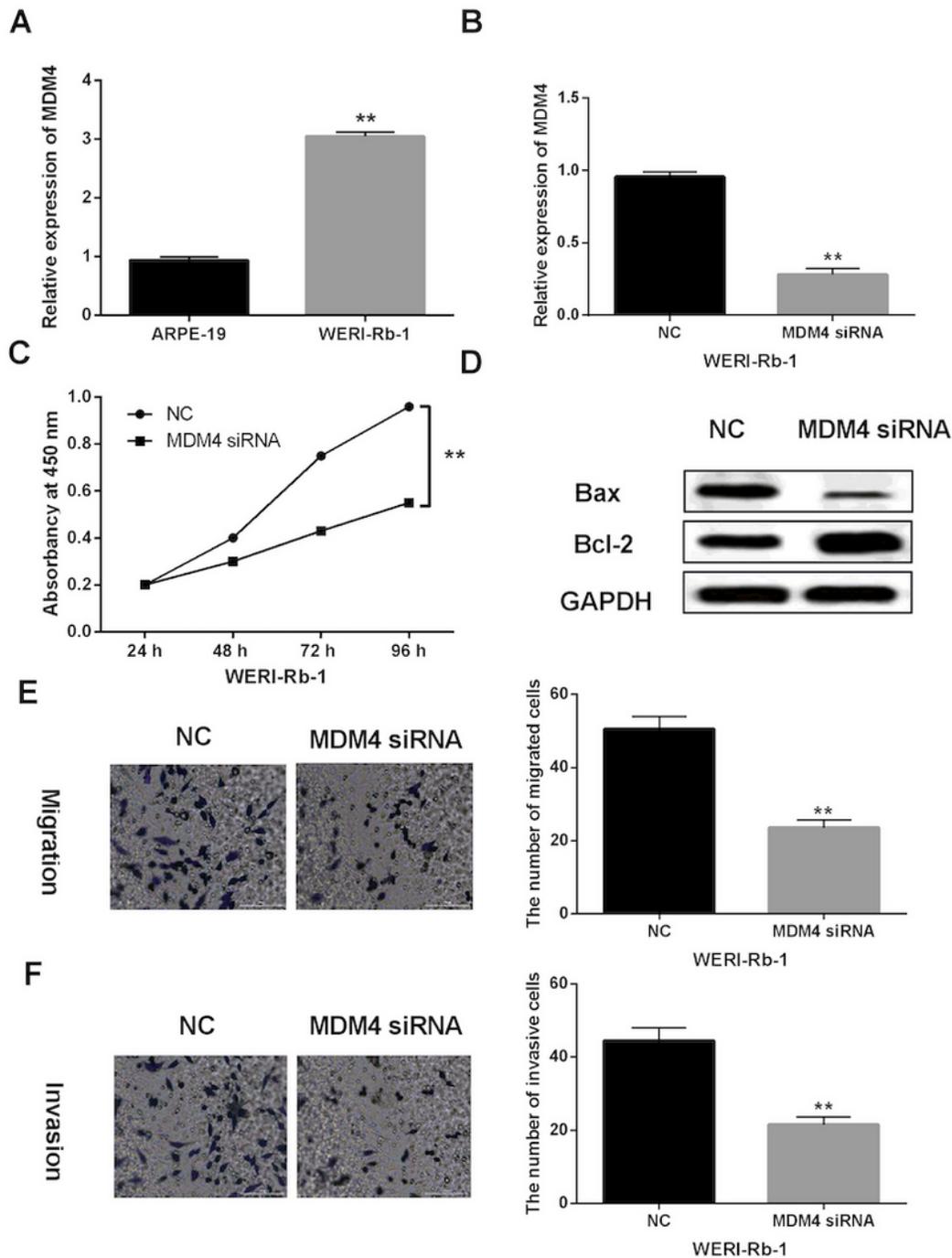


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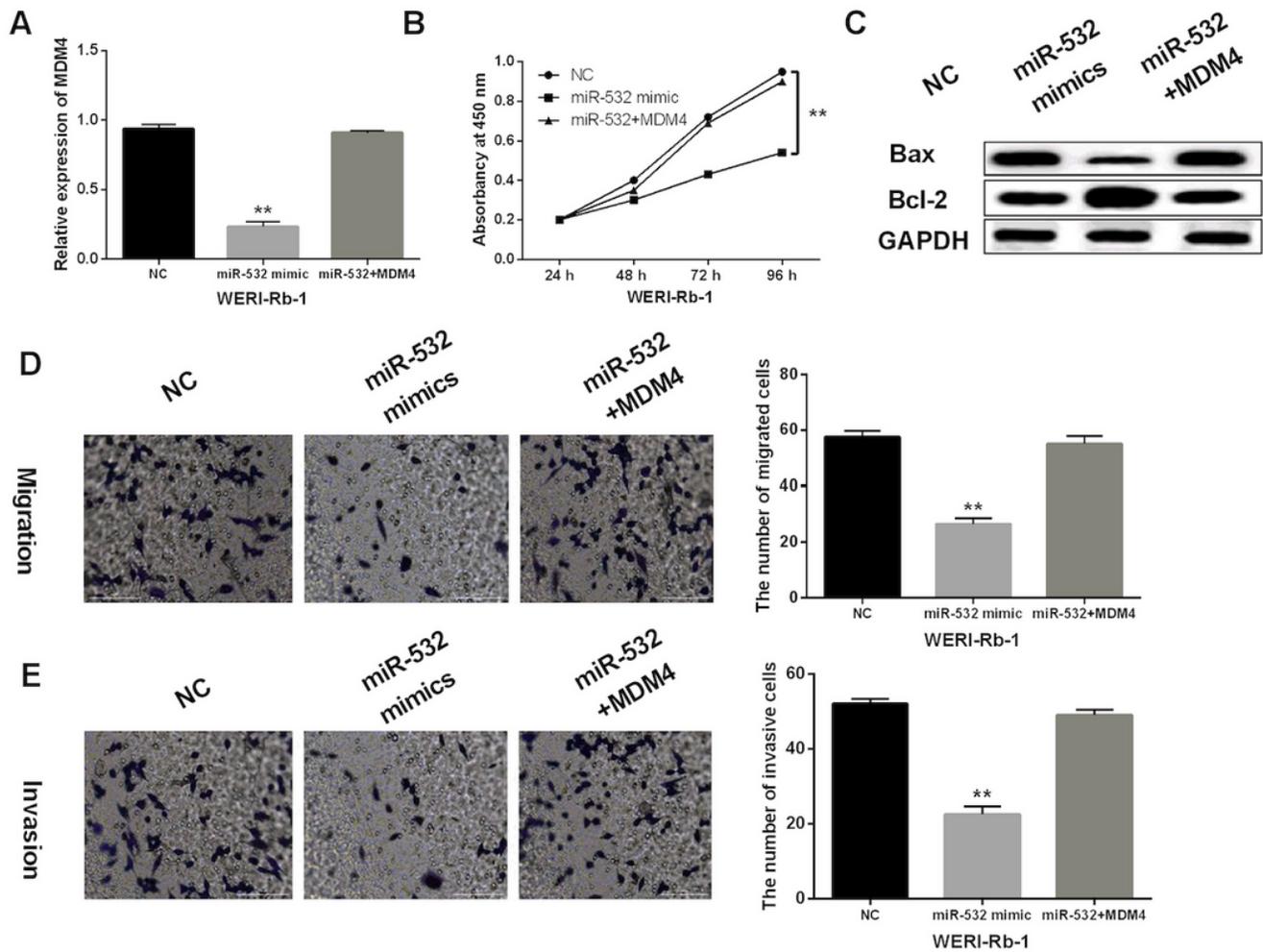
**Figure 3**

MiR-532 directly targets MDM4. (A) The binding sites between MDM4 and miR-532. (B) Luciferase reporter assay (C) MDM4 expression regulated by miR-532 mimics or inhibitor (D) The mRNA MDM4 expression in RB tissues. (E) MiR-532 was negatively correlated with MDM4 in RB tissues. \*\* P < 0.01.



**Figure 4**

Downregulation of MDM4 blocked the progression of RB. (A) MDM4 expression was measured in WERI-Rb-1 and ARPE-19 cells (B) MDM4 expression was measured in WERI-Rb-1 cells with its siRNA. (C) Cell proliferation in WERI-Rb-1 cells with MDM4 siRNA (D) MDM4 regulated protein expressions of Bax and Bcl-2 in WERI-Rb-1 cells. (E, F) Cell migration and invasion in WERI-Rb-1 cells with MDM4 siRNA \*\*  $P < 0.01$



**Figure 5**

The interaction between miR-532 and MDM4 was involved in RB progression. (A) MDM4 expression was observed in WERI-Rb-1 cells containing miR-532 mimics and MDM4 vector. (B) Cell proliferation in WERI-Rb-1 cells containing miR-532 mimics and MDM4 vector (C) Bax and Bcl-2 in WERI-Rb-1 cells containing miR-532 mimics and MDM4 vector (D, E) Cell migration and invasion in WERI-Rb-1 cells containing miR-532 mimics and MDM4 vector \*\* P < 0.01