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ORIGINAL RESEARCH

microRNA-769 is downregulated in colorectal cancer and inhibits cancer progression by directly targeting cyclin-dependent kinase 1

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Background: In recent years, microRNAs (miRNAs) have been found to be aberrantly expressed in colorectal cancer (CRC). The deregulation of miRNAs is implicated in the formation and progression of CRC, and participates in the regulation of a wide range of biological behaviors. Considering the crucial role of miRNAs in CRC, miRNAs are thought to have significant promise in the diagnosis and therapy of patients with this malignancy.

Material and methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to detect miR-769 expression in CRC tissues and cell lines. MTT assay and flow cytometry analysis were used to determine the effects of miR-769 upregulation in CRC cell proliferation and apoptosis, respectively. The influence of miR-769 overexpression in CRC cell migration and invasion was evaluated through migration and invasion assays. Notably, the possible mechanisms underlying the action of miR-769 in CRC cells were explored.

Results: In the present study, miR-769 was frequently found to be poorly expressed in CRC tissues and cell lines. Functional assays showed that recovery of miR-769 expression suppressed CRC cell proliferation, migration, and invasion, increased cell apoptosis in vitro, and inhibited tumor growth in vivo. Cyclin-dependent kinase 1 (CDK1) was the direct target of miR-769 in CRC cells. CDK1 was overexpressed in CRC tissue samples and negatively correlated with miR-769 expression. In addition, CDK1 inhibition imitated the tumor suppressor activity of miR-769 in CRC cells, and restoration of CDK1 expression partially abolished the tumor-suppressing roles of miR-769 in malignant CRC cells.

Conclusion: The results of this study demonstrated that miR-769 was downregulated in CRC and directly targeted CDK1 to be implicated in the regulation of CRC cell proliferation, apoptosis, migration and invasion. Thus, the miR-769/CDK1 axis might be an effective therapeutic target for treating patients with CRC.

Keywords: colorectal cancer, microRNA-769, proliferation, apoptosis, metastasis, cyclin-dependent kinase 1

Introduction

Colorectal cancer (CRC) is the third most common malignant tumor and fourth most common cause of cancer related deaths worldwide.[1] In the past few decades, approximately one million new CRC cases have been diagnosed, and half a million patients with CRC die every year worldwide.[2] Currently, surgery, adjuvant chemotherapy, and radiotherapy are the primary techniques for treating patients with CRC.[3] Despite the advances in diagnoses and therapeutic approaches, the prognosis of patients with CRC remains poor, especially in patients diagnosed at the advanced stages of the disease.[4] Two-thirds of patients with CRC exhibit local recurrence or distant metastasis after

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surgical resection.[5] Accumulated evidence demonstrates that genetic and epigenetic alterations are involved in the genesis and development of CRC; however, the precise molecular mechanisms related to the malignant progression of CRC are complicated and remain largely unknown.[5] Therefore, elucidating the mechanisms of CRC pathogenesis might be helpful in identifying novel therapeutic methods and improving clinical outcomes in patients with this malignancy.

microRNAs (miRNAs) are a group of evolutionarily conserved noncoding short RNAs containing 18–25 nucleotides.[6] The primary role of miRNAs is to reduce gene expression through imperfect or perfect hybridization with the 3′-untranslated regions (UTRs) of their target genes, resulting in either mRNA degradation or suppression of mRNA translation.[7] Approximately 30%–50% of human protein-coding genes are believed to be modulated by miRNAs.[8] Particularly, miRNAs involved in tumorigenesis and tumor development have been extensively characterized.[9–11] miRNAs can play tumor-suppressing or oncogenic roles and are implicated in the regulation of multiple biological behaviors such as cell proliferation, cell cycle, apoptosis, migration, metastasis, and resistance to radiotherapy and chemotherapy.[12–14] In particular, various miRNAs have been reported to be upregulated or downregulated in CRC, and their aberrant expression plays a crucial role in CRC occurrence and development.[15,16] These results highlight the importance of miRNAs in the diagnosis and management of patients with CRC.

miR-769 was reported to be downregulated in small-cell lung cancer and was upregulated in neuroblastoma;[17,18] however, its expression pattern, function, and underlying mechanisms in CRC have not been completely elucidated. Therefore, we attempted to measure miR-769 expression in CRC, examine the regulation of miR-769 on the malignant behaviors of CRC, and explore the possible mechanisms in CRC. The results of this study have revealed the crucial role of miR-769 in the initiation and progression of CRC and have underscored its importance in the diagnosis and treatment of patients with this disease.

Materials and methods

Clinical samples and ethics committee

CRC tissues and adjacent normal tissues (ANTs) were obtained from 47 patients who received surgical resection at the Shanghai Eighth People's Hospital between May 2014 and March 2017. None of the patients had undergone adjuvant chemotherapy or radiotherapy before the specimens were collected. All tissues were snap-frozen in liquid nitrogen, followed by storage at −80°C until further use. The Ethics Committee of Shanghai Eighth People's Hospital approved this study, and it was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Shanghai No Eighth People's Hospital. Written informed consent was obtained from all patients enrolled in the study.

Cell culture

In total, four CRC cell lines (HT29, HCT116, SW480, and SW620) and a normal human colon epithelium cell line (FHC) were ordered from the American Type Culture Collection (Manassas, VA, USA). DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco-Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to culture the cell lines. All cells were cultured at 37°C in a humidified condition with 5% CO_2.

Transfection

miR-769 mimics, negative control miRNA mimics (miR-NC), small interfering RNA against CDK1 (CDK1 siRNA), and negative control siRNA (NC siRNA) were obtained from Shanghai GenePharma Co. Ltd. (Shanghai, People's Republic of China). CDK1 overexpression vector pCMV-CDK1 and empty control vector pCMV were constructed by Guangzhou RiboBio Co. Ltd. (Guangzhou, People's Republic of China). The cells were plated in 6-well plates with antibiotic-free culture medium one day before transfection and transfected with the above mimics, siRNA or plasmid, using Lipofectamine 2000 (Invitrogen Life Technologies-Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's guidelines.

RNA extraction and reverse transcription-quantitative PCR

Total RNA was isolated from tissue samples and cultured cells using the TRIzol reagent (Invitrogen Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For quantifying miR-769 expression, total RNA was converted into first-strand cDNA using a TaqMan miRNA reverse transcription kit (Applied Biosystems-Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, quantitative PCR (qPCR) was performed using a TaqMan microRNA assay kit (Applied Biosystems-Thermo Fisher Scientific Inc., Waltham, MA, USA). miR-769 expression was normalized to that of U6 snRNA. For determining miR-769 expression, first-strand cDNA synthesis was conducted using a PrimeScript™ RT reagent kit, followed by qPCR using a SYBR Premix Ex Taq master mix (both from Takara Biotechnology Co. Ltd., Dalian, People's Republic of China). GAPDH was used for normalization of CDK1 mRNA level. All data were analyzed using the $2^{-\Delta\Delta Ct}$ method.[19] Each sample was analyzed in triplicate and this assay was repeated three times.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay

Twenty-four hours after transfection, the cells were trypsinized, resuspended, and plated in 96-well plates with a density of 3×10^3 cells per well. The cells were then incubated at 37°C in the presence of 5% CO_2 for 0, 24, 48, and 72 hours after implantation. The MTT assay was performed at each time point to detect cell proliferation. Briefly, 20 µL of 5 mg/mL MTT (Sigma, St Louis, MO, USA) was added into each well and incubation was continued for additional 4 hours. The culture medium containing MTT solution was discarded carefully, followed by addition of 150 µL dimethyl sulfoxide (Sigma, St Louis, MO, USA). Finally, a microplate reader was used to detect absorbance at 490 nm. All the experiments were performed in triplicate, and repeated at least three times.

Flow cytometry analysis of cell apoptosis

The rate of apoptosis was determined using an annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (BioLegend, San Diego, CA, USA) in accordance with the manufacturer's protocol. Briefly, the transfected cells were harvested by trypsinization after 48 hours of incubation, washed thrice with cold P PBS (Gibco-Thermo Fisher Scientific Inc., Waltham, MA, USA), and then resuspended in 100 µL binding buffer. Subsequently, the cells were stained with 5 µL each of annexin V-FITC and propidium iodide at room temperature in the dark for 20 minutes. Finally, the stained cells were subjected to detection of apoptotic cells using a flow cytometer (FACScan™, BD Biosciences, Franklin Lakes, NJ, USA). The assay was performed in triplicate, and repeated at least three times.

Migration and invasion assays

Migration and invasion assays were used to assess the migratory and invasive ability of CRC cells. Transwell chambers (8 µm) containing Matrigel® (both from BD Biosciences, Franklin Lakes, NJ, USA) were used for the invasion assay, whereas the cell migration assay was performed using Transwell chambers without Matrigel®. After 48 hours incubation, the transfected cells (5×10^4) in FBS-free DMEM were inoculated in the upper chambers. The bottom chambers were covered with 600 µL DMEM containing 20% FBS, which acted as the nutritional attractant. After 24 hours of incubation at 37°C with 5% CO_2, non-migrated and non-invasive cells were removed, whereas cells that invaded the filter membranes were fixed with 4% paraformaldehyde and stained with 0.05% crystal violet (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). Cells in at least five randomly selected visual fields were counted and expressed as the average number of cells per field of view using an inverted microscope (IX83; Olympus, Tokyo, Japan). Each assay was performed in triplicate, and repeated at least three times.

Xenograft experiment

In total, eight BALB/c nude mice (4-week-old) were ordered from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, People's Republic of China). All the mice were maintained under special pathogen-free conditions. Cells were transfected with miR-769 mimics or miR-NC, and cultured at 37°C with 5% CO_2. Following 24 hours incubation, transfected cells were subcutaneously injected into the hind flanks of BALB/c nude mice. The width and length of tumor xenografts were detected using Vernier calipers. Tumor volumes were figured out based on the following equation: tumor volume =1/2×tumor length×tumor width. At 30 days after injection, all BALB/c nude mice were sacrificed and the formed xenografts were excised and weighed. All experimental procedures were approved by the Ethics Review Committee of Shanghai Eighth People's Hospital, and were performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Shanghai Eighth People's Hospital.

Bioinformatics prediction

To predict the potential targets of miR-769, bioinformatic analysis was performed using TargetScan 7.1 (http://www.targetscan.org/) and miRDB (http://mirdb.org/). The analysis showed that CDK1 might be a putative target of miR-769.

Luciferase reporter assay

The wild-type (WT) and mutant (MUT) 3′-UTR of CDK1 were amplified by Shanghai GenePharma and cloned into the pMIR-REPORT plasmid (Promega, Madison, WI, USA). The chemically synthesized luciferase reporter vectors were named as pMIR-CDK1-3′-UTR Wt and pMIR-CDK1-3′-UTR MUT, respectively. The cells were plated in 24-well plates one night prior to transfection. Transient co-transfection with either the luciferase reporter vector and miR-769 mimics or miR-NC was performed using Lipofectamine 2000. Forty-eight hours after transfection, the activities of Firefly and Renilla luciferases were determined using the dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla luciferase activity. This assay was performed in triplicate, and repeated at least three times.

Western blot analysis

The transfected cells were washed thrice with PBS and were lysed in radioimmunoprecipitation assay lysis buffer

(Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). Protein concentration was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). Equal quantities of protein were electrophoretically separated on a 10% polyacrylamide gel, and transferred onto polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). The membranes were blocked with 5% dried skimmed milk diluted in Tris-buffered saline containing 0.1% Tween-20 at room temperature for 2 hours and incubated at 4°C with primary antibodies against CDK1 (ab18; 1:1,000 dilution; Abcam, Cambridge, UK) or GAPDH (ab9482; 1:1,000 dilution; Abcam, Cambridge, UK). Next day, a horseradish peroxidase-conjugated secondary antibody (ab205719; 1:5,000 dilution; Abcam, Cambridge, UK) was added and incubated at room temperature for 2 hours. Finally, the protein bands were visualized using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal reference. This assay was performed in triplicate, and repeated at least three times.

Statistical analysis

All data were analyzed using the SPSS 19.0 statistic software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to compare the differences between t oups. One-way analysis of variance was adopted evalua the differences between multiple groups, follow d by Newman-Keuls post hoc test. The lationsh between miR-769 and CDK1 mRNA lev ere determ ed by Spearman's correlation analysis. *P*-value <0.05 was considered statistically significant.

Results

miR-769 is downregulated in CRC tissues and cell lines

To determine the role of miR-769 in CRC progression, we first detected miR-769 expression in 47 pairs of CRC tissues and ANTs. Reverse transcription-qPCR (RT-qPCR) showed that miR-769 expression was significantly reduced in CRC tissues compared to in ANTs (1A, $P<0.05$). Consistent with the results obtain with tissu pecimens, miR-769 expression was lower in l four teste CRC cell lines (HT29, HCT116, SW4 , and S 20) tha that in the normal human colon epi elium c l line ( ) (Figure 1B, $P<0.05$). These results s gest hat low miR-769 expression may be closely r ted wit RC dev opment.

miR-769 upregulation attenuates proli ion, indu es apoptosis, and pr nibits metastasis of CRC cells in vitro

We lected HCT 6 and SW480 cell lines, which exhibited the lo st miR 69 expression among the four CRC cell lines, to investigate the role of miR-769 in CRC. miR-769 mimics miR-NC was transfected into HCT116 and SW480 cells, and the relative miR-769 expression in the two cell lines was verified by RT-qPCR analysis. Results showed that miR-769 was markedly overexpressed in miR-769 mimic-transfected HCT116 and SW480 cells (Figure 2A, $P<0.05$). The MTT assay was used to explore the biological effect of

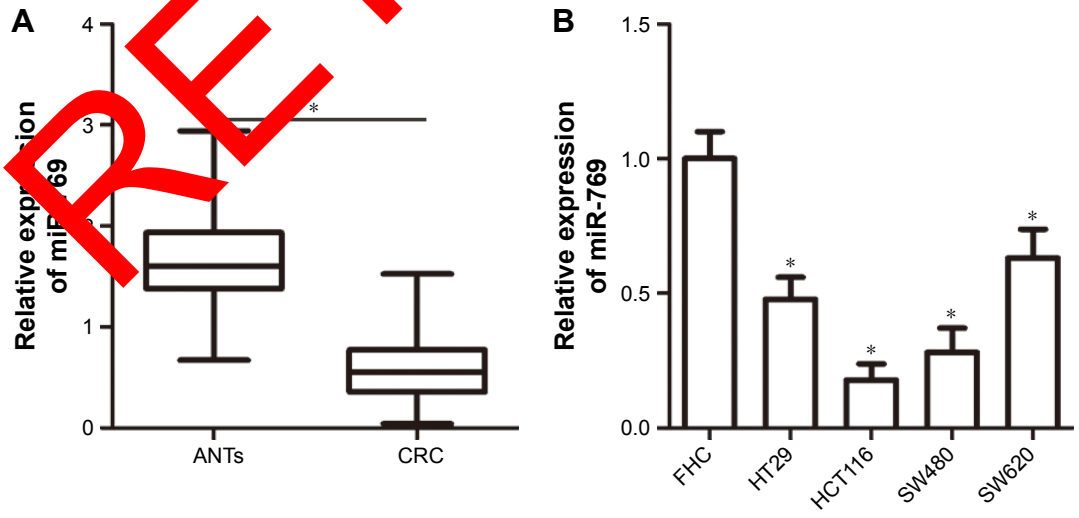

Figure 1 Reduced miR-769 expression in CRC tissues and cell lines.
Notes: (**A**) RT-qPCR analysis was performed to detect miR-769 expression in 47 pairs of CRC tissues and ANTs. *$P<0.05$. ANTs. (**B**) Relative miR-769 expression in four CRC cell lines (HT29, HCT116, SW480, and SW620) and a normal human colon epithelium cell line (FHC) was assessed using RT-qPCR. *$P<0.05$. FHC.
Abbreviations: ANTs, adjacent normal tissues; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR.

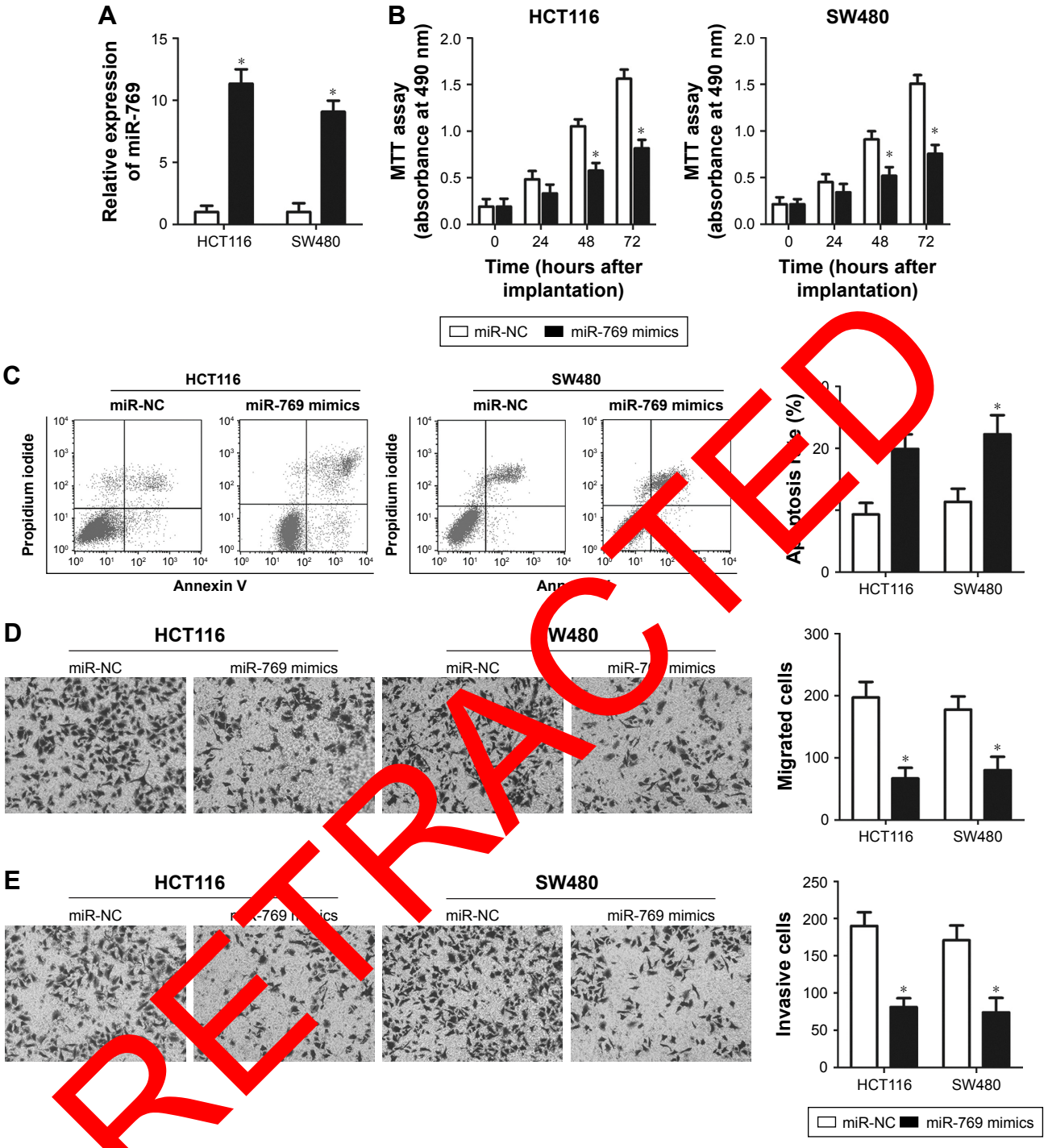

Figure 2 miR-769 regulates CRC cell proliferation, apoptosis, migration, and invasion in vitro.
Notes: (**A**) miR-769 was markedly upregulated in HCT116 and SW480 cells after transfection with miR-769 mimics. *$P<0.05$. miR-NC. (**B**) MTT assay after the transfection with miR-769 mimics or miR-NC in HCT116 and SW480 cells. *$P<0.05$. miR-NC. (**C**) Flow cytometry analysis was used to determine the apoptosis rate of HCT116 and SW480 cells that were transfected with miR-769 mimics or miR-NC. *$P<0.05$. miR-NC. (**D**, **E**) Migration and invasion assays were used to determine the migratory and invasive capacities of HCT116 and SW480 cells transfected with miR-769 mimics or miR-NC, respectively. *$P<0.05$. miR-NC.
Abbreviations: CRC, colorectal cancer; miR-NC, negative control miRNA mimics.

miR-769 overexpression on CRC cell proliferation. Ectopic miR-769 expression evidently reduced the proliferative ability of HCT116 and SW480 cells relative to the miR-NC group (Figure 2B, $P<0.05$). To elucidate the regulatory effect of miR-769 in cell apoptosis, flow cytometry analysis was performed and the percentage of apoptotic cells was obviously increased in HCT116 and SW480 cells treated with miR-769 mimics (Figure 2C, $P<0.05$). Furthermore, migration and invasion assays were performed to determine the role of miR-769 on CRC cell metastasis. Results showed

that miR-769 upregulation dramatically suppressed the migratory (Figure 2D, $P<0.05$) and invasive (Figure 2E, $P<0.05$) capacities of HCT116 and SW480 cells. Taken together, the above results suggest that miR-769 may play tumor suppressive roles in CRC progression.

CDK1 is a direct target of miR-769 in CRC cells

To understand the mechanisms underlying the tumor suppressor activity of miR-769 in CRC, bioinformatic analysis was performed to identify the putative targets of miR-769. According to bioinformatic prediction, miR-769 is partially complementary to the 3′-UTR of CDK1 (Figure 3A). To further confirm this prediction, luciferase reporter assay was performed to determine whether miR-769 could directly recognize and interact with the 3′-UTR of CDK1. Results showed that miR-769 overexpression decreased the luciferase activity of the reporter carrying the WT 3′-UTR sequences in HCT116 and SW480 cells (Figure 3B, $P<0.05$); however, miR-769 mimics did not affect the luciferase activity when the binding sequences for miR-769 in the CDK1 3′-UTR were mutated. To determine whether miR-769 can modulate the expression of endogenous CDK1, RT-qPCR and Western blot analysis were employed to assess CDK1 expression in HCT116 and SW480 cells transfected with miR-769 mimics or miR-NC. We observed that both the mRNA (Figure 3C, $P<0.05$) and protein (Figure 3D, $P<0.05$) levels of CDK1 in HCT116 and SW480 cells were significantly reduced after transfection with miR-769 mimics. In summary, we demonstrated that CDK1 is a direct target of miR-769 in CRC cells.

CDK1 expression correlates inversely with miR-769 expression in CRC tissues

We next attempted to examine the association between miR-769 and CDK1 in clinical CRC tissues. CDK1 mRNA was detected in 30 CRC tissues and ANTs. Higher CDK1 mRNA level was observed in CRC tissues than in ANTs (Figure 4A, $P<0.05$). Next, we determined CDK1 protein level in several pairs of CRC tissues and ANTs by Western blot analysis, and observed that CDK1 protein was significantly upregulated in CRC tissues than in ANTs (Figure 4B and C, $P<0.05$). Furthermore, we demonstrated an inverse correlation between miR-769 and CDK1 mRNA levels in clinical CRC tissues using Spearman's correlation analysis (Figure 4D; $r=-0.5336$, $P=0.0001$). These results further supported the conclusion that CDK1 is a direct target of miR-769 in CRC cells.

CDK1 inhibition imitates the tumor suppressive roles of miR-769 mimics in CRC cells

Our aforementioned results demonstrated that miR-769 restricted the development of CRC, and CDK1 was the direct target of miR-769. Next, we attempted to determine whether miR-769 affected proliferation, apoptosis, migration, and invasion of CRC cells via CDK1 inhibition. We knocked down CDK1 expression in HCT116 and SW480 cells using the CDK1 siRNA. Western blot analysis showed that CDK1 siRNA efficiently knocked down endogenous CDK1 expression in HCT116 and SW480 cells (Figure 5A, $P<0.05$). As expected, CDK1 inhibition suppressed proliferation (Figure 5B, $P<0.05$), promoted apoptosis (Figure 5C, $P<0.05$), and decreased migration (Figure 5D, $P<0.05$) and invasion (Figure 5E, $P<0.05$) of HCT116 and SW480 cells. These results indicated that the biological roles of CDK1 inhibition were consistent with the phenotype observed after miR-769 upregulation in CRC cells, further suggesting that miR-769 prohibited the aggressive behaviors of CRC cells by inhibiting CDK1.

CDK1 mediates miR-769-induced inhibition of CRC progression

Rescue experiments were conducted to further evaluate whether CDK1 mediates the tumor-suppressing roles of miR-769 in CRC cells. CDK1 overexpression vector lacking the 3′-UTR was transfected into HCT116 and SW480 cells overexpressing miR-769 to recover CDK1 expression. Western blot analysis confirmed that CDK1 protein level was reduced in miR-769-overexpressing HCT116 and SW480 cells and expression was re-established after co-transfection with pCMV-CDK1 (Figure 6A, $P<0.05$). The MTT assay showed that reintroduction of CDK1 expression partially rescued the miR-769-mediated inhibition of HCT116 and SW480 cell proliferation (Figure 6B, $P<0.05$). Analysis of cell apoptosis revealed that CDK1 restoration in HCT116 and SW480 cells might re-establish the miR-769-induced increase in apoptosis (Figure 6C, $P<0.05$). Furthermore, restored CDK1 expression blocked the suppressive effects of miR-769 on migration (Figure 6D, $P<0.05$) and invasion (Figure 6E, $P<0.05$) of HCT116 and SW480 cells. These results suggest that miR-769 exerts its anticancer effects in CRC, at least partially, by repressing CDK1.

miR-769 inhibits the tumor growth of CRC in vivo

Xenograft experiments were further performed to explore the effect of miR-769 overexpression in CRC tumor growth

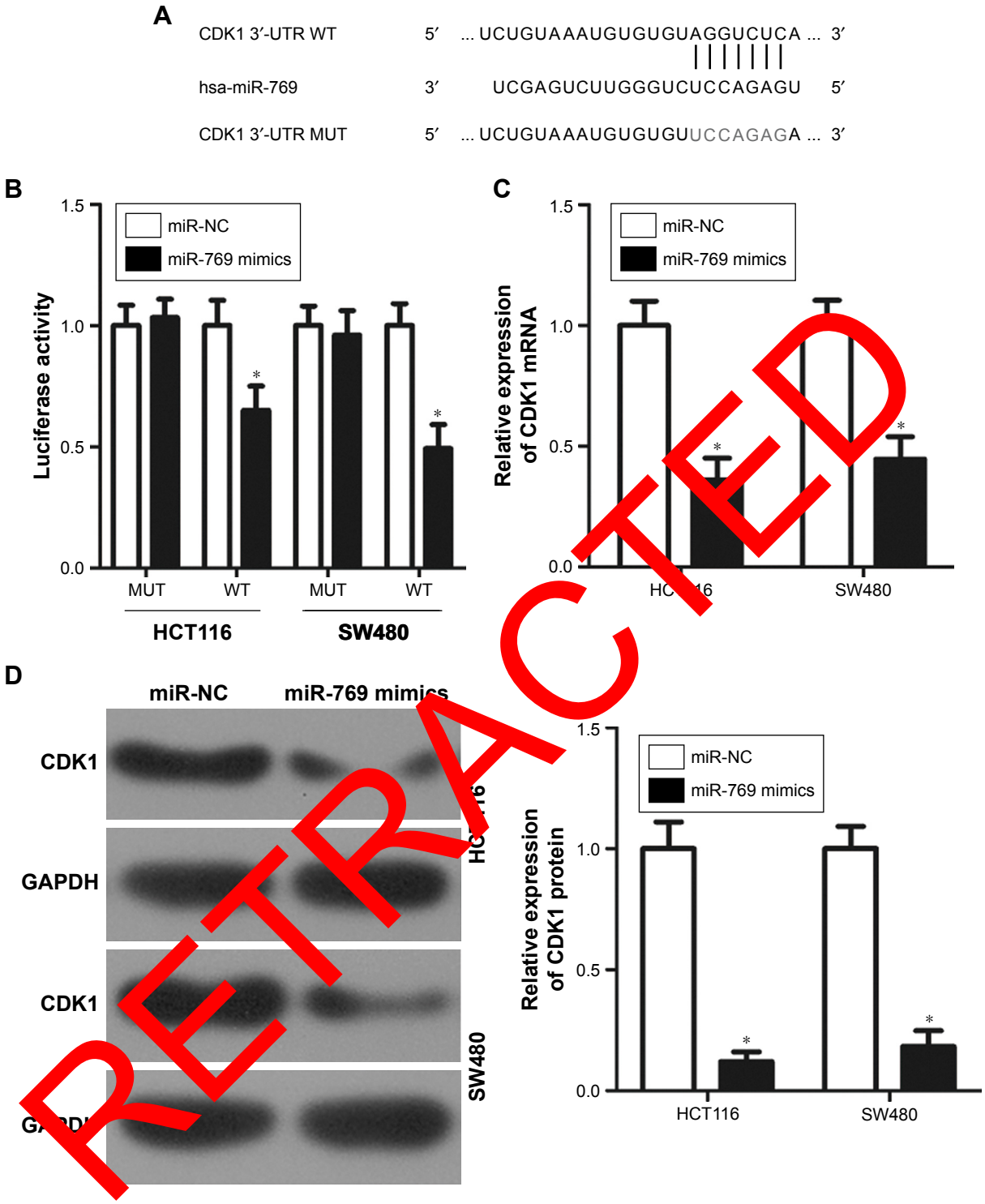

Figure 3 Identification of CDK1 as a direct target of miR-769 in CRC cells.

Notes: (**A**) Sequence alignment of miR-769 and the 3′-UTR of CDK1. The mutant binding sequences in the 3′-UTR of CDK1 for miR-769 are also shown. (**B**) HCT116 and SW480 cells were co-transfected with miR-769 mimics or miR-NC, and luciferase reporter plasmid carrying the WT or MUT 3′-UTR sequences. *P<0.05. miR-NC. (**C**, **D**) RT-qPCR and Western blot analysis was performed to determine CDK1 mRNA and protein levels in HCT116 and SW480 cells after transfection with miR-769 mimics or miR-NC. *P<0.05. miR-NC.

Abbreviations: CDK1, cyclin-dependent kinase 1; miR-NC, negative control miRNA mimics; miRNA, microRNAs; MUT, mutant; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type.

in vivo. miR-769 mimics or miR-NC transfected SW480 cells were injected into the flanks of nude mice. The volume of tumor xenografts was significantly decreased in the miR-769 mimics group compared to that in the miR-NC group (Figure 7A and B, P<0.05). At 30 days, all BALB/c nude mice were sacrificed, and the formed xenografts were excised and weighed. The tumour weight of the miR-769 mimics groups was obviously lower than that of the miR-NC groups

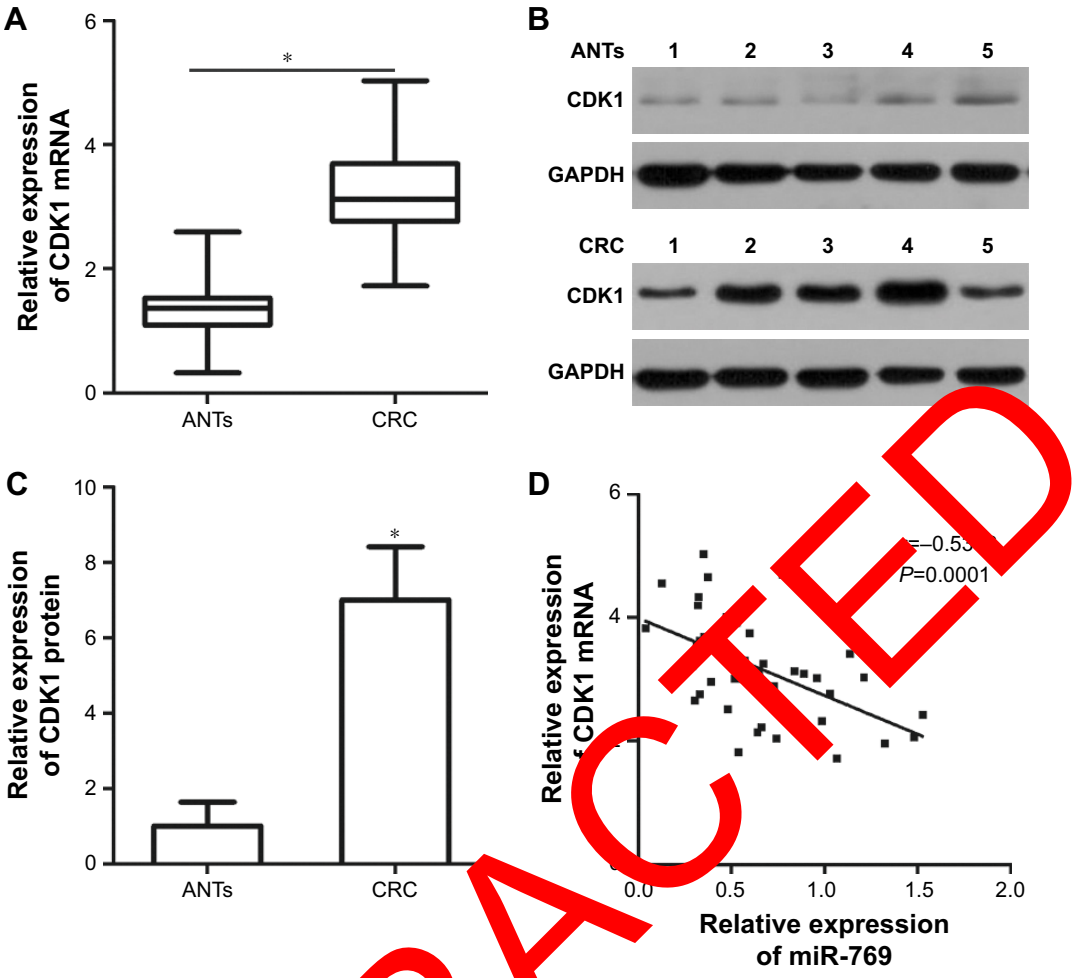

Figure 4 CDK1 is overexpressed in CRC tissue samples and negatively correlates with miR-769 expression.
Notes: (**A**) CDK1 mRNA level was detected in clinical CRC tissues by RT-qPCR analysis. $^{*}P<0.05$. ANTs. (**B**, **C**) Western blot analysis was used to measure CDK1 protein level in several pairs of CRC tissues and ANTs. $^{*}P<0.05$ ANTs. (**D**) The correlation between miR-769 and CDK1 mRNA expression in clinical CRC tissues was assessed by Spearman's correlation analysis. $r=-0.5336$, $P=0.0001$.
Abbreviations: ANTs, adjacent normal tissues; CDK1, cyclin-dependent kinase 1; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR.

(Figure 7C, $P<0.05$). Additionally, miR-769 expression in the tumor xenografts was determined using RT-qPCR. The results showed that miR-769 expression was noticeably upregulated in the tumor xenografts that were injected with miR-769 mimics (Figure 7D, $P<0.05$). Furthermore, Western blot analysis was applied to detect CDK1 expression in the tumor xenografts. It was observed that expression level of CDK1 was significantly downregulated in the xenograft tumour tissues upon miR-769 overexpression (Figure 7E). These results suggest that miR-769 directly targets CDK1 to inhibit CRC tumour growth in vivo.

Discussion

Recent studies have shown that miRNAs are aberrantly expressed in CRC.[20–22] miRNA deregulation is implicated in the formation and progression of CRC, and participates in the regulation of a wide range of biological behaviors.[23–25] Considering their crucial role in CRC, miRNAs hold significant promise in the diagnosis and therapy of patients with this malignancy. In the present study, we demonstrated that miR-769 was downregulated in both CRC tissues and cell lines. Functional studies showed that rescue of miR-769 expression inhibited proliferation, increased apoptosis, and decreased metastasis of CRC cells in vitro as well as restricted the tumor growth in vivo. Importantly, CDK1 was identified as a direct and functional target of miR-769 in CRC cells. These results supported the conclusion that miR-769 was downregulated in CRC and targeted CDK1 to inhibit CRC development, suggesting that this miRNA might represent a valuable target for treating patients with this disease.

miR-769 level was reduced in non-small-cell lung cancer tissues and cell lines. Reduced miR-769 expression correlated strongly with the clinical stage and lymph node metastasis of patients with non-small-cell lung cancer. Non-small-cell

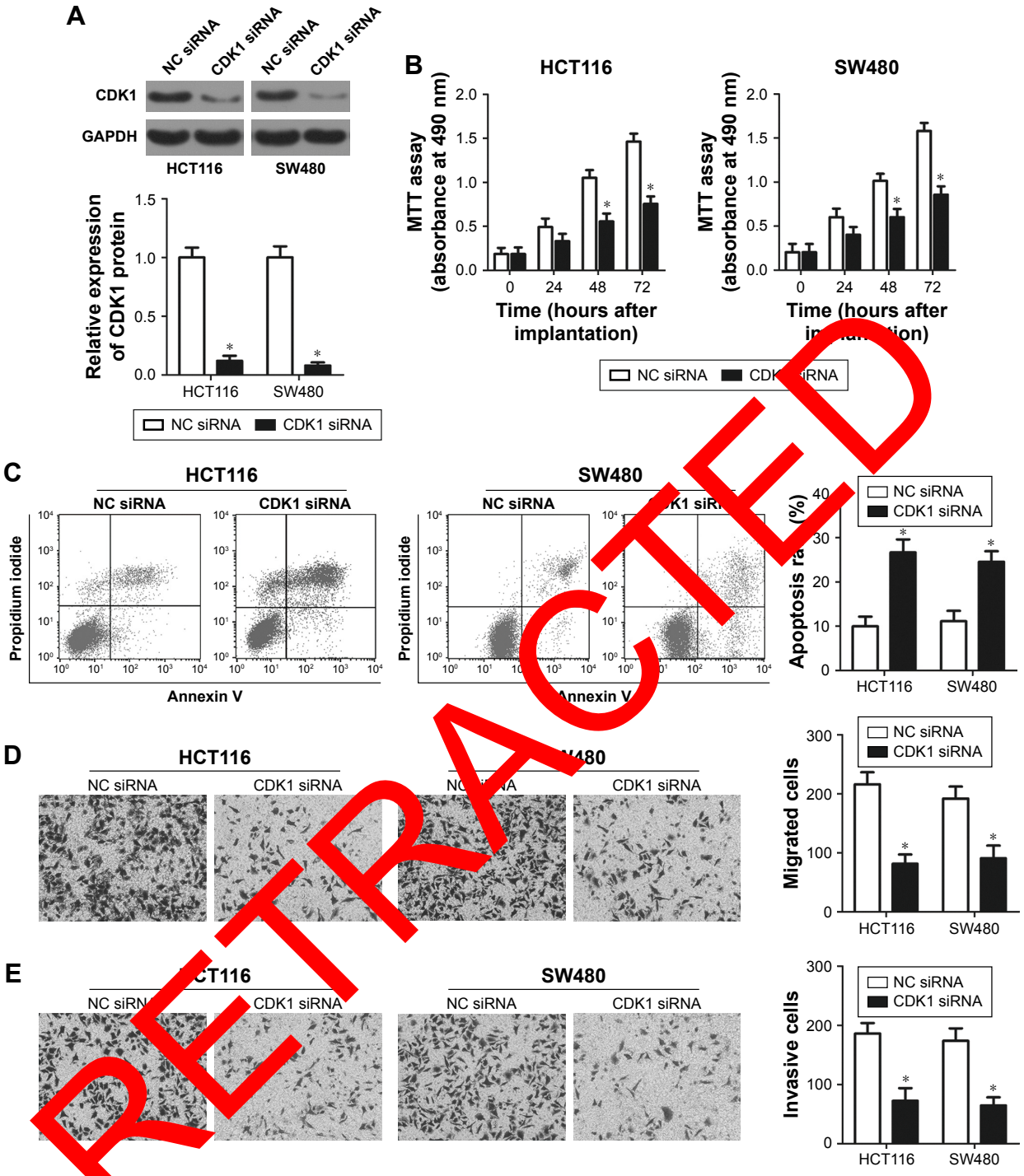

Figure 5 CDK1 knockdown inhibits HCT116 and SW480 cell proliferation, promotes apoptosis, and restricts migration and invasion.

Notes: (**A**) CDK1 protein level in CDK1 siRNA or NC siRNA-transfected HCT116 and SW480 cells was determined using Western blot analysis. *$P<0.05$. NC siRNA. (**B**, **C**) Proliferation and apoptosis of HCT116 and SW480 cells after transfection with CDK1 siRNA or NC siRNA were evaluated using the MTT assay and flow cytometry analysis, respectively. *$P<0.05$. NC siRNA. (**D**, **E**) Cell migration and invasion were detected by migration and invasion assays in HCT116 and SW480 cells after CDK1 siRNA or NC siRNA transfection. *$P<0.05$. NC siRNA.

Abbreviations: ANTs, adjacent normal tissues; CDK1, cyclin-dependent kinase 1; CRC, colorectal cancer; NCsiRNA, negative control siRNA; RT-qPCR, Reverse transcription-quantitative PCR; siRNA, small interfering RNA.

lung cancer patients with low miR-769 expression had poorer prognosis than that patients with high miR-769 level.[17] Functional studies revealed that miR-769 may act as a tumor suppressor in non-small-cell lung cancer by affecting cell growth and metastasis, both in vitro and in vivo.[17] In contrast, miR-769 was upregulated in melanoma and promoted cell growth and colony formation.[18] These contradictory observations indicate that the expression pattern and biological roles

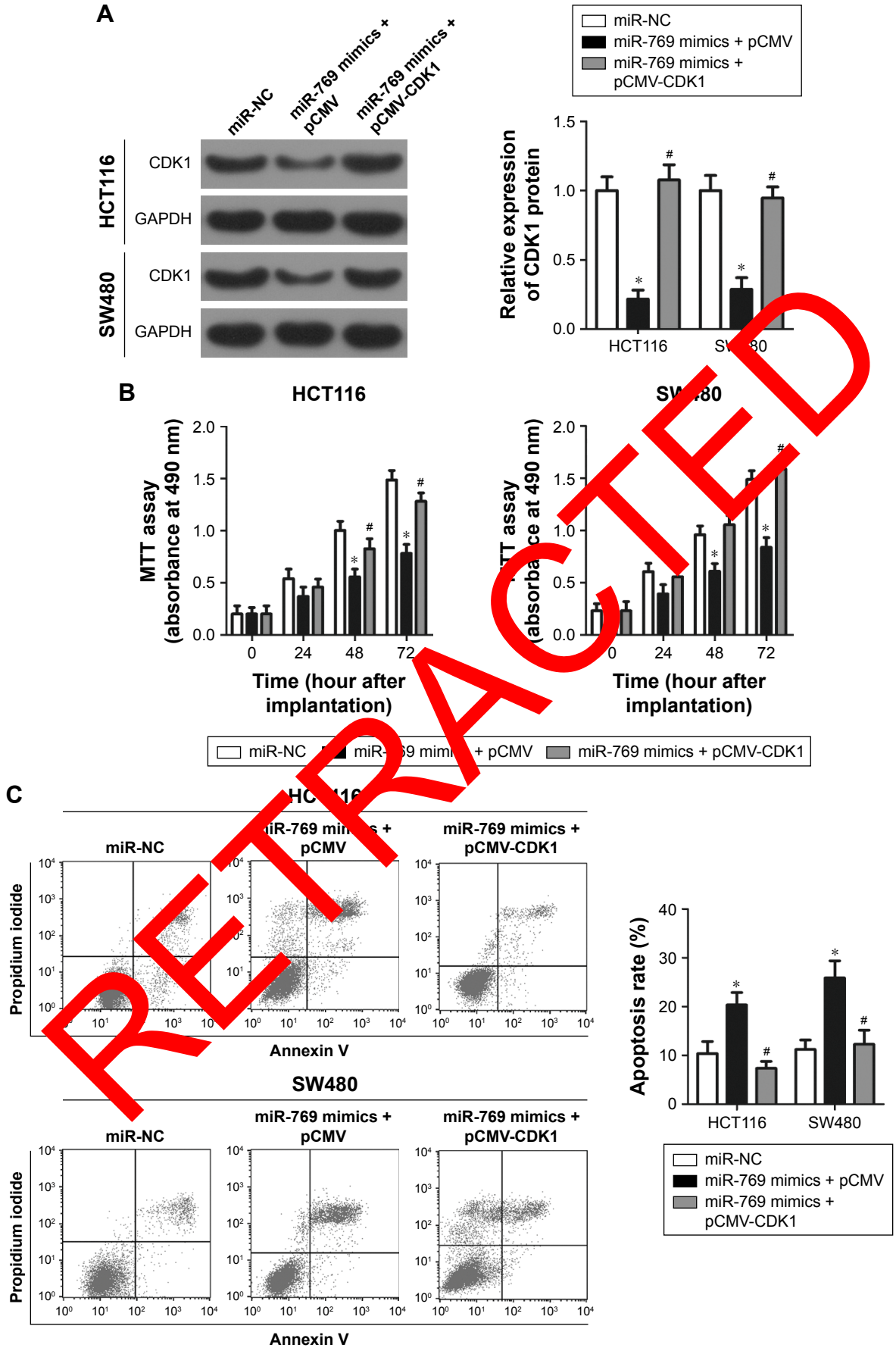

Figure 6 (*Continued*)

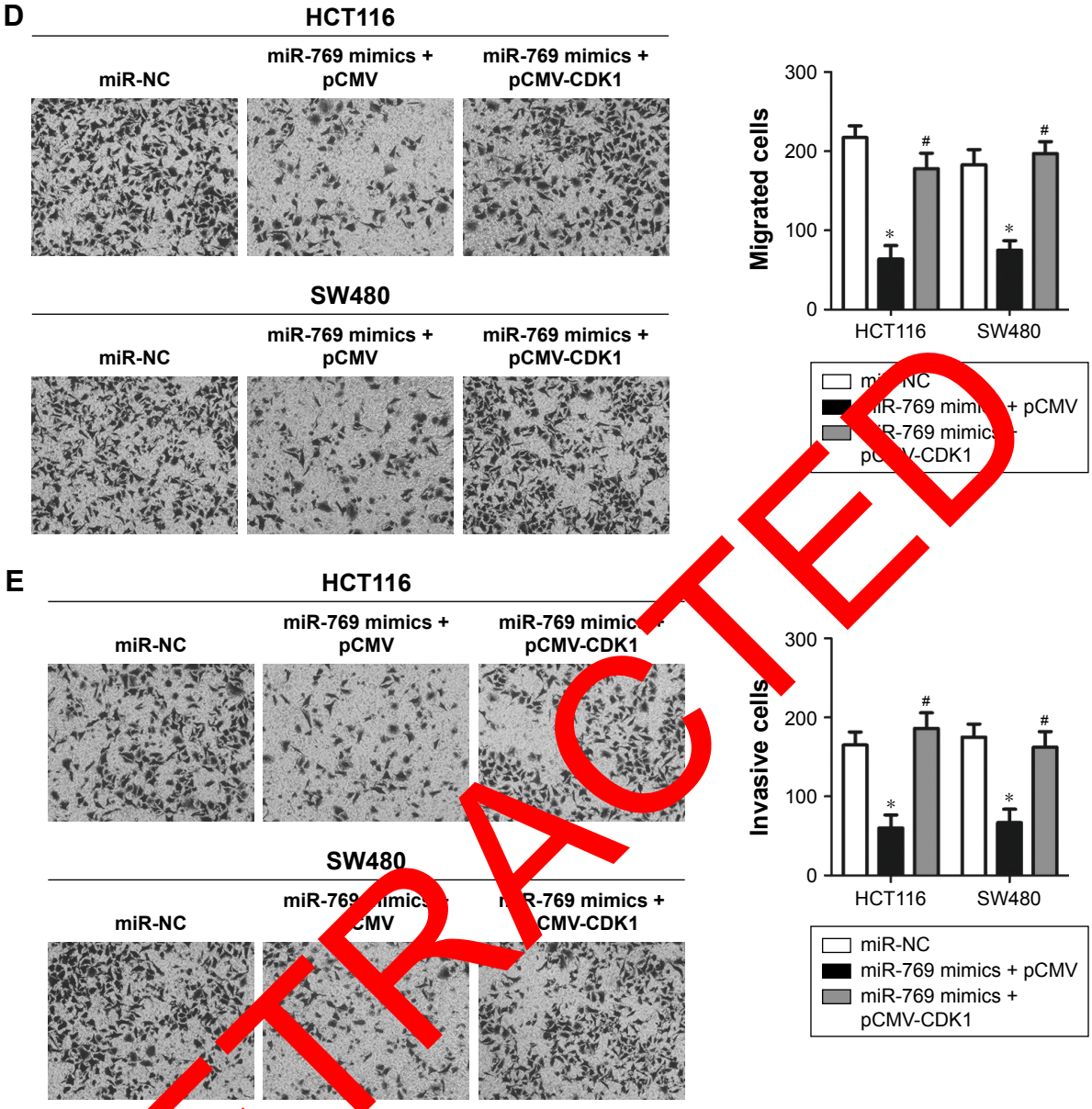

Figure 6 Restoring CDK1 expression reverses the effects of miR-769 in CRC cells.

Notes: miR-769 mimics, alone or with pCMV-CDK1 or pCMV, were co-transfected into HCT116 and SW480 cells. After different incubation time points, the transfected cells were used in the following experiments. (**A**) Western blot analysis of CDK1 protein expression in above-mentioned cells. *$P<0.05$. miR-NC. #$P<0.05$. miR-769 mimics + pCMV. (**B**, **C**) MTT assay and flow cytometry analysis of the proliferative ability and apoptosis rate of the above-mentioned cells. *$P<0.05$. miR-NC. #$P<0.05$. miR-769 mimics + pCMV. (**D**, **E**) Migratory and invasive abilities of the indicated cells were determined using migration and invasion assays. *$P<0.05$. miR-NC. #$P<0.05$. miR-769 mimics + pCMV.

Abbreviations: CDK1, cyclin dependent kinase 1; CRC, colorectal cancer; miR-NC, negative control miRNA mimics.

of miR-769 in malignant tumors exhibit tissue specificity. Hence, miR-769 may potentially act as a diagnostic biomarker and therapeutic target for patients with these specific types of cancer in the future.

TGFBR1 and GSK3B have been demonstrated to be direct targets of miR-769.[17,18] Validation of the direct targets of miR-769 is essential for understanding its detailed role in CRC and may be useful in identifying promising therapeutic approaches. Therefore, we investigated the molecular mechanisms responsible for the tumor suppressor activity of miR-769 in CRC cells. First, bioinformatic analysis showed that miR-769 is partially complementary to the 3′-UTR of CDK1. Second, miR-769 can directly target the 3′-UTR of CDK1 and decrease its expression in CRC cells. Third, CDK1 was upregulated in clinical CRC tissues, which was inversely related to miR-769 expression. Fourth, CDK1 knockdown was able to simulate the tumor suppressive roles of miR-769 in CRC cells. Furthermore, restoration of CDK1 expression partially abolished the tumor-suppressing activity of miR-769 in CRC cells. These results provided adequate evidence to

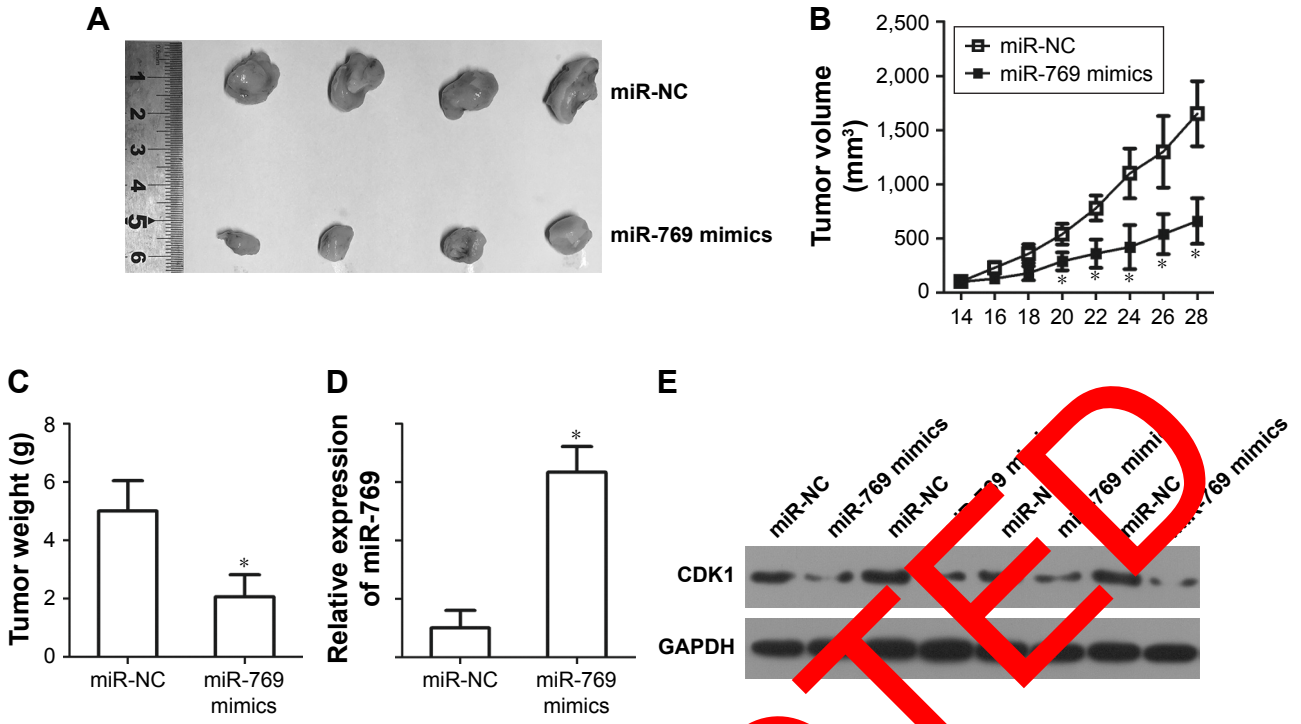

Figure 7 miR-769 inhibits CRC cell growth in vivo.

Notes: (**A**) The representative images of the miR-769 mimics and miR-NC tumor xenografts. (**B**) The volume of tumor xenografts from the miR-769 mimics and miR-NC groups was determined after inoculation. *$P<0.05$ vs miR-NC. (**C**) Nude mice were sacrificed at 30 days after injection. The weights of tumor xenografts were weighted. *$P<0.05$ vs miR-NC. (**D**) RT-qPCR analysis was utilized to determine miR-769 expression in the tumor xenografts. *$P<0.05$ vs miR-NC. (**E**) Expression level of CDK1 protein in the tumor xenografts was detected through Western blot analysis.

Abbreviations: CDK1, cyclin-dependent kinase 1; CRC, colorectal cancer; miR-NC, negative control; miR-769 mimics.

suggest that CDK1 is a direct target of miR-769, and that CDK1 downregulation is essential for the tumor suppressing roles of miR-769 in CRC cells.

CDK1 is a member of the serine/threonine kinase family and consists of a catalytic kinase subunit and cyclin protein partners.[26] It plays important roles in spindle morphogenesis and mitosis.[27] Several studies have reported that CDK1 is upregulated in various human cancers, including breast cancer,[28] endometrial cancer,[29] epithelial ovarian cancer,[30] and lung cancer.[31] CDK1 is overexpressed in CRC, and its expression correlated significantly with lymph node metastasis.[32] CDK1 was demonstrated to be an independent prognostic factor for predicting the therapeutic outcomes of patients with CRC.[33,34] Functional analyses revealed that CDK1 contributes to the genesis and progression of CRC, and is involved in several biological processes, such as cell proliferation, apoptosis, metastasis, and sensitivity to chemotherapy and radiotherapy.[35–38] The present study demonstrated that miR-769 targeted CDK1 to inhibit the malignant behavior of CRC cells. These observations suggest that miR-769-based molecular targeted therapy against CDK1 might be an effective therapeutic technique for patients with CRC.

Conclusion

The results of this study revealed that miR-769 was downregulated in CRC, and its upregulation inhibited cancer progression by directly targeting CDK1. Understanding the role of miR-769 in the suppression of CRC development will improve our understanding of CRC biology, and restoration of miR-769 expression may represent a novel therapeutic approach for managing patients with this malignant tumor.

Disclosure

The authors report no conflicts of interest in this work.

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