Am J Transl Res 2022;14(3):1592-1605
www.ajtr.org /ISSN:1943-8141/AJTR0137442

Original Article

Long non-coding RNA UCA1 promotes retinoblastoma progression by modulating the miR-124/c-myc axis

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Received July 17, 2021; Accepted February 12, 2022; Epub March 15, 2022; Published March 30, 2022

Abstract: The long non-coding RNA (lncRNA), urothelial carcinoma-associated 1 (UCA1), belongs to cancer-related lncRNAs implicated in various carcinomas, including colorectal and gastric cancers. Nonetheless, the role and underlying mechanisms of UCA1 in retinoblastoma are still unclear. This study found that UCA1 expression in retinoblastoma tissues and cells was dramatically upregulated relative to that of healthy controls. Functionally, UCA1 knockdown could suppress retinoblastoma cells' proliferation, migration and invasion, and facilitate their apoptosis. Knockdown of UCA1 also retarded the growth of xenograft tumors *in vivo*. Mechanistically, UCA1 promoted c-myc expression through sponging miR-124. miR-124 inhibition or c-myc overexpression partially reversed the effects of UCA1 knockdown on retinoblastoma cells. Overall, lncRNA UCA1 may exert an oncogenic effect on retinoblastoma progression through the miR-124/c-myc axis, which might serve as a promising retinoblastoma treatment target.

Keywords: Retinoblastoma, urothelial carcinoma-associated 1, miR-124, c-myc, tumorigenesis

Introduction

Retinoblastoma (RB) is the most common primary intraocular malignant tumor in children and is triggered by RB1 gene mutations [1]. It has an incidence of about 1/15000 worldwide, and takes up about 3% of pediatric malignancies [2]. The RB mortality rate in developing countries is approximately 50%-70% [3]. Treatment for RB includes enucleation, locally targeted treatment (cryotherapy, laser therapy, and brachytherapy), radiation therapy, and chemotherapy. Despite the various treatment options, they are associated with many side effects and complications, including multidrug resistance and chemotherapy insensitivity, which seriously impact their therapeutic effects [4]. Therefore, further investigation of the molecular mechanisms underlying RB occurrence will promote the advances of new diagnosis and treatment targets, thereby improving the effectiveness of RB treatment.

The dysregulation of long non-coding RNAs (lncRNAs) plays a significant regulatory role in multiple cancers [5-7]. Several studies have reported that lncRNAs were relevant to RB carcinogenesis and metastasis. For instance, NEAT1 (lncRNA nuclear paraspeckle assembly transcript 1), which is correlated with poor prognosis of RB, accelerates retinoblastoma cell (RC) progression by targeting miR-124 [8]. LncRNA-H19, as an oncogene in RCs (Y79 and WERI-Rb1 cells), promotes RCs' viability, migration as well as invasion, and inhibits apoptosis by upregulating the miR-143/runt-related transcription factor 2 axis and inhibiting the PI3K/AKT/mTOR pathways [9]. The lncRNA, small nucleolar RNA host gene 16, is increased in retinoblastoma tissues (RTs) and cells, and modulates RB progression through sponging miR-140-5p [10]. Therefore, lncRNAs may assume a critical modulatory part in RB.

The lncRNA, UCA1, is considered to be a cancer-related lncRNA, and was first discovered in human bladder carcinoma [11]. The dysregulation of UCA1 has been associated with several human malignancies, including hepatocellular carcinoma (HCC), gastric cancer and colorectal

cancer as well as esophageal squamous cell cancer [12]. Further, the UCA1 upregulation in HCC tissues and cell lines promotes HCC cells' proliferation and migration, and inhibits the apoptosis [13], which is relevant to poor prognosis and advanced clinicopathologic features in colorectal cancer, gastric cancer and esophageal squamous cell cancer [14-16]. Recently, UCA1 was found to be highly expressed in RB and significantly upregulated in chemo-resistant RTs and multi-drug resistant retinoblastoma cell lines (RCLs). The upregulation of UCA1 predicts unfavorable overall survival (OS) and promotes RC migration and invasion by miR-513a-5p/Stathmin 1 (STMN1) axis [17]. However, the relevant mechanism of lncRNA UCA1 in RB carcinogenesis is unclear.

miRNAs belong to the most widely studied non-coding RNAs that can interact directly with lncRNAs and regulate cell processes, including individual development, body metabolism, and tumorigenesis [18, 19]. Liu et al. indicated that the decreased miR-124 expression was relevant to shorter OS in patients with gastric cancer [20]. Additionally, miR-124 repressed gastric cancer cells' migration and invasion through acting on Rac1 and SP1. MiR-124 exhibits anti-invasion properties in pancreatic ductal adenocarcinoma [21] by negatively regulating integrin subunit alpha 3 (ITGA3) and integrin subunit beta 1 (ITGB1), and suppresses the metastasis of melanoma cells by directly targeting the receptor of activated C kinase 1 (RACK1) [22]. In RB, miR-124 has been confirmed as a well-known tumor inhibitor [23].

The miR-124 level was found to be low in both RTs and cell lines. The miR-124 overexpression can suppress RC migration and invasion, and facilitate apoptosis [24]. UCA1 assume an oncogenic role in many tumor cells through modulating miRNAs. In particular, UCA1 facilitates cell development through suppressing miR-124 and upregulating ITGB1 expression in nasopharyngeal carcinoma [25]. UCA1 also promotes the chondrogenic differentiation of human bone marrow mesenchymal stem cells through the miRNA-124-3p/SMAD family member 4 (SMAD4) axis [26]. However, the correlation between UCA1 and miR-124 in RB remains unclear. C-myc is a common oncogene and miR-124 could directly target it [27]. C-myc is upregulated in many cancer cells. In RB, c-myc knockdown promotes cell apoptosis and suppresses cell proliferation [28]. However, more research is needed to assess the association among UCA1, miR-124, and c-myc in RB.

The study sought to reveal the clinical role of lncRNA UCA1 in RB, and explore the underlying molecular mechanisms of the oncogenic role of UCA1 in RB progression. UCA1 in RTs and cell lines was detected to be increased. Further, UCA1, as a ceRNA, increased c-myc expression by sponging miR-124, resulting in enhanced cell proliferation. These observations shed new light on the mechanisms underlying RB progression and provide a basis for the development of novel RB treatment targets.

Materials and methods

Tissue specimens

Tumor specimens and adjacent noncancerous tissues were collected from 15 RB patients who were treated at the Second Affiliated Hospital of Chongqing Medical University from January 2018 to January 2020.

Inclusion criteria: Newly diagnosed RB, previously untreated; life expectancy of at least 10 weeks; and adequate renal function as measured by a serum creatinine level ≤3× normal for age. The informed consent was provided by legal guardians, which indicated that they were informed of the details of the study.

Exclusion Criteria: patients that received treatment before, or suffered from metastatic disease or orbital involvement.

The study included 9 males and 6 females (age, 6-60 months old; average age, 28 months old). Three patients had optic nerve/choroid/orbital metastasis, and 12 had local lesions without metastasis. The study has been approved by the Research Ethics Committee (the Second Affiliated Hospital, Chongqing Medical University), China (approval number 2019-270). The legal guardians signed informed consent before testing. Baseline data for patients are presented in **Table 1**. High and low expressions of this gene are relative concepts. According to a previous study by Wu et al. [29], RTs were classified to two groups. By comparing the relative UCA1 expression level in RTs compared with normal controls, the

Table 1. Expression of lncUCA1 and the clinicopathological features of RB patients

LncUCA1 expression			
Feature	n	low	high
All cases	15	3	12
Age			
<2	8	2	6
≥2	7	1	6
Gender			
Male	9	1	8
Female	6	2	4
Tumor size (mm)			
<3	0		
≥3	15	3	12
TNM stage			
I and II	0		
III and IV, V	15	3	12
The Optic nerve/Choroid/Orbit metastasis			
Negative	12	3	9
Positive	3		3

Table 2. Primers used for qPCR

Primer	Sequence
LncRNA UCA1	F: 5'-CTTAGGCTGGCAACCATCAGATCC-3'
	R: 5'-GTGTTGTCCTGGATCTGGTCTG-3'
c-myc	F: 5'-GCCAACTACTCTACTGTGGATT-3'
	R: 5'-ATGCAGATGACTGTGTCGTT-3'
GAPDH	F: 5'-TGGCCTGACCC AACTATGAT-3'
	R: 5'-GCACTTTTTGCCCTTCTTAATGTT-3'
miR-124	F: 5'-TAAGGCACGCGGTGAATG-3'
	R: 5'-GCAGGGTCCGAGGTATTC-3'
U6	F: 5'-GCTTCGGCAGCACATATACT-3'
	R: 5'-GTGCAGGGTC CGAGGTATTC-3'

cases could be divided into high and low expression groups. In RTs compared with normal controls, an expression difference of more than two-fold was defined as high expression.

Cell lines and culture

Human cell line RCL Y79, was provided by American Type Culture Collection, ATCC. WERI-Rb1 and ARPE-19 cells were provided by iCell Bioscience Inc. (Shanghai, China). Cells were cultivated in RPMI-1640 medium with 20% FBS (ExCell Bio, China), 1% penicillin, and streptomycin (Gibco), and incubated at 37°C in a damp under the condition with 5% CO_2.

Cell transfection and selection of stably transfected cell lines

To stabilize the knockdown of UCA1, a short hairpin shRNA against UCA1 (sh-UCA1 GGACAACACAAAGTATGTT) and its negative control (sh-NC TTCTCCGAACGT-GTCACGT) were synthesized and inserted into the lentiviral vector by Genechem (Shanghai, China). The transfected cells were selected with a culture medium containing puromycin for approximately 4 weeks. MiR-124 mimics (dsRNA oligonucleotides), miR-124 inhibitors (single-stranded chemically modified oligonucleotides), and the relevant negative controls (miR-124 nc) were provided by GenePharma (Shanghai, China). The miR-124 mimic sequence was 5'-UAAGGCA-CGCGGUGAAUGCC-3', and the negative control sequence was 5'-UUGUACUACAC-AAAAGUACUG-3'. The inhibitor sequence was 5'-CGUGUUCACAGCGGACCUUGAU-3', and the negative control sequence was 5'-CAGUACUUUUGUGUAGUACAA-3'. Full-length human c-myc cDNA (NM_001354870.1.) was synthesized by GenePharma and cloned into the pcDNA3.1. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for transfection as the relevant guidance.

qRT-PCR

Total RNA was separated from cells and RTs with TRIzol. The cDNAs of UCA1 and c-myc were transcribed through an M-MLV Reverse Transcription Kit (Invitrogen). miR-124 was reverse-transcribed and measured using a Hairpin-it™ miRNA RT-PCR Quantitation Kit (GenePharma). The cDNAs were detected on an ABI 7500 RT-PCR system through a SYBR Green master mix (Invitrogen). Values were calculated through the $2^{-\Delta\Delta Ct}$ method with GAPDH and U6 as the internal reference. The primers used for qPCR were listed in **Table 2**.

CCK-8 assay

Cell proliferation assay was conducted with CCK-8 (Dojindo, Japan). Cells (5×10^3 cells/well) were inoculated into 96-well plates. CCK-8 solution (10 μL) was supplemented at 1, 2, 3, and 4 d, and cells were incubated in an incubator with 5% CO_2 for 60 min at 37°C. Ab-

sorbance at 450 nm was determined through a microplate reader.

Colony formation assay (CFA)

Cells with a low density (500 cells per dish) were inoculated in a 6 cm dish and cultivated for 2 weeks. When the colonies were visible to the naked eye, the culture was terminated. Cell colonies were then rinsed with PBS and fixed with 4% paraformaldehyde for 0.5 h. Thereafter, cell colonies were exposed to crystal violet staining (0.1%, 1 mg/mL; Beyotime, Shanghai, China), and colonies with over 50 cells were manually counted under a microscope.

Transwell assay

Transwell assays were conducted to evaluate cell migration and invasion ability, and 24-well chambers with 8 μm pores (Corning Costar, MA, USA) were used. Briefly, the cells (1×10^4 cells/well) were suspended in 200 μL medium without serum and inoculated in the upper chamber. Thereafter, 600 μL normal medium was supplemented to the lower chamber. After incubated for 1 d at 37°C, the cells which migrated to the lower chamber were counted through BX53 microscope. In the invasion assays, the chambers were pre-coated with 30 μg Matrigel (BME, 3432-005-01P, R&D, USA), and the other operations were the same as those in the migration assay.

Cell apoptosis assay

Cell apoptosis was measured through annexin V-FITC/PI kit (4A Biotech, Beijing, China) as per manufacturer's instructions. In brief, at 2 d following transfection, cells were harvested, rinsed with PBS, resuspended in binding buffer, and stained with annexin V-FITC/PI. After incubation for 60 min away from light at RT, the proportion of apoptotic cells was detected through NovoCyte (ACEA Biosciences Inc., CA, USA).

Dual-luciferase report assay (DLRA)

The binding site sequences of miR-124 with UCA1 or c-myc and their corresponding mutant sequences were amplified through PCR and cloned into a pSI-Check2 dual-luciferase vector (Hanbio Biotechnology Co., Ltd, Shanghai, China) to establish luciferase reporter vectors (lncRNA UCA1-WT/MUT and c-myc-WT/MUT). The vectors and miR-124 mimic/NC were co-transfected into cells through Lipofectamine 3000 reagent (Invitrogen). After transfection for 2 h, luciferase activity was determined through a DLRA system (Promega, Madison, WI, USA).

Western blotting (WB)

Total protein was isolated through radioimmunoprecipitation assay buffer containing 10% PMSF. Protein concentration was determined through BCA Assay Kit (Beyotime, Beijing, China). Thereafter, 30 μg of protein was supplemented and isolated through 10% PAG. The target proteins were added onto a PVDF membrane (Millipore, Bedford, MA, USA) by SDS-PAGE, which was incubated with primary antibodies (1:1000) against c-myc (ab32072, Abcam) and β-actin (ab8226, Abcam) overnight at 4°C. After blocking with skim milk, the membrane was incubated with a 1:5000 anti-rabbit secondary antibody conjugated with HRP (WLA024, Wanleibio, Shenyang, China) at RT. The membrane density was measured using a chemiluminescence digital image system (Bio-Rad).

Xenografts in nude mice transfected with lentivirus

Twelve female BALB/c nude mice (female, age, 28-42 d) were obtained from Beijing Animal Experimental Center, China, and raised in the Animal Center of Chongqing Medical University, China. Mice were arbitrarily classified to two groups and administered a 200-μL cell suspension (1×10^7 Y79 cells with stable sh-UCA1/sh-NC transfection) in the right flanks. To stabilize transfection, the transfection was mediated by GeneCopoeia eGFP (LPP-EGFP-LV105, 10^8 TU/ml) [30, 31]. One week later, tumor size was recorded every 4 days and obtained through the equation $V = (\text{shortest diameter})^2 \times (\text{longest diameter})/2$. Mice were sacrificed 28 days after injection by an intraperitoneal injection of barbiturate. The dose was three times the usual anesthesia dosage (≥150 mg/kg). The tumors were then excised and stored for further analysis. Animal experiments have been approved by the Animal Ethics Committee (Chongqing Medical University, China).

Data analysis

The results were displayed as mean ± SD. Data was analyzed through GraphPad Prism 5.0. The

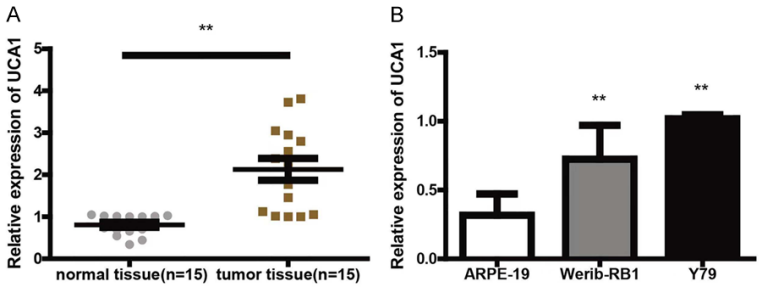

Figure 1. Upregulation of the lncRNA, UCA1, in retinoblastoma tissues and cell lines. A. The relative expression of UCA1 in 15 human retinoblastoma and adjacent noncancerous tissues. B. The relative expression of UCA1 in retinoblastoma cell lines (Y79 and WERI-Rb1) and ARPE-19. $^{**}P<0.01$; n=15.

variance between the two groups or multiple groups was analyzed through Student's t-test or ANOVA with post hoc Bonferroni test. The association among UCA1, miR-124, and c-myc expression in RTs was estimated through Pearson correlation analysis. $P<0.05$ indicated statistical significance.

Results

UCA1 exhibited high expression in RTs and RCLs

The lncRNA UCA1 level was detected in 15 pairs of human RTs and adjacent noncancerous tissues. UCA1 expression in RTs was dramatically higher relative to that in adjacent noncancerous tissues (**Figure 1A**). The UCA1 expression in RCs was also higher than that in ARPE-19 cells (**Figure 1B**). These findings indicated that UCA1 might be implicated in RB.

lncRNA UCA1 knockdown suppressed RC proliferation, migration and invasion and facilitated apoptosis

The role of UCA1 in RCs was explored by transfecting shRNA against lncRNA UCA1 and its negative controls (sh-UCA1 and sh-NC). These shRNAs were transfected into Y79 cells and WERI-Rb1 cells to knock down UCA1. qRT-PCR revealed that UCA1 expression was decreased after transfection with sh-UCA1 (**Figure 2A**). Further, CCK8 and colony formation assays confirmed that the proliferation of UCA1-knockdown cells was significantly suppressed relative to that of control cells (**Figure 2B**, **2C**). MeUCA1 knockdown inhibited clone numbers, Y79 and WERI-Rb1 cells' migration and invasion (**Figure 2D-I**). However, downregulation of UCA1 significantly promoted the apoptosis of RCs relative to the control (**Figure 2J**, **2K**). These findings indicate that UCA1 knockdown inhibited RC proliferation, clone numbers, migration, and invasion and promoted apoptosis, which is consistent with the conclusions reported before [17].

UCA1 targeted and negatively regulated miR-124

According to previous studies, lncRNAs are capable of competitively binding with miRNAs and alleviate the inhibitory roles of miRNAs in their targets to exert regulatory effects. Bioinformatic analysis was performed using TargetScan prediction to elucidate the carcinogenic effect of UCA1 on RB. Based on the findings, miR-124 may act as a candidate target of lncRNA UCA1 (**Figure 3A**). To verify the prediction, UCA1 3'UTR luciferase reporters were co-transfected with miR-124 mimics or miRNA-NC into RCs. Luciferase activity was found to be reduced in cells co-transfected with a UCA1-WT reporter and miR-124 mimic relative to miRNA-NC. Further, no significant decrease in UCA1-MUT reporters was detected (**Figure 3B**, **3C**). miR-124 expression was found to be decreased in the two RCLs relative to the matched controls, thereby aligning with the findings of previous studies (**Figure 3D**) [22]. UCA1 knockdown dramatically upregulated the miR-124 expression in RCs ($P<0.05$; **Figure 3E**). However, UCA1 was markedly elevated in WERI-Rb1 cells treated with miR-124 inhibitor transfection. The UCA1 level was dramatically decreased in Y79 cells with miR-124 mimic transfection (**Figure 3F**). These findings suggest that UCA1 may negatively regulate miR-124 by directly targeting it.

MiR-124 suppression eliminated the effects of lncRNA UCA1-knockdown on RCs

To determine the effects of UCA1/miR-124 axis in RC proliferation, clone numbers, migration, invasion, and apoptosis, loss-of-function and rescue experiments were conducted. Sh-UCA1 was co-transfected with specific miR-124 inhibitors into RCs. Thereafter, cell proliferation, migration, invasion as well as apoptosis were assessed. CCK8, colony formation, and Transwell assays revealed that miR-124 inhibition could counteract the suppressive

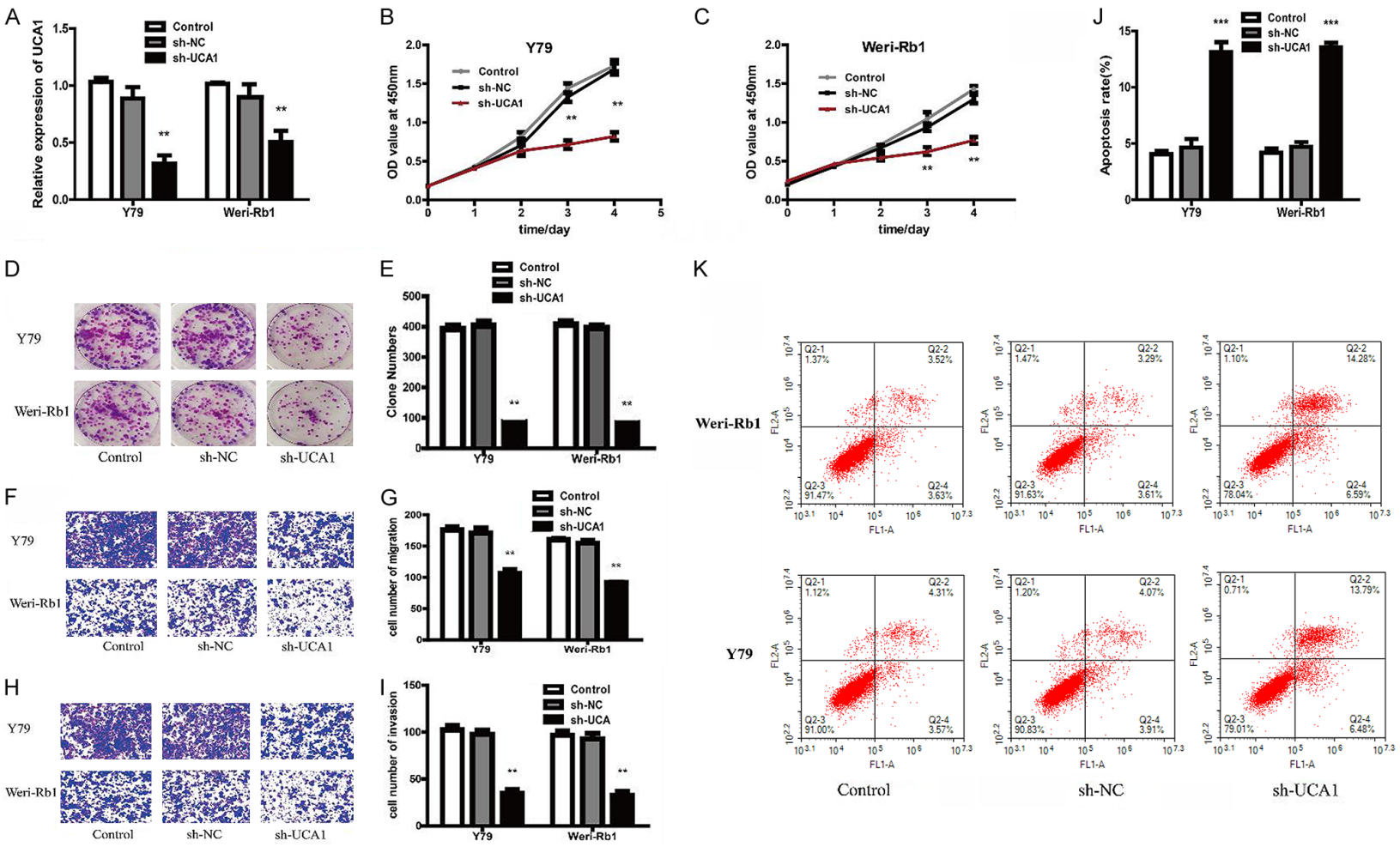

Figure 2. Knockdown of the lncRNA, UCA1, inhibits the proliferation, clone numbers, migration, and invasion of retinoblastoma cells, and promotes the apoptosis of retinoblastoma cells. (A) UCA1 expression was decreased in WERI-Rb1 and Y79 cells after transfection with specific shRNAs targeting the lncRNA, UCA1. Effect of UCA1 silencing on the proliferation (B, C), clone numbers (D, E), migration and invasion (F-I), and apoptosis (J, K) of retinoblastoma cells. $^{*}P<0.05$, $^{**}P<0.01$; n=6, magnification 40×, scale bar 100 μm.

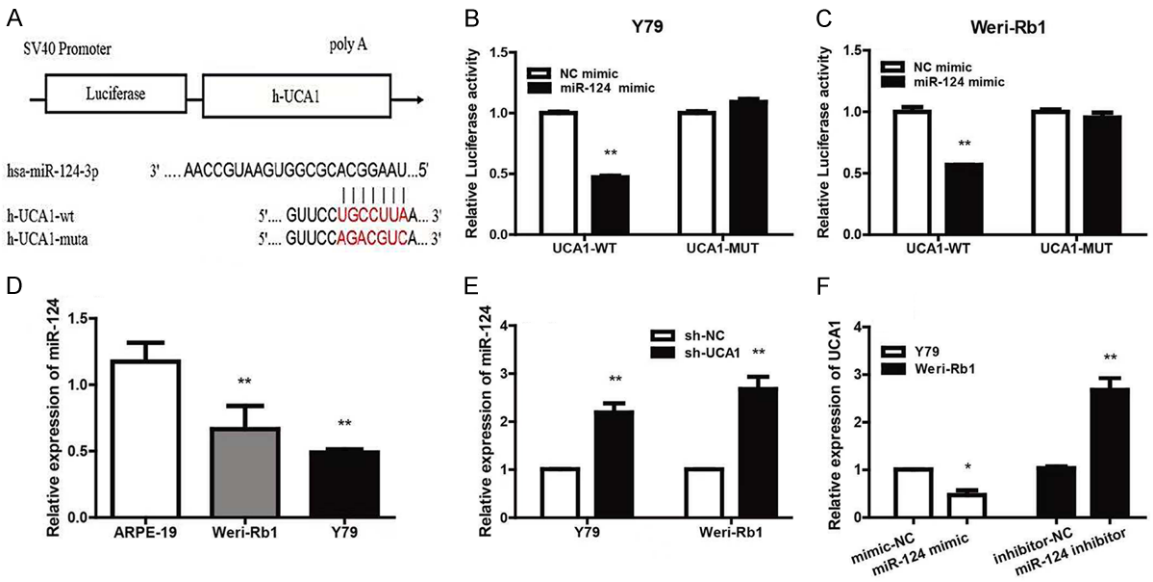

Figure 3. The lncRNA, UCA1, interacted with miR-124 in retinoblastoma cells. A. The potential binding sequence of UCA1 and miR-124 predicted by TargetScan. B, C. Dual-luciferase activity assay revealed that miR-124 may directly target UCA1 in Y79 and WERI-Rb1 cells. D. Expression levels of miR-124 in retinoblastoma cells. E. Expression levels of miR-124 in Y79 and WERI-Rb1 cells transfected with sh-NC and sh-UCA1. F. Relative expression of c-myc in Y79 and WERI-Rb1 cells administered different treatments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; n=6.

effects of UCA1 silencing on cell proliferation, clone numbers, migration and invasion (**Figure 4A-H**). In addition, cell apoptosis assay demonstrated that the miR-124 suppression reversed the effect of UCA1 silencing on the apoptosis of RCs (**Figure 4I**, **4J**). These findings displayed that UCA1 may regulate RC development through suppressing miR-124.

C-myc may act as a target gene of miR-124 and may be regulated by lncRNA UCA1

Several studies have shown that miRNAs exerted their regulatory effects by binding with the relevant target genes. Bioinformatics analysis predicted that c-Myc may act as a promising target of miR-124 (**Figure 5A**), and DLRA verified this hypothesis. Luciferase activity was found to be decreased in cells treated with miR-124 mimics and c-myc-WT vector co-transfection. Nonetheless, no significant decrease was detected in cells transfected with the c-myc-MUT vector (**Figure 5B**, **5C**). qRT-PCR displayed that c-myc expression was very elevated in RCs relative to that of normal controls (**Figure 5D**). Besides, the c-myc level in the miR-124 mimic group was dramatically decreased. The c-myc level in miR-124 inhibition group was dramatically higher relative to that of the control group (**Figure 5E-G**). Meanwhile, c-myc expression in UCA1 knockdown group was reduced (**Figure 5H**, **5I**). The western blot results further confirmed these findings, indicating that c-myc might be a target of miR-124, and UCA1 may regulate c-myc by sponging miR-124.

UCA1 facilitated the RB progression by upregulating c-myc

To validate the regulatory effects of the UCA1/miR-124/c-myc axis in RC proliferation, clone numbers, cell migration, invasion, and apoptosis, cells were treated with sh-UCA1+pcDNA-NC or sh-UCA1+pcDNA-c-myc vectors. The c-Myc vectors were successfully transfected (Supplementary Figure 1). CCK8 assay, CFA and Transwell assay showed that the c-myc overexpression reversed the UCA1 knockdown - induced suppression in cell proliferation, migration, and invasion (**Figure 6A-H**). Moreover, the apoptosis of RCs with UCA1 knockdown was reversed by transfection with pcDNA-c-myc compared with pc-DNA-NC (**Figure 6I**, **6J**). Thus, UCA1 could facilitate RC progression through upregulating c-myc.

UCA1 knockdown suppressed RB tumor growth in vivo

The effect of UCA1 on RB progression *in vivo* was investigated. In a xenograft model, RCs (Y79) stably transfected with sh-UCA1 or sh-NC

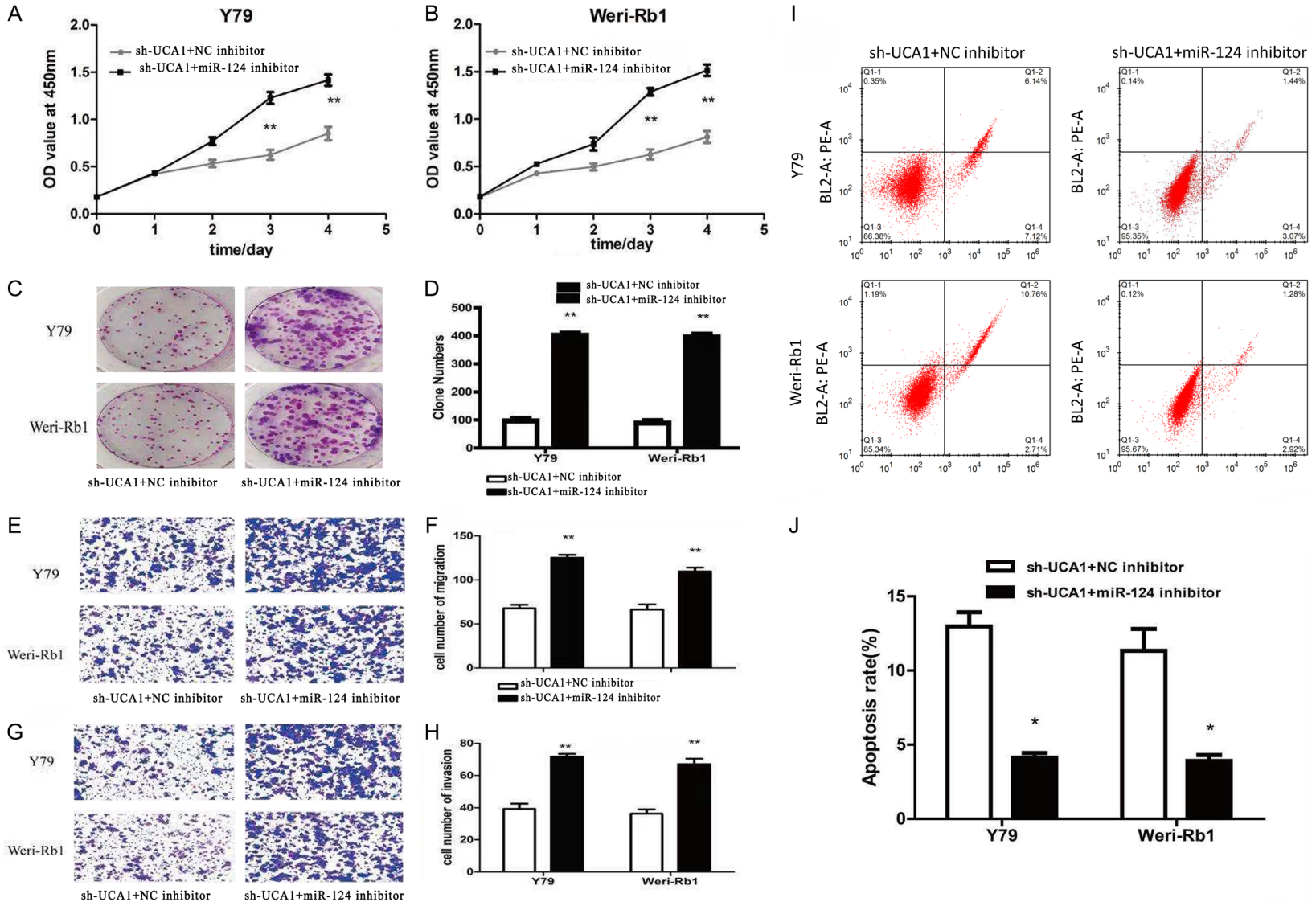

Figure 4. miR-124 inhibition reversed the effects of UCA1 knockdown on retinoblastoma cells. Cell proliferation (A, B), clone numbers (C, D), migration (E, F) and invasion ability (G, H) as well as apoptosis (I, J) were verified in Y79 and WERI-Rb1 cells transfected with sh-UCA1 and sh-UCA1+miR-124-3p inhibitor using CCK8, colony formation, Transwell, and cell apoptosis assays, respectively. $^{*}P<0.05$, $^{**}P<0.01$; n=6, magnification 40×, scale bar 100 μm.

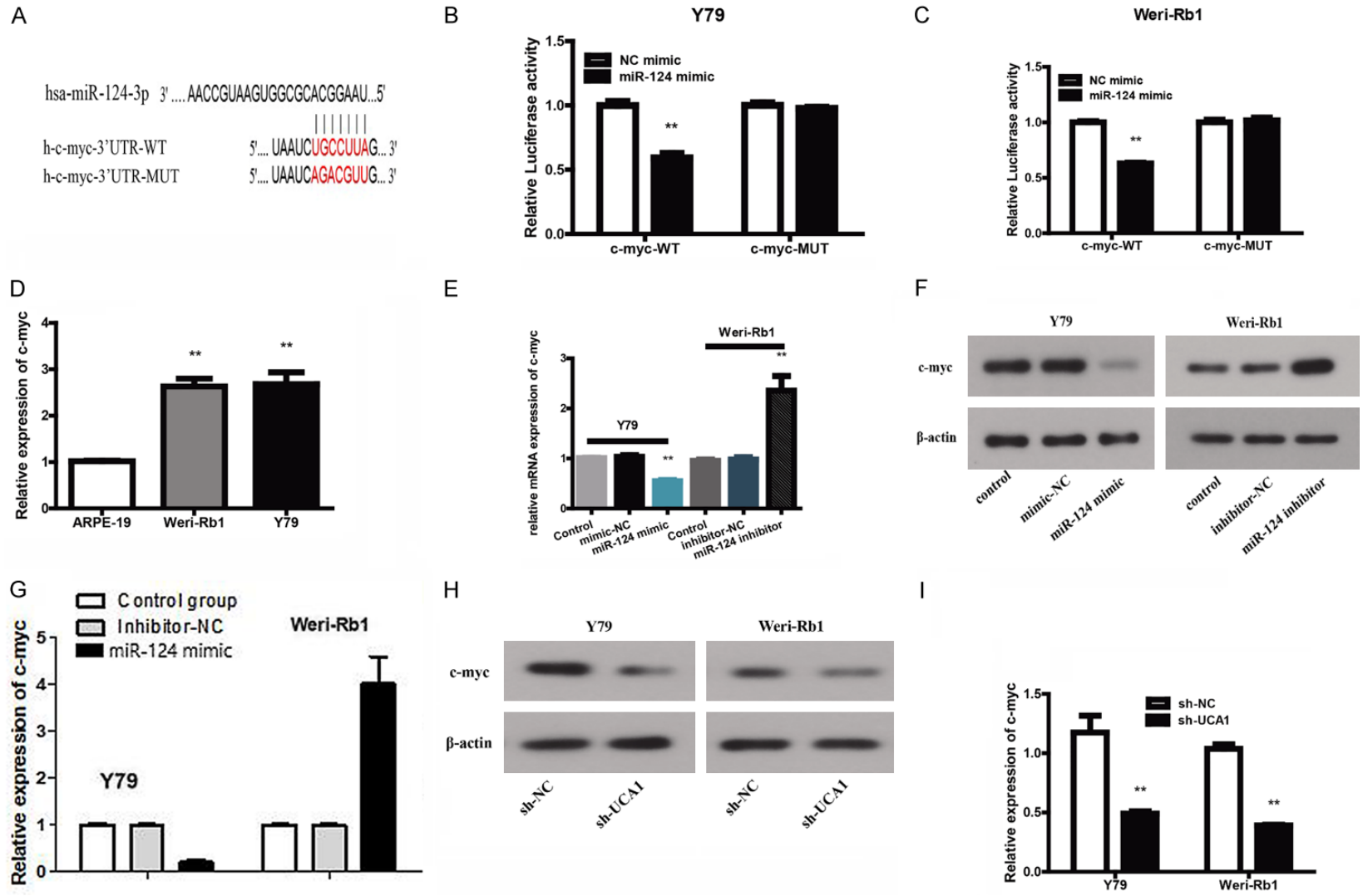

Figure 5. C-myc may be a target gene of miR-124 and may be regulated by UCA1. A. The potential binding sequence of miR-124 and c-myc predicted by TargetScan. B, C. Dual-luciferase activity assay showed that miR-124 may directly target c-myc in Y79 and WERI-Rb1 cells. D. The mRNA expression level of c-myc in retinoblastoma cells compared to corresponding normal controls. E-G. The expression level of c-myc in Y79 and WERI-Rb1 cells transfected with mimic-NC, miR-124 mimic, inhibitor-NC, and miR-124-inhibitor. H, I. The expression level of the c-myc protein in Y79 and WERI-Rb1 transfected with sh-NC and sh-UCA1. $^{**}P<0.01$, $^{***}P<0.001$; n=6.

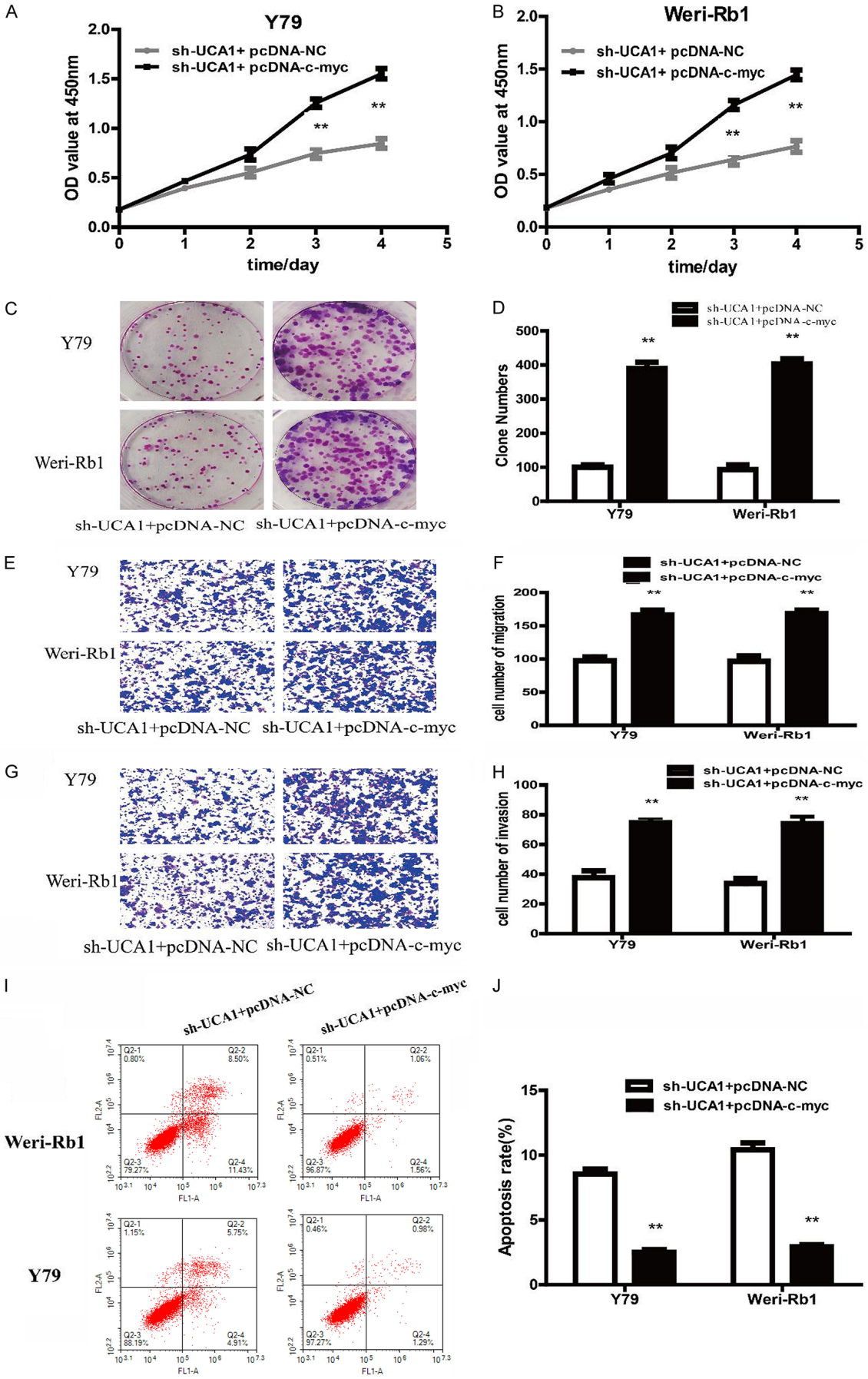
A
Y79
sh-UCA1+ pcDNA-NC
sh-UCA1+ pcDNA-c-myc
OD value at 450nm
time/day
B
Weri-Rb1
C
Y79
Weri-Rb1
sh-UCA1+pcDNA-NC
sh-UCA1+pcDNA-c-myc
D
Clone Numbers
E
F
cell number of migration
G
H
cell number of invasion
I
Q2-1 0.80%
Q2-2 8.50%
Q2-3 79.27%
Q2-4 11.43%
Q2-1 0.51%
Q2-2 1.06%
Q2-3 96.87%
Q2-4 1.56%
Q2-1 1.15%
Q2-2 5.75%
Q2-3 88.19%
Q2-4 4.91%
Q2-1 0.46%
Q2-2 0.98%
Q2-3 97.27%
Q2-4 1.29%
FL2-A
FL1-A
J
Apoptosis rate(%)

Figure 6. C-myc overexpression abolished UCA1 knockdown-induced retinoblastoma cell proliferation, clone numbers, migration, invasion, and apoptosis. Cell proliferation (A, B), clone numbers (C, D), migration and invasion ability (E-H) as well as apoptosis (I, J) were measured in Y79 and WERI-Rb1 cells transfected with sh-UCA1+pcDNA-NC and sh-UCA1+pcDNA-c-myc by CCK8, colony formation, Transwell, and cell apoptosis assays, respectively. *P<0.05, **P<0.01; n=6, magnification 40×, scale bar 100 μm.

were subcutaneously injected into nude mice. The excised tumors are displayed in **Figure 7A**, and the tumor growth rate and weight were recorded. As displayed in **Figure 7B**, **7C**, the tumor growth rate and weight of the UCA1 silencing group were dramatically lower than those of control group. Besides, the UCA1 and c-myc expression in lncRNA UCA1-knockdown group were dramatically reduced relative to those of control group. Nonetheless, the miR-124 level was upregulated in UCA1-knockdown group relative to that in control group (**Figure 7D-F**). The correlation analysis showed that UCA1 was inversely related to miR-124 (**Figure 7G**); Further, miR-124 expression was significantly decreased, and c-myc expression was significantly increased in RTs compared with that in matched controls (**Figure 7H**, **7I**). Pearson correlation analysis displayed that c-myc expression was inversely related to miR-124 expression (**Figure 7J**). C-myc expression was positively related to UCA1 in RTs (**Figure 7K**). These findings suggest that UCA1 might facilitate RB development *in vivo* by increasing c-myc and inhibiting miR-124.

Discussion

Previous work has shown that the dysregulated expression of lncRNAs might assume an important part in several cancers, including RB. For example, the lncRNA NEAT1 is elevated in RTs and cell lines. The overexpression of NEAT1 promotes RC growth by regulating the miR-204-CXCR4 axis [32]. PVT1, as an onco-lncRNA in RB, acts by sponging miR-488-3p [33]. Simultaneously, MEG3 [34] and MT1JP [35] were found to be downregulated in RTs and cells and play a suppressive role in tumors. The lncRNA, UCA1, is a bladder cancer-specific lncRNA that plays an essential role in tumorigenesis as a carcinoembryonic gene [36]. A recent study reported that UCA1 was dramatically increased in chemo-resistant RTs and multi-drug resistant RCLs, and predicted poor OS. Furthermore, UCA1 increased the proliferation and multi-drug resistance of RCs by downregulating miR-513a-5p [17]. Nevertheless, the role of UCA1 in RC growth is still unclear.

The UCA1 upregulation in RTs and cell lines was observed in this study, which aligned with previously published data [17]. Further, UCA1 knockdown was shown to suppress cell proliferation, migration, and invasion, and facilitate the apoptosis of RCs. *In vivo* experiments also revealed that silencing UCA1 significantly retarded tumor size and weight. These observations displayed that UCA1 might assume an oncogenic role in RB.

Bioinformatics predictions revealed that miR-124 may act as a candidate target of UCA1. In this study, the miR-124 expression was reduced in RTs and cell lines, and was negatively correlated with UCA1, thereby aligning with the findings of previous studies [24]. UCA1 silencing increased the expression of miR-124, while the miR-124 mimic and inhibitor significantly inhibited and increased the expression of UCA1. Rescue experiments revealed that the effects induced by UCA1 knockdown, on RC proliferation, migration, invasion, and apoptosis could be reversed by miR-124 inhibition. Thus, UCA1 may target and negatively regulate miR-124 in RCs. Bioinformatics prediction showed that c-Myc might be a target geneof miR-124.

C-myc is relevant to numerous cellular processes, including cell cycle, apoptosis, autophagy, and carcinogenesis [37]. C-myc has been reported to be an oncogene and is upregulated in several tumors, such as HCC [38], colon carcinoma [39], and glioma [40]. Recent studies have shown that c-myc was increased in RCLs and assumed an oncogenic role in RB development. Accordingly, the overexpression of c-Myc promotes RC proliferation and inhibits apoptosis [41]. In this study, c-myc expression was elevated in RTs and cells, assuming an oncogenic role of c-myc.

The correlation of c-Myc expression and miR-124 was assessed in this study. The miR-124 overexpression suppressed the mRNA and protein expression levels of c-myc in RCs. Luciferase reporter assay results revealed that miR-124 could directly interact with the c-myc 3'-UTR. These findings suggest that c-Myc might act as a regulatory target of miR-124.

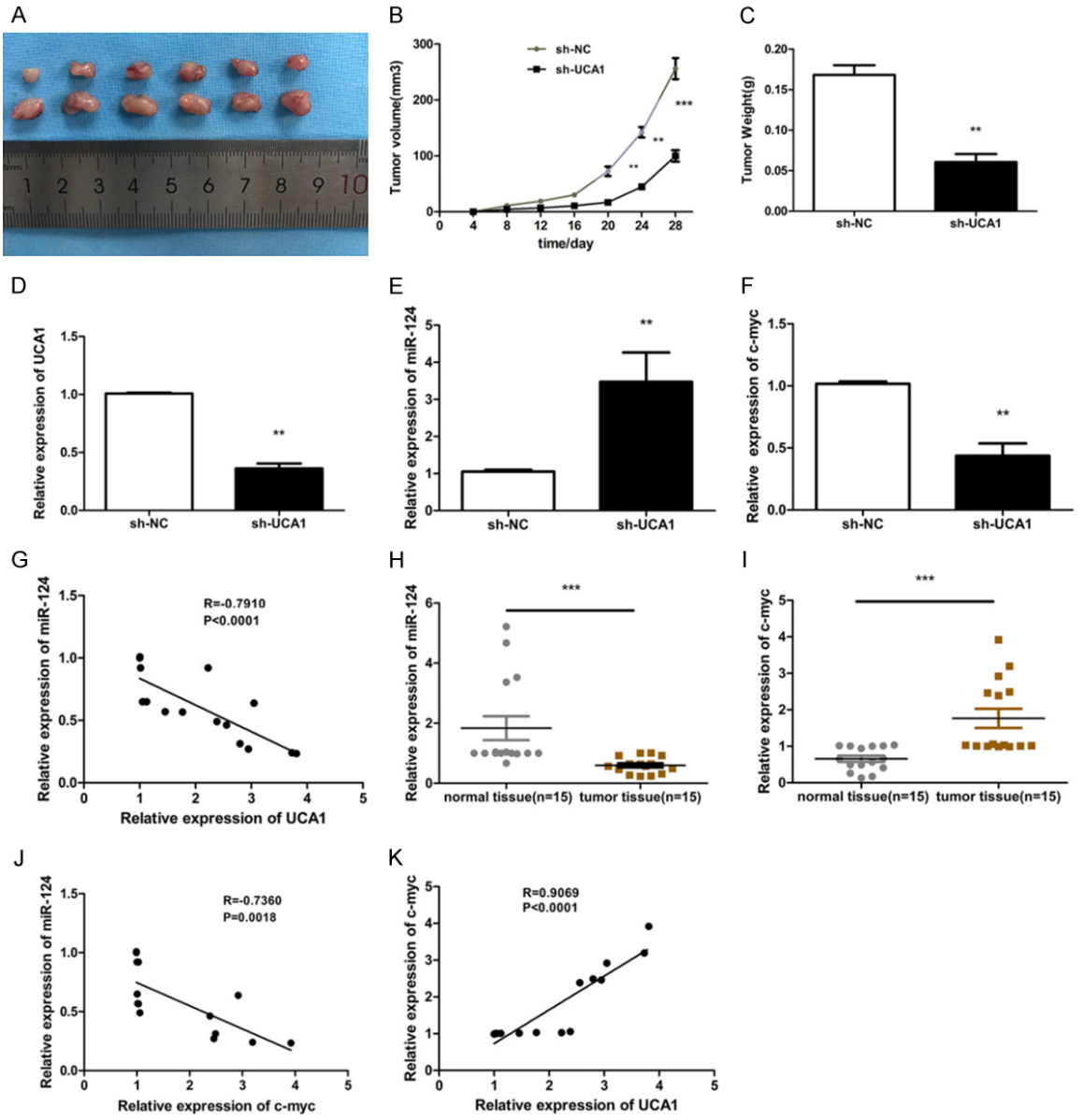

Figure 7. UCA1 knockdown repressed tumor growth *in vivo*. (A) Excised tumor of xenograft mice. (B) Tumor growth curve for 28 days. (C) Tumor weight. The expression level of UCA1 (D), miR-124 (E), and c-myc (F) of xenograft tumor. (G) UCA1 was negatively correlated with miR-124 (r=-0.7910, P<0.0001). (H) Expression level of miR-124 in retinoblastoma tissues compared to that in normal controls. (I) Expression level of c-myc in retinoblastoma tissues compared to that in normal controls. (J) C-myc was negatively correlated with miR-124 (*r*=-0.7360, *P*<0.0001). (K) C-myc was positively correlated with UCA1 (*r*=0.9069, *P*<0.0001). $^{*}P<0.05$, $^{**}P<0.01$; n=6.

Similarly, UCA1 silencing decreased the mRNA and protein expressions of c-myc in RCs. Pearson correlation analysis revealed that c-myc was positively related to UCA1 and inversely related to miR-124 in RTs (Supplementary Figure 2).

The miR-124 inhibitor reversed the suppressed c-myc expression level induced by UCA1 silencing, suggesting that UCA1 may function through regulating c-myc via miR-124 sponging. Nonetheless, CCK8, CFA, Transwell, and apoptosis assays displayed that the overexpression of c-myc reversed RC proliferation, clone numbers, migration, invasion, and apoptosis induced by UCA1 silencing. The results indicated that UCA1 silencing may inhibit RC development by modulating miR-124/c-myc axis.

The incidence of RB is relatively low, and many cases remain undiagnosed due to infrequent

visits. As a result, there is a low number of RB cases, despite presumption of a high prevalence [2]. In this study, data were obtained from only 15 patients with eyeball enucleation due to the low availability of cases. Further, no systemic metastases were found in any of these cases. Nonetheless, follow-up after enucleation is difficult because of the lack of patient compliance. Therefore, this study had two limitations: (i) a small number of cases and (ii) lack of data on prognosis and survival curve analysis.

In conclusion, UCA1 expression is elevated in RTs and cell lines, and UCA1 might assume an oncogenic role in the regulation of RC progression. Mechanistically, UCA1 may modulate RC progression by regulating c-myc via sponging miR-124. These findings may provide an experimental basis for further identification of the therapeutic targets of RB.

Disclosure of conflict of interest

None.

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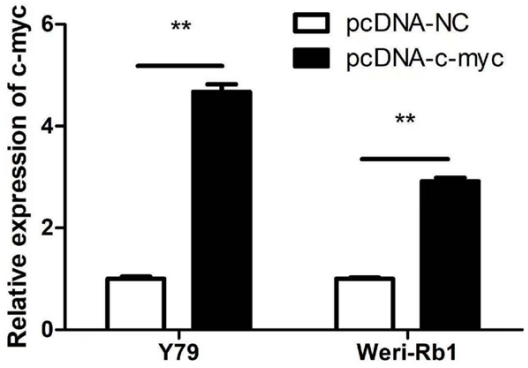
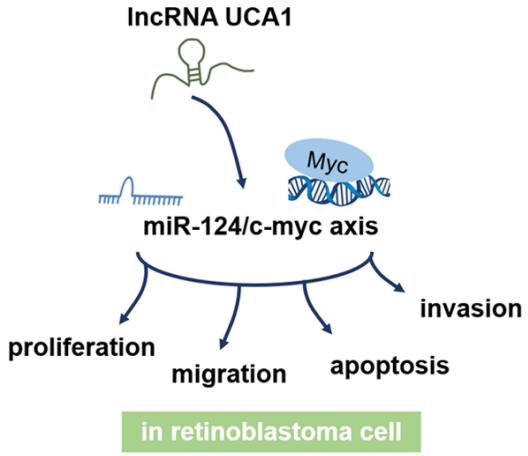
References

[1] Knudson AG Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971; 68: 820-823.
[2] Ortiz MV and Dunkel IJ. Retinoblastoma. J Child Neurol 2016; 31: 227-236.
[3] Rajeshuni N, Whittemore AS, Ludwig CA, Mruthyunjaya P and Moshfeghi DM. Racial, ethnic, and socioeconomic disparities in retinoblastoma enucleation: a population-based study, SEER 18 2000-2014. Am J Ophthalmol 2019; 207: 215-223.
[4] Fruci D, Cho WC, Nobili V, Locatelli F and Alisi A. Drug transporters and multiple drug resistance in pediatric solid tumors. Curr Drug Metab 2016; 17: 308-316.
[5] Bhan A, Soleimani M and Mandal SS. Long noncoding RNA and cancer: a new paradigm. Cancer Res 2017; 77: 3965-3981.
[6] Fatima R, Akhade VS, Pal D and Rao SM. Long noncoding RNAs in development and cancer: potential biomarkers and therapeutic targets. Mol Cell Ther 2015; 3: 5.
[7] Ponting CP, Oliver PL and Reik W. Evolution and functions of long noncoding RNAs. Cell 2009; 136: 629-641.
[8] Wang L, Yang D, Tian R and Zhang H. NEAT1 promotes retinoblastoma progression via modulating miR-124. J Cell Biochem 2019; 120: 15585-15593.
[9] Qi D, Wang M and Yu F. Knockdown of lncRNA-H19 inhibits cell viability, migration and invasion while promotes apoptosis via microRNA-143/RUNX2 axis in retinoblastoma. Biomed Pharmacother 2019; 109: 798-805.
[10] Xu C, Hu C, Wang Y and Liu S. Long noncoding RNA SNHG16 promotes human retinoblastoma progression via sponging miR-140-5p. Biomed Pharmacother 2019; 117: 109153.
[11] Wang XS, Zhang Z, Wang HC, Cai JL, Xu QW, Li MQ, Chen YC, Qian XP, Lu TJ, Yu LZ, Zhang Y, Xin DQ, Na YQ and Chen WF. Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma. Clin Cancer Res 2006; 12: 4851-4858.
[12] Liu C, Jin J, Shi J, Wang L, Gao Z, Guo T and He Y. Long noncoding RNA UCA1 as a novel biomarker of lymph node metastasis and prognosis in human cancer: a meta-analysis. Biosci Rep 2019; 39: BSR20180995.
[13] Qin LT, Tang RX, Lin P, Li Q, Yang H, Luo DZ, Chen G, He Y and Li P. Biological function of UCA1 in hepatocellular carcinoma and its clinical significance: investigation with in vitro and meta-analysis. Pathol Res Pract 2018; 214: 1260-1272.
[14] Liu X, Liu X, Qiao T and Chen W. Prognostic and clinicopathological significance of long noncoding RNA UCA1 in colorectal cancer: results from a meta-analysis. Medicine (Baltimore) 2019; 98: e18031.
[15] Gao S, Zhao ZY, Wu R, Zhang Y and Zhang ZY. Prognostic value of long noncoding RNAs in gastric cancer: a meta-analysis. Onco Targets Ther 2018; 11: 4877-4891.
[16] Aalijahan H and Ghorbian S. Clinical application of long non-coding RNA-UCA1 as a candidate gene in progression of esophageal cancer. Pathol Oncol Res 2019; 26: 1441-1446.
[17] Yang L, Zhang L, Lu L and Wang Y. lncRNA UCA1 increases proliferation and multidrug resistance of retinoblastoma cells through downregulating miR-513a-5p. DNA Cell Biol 2020; 39: 69-77.
[18] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[19] Cheng AM, Byrom MW, Shelton J and Ford LP. Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 2005; 33: 1290-1297.

[20] Liu F, Hu H, Zhao J, Zhang Z, Ai X, Tang L and Xie L. miR-124-3p acts as a potential marker and suppresses tumor growth in gastric cancer. Biomed Rep 2018; 9: 147-155.
[21] Idichi T, Seki N, Kurahara H, Fukuhisa H, Toda H, Shimonosono M, Yamada Y, Arai T, Kita Y, Kijima Y, Mataki Y, Maemura K and Natsugoe S. Involvement of anti-tumor miR-124-3p and its targets in the pathogenesis of pancreatic ductal adenocarcinoma: direct regulation of ITGA3 and ITGB1 by miR-124-3p. Oncotarget 2018; 9: 28849-28865.
[22] Shen C, Hua H, Gu L, Cao S, Cai H, Yao X and Chen X. miR-124 functions as a melanoma tumor suppressor by targeting RACK1. Onco Targets Ther 2019; 12: 9975-9986.
[23] Huang J, Yang Y, Fang F and Liu K. MALAT1 modulates the autophagy of retinoblastoma cell through miR-124-mediated stx17 regulation. J Cell Biochem 2018; 119: 3853-3863.
[24] Liu S, Hu C, Wang Y, Shi G, Li Y and Wu H. miR-124 inhibits proliferation and invasion of human retinoblastoma cells by targeting STAT3. Oncol Rep 2016; 36: 2398-2404.
[25] Liu C, Zhang H and Liu H. Long noncoding RNA UCA1 accelerates nasopharyngeal carcinoma cell progression by modulating miR-124-3p/ITGB1 axis. Onco Targets Ther 2019; 12: 8455-8466.
[26] Shu T, He L, Wang X, Pang M, Yang B, Feng F, Wu Z, Liu C, Zhang S, Liu B, Wang Q and Rong L. Long noncoding RNA UCA1 promotes chondrogenic differentiation of human bone marrow mesenchymal stem cells via miRNA-145-5p/SMAD5 and miRNA-124-3p/SMAD4 axis. Biochem Biophys Res Commun 2019; 514: 316-322.
[27] Taniguchi K, Sugito N, Kumazaki M, Shinohara H, Yamada N, Matsuhashi N, Futamura M, Ito Y, Otsuki Y, Yoshida K, Uchiyama K and Akao Y. Positive feedback of DDX6/c-Myc/PTB1 regulated by miR-124 contributes to maintenance of the Warburg effect in colon cancer cells. Biochim Biophys Acta 2015; 1852: 1971-1980.
[28] Shang Y. LncRNA THOR acts as a retinoblastoma promoter through enhancing the combination of c-myc mRNA and IGF2BP1 protein. Biomed Pharmacother 2018; 106: 1243-1249.
[29] Wu S, Gu Z, Wu Y, Wu W, Mao B and Zhao S. LINC00324 accelerates the proliferation and migration of osteosarcoma through regulating WDR66. J Cell Physiol 2020; 235: 339-348.
[30] Gong Y, Wang H and Xia H. Stable transfection into rat bone marrow mesenchymal stem cells by lentivirus-mediated NT-3. Mol Med Rep 2015; 11: 367-373.
[31] Fan SP, Li XH, Shi CX, Fan CX and Ye FG. Lentivirus-mediated BMP-2 overexpression plasmid transfection into bone marrow mesenchymal stem cells combined with silk fibroin scaffold for osteoblast transformation. Zhongguo Gu Shang 2019; 32: 853-860.
[32] Zhong W, Yang J, Li M, Li L and Li A. Long noncoding RNA NEAT1 promotes the growth of human retinoblastoma cells via regulation of miR-204/CXCR4 axis. J Cell Physiol 2019; 234: 11567-11576.
[33] Wu XZ, Cui HP, Lv HJ and Feng L. Knockdown of lncRNA PVT1 inhibits retinoblastoma progression by sponging miR-488-3p. Biomed Pharmacother 2019; 112: 108627.
[34] Gao Y and Lu X. Decreased expression of MEG3 contributes to retinoblastoma progression and affects retinoblastoma cell growth by regulating the activity of Wnt/β-catenin pathway. Tumour Biol 2016; 37: 1461-1469.
[35] Bi LL, Han F, Zhang XM and Li YY. LncRNA MT1JP acts as a tumor inhibitor via reciprocally regulating Wnt/β-Catenin pathway in retinoblastoma. Eur Rev Med Pharmacol Sci 2018; 22: 4204-4214.
[36] Avgeris M, Tsilimantou A, Levis PK, Rampias T, Papadimitriou MA, Panoutsopoulou K, Stravodimos K and Scorilas A. Unraveling UCA1 lncRNA prognostic utility in urothelial bladder cancer. Carcinogenesis 2019; 40: 965-974.
[37] Miller DM, Thomas SD, Islam A, Muench D and Sedoris K. c-Myc and cancer metabolism. Clin Cancer Res 2012; 18: 5546-5553.
[38] Peng N, Li J, He J, Shi X, Huang H, Mo Y, Ye H, Wu G, Wu F, Xiang B, Zhong J, Li L and Zhu S. c-Myc-mediated SNRPB upregulation functions as an oncogene in hepatocellular carcinoma. Cell Biol Int 2020; 44: 1103-1111.
[39] Mármol I, Sánchez-de-Diego C, Pradilla Dieste A, Cerrada E and Rodriguez Yoldi MJ. Colorectal carcinoma: a general overview and future perspectives in colorectal cancer. Int J Mol Sci 2017; 18: 197.
[40] Liu N, Wang Z, Liu D and Xie P. HOXC13-AS-miR-122-5p-SATB1-C-Myc feedback loop promotes migration, invasion and EMT process in glioma. Onco Targets Ther 2019; 12: 7165-7173.
[41] Wang X, Zhang X, Han Y, Wang Q, Ren Y, Wang B and Hu J. Silence of lncRNA ANRIL represses cell growth and promotes apoptosis in retinoblastoma cells through regulating miR-99a and c-Myc. Artif Cells Nanomed Biotechnol 2019; 47: 2265-2273.



Supplementary Figure 1. Expression levels of c-myc in Y79 and WERI-Rb1 cells transfected with pcDNA-NC and pcDNA-c-myc. $^{**}P<0.01$; n=9.



Supplementary Figure 2. Schematic.