



miR-28-5p inhibits carcinogenesis in colon cancer cells and is necessary for erastin-induced ferroptosis

Jin-Cui Hu¹, Ting-Pei Zhu¹, Yu-Chang Gui¹, Zhi-Biao Tan¹, Ru-Qiong Wei¹, Bang-Li Hu², Jian-Wen Xu¹

¹Department of Rehabilitation Medicine, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, China; ²Department of Research, Guangxi Medical University Cancer Hospital, Nanning 530021, China

Contributions: (I) Conception and design: JW Xu, BL Hu; (II) Administrative support: None; (III) Provision of study materials or patients: JC Hu, TP Zhu, YC Gui, ZB Tan; (IV) Collection and assembly of data: JC Hu, TP Zhu; (V) Data analysis and interpretation: JC Hu, RQ Wei; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Jian-Wen Xu, Department of Rehabilitation Medicine, The First Affiliated Hospital of Guangxi Medical University, Shuangyong Road 6, Nanning 530021, China. Email: xujianwen@gxmu.edu.cn.

Background: Ferroptosis is a newly discovered type of regulated cell death, the underlying mechanisms of which need to be further illuminated. The regulatory activity of *miR-28-5p* in ferroptosis in colon cancer cells is currently unclear. This study set out to investigate the effect of *miR-28-5p* on ferroptosis in colon cancer cells and determine its underlying mechanism.

Methods: Biochemical Kits were used to measure iron concentration, malondialdehyde (MDA) concentration, glutathione (GSH) concentration and glutathione peroxidase (GPX) vitality. Cell counting kit 8 (CCK8) assays were conducted to evaluate cell viability. Flow cytometry was conducted to assess apoptosis. Transwell™ assays were used to measure the migratory and invasive abilities of HCT116 cells. Western blotting was used to measure the protein relative expression of NEDD4 binding protein 1 (N4BP1). Quantitative real-time polymerase chain reaction (RT-PCR) was used to measure the RNA relative expression of N4BP1 and *miR-28-5p*.

Results: Ferroptosis was induced in HCT116 cells by erastin in a dose- and time-dependent manner, which caused significant inhibition of proliferation, migration, and invasion in HCT116 cells; however, there was no obvious effect on apoptosis. *miR-28-5p* expression was decreased in colon cancer cells compared with the normal colon cells but was upregulated in erastin-treated HTC116 cells. Additionally, when overexpressed via the transfection of *miR-28-5p* mimics, *miR-28-5p* had an inhibitive effect on proliferation, migration, and invasion, while promoting apoptosis, in HCT116 cells. erastin-induced ferroptosis was also increased by *miR-28-5p* overexpression. Compared with normal colon cells, following erastin treatment, NEDD4 binding protein 1 (N4BP1) expression was increased in colon cancer cells and further decreased in HTC116 cells. *miR-28-5p* overexpression also inhibited *N4BP1* mRNA and protein expression in HTC116 cells.

Conclusions: *miR-28-5p* plays an important role in ferroptosis by targeting *N4BP1* and could serve as a potential therapeutic approach for colon cancer.

Keywords: *miR-28-5p*; colon cancer cells; ferroptosis; *N4BP1*; erastin

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Introduction

Colorectal cancer (CRC) is an extremely common form of gastrointestinal disease. In developed countries, CRC claims the second highest number of adult lives of any

cancer (1). For CRC patients, especially those diagnosed at later stages, chemotherapy is the most common treatment. However, in the clinical setting, many chemotherapeutic drugs, including cisplatin and oxaliplatin, are associated

with acquired resistance and toxicity, which lead to failure in cancer patient management (2,3). Therefore, a new approach which can overcome the issues posed by chemotherapeutic drug resistance and toxicity is desperately needed to improve treatment for patients with CRC.

Ferroptosis, a recently identified form of regulated cell death, occurs when iron-dependent lipid peroxide accumulates, thus employing a different method to those of apoptosis and necrosis (4,5). Ferroptosis plays a crucial role in the pathogenesis of a number of diseases, including cancer (6,7). Therefore, triggering ferroptosis might offer a novel therapeutic strategy for treating such diseases. A previous study showed that ferroptosis helped to facilitate RSL3-induced cell death in CRC cells, and is perhaps a pervasive and dynamic form of cell death in cancer therapy (8). However, the underlying mechanisms of ferroptosis are largely unknown and need to be further illuminated.

miRNAs are single-stranded, small non-coding RNA molecules that can be oncogenic or can take up a tumor-suppressive role and are known to participate in the initiation, progression, and metastasis of various types of cancer (9). *miR-28-5p* has been shown to target a number of cancer-associated genes and participates in cancer cell proliferation, migration, invasion, and epithelial-mesenchymal transition (10). A previous investigation demonstrated that in comparison with normal colon samples, CRC samples exhibited downregulation of *miR-28-5p*, and that the expression levels of cyclin D1 (CCND1) and homeobox B3 (HOXB3) are inhibited when *miR-28-5p* is overexpressed (11). However, the role of *miR-28-5p* in ferroptosis in CRC cells is not yet understood. In the current study, we explored the effect of erastin, an inducer of ferroptosis, in colon cancer cells and examined the role of *miR-28-5p* in colon cancer cells undergoing ferroptosis, with the aim of revealing the regulatory mechanism of miRNAs in ferroptosis in CRC cells.

Methods

Cell culture

The human colon cancer lines HCT116 and SW480 and the human colon line NCM460 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (FBS; Gibco) and 100 U/mL penicillin-100 µg/mL streptomycin (Solarbio, Beijing,

China) were used for cell culture. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Iron, malondialdehyde (MDA), and glutathione (GSH) concentration and glutathione peroxidase (GPX) vitality assay

An Iron Assay Kit (Baiaolaibo, Beijing, China) was used to measure relative iron concentration in cell lysates. The MDA concentration was detected with a Lipid Peroxidation Assay Kit (Sigma, St Louis, Mo, USA). A Glutathione Assay Kit (Solarbio, Beijing, China) was employed to assess the GSH concentration. GPX vitality was assessed by GPX vitality assay (Leagene, Beijing, China). All assays were performed according to the manufacturer's instructions.

Transfection of *miR-28-5p* mimics and inhibitors

The effect of *miR-28-5p* on HCT116 cells was evaluated by transfecting *miR-28-5p* mimics or inhibitors purchased from Bioneer (Shanghai, China). Cells were plated with RPMI-1640 with 10% FBS without antibiotics in 6-well plates at a density of 2×10^5 cells/well for 24 h. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was then used to transfect the cells in accordance with the manufacturer's protocol. *miR-28-5p* mimics and *miR-28-5p* inhibitors were used at final concentrations of 80 and 100 pM, respectively, in antibiotic-free Opti-minimum essential medium (Opti-MEM, Gibco, Grand Island, NY, USA). The medium was discarded and replaced with RPMI-1640 (10% FBS, 20 µmol/L erastin) 6 h later. The extraction of total RNA and protein was performed 48 h after transfection.

Cell viability assays

CCK8 assays were conducted to evaluate cell viability. Briefly, HCT116 cells were seeded at 10^4 cells/well in 96-well plates in 0.1 mL RPMI-1640 for 12, 24, and 48 h. At each time point, 10 µL of CCK8 solution (5 mg/mL) was added, before the cells were incubated for 2 h at 37 °C. A microplate reader (Eppendorf, Hamburg, Germany) was used to read the absorbance was read at 490 nm.

Cell apoptosis assays

Flow cytometry was conducted to assess apoptosis. The Annexin V-fluorescein isothiocyanate (FITC) Apoptosis

Detection kit (BD, San Jose, CA, USA) was used to measure the rate of apoptosis. Trypsin [without ethylene diamine tetraacetic acid (EDTA)] was used to collect cells, which were centrifuged at 1,000 ×g for 5 min at room temperature. Next, the cells were washed twice with phosphate buffer saline (PBS), resuspended in 100 µL Annexin V-FITC Binding Buffer, and mixed with 5 µL Annexin V-FITC and 5 µL propidium iodide. The cells were subsequently stained for 15 min in darkness. Then, 400 µL of Annexin V-FITC Binding Buffer was added and the cells were kept in the dark and examined by flow cytometry. Cell Quest™ (BD) was used to analyze the results.

Migration and invasion assays

Transwell™ assays (Corning, NY, USA) were used to measure the migratory and invasive abilities of HCT116 cells. To coat the surface of the polycarbonate membrane, serum-free medium, with or without the addition of 50 µL Matrigel™ (Corning) was used to wash the upper chamber of the Transwell, into which cells (10⁵) in 0.2 mL of serum-free RPMI-1640 were seeded. Meanwhile, 0.5 mL of RPMI-1640 supplemented with 10% fetal bovine serum was put into the lower chamber. The membrane was incubated at 37 °C for 48 h, before the removal of cells from the upper chambers. Finally, crystal violet was used to stain the invaded cells in the lower chambers, and they were observed and counted using a high-power microscope.

Western blotting assays

For Western blotting analysis, radio immunoprecipitation assay (RIPA) lysis buffer (100 µL) was used to extract the lysates of total cellular proteins. Next, 50 µg of lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotting was performed to transfer them to nitrocellulose membranes. The membranes were blocked for 1 h with 5% blocking solution before being incubated overnight at 4 °C with primary antibodies purchased from Abcam Biotechnology [N4BP1: 1:2,000 dilution; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 1:5,000 dilution]. The membranes were then washed with tris buffered saline tween (TBST) three times and incubated with goat anti-rabbit IgG secondary antibodies (1:3,000; Abcam, Cambridge, UK) for 1 h. Immunoreactivity was measured using a two-color IR imaging system (Odyssey, Lincoln, USA).

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

The extraction of total cellular RNA was performed using 1 mL Nuclezol™ Reagent (Macherey-Nagel, Wiesbaden, Germany) in line with the instructions of the manufacturer. Then, 3.75 µL of RNA was reverse-transcribed to cDNA in a 10-µL reaction using a miRNA Reaction kit (Takara, Tokyo, Japan). A 7500 Real-Time PCR system (Applied Biosystems, Fostercity, CA, USA) was employed to carry out RT-PCR. The PCR program was as follows: 40 cycles of 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 34 s. Each experiment was performed in triplicate. Gene expression was normalized to that of U6.

Primer sequences

The primer sequences of *miR-28-5p* were: forward 5'-CGC AAG GAG CTC ACA GTC TAT TGA G-3'; reverse: mRQ3' primer (Takara, Tokyo, Japan). The primer sequences of *N4BP1* were: forward 5'-CAC CTT CTG TTG CCT CTC CAA GTC-3'; reverse 5'-GGG TTC TGG CTG GTG TAA AC-3'.

Statistical analyses

All statistical analyses were performed using SPSS 17.0 (IBM Corp, Armonk, NY, USA). Data were expressed as the mean ± standard deviation. One-way analysis of variance with least square difference test was used to analyze difference. A P value <0.05 was considered to represent statistical significance.

Results

Erastin induces ferroptosis, but not apoptosis, and inhibits proliferation, migration, and invasion in CRC cells

To identify the optimal treatment conditions for erastin, HCT116 cells were treated with different concentrations of erastin at different timepoints. By evaluating the indicators of cell ferroptosis, including Fe²⁺, MDA, GSH, and GPX, 20 µmol/L Erastin for 48 h was revealed to considerably induce ferroptosis in HCT116 cells compared to other concentrations and times (*Figure 1*), indicating that this concentration and time were the optimal conditions for erastin treatment of HCT116 cells.

Subsequently, proliferation, migration, and invasion

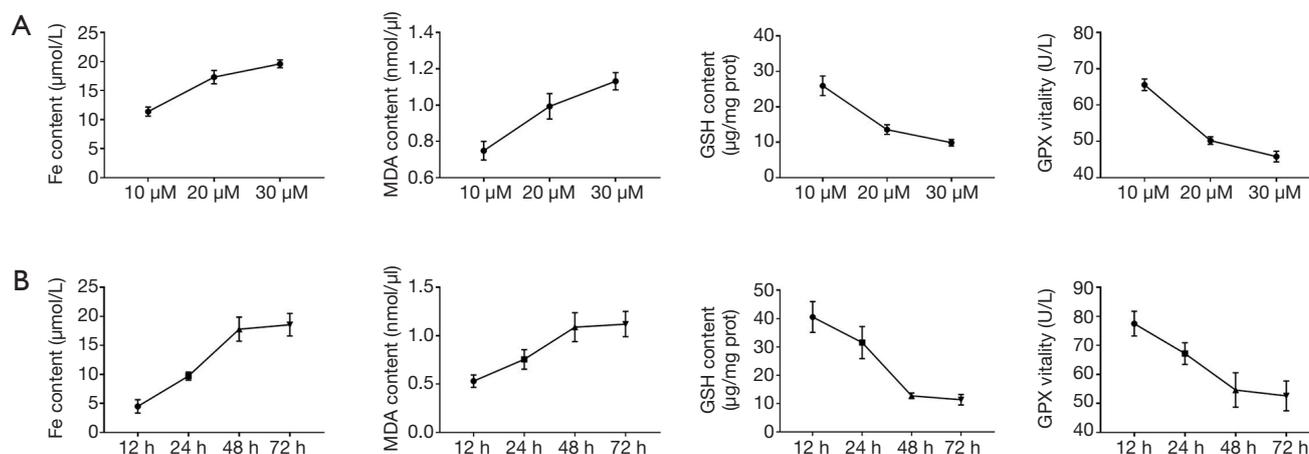


Figure 1 Erastin induced ferroptosis at different concentrations and time points. (A) Relative iron, MDA, GSH, and GPX concentration in HCT116 cells treated with erastin at different doses. (B) Relative iron, MDA, GSH, and GPX concentration in HCT116 cells treated with erastin at different time points. MDA, malondialdehyde; GSH, glutathione; GPX, glutathione peroxidase.

in erastin-treated HCT116 cells were assessed after treatment with erastin at 20 μmol/L for 48 h. Ferrostatin-1, a ferroptosis inhibitor, was used as a negative control. The results of the CCK8 assay showed that erastin greatly inhibited HCT116 cell proliferation compared to the control and ferrostatin-1 treatment (Figure 2A). The Transwell assay also revealed that the migratory and invasion abilities of HCT116 cells were suppressed after erastin treatment compared to the control and ferrostatin-1 treatment (Figure 2B,C).

To explore the influence of erastin on HCT116 cell apoptosis, flow cytometry was performed to measure the apoptosis rate. No significant difference was observed in the apoptosis rate between the cells treated with erastin, the control, or ferrostatin-1, suggesting that erastin has little effect on apoptosis in HCT116 cells (Figure 2D). Collectively, these results demonstrated that erastin induces ferroptosis but not apoptosis in HCT116 cells, and inhibits their proliferation, migration, and invasion.

miR-28-5p is downregulated in CRC cells

RT-PCR was used to examine *miR-28-5p* expression in two colon cancer cell lines, HCT116 and SW480, and one normal human colon line, NCM460. The expression levels of *miR-28-5p* were significantly lower in both HCT116 and SW480 cells than in NCM460 cells ($P < 0.01$) (Figure 3), which confirmed that *miR-28-5p* was downregulated in colon cancer cells. In addition, the expression of *miR-28-*

5p was found to be lower in HCT116 cells than in SW480 cells; therefore, HCT116 cells were selected for subsequent experiments.

Overexpression of miR-28-5p inhibits proliferation, migration, and invasion in CRC cells

The effect of *miR-28-5p* in HCT116 cells, was investigated by transfecting HCT116 cells with *miR-28-5p* mimics or inhibitors and assessing proliferation, migration, invasion, and apoptosis. As shown in Figure 4A, cell proliferation was significantly inhibited by the transfection of *miR-28-5p* mimics, whereas proliferation was increased by *miR-28-5p* inhibitors. The Transwell assay revealed that migration and invasion were suppressed after *miR-28-5p* mimics were transfected but enhanced after *miR-28-5p* inhibitors were transfected (Figure 4B,C). Flow cytometry also showed that the apoptosis rate was elevated in HCT116 cells after they were transfected with *miR-28-5p* mimics but decreased after the cells were transfected with *miR-28-5p* inhibitors (Figure 4D).

Overexpression of miR-28-5p promotes erastin-induced ferroptosis in CRC cells

Erastin treatment elevated the levels of *miR-28-5p* expression in HCT116 cells, which indicated that *miR-28-5p* is increased in HCT116 cells undergoing ferroptosis (Figure 5A).

To explore the role of *miR-28-5p* in erastin-induced

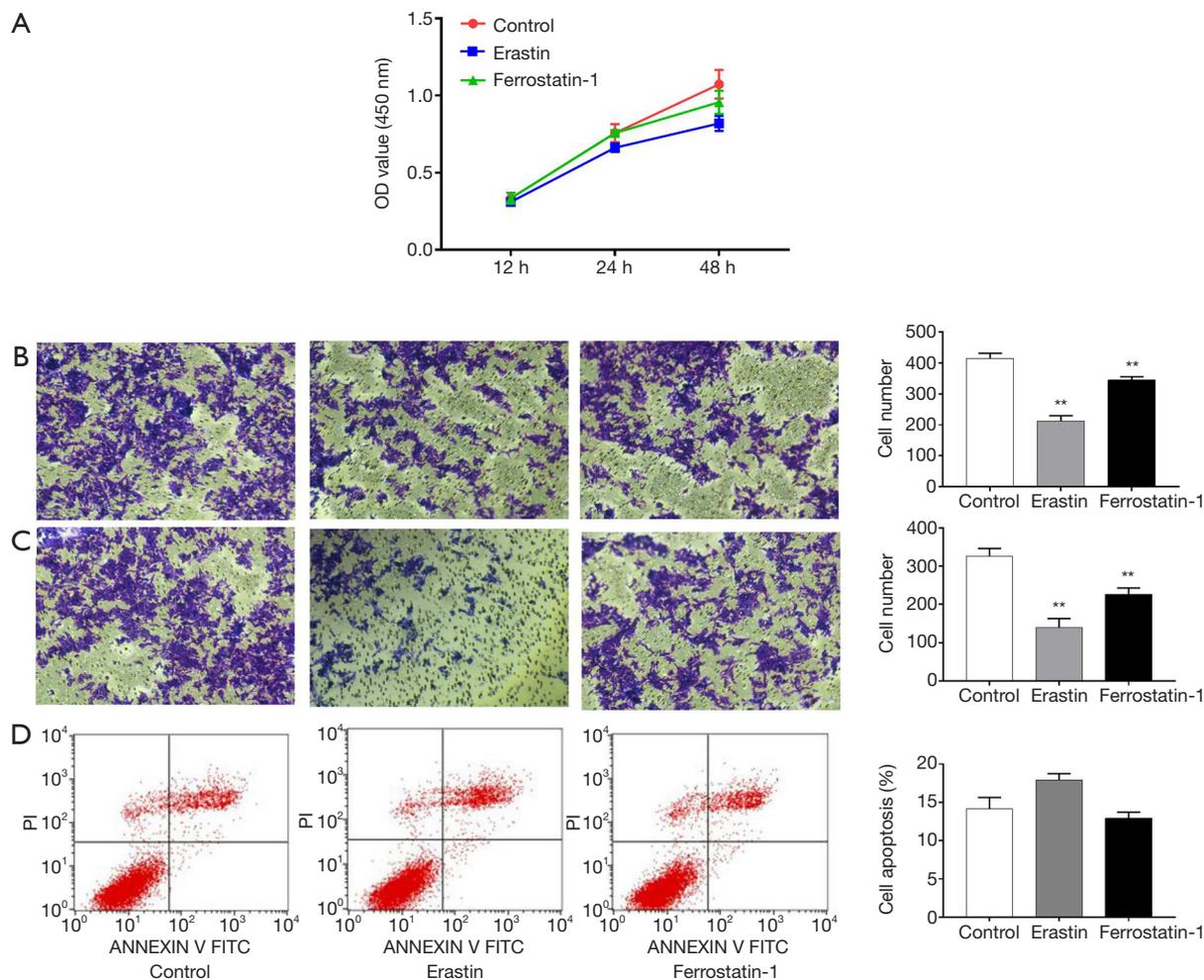


Figure 2 Erastin inhibited proliferation, migration, and invasion in CRC cells, but not apoptosis. (A) Viability of HCT116 cells treated with erastin using the CCK8 assay. (B) Migration in HCT116 cells treated with erastin using Transwell assay. (C) Invasion in HCT116 cells treated with erastin using Transwell assay. (D) Apoptosis in HCT116 cells treated with erastin using flow cytometry. Magnification: 100x. **, P<0.01. CRC, colorectal cancer; CCK8, cell counting kit 8.

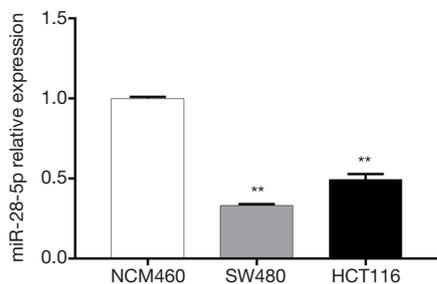


Figure 3 Expression of *miR-28-5p* in NCM460, SW480, and HCT116 cells. **, P<0.01.

ferroptosis in HCT116 cells, *miR-28-5p* mimics or inhibitors were transfected into HCT116 cells for 24 h, and the cells were then treated with erastin. RT-PCR was used to assess *miR-28-5p* expression. After the transfection of *miR-28-5p* mimics, *miR-28-5p* expression was significantly increased (P<0.01), whereas *miR-28-5p* expression was decreased after *miR-28-5p* inhibitors were transfected (Figure 5B).

The Fe, MDA, GSH, and GPX content in the HCT116 cells were measured after the transfection of mimics or

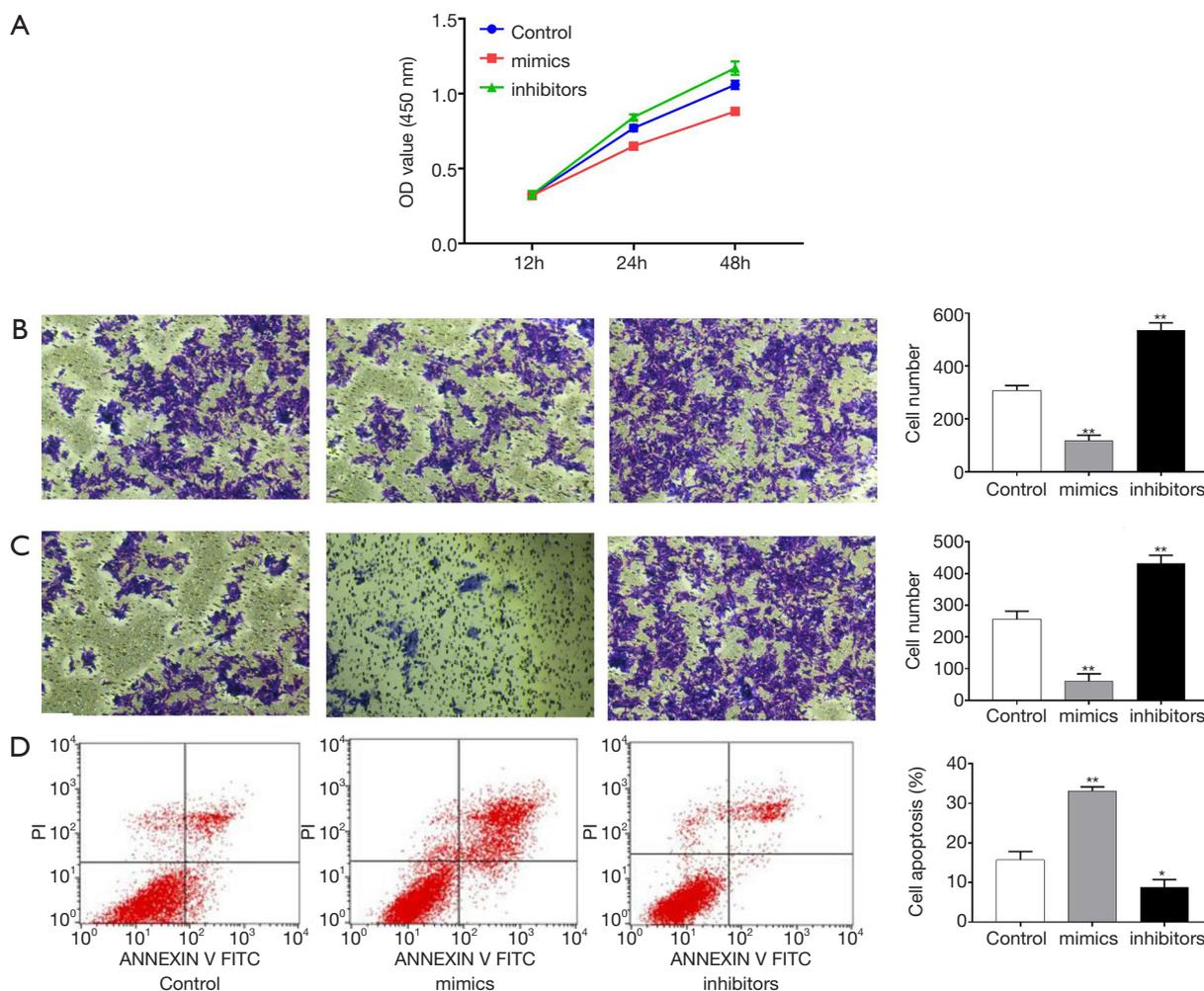


Figure 4 Overexpression of *miR-28-5p* inhibited the proliferation, migration, and invasion of CRC cells. (A) Viability of HCT116 cells transfected with *miR-28-5p* mimics or inhibitor using the CCK8 assay. (B) Migration in HCT116 cells transfected with *miR-28-5p* mimics or inhibitor using Transwell assay. (C) Invasion in HCT116 cells transfected with *miR-28-5p* mimics or inhibitor using Transwell assay. (D) Apoptosis in HCT116 cells transfected with *miR-28-5p* mimics or inhibitors using flow cytometry. **, $P < 0.01$. CRC, colorectal cancer.

inhibitors and treatment with erastin for 48 h. GSH and GPX were significantly decreased, while Fe and MDA were increased after the transfection of *miR-28-5p* mimics; however, transfection of *miR-28-5p* inhibitors did not significantly affect the content of MDA, GSH, or and GPX in erastin-treated cells (Figure 5C,D,E,F). These results showed that *miR-28-5p* overexpression could promote ferroptosis in HCT116 cells.

miR-28-5p decreases *N4BP1* levels after Erastin treatment in CRC cells

A previous study showed that *miR-28-5p* decreases the

levels of *N4BP1* in two ovarian cancer cell lines, indicating that *N4BP1* is a target gene of *miR-28-5p* (10). However, whether *miR-28-5p* regulates *N4BP1* in CRC has not been established. To evaluate regulation of *N4BP1* by *miR-28-5p* in erastin-treated CRC cells, we first measured the expression of *N4BP1* in NCM460, SW480, and HCT116 cells using RT-PCR. *N4BP1* was significantly increased in SW480 and HCT116 cells compared to NCM460 cells (Figure 6A), suggesting that the expression of *N4BP1* was also increased in colon cancer cells. Next, we assessed the levels of *N4BP1* in erastin-treated HCT116 cells; *N4BP1* expression was decreased after treatment with erastin (Figure 6B).

To determine the association between *miR-28-5p* and

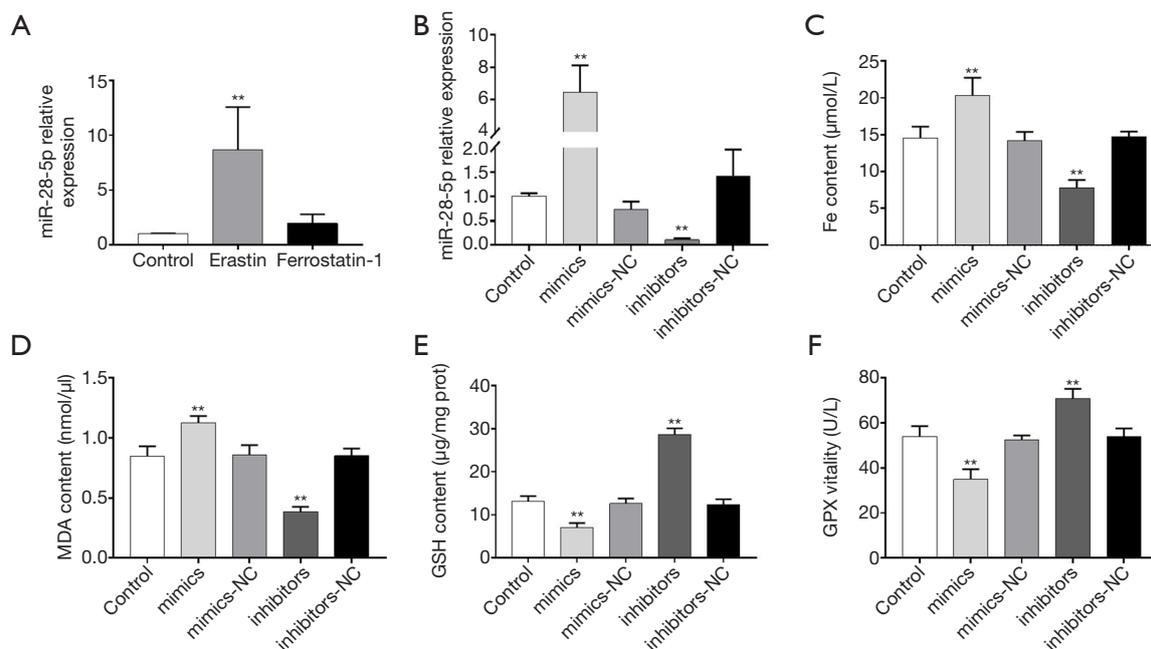


Figure 5 Overexpression of *miR-28-5p* promote erastin-induced ferroptosis in CRC cells. (A) Expression of *miR-28-5p* in HCT116 cells treated with erastin. (B) Expression of *miR-28-5p* in HCT116 cells transfected with *miR-28-5p* mimics or inhibitors. Changes in Fe (C), MDA (D), GSH (E), and GPX (F) expression in HCT116 cells treated with erastin and transfected with *miR-28-5p* mimics or inhibitors. **, $P < 0.01$. CRC, colorectal cancer; MDA, malondialdehyde; GSH, glutathione; GPX, glutathione peroxidase.

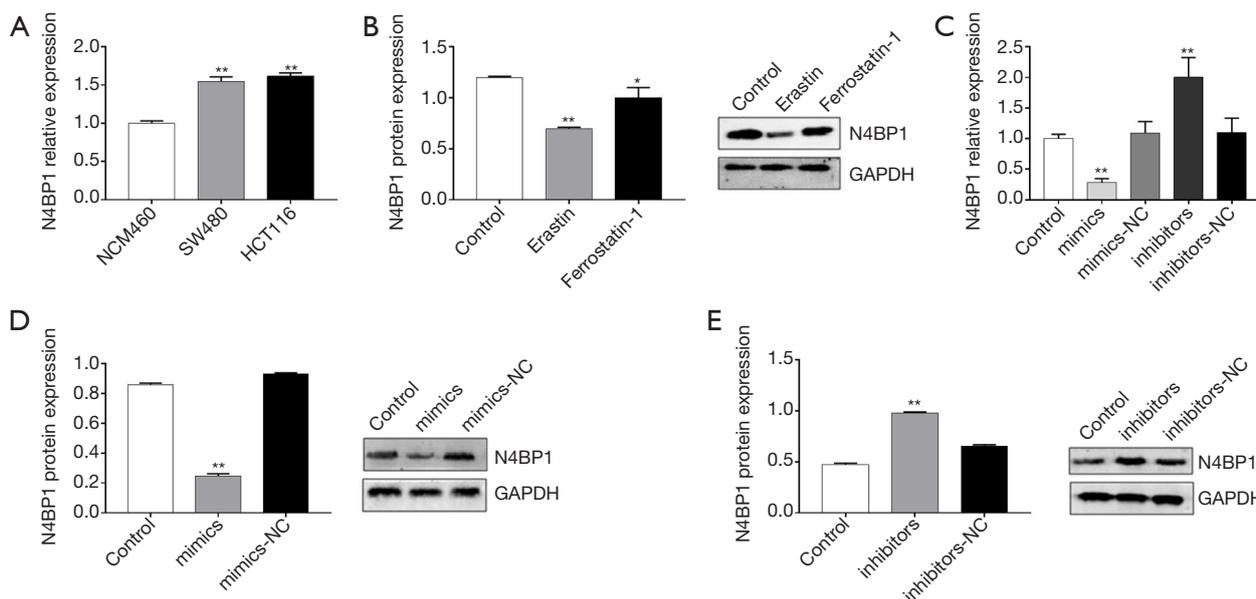


Figure 6 *miR-28-5p* decreased *N4BP1* levels in CRC cells after erastin treatment. (A) Expression of *N4BP1* in NCM460, SW480, and HCT116 cells. (B) Expression of *N4BP1* in HCT116 cells treated with erastin. (C) Expression of *N4BP1* mRNA in HCT116 cells treated with erastin and transfected with *miR-28-5p* mimics or inhibitors. Expression of *N4BP1* protein in HCT116 cells treated with erastin and transfected with *miR-28-5p* mimics (D) or inhibitors (E). * $P < 0.05$, ** $P < 0.01$. CRC, colorectal cancer; *N4BP1*, NEDD4 binding protein 1.

N4BP1 in HCT116 cells, we transfected *miR-28-5p* mimics and inhibitors into HCT116 cells before erastin treatment with, and the expression of *N4BP1* was tested by RT-PCR and Western blotting. Both the mRNA and protein expression of *N4BP1* were significantly decreased in cells transfected with *miR-28-5p* mimics compared to those transfected with inhibitors ($P < 0.05$). These results demonstrated that *miR-28-5p* downregulates *N4BP1* expression in HCT116 cells treated with erastin (Figure 6C,D,E).

Discussion

The induction of regulated non-apoptotic cell death has been put forward as an alternative approach for the elimination of drug-resistant cancer cells (12). Ferroptosis, a newly identified type of regulated cell death, occurs when iron-based lipid reactive oxygen species (ROS) accumulates (13). Because of genetic alterations and aberrant proliferation, migration, and invasion, the levels of ROS-related oxidative stress displayed by cancer cells are consistently high. Therefore, agents affecting ROS metabolism can influence tumor cell growth (14). Erastin has been shown to eliminate rat sarcoma viral oncogene-mutated cancer cells via the induction of ferroptosis (15,16). However, the molecular mechanisms of ferroptosis regulation have not been fully explored.

In this study, we examined the effect of erastin on colon cancer cells and observed that it induces ferroptosis in a dose- and time- dependent manner. However, as in other cancer cells, erastin failed to induce apoptosis at any time point or dose. More importantly, we observed that proliferation, migration, and invasion are decreased in erastin-treated cells, suggesting that the induction of ferroptosis might serve as a treatment strategy for colon cancer.

Although previous studies have described some molecular mechanisms of ferroptosis in cancer, few have reported the regulatory role of miRNAs in ferroptosis in cancer cells. Recently, Niu *et al.* (17) reported that the miR-103a-3p/glutaminase 2 (GLS2) axis was involved in the regulation of physcion 8-O- β -glucopyranoside-triggered ferroptosis and suppressed carcinogenesis *in vitro* and *in vivo*. Another study showed that miR-17-92 asserts a protective effect over endothelial cells after erastin-induced ferroptosis and promotes acyl-CoA synthetase4 (ACSL4) expression (18). In addition, miR-9 and miR-137 have been shown to regulate ferroptosis in melanoma through their target genes *in vivo* and *in vitro* (19,20).

miR-28-5p is an intragenic miRNA with aberrant expression in several cancers. Increased *miR-28-5p* expression

has been found in ovarian (10), esophageal (21), and cervical cancer (22) and low expression has been observed in hepatocellular carcinoma (23), renal cell carcinoma (24), and CRC (11), suggesting *miR-28-5p* takes up different roles during carcinogenesis. A high level of *miR-28-5p* expression in CRC has been shown to be a predictor of short-term relapse in node-negative patients and of poor overall survival in patients with no metastasis (25). Using *in vivo* and *in vitro* experiments, Wu *et al.* observed that *miR-28-5p* could negatively regulate SSRP1 expression, thereby inhibiting CRC progression (26). However, no study has reported the activity of *miR-28-5p* in ferroptosis.

To examine the effect of *miR-28-5p* on the phenotype of CRC cells and its regulation in ferroptosis, we overexpressed and inhibited *miR-28-5p* in HCT116 cells treated with erastin and observed *miR-28-5p* overexpression significantly inhibited proliferation, migration, and invasion and also promoted ferroptosis, indicating that overexpression of *miR-28-5p* might prove useful in treating CRC. To explore the molecular mechanism behind the regulatory involvement of *miR-28-5p* in ferroptosis, we identified *N4BP1* as a target of *miR-28-5p* in CRC and showed that *N4BP1* was decreased in erastin-treated HCT116 cells. Moreover, *N4BP1* expression was induced by *miR-28-5p* suppression and was downregulated by *miR-28-5p* overexpression. Overall, our findings demonstrate the important regulatory role *miR-28-5p* plays in ferroptosis in CRC cells via the regulation of *N4BP1*.

Limitation

Only HCT116 cell line is selected in the study of tumor cell function, we will consider using a variety of cells to test our conclusions in the future. Luciferase reporter gene to analyze the direct interaction between *miR-28-5p* and *N4BP1* is in our next research plan.

Conclusions

The present study discovered that *miR-28-5p* promoted ferroptosis in CRC cells by targeting *N4BP1*. These findings highlight the significance of miRNA in ferroptosis and present *miR-28-5p* as a key regulator of ferroptosis in colon cancer.

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Footnote

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tcr-20-1809>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-1809>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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