

# Oncogenic *cAMP* responsive element binding protein 1 is overexpressed upon loss of tumor suppressive miR-10b-5p and miR-363-3p in renal cancer

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**Abstract.** Renal cell carcinoma (RCC) is the most common kidney cancer in adults and has a poor prognosis. *cAMP responsive element binding protein 1* (*CREB1*) is a proto-oncogenic transcription factor involved in malignancies of various organs. However, its functional role(s) have not yet been elucidated in RCC. We investigated the expression pattern, function and regulation of *CREB1* in RCC. *CREB1* was overexpressed in the RCC tissues and cell lines. Downregulation of *CREB1* inhibited RCC tumorigenesis by affecting cell proliferation, migration and apoptosis. Multiple computational algorithms predicted that the 3'-untranslated region (3'-UTR) of human *CREB1* mRNA is a target for miR-10b-5p and miR-363-3p. Luciferase reporter assay, qPCR and western blot analysis confirmed that miR-10b-5p and miR-363-3p bind directly to the 3'-UTR of *CREB1* mRNA and inhibit mRNA and protein expression of *CREB1*. qPCR data also revealed a significantly lower expression of miR-10b-5p and miR-363-3p in RCC tissues. Introduction of miR-10b-5p and miR-363-3p mimics led to suppressed expression of *CREB1* and inhibited

cell proliferation, migration and apoptosis reduction. Taken together, we propose that *CREB1* is an oncogene in RCC and that upregulation of *CREB1* by loss of tumor suppressive miR-10b-5p and miR-363-3p plays an important role in the tumorigenesis of RCC.

## Introduction

Renal cancer is one of the 14 most frequent cancers worldwide and its incidence as well as mortality rate have plateaued in Western countries during the past decade. In developing countries, the incidence of tumors of the kidney has increased (1,2). Renal cell carcinoma (RCC) is the most common renal cancer in adults. In the United States, the rate of RCC has increased by 1.7% per year for the past 10 years (3). In China, kidney cancer incidence has increased with an average annual growth rate of 6.5% in the past 20 years, and kidney cancer-related deaths have surpassed bladder cancer moving to the first place in cancers of the urinary system (4,5). RCC has a poor prognosis due to a lack of early-warning signs and symptoms and resistance to radiotherapy and chemotherapy (6). Therefore, it is important to identify novel biomarkers for early detection, diagnosis and personalized therapy.

Transcription factors are key regulators of the pattern of gene expression and directly regulate biological processes such as cell growth, proliferation, survival, self-renewal and invasion. By driving the expression of key target genes, these oncogenic transcription factors play a central role in tumor pathogenesis (7). *cAMP responsive element binding protein 1* (*CREB1*) is a proto-oncogenic transcription factor. Aberrant expression of *CREB1* has been found in acute myeloid leukemia (AML) (8), non-small cell lung carcinoma (NSCLC) (9), breast cancer (10), gliomas (11), mesotheliomas (12) and endocrine tumors (13,14). As a potent oncogene, through gene amplification, chromosome translocation, interaction with viral oncoproteins and inactivation of tumor-suppressor genes (15), *CREB1* promotes tumorigenesis by significantly impacting growth, prolifera-

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tion, survival, metastasis and invasion of tumor cells (7,12). In response to various stimuli such as growth factors, neurotransmitters, stress signals and other agents that elevate intracellular *cAMP* or  $\text{Ca}^{2+}$  levels, *CREB* is activated through phosphorylation at Ser133 and/or nuclear translocation of transducer of regulated *CREB* activity (TORC) coactivators (15). In addition, activated *CREB1* turns on the transcription of more than 5,000 target genes such as *c-fos* (16), cell cycle regulatory genes such as cyclin A1 and cyclin D2 (17,18), and other genes related to glucose homeostasis, growth factor-dependent cell survival and neuronal activities such as memory and learning (19-21). However, the oncogenic role of *CREB1* has not been investigated in RCC.

MicroRNAs (miRNAs) play important roles in many biological processes including tumorigenesis by regulating ~50% of human protein-coding genes (22). Depending on their target genes, miRNAs can function as both oncogenes and tumor-suppressor genes by affecting cell differentiation, migration, growth, proliferation, apoptosis and metabolism (22-24). Generally, miRNAs, as a negative regulator, bind to a partially complementary sequence usually located in the 3'-untranslated region (3'-UTR) of their target mRNA and inhibit its translation (25). By targeting the most common genes involved in RCC tumorigenesis including the *VHL/HIF*, *VEGF* and *mTOR* pathway, miRNAs play crucial roles in RCC initiation and development (26,27).

In the present study, we showed that the cell proliferation, migration and apoptosis reduction of human RCC cell lines were suppressed by knockdown of *CREB1*. Furthermore, using computational prediction followed by experimental confirmation, miR-10b-5p and miR-363-3p were found to bind directly to the 3'-UTR of *CREB1* and downregulate *CREB1*. In human samples, miR-10b-5p and miR-363-3p expression was downregulated while *CREB1* expression was upregulated, and a negative correlation was found between miR-10b-5p and miR-363-3p and *CREB1* expression. Finally, the oncogenic activity of *CREB1* was decreased by miR-10b-5p and miR-363-3p which showed a similar function to si-*CREB1*.

## Materials and methods

**Clinical specimens.** A total of 35 paired fresh RCC specimens and adjacent normal tissues (ANTs) were collected from the Peking University Shenzhen Hospital (Shenzhen, Guangdong, China). All patients were informed in regards to the purposes of this research and provided written consent. This study was approved by the Institutional Review Board and Ethics Committee of Peking University Shenzhen Hospital, China. All the RCC samples were diagnosed as RCC pathologically without chemotherapy or radiotherapy before surgery, and the ANTs were collected from the normal region  $\geq 5$  cm outside the tumor range. All specimens were immediately frozen in liquid nitrogen following surgical resection for further study. The clinicopathological information of the patients is documented in Table I.

**Cell culture and cell transfection.** Human RCC cell lines, including 786-O, ACHN and Caki-2, and cervical cancer cell line HeLa were obtained from the American Type Culture

Table I. Clinicopathological features of the RCC patients.

Characteristics	Data
Mean age (range), in years	49 (25-71)
Gender, no. of patients	
Male/female	23/12
Histological type, no. of patients	
Clear cell/papillary	32/3
pT-stage, no. of patients	
T1/T2/T3+T4	19/14/2
Fuhrman grade, no. of patients	
I/II/III/IV	10/18/5/2
AJCC clinical stages, no. of patients	
I/II/III+IV	21/11/3

cpT, primary tumor; AJCC, American Joint Committee on Cancer.

Collection (ATCC, Manassas, VA, USA). The human embryo kidney cell line 293T (293T) was purchased from the Type Culture Collection of the Chinese Academy of Medical Sciences, Beijing, China. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA), with 10% fetal bovine serum (Gibco), 1% antibiotics (100  $\mu\text{g}/\text{ml}$  penicillin and 100  $\text{mg}/\text{ml}$  streptomycin sulfates) and 1% glutamate (Gibco), and then incubated at 37°C in a humidified chamber containing 5%  $\text{CO}_2$ .

For the downregulation of *CREB1*, two siRNAs targeting *CREB1* (si-*CREB1a* and si-*CREB1b*) were designed and purchased from GenePharma (Shanghai, China). A negative control (NC) (siRNA-NC, si-NC), positive control (siRNA-GAPDH) and Fam-labeled siRNA-NC (siRNA-NC-FAM) used in this study were also designed and purchased from GenePharma. For the overexpression of miR-10b-5p and miR-363-3p, miR-10b-5p and miR-363-3p mimic oligoribonucleotides and NC were all chemically synthesized and purchased from GenePharma. When the cells reach 60-80% confluency, they were transfected with si-*CREB1a*, si-*CREB1b*, si-NC, siRNA-GAPDH, siRNA-NC-FAM, miR-10b-5p and miR-363-3p mimics or the NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), which were mixed in Opti-MEM® I reduced serum medium (Gibco). Then, fluorescence microscopy and the quantitative real-time PCR (qPCR) were used to verify and quantify transfection efficiency. The sequences of all the siRNAs and RNAs are summarized in Table II.

**RNA extraction, reverse transcription and qPCR.** The total RNA of cells and tissue samples was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA samples with 260/280 ratios of 1.8-2.0 were used for further experiments. Total RNA was converted into cDNA by using the miScript II RT kit (Qiagen, Valencia, CA, USA) or PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan). qPCR analysis was used to detect the expression level of *CREB1* using SYBR® Premix Ex Taq II (Takara) and the expression level of miR-10b-5p and

Table II. Sequences of the primers, siRNAs and RNAs.

Name	Sequence	
<i>CREB1</i>	F: 5'-GCCTGCTATACCTGCCATTG-3'	R: 5'-ACCCGATTCCGATTTGCTTAG-3'
GAPDH	F: 5'-GGAGCGAGATCCCTCCAAAT-3'	R: 5'-GGCTGTTGTCATACTTCTCATGG-3'
si- <i>CREB1a</i>	F: 5'-GAGAGAGGUCCGUCUAAUG-3'	R: 5'-CAUUAGACGGACCUCUCUC-3'
si- <i>CREB1b</i>	F: 5'-AUUGUUAGCCAGCUGUAUUG-3'	R: 5'-CAAUACAGCUGGCUAACAAU-3'
siRNA-NC	F: 5'-UUCUCCGAACGUGUCACGUTT-3'	R: 5'-ACGUGACACGUUCGGAGAATT-3'
siRNA-GAPDH	F: 5'-GUAUGACAACAGCCUCAAGTT-3'	R: 5'-CUUGAGGCUGUUGUCAUACTT-3'
siRNA-NC-FAM	F: 5'-UAUGUAGCUAACAAGGUGCCA-3'	R: 5'-UUCUCCGAACGUGUCACGUTT-3'
miR-10b-5p <sup>a</sup>	F: 5'-TACCCTGTAGAACCGAATTTGTG-3'	
miR-363-3p <sup>a</sup>	F: 5'-AATTGCACGGTATCCATCTGTA-3'	
U6	F: 5'-CTCGCTTCGGCAGCACA-3'	R: 5'-ACGCTTCACGAATTTGCGT-3'
miR-10b-5p mimics	F: 5'-UACCCUGUAGAACCGAAUUUGUG-3'	R: 5'-CAAAUUCGGUUCUACAGGGUAUU-3'
miR-363-3p mimics	F: 5'-AAUUGCACGGUAUCCAUCUGUA-3'	R: 5'-CAGAUGGAUACCGUGCAAUUUU-3'
NC	F: 5'-UUCUCCGAACGUGUCACGUTT-3'	R: 5'-ACGUGACACGUUCGGAGAATT-3'

<sup>a</sup>The reverse primer was provided by the miScript SYBR®-Green PCR kit (Qiagen, Valencia, CA, USA). F, forward; R, reverse.

miR-363-3p using miScript SYBR®-Green PCR kit (Qiagen), respectively, according to the manufacturer's instructions on the LightCycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). GAPDH and U6 were used as the internal control. Their expression levels were shown as fold differences relative to GAPDH and U6, which was based on the equation: Relative expression =  $2^{-\Delta\Delta C_t}$ ,  $\Delta\Delta C_t = (\text{mean}C_{t_{\text{cancer}}} - \text{mean}C_{t_{\text{control}}}) - (\text{mean}C_{t_{\text{normal}}} - \text{mean}C_{t_{\text{control}}})$ . The primers used for this study are shown in Table II. All reactions were run in triplicate.

**Cell proliferation assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, 5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was used to analyze the cell proliferation according to the manufacturer's protocol. Human RCC cells (786-O and ACHN) were seeded into 96-well culture plates at a cell density of 5,000 cells/well and transfected with either 13.3 pmol siRNAs or miRNAs for each well. Cell growth was assayed by addition of 20  $\mu$ l of MTT to each well, and the plate was incubated at 37°C for 4-6 h. Later, the reaction was stopped by addition of 120  $\mu$ l DMSO (Sigma-Aldrich). After shaking for 30 min at room temperature, the optical density (OD) at the wavelength of 490 nm (with 630 nm as the reference wave length) of each sample was measured with an enzyme immunoassay instrument (Bio-Rad, Hercules, CA, USA). The OD values were measured for 3 days at every 24 h interval. All assays were carried out in triplicate.

**Cell migration assay.** For the Transwell migration assays,  $1 \times 10^4$  786-O or ACHN cells were harvested 24 h post-transfection. They were then plated into the upper chambers (24-well insert, pore size 8  $\mu$ m; Corning, Inc., Corning, NY, USA) with 100  $\mu$ l serum-free DMEM. The lower chambers were filled with 500  $\mu$ l DMEM containing 10% fetal bovine serum. The cells were then cultured at 37°C in a humidified chamber

containing 5% CO<sub>2</sub>. Two days later, the cells on the surface of the upper chamber were wiped off with cotton gently. Cells under the surface of the lower chamber were washed with PBS, fixed with paraformaldehyde for 25 min, stained with 0.1% crystal violet for 25 min, and then washed with PBS tenderly three times.

Wound scratch assay was also used to examine the migration of RCC cells. Approximately  $3 \times 10^5$  786-O or ACHN cells were seeded in 12-well plates one day before the transfection. The cells were transfected when they grew to reach 80-90% confluency, and a sterile 200- $\mu$ l pipette tip was used to scrape a clear line through the cell monolayer 6 h post-transfection. Then, the medium was changed with serum-free DMEM. Images of the scratches were acquired with a digital camera system at 0 and 24 h. Experiments were run in three independent repeats in triplicate and analyzed in a double-blinded manner by at least two observers.

**Cell apoptosis assay.** For cell apoptosis analysis,  $2 \times 10^5$  786-O or ACHN cells were seeded in 6-well plates and transfected with 200 pmol siRNAs/miRNAs for 6 h. Forty-eight hours post-transfection, the cells, including floating cells, were harvested, washed twice with 4°C PBS and resuspended in 100  $\mu$ l 1X binding buffer and stained with 5  $\mu$ l of propidium iodide (PI) and 5  $\mu$ l of Annexin V-FITC (Invitrogen) for 15 min at room temperature. Flow cytometry (EPICS, XI-4; Beckman, CA, USA) was used to quantify the percentage of apoptotic cells within 30 min after staining and 400  $\mu$ l 1X binding buffer was added to each sample before measurement. Each experiment was conducted at least three times.

**Target gene prediction.** Prediction of miRNAs that target *CREB1* was performed by computational algorithms due to their base-pairing rules between miRNA and mRNA target sites, location of binding sequences within the target's 3'-UTR, and conservation of target binding sequences within related

genomes. In our study, miRNAs that target *CREB1* were predicted by miRanda (<http://mirdb.org/miRDB/index.html>), TargetScanRelease 6.2 (<http://www.targetscan.org>), microRNA (<http://www.microrna.org>), and miRWalk (<http://www.umh.uni-heidelberg.de/apps/zmf/mirwalk>). Putative miRNAs targeting *CREB1* predicted by all four algorithms were accepted and candidates were chosen based on experimental confirmation.

**Plasmid construction and luciferase reporter assay.** Luciferase reporter plasmids within the *CREB1* 3'-UTR fragment (500 bp) that containing target sequences of miR-10b-5p and miR-363-3p were constructed to confirm that miR-10b-5p and miR-363-3p bind directly to the 3'-UTR of *CREB1* and inhibit its translation. The miRNA target sequences were inserted between the *XhoI*-*NotI* restriction sites in the 3'-UTR of the *hRluc* gene in the psiCHECK<sup>TM</sup>-2 luciferase vector (Promega, Madison, WI, USA), generating the wild-type (WT) of psiCHECK<sup>TM</sup>-2-3'UTR. The primer sequences for the 3'-UTR of *CREB1* mRNA containing the miR-10b-5p binding site (forward primer, 5'-CCGCTCGAGTGAAGAGTTGTGAGTAAATAGTTC-3' and reverse primer, 5'-AAGGAAAAAAGCGGCCGCCCTGTTTATAATATCAACTTGCATC-3') and containing the miR-363-3p binding site (forward primer, 5'-CCGCTCGAGCTCAAGAAATTTTCAACGCCAG-3' and reverse primer, 5'-AAGGAAAAAAGCGGCCGCGTTGAA CACATACAGAACTGAATTA-3') were designed. Then, we mutated the potential binding sites by exchanging the G and T, A and C, and inserted the mutated sequences in psiCHECK<sup>TM</sup>-2-3'UTR (MUT) (Fig. 6A and C). These short fragments were all cloned into psiCHECK-2 luciferase vector, respectively, and all the constructs were verified by sequencing.

For the luciferase reporter assay, 0.2  $\mu$ g luciferase reporter vectors, together with 20 pmol miR-10b-5p mimics, miR-363-3p mimics and negative control, were transfected into 786-O, ACHN, HeLa and 293T cells using Lipofectamine 2000. Luciferase activity was detected using the dual luciferase assay system (Promega) on the Modulus<sup>TM</sup> Single Tube Multimode Reader (Bio-Systems International, Beloit, WI, USA), according to the manufacturer's instructions 24 h after transfection. Normalized data were calculated as the quotient of *Renilla/Firefly* luciferase activities. The experiments were performed in duplicate and repeated at least three times.

**Immunohistochemistry (IHC).** Immunohistochemical assay of *CREB1* was performed in 20 RCC paraffin-embedded tissues according to standard procedures. The 5- $\mu$ m sections were dewaxed in xylene, rehydrated in a descending series of ethanol, incubated in 3% hydrogen peroxide solution for 20 min, boiled in 0.01 M citrate buffer (pH 6.0) for antigen retrieval, and treated in 10% bovine serum albumin for 30 min at 37°C to block non-specific protein binding. Then the sections were incubated in *CREB1* mouse monoclonal antibody (1:100, ab178322; Cambridge, MA, USA) overnight at 4°C. Subsequently, the sections were rinsed with PBS and treated with the anti-rabbit IHC kit (Maixin Bio, Fuzhou, China) at 37°C for 30 min, followed by staining with a DAB kit (Maixin Bio), for 4 min and counterstained with hematoxylin. Negative controls were performed with omission of the primary antibodies.

**Protein extraction and western blot analysis.** The frozen fresh samples and collected cells of human RCC cell lines (786-O and ACHN) seeded in a 6-well plate were homogenized in three volumes of RIPA buffer (Sigma-Aldrich) on ice. The protein concentration was quantified using Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA), and protein samples (60  $\mu$ g) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% fat-free milk at room temperature for 2 h, the membranes were incubated in 5% fat-free milk containing *CREB1* (1:100, ab178322; Abcam) or  $\beta$ -actin (1:5,000, NB600-532; Novus, Littleton, CO, USA) antibody overnight at 4°C. The membranes were washed three times with TBST (Tris-buffered saline with 0.05% Tween) and incubated for 1 h with horseradish peroxidase (HRP)-linked secondary antibody (1:10,000; EarthOx, LLC, USA) at room temperature. The protein bands were detected with the chemiluminescence phototube-HRP kit (WBKLS0500; Millipore) and exposure to Kodak film.

**Statistical analysis.** All data are expressed as the mean  $\pm$  SD from at least three separate experiments. All data were analyzed by SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). The clinicopathological information of the patients was analyzed by Chi-square test, while other data were determined by the Student's t-test. Results were considered statistically significant with two-tailed  $P < 0.05$ .

## Results

**Validation of the upregulation of *CREB1* in the RCC tissues and cell lines by qPCR.** To determine the mRNA expression level of *CREB1*, qPCR was performed in 35 paired RCC tissues and ANTs. As shown in Fig. 1A, the expression of *CREB1* was significantly upregulated in the RCC tissues compared with ANTs. We also analyzed the expression of *CREB1* in RCC cell lines (786-O, ACHN and Caki-2) and 293T. As shown in Fig. 1B, the expression level of *CREB1* was significantly higher in the ACHN, 786-O and Caki-2 cells ( $P < 0.01$ ) compared with the level in the 293T cells.

**Protein expression of *CREB1* in the RCC tissues and ANTs.** *CREB1* was discovered to be upregulated in many human cancers including leukemia (8), breast cancer (10), gliomas (11) and lung cancer (9). However, the protein expression of *CREB1* in renal cancer remains unknown. Therefore, we performed IHC and western blot analysis to determine the protein expression of *CREB1* in 20 pairs of RCC sections and 6 pairs of fresh tissues, respectively. The results of the IHC assay showed that *CREB1* was located in the nuclei of the cells and overexpression of *CREB1* was observed in 80% (16/20) of the RCC sections (Fig. 1C). The western blot assay also showed that *CREB1* was upregulated in all paired RCC tissues and human RCC cell lines (786-O and ACHN) compared with the ANTs and the 293T cell line, with  $\beta$ -actin as the internal control (Fig. 1D and E). No relationship was found between the protein expression level of *CREB1* and clinicopathologic variables due to the limited number of cases.

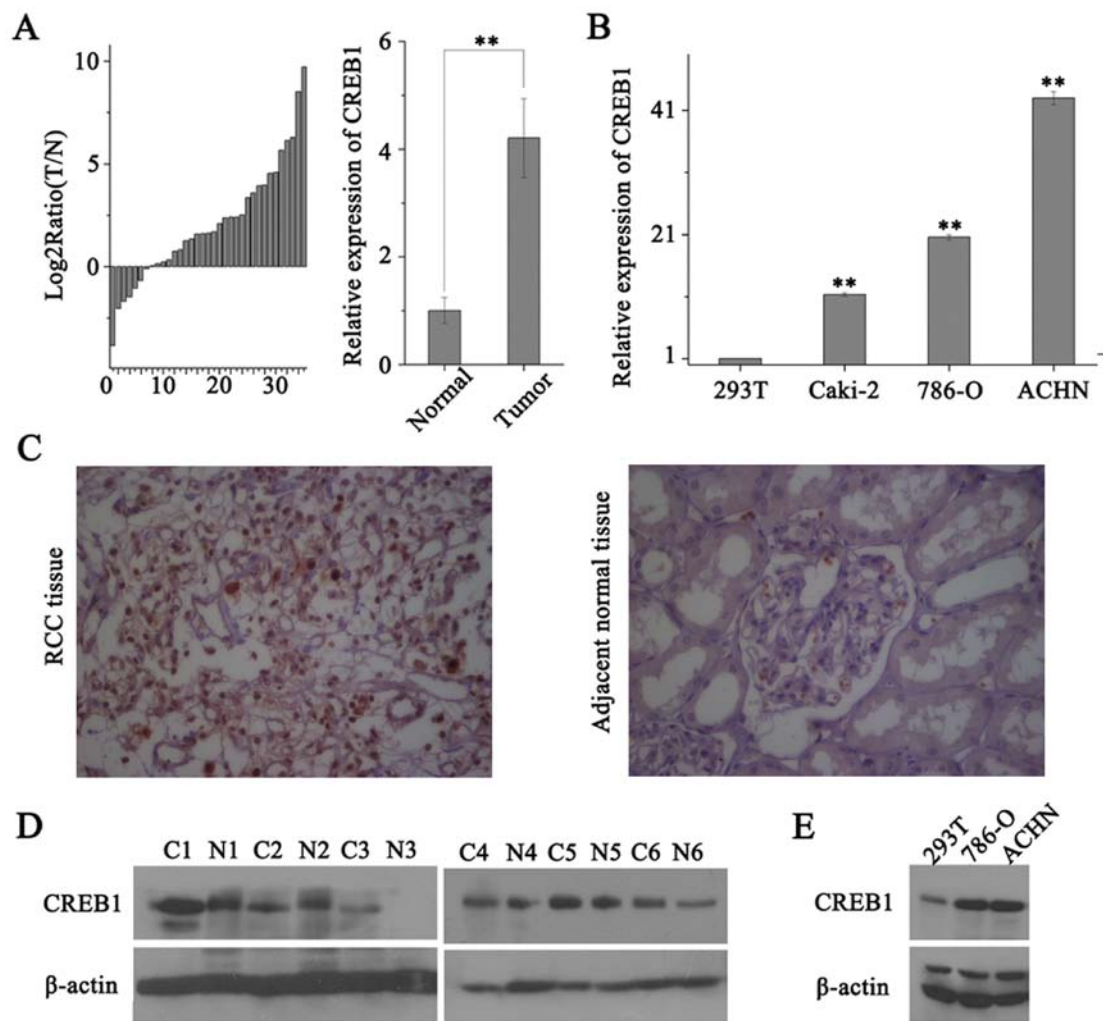


Figure 1. Overexpression of *CREB1* in RCC tissues and cell lines. The mRNA expression of *CREB1* in 35 paired RCC tissues and adjacent normal tissues (A) and human RCC cell lines (786-O, ACHN and Caki-2) and the 293T cell line (B). The protein expression of *CREB1* in paired RCC and adjacent normal tissues as shown by immunohistochemistry (C) and western blot analysis (D); and the protein expression of *CREB1* in human RCC cell lines (786-O and ACHN) and the 293T cell line (E). \*\* $P < 0.01$ .

*Silencing of CREB1 inhibits cell proliferation and migration and induces cell apoptosis in vitro.* To explore the function of *CREB1* in renal cancer, two siRNAs to downregulate *CREB1* were designed and confirmed. Through transient transfection efficiency validation of the siRNAs (Fig. 2), we verified and chose si-*CREB1a* as the most effective siRNA targeting *CREB1*. Cell proliferation, migration and apoptosis of 786-O and ACHN cells were evaluated after transfection with si-*CREB1a* (si-*CREB1*) by MTT assay, Transwell migration assay, wound scratch assay and flow cytometry.

The results of the MTT assay revealed that downregulation of *CREB1* suppressed cell proliferation. As shown in Fig. 3A, the cell proliferation of 786-O and ACHN cells was reduced by 8.15% ( $P < 0.05$ ), 16.20% ( $P < 0.01$ ), 32.51% ( $P < 0.01$ ) and 9.51% ( $P < 0.05$ ), 22.12% ( $P < 0.01$ ), 31.41% ( $P < 0.05$ ), respectively, after transfection of si-*CREB1* at 24, 48 and 72 h, compared with the cells transfected with si-NC, respectively. Transwell migration and wound scratch assays showed the same result. Inhibition of *CREB1* attenuated the migratory ability of 786-O and ACHN cells. Transwell migration assay showed that the number of migrated cells was decreased by 89.98% ( $P < 0.01$ )

in the 786-O cells and by 90.57% ( $P < 0.01$ ) in the ACHN cells (Fig. 3B). Wound scratch assay showed that the inhibition rate of migration was 40.07% for the 786-O cells ( $P < 0.01$ ) and 49.61% for the ACHN cells ( $P < 0.001$ ) (Fig. 3C). Compared with the cells transfected with si-NC, the average cell apoptosis rate was increased significantly in the cells transfected with si-*CREB1*, with 6.36 vs. 10.09% in the 786-O cells ( $P < 0.05$ ) and 6.16 vs. 12.88% in the ACHN cells ( $P < 0.01$ ) (Fig. 3D). All the data suggest that *CREB1* is an oncogene and affects cellular proliferation, migration and apoptosis.

*CREB1 is targeted by miR-10b-5p and miR-363-3p.* Bioinformatic analyses (miRanda, TargetScan Release 6.2, microRNA and miRWalk) were used to determine the potential miRNAs that target *CREB1*. miR-10b-5p and miR-363-3p, were predicted by all four algorithms simultaneously and were selected as candidates for further study (Fig. 4A and B). Furthermore, miR-10b-5p and miR-363-3p were previously found to be downregulated in RCC tissues compared with ANTs or downregulated in metastatic compared with primary RCCs by all the high-throughput screens (28-33).

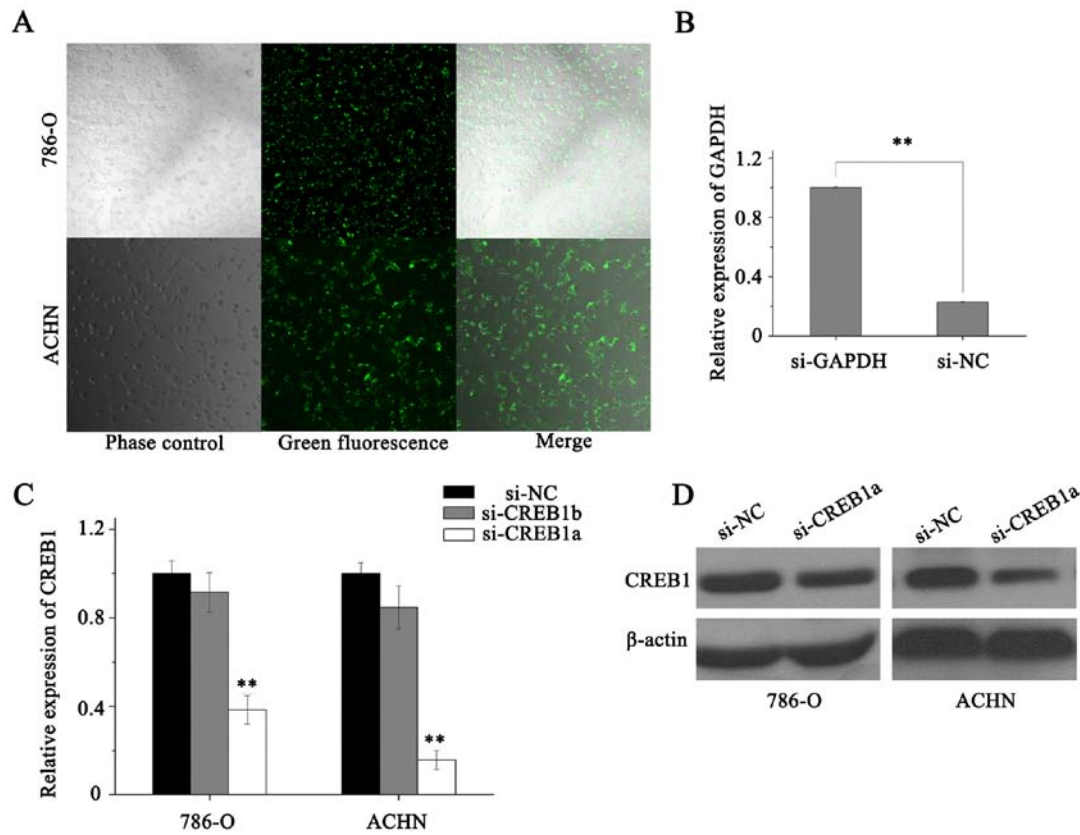


Figure 2. Validation of the silencing efficiency of *CREB1*. (A) Phase-contrast and green fluorescence images were captured from the same field. (B) The positive control of si-*GAPDH* was performed to guarantee the efficiency of the systemic experiments. (C) The relative expression of *CREB1* mRNA in 786-O and ACHN cells transfected with si-*CREB1a*, si-*CREB1b* or si-NC. (D) The protein expression of *CREB1* in 786-O and ACHN cells transfected with si-*CREB1a* or si-NC. \*\* $P < 0.01$ .

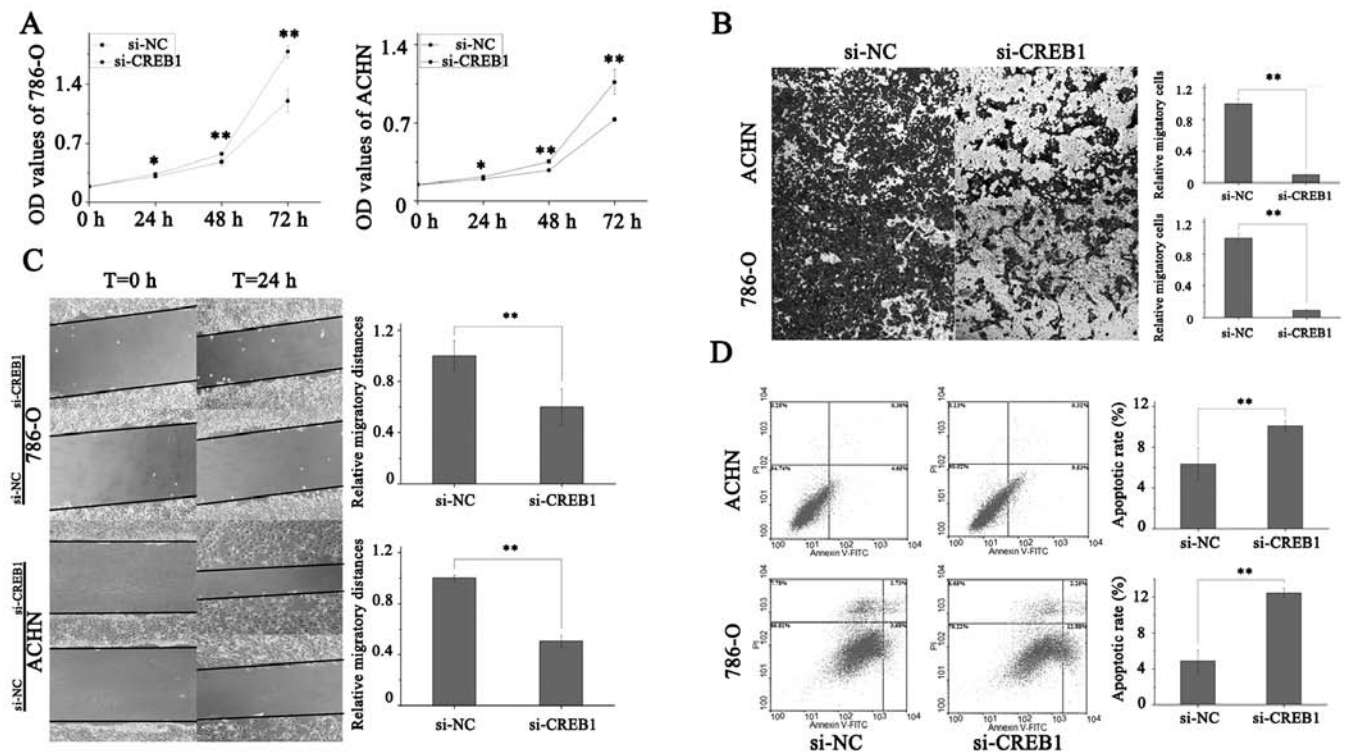


Figure 3. si-*CREB1* inhibits neoplastic phenotypes of 786-O and ACHN cells. After introduction of si-*CREB1* or si-NC, cell proliferation was measured by MTT assay (A), and cell migration was evaluated by Transwell migration (B) and wound scratch assays (C), and cell apoptosis was quantified by flow cytometry (D). \* $P < 0.05$ , \*\* $P < 0.01$ .



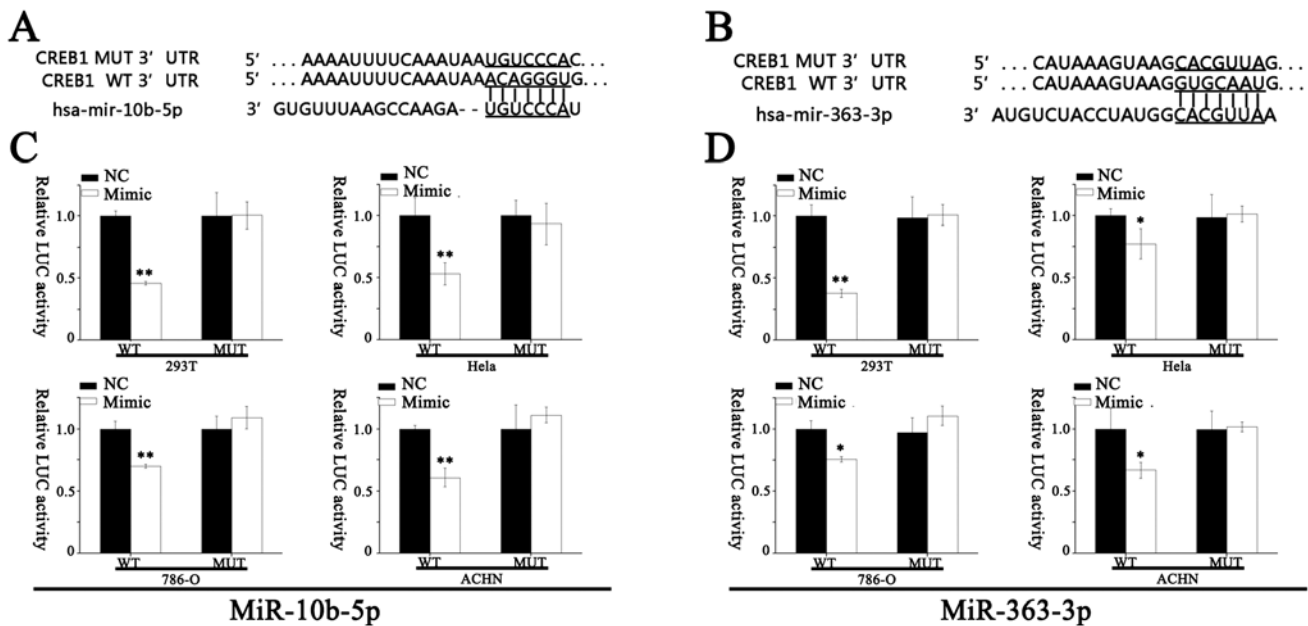


Figure 4. miR-10b-5p and miR-363-3p target CREB1. (A and B) Four fragments of CREB1 3'-UTR were constructed, which contained the wild-type (WT) potential binding sites of miR-10b-5p and miR-363-3p and the corresponding mutated sequence (MUT). (C and D) The psiCHECK<sup>TM</sup>-2 luciferase constructs containing WT or MUT sequence were transfected together with miR-10b-5p, miR-363-3p mimics and NC into 786-O and ACHN cells. Luciferase activity was measured by the dual luciferase assay system. \*P<0.05, \*\*P<0.01.

To determine whether *CREB1* is directly regulated by miR-10b-5p and miR-363-3p, a luciferase reporter assay was performed in 293T, HeLa, 786-O and ACHN cells. As shown in Fig. 4C, the relative luciferase activity of wild-type plasmids containing the putative binding site was significantly decreased when transfected with miR-10b-5p mimics in the 293T (P=0.001), HeLa (P=0.005), 786-O (P=0.009) and ACHN cells (P=0.006) while no notable reduction was observed in the mutant groups. As shown in Fig. 4D, when transfected with the miR-363-3p mimics, identical results were also obtained in the 293T (P=0.004), HeLa (P<0.05), 786-O (P<0.05) and ACHN cells (P<0.05), suggesting that miR-10b-5p and miR-363-3p may suppress the expression of *CREB1* by targeting the putative binding site in the 3'-UTR.

*CREB1* is downregulated by miR-10b-5p and miR-363-3p in RCC cell lines and tissues. To validate the results of the luciferase reporter assay, we performed qPCR and western blot analysis to quantify the mRNA and protein levels of *CREB1* in the 786-O and ACHN cells 48 h after transfection with miR-10b-5p and miR-363-3p mimics. miR-10b-5p and miR-363-3p mimics significantly downregulated the mRNA and protein levels of *CREB1* (Fig. 5A and B). To further explore the relationship of the expression pattern between *CREB1* and miR-10b-5p and miR-363-3p, we quantified the expression levels of miR-10b-5p and miR-363-3p in the 35 paired RCC tissues and ANTs by qPCR. We analyzed the fold-change, Log2Ratio(T/N), of *CREB1* and miR-10b-5p and miR-363-3p in each paired RCC tissue and ANT and we found that the expression level of *CREB1* decreased when miR-10b-5p and miR-363-3p expression increased, suggesting that *CREB1* was properly regulated by miR-10b-5p and miR-363-3p in the tissue samples (Fig. 5C). As shown in Fig. 6A and B, the

results also showed that miR-10b-5p and miR-363-3p expression levels were significantly lower in the RCC tissues than the levels in the ANT, respectively.

*Introduction of miR-10b-5p and miR-363-3p suppresses cell proliferation, migration and apoptosis.* To further confirm that lower expression levels of miR-10b-5p and miR-363-3p mediate the upregulation of *CREB1* to promote tumorigenicity, we introduced exogenous miR-10b-5p and miR-363-3p into human RCC cell lines (786-O and ACHN) and performed MTT and cell migration assays and apoptosis analysis. Forty-eight hours after transfection, the transient transfection efficiency was verified by qPCR (Fig. 7). Then MTT assay showed that overexpression of miR-10b-5p significantly decreased the cell proliferation of 786-O cells by 3.72% (P<0.05), 13.99% (P<0.01), 16.75% (P<0.01) and ACHN cells by 16.86% (P<0.05), 18.16% (P<0.01), 32.19% (P<0.01), at 24, 48 and 72 h post-transfection, respectively (Fig. 8A). Transwell migration assay found that the number of migratory cells was suppressed by 45.65% (P<0.05) and 56.28% (P<0.05) (Fig. 8B) and the migratory distances were inhibited by 28.73% (P<0.05) and 30.97% (P<0.01) (Fig. 8C) for 786-O and ACHN cells, respectively, after transfection of miR-10b-5p mimics. For the apoptosis assay, upregulated expression of miR-10b-5p promoted the mean early apoptotic rate from 4.77 to 13.20% (P<0.05) for 786-O cells and from 10.31 to 24.04% (P<0.05) for ACHN cells (Fig. 8D).

The proliferation ability of the 786-O and ACHN cells was attenuated identically by 6.70% (P<0.05), 13.22% (P<0.01), 19.79% (P<0.01) and 10.53% (P<0.05), 15.17% (P<0.01), 24.14% (P<0.01) due to introduction of miR-363-3p, respectively (Fig. 9A). After transfection of miR-363-3p mimics, for 786-O and ACHN cells, respectively, the migratory cells were

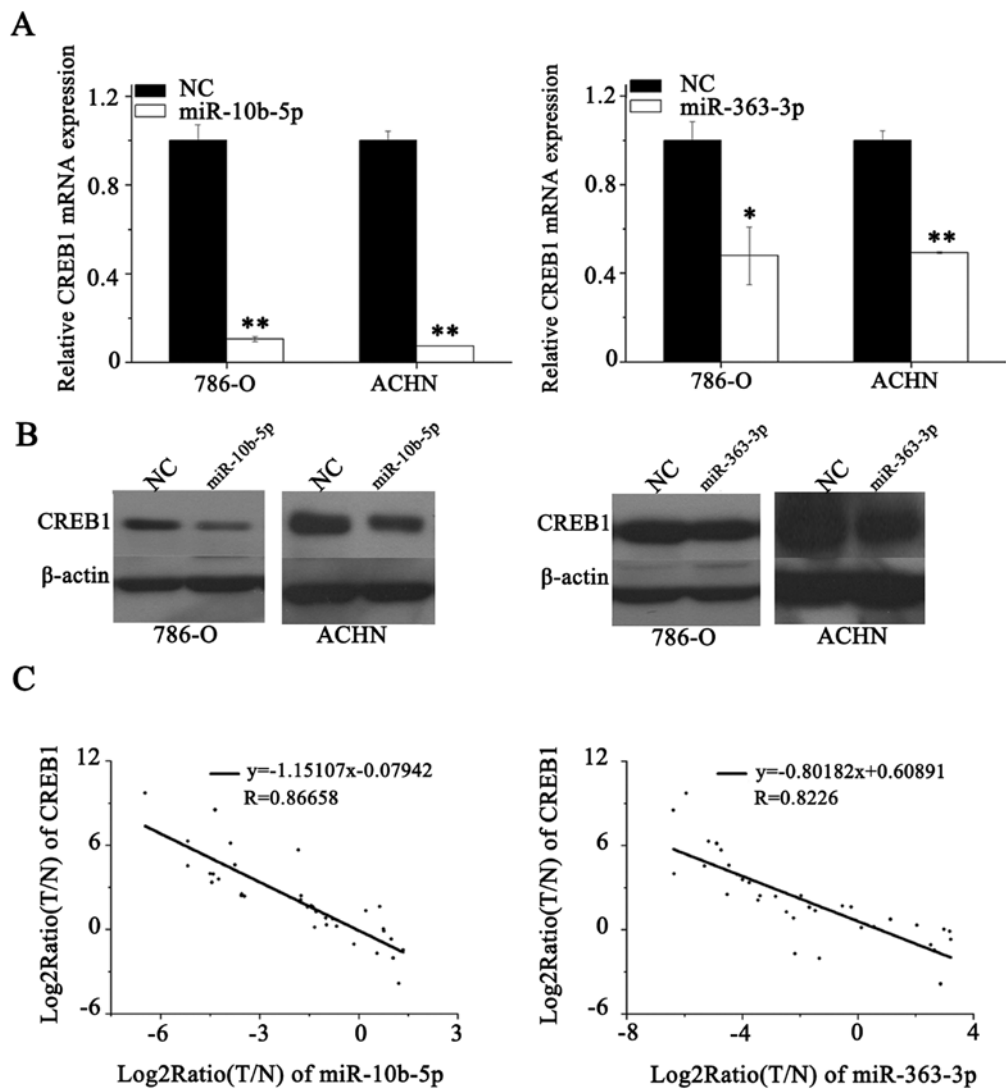


Figure 5. miR-10b-5p and miR-363-3p downregulate the expression of *CREB1*. (A and B) qPCR and western blot analysis indicate the effect of miR-10b-5p and miR-363-3p on *CREB1* expression at the mRNA and protein levels in 786-O and ACHN cells after transfection of miR-10b-5p mimics, miR-363-3p mimics and NC. (C) The mRNA expression level of *CREB1* was negatively correlated with miR-10b-5p and miR-363-3p in the 35 paired RCC tissues and ANTs by qRT-PCR. Relative expression, Log2Ratio (T/N), method was used to analyze the data. \* $P < 0.05$ , \*\* $P < 0.01$ .

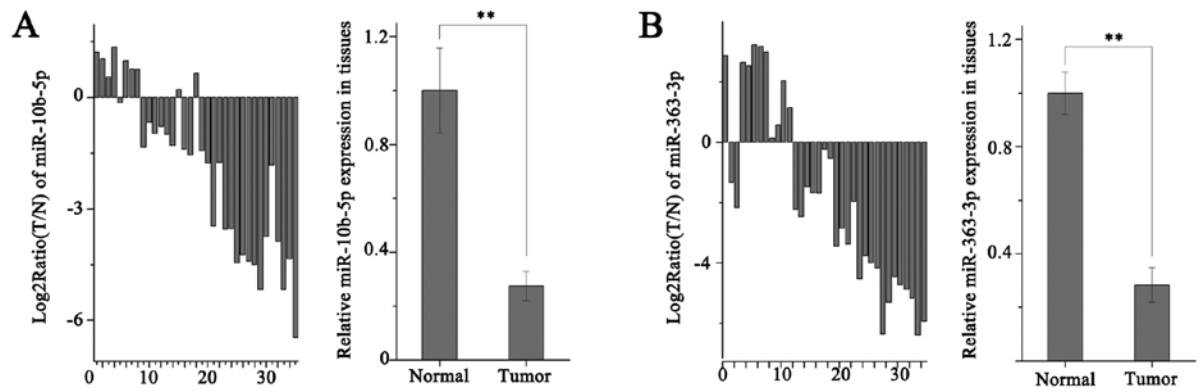


Figure 6. miR-10b-5p and miR-363-3p are downregulated significantly in RCC tissues. qPCR of miR-10b-5p (A) and miR-363-3p (B) expression relative to U6 expression in the 35 paired RCC tumor samples and ANTs. \*\* $P < 0.01$ .

suppressed by 17.95% ( $P < 0.05$ ) and 25.65% ( $P < 0.05$ ) (Fig. 9B) and the migratory distances were inhibited by 16.17% ( $P < 0.05$ ) and 30.02% ( $P < 0.01$ ) (Fig. 9C) due to ectopic expression of

miR-363-3p. miR-363-3p mimics induced the mean early apoptotic rate from 4.56 to 9.58% ( $P < 0.05$ ) for 786-O cells and from 11.61 to 18.84% ( $P < 0.01$ ) for ACHN cells (Fig. 9D).



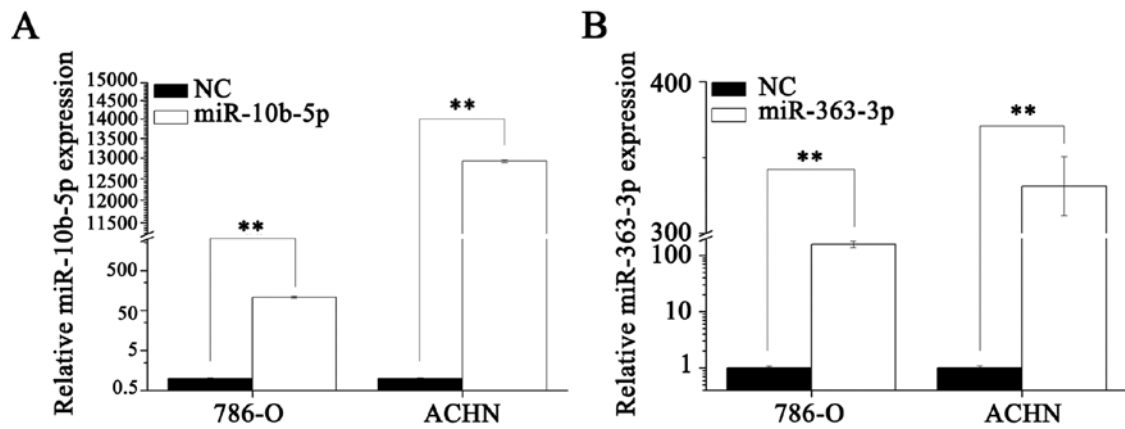


Figure 7. Validation of the upregulation of miR-10b-5p and miR-363-3p. The relative expression levels of miR-10b-5p (A) and miR-363-3p (B) in 786-O and ACHN cells transfected with miR-10b-5p mimic, miR-363-3p mimics or NC. \*\* $P < 0.01$ .

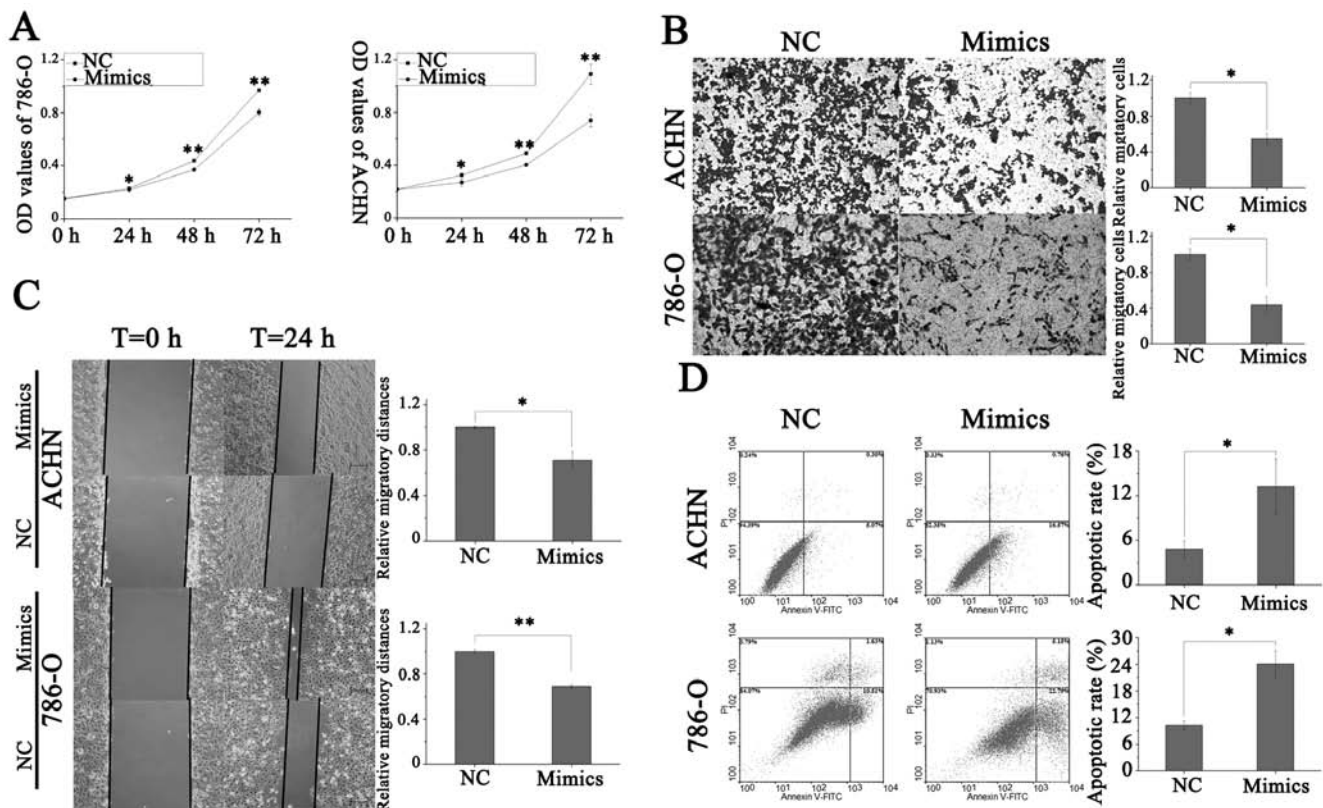


Figure 8. miR-10b-5p mimics suppress the proliferation, migration and apoptosis reduction in the RCC cell lines. MTT assay (A), Transwell migration assay (B), wound scratch assay (C) and cell apoptosis assay (D) were performed to examine proliferation, migration and apoptosis of the 786-O and ACHN cells after transfection with miR-10b-5p mimics and NC. \* $P < 0.05$ , \*\* $P < 0.01$ .

All the above data revealed that miR-10b-5p and miR-363-3p mimics exert a function similar to si-*CREB1* by inhibiting cell proliferation and migration and inducing cell apoptosis, suggesting that low miR-10b-5p and miR-363-3p expression-mediated upregulation of *CREB1* in RCC tissues and cell lines restores tumorigenicity.

## Discussion

The *CREB1* gene is localized at human chromosome 2q32.3-q34, a region known to be a hot spot for translocations

or deletions in various diseases including carcinomas (34). Chromosomal translocations or deletions frequently result in the activation of cellular oncogenes, such as oncogene *c-MYC* in human B cell and plasma cell neoplasms (35). In the present study, we showed that *CREB1* expression was upregulated in human RCC cell lines and RCC tissues at the mRNA and protein levels.

To further determine the roles of *CREB1* in RCC development, we performed MTT and cell migration assays and cell apoptosis analysis after knockdown of *CREB1*. The results revealed that downregulation of *CREB1* expression resulted

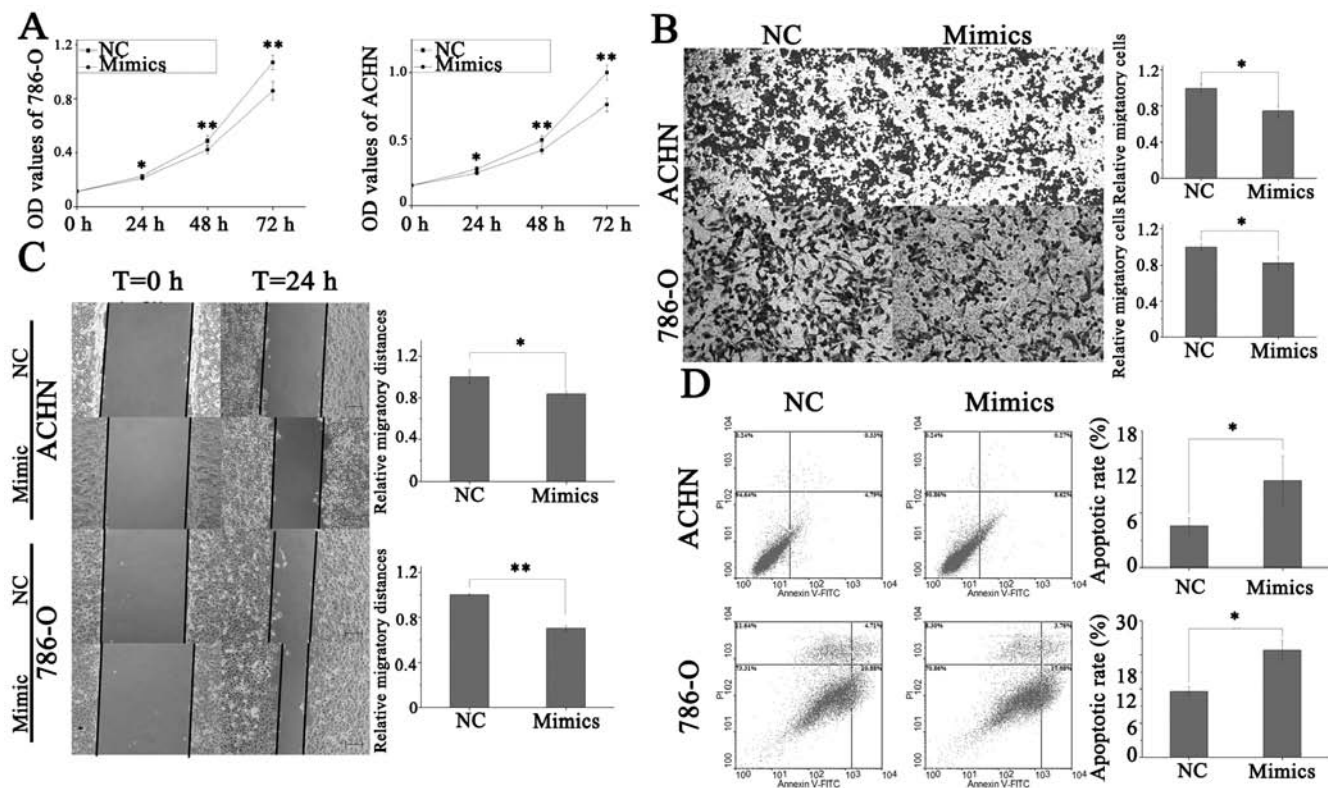


Figure 9. Effects of miR-363-3p mimics on RCC neoplastic phenotypes. MTT assay (A), Transwell migration assay (B), wound scratch assay (C) and cell apoptosis analysis (D) were performed to examine the proliferation, migration and apoptosis of the 786-O and ACHN cell after transfection with the miR-363-3p mimics and NC. \* $P < 0.05$ , \*\* $P < 0.01$ .

in the suppression of neoplastic phenotypes of RCC cells. It has been reported that overexpression of *CREB1* promotes the growth and survival of myeloid and glioma cells (8,36). *CREB1* is critical in *intercellular adhesion molecule-3 (ICAM-3)*-induced cellular radioresistance and cancer cell proliferation in NSCLC cell lines (9). It has also been confirmed that *CREB1* binds to the promoter region and causes overexpression of *LAPTM4B* and is involved in the occurrence and progression of breast cancer (10). In the present study, we showed for the first time that inhibition of *CREB1* strongly suppressed the tumorigenic activity including proliferation, migration and apoptosis of RCC cells.

Oncogenes are activated by overexpression or inhibition of degradation at the mRNA and protein levels to promote tumor development. In leukemia, *CREB* gene copies were amplified through an unknown mechanism (8). In clear cell sarcoma (37), angiomatoid fibrous histiocytoma (38) and mucoepidermoid carcinoma (39), fusion oncoproteins comprising *CREB* or *ATF1* activated the expression of *CREB*-regulated genes. In adult T cell leukemia and liver cancer, viral oncoproteins Tax of *HTLV-I* and HBx of hepatitis B virus physically interact with and activate *CREB* by enhancing DNA binding and/or coactivator recruitment (40-43). Inactivating mutations of tumor-suppressor gene, *LKB1* in Peutz-Jeghers syndrome and *PDE11A* in adrenocortical hyperplasia, induce the activation of *CREB* (13,44). Therefore, the *CREB1* upregulation in RCC may be caused by different mechanisms.

Previous studies have reported that many tumor-suppressive miRNAs inhibit tumorigenesis by targeting *CREB1* in various

types of cancers (45-50). *CREB1* is targeted and downregulated by miR-9, miR-1224-5p and miR-200b in gliomas (47,50,51). Oncogenic *CREB1* is also regulated negatively by tumor suppressive miR-181b and miR-182 in gastric cancer (45,46) and targeted by miR-433 in liver cancer (49). In AML, miR-34b downregulation was shown to induce *CREB* overexpression, thereby causing leukemia proliferation *in vitro*, *in vivo* and in the clinic (48,52). However, the relationship between *CREB1* and miRNAs remains unknown in RCC. Multiple computational algorithms were used to search for miRNAs predicted to suppress *CREB1* expression. We identified the seed region matched between miR-10b-5p and miR-363-3p and the 3'-UTR of the *CREB1* gene. Luciferase reporter assays confirmed that miR-10b-5p and miR-363-3p target *CREB1* directly. Moreover, ectopic expression of miR-10b-5p and miR-363-3p suppressed *CREB1* expression, thereby inhibited cell proliferation, migration and apoptosis reduction. Thus, a low level of miR-10b-5p and miR-363-3p is supportive for RCC tumorigenesis by way of loss of *CREB1* downregulation.

miR-10b-5p and miR-363-3p have been found dysregulated and to function in various cancers, especially in RCC. In RCC, 4 previous microarray profiling assays showed that miR-10b-5p was downregulated significantly in RCC tissues and metastatic RCC tissues compared with normal kidney tissues and primary RCC tissues (29-32,53,54). miR-363-3p is part of the miR-29a family and is highly conservative in the process of evolution (55). Previous studies revealed that inhibition of miR-363-3p mediated invasion and metastasis of head and neck cancer by inducing podoplanin (56), promoted

proliferation and G1 to S progression of hepatocellular cancer by targeting *SIPRI* and *c-MYC* (57,58). In RCC, miR-363-3p was reported to be significantly downregulated by microarray chip studies (28,33). These data suggest that miR-10b-5p and miR-363-3p are potential biomarkers for RCC tumorigenesis. In relation to this observation, a recent report indicated that miR-10b-5p and miR-363-3p are notably downregulated in tumors of RCC patients who developed tumor relapse (54). This result strongly suggests that miR-10b-5p and miR-363-3p possess tumor suppressive function in RCC development.

Furthermore, to validate that lower expression levels of miR-10b-5p and miR-363-3p mediate the upregulation of *CREB1* to promote RCC tumorigenicity, the RNA of the 35 paired clinical specimens was reversed to cDNA to compare the expression level of miR-10b-5p and miR-363-3p between RCC tissues and ANTs. In contrast, while expression of *CREB1* in RCC tissues was significantly higher than that in ANTs, miR-10b-5p and miR-363-3p were significantly expressed at lower levels in the RCC tissues. Through upregulation of miR-10b-5p and miR-363-3p, we confirmed the tumor suppressive functions of miR-10b-5p and miR-363-3p by MTT, wound scratch, transwell migration and cell apoptosis assays.

In conclusion, *CREB1* is overexpressed and has an oncogenic activity in RCC. miR-10b-5p and miR-363-3p suppress *CREB1* mRNA and protein expression via the conserved seed region of miR-10b-5p and miR-363-3p within the 3'-UTR of *CREB1* mRNA. Importantly, the expression of miR-10b-5p and miR-363-3p was correlated inversely with the *CREB1* expression level in 35 paired RCC tissues. In addition, upregulation of miR-10b-5p and miR-363-3p mediated downregulation of *CREB1* which inhibited RCC tumorigenicity. Taken together, a novel miRNA low expression-dependent upregulation of *CREB1* expression is important for the initiation and development of RCC. However, one future challenge will be to elucidate the miRNA and *CREB1*-mediated regulatory networks in RCC.

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