The Effects of *TRAF6* on Growth and Progression in Colorectal Cancer are Regulated by *miRNA-140*

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Background and Aim: Some studies have confirmed that *miRNA-140* exhibits a suppressive role in gastric cancer, Wilms' tumor. However, the function of *miRNA-140* in colorectal cancer has not been completely elucidated. The present study aims to verify *TRAF6* as the targeted gene by *miRNA-140* which was investigated in colorectal cancer tissues and cells, and its effects on the biological characteristics of colorectal cancer cells were determined, in order to provide an experimental and theoretical basis for the application of *TRAF6* in the treatment of colorectal cancer.

Methods: qPCR analyzed *miRNA-140* expression levels in colorectal cancer tissues, normal colorectal cancer tissues and colorectal cells including SW480 and HCT116 cancer cells and FHC normal colorectal epithelial cells. A serial biological experiment analyzed *miRNA-140* effects on cell proliferation, migration and invasion capacities in SW480 and HCT116 cells. *miRNA* targeting gene prediction and a dual luciferase assay were used to analyze *miRNA-140*-targeted *TRAF6*. qPCR and Western blot analyzed *miRNA-140* effects on the mRNA and protein expression of *TRAF6*. Western blot analyzed *miRNA-140* effects on NF- κ B/c-jun signaling pathways. Animal studies were performed to investigate the effects of *miRNA-140* on colorectal cancer implantation tumor growth. Immunohistochemistry analyzed *TRAF6* expression in animal experimentation tumors.

Results: *miRNA-140* expression is lower in colorectal cancer tissues and colorectal cancer cells. Over-expression of *miRNA-140* inhibited the proliferation, migration and invasion capacities of colorectal cancer cells. *miRNA-140* targeted the *TRAF6* mRNA 3'UTR area and decreased *TRAF6* protein expression. *miRNA-140* suppressed p-NF- κ B/p-c-jun proteins expression. *miRNA-140* inhibited colorectal cancer implantation tumor growth in the mice model.

Conclusion: *miRNA-140* targeting *TRAF6* affects the progression and growth of colorectal cancer, the mechanism could be *miRNA-140* decreasing the *TRAF6* expression effects on the NF- κ B/c-jun signaling pathways.

Keywords: *TRAF6*, *miRNA-140*, NF- κ B/c-jun signaling pathways, progression, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer-related disease and cause of death in the United States.¹ The American Cancer Society evaluated that more than 140 thousands new CRC patients will be diagnosed and more than 50 thousands CRC patients will die because of this cancer in 2019.² Surgical resection is still the most effective and most possible curative treatment for CRC.^{3,4} However, many CRC patients develop recurrences. It is necessary to study the molecular mechanisms that effect the growth and progression of CRC, as these may be potential therapeutic targets for CRC treatment.

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The tumor necrosis factor receptor-associated factor-6 (*TRAF6*) family includes a family of seven members *TRAF1-7*. The TRAFs family play key roles in regulating cell survival and cell death.^{5,6} *TRAF6* has been proved to be involved in cancer progression.^{7,8} Luo et al⁷ reported that *TRAF6* is involved in regulating melanoma invasion and metastasis. Han et al⁸ indicated that *TRAF6* promotes the invasion and metastasis and predicts a poor prognosis in gastric cancer. *TRAF6* can promote the metastasis of esophageal squamous cell carcinoma and tumorigenicity of pancreatic cancer cells.^{9,10} Our previously study also showed that *TRAF6* promotes the progression and growth of colorectal cancer.¹¹ However, the regulation mechanisms of *TRAF6* in cancer cells is not fully understood, and why *TRAF6* expression is upregulated in colorectal cancer cells remains unclear. Therefore, research regarding the regulation of *TRAF6* expression may have vital theoretical significance and practical value.

In recent years, microRNA (miRNA) has been discovered to have the ability of regulating gene expression.^{12,13} miRNAs are small, single stranded, non-coding RNAs consisting of 20–22 nucleotides, which play significant roles in many biological processes by regulating gene expression at the post-translational level.¹⁴ The mechanisms by which miRNAs work is binding to the complementary sites in the 3' untranslated region (3'UTR) of the targeting genes which induced degradation of the transcript or translational repression.¹⁵ miRNAs are involved in many physiological processes, including cell proliferation, cell apoptosis, cell cycle and cell differentiation.^{16,17}

Studies revealed that *miRNA-140* is a tumor suppressor in several types of human cancer.^{18–20} We carried out bioinformatics analysis using Targetscan and starBase related prediction software.^{21,22} The bioinformatics analysis showed that *TRAF6* was a *miRNA-140* targeted gene. Therefore, the present study aimed to determine the role of *miRNA-140* regulating *TRAF6* in colorectal cancer in vitro and in vivo.

Patients and Methods

Patients with CRC Tissues Specimens

5 pairs of fresh cancer specimens and normal colorectal tissues were collected from patients with CRC by surgical excision. The specimens and tissues were immediately cut into 1 mm³ fragments and then cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Life Technologies), 1% penicillin

(Sigma Millipore, Louis, USA) and incubated at 37 °C under 95% air and 5% CO₂ for 48 h. All patients provided informed consent and the experiment was conducted in accordance with the Declaration of Helsinki. The present study was approved by the Ethics Committee of The First Hospital Affiliated to Fujian Medical University.

Cell Culture

The CRC cell lines SW480 and HCT116 were purchased from the Shanghai Institute for Biological Science (Shanghai, China). Both cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, both Life Technologies; Thermo Fisher Scientific, Inc.). The FHC (normal colonic epithelial cells) were obtained from Biological Science and kept in Dulbecco's modified Eagle's media (DMEM)/F12 containing 10% FBS, 10 ng/mL cholera toxin, 10 mM HEPES, insulin and transferring both at 0.005 mg/mL plus 100 ng/mL hydrocortisone medium. The SW480, HCT116 and FHC cell lines were incubated at 37 °C under 95% air and 5% CO₂.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol and quantified by UV 260/280 nm, as described previously.^{23,24} Total RNA (2 µg) and concentrations of sample RNA were reverse transcribed to cDNA in a final volume of 20 µL using the AVM First Strand cDNA synthesis kit (Invitrogen) following the manufacture's instructions. The primer sequences were as follows: *miRNA-140* forward, 5'-ACACTCCAGCTGGGTACCACAGGG-3' and reverse, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCGTG GTT-3'. U6 forward, 5'-CTCGCTTCGGCAGCACA-3', and reverse, 5'-AAACGCTTCACGAATTTGCGT-3'. They were used as cDNA templates for qPCR. The qPCR primers used for amplification areas were as follows: *miRNA-140*, forward 5'-TCGGCAATTCAGTTGAGCCGTGGT T-3', reverse 5'-ACACTCCAGCTGGGTA CCACAGGG-3' and *TRAF6*, forward 5'-ATGCGGCC ATAGGTTCTGC-3', reverse 5'-TCCTCAAG ATGTCTCAG TTCCAT-3'. All qPCRs were performed with the Thermo Scientific SYBR Green qPCR kit on an Applied Biosystems StepOne Real-time PCR System. PCR conditions were: 95 °C for 2 min, 95 °C for 15 sec, and 60 °C for 30 sec for 40 cycles. All gene transcripts were quantified by the 2^{-ΔΔCt} method.

miRNA-140 Oligonucleotides, Vectors

The miRNA oligonucleotides including the mimic of *miRNA-140* as well as their corresponding negative controls (NC) were commercially obtained from Genechem Co. Ltd. (Shanghai, China). pGL4.1-wt*TRAF6* (Wild type *TRAF6* 3'UTR sequence) or pGL4.1-mut*TRAF6* (Mutant *TRAF6* 3'UTR sequence) were stored by the Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University.

miRNA-140 Oligonucleotides Transfection

The transfection with *miRNA-140* NC or *miRNA-140* mimic of SW480 and HCT116 cells was performed with Lipofectamine 2000 (Life Technologies). At 48 h following transfection, the transfected SW480 and HCT116 cells were used for other experimental assays.

Cell Proliferation and Invasion Assays

Colorectal cancer cells which were treated using *miRNA-140* oligonucleotides are planted in 96-well plates at a density of 1500 each well, 3 replicate wells per experimental group. These cells were cultured in RPMI-1640 medium with 10% fetal bovine serum in a 5% CO₂, 37 °C incubator for 1, 2, 3, 4 and 5 days, then add 10% CCK-8 (Cell Counting kit-8, dojindo, Kumamoto, Japan) serum-free medium, counted by microplate reader (BioTek, Winooski, VT, USA) after continuing to culture for 1 h, as described previously.¹¹

For cell invasion assay, 5×10⁴ CRC cells with *miRNA-140* oligonucleotides transfection were seeded into the upper chamber of 8 μm pore transwells (Corning, NY 14831, USA) coated with Matrigel (BD Bioscience, USA). A medium including 10% FBS was added to the lower chamber. Experiments were performed as described previously.¹¹

Cell Migration and Wound Healing Assays

For the migration assay, the Transwell migration chamber experiment was used without Matrigel, and experiment steps were the same as the invasion assay.

For wound healing assay, CRC cells with *miRNA-140* oligonucleotide transfection were implanted into 6-well plates. The experiments were performed as previously described.¹¹

Dual-Luciferase Reporter Assay

SW480 and HCT116 cells were seeded at a density of 2×10⁵ cells per well in 12-well plates. *miRNA-140* oligonucleotide

transfection was performed by use of Lipofectamine 2000 (Life Technologies), according to the manufacturer's protocol. SW480 and HCT116 cells were co-transfected with *miRNA-140* oligonucleotides, pRL-TK (Promega Corporation) and pGL4.1-wt*TRAF6* or pGL4.1-mut*TRAF6*. Cells were lysed 48 h after transfection. A total of 20 μg of cell lysate was used for the intracellular luciferase activity, following the manufacturer's protocol of the Dual-Luciferase reporter Assay system (Promega Corporation). The Renilla luciferase expression vector pRL-TK was used for normalization. Luminescence measurements were performed on a luminometer (Orion II Microplate Luminometer; Berthold Detection Systems GmbH). Each transfection was performed in duplicate and data are expressed as the mean ± standard deviation of three separate experiments.

Western Blot Analysis

The experiments and results analysis were carried out as described previously.^{23,25} The following primary antibodies were used in this study under the listings, respectively: *TRAF6* (1:1000, Affinity), GAPDH (1:1000, Abcam), NF-κB (1:1000, Affinity), p-NF-κB (1:1000, Abcam), c-jun (1:1000, Bioss) and p-c-jun (1:1000, Bioss).

Animal Experiment

Animal experiments were performed in the Animal Experimental Center of Fujian Medical University and approved by the Animal Protection and Use Committee of Fujian Medical University. The animal experiments followed the guidelines of the 5 freedoms-animal welfare standards. 4×10⁶ HCT116 cells which were transfected with *miRNA-140* NC or *miRNA-140* mimic oligonucleotides using lipofectamine2000 were injected into the right armpit of 4- to 6-week old BALB/c nu/nu nude mice. Mice were housed in a barrier facility on a high efficiency particulate arrestance (HEPA)-filtered rack under standard conditions of 12-hour light/dark cycles. The animals were fed an autoclaved laboratory rodent diet. Tumor length, width and mouse body weight were measured twice in a week. Tumor volume was calculated by following formula: tumor volume (mm³)=length (mm)×width (mm)×width (mm)×1/2. Data was presented as mean±SD. After 4 weeks, mice were humanely sacrificed and the tumors were removed, then the tumors were weighed and photographed. The tumors were soaked in formalin for further experiments.

Immunohistochemistry (IHC) and Evaluation

The IHC and assessment were carried out as previously described.²⁵ The following primary antibody used was *TRAF6* antibody (1:250 affinity). The expression of *TRAF6* was evaluated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc. USA). Semiquantitatively analyzed the expression of *TRAF6* in each group using Mean Optical Density (MOD) = the Integral Optical Density (IOD)/the positive area.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, CA, USA). Data were analyzed by Student's *t*-test. The data were expressed as the mean $\pm$ standard deviation. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

The Expression Levels of miRNA-140 in Colorectal Cancer Tissues and Cells

We determined the *miRNA-140* expression in CRC tissues and normal paired colorectal tissues which were cultured in a medium for 48 h. *miRNA-140* is down-regulated in the CRC tissues compared with normal colorectal tissues (Figure 1A). And *miRNA-140* expression is lower in SW480 and HCT116 cells than FHC cells which is normal for colorectal epithelial cells (Figure 1B).

Over-Expression of miRNA-140 Suppressed the Proliferation, Migration and Invasion of SW480 and HCT116 Cells

To determine the influence of *miRNA-140* on the colorectal cancer cells, we evaluated the proliferation, migration and invasion capacities using a series of cell biology assays in vitro. As shown in Figure 2A and B, *miRNA-140* mimic transfection markedly enhanced the *miRNA-140* expression in SW480 and HCT116 cells, respectively. Over-expression of *miRNA-140* significantly decreased cell proliferation capacity in SW480 and HCT116 cells (Figure 2C and D). Wound healing and cell migration assays showed that over-expression of *miRNA-140* significantly inhibited SW480 and HCT116 cells migration, respectively, compared with *miRNA-140* NC controls (Figure 2E–H and I–K). The invasion transwell assays showed that over-expression of *miRNA-140* could significantly weaken the invasive abilities of SW480 and HCT116 cells (Figure 2L–N). These data indicated that over-expression *miRNA-140* could inhibit CRC growth and progression in vitro.

miRNA-140 Post-Transcriptionally Regulates TRAF6 Expression

To further investigate the reason of *miRNA-140* regulating the expression of *TRAF6*, we carried out bioinformatics analysis using related prediction software. As illustrated in

Figure 1 The expression of *miRNA-140* is down-regulated in the colorectal cancer and cells. (A) The *miRNA-140* expressions were decreased in colorectal cancer tissues (T) compared with normal colorectal tissues (N). (B) The *miRNA-140* expression was significantly lower in SW480 and HCT116 cells than that in FHC cells which is the normal colorectal epithelial cell. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2 Over-expression of *miRNA-140* suppressed the proliferation, migration and invasion of SW480 and HCT116 cells. (A and B) *miRNA-140* mimic transfection were markedly increased the expression of *miRNA-140* compared with *miRNA-140* NC transfection in SW480 and HCT116 cells, respectively. (C and D) CCK-8 assays showed that over-expression of *miRNA-140* significantly inhibited the proliferation ability of SW480 and HCT116 cells. (E, F, G and H) Wound healing experiments showed that over-expression of *miRNA-140* significantly inhibited the migration of SW480 and HCT116 cells. (I, J and K) Transwell migration assays reported that over-expression of *miRNA-140* significantly suppressed the migration of SW480 and HCT116 cells. (L, M and N) Transwell invasion assays reported that over-expression of *miRNA-140* significantly suppressed the invasion of SW480 and HCT116 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3A, a putative binding site for *miRNA-140* was identified in the 3'UTR of *TRAF6* mRNA. *miRNA-140* over-expression inhibited wild-type *TRAF6* 3'UTR-

luciferase pGL4.1-wt*TRAF6* reporter vector in SW480 and HCT116 cells (Figure 3B and D). *miRNA-140* over-expression did not affect the luciferase activity of

Figure 3 *miRNA-140* inhibited *TRAF6* expression through targeting the 3' UTR area. (A) The wild-type 3'UTR of *TRAF6* mRNA includes a putative *miRNA-140* binding site. The mutant sequence was indicated with green letters. (B) Relative luciferase activity in SW480 cells co-transfected with *miRNA-140* NC, *miRNA-140* mimic and pGL4.1-wt*TRAF6* reporter vectors. (C) Relative luciferase activity in SW480 cells co-transfected with *miRNA-140* NC, *miRNA-140* mimic and pGL4.1-mut*TRAF6* reporter vectors. (D) Relative luciferase activity in HCT116 cells co-transfected with *miRNA-140* NC, *miRNA-140* mimic and pGL4.1-wt*TRAF6* reporter vectors. (E) Relative luciferase activity in HCT116 cells co-transfected with *miRNA-140* NC, *miRNA-140* mimic and pGL4.1-mut*TRAF6* reporter vectors. ** $p < 0.01$.

pGL4.1-mut*TRAF6* reporter vector in SW480 and HCT116 cells (Figure 3C and E).

Over-Expression of *miRNA-140* Inhibited *TRAF6* Expression

To determine the effects of *miRNA-140* over-expression, we tested the mRNA and protein expression levels of *TRAF6*, after transfection *miRNA-140* NC and *miRNA-*

140 mimic for 48 h. The *TRAF6* mRNA was significantly inhibited by *miRNA-140* mimic transfection compared with *miRNA-140* NC transfection in SW480 and HCT116 cells (Figure 4A and C). Consistent with the *TRAF6* mRNA situation, *TRAF6* protein expression was also markedly suppressed by *miRNA-140* mimic transfection compared with *miRNA-140* NC transfection in SW480 and HCT116 cells (Figure 4B and D).

Figure 4 Over-expression of *miRNA-140* inhibited *TRAF6* expression in colorectal cancer cells. **(A)** qPCR analysis of the expression after transfection *miRNA-140* NC and *miRNA-140* mimic in SW480 cells, the *TRAF6* mRNA expression levels were significantly inhibited by over-expression of *miRNA-140*. **(B)** Western blot analysis of the expression after transfection *miRNA-140* NC and *miRNA-140* mimic in SW480 cells, the *TRAF6* protein expression levels were significantly inhibited by over-expression of *miRNA-140*. **(C)** qPCR analysis of the expression after transfection *miRNA-140* NC and *miRNA-140* mimic in HCT116 cells, the *TRAF6* mRNA levels were significantly inhibited by over-expression of *miRNA-140*. **(D)** Western blot analysis of the expression after transfection *miRNA-140* NC and *miRNA-140* mimic in HCT116 cells, the *TRAF6* protein levels were significantly inhibited by over-expression of *miRNA-140*. * $p < 0.05$; ** $p < 0.01$.

Over-Expression of *miRNA-140* Suppressed the NF- κ B/c-Jun Signaling Pathway Proteins Expression

Because over-expression of *miRNA-140* inhibited the *TRAF6* expression, we next determined the expression of NF- κ B/c-jun signaling pathway which was regulated by *TRAF6*. After transfection of HCT116 cells with *miRNA-140* NC and *miRNA-140* mimic for 48 h, Western blot analysis showed that p-NF- κ B and p-c-jun proteins were significantly inhibited by the *miRNA-140* over-expression compared with *miRNA-140* NC controls (Figure 5A and B). However, the proteins expression of NF- κ B and c-jun were not changed in

both *miRNA-140* NC and *miRNA-140* mimic over-expression (Figure 5A and B).

Over-Expression of *miRNA-140* Inhibited the Growth of Colorectal Cancer in vivo

To determine the effects of over-expression of *miRNA-140* on colorectal cancer in vivo, 4×10^6 *miRNA-140* NC or *miRNA-140* mimic HCT116 cells were injected into the right armpit of nude mice, respectively. After 4 weeks, we sacrificed the mice and found that the tumor growths were significantly inhibited in the *miRNA-140* mimic group compared with *miRNA-140* NC group (Figure 6A). Similarly, the weight of

Figure 5 Over-expression of *miRNA-140* decreased the activity of NF-κB/c-jun signaling pathways in HCT116 cells. **(A)** Western blot analysis found that over-expression of *miRNA-140* markedly inhibited the p-NF-κB/p-c-jun proteins expression. **(B)** The histogram of NF-κB/c-jun signaling pathway is derived from A. ** $p < 0.01$.

tumors in the *miRNA-140* mimic group were lighter than that in the *miRNA-140* NC group (Figure 6B). We made a tumor volume growth curve, and found that the speed of growth of tumors in the *miRNA-140* mimic group were significantly suppressed compared with the *miRNA-140* NC group (Figure 6C). We further carried out immunohistochemistry of tumors and found that the expression of *TRAF6* was significantly suppressed in the *miRNA-140* mimic group compared with *miRNA-140* NC group (Figure 6D and E).

Discussion

TRAF6 is an important signal transduction molecule of the toll-like receptor (TLR) pathway, which plays a key role in innate and adaptive immunity.^{26,27} Recently studies have showed that *TRAF6* functions as a cancer gene and has involvement in tumor malignancy.^{7,8,28,29} Our previous study also showed that *TRAF6* promotes the progression and growth of colorectal cancer.¹¹ In our previous study, we found that if we tested the *miRNA-140* expression directly its expression levels were higher in colorectal cancer tissues than that in normal colorectal tissue. So in this study, we cultured the colorectal cancer tissues and normal colorectal tissues in the medium for 48 h. Then we determined that the expression levels of *miRNA-140* in colorectal cancer tissue was lower than colorectal normal tissues. The reason may be LPS in the colorectal cancer tissues increased the expression of *miRNA-140*, because we previously found that LPS concentration in the

colorectal cancer tissues was higher than that in normal cancer tissues.³⁰ Consistent with cell lines *miRNA-140* expression, we found *miRNA-140* expression was highest in FHC cells than in SW480 and HCT116 cells.

Liu et al¹⁸ reported that *miRNA-140* suppressed the proliferation and progression of Wilms' tumor. Takata et al¹⁹ showed that *miRNA-140* inhibited liver tumor progression. In the present study, we found that *miRNA-140* inhibited the proliferation, migration and invasion in colorectal cancer. As we expected, over-expression of *miRNA-140* inhibited the growth of colorectal cancer in vivo.

miRNAs are a class of evolutionarily conserved, non-protein coding RNAs, which are found in numerous organisms and tissues.^{31,32} miRNAs has been thought a novel method of gene expression regulation and through the transcriptional regulation regulated gene expression levels.^{12,13} To further investigate the mechanisms of how *miRNA-140* inhibited the progression of colorectal cancer, we found *miRNA-140* targeted the 3'UTR of *TRAF6*, then made the *TRAF6* mRNA degradation.

Recently studies have revealed that *TRAF6* could activate the NF-κB/c-jun signaling pathway. Song et al³³ reported that *TRAF6* activates NF-κB/c-jun, because *TRAF6* and NF-κB-inducing kinase (NIK) bind indirectly. Some studies showed *TRAF6* could be recruited to other signaling pathways (IL-1, IL-6, IL-8 and LPS) as a very important adapter protein to activate the NF-κB/c-jun signaling pathway.^{34–36} In our previous study, we also found

A miRNA-140 NC miRNA-140 mimic **B** **C** **D**

miRNA-140 NC

miRNA-140 mimic

E

Figure 6 Over-expression of *miRNA-140* suppressed growth of colorectal cancer in vivo. **(A)** Tumors in nude mice were harvested 4 weeks after initiation. The *miRNA-140* mimic group tumor sizes were smaller than those in the *miRNA-140* NC group. **(B)** After tumors were harvested, the weights of tumors were weighed, the *miRNA-140* mimic group tumor weights were significantly reduced compared with *miRNA-140* NC group. **(C)** The growth curves of tumors showed that the *miRNA-140* mimic group tumors volume were markedly inhibited compared with the *miRNA-140* NC group. **(D)** Immunohistochemistry analysis of expression of *TRAF6* in subcutaneous xenograft tumors showed *TRAF6* expression in *miRNA-140* mimic group were decreased compared with *miRNA-140* NC group. **(E)** The histogram of MOD of *TRAF6* expression originated from **(D)**. * $p < 0.05$, ** $p < 0.01$.

that *TRAF6* could inhibit the NF- κ B/c-jun signaling pathway.¹¹ So, we determined the change of the NF- κ B/c-jun signaling pathway after transfection with *miRNA-140*

NC and mimic for 48 h. Consistent with our expectations, we found that the NF- κ B/c-jun signaling pathway was inhibited.

Figure 7 Schematic diagram of *miRNA-140* regulation mechanism of *TRAF6*. Briefly, *miRNA-140* binds with the *TRAF6* mRNA 3'UTR area, then makes the *TRAF6* mRNA degrade. Therefore, *TRAF6* protein expression is reduced which reduces the activity of NF-κB/c-jun signaling pathways and decreases NF-κB/c-jun regulated related genes expression.

Conclusion

In the present study, we found that *miRNA-140* inhibited the proliferation, migration and invasion in colorectal cancer. Over-expression of *miRNA-140* inhibited the growth of colorectal cancer in vivo. The mechanism studies found that *miRNA-140* binds to *TRAF6* mRNA 3'UTR, then caused *TRAF6* mRNA degradation and *TRAF6* protein expression decreased which reduced the activities of NF-κB/c-jun signaling pathways, influencing the expression of related genes. The relevant schematic is shown in Figure 7.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA cancer J clin.* 2018;68(1):7–30.
2. Wang Z, Yip LY, Lee JHJ, et al. Methionine is a metabolic dependency of tumor-initiating cells. *Nat med.* 2019;25(5):825–837.
3. Andre T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med.* 2004;350(23):2343–2351.
4. Yang AD, Fan F, Camp ER, et al. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res.* 2006;12(14 Pt 1):4147–4153.
5. Lin FT, Lin VY, Lin VT, Lin WC. TRIP6 antagonizes the recruitment of A20 and CYLD to TRAF6 to promote the LPA2 receptor-mediated TRAF6 activation. *Cell Discovery.* 2016;2.
6. Panda S, Nilsson JA, Gekara NO. Deubiquitinase MYSM1 Regulates Innate Immunity through Inactivation of TRAF3 and TRAF6 Complexes. *Immunity.* 2015;43(4):647–659.
7. Luo Z, Zhang X, Zeng W, et al. TRAF6 regulates melanoma invasion and metastasis through ubiquitination of Basigin. *Oncotarget.* 2016;7(6):7179–7192.
8. Han F, Zhang L, Qiu W, Yi X. TRAF6 promotes the invasion and metastasis and predicts a poor prognosis in gastric cancer. *Pathol Res Pract.* 2016;212(1):31–37.
9. Han Q, Yao F, Zhong C, Zhao H. TRAF6 promoted the metastasis of esophageal squamous cell carcinoma. *Tumour Biology.* 2014;35(1):715–721.
10. Li J-A, Kuang T, Pu N, et al. TRAF6 regulates YAP signaling by promoting the ubiquitination and degradation of MST1 in pancreatic cancer. *Clinical and Experimental Medicine.* 2019;19(2):211–218. doi:10.1007/s10238-018-00543-6

11. Zhu G, Cheng Z, Huang Y, et al. TRAF6 promotes the progression and growth of colorectal cancer through nuclear shuttle regulation NF- κ B/c-jun signaling pathway. *Life Sci.* **2019**;235:116831. doi:10.1016/j.lfs.2019.116831
12. Yeh CH, Moles R, Nicot C. Clinical significance of microRNAs in chronic and acute human leukemia. *Mol Cancer.* **2016**;15(1):37.
13. Peng S, Gao D, Gao C, Wei P, Niu M, Shuai C. MicroRNAs regulate signaling pathways in osteogenic differentiation of mesenchymal stem cells (Review). *Mol Med Rep.* **2016**;14(1):623–629.
14. Fadaka AO, Pretorius A, Klein A. Biomarkers for Stratification in Colorectal Cancer: microRNAs. *Cancer Control.* **2019**;26(1):1073274819862784.
15. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol.* **2010**;10(2):111–122.
16. Gammell P. MicroRNAs: recently discovered key regulators of proliferation and apoptosis in animal cells: identification of miRNAs regulating growth and survival. *Cytotechnology.* **2007**;53(1–3):55–63.
17. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet.* **2004**;5(7):522–531.
18. Liu Z, He F, OuYang S, et al. miR-140-5p could suppress tumor proliferation and progression by targeting TGFBR1/SMAD2/3 and IGF-1R/AKT signaling pathways in Wilms' tumor. *BMC Cancer.* **2019**;19(1):405.
19. Takata A, Otsuka M, Yoshikawa T, et al. MicroRNA-140 acts as a liver tumor suppressor by controlling NF- κ B activity by directly targeting DNA methyltransferase 1 (Dnmt1) expression. *Hepatology.* **2013**;57(1):162–170.
20. Wolfson B, Eades G, Zhou Q. Roles of microRNA-140 in stem cell-associated early stage breast cancer. *BMC Cancer.* **2014**;6(5):591–597.
21. Chen B, Liu B. MiRNA-381 inhibits the invasion of renal carcinoma and the underlying mechanisms. *J Central South Univ Med Sci.* **2015**;40(10):1053–1059.
22. Li D, Bao P, Yin Z, et al. Exploration of the involvement of LncRNA in HIV-associated encephalitis using bioinformatics. *PeerJ.* **2018**;6:e5721.
23. Zhu G, Ye J, Huang Y, et al. Receptor-interacting protein-1 promotes the growth and invasion in gastric cancer. *Int J Oncol.* **2016**;48(6):2387–2398.
24. Zhu G, Du Q, Wang X, Tang N, She F, Chen Y. TNF- α promotes gallbladder cancer cell growth and invasion through autocrine mechanisms. *Int J Mol Med.* **2014**;33(6):1431–1440.
25. Zhu G, Chen X, Wang X, et al. Expression of the RIP-1 gene and its role in growth and invasion of human gallbladder carcinoma. *Cellular Physiol Biochem.* **2014**;34(4):1152–1165.
26. Zhang J, Zhu Y, Chen Z, Li C, Zhao X, Kong X. Molecular cloning and expression analysis of MyD88 and TRAF6 in Qihe crucian carp *Carassius auratus*. *Fish Shellfish Immunol.* **2019**;87:829–838.
27. Wang P, Zhang Z, Xu Z, Guo B, Liao Z, Qi P. A novel invertebrate toll-like receptor with broad recognition spectrum from thick shell mussel *Mytilus coruscus*. *Fish Shellfish Immunol.* **2019**;89:132–140.
28. Yang WL, Wang J, Chan CH, et al. The E3 ligase TRAF6 regulates Akt ubiquitination and activation. *Science.* **2009**;325(5944):1134–1138.
29. Zhang X, Wu L, Xiao T, et al. TRAF6 regulates EGF-induced cell transformation and cSCC malignant phenotype through CD147/EGFR. *Oncogenesis.* **2018**;7(2):17.
30. Zhu G, Huang Q, Huang Y, et al. Lipopolysaccharide increases the release of VEGF-C that enhances cell motility and promotes lymphangiogenesis and lymphatic metastasis through the TLR4-NF- κ B/JNK pathways in colorectal cancer. *Oncotarget.* **2016**;7(45):73711–73724.
31. Zhu Z, Tang J, Wang J, Duan G, Zhou L, Zhou X. MiR-138 Acts as a Tumor Suppressor by Targeting EZH2 and Enhances Cisplatin-Induced Apoptosis in Osteosarcoma Cells. *PLoS One.* **2016**;11(3):e0150026.
32. Wu S, Du X, Wu M, Du H, Shi X, Zhang T. MicroRNA-409-3p inhibits osteosarcoma cell migration and invasion by targeting catenin- δ 1. *Gene.* **2016**;584(1):83–89.
33. Song HY, Regnier CH, Kirschning CJ, Goeddel DV, Rothe M. Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor- κ B and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A.* **1997**;94(18):9792–9796.
34. Schwandner R, Yamaguchi K, Cao Z. Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction. *J Exp Med.* **2000**;191(7):1233–1240.
35. Ye X, Wu H, Sheng L, et al. Oncogenic potential of truncated RXR α during colitis-associated colorectal tumorigenesis by promoting IL-6-STAT3 signaling. *Nat Commun.* **2019**;10(1):1463.
36. Li ZW, Sun B, Gong T, et al. GNAI1 and GNAI3 Reduce Colitis-Associated Tumorigenesis in Mice by Blocking IL6 Signaling and Down-regulating Expression of GNAI2. *Gastroenterology.* **2019**;156(8):2297–2312.

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