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Serum miR-129 functions as a biomarker for colorectal cancer by targeting estrogen receptor (ER) β

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Received June 19, 2016, accepted August 12, 2016

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Pharmazie 72: 107–112 (2017) *doi: 10.1691/ph.2017.6718*

Aberrantly expressed miRNAs widely participate in the signaling cascades of colorectal carcinogenesis. The present study aimed to identify a potential miRNA that serves as effective biomarker for colorectal cancer (CRC). The expression of estrogen receptor β (ERβ) was explored using immunohistochemistry. The possible miRNAs targeting ERβ were predicted by TargetScan, and their expression patterns were validated using real time PCR. Dual luciferase reporter assays were performed to determine the potential binding of miR-129 in the 3' untranslated region (3'UTR) of ERβ. In vitro scratch assays and flow cytometry assays were conducted to determine the role of miR-129 on colon cancer cell migration and apoptosis. Proteins related to cell proliferation were determined using western blots. Compared with adjacent non-cancer tissues, the protein level of ERβ was significantly decreased in CRC tissues, and compared with NC the level of miR-129 was significantly increased in blood and tissue samples. Dual luciferase reporter assays demonstrated that ERβ was a direct target gene of miR-129. Further study showed that inhibition of miR-129 decreases HCT116 cell migration and enhances cell apoptosis. More importantly, we found that the silencing of ERβ significantly decreased the activation of caspase3 but increased the protein expression of PCNA. Interestingly, miR-129 inhibitor-induced protein expression pattern changes could be reversed by the siRNA targeting ERβ. The high expression level of circulating miR-129 in the tissue and blood samples of CRC patients contributes to aberrant colon cancer cell proliferation and migration mainly by targeting ERβ.

1. Introduction

Among both men and women, colorectal cancer (CRC) is one of the most common malignant tumors worldwide (O'Connell et al. 2004). Although the treatment methods have been significantly improved, CRC is still a leading cause of cancer-related death in males and females (Siegel et al. 2012). It is estimated that almost 10% of all reported cancer cases are attributable to CRC-related mortality worldwide (Torre et al. 2015). Compared with men and younger women (18–44 years old), older women (>50 years old) have a higher incidence of colon cancer (Hansen et al. 2013). Studies have indicated that the hormone estrogen plays a key role in the progression of CRC (Breynaert et al. 2008; Beaugerie et al. 2013). Moreover, estrogen treatment also suppresses colonocyte growth in non-malignant mouse cell lines and decreases abnormal crypt foci in mice (Weige et al. 2009).

Estrogen plays a key role in maintaining women's health, mainly through two nuclear receptors, estrogen receptors α (ERα) and β (ERβ) (Honma et al. 2013). Estrogen can bind the two receptors, which function as transcription factors by post-transcriptionally modulating the expression of target genes (Wu et al. 2012; Passarelli et al. 2013). ERα is found to be upregulated in breast cancer, thereby enhancing cell proliferation (Giroux et al. 2008). However, the expression of ERα is relatively low in colon cancer, while ERβ is predominantly expressed in normal colonic epithelium (Saleiro et al. 2012). During the progression of CRC, the expression of ERβ is found to be significantly suppressed (Hartman et al. 2009). Polymorphism in the ERβ gene exerts a deep influence on colon cancer risk and survival (Tu et al. 2012). Multiple studies have suggested a protective role of ERβ in colon cancer (Hartman et al. 2009; Bielecki et al. 2011). Thus, it is important to explore the underlying molecular mechanism of ERβ activity in colon cancer.

MicroRNAs (miRs) are small non-coding RNAs with approximately 22 nucleotides (Arndt et al. 2009). Compared to normal colonic epithelium, aberrant expression of miRNAs has been widely identified in CRC tissues (Arndt et al. 2009). At present, accumulating evidence has suggested that abnormal expression of many miRNAs has been found in the blood and/or tissues of CRC cases, including miR-20, miR-21, miR-17-5p, miR-15b, miR-181b, miR-191 and miR-200c (Wang et al. 2010, Peacock et al. 2012). The wide distribution of miRNAs in serum and plasma render them ideal candidates as non-invasive biomarkers for the early detection of disease progression (Jia et al. 2014).

The aim of the current study is to investigate the relationships of ERβ and miRNA in patient tissue/serum samples. We find that the level of miR-129 was significantly upregulated in the tissue/serum samples of CRC patients. More importantly, through binding the 3' untranslated region of ER-β, miR-129 acts as an oncogene in the progression of CRC.

2. Investigations, results and discussion

2.1. Upregulation of miR-129 in the tissues and blood samples of CRC patients

First, we explored the expression of ERβ in the CRC tissues. Compared with adjacent non-cancer tissues, the protein level of ERβ was significantly decreased in CRC tissues (Fig. 1A). Then, we extended this study to further identify the potential miRNAs that could bind the 3'UTR of ERβ. According to TargetScan (release 7.0), ten miRNAs were predicted to target the 3'UTR of ERβ. Then, we explored their expression pattern in the CRC tissues. Compared to the adjacent non-cancer tissues, as shown in Fig. 1B, the levels of miR-129, miR-16 and miR-6383 in the CRC tissues were significantly increased. Last, we determined their expression changes in the serum of CRC patients. Strikingly, compared with NC (Fig. 1C), the level of miR-129 was the most significantly increased miRNA

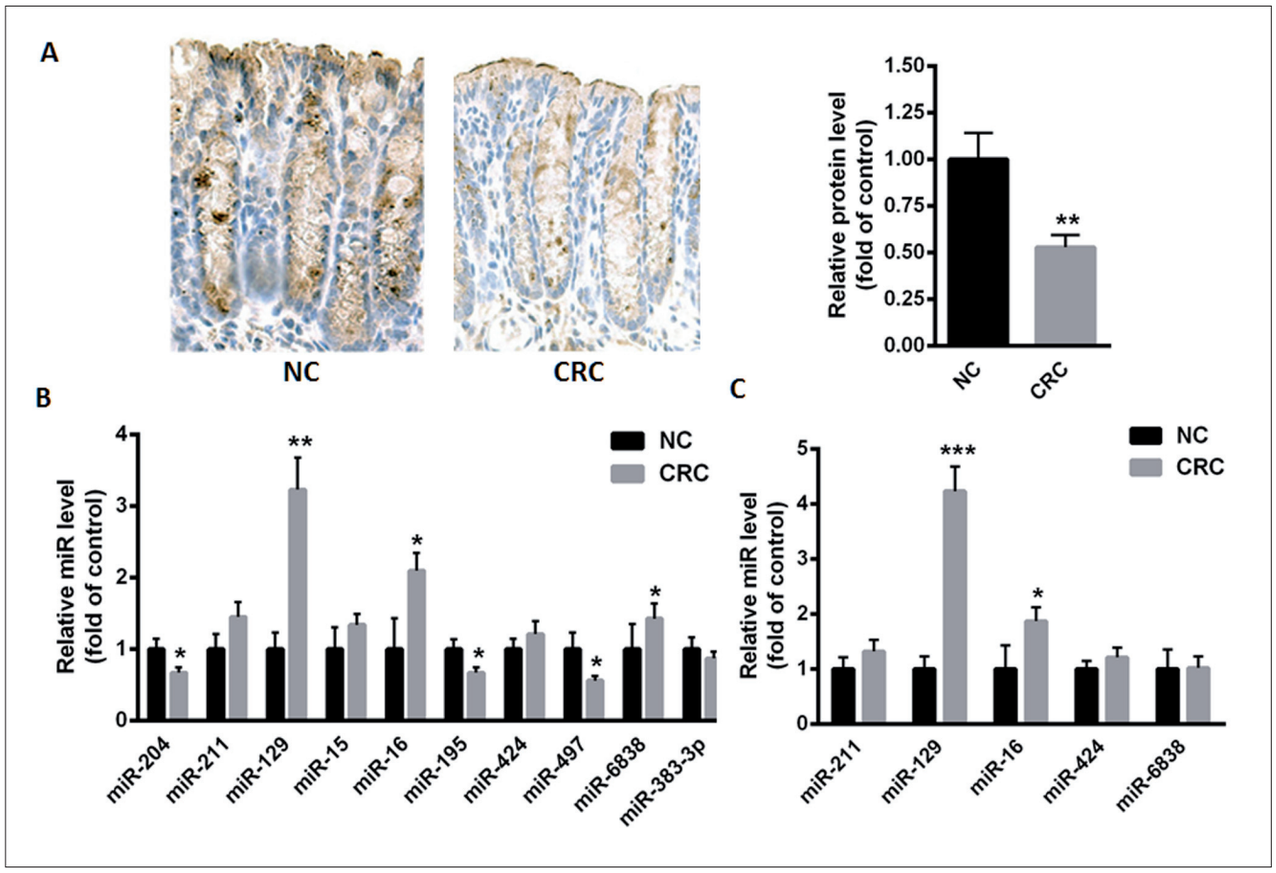

Fig. 1: Upregulation of miR-129 in the tissues and blood samples of CRC patients. (A) Compared with adjacent non-cancer tissues, the protein level of ERβ was significantly decreased in CRC tissues. (B) Compared to adjacent non-cancer tissues, the levels of miR-129, miR-16 and miR-6383 in CRC were significantly increased. (C) Compared with NC, the level of miR-129 was most significantly increased in CRC patients' blood samples. Data are presented as the mean ± SE. n=10 for each group, *P<0.05, **P<0.01.

in the CRC patients' blood samples. Thus, the present study mainly focused on the possible role of miR-129 in the progression of CRC.

2.2. *ERβ is a direct target gene of miR-129*

Then, the possible binding site of miR-129 in the 3'UTR of ERβ was cloned into pmirGLO plasmid (Fig. 2A). Dual luciferase reporter assay demonstrated that miR-129 significantly suppressed the luciferase activity of pmirGLO-ERβ-3'UTR (Fig. 2B). Furthermore, a point mutation was introduced in the possible binding sites of miR-129 on the 3'UTR of ERβ, and no changes of luciferase activity were observed for pmirGLO-ERβ-3'UTR-Mut (Fig. 2B). Meanwhile, a western blot assay showed that overexpression of miR-129 markedly suppressed the protein expression of ERβ, while inhibition of miR-129 significantly enhanced the protein level of miR-129 in HCT116 cells (Fig. 2C and 2D). These data indicated that ERβ was a target gene of miR-129 in HCT116 cells.

2.3. *Inhibition of miR-129 decreases HCT116 cell migration and enhances cell apoptosis*

We further explored the potential role of miR-129 on HCT116 cell migration and apoptosis. An *in vitro* scratch assay showed that transfection of miR-129 markedly enhanced the migration capacity of HCT116 cells, while miR-129 inhibitor decreased HCT116 cell migration after 48 h (Fig. 3A). To further explore whether miR-129 exerts its role through ERβ, a specific siRNA targeting ERβ was selected (Fig. 3B). As shown in Fig. 3C, inhibition of miR-129 significantly enhanced the apoptotic cells (Fig. 3C). In contrast, miR-129 inhibitor-induced cell apoptosis could be markedly abolished with knockdown of ERβ (Fig. 3C). These data indicated that inhibition of miR-129 decreases HCT116 cell migration and enhances cell apoptosis mainly by targeting ERβ.

2.4. *The oncogenic role of miR-129 in colon cancer cells by targeting ERβ*

Furthermore, PCNA and c-caspase3, proteins related to cancer cell apoptosis, were studied. As shown in Fig. 4A, transfection of miR-129 inhibitor suppressed the expression of PCNA, but increased the activation of caspase3, indicating a protective effect of miR-129 inhibition in HCT116 cells. More importantly, we found that the silencing of ERβ significantly decreased the activation of caspase3 but increased the protein expression of PCNA, thereby contributing to the abnormal proliferation of HCT116 colon cancer cells (Fig. 4B). Interestingly, the miR-129 inhibitor-induced protein expression pattern changes could be reversed by the siRNA targeting ERβ (Fig. 4B). These findings showed the oncogenic role of miR-129 in colon cancer cells by targeting ERβ. Increasing studies have suggested that sex hormones play a key role in the progression of colorectal cancer (Lin et al. 2013). It was reported that those who had received oral contraceptives demonstrated a decreased colorectal cancer risk of approximately 19% (OR, 0.81; 95% CI, 0.72–0.92) (Bosetti et al. 2009). Reproductive factors, including age at menarche, parity, and age at menopause, are widely reported to correlate with CRC risks (Lin and Giovannucci 2010). ERβ is accepted as the major form of estrogen receptor in the large intestine. In CRC patients, loss of ERβ expression is closely related to the poor differentiation of tumors and more advanced cancer stages (Clendenen et al. 2009). Thus, it is of great importance to explore the underlying mechanism by which the expression of ERβ is suppressed in CRC patients.

The present study explored the expression of ERβ in the tissues of CRC patients. Compared to non-cancer adjacent tissue, the protein level of ERβ was markedly decreased in the cancerous tissues of CRC patients. Because aberrantly expressed miRNAs widely participate in the signaling cascades of colorectal carcinogenesis, we

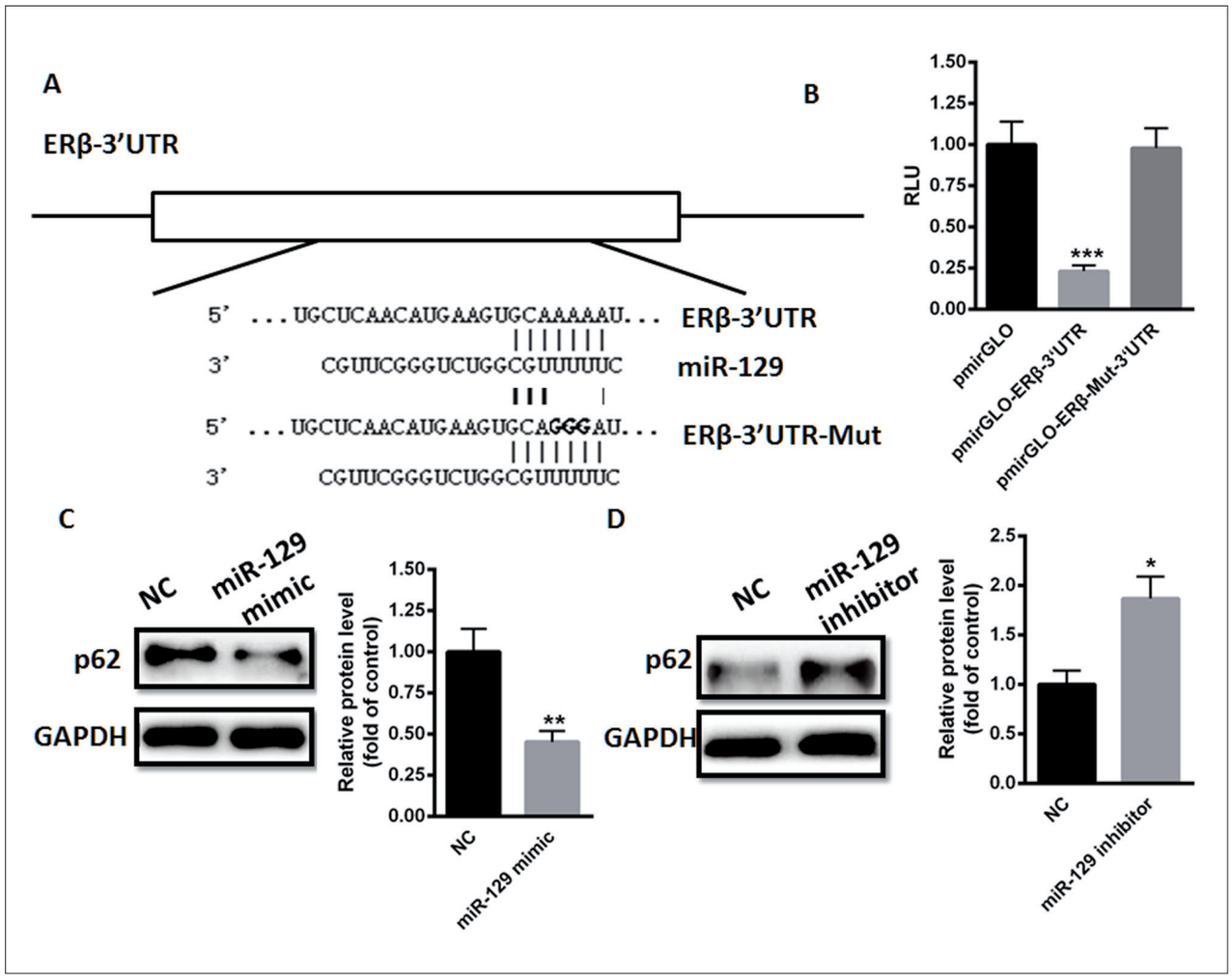

Fig. 2: ERβ is a direct target gene of miR-129. (A) The binding site of miR-129 on the 3'UTR of ERβ was predicted by TargetScan (release 7.0). (B) A dual luciferase reporter assay demonstrated that miR-129 significantly suppressed the luciferase activity of pmirGLO-ERβ-3'UTR. (C) Overexpression of miR-129 markedly suppressed the protein expression of ERβin HCT116 cells. (D) Inhibition of miR-129 significantly enhanced the protein level of miR-129 in HCT116 cells. Data are presented as the mean ± SE. n=6 for each group, *P<0.05, **P<0.01.

further explored the expression of possible miRNAs targeting ERβ. Here, we identified miR-129, which was significantly increased in both the tissue and blood samples of CRC patients. Unfortunately, no study has been conducted on the possible role of miR-129 in CRC progression, although its aberrant expression has been widely described in different tumors. Our results first demonstrated that ERβ was a target gene of miR-129, suggesting a potential oncogenic role of miR-129 in colon cancer cell migration and proliferation.

Furthermore, many studies have tried to elucidate the diagnostic role of circulating miRNAs in the outcome or response to chemotherapy among CRC patients (Bauer and Hummon 2012; Ma et al. 2012). For instance, high levels of circulating miR-17-3p and miR-106a were found to be correlated with poor survival, independent of other clinical factors, suggesting the possibility of these miRNAs as noninvasive prognostic biomarkers for CRC (Li et al. 2015). These serum markers were suggested to effectively monitor the cancer progression of CRC patients (Mitchell et al. 2008). More importantly, it may be possible that antagomirs that target these two miRNAs could be used as therapeutic agents in patients with tumors with high expression levels. In this study, we found that upregulation of miR-129 significantly suppressed the expression of ERβ, thereby enhancing colon cancer cell proliferation and migration, thus indicating their tumor invasive role in CRC patients.

In conclusion, we found that a circulating miRNA, miR-129, was upregulated in the tissue and blood samples of CRC patients. The high expression level of circulating miR-129 contributes to aberrant colon cancer cell proliferation and migration mainly by targeting ERβ.

3. Experimental

3.1. Subjects

A total of 18 female patients (age range 49 to 67 years, with a mean age of 56 years) diagnosed with CRC by colonoscopy at Tengzhou Central People's Hospital were enrolled in this study. These patients were randomly chosen from the patient pool of the hospital's gastrointestinal clinic; none of them received estrogen replacement therapy. Blood was taken half an hour after colonoscopy. During colonoscopy, four or five samples from cancer tissues and adjacent non-cancer tissues, were obtained and stored in liquid nitrogen. All tissue samples were reviewed and evaluated by two different gastrointestinal pathological experts. The study was approved by the ethics committee of the Shandong Provincial Cancer Hospital and informed consent was obtained from all study subjects.

3.2. Cell culture

Cell lines (HCT116) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 IU/ml) at 37 °C in a humidified atmosphere containing 5% CO_2.

3.3. Immunohistochemistry

Paraffin-embedded samples were deparaffinized in the xylene substitute, Pro-Par Clearant (Anatech) and rehydrated in graded ethanol and water. After washing with phosphate buffered saline (PBS), sections were blocked with 5% serum for 30 min at room temperature and then incubated with rabbit anti-ERβ polyclonal antibody

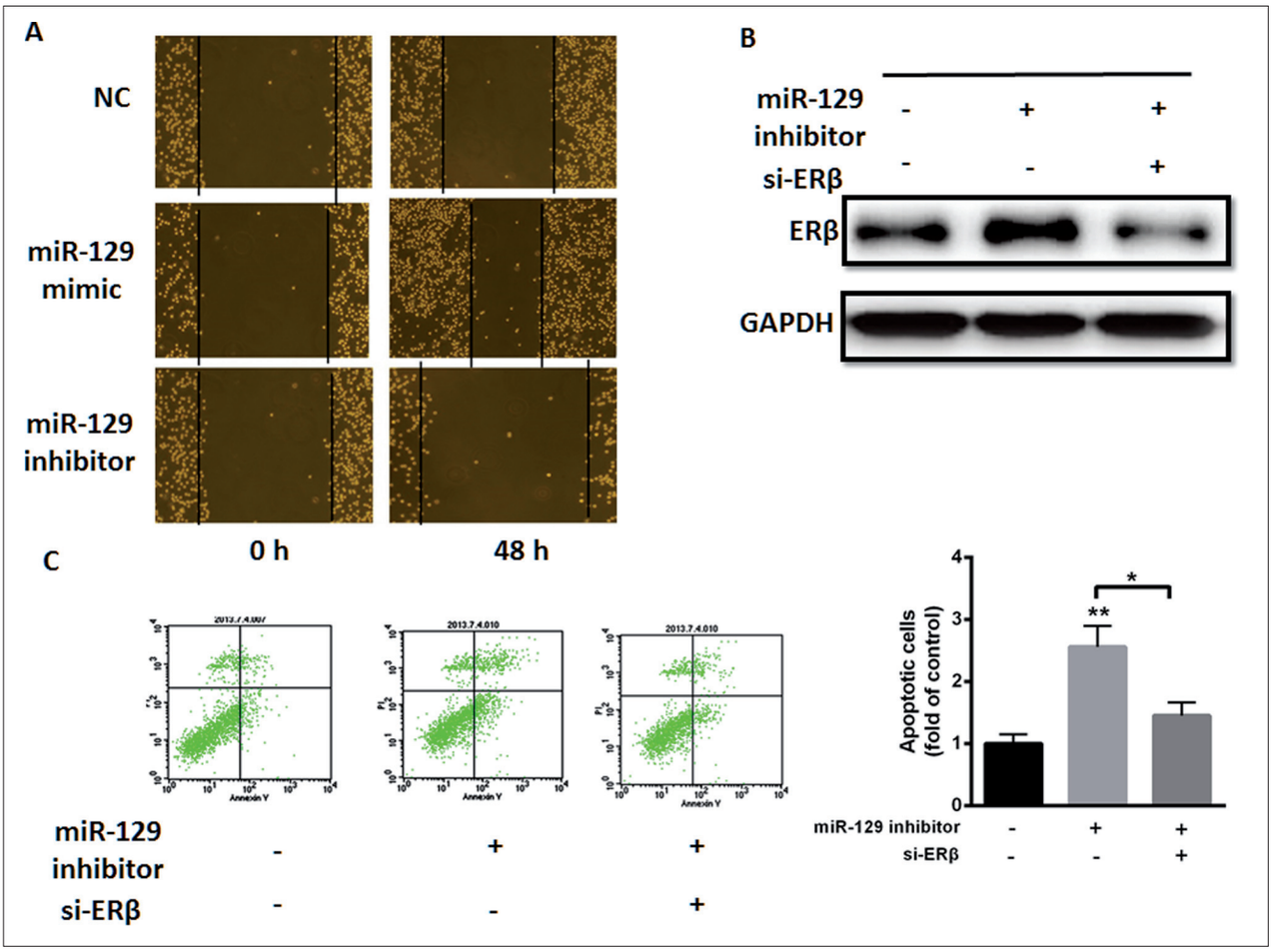

Fig. 3: Inhibition of miR-129 decreases HCT116 cell migration and enhances cell apoptosis mainly by targeting ERβ. (A) An in vitro scratch assay showed that miR-129 inhibitor decreased HCT116 cell migration after 48 h. (B) A western blot assay showed that ERβ was effectively silenced by a specific siRNA. (C) A flow cytometry assay demonstrated that miR-129 inhibitor-induced cell apoptosis could be markedly abolished with knockdown of ERβ. Data are presented as the mean ± SE. n=6 for each group, *P<0.05, **P<0.01.

(1:50) (Abgent, San Diego, CA) overnight at 4 °C. The slides were sealed at room temperature for approximately 1 h in the dark. Then, the fluctuation of fluorescence intensity was examined using a fluorescence microscope. The tissues were incubated for 10 min in 3% bovine serum albumin to block unspecific binding and treated over night with a 1:50 dilution of in-house anti-ERβ antibody in a humid chamber. After washing with PBS, sections were incubated with secondary antibody (biotin-labeled goat anti-mouse IgG, 1:200) for 1 h at 4 °C, washed with PBS and incubated with horseradish peroxidase conjugated streptavidin (1:200) for 1 h at RT, and then with diaminobenzidine (DAB)/H_2O_2 for 15 min at RT. After dehydration in gradient alcohol, and transparentizing in xylene, the sections were mounted with glycerol and observed under a microscope. In control sections, the primary antibody was replaced with 1% calf serum. The Olympus BX41 microscope and MicroSuite Basic Edition software from Olympus were used for visualization and imaging of the tissues.

3.4. Transient transfection

First, 6×10^5 cells were equally seeded in the 6-well plates with 2 ml RPMI-1640, supplemented with 10% FBS. Meanwhile, miR-129 mimic, inhibitor, or miR negative control (Genepharma) were mixed with HiPerFect Transfection Reagent (QIAGEN) and incubated at room temperature for 10 min. Then, the complex was transfected into HCT116 cells for 48 h.

3.5. RNA extraction and real-time PCR

The total RNA from HCT116 cells was rigorously extracted with Trizol (Invitrogen), according to the manufacturer's instructions. The concentration and purity of the RNA samples were assayed by absorbent density analysis on OD_{260}/OD_{280}.
To obtain the cDNA sequence of the specific miR, 2 μg of the total RNA was reversely transcribed using Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems), with specific primers for miR-129 and U6 (Shanghai Sangon Technology). To quantify the miR-129, a quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a Bio-Rad iCycler iQ Real-Time PCR Detection System. The PCR amplifications were performed in a 10 μl reaction system containing 5 μl of SYBR Green Supermix, 0.4 μl of forward primer, 0.4 μl of reverse primer, 2.2 μl of ddH_2O and 2 μl of template cDNA. The thermal cycling conditions were a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative level of miR-129 was determined using the 2-delta delta Ct analysis method. We chose U6 as the endogenous control. The nucleotide primers used for reverse transcription were as follows (5'-3'): miR-129-5p, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACTGGAAGAC; U6, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATATG. The primers used for real-time PCR were as follows (5'-3'): miR-129-5p forward, GCGCTAAAGTGCTTATGGAAGAC; U6 forward, GCGCGTCGTGAAGCGTTC; Universal reverse primer, GTGCAGGGTCCGAGGT.

3.6. Protein extraction and Western blot analysis

Proteins were extracted from the HCT116 cells in RIPA buffer (1% Triton X-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China), supplemented with a protease and phosphatase inhibitor cocktail (Sigma). The mixed protein from the cell lysates were separated by 10% SDS-PAGE and transferred electrophoretically to a PVDF membrane. After soaking with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following specific primary antibodies: anti-ERβ, anti-Proliferating Cell Nuclear Antigen (PCNA), anti-cleaved caspase3 and anti-GAPDH (Cell signaling). After incubating for 24 h, the corresponding HRP-conjugated anti-rabbit or mouse IgG secondary antibodies (Abmart, all at a 1:5000) were subsequently applied and immunodetection was achieved using the ECL plus detection system (Millipore), according to the manufacturer's instructions. The housekeeping gene GAPDH was used as the internal control.

3.7. Luciferase target assay

The 3' untranslated region (UTR) of ERβ, containing the predicted target site for miR-129, was cloned into the pmirGLO (Promega) luciferase reporter vector, which had been cleaved at SacI and XhoI sites. Details of the PCR procedures are described as follows: a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 45 s, and 72 °C for 30 s. The mutant was cloned using the Fast Mutagenesis System (TransGen Biotech, Beijing, China).

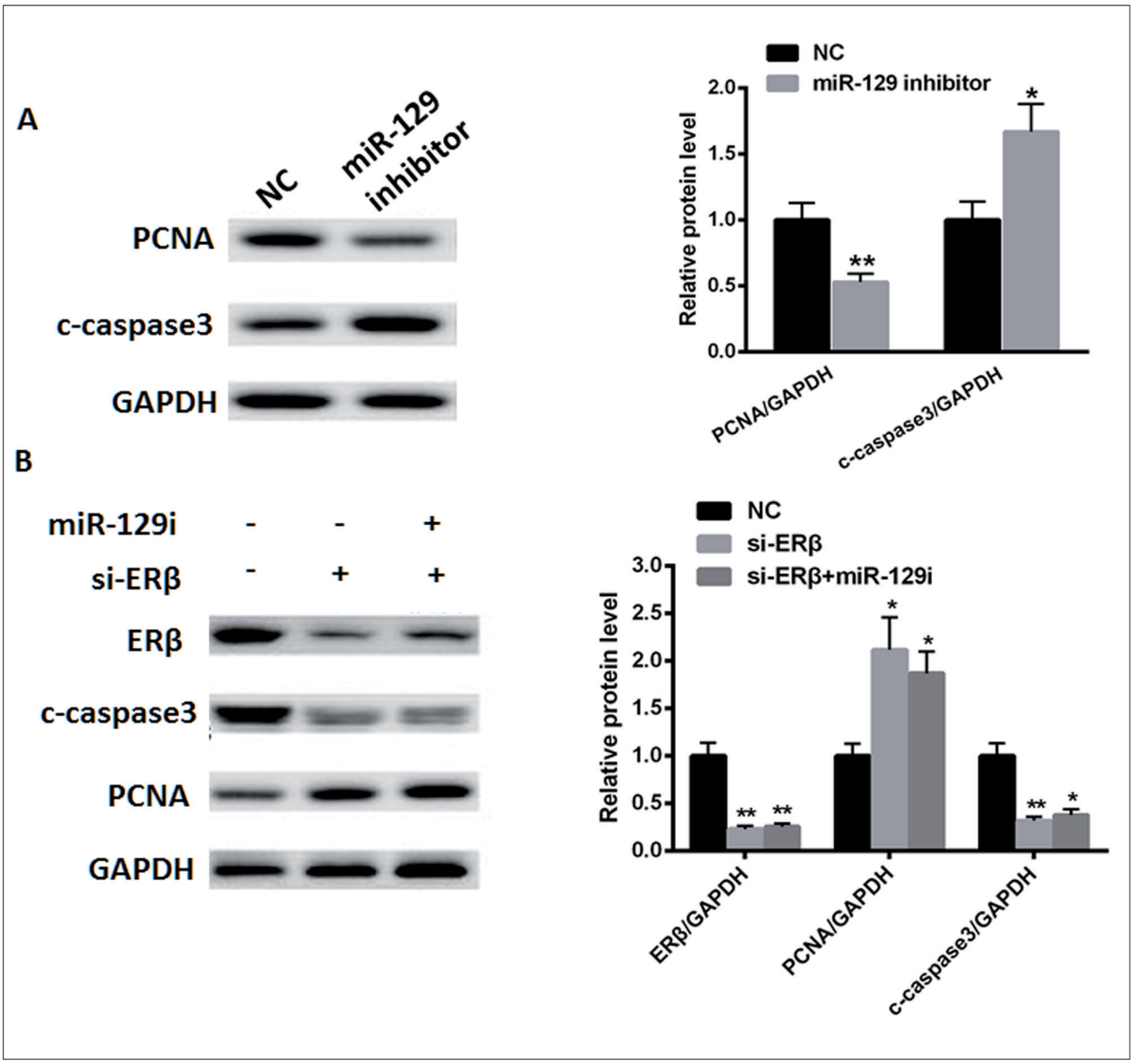

Fig. 4: The oncogenic role of miR-129 in colon cancer cells by targeting ERβ. (A) Transfection of miR-129 inhibitor suppressed the expression of PCNA but increased the activation of caspase3. (B) The miR-129 inhibitor-induced protein expression pattern changes could be reversed by siRNA targeting ERβ. Data are presented as the mean ± SE. n=6 for each group, *P<0.05, **P<0.01.

Before conducting the luciferase reporter assay, 5× 10^4 cells per well were seeded in 24-well plates in 500 µl of media and cultured for 18 h. The cells were transfected with the modified firefly luciferase vector (500 ng/µl) mixed with Vigofect transfection reagent, strictly according to the manufacturer's instructions. After continuous exposure for 48 h, the luciferase activities from firefly and renilla were measured with the Dual-Luciferase Reporter Assay System (Promega). We used renilla activity as the normalized parameter.

3.8. Inhibition of ERβ expression by RNA interference

Before transfection, 1×10^5 cells per well were seeded in a 6-well plate for application. Then, the siRNA targeting ERβ or negative control, which was purchased from Genepharma, was transfected into cells for 48 h using HiPerFect Transfection Reagent (QIAGEN), as described above. The siRNA sequence was 5' GGAAUGAACAUGAGCCGCAATT 3'.

3.9. Cell cycle and apoptosis analysis by flow cytometry

Cells were fixed in 70% ethanol and were permeabilized in Triton-X 100. Then, the cells were washed three times with PBS (5 min/time) and further digested with RNAse A. Subsequently, the cells were stained with propidium iodide. Apoptotic cells were measured with an Annexin V/FITC Kit (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions, and analyzed by flow cytometry.

3.10. Migration assay

Cell migration was determined using in vitro scratch assays. Cells were seeded in 12-well plates for 24 h. Then, an artificial gap was created in the confluent cell monolayer with a pipette tip. After transfection with miR-129 mimic, inhibitor or NC for 48 h, the cells were washed twice with pre-warmed PBS to remove the debris and to smooth the edges of the scratch. The initial images of the scratch (0 h) and final images of the scratch (48 h) were taken with an Olympus 1X51 inverted microscope. The distance of migration and area covered by migrating cells were analyzed quantitatively from the acquired images by using Olympus cellSens® digital imaging software and ImageJ software, respectively.

3.11. Statistical analysis

Data are presented as the mean ± SE from 3 independent experiments or 5 mice. Statistical analysis was performed using Student's t test, and $P < 0.05$ was considered statistically significant.

Conflicts of interest: None declared.

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