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MicroRNA-32 inhibits the proliferation, migration and invasion of human colon cancer cell lines by targeting E2F transcription factor 5

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Abstract. – OBJECTIVE: MicroRNAs to be essential therapeutic targets for the treatment of diseases such as cancer. Colon cancer is one of the prevalent cancers and causes tremendous human mortality. The treatment of colon cancer is limited by late diagnosis and lack of efficient therapeutic targets. Herein, the therapeutic potential of the miR-32 was explored in colon cancer.

PATIENTS AND METHODS: Expression analysis was carried out by quantitative Real-time polymerase chain reaction (qRT-PCR). Transfections were performed by Lipofectamine® 2000 reagent. The cell counting kit 8 (CCK-8) assay was used to determine cell viability. The 4′, 6-diamidino-2-phenylindole (DAPI), Annexin V-FITC/Propidium Iodide (PI) assays were used for the detection of apoptosis. Transwell assays were used to determine cell migration and invasion. The expression of the proteins was detected by Western blotting.

RESULTS: The results showed that the expression of miR-32 was aberrantly downregulated in all colon cancer cells. Overexpression of miR-32 caused significant ($p<0.01$) decline in the viability of SW-948 cells via induction of apoptosis. The induction of apoptosis was also accompanied by the up-regulation of Bax and down-regulation of Bcl-2 expression. Overexpression of miR-32 also caused the arrest of the SW-498 cells in the G2/M phase of cell cycle and inhibited their migration and invasion. Target prediction analysis revealed E2F transcription factor 5 (E2F5) to be a potential target of miR-32 which was confirmed by dual luciferase reporter assay. The expression of E2F5 was significantly ($p<0.01$) upregulated in the colon cancer cells. The overexpression of miR-32 caused a considerable decline in the expression of E2F5 in the SW-948 cells. E2F5 silencing also inhibited the growth of the SW-948 cells via induction of apoptosis and G2/M cell cycle arrest. MiR-32 overexpression also inhibited the migration and invasion of the SW-948 cells. However, rescue assays revealed E2F5 to be essential for the tumor suppressive effects of miR-32.

CONCLUSIONS: The findings of the present study reveal that miR-32 acts as a tumor suppressor in colon cancer cells and may have therapeutic implications in colon cancer treatment.

Key Words:
Colon cancer, MicroRNA-32, Apoptosis, Cell cycle arrest.

Introduction

Colon cancer is the fourth prevalent cause of cancer-related mortality worldwide. With 1.4 million new cases of colon cancer reported annually, colon cancer is the third most common type of cancer throughout the world[1,2]. Although the incidence of colon cancer has declined to some extent, it is believed to increase by 60% till 2030[3]. The late diagnosis, dearth of the potent and safer chemotherapeutic drugs and efficient therapeutic targets form an obstacle in the treatment of colon cancer[4]. Researches carried out previously on microRNAs (miRs) have provided strong evidence about the involvement of miRs in almost all types of cancers and malignancies. The advent of microarray and next-generation sequencing has greatly allowed to understand the relation between miRs and cancer[5]. The miRs are generally 22 nucleotides in length, non-coding RNAs molecules that regulate the expression of genes post-transcriptionally[6]. They are evolutionarily conserved and perform their functions in almost all biological processes[7]. More than

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2500 miRs have been identified in human so far and the list is still growing[8]. The expression of several miRs has been reported to be dysregulated in cancers. Generally the expression of miRs is repressed in cancerous tissues, however, many miRs have also been reported to be overexpressed in cancer[9]. It has been reported that therapeutic application of miRs may prove an amazing and essential strategy to interfere with molecular mechanisms underlying cancer[10]. Therefore, several miRs have been studied for their therapeutic potential and miR-32 is one such candidate. While miR-32 has been shown to promote the growth of cancers, it has also been reported to act as a tumor suppressor in many cancer types[11-13]. For instance, miR-32 has been shown to promote the proliferation of breast and hepatocellular carcinoma[11,12] and acts as a tumor suppressor in human oral squamous cell carcinoma and uveal melanoma[13]. The overexpression of miR-32 has also been shown to regulate the induction of apoptosis in myeloid leukemia cells[14]. The expression of miR-32 has been shown to exhibit a correlation with progression of tumor development and survival of lung carcinoma patients[15]. Nonetheless, the role and therapeutic potential of miR-32 is still largely unknown in colon cancer. This study was therefore undertaken to investigate the therapeutic potential of miR-32 in human colon cancer. Moreover, for the first time, the study reports the tumor suppressive role of miR-32 in colon cancer via suppression of E2F transcription factor 5 (E2F5).

Patients and Methods

Cell Culture

The normal colon cell line CCD-18Co and the colon cancer cell lines (HT-29, SW-948, RKO, and SW480) were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cell lines were subjected to culturing in cell Park Memorial Institute-1640 (RPMI-1640, Gibco, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were cultured in CO_2 incubation chamber at 37 °C. The study was approved by the Research Ethics Committee of The NO.1 hospital of Jilin University (Changchun, Jilin) under approval No. HJU-A/20198.

Expression Analysis

The TRIzol reagent (Invitrogen, Carlsbad, USA) was used for the extraction of RNA from the colon cancer cell lines. This was followed by purification of the RNA by using Mini Kit (Qiagen, Hilden, Germany). The complementary DNA was then synthesized with the miScript Reverse Transcription Kit (Qiagen, Germany). Afterward, the cDNA was amplified by using SYBR Premix Ex TaqII (TaKaRa, Otsu, Shiga, Japan). The expression was estimated by $2^{-\Delta\Delta Ct}$ method and actin was used as the internal control.

Transfection

The SW-948 cells were cultured to 80% confluence and then 10 pmol negative control (NC) and miR-32-2 mimics, E2F5 from Shanghai GenePharma (Shanghai, China; 10 pmol), small interfering (si)- RNA-E2F5 and pcDNA-E2F5 (2 pmol; Taijin Saier Biotechnology, Inc., Xiaozhan, China) were transfected using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol.

Cell Viability Assay

Cell Counting Kit 8 (CCK-8) assay was used for the determination of the cell viability. In brief, the transfected SW-948 cells were seeded in 96-well plates and incubated at 37°C for 24 h and subjected to treatment with 10 microtiters of CCK-8 solution. The cells were then again subjected to incubation for 2 h at 37° in a humidifier (5% CO_2/95% O_2). OD_{450} was taken at different time intervals (0, 12, 24, 48, and 96 h) with the help of a microplate reader.

Apoptosis Assays

The transfected colon cancer SW-948 cells (0.6×10^6) were seeded in 6 well plates and subjected to incubation for 12 h at 37°C. As the cells sloughed off, 10 µl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with cover slips and examined with a fluorescent microscope. Annexin V/PI staining of the SW-948 cells was performed as described previously[16]. In brief, ApoScan kit was used to determine the apoptotic SW-948 cell percentage. The transfected SW-948 cells (5×10^5 cells per well) were incubated for 24 h at 37°C. This was followed by the staining of these cells with annexin V-FITC or PI. The percentage of apoptotic SW-948 cells was then determined by flow cytometry.

Bromodeoxyuridine (BrdU) Incorporation and Flow Cytometry Analysis

The two color-flow cytometric analysis was employed for the estimation of the SW-948 cell cycle distribution profile. Approximately 1× 10^6 SW-948 transfected cells were pulsed with 10 µl BrdU for 1 h and then harvested with trypsinization. The cells were then subjected to fixation with ethanol (70%) at 4°C for 24 h. Thereafter, the cells were treated for 25 min with HCl (2 N) containing Triton X-100 (0.5%). Sodium borate (0.1 M, pH 8.5) was added to neutralize the residual acid. This was followed by incubation of the cells with anti-BrdU-FITC antibody for 24 h, at 4°C. The cells were then subjected to incubation with Propidium Iodide (PI), washed with PBS and then finally analyzed by flow cytometry.

Cell Migration and Invasion Assay

Following transfection of the colon SW-948 cells, they were subjected to culturing in a serum-free medium for 12 h. The cell density was adjusted to 4 × 10^5 cells/ml. Transwell chamber having 8 µm pore size was utilized for the 2 ll plates. Around 500 µl of RPMI-500 media mented with fetal bovine serum (FBS) (10 as put into the lower chamber while as 250 µl o l culture suspension was put into lower cham and subjected to incubation for around 24 h. T cells that moved to the lower ere subjected to fixation with glacial tic ac nd subsequently stained with cr violet 25 min. Around 10 randomly se fields determine cell migrati T ethodology was used for the d ination ll invasion except that the ce ere covered atrigel.

Target Iden atio d Dual Luciferas Assay

The p 2 targets were fied by online TargetS softw re version 7.2 (http://www.targetsc g). To y out the dual luciferase assay vild (WT) d the mutated (MUT) E2F5 nding were cloned into the downstre ion e luciferase gene in the REPO erase vector (Invitrogen, Car d, CA, U The SW-948 cells were then subj d to co-transfection with WT and MUT PG UTR vectors and the miR-32 ics. The luciferase activity was determined the help of a Luciferase Reporter Assay kit (a Corporation, Madison, WI, USA) in acco nce with the manufacturer's protocol and Renilla luciferase activity was employed to normalize the data.

Western Blotting

The SW-948 cells were cul for 24 h and then harvested by centrifugation. lls were washed twice with ice-co PBS a d in RIPA lysis buffer. Bradf ssay was us termine the protein co . From ch samp µg of protein was l d on S PAGE before being shifted to po lide fluori em-brane. The mem ne hen s ted to treatment with 3 and th os primary antibodies a Thereafter, were treated with a econdary an odies and the proteins intere visualized by enhanced chemiluminescence (eagent.

Statistical Analysis

Data are show as mean ± SD. Statistical analy was done u Students *t*-test with GraphPrism 7 sof re (La Jolla, CA, USA). For c isons b en more than two samples, one followed Tukey's post-hoc test was used for statistical analysis. Values of $p <$ 0.01 were taken as indicative of statistical significence.

Results

MiR-32 is Downregulated in Human Colon Cancer Cells

The miR-32 expression was determined in one normal (CDD-18Co) and four colon cancer cell lines by qRT-PCR (Figure 1A). The results showed that miR-32 is significantly downregulated ($p < 0.01$) in all the colon cancer cell lines. Furthermore, miR-32 was found to be downregulated in colon cancer cell lines by up to 8 fold relative to the normal cell CCD-18Co line. The lowest expression was observed in case of the SW-948 cell line.

MiR-32 Induces Apoptosis and Cell Cycle Arrest of SW-948 Cells

Since miR-32 was found to be downregulated in colon cancer cell lines, we sought to know the effects of miR-32 overexpression on SW-948 cells. Therefore, the SW-948 cells were transfected with either the miR-32 mimics or NC (negative control). The overexpression of miR-32 in SW-948 cells was validated by the qRT-PCR. Overexpression of miR-32 in SW-948 caused around 6.3 fold upregulation

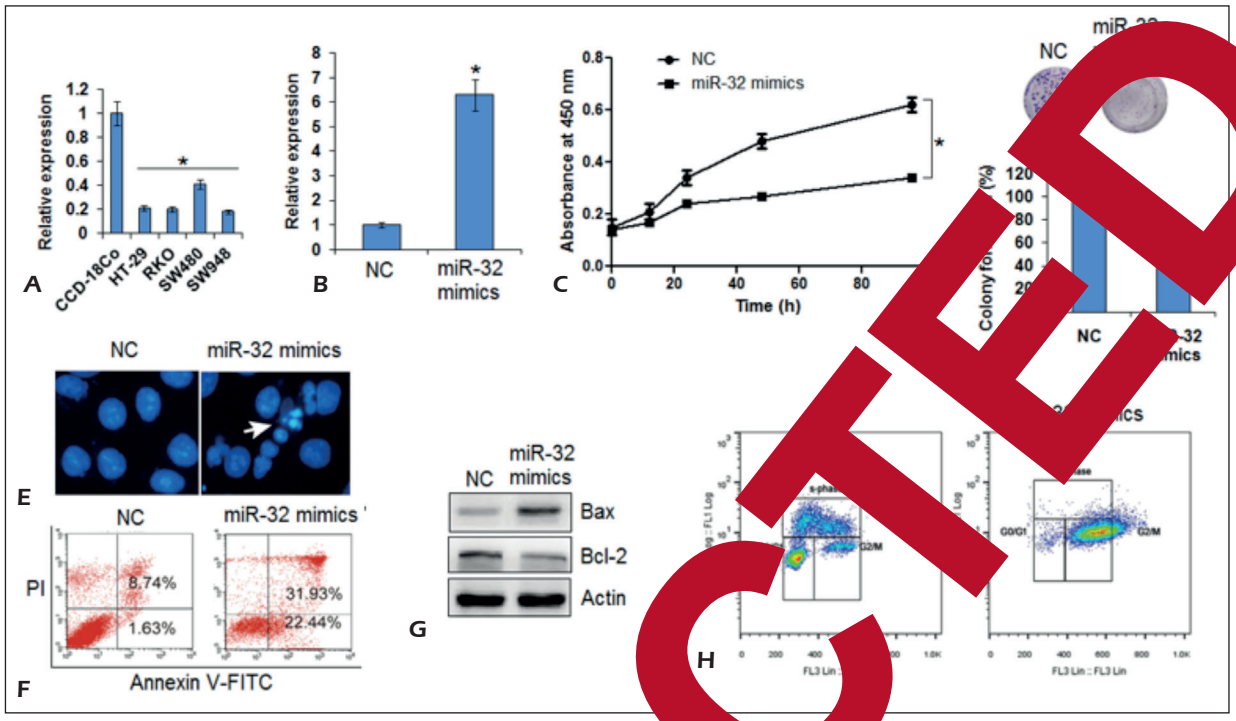


Figure 1. MiR-32 inhibits the growth of colon cancer cells by trigger[illegible] cell cycle arrest. **A**, Expression profile of miR-32 in colon cancer and normal cell lines. **B**, Overexpression of miR[illegible]48 cells. **C**, Cell viability of NC and miR-32 mimics transfected SW-948 cells at indicated ti[illegible]. **D**, Colony formation of NC and miR-32 mimics transfected SW-948 cells. **E**, DAPI staining of NC and miR-32[illegible] SW-948 cells (magnification 200X). **F**, AO/EB staining of NC and miR-32 mimics transfected SW-948 cel[illegible] Expre[illegible]nd Bcl-2 in NC or miR-32 mimics transfected SW-948 cells. **H**, Cell cycle analysis of NC or miR-32[illegible]ics t[illegible]ect[illegible]-948 cells. The experiments were carried out in triplicates and presented as mean ± SD (*p<0.01).

of miR-32 (Figure 1B). The res[illegible]-8 assay[illegible]ony formation of the SW-948 cells (Figure 1C and showed that overexpression [illegible]R-32[illegible]sed sig-[illegible]D). The DAPI staining results showed that miR-32 nificant ($p < 0.01$) decline[illegible]e viabil[illegible]nd col-[illegible] induced apoptosis in the SW-948 cells (Figure 1E).

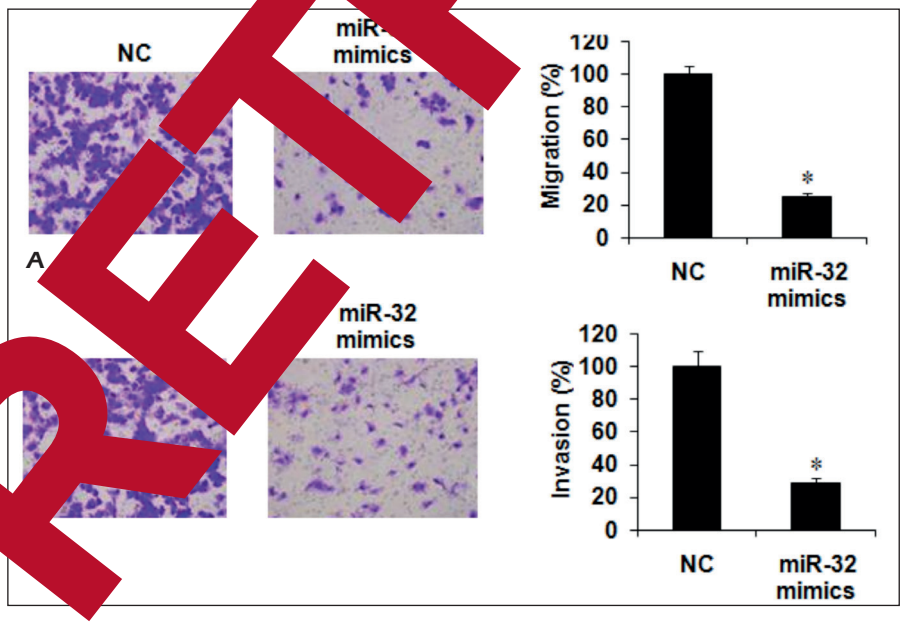


Figure 2. MiR-32 inhibits the migration and invasion of SW-948 cells. **A**, Transwell assay showing cell migration in NC or miR-32 mimics transfected SW-948 cells. **B**, Transwell assay showing cell migration in NC or miR-32 mimics transfected SW-948 cells (magnification 200X). The experiments were carried out triplicates and presented as mean ± SD (*p<0.01).

The percentage of apoptotic cells was found to increase from 1.63% in NC to 22.44% in miR-32 mimics transfected cells as indicated by the annexin V/PI assay (Figure 1F). The miR-3 triggered apoptosis was accompanied by upregulation of the Bax and downregulation of Bcl-2 in the SW-948 cells (Figure 1G). Next, the effects of miR-32 overexpression were also evaluated on the cell cycle phase distribution of the SW-948 cells. It was found that miR-32 overexpression caused the arrest of the SW-948 cells in the G_2/M phase of the cell cycle (Figure 1H).

MiR-32 Inhibits The Migration and Invasion of the SW-948 Cells

The effects of miR-32 were also examined on the migration and invasion of the SW-948 colon cancer cells by transwell assays. The results showed that the migration and invasion of the SW-948 colon cancer were significantly ($p < 0.01$) reduced in the miR-32 mimics transfected cells (Figure 2A and B). The SW-948 cell migration was inhibited by up to 75 % and the invasion was inhibited by 72% in miR-32 transfected cells relative to the NC-transfected SW-948 cells.

MiR-32 Targets E2F5 in Colon Cancer Cells

TargetScan analysis revealed E2F5 as the p tential target of miR-32 (Figure 3A). The qR PCR analysis showed E2F5 nificantly upregulated in all the co cance ll lines (Figure 3B). However, ov pressio miR-32 in the SW-948 cell cau nific ulation of the E2F5 ells ( ). Dual luciferase assay furth onfirme 5 to be the target of miR-32 (re 3D). Nex ught to know about the f E2F5 silen on the proliferation of th 948 cells (Figure 3E). The results howed that t cing of E2F5 expression ed significant (01) decline in the via and olony formati of the SW-948 cells ure 3F 3G) and the inhibitory effects we inly to indu n of apoptosis which was a ted wi pregulation of Bax and d wnregu of B (Figure 3H-J). The cell analys ealed that E2F5 silencing also gers the st of the SW-948 cells at the G_2/M hase of the cell cycle (Figure 3K). The ef silencing were evaluated on the ation and invasion of the SW-498 cells and as found that E2F5 silencing caused a significa rease in the migration and invasion of the SW- cells (Figure 4A and 4B).

E2F5 is Essential for the Tumor Suppressive Effects of MiR-32

We sought to know if the over ession E2F5 could reverse the inhibitory cts of miR-32 on the proliferation of the 8 8 cells. The results showed that E2F5 could al mpletely reverse the effects of miR overex n o the proliferation of the 498 cells i that E2F5 is essential he tu r suppre effects of the miR-32 ure 5).

Discuss

Colon devastating c er and its incidence pecte crease dramatically in the upcoming years[17]. T ical outcome is unsatisfa d treatment gies have a number o s. The currently available chemotherapeu- agents have a erse effects and the efficient rapeutic targ re lacking[18]. It has been re- d that miR modulate the expression of t ority of an genes and are involved in a w cellular processes[19]. Because of the importance of miRNAs in cellular and physiological processes, several studies have revealed ial of miRNAs as therapeutic targets[20]. ere the role and therapeutic potential of the miR-32 were investigated in colon cancer. It was found that miR-32 is aberrantly downregulated in the colon cancer cells. Previous studies have indicated that the dysregulated expression of miR-32 may be correlated with the development and progression of non-small cell lung cancer and survival of lung cancer patients[15]. Moreover, miR-32 has been shown to be dysregulated in gastric cancer cells and also suppresses their proliferation and invasion[21]. Overexpression of miR-32 caused a significant reduction in the proliferation rate of the SW-948 colon cancer cells via induction of apoptotic cell death and cell cycle arrest. The overexpression of miR-32 also caused a decrease in the migration and invasion of the SW-948 cells. Studies carried out previously have shown miR-32 to be involved in regulating apoptosis in myeloid leukemia[14]. MiR-32 has also been shown to regulate the migration and invasion of the hepatocellular carcinoma[22]. *In silico* analysis indicated E2F5 to be the potential target of miR-32. We observed that E2F5 is highly upregulated in the colon cancer and miR-32 overexpression could suppress the expression of E2F5. Furthermore, E2F5 silencing could also inhibit the growth of SW-948 colon cancer cells via induction of apop-

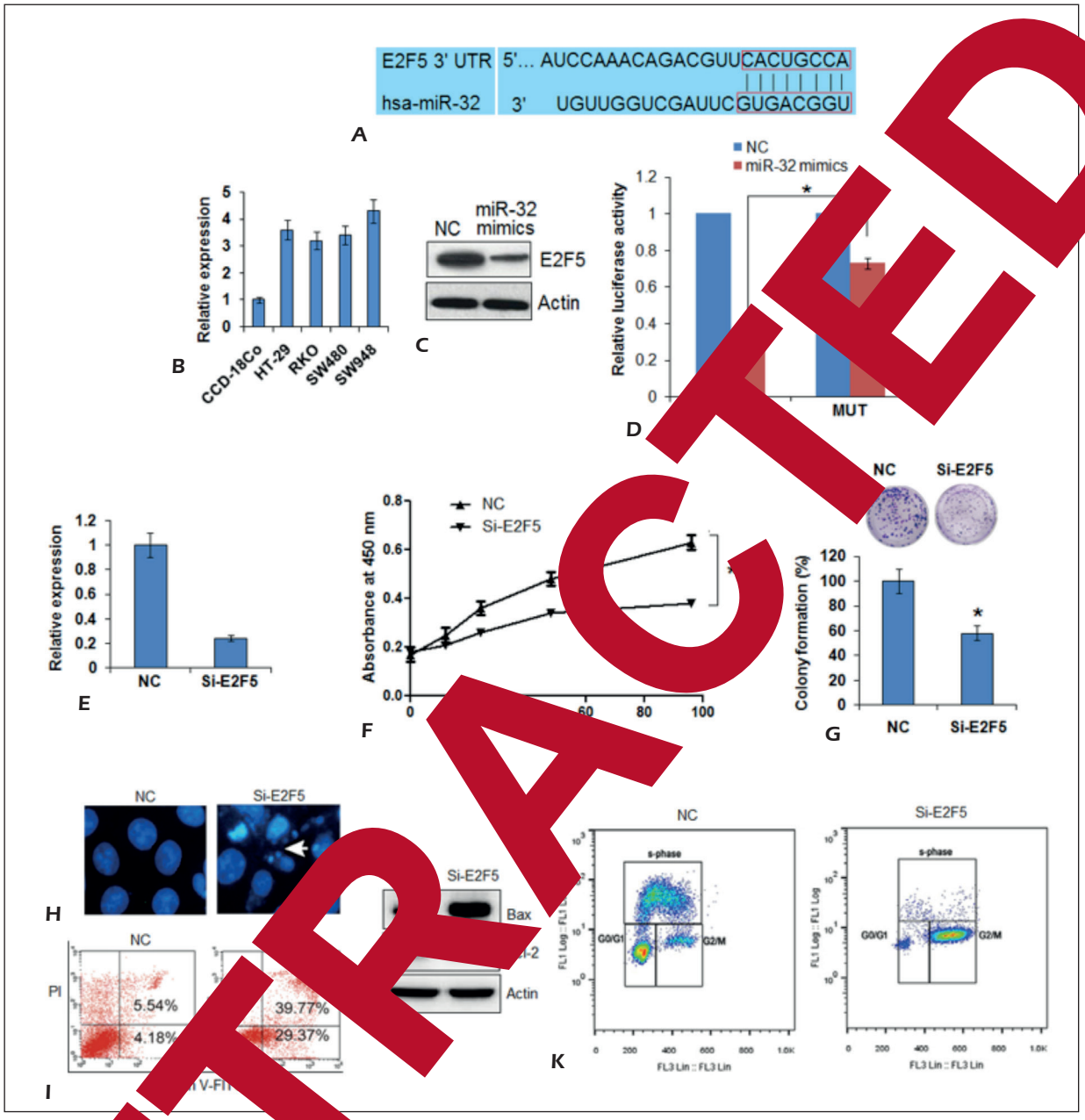


Figure 3. ...2 exerts effects via s... on of E2F5. **A**, TargetScan analysis showing E2F5 as the target of miR-32. **B**, Upregulated expressio... E2F5 in ...ormal and colon ...ncer cell lines. **C**, Western blots showing the expression of E2F5 in NC or miR-32 mimics transfe... W-948 ... **D**, Dual luciferase assay. **E**, Silencing of E2F5 in NC and Si-E2F5 transfected SW-948 cells. **F**, CCK-8 assay show... e viabil... the NC ...nd Si-E2F5 transfected SW-948 cells. **G**, Colony formation of NC and Si-E2F5 transfected cells. **H**, DAP... ... ...nd Si-E2... ...ansfected SW-948 cells (magnification 200X). **I**, Annexin V/PI staining of NC and Si-E2F5 transfected S... ... **J**, Exp... ...n of Bax and Bcl-2 in NC and Si-E2F5 transfected SW-948 cells. The experiments were carried out ...licates a... ...ted a... ...n ± SD (*$p < 0.01$). **K,** Cell cycle analysis of NC or Si-E2F5 transfected SW-948 cells.

to... ...e arrest similar to that of miR-32 ...expression. Next, E2F5 overexpression was ...d to almost completely rescue the effects of ...overexpression on the proliferation of the SW-... cells. E2F5 is considered an important transcription that has been shown to regulate the several cell cycle and apoptosis-related process[23]. The suppression of E2F5 has also been shown to induce cell cycle arrest of the pancreatic cancer cells and also suppress their migration[24].

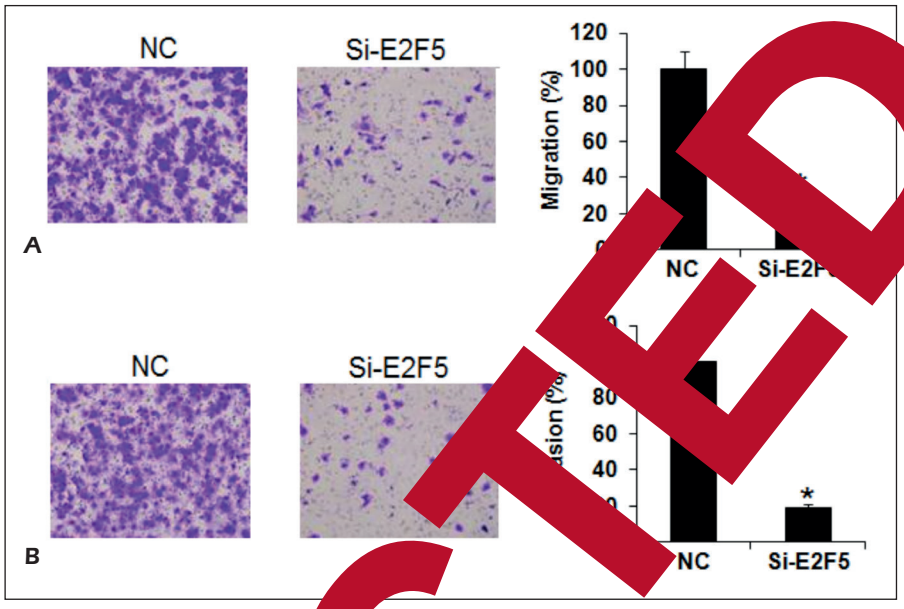


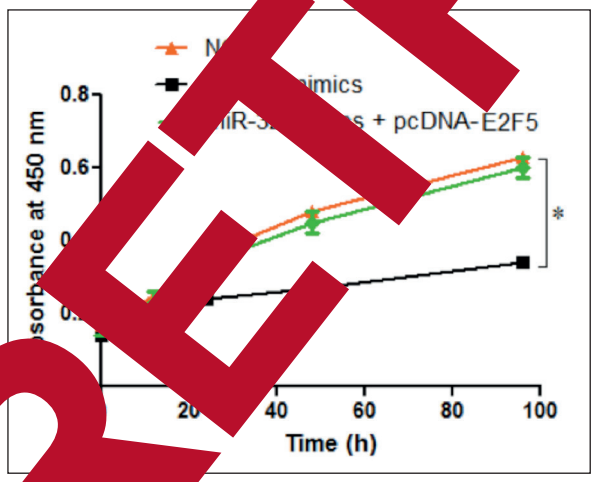
Figure 4. Silencing of E2F5 inhibits the migration and invasion of SW-948 cells. **A**, Transwell assay showing cell migration in NC or Si-E2F5 transfected SW-948 cells. **B**, Transwell assay showing cell migration of NC or Si-E2F5 transfected SW-948 cells (magnification 200X). The experiments were carried out triplicates and presented as mean ± SD (*p<0.01).

Conclusions

We revealed that miR-32 acts as a tumor suppressor and inhibits the growth of colon [illegible] cells via induction of cell cycle arrest. Th[illegible] miR-32 may act as a potent therapeutic ta[illegible] for anticancer drugs and may prove beneficial [illegible] treatment of cancers including colon cancer.

Conflict of Interests

The Authors declare that they h[illegible]o conflic[illegible]terests.



[illegible]re 5. Overexpression of E2F5 reverse the growth inhib-[illegible]effects of miR-32 overexpression on the proliferation of [illegible]olon cancer cells. The experiments were carried out tripli[illegible]nd presented as mean ± SD (*p<0.01).

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