

# Long noncoding RNA *SNHG14* promotes the aggressiveness of retinoblastoma by sponging microRNA-124 and thereby upregulating STAT3

XIAOWEN SUN<sup>1</sup>, HUI SHEN<sup>1</sup>, SHUBIN LIU<sup>2</sup>, JING GAO<sup>3</sup> and SHUYAN ZHANG<sup>1</sup>

<sup>1</sup>Department of Ophthalmology, People's Hospital of Rizhao, Rizhao, Shandong 276800; <sup>2</sup>Department of Oncology, Binzhou People's Hospital, Binzhou, Shandong 256610; <sup>3</sup>Department of Ophthalmology, Weifang Ophthalmic Hospital, Weifang, Shandong 261041, P.R. China

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**Abstract.** A long noncoding RNA called small nucleolar RNA host gene 14 (*SNHG14*) has been validated as a key regulator of cellular processes in multiple types of human cancer. However, to the best of our knowledge, the expression status and specific roles of *SNHG14* in retinoblastoma (RB) have not been studied. The aims of the present study were to determine the expression status of *SNHG14* in RB, assess the effects of *SNHG14* on malignant characteristics of RB cells and investigate the mechanisms of action of *SNHG14* in RB. *SNHG14* expression levels in RB tissue samples and cell lines were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cell proliferation, apoptosis, migration and invasion *in vitro*, and tumor growth *in vivo* were quantitated by the Cell Counting Kit-8 assay, flow cytometry, migration and invasion assays, and mouse tumor xenograft experiments, respectively. The target microRNA (miRNA) of *SNHG14* was predicted by bioinformatics analysis and was subsequently validated by a luciferase reporter assay, RNA immunoprecipitation (RIP) assay, RT-qPCR, and western blot analysis. *SNHG14* was identified to be significantly overexpressed in RB tissues and cell lines. *SNHG14* overexpression was markedly associated with the intraocular international retinoblastoma classification stage, optic nerve invasion, and differentiation grade among patients with RB. The patients in the *SNHG14* high-expression group exhibited shorter overall survival compared with the *SNHG14* low-expression group. Functional analysis revealed that *SNHG14* silencing inhibited cell proliferation, migration

and invasion, and increased apoptosis *in vitro*, and decreased tumor growth *in vivo*. *SNHG14* directly interacted with, and functioned as a competing endogenous RNA (ceRNA) of, miR-124, consequently upregulating signal transducer and activator of transcription 3 (STAT3). miR-124 inhibition and STAT3 expression recovery attenuated the effects of the *SNHG14* silencing on RB cells. In conclusion, *SNHG14* served as a ceRNA to upregulate STAT3 by sponging miR-124. Therefore, targeting the *SNHG14*/miR-124/STAT3 pathway may be an effective therapeutic strategy against RB.

## Introduction

Retinoblastoma (RB), a malignant tumor derived from photoreceptor precursor cells, is the most common intraocular human cancer among infants and children (1). It accounts for ~4% of all pediatric human malignant tumors (2). The typical clinical manifestations of RB are leukocoria and strabismus, and its morbidity rate ranges from 1/15,000-1/20,000 among live births worldwide (3). At present, the primary therapeutic techniques for patients with RB are enucleation, laser photocoagulation, chemotherapy and focal therapy (4). The pathogenesis of RB is a complicated process that involves gene mutations, activation of oncogenes, and inactivation of tumor suppressor genes (5-7); however, the detailed mechanism is yet to be determined. Although the primary option for RB management has yielded satisfactory results for the patients, long-term survival is poor among the majority of patients with the RB diagnosed at advanced stages (8). Therefore, further studies on the mechanisms of the genesis and progression of RB are urgently required for identifying early interventions and therapeutic targets.

Long noncoding RNAs (lncRNAs) measure >200 nucleotides long and have no protein-coding ability (9). They can regulate gene expression at epigenetic, transcriptional and post-transcriptional levels (10). lncRNAs have been demonstrated to exert regulatory effects on the initiation and progression of various human cancer types (11-13). Particularly, a number of lncRNAs are dysregulated in RB and serve either tumor-suppressive or oncogenic roles. For example, small nucleolar RNA host gene 16 (14), homeobox A11 antisense

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*Correspondence to:* Professor Shuyan Zhang, Department of Ophthalmology, People's Hospital of Rizhao, 126 Taian Road, Rizhao, Shandong 276800, P.R. China  
E-mail: zeyeye01@163.com

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RNA (15), and FEZ family zinc finger 1 antisense RNA 1 (16) are overexpressed in RB and promote its aggressive characteristics. By contrast, Pvt1 oncogene (17), metallothionein 1J, pseudogene (18), and H19 imprinted maternally expressed transcript (19) are expressed weakly and inhibit RB carcinogenesis. Considering the important activities of lncRNAs in RB, these polynucleotides may be potential targets for the diagnosis, therapy and prognosis of RB.

MicroRNAs (miRNAs) are a class of noncoding small RNAs that act as gene expression regulators and have been studied extensively in the last decade (20). miRNAs participate in the modulation of gene expression by directly interacting with the 3'-untranslated region of their target mRNAs, thereby inhibiting translation or facilitating mRNA degradation (21). miRNAs are widely expressed in eukaryotes and are involved in a variety of pathological processes, including tumorigenesis and tumor progression (22,23). In recent years, an increasing number of studies revealed that a variety of miRNAs are aberrantly expressed and perform an important function in the malignancy of RB (24-26). Therefore, miRNAs involved in RB carcinogenesis and progression should be explored to facilitate the identification of effective diagnostic biomarkers and therapeutic strategies.

An lncRNA called small nucleolar RNA host gene 14 (*SNHG14*) has been validated as a key regulator of cellular processes in multiple types of human cancer (27-35). However, to the best of our knowledge, the expression status and detailed roles of *SNHG14* in RB have not been determined. The present study first investigated whether *SNHG14* is dysregulated in RB by measuring its expression in RB tissue samples and cell lines. Secondly, functional assays were performed to determine the biological activities of *SNHG14* in RB cells. Third, the regulatory mechanisms of action of *SNHG14* in RB were explored. These analyses identified the involvement of the *SNHG14*/miR-124/signal transducer and activator of transcription 3 (STAT3) pathway in RB and may have revealed a new mechanism underlying RB carcinogenesis and progression. Among the miRNAs that may interact with *SNHG14*, miR-124 was selected for validation as it has been demonstrated to be downregulated in RB and to exert tumor-suppressive actions (36).

## Materials and methods

**Patients and tissue samples.** In total, 43 RB tissue samples were obtained from patients (mean age, 11 years; age range, 1-34 years; 21: 22 male: Female ratio) with RB between February 2012 and May 2014 at the People's Hospital of Rizhao. Normal retinas were collected from 11 patients (mean age, 28 years; age range, 17-49 years; 7: 4 male: Female ratio) with globe rupture between April 2012 and August 2016. All the patients were treated with enucleation and had not received preoperative radiotherapy or chemotherapy. Patients that had been treated with radiotherapy, chemotherapy or other anti-cancer therapies were excluded from the study. International Intraocular Retinoblastoma Classification (IIRC) was utilized to classify the severity of RB (37). Following surgical resection, all tissue samples were immediately immersed into liquid nitrogen and stored at -80°C for subsequent analysis. The present study was conducted with the approval of the Clinical

Research Ethics Committee of People's Hospital of Rizhao and following the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients or their parents/legal guardians prior to surgical resection.

**Cell lines and transient transfection.** A total of 3 RB cell lines (Y79, SO-RB50 and Weri-RB-1) and the normal retinal pigmented epithelial ARPE-19 cell line were purchased from the American Type Culture Collection and were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of a penicillin-streptomycin solution (all from Gibco; Thermo Fisher Scientific, Inc.).

To induce *SNHG14* knockdown, small interfering RNA (siRNA) targeting *SNHG14* (si-SNHG14) and its negative control (NC) nonsense sequence (si-NC) were chemically synthesized by Guangzhou RiboBio Co., Ltd. The si-SNHG14 sequence was 5'-GCACAAUAUCUUUGAACUA-3' and the si-NC sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. An miR-124 agomir (agomir-124), NC agomir (agomir-NC), miR-124 antagomir (antagomir-124), and antagomir-NC were purchased from Shanghai GenePharma Co., Ltd. The agomir-124 sequence was 5'-CCGUAAGUGGCGCACGGA AU-3' and the agomir-NC sequence was 5'-UUGUACUAC ACAAAGUACUG-3'. The antagomir-124 sequence was 5'-GGCAUUCACCGCGUGCCUUA-3' and the antagomir-NC sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. The empty pcDNA3.1 vector and pcDNA3.1 carrying the full-length *STAT3* sequence (pcDNA3.1-STAT3, hereafter: Pc-STAT3) were purchased from Shanghai GeneChem Co., Ltd. The aforementioned siRNAs (100 pmol), agomir (50 nM), and/or plasmid (4 µg) were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Transfected cells were cultured at 7°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR), flow cytometry analysis, migration and invasion assays were performed at 48 h post-transfection. A Cell Counting Kit-8 (CCK-8) assay and western blot analysis were conducted at 24 and 72 h after transfection, respectively. The mouse xenograft tumor model was constructed after 24 h culture.

**RT-qPCR.** TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for total RNA isolation from tissues or cells. To determine miR-124 expression, total RNA was reverse-transcribed into complementary DNA using the miScript Reverse Transcription kit (Qiagen GmbH). The temperature protocols for reverse transcription were as follows: 37°C for 60 min, 95°C for 5 min and maintenance at 4°C. Following this step, the complementary DNA was analyzed by qPCR using the miScript SYBR-Green PCR kit (Qiagen GmbH). The thermocycling conditions were as follows: 95°C for 2 min, followed by 95°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, for 40 cycles. Small nuclear RNA U6 served as the endogenous control for miR-124 expression. To examine *SNHG14* expression, the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) and the SYBR Premix Ex Taq™ kit (Takara Biotechnology Co., Ltd.) were utilized for reverse transcription and qPCR, respectively.

The temperature protocols for reverse transcription were as follows: 37°C for 15 min and 85°C for 5 sec, and the thermocycling conditions for the qPCR step were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. *SNHG14* expression was normalized to that of *GAPDH*. All reactions were carried out on the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, Inc.). Relative gene expression was analyzed using the  $2^{-\Delta\Delta C_q}$  method (38).

The primers were designed as follows: miR-124 forward, 5'-GCTAAGGCACGCGGTG-3'; miR-124 reverse, 5'-GTG CAGGGTCCGAGGT-3'; U6 forward, 5'-CTCGCTTCGGCA GCACATATACT-3'; U6 reverse, 5'-ACGCTTCACGAATTT GCGTGTC-3'; *SNHG14* forward, 5'-GGGTGTTTACGTAGA CCAGAACC-3'; *SNHG14* reverse, 5'-CTTCCAAAAGCC TTCTGCCTTAG-3'; *GAPDH* forward, 5'-GCACCGTCA AGGCTGAGAAC-3'; and *GAPDH* reverse, 5'-AGGGATCTC GCTCCTGGAA-3'.

**CCK-8 assay.** Transfected cells were collected at 24 h post-transfection and seeded at 2,000/well in 96-well plates. The CCK-8 assay was conducted to measure cellular proliferation at 4 time points: 0, 24, 48 and 72 h after seeding. At these time points, the culture medium was replaced with fresh DMEM containing 10  $\mu$ l CCK-8 solution (Dojindo Molecular Technologies, Inc.), followed by incubation for 2 h at 37°C and 5% CO<sub>2</sub>. The optical density value was then detected at a wavelength of 450 nm. Each assay was performed in triplicate, and every group was analyzed in 3 replicate wells.

**Flow-cytometric analysis of apoptosis.** A total of 1.5x10<sup>6</sup> transfected cells were incubated with 0.25% trypsin, harvested and rinsed twice with pre-chilled PBS. Then, the apoptotic rate was evaluated with the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BioLegend, Inc.). Briefly, transfected cells were resuspended in 100  $\mu$ l binding buffer prior to counterstaining with 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide solution. The stained cells were analyzed on a flow cytometer (FACScan; BD Biosciences). Data were analyzed using CellQuest™ software v.5.1 (BD Biosciences).

**Migration and invasion assays.** A total of 5x10<sup>4</sup> cells were resuspended in 200  $\mu$ l FBS-free DMEM and were subjected to the migration and invasion assays. For the migration assay, the cells were inoculated into the upper chambers of Transwell inserts (8  $\mu$ m pore size; Corning Incorporated), while Matrigel (BD Biosciences)-precoated Transwell inserts were employed for determining the invasive ability and were loaded with equal numbers of cells.

A total of 50  $\mu$ l Matrigel was evenly smeared onto the upper chambers of the Transwell inserts and incubated at 37°C for 1 h. The complete culture medium (supplemented with 20% of FBS) was added into the lower chambers and functioned as a chemoattractant. After 24 h incubation, the non-traversing cells remaining on the upper surface of the insert were gently wiped off with a cotton swab. The migratory and invasive cells were fixed in 4% formaldehyde at room temperature for 15 min and stained in a 0.1% crystal violet solution at room temperature for 15 min. Following extensive washing (5 min), the migratory and invasive cells were counted in 5 randomly

selected visual fields under an IX71 inverted light microscope (Olympus Corporation) at magnification, x200.

**Mouse xenograft tumor model.** All experimental procedures involving animals were conducted in accordance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals (39,40) and were approved by the Animal Care Committee of People's Hospital of Rizhao. A total of 6 female BALB/c mice (average weight, 20 g; age, 4-6 weeks) were purchased from the Animal Experimental Center of Jilin University and were randomly assigned to two groups: Si-*SNHG14* and si-NC. In the si-*SNHG14* group, a total of 5x10<sup>6</sup> si-*SNHG14*-transfected cells were injected subcutaneously into the flank of the mice, whereas si-NC-transfected cells were injected into the flank of mice in the si-NC group. Each group contained 3 nude mice. The animals were maintained under specific pathogen-free conditions at 25°C and 50% humidity, with a 10: 14 light: Dark cycle and *ad libitum* food/water access. The width and length of the resultant tumor xenografts were monitored with an interval of 4 days, and their volume was calculated via the following formula: Volume = (length x width<sup>2</sup>)/2. A total of 4 weeks after injection, all mice were euthanized via cervical dislocation; tumor xenografts were then excised and weighed. The humane endpoints used in the study included: Tumor diameter >15 mm; tumor ulceration; and extreme weight loss. None of these endpoints were observed in the mice during the study period.

**RNA immunoprecipitation (RIP) assay.** This assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (cat. no. 17-700; EMD Millipore). In brief, cells were incubated with RIP lysis buffer, and the obtained cellular lysates were next probed with magnetic beads conjugated with a human anti-AGO2 antibody or control IgG (1:5,000 dilution; cat. no. 03-110; EMD Millipore). The cell lysates were then treated with proteinase K buffer (150  $\mu$ l) at 55°C for 30 min to digest the protein. The expression of *SNHG14* and miR-124 in RIP-derived immunoprecipitated RNA was measured by RT-qPCR, as aforementioned.

**Bioinformatics analysis.** starBase 3.0 (<http://starbase.sysu.edu.cn/>), an open-source platform for studying the lncRNA-miRNA interactions, was applied to search for the target(s) of *SNHG14*.

**Luciferase reporter assay.** The fragments of *SNHG14* containing predicted wild-type (wt) and mutant (mut) miR-124-binding sequences were amplified by Shanghai GenePharma Co., Ltd., and inserted into the luciferase reporter gene of the pmirGLO vector (Promega Corporation), producing the reporter plasmids *SNHG14*-wt and *SNHG14*-mut, respectively. Following this, the generated plasmids were co-transfected with either agomir-124 or agomir-NC into RB cells using Lipofectamine 2000. After 48 h of incubation, firefly luciferase activity was normalized to that of *Renilla* luciferase as determined with the Dual-Luciferase® Reporter Assay kit (Promega Corporation).

**Western blot analysis.** Radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) was used for the extraction of total protein. The concentration of total protein was

quantified via the BCA kit (Beyotime Institute of Biotechnology). Equal amounts of proteins (30  $\mu$ g) were loaded, separated by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (EMD Millipore), followed by blocking for 2 h with 5% fat-free milk at room temperature and probing with primary antibodies overnight 4°C. Next, after 2 h incubation with a goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (cat. no. sc-516102; 1:5,000 dilution; Santa Cruz Biotechnology, Inc.) at room temperature, the protein signals were detected using the Pierce™ ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). The primary antibodies used in this assay included anti-STAT3 (cat. no. sc-8019; 1:1,000 dilution; Santa Cruz Biotechnology Inc.) and anti-GAPDH (cat. no. sc-47724; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.). GAPDH served as an endogenous control for the quantification of STAT3 protein expression. Quantity One software v.4.62 (Bio-Rad Laboratories, Inc.) was used for densitometry analysis.

**Statistical analysis.** Data were presented as mean  $\pm$  standard deviation, and analyzed using SPSS statistics software (version 21.0; IBM Corp.). The comparisons between two groups were conducted via Student's t-test, whereas comparisons between multiple groups were performed by one-way analysis of variance followed by Bonferroni's post hoc test. The association between *SNHG14* expression and clinical parameters among the patients with RB was assessed by the  $\chi^2$  test. Spearman's correlation between *SNHG14* and miR-124 expression levels in RB tissues was calculated next. Survival curves were created by Kaplan-Meier analysis and compared using the log-rank test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

***SNHG14* expression is high in RB tumors and cell lines.** The expression of *SNHG14* in the RB tissue samples and normal retinas was examined by RT-qPCR. The results indicated that the expression of *SNHG14* was increased in RB tissue samples compared with in normal retinas (Fig. 1A). In addition, *SNHG14* expression was examined in 3 RB cell lines: Y79; SO-RB50; and Weri-RB-1. The normal retinal pigmented epithelial ARPE-19 cell line served as the control. As compared with the ARPE-19 cells, all 3 RB cell lines exhibited marked *SNHG14* upregulation (Fig. 1B). All of the patients in the study cohort were assigned to either an *SNHG14* high-expression or *SNHG14* low-expression group based on the median level of *SNHG14* among the RB tissue samples, which served as the cutoff value (median *SNHG14* expression in RB tissues; 2.21). The correlation between *SNHG14* levels and clinical parameters of the patients with RB was investigated. The analysis indicated that an increased level of *SNHG14* was significantly associated with the IIRC stage ( $P=0.004$ ), optic nerve invasion ( $P=0.006$ ) and differentiation grade ( $P=0.031$ ; Table I). Notably, the patients with RB in the *SNHG14* high-expression group exhibited shorter overall survival times compared with the patients in the *SNHG14* low-expression group (Fig. 1C;  $P=0.032$ ). These results implied that *SNHG14* may be implicated in RB carcinogenesis.

***Silencing of SNHG14* inhibits RB cell proliferation, migration and invasion, and promotes apoptosis.** As *SNHG14* was

Table I. Associations between *SNHG14* expression and clinicopathological parameters of patients with retinoblastoma.

Parameters	<i>SNHG14</i> expression		P-value
	High (n=22)	Low (n=21)	
Age, years			0.537
<3	15	12	
$\geq 3$	7	9	
Sex			0.763
Male	10	11	
Female	12	10	
IIRC stage			0.004
Early stage (A, B, C)	3	12	
Advanced Stage (D, E)	19	9	
Optic nerve invasion			0.006
Negative	7	16	
Positive	15	5	
Differentiation grade			0.031
Well/moderately	9	16	
Poorly/undifferentiated	13	5	

*SNHG14*, small nucleolar RNA host gene 14.

identified to be markedly overexpressed in the Y79 and Weri-RB-1 cell lines, these 2 cell lines were selected for subsequent experiments. To examine the functions of *SNHG14* in RB progression, si-*SNHG14* was transfected into the Y79 and Weri-RB-1 cells to silence endogenous *SNHG14* expression. The transfection efficiency was verified by RT-qPCR, which indicated that *SNHG14* was knocked down efficiently in the Y79 and Weri-RB-1 cells following transfection with si-*SNHG14* (Fig. 2A). The CCK-8 assay was performed to quantify the effect of *SNHG14* silencing on RB cell proliferation, and it was revealed that the downregulation of *SNHG14* inhibited the proliferation of the Y79 and Weri-RB-1 cells (Fig. 2B). The apoptosis status of the *SNHG14*-deficient Y79 and Weri-RB-1 cells was examined via flow cytometry. Silencing of *SNHG14* enhanced the apoptosis levels of the Y79 and Weri-RB-1 cells (Fig. 2C). Furthermore, the migration and invasion assays revealed that the migratory (Fig. 2D) and invasive (Fig. 2E) abilities were markedly suppressed by *SNHG14* silencing. Overall, these results suggested that *SNHG14* knockdown inhibited the malignant properties of RB cells *in vitro*.

***SNHG14* functions as a molecular sponge of miR-124 in RB cells.** Previous studies have indicated that lncRNAs are implicated in cancer progression by acting as competing endogenous RNAs (ceRNAs) on certain miRNAs, thereby relieving the miRNA-induced repression of their target genes (41-43). To elucidate the mechanisms by which *SNHG14* affected the malignant properties of RB cells, bioinformatics analysis was conducted to predict the miRNA(s) that can directly interact with *SNHG14*. Among

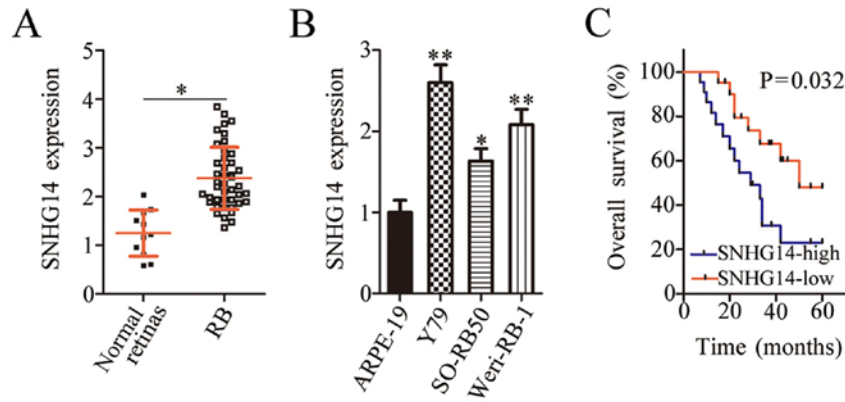


Figure 1. Upregulation of *SNHG14* is associated with poor patient survival in RB. (A) Relative *SNHG14* expression was measured by RT-qPCR assay in RB tissue samples (n=43) and normal retinas (n=11). \*P<0.05 vs. normal retinas. (B) RT-qPCR was performed to determine *SNHG14* expression in 3 RB Y79, SO-RB50 and Weri-RB-1 cell lines and the normal retinal pigmented epithelial ARPE-19 cell line. \*P<0.05 and \*\*P<0.01 vs. ARPE-19 cells. (C) Results from the Kaplan-Meier analysis of the overall survival of patients with RB were evaluated by the log-rank test. (P=0.032). *SNHG14*, small nucleolar RNA host gene 14; RB, retinoblastoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

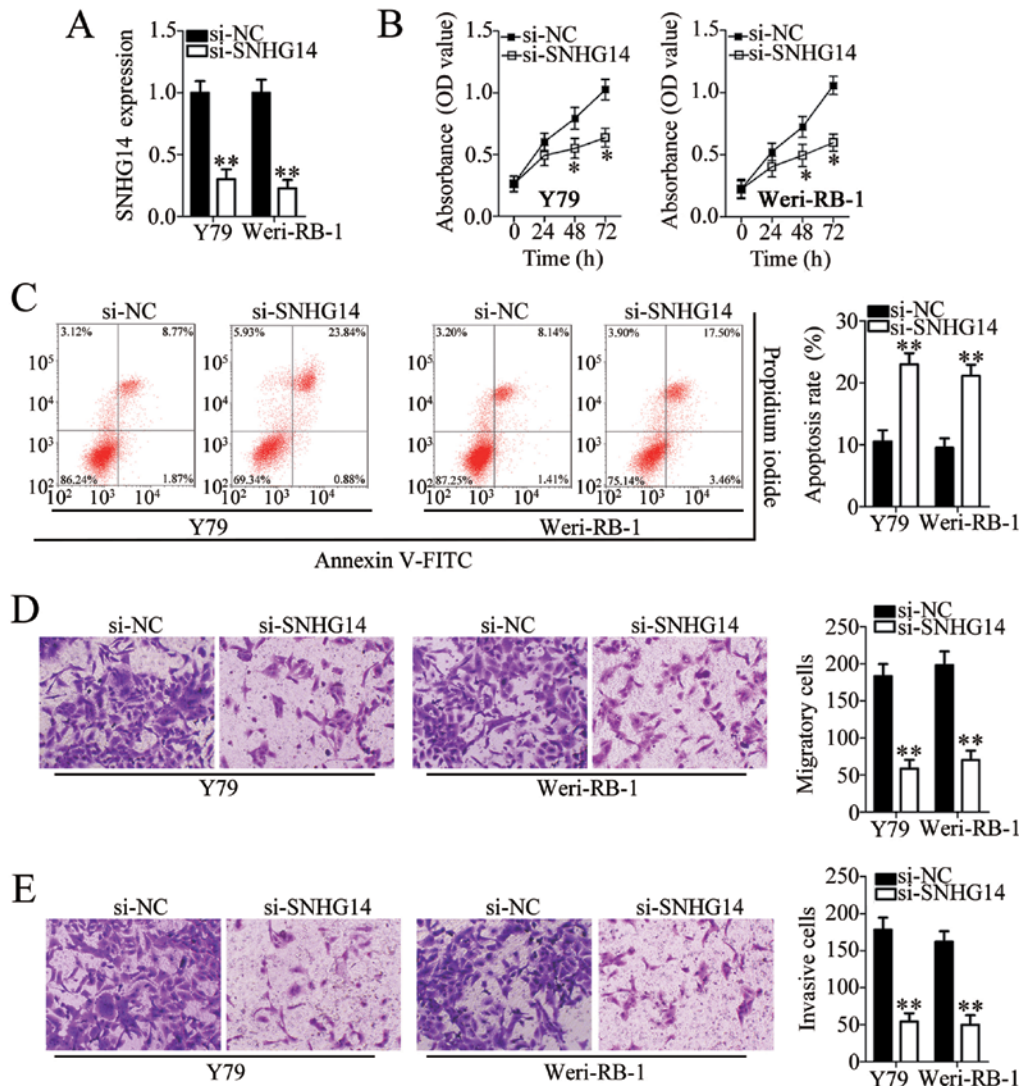


Figure 2. Silencing of *SNHG14* represses the proliferation, migration, and facilitates the apoptosis of Y79 and Weri-RB-1 cells. (A) Y79 and Weri-RB-1 cells were transfected with either si-SNHG14 or si-NC. Expression of *SNHG14* was analyzed at 48 h post-transfection. \*\*P<0.01 vs. the si-NC group. (B) Proliferation of *SNHG14*-deficient Y79 and Weri-RB-1 cells was quantitated in the Cell Counting Kit-8 assay. \*P<0.05 vs. the si-NC group. (C) Flow cytometry was utilized to measure the apoptotic rates of Y79 and Weri-RB-1 cells following either si-SNHG14 or si-NC transfection. \*\*P<0.01 vs. the si-NC group. (D and E) The levels of migration and invasion in the *SNHG14* knockdown Y79 and Weri-RB-1 cells were assessed using (D) Transwell migration and (E) invasion assays. Magnification, x200 magnification. \*\*P<0.01 vs. the si-NC group. *SNHG14*, small nucleolar RNA host gene 14; si, small interfering RNA; NC, negative control; FITC, fluorescein isothiocyanate.

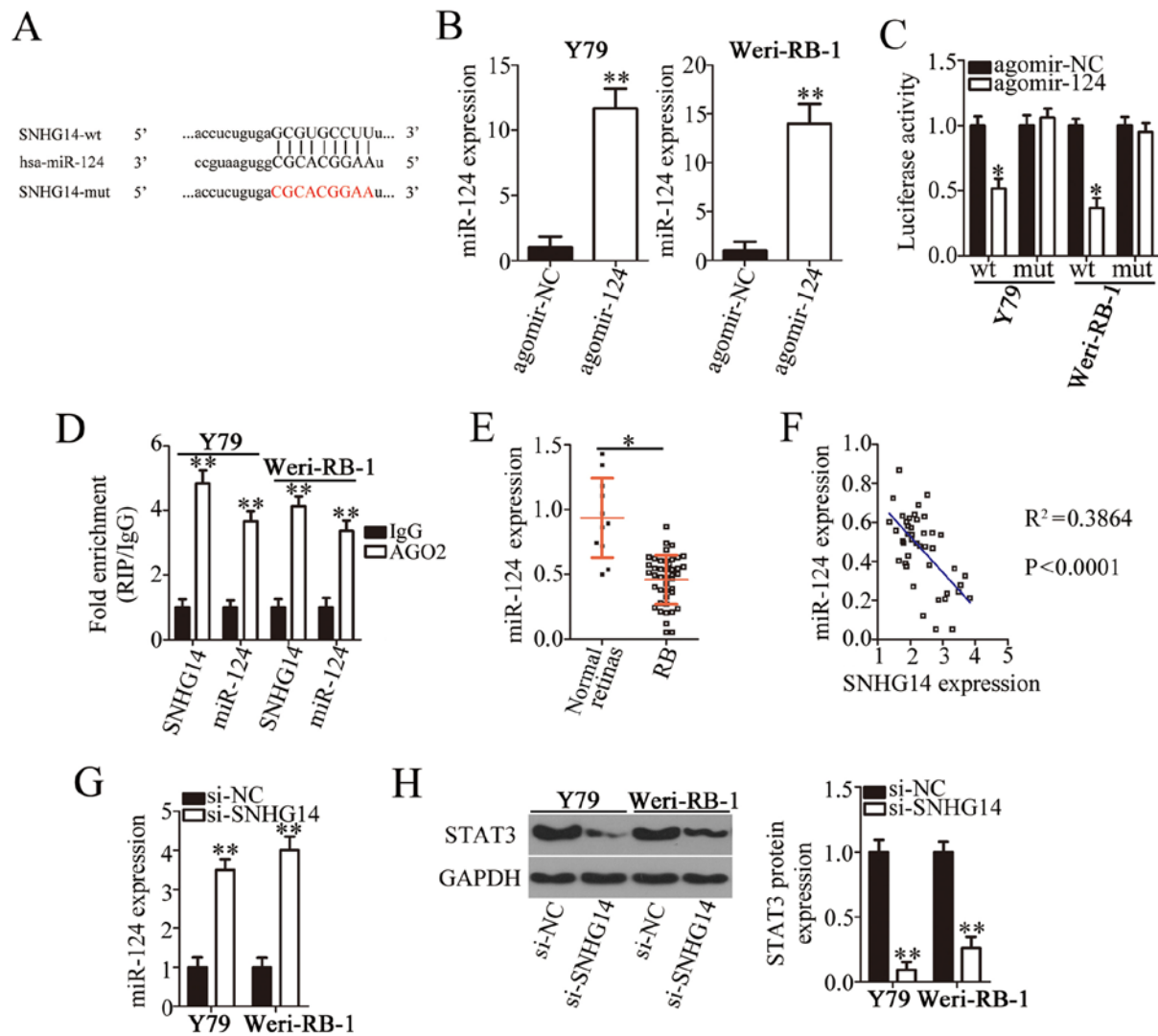


Figure 3. *SNHG14* functions as a sponge for miR-124 and thereby upregulates STAT3. (A) The predicted miR-124-binding sequences in *SNHG14* and the designed mut sequence (*SNHG14*-mut). (B) The expression of miR-124 was examined in Y79 and Weri-RB-1 cells that were transfected with either agomir-124 or agomir-NC. \*\* $P < 0.01$  vs. the agomir-NC group. (C) The luciferase reporter assay was conducted to evaluate the interaction between *SNHG14* and miR-124 in RB cells. Luciferase activities were determined in Y79 and Weri-RB-1 cells following co-transfection with either the *SNHG14*-wt or *SNHG14*-mut reporter plasmid and either agomir-124 or agomir-NC. \* $P < 0.05$  vs. the agomir-NC group. (D) The RIP assay was performed to validate the interaction between *SNHG14* and miR-124 in RB. *SNHG14* was identified to be preferentially enriched on AGO2-containing beads following the immunoprecipitation in the lysates of Y79 and Weri-RB-1 cells. \*\* $P < 0.01$  vs. the IgG group. (E) The expression of miR-124 was examined in RB tissue samples ( $n = 43$ ) and normal retinas ( $n = 11$ ). \* $P < 0.05$  vs. normal retinas. (F) The correlation between *SNHG14* and miR-124 levels in RB tissue samples was assessed by Spearman's correlation analysis.  $R^2 = 0.3864$ .  $P < 0.0001$ . (G) miR-124 expression in *SNHG14*-deficient Y79 and Weri-RB-1 cells was measured by reverse transcription-quantitative polymerase chain reaction. \*\* $P < 0.01$  vs. the si-NC group. (H) Western blot analysis was conducted to examine STAT3 protein expression in Y79 and Weri-RB-1 cells following transfection with either si-*SNHG14* or si-NC. \*\* $P < 0.01$  vs. the si-NC group. *SNHG14*, small nucleolar RNA host gene 14; miR, microRNA; STAT3, signal transducer and activator of transcription 3; wt, wild type; mut, mutant; NC, negative control; RB, retinoblastoma; RIP, RNA immunoprecipitation; si, small interfering RNA.

the candidates, miR-124 was selected for validation, as this miRNA has been demonstrated to be downregulated in RB and to exert tumor-suppressive actions (36). The putative binding site, the complementary sequence between *SNHG14* and miR-124, is presented in Fig. 3A. First, the transfection efficiency of agomir-124 was confirmed via RT-qPCR, and the results indicated that transfection with agomir-124 notably increased the expression of miR-124 in both the Y79 and Weri-RB-1 cells (Fig. 3B).

The luciferase reporter assay was performed to confirm the potential *SNHG14*/miR-124 interaction: Agomir-124 or agomir-NC and either *SNHG14*-wt or *SNHG14*-mut plasmids were co-transfected into the Y79 and Weri-RB-1 cells.

The luciferase activities of the Y79 and Weri-RB-1 cells transfected with the *SNHG14*-wt reporter plasmid were markedly decreased following miR-124 overexpression ( $P < 0.05$ ), whereas no change was observed in the cells transfected with the *SNHG14*-mut plasmid (Fig. 3C). The results from the RIP assay in the Y79 and Weri-RB-1 cells revealed that *SNHG14* was preferentially enriched on the AGO2-containing beads (Fig. 3D), suggesting that *SNHG14* is likely bound to the miR-124 RNA-induced silencing complex. Furthermore, miR-124 was much more weakly expressed in RB tissue samples in comparison with normal retinas (Fig. 3E), which was in agreement with a previous study (36). As demonstrated in Fig. 3F, Spearman's correlation analysis indicated an

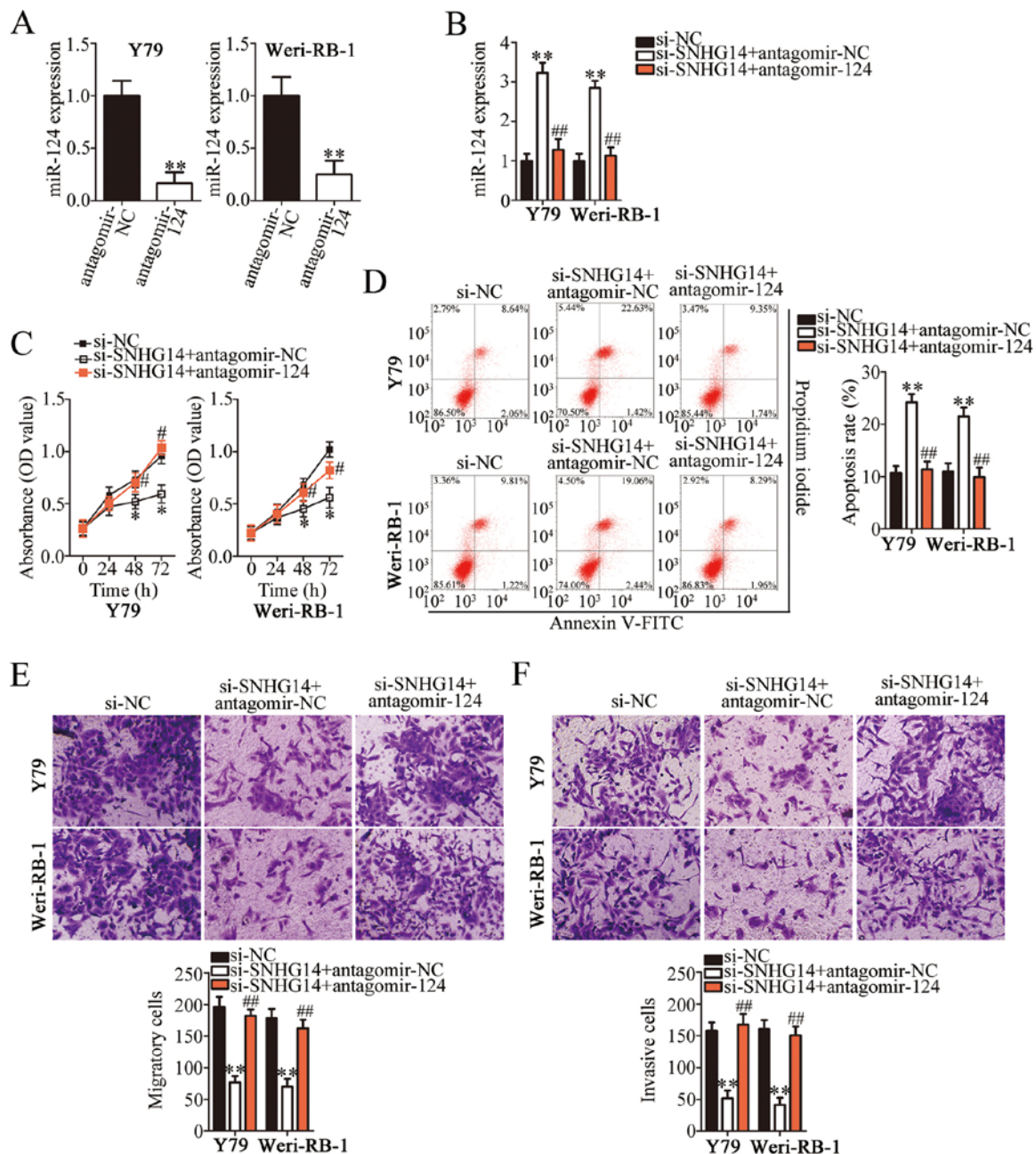


Figure 4. Inhibition of miR-124 abrogates the effect of *SNHG14* knockdown on RB cells. (A) The transfection efficiency of antagomir-124 was assessed via RT-qPCR. \*\* $P < 0.01$  vs. the antagomir-NC group. (B) Y79 and Weri-RB-1 cells were co-transfected with si-SNHG14 and either antagomir-124 or antagomir-NC. RT-qPCR analysis indicated that miR-124 inhibition abrogated the increase in miR-124 expression caused by *SNHG14* silencing. \*\* $P < 0.01$  vs. the si-NC group. ## $P < 0.01$  vs. si-SNHG14+antagomir-NC group. The (C) proliferation, (D) apoptosis, (E) migration and (F) invasion levels of Y79 and Weri-RB-1 cells treated as aforementioned were quantitated by the Cell Counting Kit-8, flow cytometry, migration and invasion assays. Microscopy images are at magnification,  $\times 200$ . The si-SNHG14-mediated decrease in cell proliferation, migration and invasion, and the increase in apoptosis were abrogated by the depletion of miR-124. \* $P < 0.05$  and \*\* $P < 0.01$  vs. si-NC group. # $P < 0.05$  and ## $P < 0.01$  vs. si-SNHG14+antagomir-NC group. miR, microRNA; *SNHG14*, small nucleolar RNA host gene 14; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; si, small interfering; FITC, fluorescein isothiocyanate.

inverse correlation between *SNHG14* and miR-124 expression levels among the RB tissue samples ( $R^2 = 0.3864$ ;  $P < 0.0001$ ). Following this, the regulatory effect of *SNHG14* on the miR-124 expression in RB cells was examined by RT-qPCR. The downregulation of *SNHG14* markedly increased miR-124 accumulation in Y79 and Weri-RB-1 cells (Fig. 3G). *STAT3* has been previously demonstrated to be a direct target gene of miR-124 in RB cells (36); accordingly, *STAT3* protein expression levels were measured in *SNHG14*-deficient Y79 and Weri-RB-1 cells. The results of the western blot analysis

indicated that a decrease in *SNHG14* expression significantly decreased the protein expression levels of *STAT3* in the Y79 and Weri-RB-1 cells (Fig. 3H). When taken together, these results suggested that *SNHG14* may act as a miR-124 sponge in RB cells and consequently increase *STAT3* expression.

*miR-124 silencing neutralizes the effects of SNHG14 knockdown on RB cells.* A series of rescue experiments were conducted to further investigate the interaction between *SNHG14* and miR-124, and to clarify the molecular events

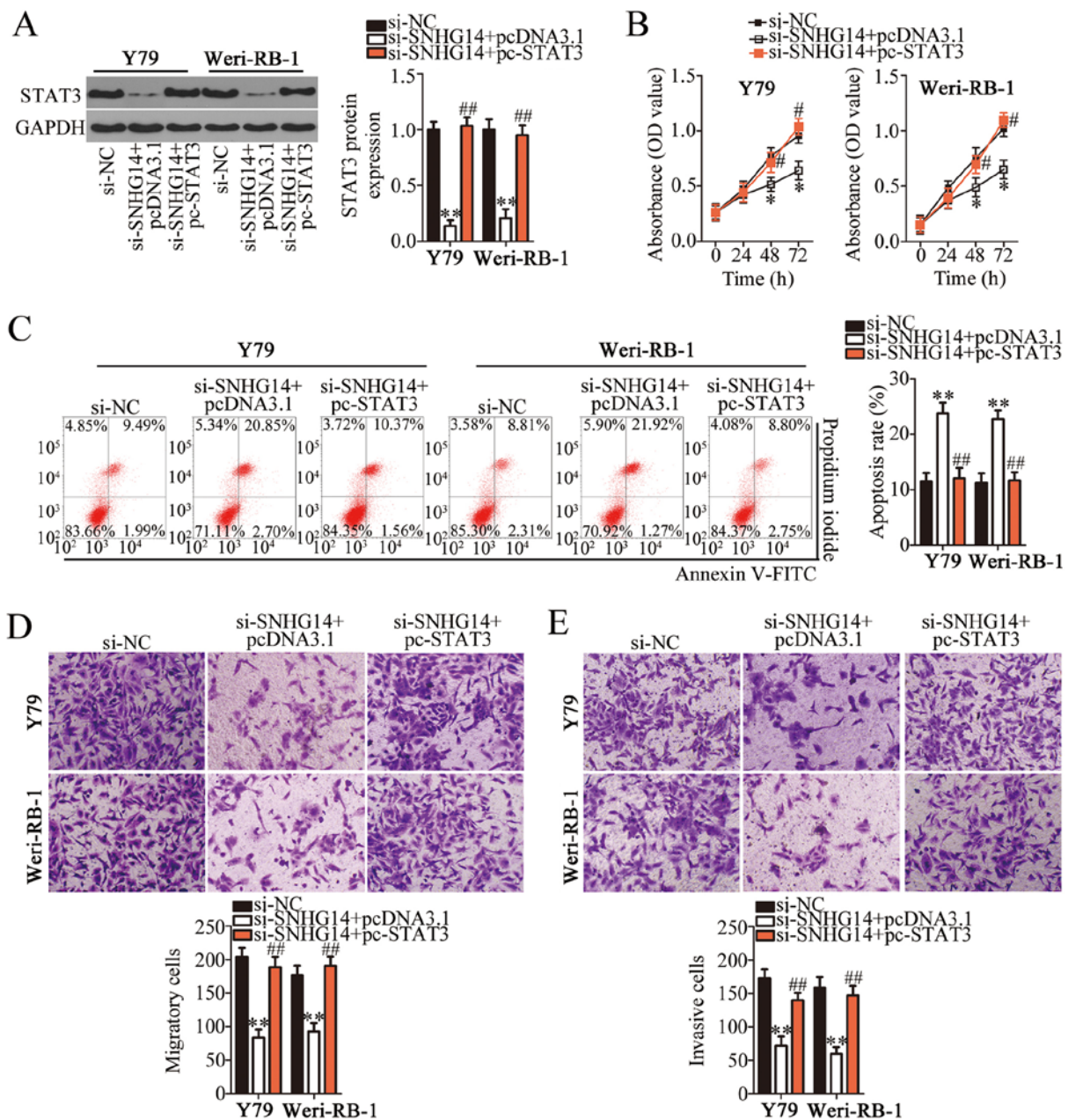


Figure 5. *SNHG14* downregulation inhibits the growth and metastasis of retinoblastoma cells *in vitro* via the miR-124/STAT3 regulatory loop. (A) si-SNHG14 together with either pc-STAT3 or pcDNA3.1 was introduced into Y79 and Weri-RB-1 cells. The protein level of STAT3 was estimated by western blot analysis. \*\* $P < 0.01$  vs. the si-NC group. ## $P < 0.01$  vs. si-SNHG14+pcDNA3.1 group. (B) Cell Counting Kit-8, (C) flow cytometry, (D) migration and (E) invasion assays (x200 magnification) were performed to determine the proliferation, apoptosis, migration, and invasion levels, respectively, of Y79 and Weri-RB-1 cells following co-transfection with si-SNHG14 and either pc-STAT3 or pcDNA3.1. Recovery of STAT3 expression attenuated the effect of the *SNHG14* knockdown on the proliferation, apoptosis, migration, and invasion levels of Y79 and Weri-RB-1 cells. Microscopy images are at magnification, x200. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the si-NC group. # $P < 0.05$  and ## $P < 0.01$  vs. si-SNHG14+pcDNA3.1 group. *SNHG14*, small nucleolar RNA host gene 14; miR, microRNA; STAT3, signal transducer and activator of transcription 3; si, small interfering; OD, optical density; FITC, fluorescein isothiocyanate.

underlying the oncogenic roles of *SNHG14* in RB cells. si-SNHG14 was co-transfected with either antagomir-124 or antagomir-NC into the Y79 and Weri-RB-1 cells. Firstly, it was confirmed that transfection with antagomir-124 resulted in efficient miR-124 silencing in the Y79 and Weri-RB-1 cells (Fig. 4A). The upregulation of miR-124 induced by *SNHG14* knockdown was identified to be attenuated in the Y79 and Weri-RB-1 cells that were co-transfected with antagomir-124 (Fig. 4B). Subsequent functional experiments revealed that *SNHG14* silencing inhibited Y79 and Weri-RB-1 cell proliferation (Fig. 4C), induced their apoptosis (Fig. 4D), and

attenuated their migratory (Fig. 4E) and invasive (Fig. 4F) capabilities. Conversely, these si-SNHG14-mediated effects on cell proliferation, apoptosis, migration and invasion were reversed by antagomir-124 co-transfection. These data suggested that *SNHG14* performs its oncogenic functions during RB progression via the downregulation of miR-124.

*Downregulation of SNHG14 inhibits the malignant characteristics of RB cells in vitro via the miR-124/STAT3 regulatory loop.* To further clarify the participation of the *SNHG14*/miR-124/STAT3 axis in the malignancy of RB,



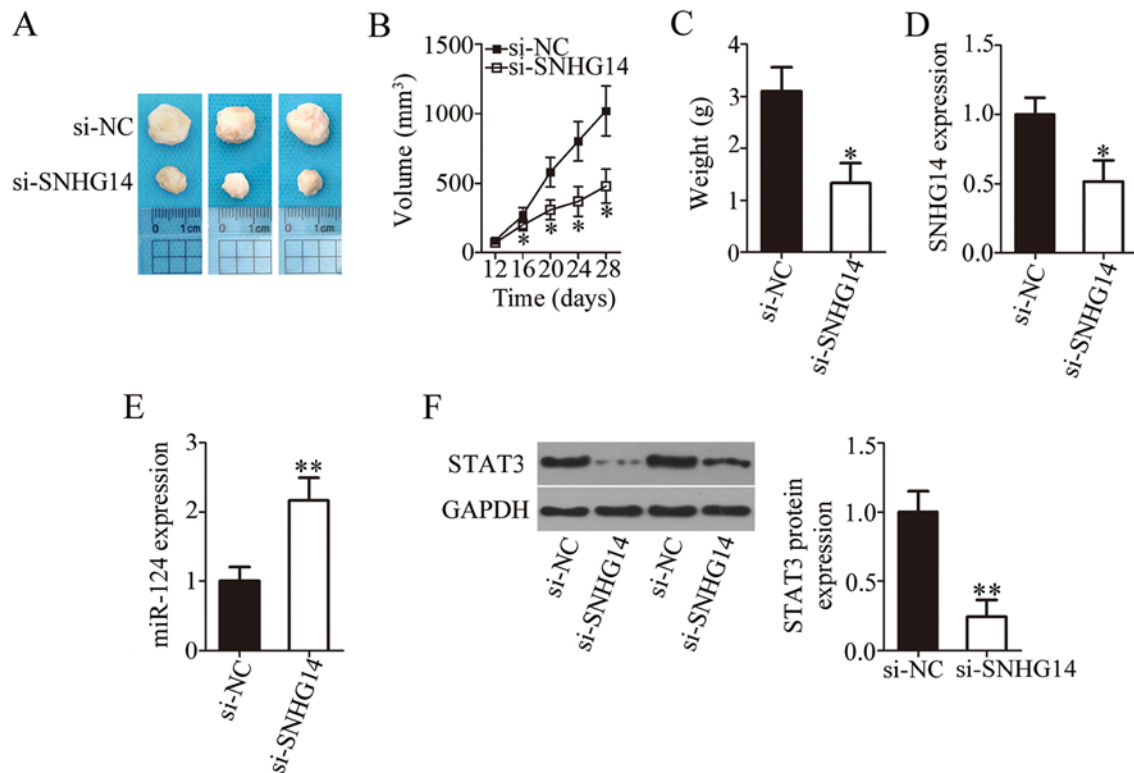


Figure 6. *SNHG14* silencing inhibits tumor growth *in vivo* via the miR-124/STAT3 regulatory axis. (A) Representative images of tumor xenografts following subcutaneous injection of Y79 cells transfected with either si-SNHG14 or si-NC. (B) The volume of tumor xenografts was monitored every 4 days. The tumor growth curves of nude mice were then constructed. \* $P < 0.05$  vs. si-NC group. (C) Average weights of tumor xenografts were measured in the si-SNHG14 and si-NC groups. \* $P < 0.05$  vs. the si-NC group. The expression of (D) *SNHG14* and (E) miR-124 was determined by reverse transcription-quantitative polymerase chain reaction analysis in the tumor xenografts derived from the si-SNHG14 and si-NC groups. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the si-NC group. (F) Western blot analysis of STAT3 protein expression in the tumor xenografts. \*\* $P < 0.01$  vs. the si-NC group. *SNHG14*, small nucleolar RNA host gene 14; miR, microRNA; STAT3, signal transducer and activator of transcription 3; si, small interfering RNA; NC, negative control.

si-SNHG14, in combination with either STAT3 overexpression plasmid pc-STAT3 or the empty pcDNA3.1 vector, was introduced into the Y79 and Weri-RB-1 cells. The results of the western blot analysis indicated that si-SNHG14 significantly decreased the protein expression of STAT3 in the Y79 and Weri-RB-1 cells, but co-transfection with pc-STAT3 abrogated this effect (Fig. 5A). Furthermore, recovery of STAT3 expression attenuated the effects of *SNHG14* silencing on Y79 and Weri-RB-1 cell proliferation (Fig. 5B), apoptosis (Fig. 5C), migration (Fig. 5D) and invasion (Fig. 5E) *in vitro*. Therefore, *SNHG14* participates in RB carcinogenesis *in vitro* by serving as a ceRNA that sponges miR-124, consequently inhibiting the miR-124-induced repression of its direct target gene *STAT3*.

**Decrease in *SNHG14* expression inhibits RB tumor growth *in vivo*.** The mouse xenograft tumor model was constructed to assess the biological effect of *SNHG14* on the *in vivo* tumor growth of RB cells. Y79 cells transfected with either si-SNHG14 or si-NC were subcutaneously inoculated into the flank of nude mice. On day 28, all the mice were euthanized, and a representative image of the formed tumor xenografts is presented in Fig. 6A. The volume of the tumor xenografts was markedly decreased in the si-SNHG14 group compared with in the si-NC group (Fig. 6B). In addition, the mean tumor weight was decreased in the si-SNHG14 group compared with the si-NC group (Fig. 6C). RT-qPCR analysis suggested that the tumor xenografts derived from si-SNHG14-transfected Y79

cells presented decreased *SNHG14* (Fig. 6D) and increased miR-124 (Fig. 6E). Western blot analysis revealed that the levels of STAT3 protein were decreased in the tumor xenografts obtained from the si-SNHG14 group (Fig. 6F). These results suggested that *SNHG14* downregulation inhibited the tumor growth of RB cells *in vivo*, and that this effect was mediated by the upregulation of miR-124 and downregulation of STAT3.

## Discussion

Previous evidence has highlighted the aberrant expression of lncRNAs in RB, and that these aberrations serve crucial roles in the RB carcinogenesis and progression (44-46). lncRNAs are implicated in the regulation of a wide range of biological activities and perform either tumor-suppressive or oncogenic functions (17,47,48). Therefore, the therapies that target lncRNAs hold promise for the management of RB. In the present study, we hypothesized that a known cancer-associated lncRNAs, *SNHG14*, may serve crucial roles in the modulation of RB progression. Therefore, *SNHG14* expression levels were measured in RB tumors and its clinical value was assessed among patients with RB. Then, the effect of *SNHG14* on the malignant characteristics of RB cells *in vitro* and *in vivo* was examined in a series of functional experiments. Finally, a systemic approach was applied to explore the mechanisms behind the oncogenic activities of *SNHG14* in RB *in vivo*.

*SNHG14* expression is low in glioma tissues and cell lines (27). Conversely, *SNHG14* expression is high in non-small cell lung cancer (NSCLC) cells (28,49,50), and *SNHG14* upregulation is associated with tumor size and TNM stage (28). Patients with NSCLC featuring high *SNHG14* expression exhibit shorter overall survival and recurrence-free survival times compared with the patients with low *SNHG14* expression (28). *SNHG14* is known to be overexpressed in cervical cancer and to be significantly associated with tumor stage, lymph node metastasis and shorter overall survival (29,30). *SNHG14* also exhibits high expression levels in ovarian (31,32), breast (33), bladder (34) and gastric cancer (35). Nevertheless, to the best of our knowledge, the expression profile and clinical significance of *SNHG14* in RB have never been determined. The present study identified that *SNHG14* was overexpressed in RB tissues and cell lines. *SNHG14* overexpression was associated with the IIRC stage, optic nerve invasion, differentiation grade and poor clinical outcomes among the patients with RB. These results suggest that *SNHG14* is a potential biomarker for the diagnosis and prognosis of RB.

The biological functions of *SNHG14* have been well studied in a range of cancer types. *SNHG14* sponges miR-92a-3p to exert tumor-suppressive effects on the progression of glioma by inhibiting cell viability and invasion and by promoting apoptosis (27). By contrast, *SNHG14* was validated as an oncogenic lncRNA in NSCLC and identified to participate in the regulation of cell proliferation, cell cycle, apoptosis, colony formation and gefitinib chemosensitivity *in vitro*, and tumor growth *in vivo* (28). These effects are mediated by the ceRNA effect on miR-340 (28), by ATP binding cassette subfamily B member 1 upregulation via the sponging of miR-206-3p (49) and by affecting the regulatory loop (50). In cervical cancer, *SNHG14* silencing decreased tumor cell viability, proliferation, migration and invasion, and facilitated apoptosis through modulation of the miR-206/tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta axis and activation of the Janus kinase-STAT pathway (29,30). Besides, *SNHG14* exerts oncogenic actions on the malignancy of ovarian (31,32), breast (33), bladder (34), and gastric (35). However, data on the detailed involvement of *SNHG14* in the malignant properties of RB is limited. In the present study, functional assays revealed that the depletion of *SNHG14* decreased the levels of RB cell proliferation, migration and invasion *in vitro*, promoted apoptosis *in vitro* and inhibited tumor growth *in vivo*.

Studies investigating the mechanisms of action of *SNHG14* on RB malignancy are crucial for understanding the roles of *SNHG14* in RB pathogenesis and for the identification of effective therapeutic targets. In the present study, *SNHG14* was identified to serve as a molecular sponge of miR-124 in RB cells, thereby upregulating STAT3. miR-124 expression has been demonstrated to be low in RB tumors and cell lines (36). miR-124 exerts tumor-suppressive effects on RB progression by repressing RB cell proliferation, migration and invasion, and by inducing apoptosis (36). Several lncRNAs, including nuclear paraspeckle assembly transcript 1 (51), X inactive specific transcript (52) and metastasis associated lung adenocarcinoma transcript 1 (53,54), have been identified to sponge miR-124 and to contribute to RB aggressiveness *in vitro* and *in vivo*. The results of the present study revealed that inhibition of miR-124 abrogated the *SNHG14* knockdown-induced

suppression of RB cell proliferation, migration and invasion, and reversed the pro-apoptotic effects of the *SNHG14* knockdown on RB cells.

A mechanistic investigation has confirmed *STAT3* as a direct target gene of miR-124 in RB cells (36). *STAT3*, a key transcription factor in the STAT family, is a signal mediator that is activated by various cytokines, growth factors and interferons (55). It is overexpressed in RB, performs oncogenic roles during RB progression, and is involved in the regulation of multiple physiological and pathological processes in tumor development (56-59). The results of the present study demonstrated clearly that *STAT3* expression is directly controlled by the *SNHG14*/miR-124 regulatory loop in RB.

The present study has certain limitations. The effect of *SNHG14* on the metastasis of RB cells *in vivo* was not examined directly. This will be addressed future studies.

In conclusion, the present study demonstrated that *SNHG14* was overexpressed in RB, and that it was associated with poor clinical outcomes among patients with RB. *SNHG14* serves as an oncogenic lncRNA in RB cells *in vitro* and *in vivo*. The regulatory role of *SNHG14* in RB malignancy is partly mediated by its function as a ceRNA of miR-124, and consequent upregulation of *STAT3*. These results may offer a novel perspective on the targeted therapy of RB.

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#### Availability of data and materials

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

#### Authors' contributions

SZ and XS designed the study. XS and HS conducted RT-qPCR, CCK-8 assay, RIP assay and luciferase reporter assay. Flow cytometric analysis, and migration and invasion assays were carried out by SL. JG constructed the mouse xenograft tumor model and conducted western blotting. Statistical analysis was conducted by SZ. All authors read and approved the final manuscript.

#### Ethics approval and informed consent

The present study was conducted with the approval of the Clinical Research Ethics Committee of People's Hospital of Rizhao and was performed following the principles of the Declaration of Helsinki. All participants provided written informed consent for participation prior to surgical resection. All experimental procedures involving animals were conducted in accordance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals and were approved by the Animal Care Committee of People's Hospital of Rizhao.

## Patient consent for publication

All participants provided written informed consent for publication prior to surgical resection.

## Competing interests

The authors declare that they have no competing interests.

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