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ORIGINAL ARTICLE

A novel role of miR-326 in colorectal carcinoma by regulating E2F1 expression

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Summary

Purpose: *Colorectal carcinoma (CRC) ranks third in incidence but second in mortality worldwide, ascertaining the pathogenesis of CRC is crucial for its treatment. Accumulating studies have shown that E2F1 is a key regulator in CRC progression, which regulates the transcription of genes engaged in DNA replication, mitosis and survival of cancer patients, however, the mechanism of these processes is not fully elucidated.*

Methods: *Here, we determined E2F1 expression in clinical CRC specimens by TCGA database analysis, Microarray immunohistochemical technique and Western blot, respectively. The expression of E2F1 was elevated in CRC tumor tissues, and the patients' total survival time was associated with the level of E2F1. Then the prediction software and meta-analysis were used to predict the miRNAs targeting E2F1. RT-qPCR, TCGA analysis and in situ hybridization experiments were utilized to determine the decreased miR-326 expression in CRC tumor tissues. Luciferase and Western blot assays determined that miR-326 directly targeted E2F1 in CRC cells. Next, CCK8, flow cytometry, Transwell and wound healing assays were used to determine the biological function of miR-326-E2F1 axis in vitro*

Results: *miR-326 overexpression significantly inhibited the viability, invasion and migration and promoted the apoptosis of CRC cells, but overexpression of both E2F1 and miR-326 in turn increased cell viability, invasion and migration and decreased cell apoptosis.*

Conclusions: *This study demonstrates the significant roles of miR-326-E2F1 in CRC progression and may represent a potential target for CRC therapy.*

Key words: *colorectal carcinoma, miRNA-326, E2F1*

Introduction

Colorectal carcinoma (CRC) is one of the most widely diagnosed malignancies worldwide [1,2]. Statistically, there were more than 1.8 million new CRC cases and 881,000 deaths in 2018, comprising about 10% of cancer cases and deaths [1]. Fully clarifying the mechanisms behind the initiation and progression of CRC and exploring more effective therapeutic approaches are urgently needed.

E2F1 is a member of E2F gene family and involved in regulating cell proliferation, cell cycle, differentiation, apoptosis, and so on [3-6]. Given its abundant functions, E2F1 is closely related to cancer development and progression [4]. Previous reports have found that E2F1 acts as a tumor promoter in different tumors such as lung cancer [7], melanoma [8], hepatocellular carcinoma [9], renal cancer [10] and colorectal cancer [11]. E2F1 deregulation is common in cancer and related to poor survival prognosis of patient [4,11,12]. Therefore, E2F1 serves as a potential target for cancer treatment, however, the molecular regulation of E2F1 expression is not fully understood. Exploring the detailed molecular mechanisms of E2F1 regulation is essential to comprehending CRC progression.

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To study the molecular mechanisms related to E2F1-related tumor development, a promising way is to study dysregulated miRNAs (miRs). MiRs are involved in various cellular processes such as tumorigenesis [13]. The differential expression of miRs during CRC development affects CRC tumorigenesis, proliferation, invasion and apoptosis [14-17]. However, there is very little research on the post-transcriptional regulation of E2F1 expression during CRC progression at present.

In this study, we determined the consistent upregulation of E2F1 in human CRC tissues and E2F1 level was significantly related to the poor prognostics of CRC patients. Then we verified the important role of E2F1 in CRC cell proliferation, apoptosis and invasion and determined E2F1 as a direct target of miR-326. *In vitro* assays demonstrated that miR-326 suppressed E2F1 expression, thereby inhibiting proliferation, invasion and migration and accelerating apoptosis of CRC cells *in vitro*.

Methods

Patient samples

14 pairs of CRC patient tissues, including human CRC tissues and normal adjacent tissues (NATs), were collected by the First People's Hospital of Lianyungang. This study was approved by the Medical Ethics Committee of the First People's Hospital of Lianyungang.

Cell lines

293T cells and human CRC cell lines, HCT116 and SW480, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) at 37°C and 5%CO_2 and supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA).

Transfections

CRC cells were seeded in 6-well plates, and when the cell confluence reached 70%, the transfections were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MiR-326 mimics or inhibitors were used to change miR-326 level, E2F1 siRNA (GenePharma, Shanghai, China) or over-expression plasmids (GenePharma, Shanghai, China) were used to alter E2F1 expression level. The cells were collected 24 h or 48 h after transfection and further used for RNA or protein analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for total RNA extraction, then reverse transcription and processing for qRT-PCR using either SYBER Green Dye (Invitrogen, Carlsbad, CA, USA) or TaqMan MicroRNA Assays (ABI) were operated as previously described [18].

Western blotting

Protein of cells and tissues was extracted with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). E2F1 protein levels were analyzed by Western blotting with anti-E2F1 antibodies (ABCAM, Cambridge, MA, USA, ab218527) according to previous protocols [8].

Luciferase reporter assay

To verify the binding of miR-326 with E2F1 3'UTR, a luciferase reporter plasmid insertion with the sequence containing the predicted miR-326 binding sites or mutated binding sites (GenePharma, Shanghai, China) was conducted. Specific experimental steps had been described previously [19,20].

Cell proliferation, apoptosis assay

Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to detect the proliferation rate of SW480 cells; moreover, the Apoptosis Detection Kit I (FITC-Annexin V, BD Biosciences, Franklin Lakes, NJ, USA) was used for measuring CRC cell apoptosis as described previously [8].

Cell invasion, migration assay

For cell invasion assay, 200 ng/mL matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used for membrane coating. After 24-h incubation, cells invading into the other side of the membrane were fixed and stained as previously described. Moreover, wound-healing assay was used to determine the migration ability of CRC cells as previously reported [19].

Statistics

Each experiment was repeated more than three times. All data were shown as mean±standard deviation (SD). Differences between two groups were analyzed by using the Student's t-test. Comparison among multiple groups was done using one-way ANOVA test followed by *post hoc* test (least significant difference). $P<0.05$ was considered as statistically significant.

Results

E2F1 deregulation in human colorectal cancer

To study the clinical significance of E2F1 in CRC, we firstly compared E2F1 expression in CRC tissues and NATs of patients from TCGA database. Analysis of results showed that E2F1 mRNA level was significantly increased in CRC tissues (Figure 1A). Besides, data analysis indicated that CRC malignancy grade rose with increasing E2F1 levels (Figure 1B). Then we detected the expression levels of E2F1 in clinical tissues from a cohort of 80 CRC patients by immunohistochemistry (IHC) staining. The results showed that E2F1 expression was greatly increased in 72/80 (90%) CRC samples (Figures 1C,1D). Notably, CRC patients with high expression

of E2F1 showed markedly shorter overall survival (Figure 1E). Then we collected another 14 pairs of fresh CRC patient samples and measured E2F1 levels by western blotting. The results showed that E2F1 expression was observably increased in CRC tissues compared with the NATs (Figure 1F). Taken together, the above results reveal that the increased E2F1 in CRC is correlated with tumor malignancy and poor prognosis, suggesting that E2F1 may play a vital role in CRC progression.

E2F1 inhibited CRC cell apoptosis and promoted invasion and migration

Then, to explore the role of E2F1 in CRC, we detected the effects of E2F1 on apoptosis, invasion and migration of SW480 cells. QRT-PCR and Western blotting showed the efficient E2F1 overexpression and knockdown (Figures 2A,2B). Flow cytometry showed that overexpressing E2F1 significantly reduced the cell apoptosis, whereas E2F1 knockdown resulted in enhanced cell apoptosis (Figure 2C). Compared with control cells, E2F1 overexpression promoted cell invasion, whereas E2F1 suppression inhibited CRC cell invasion (Figure 2D). Similar results were observed in cell migration assay (Figure 2E). These results further determined that E2F1 suppresses apoptosis and promotes invasion and migration of CRC cells.

Prediction of E2F1 as a target of miR-326

miRNAs serve as key regulatory factors in tumor development by regulating gene expression on post-transcriptional level [21]. In order to study miRNAs targeting E2F1 in colorectal cancer, Tar-

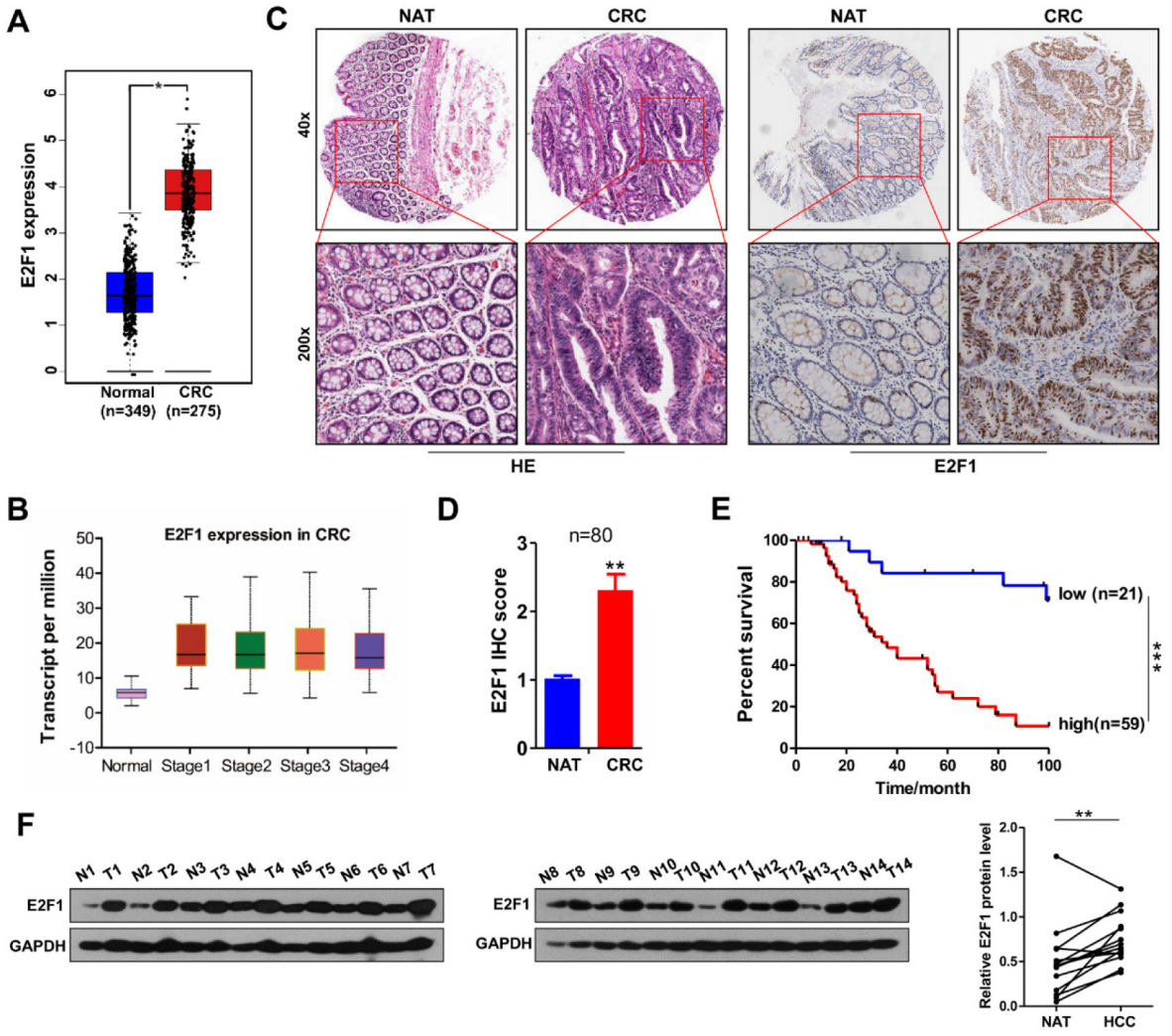

Figure 1. E2F1 deregulation in CRC. **A:** E2F1 expression in 349 normal tissues and 275 CRC tissues from TCGA database. **B:** E2F1 expression was relevant to the clinical stage of CRC in TCGA dataset. **C:** Representative images of HE staining and E2F1 IHC staining in CRC tissues and matched normal adjacent tissue (NAT) (n=80). **D:** E2F1 IHC score in CRC tissues and matched NATs (n=80). **E:** Kaplan-Meier analysis of CRC patients' survival stratified by E2F1 level. **F,G:** Western blot measured E2F1 protein levels in another 14 paired CRC and NAT samples. *p<0.05; **p<0.01; ***p<0.001.

getscan, miRWalk, OncomiR software were used [22-24]. At the same time, YM500 database was used to analyze the miRNAs markedly deregulated in CRC samples [25]. By sorting the overlaps between Targetscan, miRWalk, OncomiR and YM500 results, 23 miRs common to four sets were chosen for further verification (Figure 3A). In the 23 analyzed miRs, 16 miRs were found to be markedly decreased and 7 miRs were found to be increased in CRC compared to those in normal tissues. As the negative correlation between miRs and their target genes [26] and the increased expression of E2F1 in CRC tissues, we then detected the expressions of the most downregulated 8 miRs in 14 pairs of CRC tissues and NATs. QRT-PCR analysis found that miR-326 was the most greatly downregulated miR among the 8 miRNAs (88.1% lower in CRC tissues compared to NATs) (Figures 3B,3C). These findings were further analyzed in TCGA datasets, and it was found that miR-326 markedly decreased in CRC compared to normal tissues (Figure 3D). Likewise, *in situ* hybridization assays also validated the downregulation of miR-326 in CRC (Figure 3E). Moreover, we analyzed the negative correlation between miR-326 and E2F1 protein levels in CRC tissues using Pearson's correlation scatter plots (Fig-

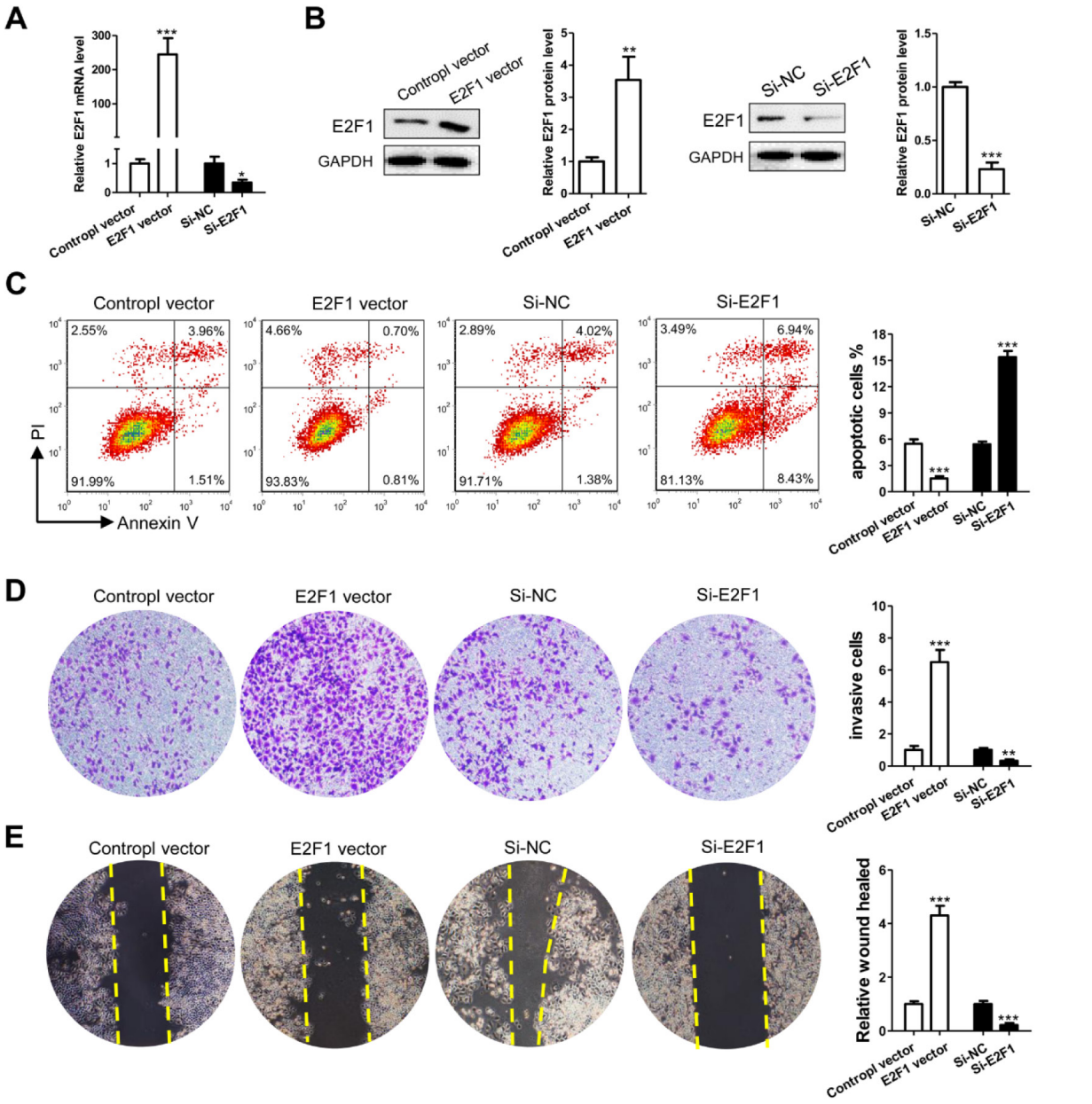

Figure 2. E2F1 serves as an oncogene in CRC. The SW480 CRC cells were transfected with control vector, E2F1 overexpression vector, control siRNA or E2F1 siRNA, respectively. **A:** QRT-PCR detected E2F1 mRNA levels. **B:** Western blotting measured E2F1 protein levels. **C:** Flow cytometry measured the levels of cell apoptosis. **D:** Transwell assay detected the cell invasive levels. **E:** Wound healing experiment analyzed the levels of cell migration. *p<0.05; **p<0.01; ***p<0.001.

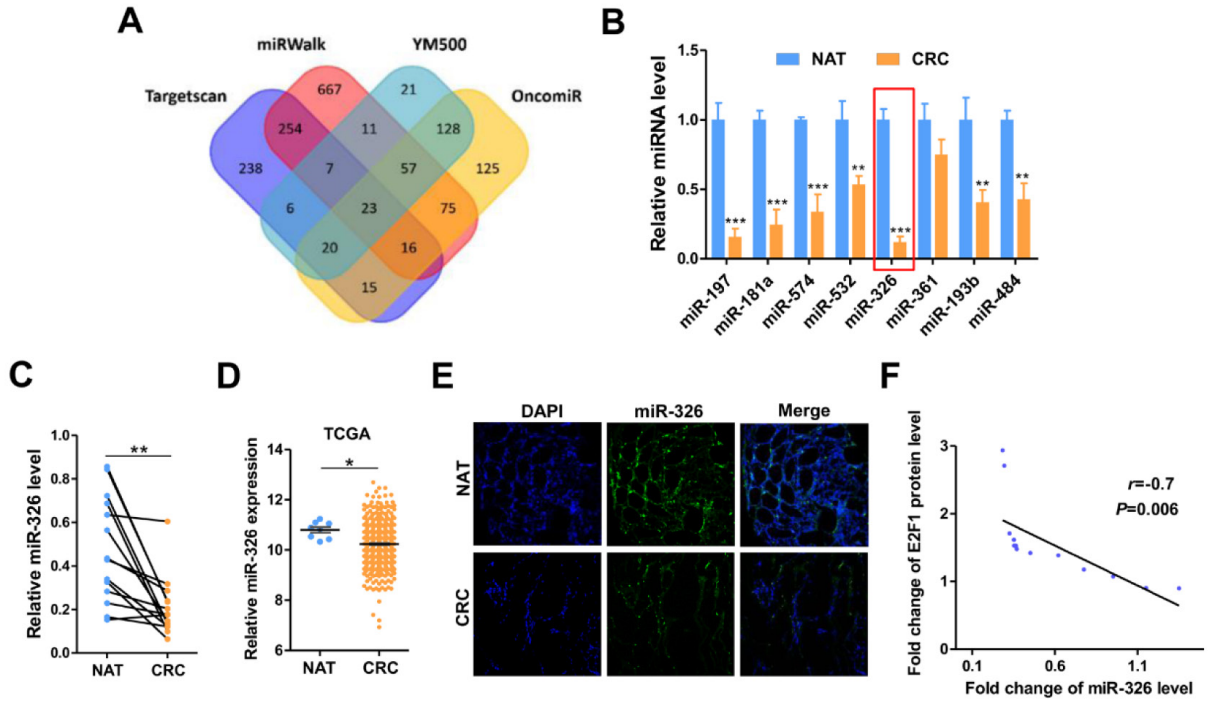

Figure 3. Prediction of E2F1 as a target of miR-326. **A:** A Venn diagram was used to search for potential miRNAs which potentially targeted E2F1 in CRC by Targetscan, miRWalk, OncomiR and YM500. **B:** QRT-PCR analyzed the expression levels of candidate miRNAs in 14 pairs of CRC tissues and NATs. **C:** qRT-PCR measured miR-326 levels in CRC tissues and NATs, n=14 in each group. **D:** Scatter plots showing relative levels of miR-326 in non-tumor (NAT), and CRC cancer tissues from TCGA patients. **E:** Representative images of miR-326 localization in CRC and NAT detected by *in situ* hybridization. **F:** Pearson's correlation scatter plot of miR-326 and E2F1 fold changes in 14 pairs of CRC tissues. *p<0.05; **p<0.01; ***p<0.001.

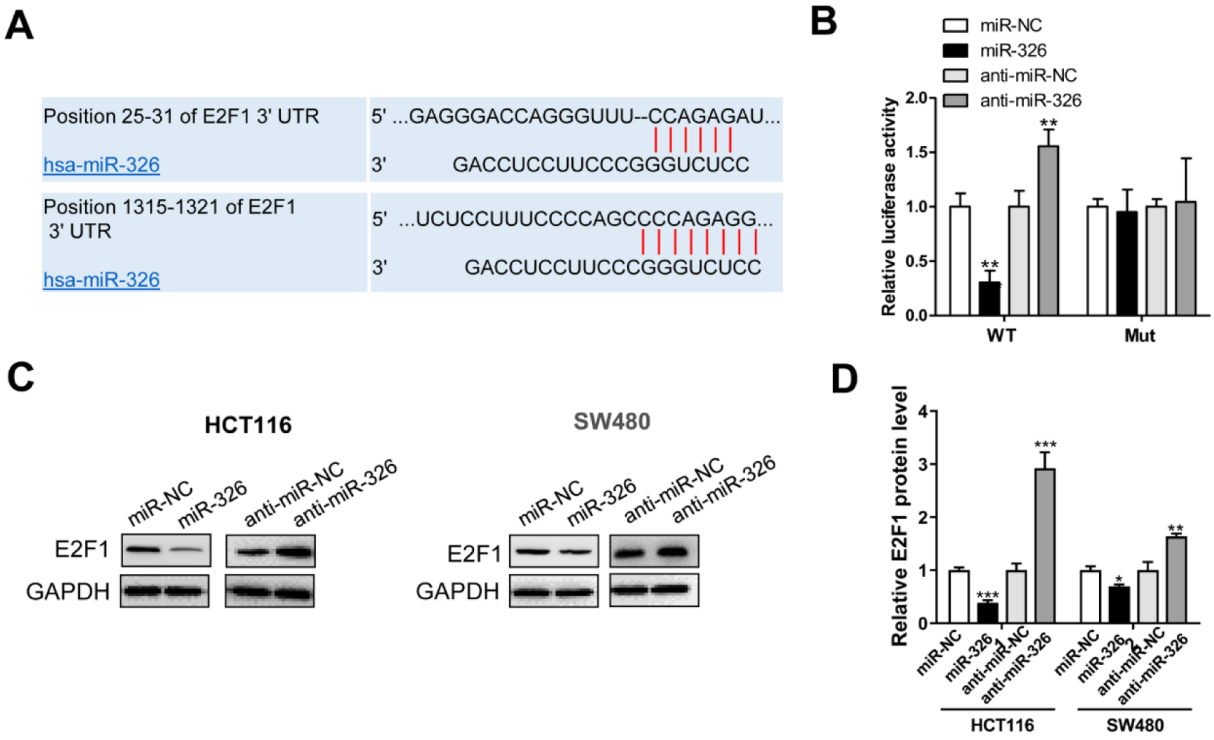

Figure 4. miR-326 directly targets E2F1. **A:** Predicted interaction between miR-326 and E2F1 3'-UTR. **B:** Relative luciferase activities in 293T cells transfected with miR-326 mimics or inhibitors. **C,D:** Western blotting detected the E2F1 protein levels in HCT116 and SW480 cells transfected with miR-326 mimics or inhibitors. **p<0.01; ***p<0.001.

ure 3F). Taken together, we speculated that E2F1 may serve as a target of miR-326 in CRC.

MiR-326 directly targeted E2F1

Next, to further explore whether miR-326 could bind with E2F1 3'-UTR (Figure 4A), a firefly reporter plasmid containing miR-326 binding sites in E2F1 3'-UTR was constructed. Then, the constructed plasmids were transfected into 293T cells along with miR-326 mimics, miR-326 inhibitors or negative controls, respectively. The results revealed that miR-326 overexpression suppressed the luciferase reporter activity, whereas miR-326 knockdown led to an increase in reporter activity (Figure 4B). Next, point mutations were introduced into sites complementary to miR-326 within E2F1 3'-UTR (Figure 4A), and luciferase assays showed that miR-326 overexpression or inhibition had no effects on luciferase activity when the binding sites were mutated (Figure 4B). Then, we transfected miR-326 mimics or inhibitors into HCT116 and SW480 cells and determined the expression of E2F1. As anticipated, overexpressing miR-326 markedly suppressed E2F1 protein levels, whereas miR-326 knockdown upregulated E2F1 levels (Fig-

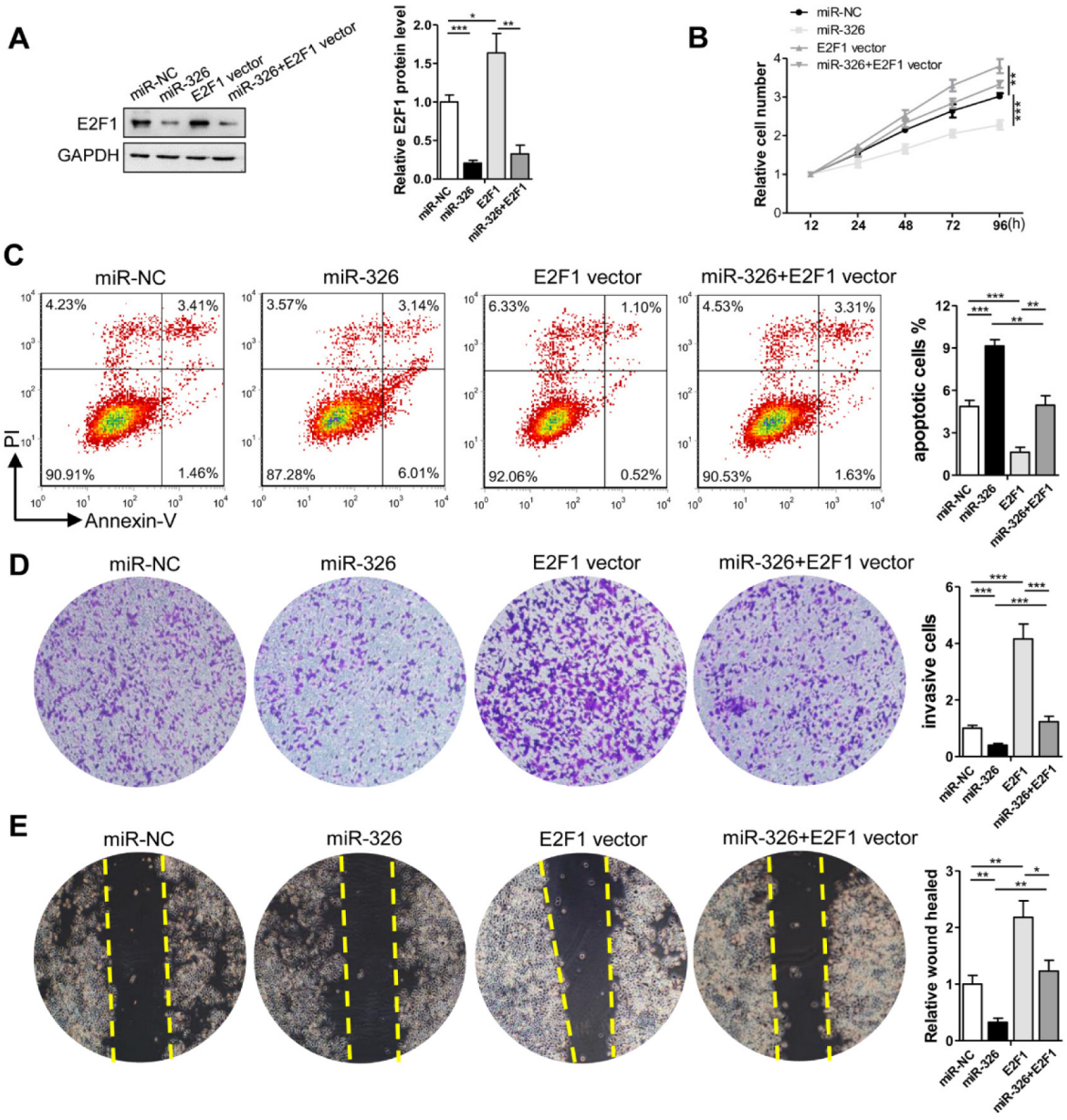

Figure 5. miR-326 inhibits CRC cell proliferation, invasion, migration and promotes apoptosis by inhibiting E2F1. **A:** Western blotting analysis of E2F1 levels in SW480 transfected with E2F1 vector or miR-326 mimics plus E2F1 vector. **B:** CCK-8 assay measured the proliferation level of CRC cells. **C:** Flow cytometry measured cell apoptosis levels. D: Transwell assay analyzed cell invasion levels. **E:** Wound healing assay detected cell migration rate. *p<0.05; **p<0.01; ***p<0.001.

ures 4C,4D). Altogether, the above findings determined that miR-326 specifically targets E2F1.

MiR-326 suppressed proliferation, invasion, migration and promoted apoptosis of CRC cells by inhibiting E2F1

The above results have proved that E2F1 plays vital roles in CRC cell apoptosis, invasion and migration. If miR-326 is a key regulator of E2F1, it should be involved in cell apoptosis, invasion, and so on. Then, we studied the biological functions of decreased E2F1 induced by miR-326 in SW480 cells (Figure 5A). CCK-8 assay showed that miR-326 upregulation suppressed SW480 cell growth, while co-expression of miR-326 with E2F1 vectors rescued the suppressive effects of miR-326 on cell proliferation (Figure 5B). Then, flow cytometry assay was used to measure the effects of miR-326-E2F1 axis on CRC cell apoptosis. The results showed that overexpressing miR-326 could promote apoptosis in SW480 cells, whereas cells overexpressing miR-326 as well as E2F1 vectors showed a lower apoptosis rate than cells transfected with miR-326 mimics, proving that miR-326–resistant E2F1 could weaken the acceleration of miR-326 on apoptosis (Figure 5C). Transwell assay showed that miR-326 upregulation led to less invasive cells than control cells, cells simultaneously transfected with miR-326 and E2F1 vectors showed a higher invasion rate than those overexpressing miR-326 alone (Figure 5D). Similar phenomena were found in cell migration assays (Figure 5E). From the above results, it is concluded that miR-326 suppresses proliferation, invasion, migration and promotes apoptosis of CRC cells by directly targeting E2F1.

Discussion

Global cancer statistics showed that there were more than 1.8 million new CRC cases and 881,000 deaths in 2018, accounting for 1/10 of the cancer cases and deaths [1]. On the whole, CRC ranks third in incidence but second in mortality. Therefore, it is urgent to further understand the molecular mechanism of CRC tumorigenesis.

E2F1 is the first-described member of the E2F family. Lots of studies have determined that E2F1 plays a vital role in regulating the development of different cancers and it is involved in various biological processes [3,5,6,9,12]. E2F1 has been reported to serve as a key regulator in CRC migration and invasion [27,28]. However, the molecular regulatory mechanism of E2F1 on CRC progression is not entirely clear. Here, we firstly identified E2F1 expression pattern and clinical significance by TCGA database, then immunohistochemical microarray and western blotting assays were used to verify the results and we found the increased expression of E2F1 was correlated with poor prognosis of CRC patients.

Recently, more and more studies showed that miRs play vital roles in cancer progression and the aberrant miR expression is correlated with tumorigenesis and disease development [13,29]. Thus miR expression could serve as an invaluable tool to diagnose tumors and a potential therapeutic target for cancer [30]. MiRs that regulate E2F1 can not only be used in treatment but also explain why E2F1 dysregulates. Here, we determine that miR-326 is a potential regulator of E2F1 and it can regulate the proliferation, apoptosis, invasion and migration of CRC cells via E2F1.

Conclusions

Altogether, our study has determined the vital role of E2F1 in CRC. More specifically, E2F1 upregulation can facilitate the proliferation, invasion and migration and inhibit apoptosis of CRC cells. Moreover, miR-326 can directly inhibit E2F1 and reverse cancer cell progression. Our results indicated that miR-326-E2F1 axis may represent a promising therapeutic target for CRC.

Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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