

Original Article

MiR-592 inhibited cell proliferation of human colorectal cancer cells by suppressing of CCND3 expression

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Abstract: Accumulating evidence shows that microRNA (miRNA) is frequently associated with multiple kinds of human cancers, including colorectal cancer (CRC). Previous studies have shown that miR-592 play critical roles in cancer cell biological processes. However, the function of miR-592 in CRC remains largely unknown. In the present study, we investigated the miR-592's role in cell proliferation of colorectal cancer. MiR-592 expression was markedly down-regulated in CRC tissues and CRC cells. Overexpression of miR-592 reduced the proliferation and anchorage-independent growth of CRC cells. Furthermore, bioinformatics analysis further revealed CCND3, a putative tumor promoter, was found to be a potential target of miR-592 in CRC. The dual-luciferase reporter gene assay results showed that CCND3 was a direct target of miR-592. Ectopic expression of miR-592 led to down-regulation of CCND3 protein, which resulted in the down-regulation of phosphorylated retinoblastoma (p-Rb). In functional assays, CCND3-silenced in miR-592-in-transfected SW48 cells have positive effect to suppress cell proliferation, suggesting that direct CCND3 suppression is required for miR-592-induced cell proliferation of CRC. We conclude that miR-592 can regulate CCND3 and function as a tumor suppressor in CRC. Therefore, miR-592 represents a potential anti-onco-miR and serves as a useful therapeutic agent for miRNA-based CRC therapy.

Keywords: miR-592, colorectal cancer, CCND3, cell proliferation

Introduction

Colorectal cancer (CRC) is one of the most common types of cancers worldwide [1]. The incidence of CRC has been experiencing a significant rise in recent years in China [2]. In the past years, major improvements have been achieved in surgery or chemotherapy treatments for CRC [3-5]. However, recurrence and metastasis are very common. Thus, there is an urgent need to elucidate the underlying molecular mechanisms of CRC and find new molecular targets for treatment of this disease.

Increasing evidences indicated that MicroRNAs (miRNAs) act as oncogenes and tumor suppressors in cancer initiation, progression and metastasis [6-10]. MiRNAs regulate gene expression through binding to 3'UTR of the target mRNA [11-13]. Multiple reports indicated that miR-592 has been shown to be one of the important determinants in cancers [14-16]. However, the relationship between CRC and the

expression of miR-592 remained incompletely understood. In this present study, we explored the role of miR-592 in CRC and found that up-regulation of miR-592 inhibited cell proliferation by suppressing cyclin D3 (CCND3). In addition, we explored the underlying mechanism of miR-592 functions in CRC. Therefore, our data demonstrated that miR-592 was indeed a proliferation suppressor in CRC, and the study also shed light on the molecular mechanisms of its anti-proliferation function through the CCND3-mediated signaling pathway.

Materials and methods

Clinical specimens

Eight paired human colorectal cancer tissues and the matched tumor adjacent tissues (TAT) were obtained from CRC patients and histopathologically diagnosed at Liaocheng People's Hospital (Liaocheng, People's Republic of China). The study was approved by the ethics

committee of Liaocheng People's Hospital (Liaocheng, People's Republic of China). Written informed consent was obtained from all patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until total RNAs or proteins were extracted.

Cell culture

Human CRC cell lines SW48, SW403, COLO205, SW620, COLO320DM, KM202L, SW480 and normal colonic cell line FHC were purchased from National Rodent Laboratory Animal Resource (Shanghai, People's Republic of China). All CRC cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100 units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA), and Normal colon FHC cells were grown in DMEM/F-12 medium with 10% FBS, 10 ng/mL cholera toxin, 5 µg/mL transferrin, 5 µg/mL insulin, 100 ng/mL hydrocortisone and extra 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cell lines were cultured in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air.

Plasmids, small interfering RNA and transfection

For ectopic expression of CCND3, CCND3 ORFs with 3'-UTR was amplified by PCR and then cloned into pGL3 Vector (Promega) downstream of the Renilla luciferase cDNA. The primers selected were as follows: CCND3-3'UTR-wt-up: 5'-CCCTGGAGAGGCCCT CTGGA-3'; CCND3-3'UTR-wt-dn: 5'-TTCCAAGAAGCCAAAGCCA-3'. miR-592 mimic, miR-592 inhibitor, miR-592-mut and negative control were purchased from GeneCopoeia (Guangzhou, China) and transfected into CRC cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

For CCND3 depletion, small interfering RNA (siRNA) was synthesized and purified by RiboBio Co. (Guangzhou, Guangdong, China). CCND3-siRNA sequences were as followed: siRNA#1: 5'-ACAGAATTGGATACATACACC-3'; siRNA#2: 5'-TAGATGGCTCCTCT CAGTACT-3'. Transfection of siRNAs were performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from cultured cells or patient samples with mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Assays for miR-592 quantification were conducted by using gene-specific TaqMan miRNA Assay Probes (Applied Biosystems, Foster City, CA). After the real-time PCR reaction, the cycle threshold (CT) data were determined using fixed threshold settings; the mean CT was determined from triplicate PCRs. A comparative CT method was used to compare each condition to the controls. The relative miR-592 expression levels after normalization to U6 small nuclear RNA were calculated using $2^{-(\text{Ct of miR-592}) - (\text{Ct of U6})}$.

Western blotting

Cell lysates were prepared in lysis buffer (Beyotime, Jiangsu, China), equal quantities of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by the transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.1% Triton X-100) containing 5% non-fat milk for two hours. The membrane was incubated overnight with using anti-CCND3 (1:500; Abcam), anti-retinoblastoma (anti-Rb, 1:1000, Cell Signaling Technology) and anti-phosphorylated Rb (anti-p-Rb, 1:1000, Cell Signaling Technology) antibodies or anti-α-tubulin antibody (Sigma, St Louis, MI). After incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma, St Louis, MI), membranes were extensively washed with TBST, immunocomplexes were visualized using the chemiluminescence (GE, USA) following the manufacturer's protocol.

MTT assay

Cell growth was examined by MTT assay, SW48 cells (2,000 cells/well) were seeded in 96-well plates in medium containing 10% FBS. Cells were stained after 1, 2, 3 and 4 days with 100 µl sterile MTT dye (0.5 mg/ml, Sigma, St Louis, MI) at 37°C for 4 h, followed by removal of the culture medium and addition of 150 µl DMSO. The absorbance at 490 nm was measured in a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA).

miR-592 inhibited cell proliferation of CRC

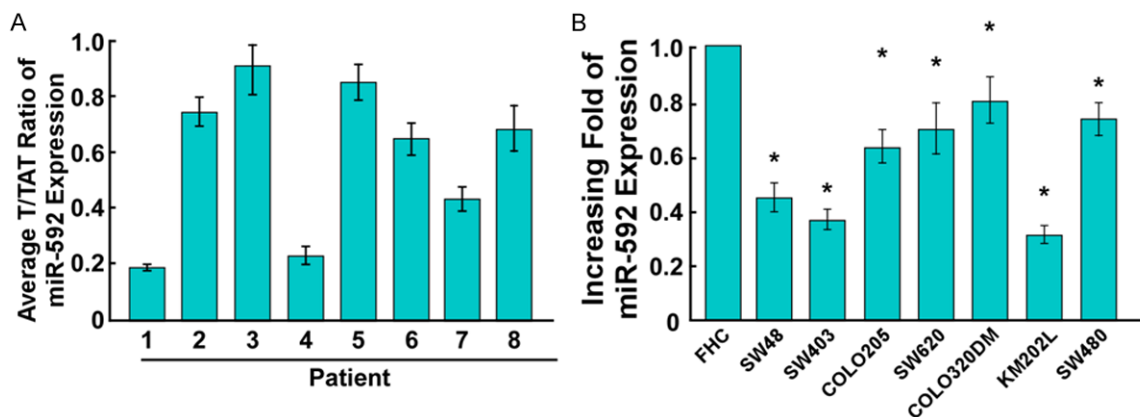


Figure 1. Expression of miR-592 in human colorectal cancer (CRC) tissues and cell lines. (A) Relative miR-592 mRNA expression levels in 8 paired primary CRC tissues (T) and the matched adjacent non-tumor tissues (TAT) from the same patient were detected by PCR analysis. (B) Real-time PCR analysis of miR-592 expression in FHC cells and CRC cell lines, including SW48, SW403, COLO205, SW620, COLO320DM, KM202L and SW480. Experiments were repeated at least three times (A and B). Each bar represents the mean of three independent experiments. * $P < 0.05$.

Colony formation assay

SW48 cells were plated 6-well plates (1000 cells per well) and incubated for 14 days. Colonies were fixed with 10% formaldehyde for 5 min and stained with 1.0% crystal violet for 1 min. The number of colonies, defined as > 50 cells/colony were counted. Three independent experiments were performed and the data was calculated using paired t test.

Anchorage-independent growth assay

One thousand cells were trypsinized and suspended in 2 ml complete medium plus 0.3% agar (Sigma, St Louis, MI). The agar-cell mixture was plated on top of a bottom layer consisting of 1% agar in complete medium. The plates were incubated in a humid atmosphere of 5% CO_2 at 37°C . Cells were incubated for 14 days at 37°C until colony formation and colonies were stained with 0.5% Crystal Violet for counting under microscope and cell colonies were photographed at an original magnification of $100\times$. Only cell colonies containing more than 50 cells were counted.

Luciferase assays

Cells (5×10^4 /well) were seeded in 24-well plates and cultured for 24 hours, cotransfected with miR-592 or miR-NC or miR-592-mut and WT 3'-UTR of CCND3 by using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). The

relative dual-luciferase activity were assayed 48 hours after transfection using the dual luciferase assay kit to the manufacturer's protocol (Promega, Wisconsin, WI, USA).

Statistical analysis

All statistical analyses except for microarray data were performed using the SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Student's t test was used to evaluate the significance of the differences between two groups of data in all the pertinent experiments. A $P < 0.05$ (using a two-tailed paired t-test) was thought to be significantly different for two groups of data.

Result

miR-592 expression was down-regulated in CRC tissues and CRC cell lines

To determine the levels of miR-592 in CRC patients samples and CRC cell lines, total RNAs were extracted from CRC tissues and cell lines, and the miR-592 expression was consistently down-regulated in the CRC tissues compared with the matched tumor adjacent tissues (**Figure 1A**), and in all 8 tested CRC cell lines showed significantly down-regulated expression of miR-592 compared to the normal colonic cell line FHC (**Figure 1B**). Collectively, these results suggested that miR-592 was abnormally down-regulated both in human CRC samples and cell lines.

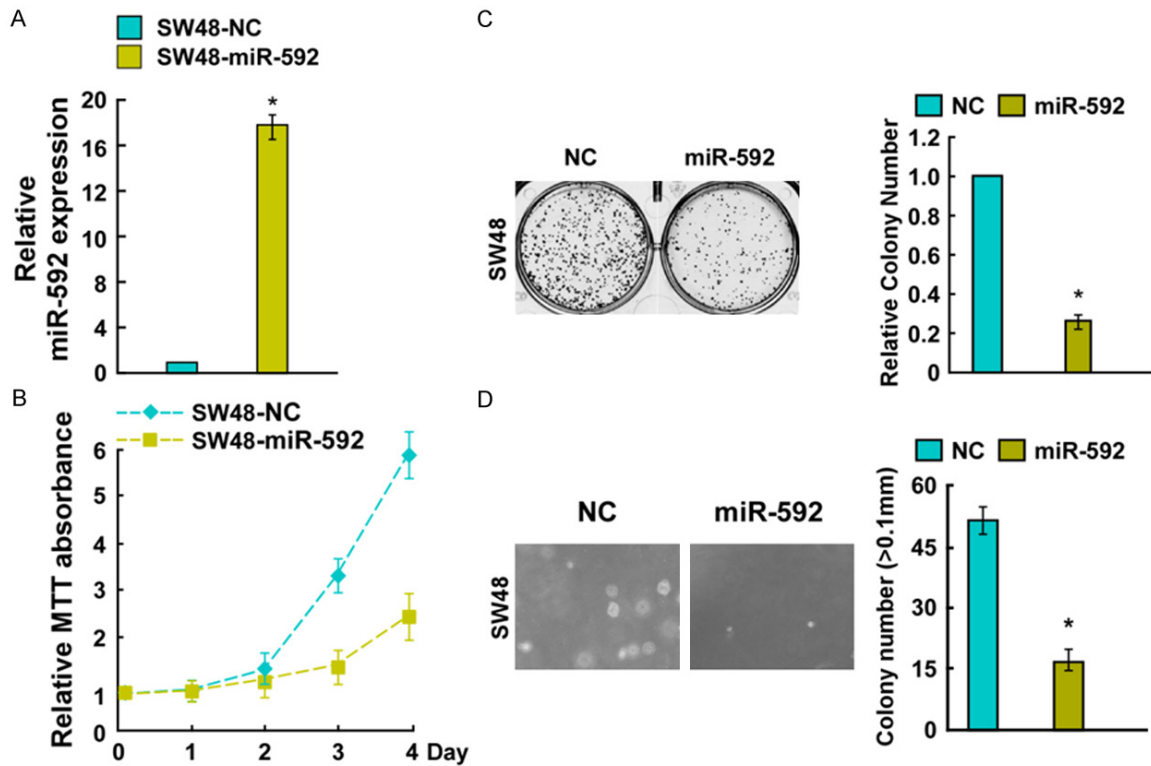


Figure 2. Up-regulation of miR-592 inhibited CRC cell proliferation. A. Validation of miR-592 expression levels after transfection by PCR analysis. B. MTT assays revealed that up-regulation of miR-592 inhibited growth of SW48 cells. C. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D. Up-regulation of miR-592 suppressed the anchorage-independent growth of SW48 cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). Each bar represents the mean of three independent experiments. * $P < 0.05$.

miR-592 inhibited CRC cell proliferation

To assess the role of miR-592 in terms of cell proliferation, we transfected the SW48 cells with miR-592 mimics, miR-592 inhibitor or the respective controls. Relative miR-592 expression was verified using qRT-PCR, transfection of miR-592 restored its expression, while miR-592-in decreased its expression, in SW48 cells (Figures 2A and 3A). In cell proliferation assay, restoration of miR-592 in SW48 cells resulted in significant suppressed of cell proliferation (Figure 2B), and this was further confirmed by a colony formation assay (Figure 2C). Strikingly, we found that enforced expression of miR-592 in SW48 cells drastically decreased their anchorage-independent growth ability (Figure 2D). In contrast, the cell growth rates and colony numbers of SW48 cells transfected with miR-592-in were significantly increased the cell growth rate than those transfected with NC (Figure 3B and 3C). In addition, the anchorage-independent growth ability of SW48 cells was

significantly enhanced in response to miR-592-in (Figure 3D). All these results showed that miR-592 inhibited cell proliferation of CRC *in vitro*.

miR-592 directly targeted CCND3 by binding to its 3'-UTR and altered levels of proteins related to cell proliferation

Increasing evidences indicate that miRNAs function mainly through inhibition of target genes, the target genes of miR-592 that functions in CRC pathogenesis were further analyzed. Potential target of miR-592 was predicted using TargetScan 6.2, we found that CCND3 was a potential target of miR-592 (Figure 4A).

To confirm whether miR-592 could regulate the expression of CCND3, SW48 cells were transfected with miR-592 mimics, miR-592-in or the respective controls, and expression of CCND3 were detected by western blotting. As predicted, the CCND3 protein expression was consistently and substantially down-regulated by miR-

miR-592 inhibited cell proliferation of CRC

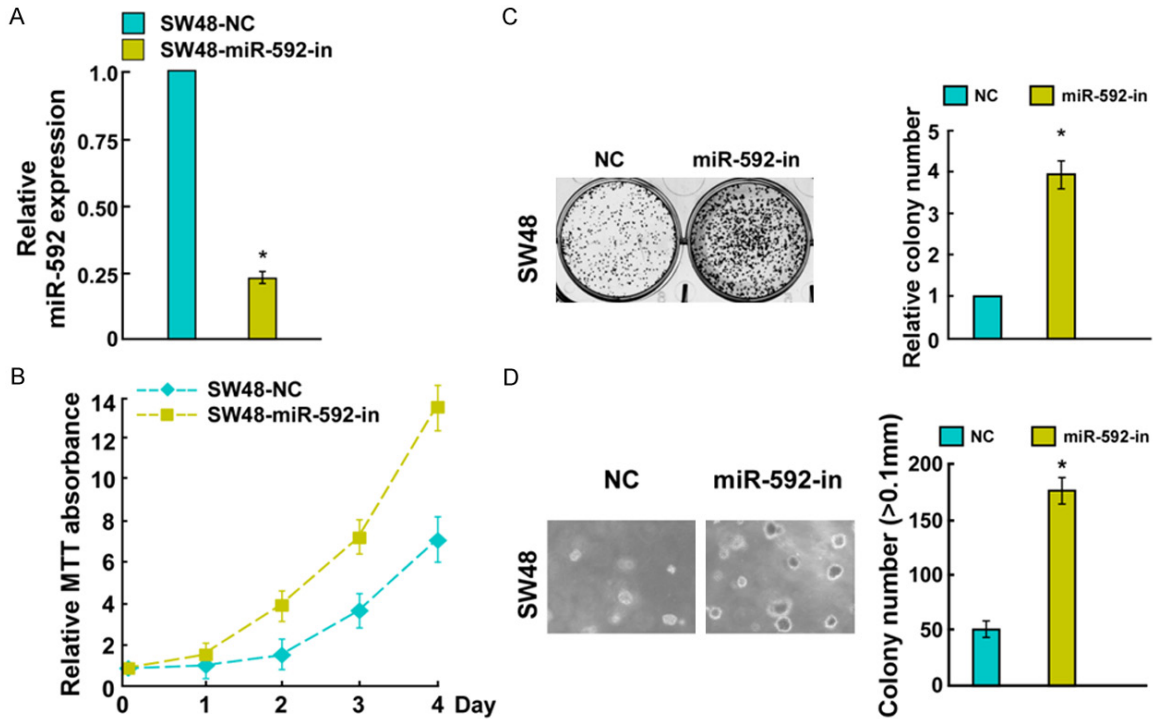


Figure 3. Inhibition of miR-592 promoted CRC cell proliferation. A. Validation of miR-592 expression levels after transfection by PCR analysis. B. MTT assays revealed that inhibition of miR-592 suppressed growth of SW48 cells. C. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D. Inhibition of miR-592 promoted the anchorage-independent growth of SW48 cells. Representative micrographs (left) and quantification of colonies that were > 0.1 mm (right). Each bar represents the mean of three independent experiments. * $P < 0.05$.

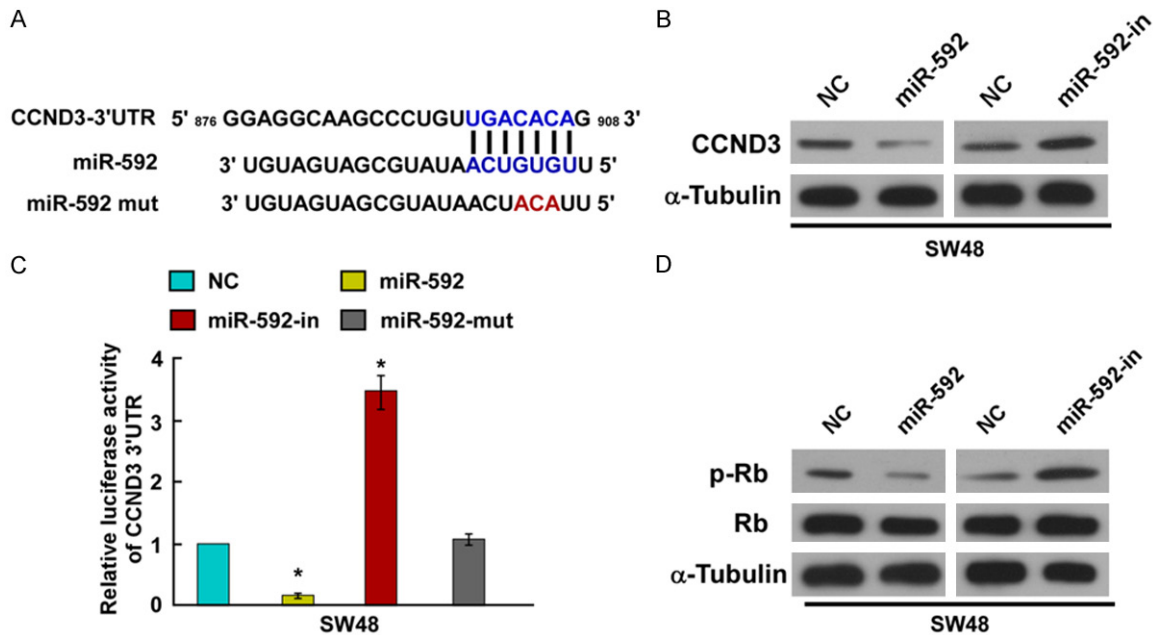


Figure 4. miR-592 suppresses CCND3 expression by directly targeting the CCND3 3'-UTR and altered levels of proteins related to proliferation in SW48 cells. A. Predicted miR-592 target sequence in the 3'-UTR of CCND3 (CCND3-3'-UTR) and positions of three mutated nucleotides (red) in the 3'-UTR of miR-592 (miR-592-mut). B. CCND3 protein expression in SW48 cells transfected with miR-592 or the miR-592 inhibitor were detected by Western blotting analysis. α -Tubulin served as the loading control. C. Luciferase reporter assay of SW48 cells transfected with the

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pGL3-CCND3-3'-UTR reporter and miR-592 or miR-592-in or miR-592-mut. D. Western blotting analysis of protein expression of p-Rb and Rb in SW48 cells. α -Tubulin was used to serve as the loading control. * $P < 0.05$.

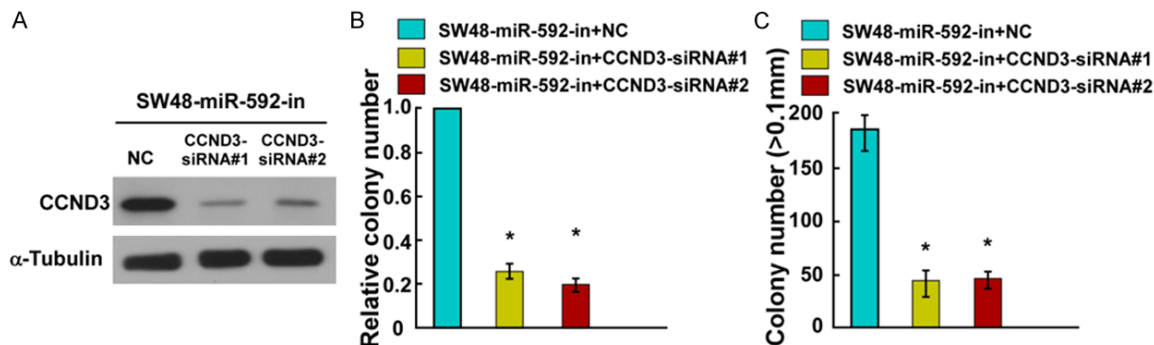


Figure 5. CCND3 up-regulation is required for miR-592-in induced proliferation of CRC cells. A. Western blot analysis verified that silencing CCND3 effectively decreased the expression of CCND3 in miR-592-in-transfected SW48 cells. B. miR-592-in-transfected SW48 cells after transfection with CCND3-siRNAs have an additive effect to inhibit the anchorage-independent growth. C. Representative quantification of colonies that were > 0.1 mm. Each bar represents the mean of three independent experiments. * $P < 0.05$.

592 (Figure 4B). Then, to examine whether miR-592 mediated-CCND3 down-regulation was through the 3'-UTR of CCND3, we created luciferase reporter plasmid with wild type targeting sequence of CCND3 mRNA (Figure 4A), which were cotransfected with miR-592mimic, miR-592-in, miR-592-mut or miR-NC for 48 h, followed by measurement of luciferase activity. As shown in Figure 4C, our results showed that the reporter plasmid with wild type targeting sequence of CCND3 mRNA caused a significant decrease in luciferase activity in cells transfected with miR-592. Meanwhile, miR-592-mut had no effect on the luciferase activity of CCND3 3'-UTR wild type. Taken together, our results demonstrated that CCND3 was a direct target of miR-592 in CRC cells.

Meanwhile, CCND3 exerts its functions by preventing activity of the cyclin D/cdk4/6 complex, which in turn may cause the inhibition of the phosphorylation of Rb and blocks cell cycle progression at G_0/G_1 phase, resulting in inhibiting cell proliferation [17]. We observed that the mRNA of the CCND3 downstream gene (Rb), in the line with above report, our data indicated that the protein expression of p-Rb were down-regulated in SW48 cells transfected with miR-592 mimic, but increased in the cells transfected with miR-592-in, relative to control cells (Figure 4D). Altogether, our results indicated that miR-592 functionally modulates cellular proliferation regulators, Cyclin D3 and Rb, thus relevant to cell proliferation.

CCND3 suppression is required for miR-592-in-induced cell proliferation in CRC

To explore the function of CCND3 in miR-592-in-transfected SW48 cell lines, CCND3-siRNAs were transfected into miR-592-in-transfected SW48 cell lines. As predicted, Western blot analysis verified that silencing CCND3 effectively decreased the expression of CCND3 in miR-592-in-transfected SW48 cells (Figure 5A). Colony formation and anchorage-independent growth assays both showed that silencing CCND3 in miR-592-in-transfected SW48 cells decreased the cell proliferation (Figure 5B and 5C). All the results suggested that further silencing CCND3 expression in SW48-miR-592-in cells could inhibit the promotion of the miR-592-in on cell proliferation of CRC. These data confirmed that miR-592-in promoted the growth of CRC by up-regulating CCND3 expression, and CCND3 suppression is required for miR-592-induced cell proliferation of CRC.

Discussion

In the current study, we focused on miR-592 which was decreased in CRC tissues and eight cell lines. Additional experiments demonstrated that restoration of miR-592 significantly decreased the proliferation of SW48 cell lines, whereas miR-592-in had the opposite effect, probably through post-translationally down-regulating CCND3 expression by targeting its mRNA 3'-UTR. The negative regulation

of CCND3 by miR-592 leads to down-regulation of Rb phosphorylation. We demonstrated that miR-592 might play essential role *via* the CCND3-mediated pathway during CRC development.

Increasing evidences indicate that microRNAs (miRNAs) are a large group of post-transcriptional gene regulators that potentially play critical roles in cell proliferation, cycle, differentiation, angiogenesis, invasion and migration of a variety of cancers. Recently, deregulation of miRNAs in cancer cells and their roles in tumorigenesis have been increasingly investigated. However, it was uncertain whether dysregulation of miR-592 was associated with the progression of CRC. Our study revealed that miR-592 is significantly down-regulated in CRC and inhibits CRC cell proliferation *in vitro* and suggested miR-592 as a candidate tumor suppressor in the pathogenesis of CRC.

CCND3 protein is critical in cell growth, proliferation, and development of cancer (18-20). The levels of CCND3 are highly expressed in most human cancers including CRC [21, 22]. XL Yuan *et al* indicated that reduction of CCND3 exerts its functions by preventing activity of the cyclin D/cdk4/6 complex, which in turn may cause the inhibition of the phosphorylation of Rb and then blocks cell cycle progression at G₀/G₁ phase, resulting in suppressing cell proliferation [17]. Rb is one key regulator of cell cycle progression, and G₁ cyclin/cdk complexes regulate cell cycle progression through the phosphorylation of Rb [23-25]. In the line with these reports, our data show that the protein expression of CCND3 and p-Rb were down-regulated in SW48 cells transfected with miR-592 mimic. Furthermore, CCND3-silenced in SW48-miR-592-in cells had a negative effect on cell proliferation, suggesting that direct CCND3 down-regulation is required for miR-592-induced CRC cell proliferation.

In conclusion, the current study revealed that miR-592 is a tumor-suppressive miRNA in CRC and that CCND3 is a novel and critical miRNA-592 target, and this implies miR-592 to be a potential mediator for novel miRNA replacement therapy.

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Disclosure of conflict of interest

None.

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