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Original Article

MiR-141 inhibits colorectal cancer cell proliferation and invasion by targeting PRKAR1A

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Abstract: Dysregulation of microRNAs (miRNAs) has been associated with the initiation and progression of oncogenesis in humans. Growing evidence has indicated that abnormal expression of microRNA-141 was associated with tumorigenesis and carcinoma progression of various cancers. In the present study, the miR-141 expression was assessed in CRC cell lines and the normal colonic cell using RTq-PCR, which demonstrated that miR-141 was significantly downregulated in CRC cells. Functional study revealed that overexpression of miR-141 significantly inhibited the proliferation, invasion and migration of CRC cells. Luciferase reporter assay validated that protein kinase A regulatory subunit 1 alpha (PRKAR1A) was a direct target of miR-141, Overexpression of miR-141 suppressed PRKAR1A protein expression, and rescued PRKAR1A attenuated the function of miR-141 in CRC cells. Taken together, our results highlight the significance of miR-141/PRKAR1A interaction in the development and progression of CRC cells, and suggest that miR-141 functions as a tumor suppressor in CRC cells. Targeting PRKAR1A may be an important mechanism involved.

Keywords: Colorectal cancer, miR-141, PRKAR1A

Introduction

CRC is the third most commonly cancer worldwide. The incidence of CRC is 4.4% in developed areas and 1.4% in developing areas [1]. Several factors, such as inflammatory bowel disease, are associated with increased risks of colorectal cancer [2]. Despite achievements in the treatment in the few past decades, approximately half of them die within 5 years [3]. The high mortality rate of CRC is related mainly to frequent tumor recurrence and metastasis after surgical resection [4]. Thus, further investigation into the underlying molecular mechanisms is of great clinical significance. Recently, increasing reports have revealed the closely relationship between dysregulated microRNAs (miRNAs) and the development of CRC, which highlights the role of miRNAs as potential biomarkers and therapeutic targets [5-7] in CRC patients.

Protein Kinase A (PKA) is the main mediator of cyclic adenosine mono-phosphate (cAMP)-Dependent signaling [8, 9]. The PKA holoenzymes contain two catalytic (C) subunits bound to homo or heterodimers of either regulatory type I (RI) or regulatory type II (RII) subunit. There are four different R subunits (Iα, Iβ and IIα, IIβ) and three different C subunits (Cα, Cβ, and Cγ), each encoded by a separate gene. Among all the subunits of PKA, RIα is studied with immense importance in cancer cases. The PRKAR1A gene on human chromosome 17q22-24 is the Gene-encoding for RIα, mutations in this gene are responsible for the multiple tumor syndrome Carney complex (CNC, Online Mendelian Inheritance in Man #160980) [10, 11]. PRKAR1A expression is increased in various human tumors and cell lines, including cancers of the breast [12, 13], ovary [14, 15], lung [16], and colon [17-19]. Furthermore, overexpression of the RIα subunit of PKA correlates with malignancy and poor prognosis in cancer patients [13-15]. Therefore, the RIα subunit of PKA is a potential target for human cancer therapy.

MiRNAs are a class of short and non-coding RNAs which negatively regulate the expression of protein-coding genes by directly binding their

3' untranslated region (3'-UTR), leading to the posttranscriptional translation inhibition or mRNA degradation [20, 21]. miRNAs regulate the expression of a wide variety of target genes, and are therefore involved in a wide range of biological processes including cell proliferation, development and differentiation [5-7]. Moreover, the widespread dysregulation of miRNAs is observed in various types of cancer [22]. In CRC, many miRNAs have been found to be involved in the regulation of multiple cellular functions, such as cell proliferation, invasion and migration [23]. For example, miR-143 and miR-145 function as tumor suppressors by inhibiting cell proliferation, invasion and metastasis [24, 25], while miR-21 acts as an important promoter of oncogenesis [26, 27]. miR-141 is a member of the miR-200 family, which has been reported to function as a tumor suppressor in many tumors including renal cell carcinoma, pancreatic cancer, and gastric cancer [28-30]. Previous studies have identified miR-200c as candidate regulator of PRKAR1A in breast cancer [31]. Although PRKAR1A and miRNAs are associated with tumorigenesis and carcinoma progression of CRC, little is known about the natural miRNAs that act on PRKAR1A. In this study, we found that PRKAR1A was directly regulated by miR-141 in CRC cells. Furthermore, we showed that miR-141 inhibited PRKAR1A expression to suppress the proliferation and invasion of CRC cells.

Materials and methods

Cell culture

Five CRC cell lines (HCT116, SW620, LOVO, HT29 and SW480) and the normal colonic cell NCM460 were purchased from American Type Culture Collection and cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 100 μM each of penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

RNA isolation and real-time quantitative PCR (RTq-PCR)

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction, and reverse transcribed using the Primer Script RT reagent Kit (TaKaRa Bio Inc., Otsu, Japan). The relative expression of PRKAR1A was detected by SYBR Green real-time quantitative polymerase chain reaction (RTq-PCR) assay (Bio-Rad Laboratories Inc., Hercules, CA, USA), and β-actin was used as internal control. The relative expression of miR-141 was determined using mirVana qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA), and small nuclear U6 RNA was used as internal control. The specific primers for miRNA-141 and U6 were purchased from GeneCopoeia(Rockville, MD, USA). All experiments were performed in at least triplicate and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Knockdown of PRKAR1A by siRNA

To knockdown PRKAR1A expression, the siRNAs (si-PRKAR1A) and negative control (si-NC) were purchased from GenePharma Company (Shang-hai, People's Republic of China). A total of 300 pmol of si-PRKAR1A or si-NC was transfected into SW480 and SW620 cells using Lipofectamine RNAi MAX Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Vector construction, lentivirus infection and cell transfection

The coding sequence of PRKAR1A was amplified and cloned into pcDNA3.1 vector, and the empty pCDNA3.1 vector was used as control. Lipofectamine 2000 Reagent (Thermo Fisher Scientific) was used for cell transfection following the manufacturer's protocol. The pre-miR-141 sequence was amplified and introduced into the PLKO.3G vector. Lentiviruses containing pre-miR-141 (miR-141) and negative control (miR-NC) were produced by GeneChem Company (Shanghai, People's Republic of China). Cells were cultured to approximately 70% confluence and then added by a concentration of 2.0×10^5 TU/well Lentiviruses containing pre-miR-141 or negative control, and RTq-PCR was performed to determinate the expression levels of miR-141 after being infected for 7 days.

Cell proliferation, invasion and migration assays

Cell proliferation was examined by Cell Counting Kit 8 (CCK8) assays. Briefly, the cell lines were

plated in 96-well plates (4,000 per well), and allowed to grow for 24, 48 and 72 h, then assessed by a colorimetric assay using CCK8 solution at 450 nm. Cell invasion ability was performed using transwell invasion chambers coated with mgatrigel (BD Biosciences, San Jose, CA, USA). 1×10^5 cells were suspended in FBS-free medium and added into the upper chamber, while medium containing 10% FBS was added to the lower chamber. After 24 hours of incubation, the cells remaining on the upper membrane were removed with cotton wool, whereas the cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged, and counted using an inverted microscope (Olympus, Tokyo, Japan). Cell migration ability was assessed by performing wound healing assays. Cells were cultured to 100% confluence, and wounds were generated using pipette tips. Then, the cells were cultured for 24 or 48 hours and the wound closure was assessed by Scion Image Software (Scion Image Beta 4.03; Scion Corporation, Frederick, MD, USA).

Luciferase reporter assays

The wild-type (WT) 3'UTR of PRKAR1A was amplified and ligated into the psiCheck-2 reporter vector (Promega Corporation, Fitchburg, WI, USA). Site-directed mutagenesis of the miR-141 seed sequence in the 3'UTR of PRKAR1A (Mut) was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Luciferase activity was detected using the Dual Luciferase Assay (Promega) according to the manufacturer's protocols. Briefly, cells were cotransfected with 0.3 μg of the reporter vectors and 40 nM miR-141 mimics or scrambled mimics. Forty eight hours after transfection, the transfected cells were lysed and the relative luciferase activity was determined using a Modulus TD20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA). All experiments were performed in triplicate. miR-141 and scramble mimics and corresponding inhibitors were purchased from RiboBio (Guangzhou, People's Republic of China).

Western blot analysis

Total cellular extracts were prepared using 200 μl of lysis buffer. Approximately 50 μg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane and incubated with the specific primary antibodies against PRK-AR1A (ab139695; Abcam, Cambridge, UK) and GAPDH (ab181602; Abcam, Cambridge, UK) overnight at 4°C. Then the membranes were washed with TBST (Tris-buffered saline with Tween-20), and probed with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1, 500 dilution; Santa Cruz) at 37°C for 30 minutes. Signals were visualized using electrochemiluminescent (ECL) substrates (EMD Millipore, Billerica, MA, USA).

Statistical analysis

All statistical analyses were performed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Experimental data were expressed as the mean ± standard deviation (SD). Statistical significance was analyzed using Student's t-test. $P<0.05$ was considered statistically significant.

Results

MiR-141 is downregulated in CRC cell lines

The expression levels of miR-141 were evaluated by RTq-PCR in five CRC cell lines (HCT116, SW620, LOVO, HT29 and SW480) and the normal colonic cell NCM460. As shown in **Figure 1A**, miR-141 was significantly decreased in CRC cell lines, and the expression of miR-141 was lower in SW480 cells compared with in other CRC cell lines, while the expression levels of PRKAR1A, a predicted target of miR-141, was significantly increased in CRC cell lines and was higher in SW480 cells than in other CRC cell lines (**Figure 1B**).

MiR-141 inhibits the proliferation, invasion and migration of CRC cells

To investigate the functional roles of miR-141 in CRC cells, we constructed miR-141 expressing lentiviral vector and infected SW480 and SW620 cells, respectively. Overexpression of miR-141 was validated by RTq-PCR (**Figure 2A**). CCK8 assays (**Figure 2B**), Transwell with matrigel assays (**Figure 2C**) and wound healing assays (**Figure 2D**) showed that miR-141 overexpression significantly inhibited cell prolifera-

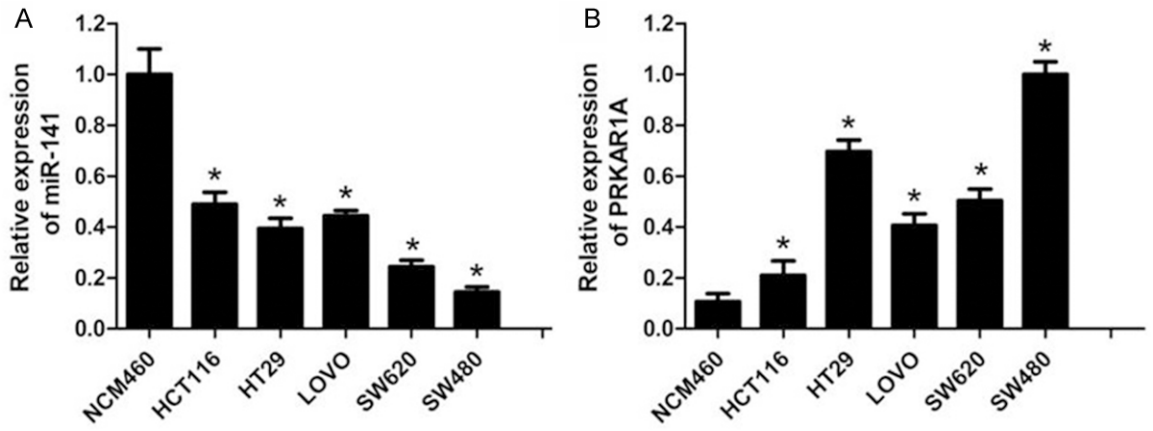


Figure 1. miR-141 is significantly down-regulated in CRC cell lines. A. The relative expression level of miR-141 in CRC cell lines and the normal colonic cell NCM460. B. The relative expression level of PRKAR1A in CRC cell lines and the normal colonic cell NCM460. *P<0.05.

tion, invasion and migration ability of SW480 and SW620 cells. In contrast, when endogenous miR-141 was silenced with inhibitor mimics, cell proliferation, invasion and migration ability was increased. These results suggest that miR-141 may be a tumor suppressor gene in CRC cells.

MiR-141 directly inhibits PRKAR1A by targeting its 3'UTR

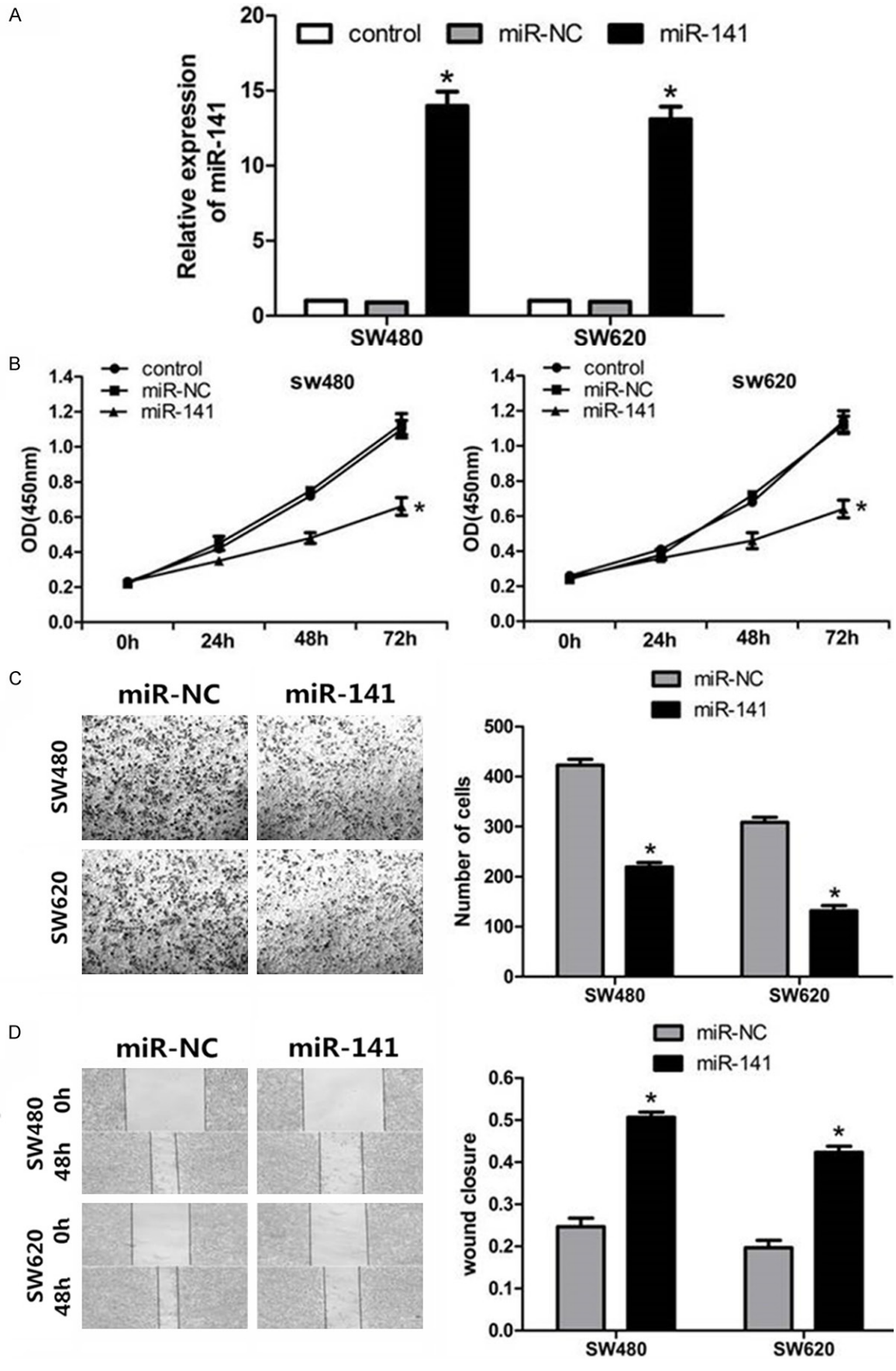
To elucidate the molecular mechanism underlying inhibitory effects of miR-141 on proliferation, invasion, and migration of CRC cells, we predicted potential targets of miR-141. Using TargetScan and miRanda tools on line, we screened several proliferation- and invasion-related genes by RT-qPCR, of which PRKAR1A expression was significantly repressed in miR-141 expressing SW480 and SW620 cells. Besides, PRKAR1A is primarily involved in the control of cell proliferation and neoplastic transformation, thus, we focused on PRKAR1A in the study. To validate if miR-141 directly target PRKAR1A 3'UTR (**Figure 3A**), luciferase reporter assays were performed in SW480 and SW620 cells, which revealed that miR-141 mimics significantly decreased the luciferase activity of wild type PRKAR1A 3'UTR reporter, but had no effect on the PRKAR1A 3'UTR reporter with mutant miR-141 binding seed sites (**Figure 3B**). RTq-PCR and western blot also validated that miR-141 mimics reduced PRKAR1A expression, and miR-141 inhibitor mimics increased PRKAR1A expression in SW480 and SW620 cells (**Figure 3C**, **3D**). Taken together, our results demonstrated that miR-141 directly inhibits PRKAR1A expression in CRC cells.

PRKAR1A is involved in miR-141 attenuated the proliferation, invasion, and migration of CRC cells

We further examined whether PRKAR1A is a substantial target of miR-141 involved in regulating the proliferation, invasion, and migration of CRC cells. Knockdown of PRKAR1A by specific siRNAs (**Figure 4A**) significantly attenuated the proliferation, invasion, and migration of SW480 and SW620 cells (**Figure 4B**, **4C**), similar to the effects of miR-141 overexpression (**Figure 2B-D**). In addition, rescued PRKAR1A lacking its 3'UTR in the miR-141 expressing SW480 and SW620 cells significantly attenuated inhibitory effects of miR-141 on the cell invasion and migration ability (**Figure 4D**). Taken together, our results demonstrated that PRKAR1A attenuates the inhibitory effects of miR-141 on the proliferation, invasion, and migration of CRC cells.

Discussion

Increasing reports have demonstrated that miRNAs are an important regulator of tumor progression and metastasis. In CRC, many miRNAs have been identified to regulate known genes that are involved in the pathology of tumorigenesis and metastasis. In the present study, we showed that miR-141 was significantly downregulated in five CRC cell lines, which was consistent with a recently published report on miR-141 in CRC cell lines [32]. In addition,


A
control
miR-NC
miR-141
Relative expression of miR-141
SW480
SW620
B
control
miR-NC
miR-141
SW480
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SW480
SW620
Number of cells
D
miR-NC
miR-141
SW480 0h
SW480 48h
SW620 0h
SW620 48h
wound closure

Figure 2. MiR-141 inhibits the proliferation, invasion and migration of CRC cells. A. SW480 and SW620 cells were infected with miR-141 or negative control (miR-NC) lentivirus, and the expression of miR-141 was analyzed by RTq-PCR. B. CCK8 assays were performed to investigate the proliferation ability of CRC cells. C. Transwell with matrigel assays were performed to investigate the invasion ability of CRC cells. D. Wound healing assays were performed to investigate the migration ability of CRC cells. *P<0.05.

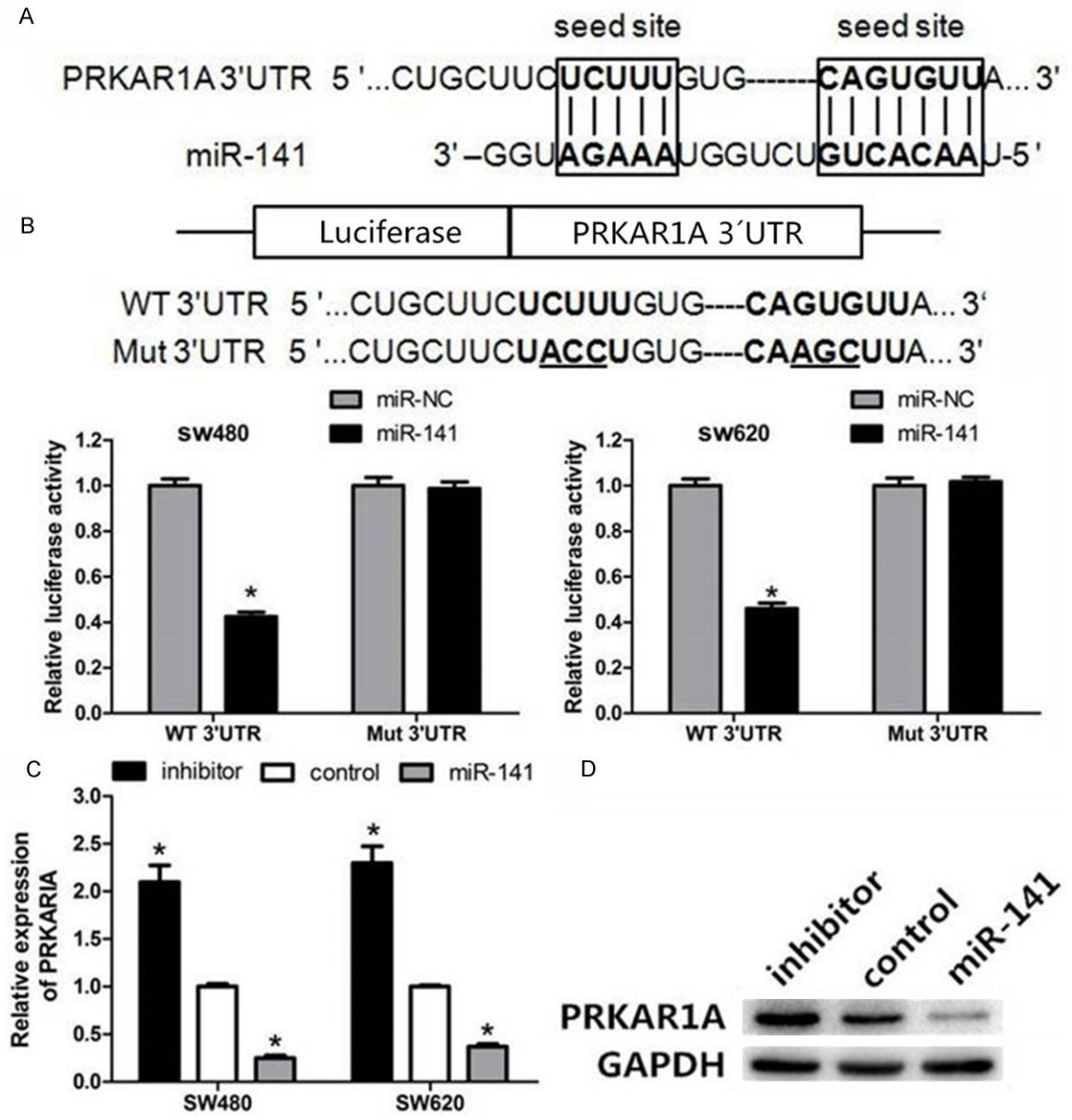
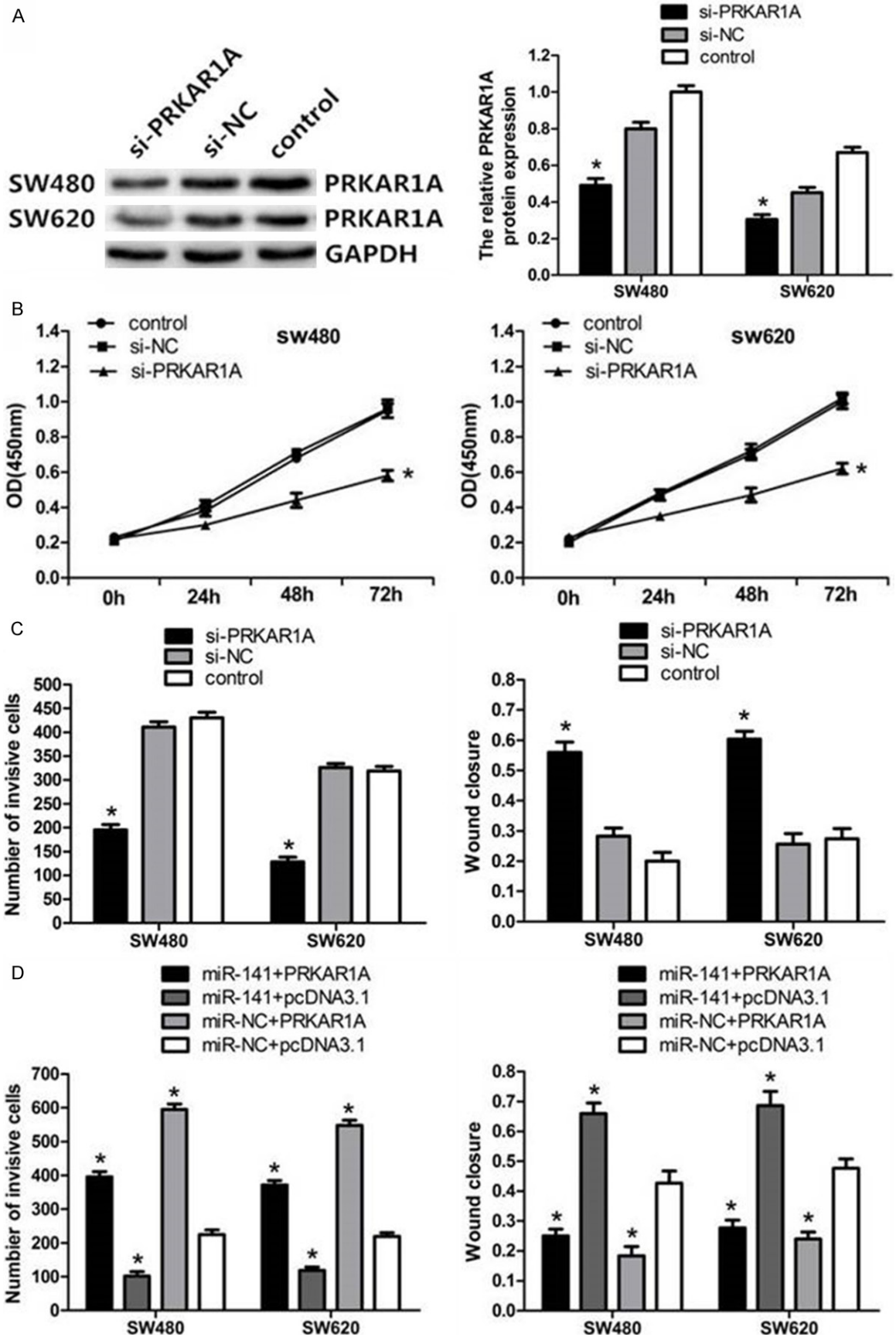


Figure 3. MiR-141 directly inhibits PRKAR1A by targeting its 3'UTR. A. The miR-141 binding site predicted in the PRKAR1A 3'UTR. B. Mutant PRKAR1A 3'UTR was generated at the seed region as indicated by the underline. A fragment of PRKAR1A 3'UTR containing wild type (WT) or mutant (Mut) of the miR-141 binding sites was cloned to the downstream of the luciferase reporter gene vector, then SW480 and SW620 cells were transfected with reporter vectors containing either wild-type or mutant PRKAR1A 3'UTR with either miR-141 mimics (miR-141) or scrambled mimics as negative control (miR-NC). Luciferase activity was determined 48 hours after transfection. C. PRKAR1A mRNA expression levels were detected by RTq-PCR in SW480 and SW620 cells transfected with miR-141 mimics (miR-141) or miR-141 inhibitor mimics (inhibitor). The untreated cells were taken as control. D. PRKAR1A protein expression level was detected by western blot in SW480 cells transfected with miR-141 mimics (miR-141) or miR-141 inhibitor mimics (inhibitor). *P<0.05.


A
si-PRKAR1A
si-NC
control
SW480
SW620
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Wound closure
D
miR-141+PRKAR1A
miR-141+pcDNA3.1
miR-NC+PRKAR1A
miR-NC+pcDNA3.1

Figure 4. PRKAR1A is involved in miR-141 attenuated the proliferation, invasion, and migration of CRC cells. A. SW480 and SW620 cells were transfected with PRKAR1A-specific small interfering RNA (si-PRKAR1A) or negative control (si-NC), and knockdown of PRKAR1A was validated by western blot. B. CCK8 assays were performed to investigate the proliferation ability of CRC cells. C. Transwell with matrigel assays and wound healing assays were performed to investigate the invasion and migration ability of CRC cells, respectively. D. SW480 and SW620 cells transfected with miR-141 mimics (miR-141) or scrambled mimics as negative control (miR-NC) were cotransfected with PRKAR1A expression plasmids lacking 3'UTR (miR-141+PRKAR1A, miR-NC+PRKAR1A) or empty pCDNA3.1 vector (miR-141+pCDNA3.1, miR-NC+pCDNA3.1). Transwell with matrigel assays and wound healing assays were performed to investigate the invasion and migration ability of CRC cells, respectively. $^{*}P<0.05$.

recent studies showed that miR-141 functioned as tumor suppressor in renal cell carcinoma [33] and breast cancer cell lines [34]. These data indicated that miR-141 may function as a tumor suppressor in CRC cell lines.

We then investigated the functional roles of miR-141 in CRC cell lines. To do so, we constructed miR-141 expressing SW480 and SW620 cells. Transwell with matrigel and wound healing assays revealed that miR-141 could inhibit cell invasion and migration ability of SW480 and SW620 cells, and CCK8 assays also found miR-141 could inhibited the proliferation of SW480 and SW620 cells. Combined with the recent observation that miR-141 inhibited the cell proliferation and invasion in gastric cancer cells [30, 35], we concluded that miR-141 acts as a tumor suppressor in CRC cells.

As miRNAs are expected to have multiple targets, we next predicted and validated the possible targets of miR-141 in CRC cells as the impact of specific miRNAs on cancer biology depends on their downstream targets. We found PRKAR1A was a direct target of miR-141 by the luciferase reporter assay. To confirm the interaction of miR-141 and PRKAR1A in CRC cells, functional study revealed that PRKAR1A knockdown significantly inhibited the proliferation, invasion, and migration ability of SW480 and SW620 cells, and rescued PRKAR1A in miR-141 expressing SW480 and SW620 cells dramatically attenuated miR-141-induced inhibition on cellular migration and invasion. Taken together, our data suggest that PRKAR1A is a functional target of miR-141 in CRC cells.

PRKAR1A is preferentially expressed in proliferating and transformed cells [36], Enhanced expression of PRKAR1A has also been shown in several human cancer tissues and cell lines, including retinoblastoma, renal and breast cancers, the transformed BT5C glioma cell line, malignant osteoblasts, and serous ovarian tumors vs. mucinous, endometroid, or clear cell lesions [37-44]. Overexpression of PRKAR1A is consistently observed in several cancer tissues and is associated with poor prognosis in patients with a variety of cancer types. Thus, uncontrolled proliferation and malignant transformation have been associated with mainly altered PRKAR1A expression. Based on the upregulation of PRKAR1A in several cancers and studies indicating that inhibition of PRKAR1A expression through antisense oligonucleotides (ODNs) resulted in the growth arrest of several tumor cell lines, PRKAR1A blockade has been considered a possible single-gene targeting approach for the treatment of certain cancers [45]. Our results demonstrated that miR-141 directly inhibits PRKAR1A as a therapeutic strategy against CRC.

In conclusion, our current findings revealed that miR-141 is downregulated in CRC cell lines and acts as a functional tumor suppressor in CRC cells. Targeting PRKAR1A may be an important mechanism involved, and inhibition of PRKAR1A may be a viable therapy for treating patients with CRC.

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Disclosure of conflict of interest

None.

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