

# Knockdown of Long Noncoding RNA (lncRNA) Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1) Inhibits Proliferation, Migration, and Invasion and Promotes Apoptosis by Targeting miR-124 in Retinoblastoma

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Evidence suggests that the long noncoding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is upregulated in cancer tissues, and its elevated expression is associated with hyperproliferation. However, the underlying mechanisms regarding the role of MALAT1 in retinoblastoma (RB) remain unclear. This study aimed to explore the functional role of MALAT1 in RB by targeting miR-124. The results showed that the expression of MALAT1 was significantly higher in the Y79 cell line than in the ARPE-19 cell line ( $p < 0.01$ ). Moreover, MALAT1 silence inhibited cell viability, migration, and invasion and promoted apoptosis in Y79 cells ( $p < 0.05$ ,  $p < 0.01$ , or  $p < 0.001$ ). miR-124 was upregulated by MALAT1 silence and hence was identified as a target of MALAT1 ( $p < 0.05$  or  $p < 0.001$ ). In addition, miR-124 suppression inhibited cell apoptosis and remarkably abolished the inhibitory effects of MALAT1 silence on cell viability, migration, and invasion ( $p < 0.05$ ,  $p < 0.01$ , or  $p < 0.001$ ). In addition, Slug was a target of miR-124 and regulated cell viability, migration, invasion, and apoptosis in Y79 cells ( $p < 0.05$ ,  $p < 0.01$ , or  $p < 0.001$ ). Further, Slug silence abolished miR-124 suppression-induced inactivation of the ERK/MAPK and Wnt/ $\beta$ -catenin pathways. Taken together, our data highlight the pivotal role of MALAT1 in RB. Moreover, the present study elucidated the MALAT1–miR-124–ERK/MAPK and Wnt/ $\beta$ -catenin signaling pathways in RB, which might provide a new approach for the treatment of RB.

**Key words:** Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1); Retinoblastoma (RB); miR-124; ERK/MAPK pathway; Wnt/ $\beta$ -catenin pathway

## INTRODUCTION

Retinoblastoma (RB) is a rare cancer of the infant retina that is diagnosed in approximately 8,000 children each year worldwide and is the most frequently seen intraocular tumor in childhood worldwide<sup>1,2</sup>. The mortality rate ranges from 50% to 70% among children diagnosed with RB in underdeveloped countries<sup>3,4</sup>. It is a genetic tumor, which occurs in two forms: sporadic (always unilateral) and hereditary (often bilateral)<sup>5</sup>. It is a curable intraocular malignancy; 95%–98% of children can recover, and more than 90% of patients can survive to adulthood<sup>6,7</sup>. Children with RB are at greater life-threatening risks, and hence it needs to be treated on an urgent basis.

MicroRNAs (miRNAs) play diverse roles in tumorigenesis<sup>8–10</sup> and in the progression of RB<sup>11</sup>. These may act as oncogenes, tumor suppressors, and modulators of tumor progression by affecting proliferation, invasion, apoptosis, and chemoresistance<sup>9,12–15</sup>. Accumulating evidence has suggested that miR-124 is related to carcinogenesis. The expression level of miR-124 was significantly suppressed in glioma<sup>16</sup>, medulloblastoma<sup>17,18</sup>, oral squamous cell carcinoma (OSCC)<sup>19</sup>, hepatocellular carcinoma (HCC)<sup>20</sup>, bladder cancer<sup>21</sup>, breast cancer<sup>22,23</sup>, and RB<sup>11</sup>.

Long noncoding RNAs (lncRNAs) are noncoding transcripts longer than 200 nucleotides. At present, research on lncRNAs is still at the beginning stage. Among

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all the human lncRNAs, <1% has been known to be functionally characterized<sup>24</sup>. Metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved mRNA-like lncRNA and was first identified with high expression in metastatic non-small cell lung cancer<sup>25</sup>. According to previous studies, MALAT1 is overexpressed in many other human malignancies, including breast, pancreas, colon, prostate, and liver cancers<sup>26</sup>. Functional studies of MALAT1 demonstrated that its deregulation influences proliferation, invasion, and/or metastasis of multiple cancer cells<sup>27-33</sup>. With these findings, it was concluded that MALAT1 is critical for cancer development. Also, lncRNAs may potentially interact with miRNAs and modulate each other's expression. However, the mechanism of MALAT1 in tumor cell growth regulation, especially in RB, is still unclear.

Our present work aimed to identify the role of lncRNA MALAT1 and miR-124 in RB development. miR-124 was found to be a tumor suppressor in RB. Therefore, in the present study, we aimed to explore the functional role of MALAT1 in RB by targeting miR-124 in RB.

## MATERIALS AND METHODS

### *Cell Culture and Treatment*

The human RB Y79 cell line and retinal pigment epithelial cell line ARPE-19 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Roswell Park Memorial Institute (RPMI)-1640 (Invitrogen, Grand Island, NY, USA) media and fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) were purchased from Life Technologies Corporation (Carlsbad, CA, USA). In brief, Y79 cells were cultured in RPMI-1640 medium supplemented with 15% heat-inactivated FBS, 0.1% ciprofloxacin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 4.5% dextrose. The cells were grown in 25-cm<sup>2</sup> culture flasks in the upright position in 10-ml aliquots of the culture medium. Incubation was performed at 37°C under a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

### *Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)*

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Corporation) according to the manufacturer's instructions. The One Step SYBR<sup>®</sup> PrimeScript<sup>®</sup> PLUS RT-RNA PCR Kit (TaKaRa, Biotechnology, Dalian, P.R. China) was used for the RT-PCR analysis to test the expression levels of MALAT1. The TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II with the TaqMan MicroRNA Assay of miR-124 and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-124 in cells and tissues.

For the test of Slug, RNA PCR Kit (AMV) Ver.3.0 (TaKaRa) was used. GAPDH used in this study for normalizing fold changes was calculated by the relative quantification ( $2^{-\Delta\Delta C_t}$ ) method.

### *Transfection and Generation of Stably Transfected Cell Lines*

Short hairpin RNA (shRNA) directed against human lncRNA MALAT1 was ligated into the U6/GFP/Neo plasmid (GenePharma, Shanghai, P.R. China) and was referred to as sh-MALAT1. For the analysis of the Slug functions, full-length Slug sequences and shRNA directed against Slug were constructed in pEX-2 and U6/GFP/Neo plasmids (GenePharma), respectively. They were referred to as pEX-Slug and sh-Slug. The Lipofectamine 3000 reagent (Life Technologies) was used for cell transfection according to the manufacturer's instructions. The plasmid carrying a nontargeting sequence was used as a negative control (NC) of sh-MALAT1 and sh-Slug, which was referred to as sh-NC. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). After approximately 4 weeks, G418-resistant cell clones were established. The miR-124 mimic, inhibitor, and their respective NC were synthesized (Life Technologies) and transfected into cells in the study. The highest transfection efficiency occurred at 48 h; thus, 72 h posttransfection was considered as the harvest time in the subsequent experiments.

### *Cell Viability Assays*

For cell viability assay,  $1 \times 10^5$  cells were seeded in duplicate in 60-mm dishes, and 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine cell viability. After incubation for 24 h, 20  $\mu$ l of MTT (0.5 mg/ml; Sigma-Aldrich) was added to each well and incubated for another 3 h at 37°C. In addition, 150  $\mu$ l of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to stop the reaction. The absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

### *Apoptosis Assay*

Cell apoptosis analysis was performed using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V staining. Briefly, cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and stained in PI/FITC-annexin V in the presence of 50  $\mu$ g/ml RNase A (Sigma-Aldrich), and then cells were incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done using a FACScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using FlowJo software.

### Migration and Invasion Assay

Cell migration was determined using a modified two-chamber migration assay with a pore size of 8  $\mu\text{m}$ . For the migration assay, cells suspended in 200  $\mu\text{l}$  of serum-free medium were seeded on the upper compartment of a 24-well Transwell culture chamber, and 600  $\mu\text{l}$  of complete medium was added to the lower compartment. After incubation at 37°C, cells were fixed with methanol. Nontraversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted.

The invasion behavior was determined using 24-well Millicell Hanging Cell Culture inserts with 8-mm polyethylene terephthalate (PET) membranes (Millipore, Bedford, MA, USA). Briefly, after the cells were treated for the indicated condition,  $5 \times 10^4$  cells in 200  $\mu\text{l}$  of serum-free DMEM medium were plated onto BD BioCoat™ Matrigel™ Invasion Chambers (8- $\mu\text{m}$  pore size polycarbonate filters; BD Biosciences, San Jose, CA, USA), and complete medium containing 10% FBS was added to the lower chamber. After processing the invasion chambers for 48 h (37°C, 5%  $\text{CO}_2$ ) in accordance with the manufacturer's protocol, the noninvading cells were removed with a cotton swab; the invading cells were fixed in 100% methanol and then stained with crystal violet solution and counted microscopically. The data are presented as the average number of cells attached to the bottom surface from five randomly chosen fields.

### Reporter Vectors Constructs and Luciferase Reporter Assay

The fragment from MALAT1 containing the predicted miR-124 binding site was amplified by PCR and then cloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to form the reporter vector MALAT1-wild-type (MALAT1-wt) and Slug-wt. To mutate the putative binding site of miR-124 in MALAT1 and Slug, the sequence of putative binding site was replaced and named as MALAT1-mutated-type (MALAT1-mt) and Slug-mt. Then the miR-124 mimic and its corresponding control were cotransfected into Y79 cells, and the Dual-Luciferase Reporter Assay System (Promega) was used for testing the luciferase activity.

### Western Blot

The protein used for Western blotting was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Guangzhou, P.R. China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The Western blot system was established using a Bio-Rad

Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. The primary antibody was incubated with the membrane at 4°C overnight, followed by a wash and incubation with the secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots, and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200  $\mu\text{l}$  of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China).

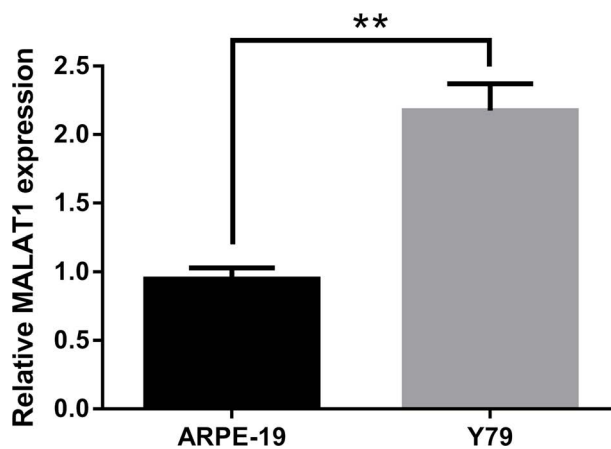
### Statistical Analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using GraphPad 6.0 statistical software (GraphPad Software, San Diego, CA, USA). The *p* values were calculated using one-way analysis of variance (ANOVA). A value of *p* < 0.05 was considered to indicate a statistically significant result.

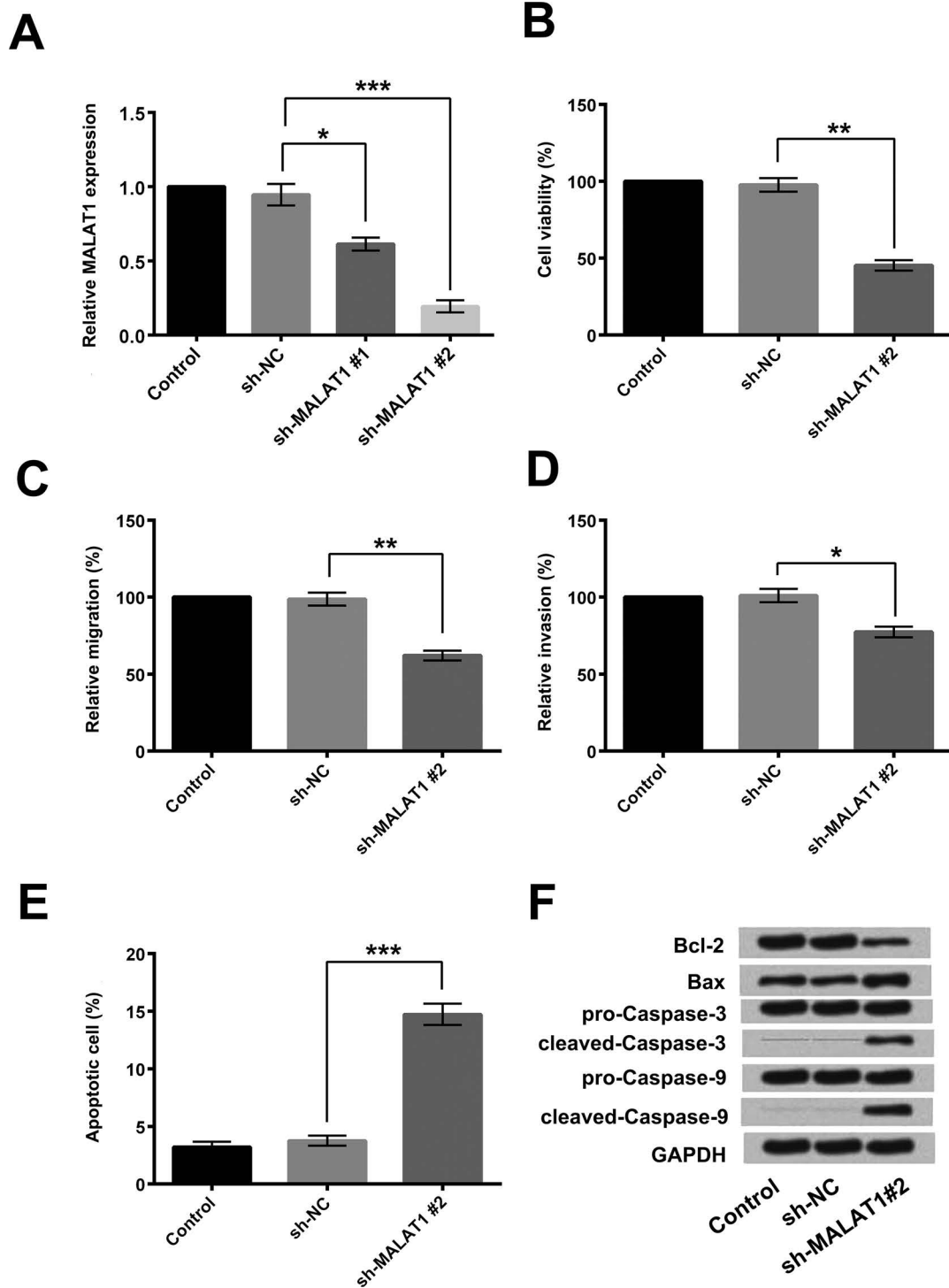
## RESULTS

### Suppression of MALAT1 Inhibited Cell Viability, Migration, and Invasion and Promoted Apoptosis in Y79 Cells

To reveal the role of MALAT1 in Y79 cells, qRT-PCR was performed to examine the expression levels of MALAT1 after transfection in the RB cell line (Y79) and the retinal pigment epithelial cell line (ARPE-19).



**Figure 1.** Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was highly expressed in retinoblastoma (RB) cells. The expression of MALAT1 in Y79 cells was significantly higher than in ARPE-19 cells. \*\**p* < 0.01.



**Figure 2.** MALAT1 silence inhibited cell viability, migration, and invasion and promoted apoptosis in Y79 cells. (A) MALAT1 silence in Y79 cells. (B) MALAT1 silence inhibited cell viability. (C) MALAT1 silence inhibited cell migration. (D) MALAT1 silence inhibited cell invasion. (E) MALAT1 silence promoted apoptosis in Y79 cells. (F) Western blotting analysis of MALAT1 silence promoted apoptosis in Y79 cells. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

MALAT1 expression level in the Y79 cells was significantly higher than in the ARPE-19 cells ( $p < 0.01$ ) (Fig. 1). Subsequently, Y79 cells were transfected with MALAT1#1 and MALAT1#2. The data of the present study revealed that MALAT1#1 and MALAT1#2 were significantly suppressed in Y79 cells compared to the NC ( $p < 0.05$  or  $p < 0.001$ ) (Fig. 2A). Moreover, MALAT1#2 displayed significant suppression levels compared to MALAT1#1. Therefore, MALAT1#2 was used in the subsequent investigations in the present study.

Y79 cells were transfected with sh-MALAT1#2, and then cell viability was examined by the MTT assay. MALAT1#2 silence significantly reduced cell viability in Y79 cells compared to the NC ( $p < 0.01$ ) (Fig. 2B). This result suggested that MALAT1#2 silence inhibited cell viability. Additionally, we used the Transwell assay to measure the migratory and invasive capacities of Y79 cells. The results showed that MALAT1#2 silence significantly reduced cell migration and invasion capacities compared with corresponding controls ( $p < 0.05$  or  $p < 0.01$ ) (Fig. 2C and D). These results indicated that MALAT1#2 silence inhibited cell metastasis in Y79 cells.

Apoptosis assay was performed to determine the apoptotic rate of Y79 cells. The results showed that MALAT1#2 silence revealed a significant increase in the apoptosis rate ( $p < 0.001$ ) (Fig. 2E). Similar results were obtained by Western blotting, which demonstrated a reduction in Bcl-2 expression, an antiapoptotic protein, and increase in Bax expression, a proapoptotic protein, in the MALAT1#2 silence group compared to the NC group

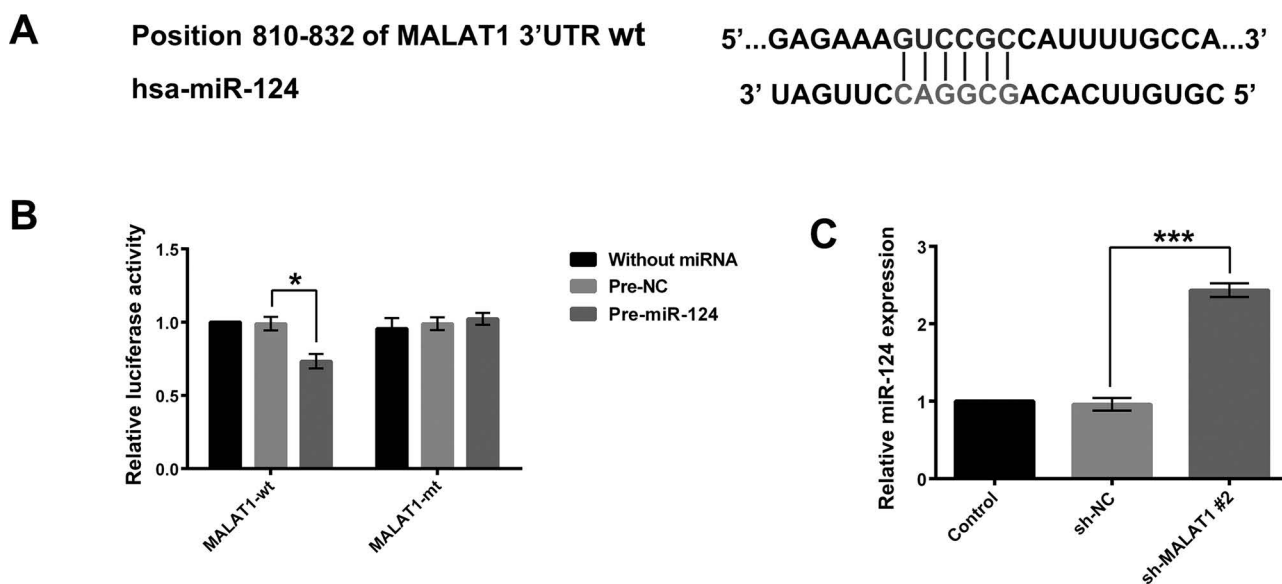
(Fig. 2F). These data suggested that MALAT1#2 silence promoted cell apoptosis in Y79 cells.

*miR-124 Was a Target of MALAT1*

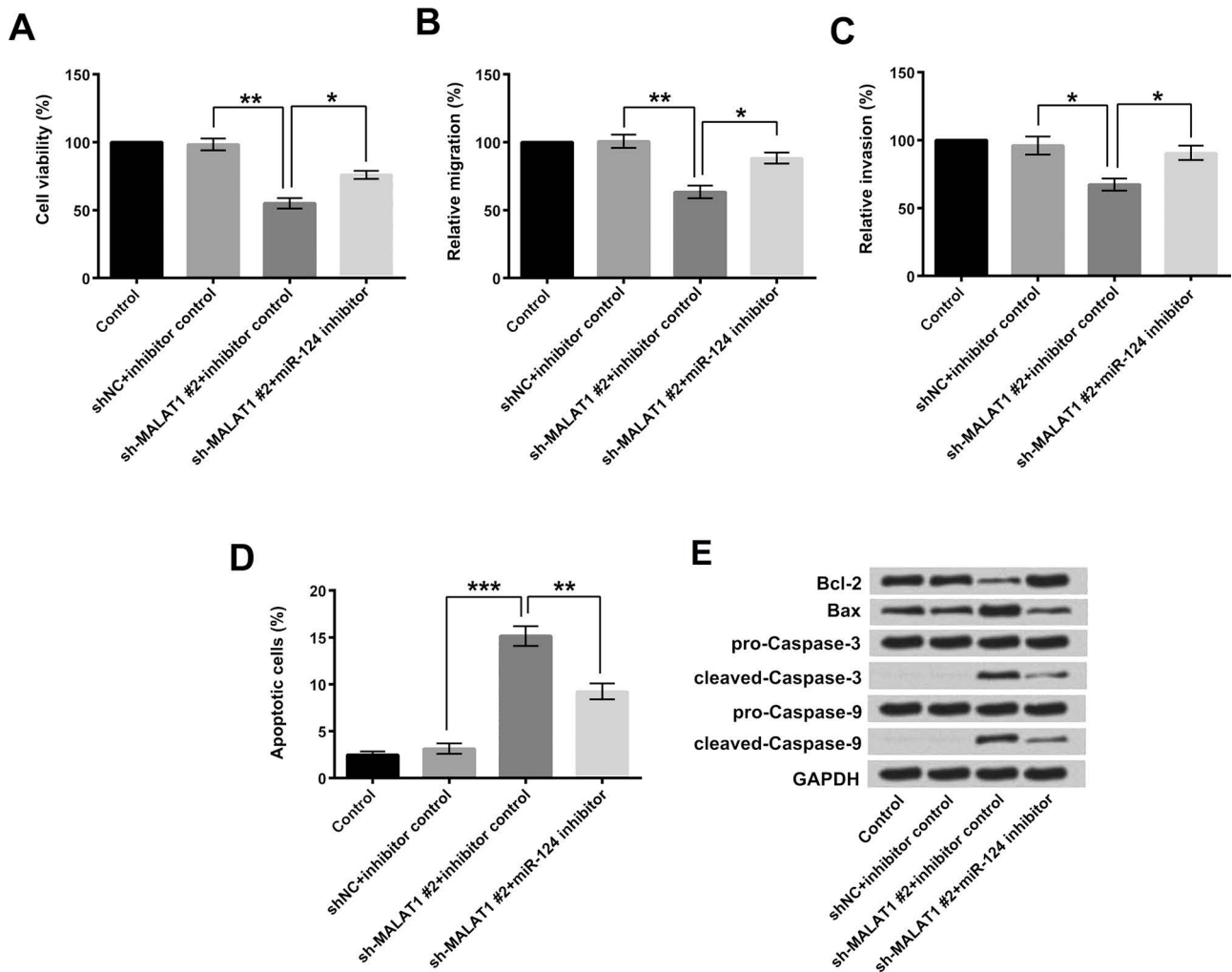
miR-124 was hypothesized to be a potential target of MALAT1. MALAT1 3'-untranslated region (3'-UTR) was highly conserved for miR-124 (Fig. 3A). To verify whether miR-124 was able to directly bind to its seed sequences in the MALAT1 3'-UTR in Y79 cells, MALAT1-wt and MALAT1-mt containing the wild-type and mutant binding sequences of miR-124 within the 3'-UTR of MALAT1 mRNA were generated, respectively (Fig. 3B). A luciferase reporter assay revealed that the luciferase activity was significantly reduced in Y79 cells when cotransfected with MALAT1-wt in miR-124 mimic compared with the NC group ( $p < 0.05$ ). The data indicate that miR-124 was able to directly bind to its seed sequences in the MALAT1 3'-UTR in Y79 cells. In addition, the result demonstrated that MALAT1#2 silence negatively regulated the expression of miR-124, which suggested that miR-124 was upregulated by the suppression of MALAT1 ( $p < 0.001$ ) (Fig. 3C). Therefore, these data indicated that miR-124 was identified as a target of MALAT1 in Y79 cells and was negatively regulated by MALAT1.

*miR-124 Suppression Abolished the Effect of MALAT1 Silencing on Cell Viability, Migration, and Invasion and Promoted Apoptosis*

miR-124 inhibitor+ sh-MALAT1#2 promoted cell viability when compared with MALAT1#2 silence



**Figure 3.** miR-124 was a target of MALAT1. (A) miR-124 was a target of MALAT1. (B) Dual-Luciferase Reporter Assay was executed to confirm that miR-124 was a target of MALAT1. (C) miR-124 was upregulated by MALAT1 silence. \* $p < 0.05$ ; \*\*\* $p < 0.001$ .



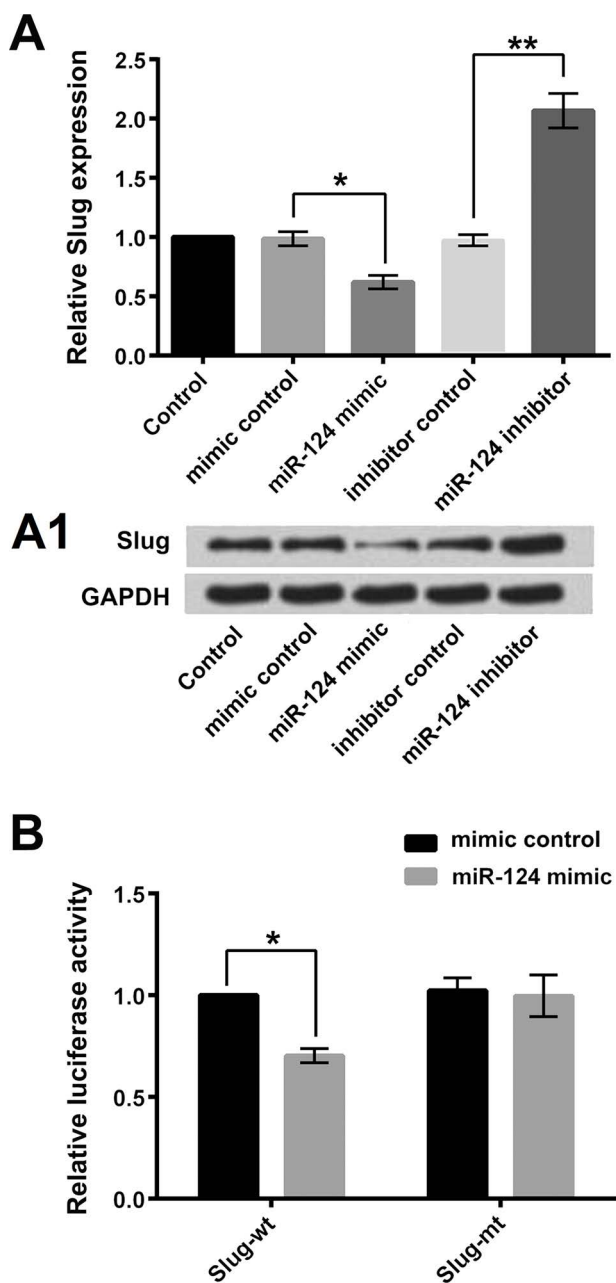
**Figure 4.** miR-124 was upregulated by MALAT1 silence, which inhibited cell viability, migration, and invasion and promoted apoptosis. (A) miR-124 was upregulated by MALAT1 silence, which inhibited cell viability; suppression of miR-124 promoted cell viability. (B) miR-124 was upregulated by MALAT1 silence, which inhibited cell migration; suppression of miR-124 promoted cell migration. (C) miR-124 was upregulated by MALAT1 silence, which inhibited cell invasion; suppression of miR-124 promoted cell invasion. (D) miR-124 was upregulated by MALAT1 silence, which promoted cell apoptosis; suppression of miR-124 inhibited cell apoptosis. (E) Western blotting analysis of miR-124 was upregulated by MALAT1 silence, which promoted cell apoptosis; suppression of miR-124 inhibited cell apoptosis. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

( $p < 0.05$ ) (Fig. 4A). In addition, miR-124 inhibitor+sh-MALAT1#2 also promoted cell migration and invasion when compared with their controls ( $p < 0.05$  or  $p < 0.01$ ) (Fig. 4B and C). The results of apoptosis assay revealed that miR-124 inhibitor+sh-MALAT1#2 inhibited cell apoptosis, and similar results were observed by Western blot analysis, which demonstrated an increased expression of Bcl-2 and reduced expression of Bax in the sh-MALAT1#2+miR-124 inhibitor ( $p < 0.01$  or  $p < 0.001$ ) (Fig. 4D and E). Thus, it could be suggested that the beneficial role of miR-124 was mediated via MALAT1 silence. These data suggested that miR-124 suppression reduced cell apoptosis and abolished the inhibitory

effects of MALAT1#2 silence on cell viability, migration, and invasion.

#### miR-124 Regulated Expression of Slug

In this context, miR-124 overexpression upregulated Slug expression, but suppression downregulated Slug expression when compared with their corresponding controls ( $p < 0.05$  or  $p < 0.01$ ) (Fig. 5A). Similar results were obtained by Western blotting analysis (Fig. 5A1). To verify whether Slug could directly bind to its seed sequences in the c-Slug 3'-UTR in Y79 cells, Slug-wt and Slug-mt containing the wild-type and mutant binding sequences of Slug within the 3'-UTR of miR-124 mRNA



**Figure 5.** Slug was a target of miR-124. (A) miR-124 negatively regulated the expression of Slug. (A1) Western blotting analysis of miR-124 negatively regulated the expression of Slug. (B) Slug was a target of miR-124. \* $p < 0.05$ ; \*\* $p < 0.01$ .

were generated, respectively. A luciferase reporter assay revealed that the luciferase activity was significantly reduced in Y79 cells when cotransfected with Slug-wt compared with the NC group ( $p < 0.05$ ). However, the luciferase activity revealed no significant difference in Y79 cells cotransfected with Slug-mt when compared with the control group (Fig. 5B). The data indicated

that Slug was able to directly bind to its seed sequences in the Slug 3'-UTR in Y79 cells. Therefore, Slug was identified as a target of miR-124 in Y79 cells.

*Slug Regulated the Cell Viability, Migration, Invasion, and Apoptosis in RB Cells*

The effects of miR-124 and Slug on the proliferation, migration, invasion, and apoptosis of RB cells were investigated. The results identified that overexpression of miR-124 downregulated the expression of Slug ( $p < 0.05$ ) (Fig. 6A). Similar results were obtained by Western blot analysis (Fig. 6A1). Moreover, overexpression of miR-124 inhibited cell viability, migration, and invasion in Y79 cells ( $p < 0.05$  or  $p < 0.01$ ) (Fig. 6B–D, respectively). Apoptosis results showed that miR-124 was downregulated by overexpression of Slug, which promoted cell apoptosis in RB cells ( $p < 0.05$ ) (Fig. 6E). Similar results were obtained by Western blot analysis, which demonstrated reduced Bcl-2 (antiapoptotic factor) expression levels and increased Bax (proapoptotic factor) expression levels in the miR-124 inhibitor+si-Slug (Fig. 6F). However, the upregulation of miR-124 overexpression on RB cell proliferation, migration, and invasion was reversed by the suppression of Slug. The data suggested that miR-124 inhibited RB cell proliferation and metastasis and promoted apoptosis in Y79 cells via targeting Slug.

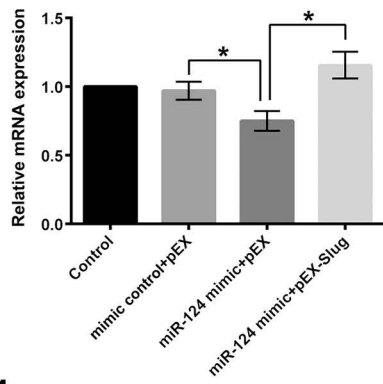
*ERK/MAPK and Wnt/ $\beta$ -Catenin Pathways*

Western blot analysis was performed to analyze the role of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and Wnt/ $\beta$ -catenin pathways in RB. The results demonstrated that overexpression of miR-124 inactivated the ERK/MAPK and Wnt/ $\beta$ -catenin pathways by downregulating Slug, which might be involved in cell viability, migration, invasion, and apoptosis in Y79 cells (Fig. 7).

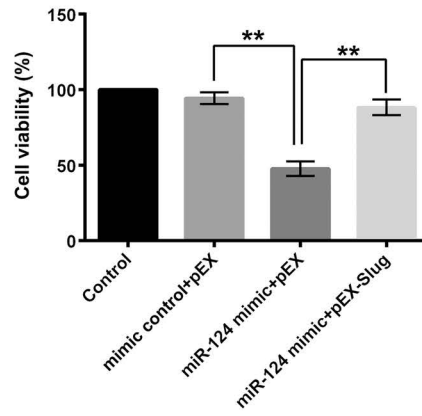
**DISCUSSION**

Studies have shown that miRNAs act either as tumor suppressors or as oncogenes in cancer<sup>12,14,15,34</sup>. To gain an insight into the roles of miR-124 in modulating RB cells, we first measured the effect of miR-124 expression in RB cells. The effects and mechanisms of lncRNA MALAT1 on the growth and apoptosis in RB were studied. Studies have shown that MALAT1 silence inhibited cell viability, migration, and invasion and promoted apoptosis in Y79 cells. Further results showed that miR-124 was upregulated by MALAT1 silence, and miR-124 was a target of MALAT1. Moreover, we found that miR-124 negatively regulated the expression of Slug, establishing Slug as a target of miR-124. Slug in turn inactivated the ERK/MAPK and Wnt/ $\beta$ -catenin pathways, which might

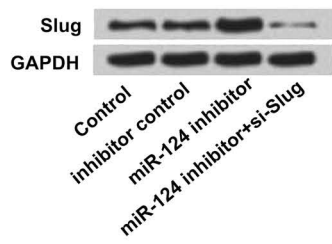
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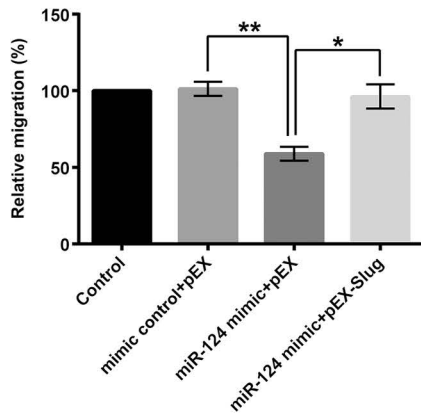
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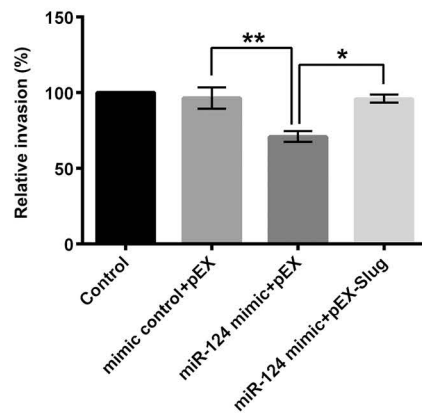
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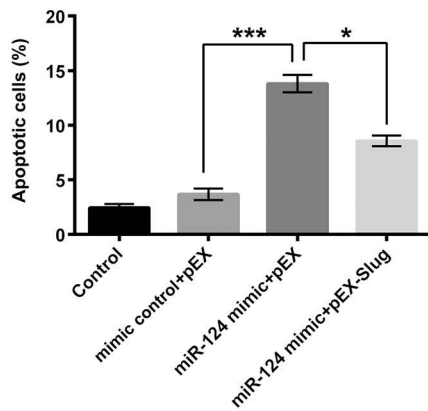
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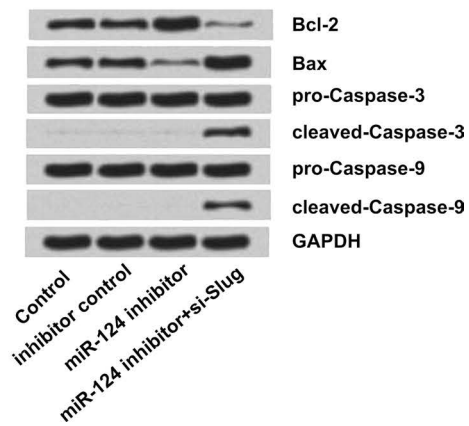
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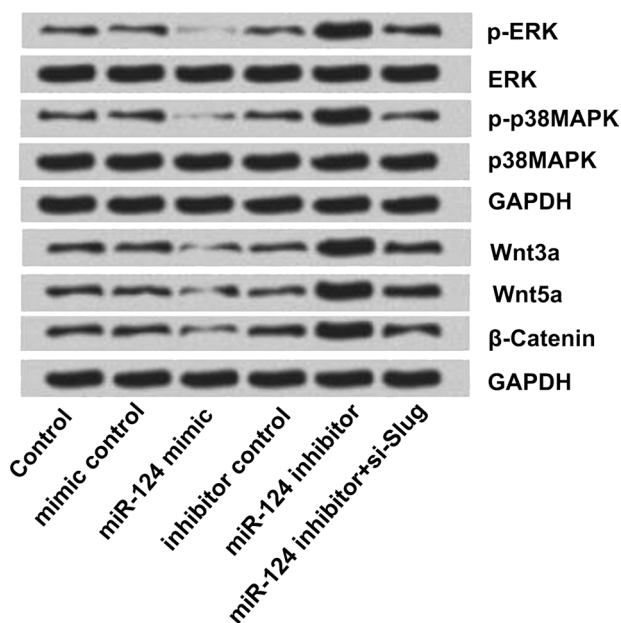


be involved in the regulation of cell viability, migration, invasion, and apoptosis in Y79 cells.

According to the study by Liu et al., the ectopic expression of miR-124 in the RB cell lines suppressed cell proliferation, migration, and invasion and induced cell apoptosis in vitro.<sup>11</sup> Our results were consistent with these findings.

lncRNAs are defined as endogenous cellular RNAs with more than 200 nucleotides in length that lack an open reading frame of significant length. lncRNAs can regulate the protein-coding genes at epigenetic, transcriptional, and posttranscriptional levels and play a central role in several physiological processes<sup>35,36</sup>. In our present work, we have found that MALAT1 expression was significantly upregulated in RB cells, and MALAT1-siRNA significantly inhibited cell proliferation after transfection into Y79 cells. Recent studies reported that MALAT1 was involved in tumor progression<sup>27,37,38</sup>. In this study, we found that MALAT1 silence upregulated miR-124 expression in RB cells, which reversed the inhibitory effect of miR-124 on tumor growth of RB cells. A previous study by Liu et al. reported that the MALAT1-miR-124-RBG2 axis was involved in the growth and invasion of HR-HPV<sup>+</sup> cervical cancer cells<sup>39</sup>. Our study found that MALAT1 expression could be regulated in RB cells treated with miR-124 overexpression.

Slug is generally associated with epithelial-mesenchymal transition (EMT) during both embryonic development and cancer metastasis<sup>23</sup>. Slug directly represses the transcription of E-cadherin, which acts as a repressor of invasion and metastasis, as well as a repressor of a subset of genes that encode cadherins, claudins, and cytokeratins to induce EMT. The Slug gene is located on human chromosome8q11.21. Comparative genomic hybridization showed the involvement of this gene in many types of cancer<sup>40</sup>. Slug is a labile protein with a short half-life. We confirmed that miR-124 directly targets Slug by binding to its 3'-UTR. Therefore, downregulation of miR-124 may contribute to the increased expression of Slug at the posttranscription level and in turn facilitate retinal carcinogenesis and progression. Moreover, this study has identified Slug as the direct target of miR-124 in RB cells; its downregulation by miR-124 increases the expression of E-cadherin, which is considered as a hallmark of epithelial cells and also a repressor of cell invasion and metastasis. These findings suggest that miR-124 plays an important role in the invasive and/or metastatic potential of RB.



**Figure 7.** Overexpression of miR-124 inactivated the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and Wnt/ $\beta$ -catenin pathways by downregulation of Slug.

Recent evidence suggested that Slug acts as a potent inducer of cell movement<sup>41</sup>. According to Chen et al., the ERK/MAPK pathway was regulated in breast cancer cells by maintaining Slug expression<sup>42</sup>. A study by Prasad et al. demonstrated that Slug-mediated E-cadherin suppression was done via the activation of the Wnt/ $\beta$ -catenin signaling pathway in invasive ductal carcinoma of the breast<sup>43</sup>. The Wnt/ $\beta$ -catenin canonical pathway has its role in carcinogenesis and also in embryonic development. It controls the embryonic processes such as cell proliferation and migration. MAPK pathways have provocative roles that impacted Wnt/ $\beta$ -catenin signaling and were observed in many cancer cells. The MAPK network involves ERK and their downstream signaling elements in Wnt/ $\beta$ -catenin signaling. In the present study, we found that Slug inactivated the ERK/MAPK and Wnt/ $\beta$ -catenin pathways, which in turn inhibited the proliferation, invasion, and migration of RB cells. Taken together, these findings suggested that lncRNA MALAT1 can be considered as a novel target of miR-124.

**FACING PAGE**

**Figure 6.** Slug regulated the cell viability, migration, invasion, and apoptosis in RB cells. (A) Overexpression of miR-124 downregulated the expression of Slug. (A1) Western blotting analysis of overexpression of miR-124 downregulated the expression of Slug. (B) Overexpression of miR-124 inhibited cell viability by downregulation of Slug. (C) Overexpression of miR-124 inhibited cell migration by downregulation of Slug. (D) Overexpression of miR-124 inhibited cell invasion by downregulation of Slug. (E) Overexpression of miR-124 promoted cell apoptosis by downregulation of Slug. (F) Western blotting analysis of overexpression of miR-124 promoted cell apoptosis by downregulation of Slug. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

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