Original Article
Resveratrol restrains colorectal cancer metastasis by regulating miR-125b-5p/TRAF6 signaling axis

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Abstract: Colorectal cancer is one of the most common malignancies with a high incidence, metastatic tendency and low 5-year survival rate. Resveratrol, a polyphenolic compound has been shown to inhibit colorectal cancer metastasis in recent studies. Its underlying molecular mechanism remains to be elucidated. Our findings demonstrated that miR-125b-5p, acting as a tumor suppressor, was conspicuously down-regulated in both colorectal cancer tissues and cell lines. The expression of miR-125b-5p negatively correlated with the expression of its direct target TNF receptor associated factor 6 (TRAF6). Both miR-125b-5p overexpression and TRAF6 knockdown inhibited metastasis of colorectal cancer cells. In addition, we uncovered that resveratrol up-regulated miR-125b-5p by increasing its stability and suppressed TRAF6-induced signal pathway in a dose/time-dependent manner. Resveratrol could significantly curtail the migration and invasion of colorectal cancer cells, which was counteracted by miR-125b-5p knockdown or TRAF6 overexpression. These results indicated that resveratrol could restrain colorectal cancer metastasis by promoting miR-125b-5p/TRAF6 signaling axis. Furthermore, lung metastasis models of colorectal cancer were constructed by tail vein injection. Down-regulation of miR-125b-5p could facilitate colorectal cancer metastasis in vivo, which could be impeded by resveratrol. In conclusion, our findings delineated the miR-125b-5p/TRAF6 signaling axis as a novel molecular mechanism underlying the metastatic process in colorectal cancer, as well as a prospective therapeutic target. Resveratrol disrupts colorectal cancer metastasis by activating miR-125b-5p/TRAF6 signal pathway and might improve the clinical outcome of colorectal cancer patients with low expression of miR-125b-5p.

Keywords: Resveratrol, miR-125b-5p, metastasis, TRAF6, colorectal cancer

Introduction

Colorectal cancer (CRC) is a common malignancy with a high morbidity and mortality [1]. Current estimations anticipate a total of 153,020 new cases to be diagnosed in 2023. Concurrently, the expected mortality associated with colorectal cancer for the same period stands at 52,550 deaths [2]. Approximately 20%, present with metastatic disease at the time of initial diagnosis. Furthermore, an estimated 25% of patients initially diagnosed with localized tumors will subsequently develop metastasis [3]. The 5-year death rate for patients with metastatic colorectal cancer is more than 80% [4]. Certainly, the advancement of scientific and technological diagnostic methods has contributed to substantial progress in the diagnosis and treatment of colorectal cancer [5]. However, in most cases, metastatic colorectal cancer remains a difficult disease to cure [6]. Indeed, the complexity and severity of metastasis in colorectal cancer necessitate continued in-depth investigation.

MicroRNA (miRNA) represents a class of endogenous small non-coding RNAs, typically comprised of 20-23 nucleotides. These minuscule

molecules have attracted considerable attention within the scientific community, largely due to their novel function as gene regulators. The intrinsic capacity of miRNAs to modulate gene expression post-transcriptionally positions them as vital participants in an array of physiological processes, ranging from cellular differentiation and proliferation to apoptosis [7]. Because of the important regulatory role in tumorigenesis and development, the miRNAs are often used as a therapeutic target for cancer. Indeed, a growing body of literature has revealed the specific role of miR-125b-5p as a key modulator in the context of breast cancer. Acting as a tumor suppressor, miR-125b-5p has been found to exert an inhibitory effect on various cancer-associated processes within tumor cells [8-11]. For example, miR-125b-5p inhibits the growth and invasion of gastric cancer by targeting RYBP [12]. MiR-125b-5p is involved in sorafenib resistance through ataxin-1-mediated epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma [13]. In the context of colorectal cancer, the miR-125b-5p/Sema4D axis has been identified as an intriguing area of focus, with available evidence pointing to its role in inhibiting both invasion and proliferation of cancer cells [9]. However, the specific molecular pathway through which the miR-125b-5p exerts its inhibitory action on colorectal cancer cells remains incompletely understood.

Resveratrol (3,5,4 '-trihydroxystilbene) belongs to the stilbene class of compounds within the polyphenol family. This naturally occurring molecule is found in a diverse array of plant sources, including but not limited to peanuts, blueberries, cranberries, beans, rhubarb, grapes, and eucalyptus [14]. Resveratrol's prevalence across a variety of plant species indeed enables its extraction in a pure form [15]. Several studies have highlighted the efficacy of resveratrol in the treatment of metabolic disorders such as diabetes [16], cardiovascular diseases [17], and various cancers [18]. Furthermore, an intriguing aspect of resveratrol's anticancer properties lies in its ability to inhibit EMT of tumor cells [19]. In our prior research, we uncovered a specific mechanism through which resveratrol may exert its anticancer effects on colorectal cancer, specifically inhibiting migration and invasion of cancerous cells. This action appeared to be mediated via the AKT/GSK-3β/SNAIL signaling pathway, a critical nexus in the regulation of various cellular functions, including cell survival, growth, and motility [20]. Its mechanism of its action remained unclear, and needed further investigation.

This study mainly revealed the relationship between resveratrol, miR-125b-5p and colorectal cancer metastasis. The expression of miR-125b-5p was down-regulated and could inhibit the metastasis in colorectal cancer. Additionally, resveratrol impeded the migration and invasion of colorectal cancer by promoting miR-125b/TRAF6 signal pathway. The metastasis induced by miR-125b-5p low-expression could be antagonized resveratrol in vivo. These results elucidated that resveratrol might be effective as an anti-metastasis drug for colorectal cancer patients.

Materials and methods

Cell culture

Human normal intestinal epithelial cell lines NCM-460 and colorectal cancer cell lines HCT-8, HCT-116, RKO, SW480, SW620 were purchased from the Cell Bank of the Committee for Typical Culture and Preservation of the Chinese Academy of Sciences (Shanghai, China). These cell lines were supplemented with 10% fetal bovine serum (FBS; PWL001, Meilunbio, Dalian, China) and 1% penicillin/streptomycin in RPMI-1640 (CR-31800-S, Cienry, Zhejiang, China) or DMEM (CR-12800-S, Cienry, China) high-glucose medium. The cells were cultured in a constant temperature incubator at 37°C and 5% carbon dioxide.

Transient transfection and reagents

The miR-125b-5p mimics, miR-125b-5p inhibitor, as well as the corresponding negative control, were synthesized by RiboBio (Guangzhou, China). Transfected cells using the riboFECT CP Transfection Kit, the specific sequences were as follows: miR-125b-5p mimics: 5'-UCCCU-GAGACCCUAACUUGUGA-3', miR-125b-5p inhibitor: 5'-UCACAAGUUAGGGUCUCAGGGA-3', miR-125b-5p mimics NC: 5'-UUUGUACUACACAA-AAGUACUG-3', miR-125b-5p inhibitor NC: 5'-CAGUACUUUUGUGUAGUACAAA-3'.

The overexpressed plasmid or siRNA of the indicator gene was transfected into cells with jetPRIME (Cat. 101000046, polyplus, France) and analyzed 48 h later. The specific sequenc-

es were as follows: si-TRAF6-1 is 5'-CGGA-AUUUCCAGGAAACUAUUTT-3', si-TRAF6-2 is 5'-UGGAUUCUACACUGGCAAATT-3', si-NC is 5'-ACTACTGAGTGACAGTAGA-3'.

RNA isolation and reverse transcription (RT)-PCR assay

Total RNA was isolated from samples using a MiPure Cell/Tissue miRNA Kit (RC201, Vazyme, Nanjing, China). RNA was reverse-transcribed into cDNA using miRNA and mRNA 1st Strand cDNA Synthesis Kit (MR201-01, Vazyme, China). The expression levels of miRNA and mRNA were detected by qRT-PCR. SYBR qPCR Master Mix (MQ101-01, Vazyme, China) was used to quantify the transcribed cDNA with the Biorad CFx96 real-time PCR System (Bio-Rad CFX Manager 3.1). The endogenous reference of miRNA was U6, and the endogenous reference of mRNA was β-actin. $2^{-\Delta\Delta Ct}$ (2-delta-CT) method was used to calculate the relative expression of related genes [21]. These primers were synthesized by Tsingke Biotechnology, and the specific sequences are shown in Table S1.

CCK8 detection

HCT-8, HCT-116 and RKO cells with 1×10^3 cells/Wells were planted in 96-well plates. After 24 h, resveratrol (cat. SRT501, MedChemExpress, New jersey, America) was added into the cells at different concentrations (0, 2, 5, 10, 20, 40, 100, 200, 400 µmol/l) and incubated for 48 h. Incubated with CCK-8 reagent (cat. HY-K0301, MedChemExpress, America) for 1 h, then measured optical density (OD) at 450 nm using a microplate reader (BioRad Laboratories, Inc.). The formula was as follows: cell survival rate (%) = (Average OD of treatment group - average OD repeats of blank group)/(average OD repeats of control group - average OD of blank group) ×100%.

Western blotting

Protein was extracted from treated cells or tissues by RIPA lysis with protease and phosphatase inhibitors. The protein concentration was determined by BCA method. Electrophoresis was performed using SurePAGE™ (M00657, GenScript, New jersey, America), a prefabricated adhesive, and bands were transferred to polyvinylidene fluoride (PVDF, ISEQ00010, Millipore, America) membranes using a rapid

membrane transfer apparatus. The primary antibody was incubated overnight with 5% skim milk prepared with TBST for 1 h. On the second day, after incubation with the second antibody for 1 h, the immune response signal was detected by enhanced chemiluminescence (ECL, E411-04, Vazyme, China) technique. The primary antibodies used in this study were GAPDH (AC033, ABclonal, Wuhan, China, 1:10000), TRAF6 (EP592Y, Abcam, Cambridge, Britain, 1:5000), SNAIL (A5243, ABclonal, China, 1:1000), AKT (#9272, Cell Signaling, Boston, America, 1:1000), p-AKT (AP0140, ABclonal, China, 1:1000), GSK-3β (#AF5016, Affinity, Texas, America, 1:1000), p-GSK-3β (AP0039, ABclonal, China, 1:1000), E-cadherin (20874-1-AP, Proteintech, Chicago, America, 1:20000), N-cadherin (A19083, ABclonal, China, 1:1000).

Colony formation assay

The cells (5×10^3) were inoculated into 6 cm petri dishes and cultured for 10 days. After various treatments, the cells were treated with methanol and 0.1% crystal violet. The colonies of more than 50 cells were counted after taking photos. Colony formation ability was represented by colony number/number of inoculated cells ×100%.

Scratch wound healing assay

To evaluate cell migration, 70 ul of the treated HCT-8, HCT-116, and RKO (6×10^4) cells were placed on either side of the scratch plugin (cat. 80206, ibidi, Martinrad, Germany) and cultured in 5% CO_2 at 37°C for 24 h before removing the plugin. Look under a light microscope and take pictures. At the same time, the culture medium was changed to a serum concentration of 1%. 24 h later, the relative distance of scratches was observed under the optical microscope and photographed, and the ImageJ software was used for evaluation.

Trans-well migration and invasion (Matrigel) experiments

For cell migration, trans-well migration analysis was performed using a 24-well chamber (cat. 3422, Corning, New York, America). The treated HCT-8 (6×10^4), HCT-116 (6×10^4) and RKO (2×10^5) cell lines were inoculated into FBS-free medium in the upper cavity, and 600 ul medium containing 10% FBS was added into the

lower cavity. After 24 h, stain was used with methanol and 0.1% crystal violet.

For cell invasion, 50 ul matrigel gel (Corning Company) diluted in a ratio of 1 to 8 with the culture medium was evenly applied to the upper chamber (24 Wells) and solidified. The treated HCT-8 (3×10^5), HCT-116 (3×10^5) and RKO (1×10^6) cell lines were inoculated into the culture medium without FBS in the upper chamber. Then 600 ul culture solution containing 10% FBS was added into the lower cavity. The cells were collected and stained 48 hours later. Finally, the cells were counted after being photographed by microscope (Olympus, Japan).

Dual luciferase reporter assay

In order to verify whether miR-125b-5p directly targets TRAF6, we designed and ordered wild-type (WT) and mutant (MUT) reporter genes with mutant TRAF6 binding sites from REPOBIO. HCT-8 and HCT-116 were implanted into 24-well plates and co-transfected with mimic miR-125b-5p or controls, and WT and MUT reporter genes were added. After 48 h transfection, the relative luciferase activity was analyzed using a dual luciferase reporting kit (cat. DL101-01, Vazyme, China).

Animal experiment

Female BALB/c non-thymus (NU/NU) nude mice aged 3 to 5 weeks were placed in the Animal Experimental Research Center of Zhejiang Chinese Medicine University. This animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medicine University. HCT-8 luciferase cells (FocusHerb, Shaanxi, China) were collected, and the cell density was adjusted to 1×10^8 cells/ml with normal saline. The tail vein of each nude mouse was inoculated with 0.1 ml cell suspension to establish the lung metastasis model of colorectal cancer [22]. Two weeks later, fluorescence imaging was performed in vivo to check whether the modeling was successful. Nude mice were randomly divided into four groups as normal control (NC) group, Resveratrol (Res) group, miR-125b-5p inhibitor (miR-125b i) group, miR-125b-5p inhibitor and Resveratrol (miR-125b i+Res) group. In the miR-125b i group and miR-125b i+Res group, the mice were injected intraperitoneally with AntagomiRs (RiboBio, China) at a dose of

100 mg/kg wt in 0.2 ml saline once every 3 days for 2 weeks. A scramble antagomiR (anta-Ctl) was used as control. In the Res group and miR-125b i+Res group, the mice were given 150 mg/kg resveratrol once a day for 2 weeks. Mice in the control group were given the same amount of normal saline once a day for 2 weeks. After the last intragastric administration and four hours of fasting and water deprivation, lung metastases were detected by in vivo fluorescence imaging. Cervical vertebrae of nude mice were removed and sacrificed. Blood from eyeballs was taken to detect liver and kidney function. Lung tissue, liver, kidney tissue and spleen tissue were taken for follow-up experiments.

In vivo fluorescence imaging

Mice were intraperitoneally injected with 150 mg/kg body weight D-luciferin Amricam potassium salt (cat. MB1834-2, meilunbio, China). In vivo imaging was performed 15 minutes later. 2 minutes before imaging, nude mice were treated by continuous inhalation of 2% isoflurane (cat. O21400, BD-Difco, New York, American) enters a coma. Using cooled charge-coupled device (CCD) Imaging system (IVIS Imaging system, PerkinElmer, America) imaging and collecting the flux of all detected photon counts within the designated region of interest within the tumor region, using the live Image software package (Xenogen, America) to calculate signal strength.

Hematoxylin and eosin (H&E) staining and immunohistochemical analysis

For hematoxylin and eosin (H&E) staining, all specimen tissues were fixed in paraformaldehyde at 4°C overnight and were cut in paraffin. Dyed with hematoxylin and eosin (H&E) (ZLI-9609, Ollie Gene Technologies) and use microscopy to observe histological changes in tumor tissue (ix71; Olympus, Japan).

For immunohistochemistry, paraffin-embedded tissue was dewaxed and antigen repaired for 30 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 solution. Block with 10% FBS blocking solution (Gicbo, America) at room temperature for 30 min, then add the primary antibody of E-cadherin, N-cadherin, SNAIL and Ki67 and incubate at 4°C overnight. PBS was used as negative control instead of prima-

ry antibody. Finally, DAB (Kanglang, China) was used for color development. Immunohisto-chemical scoring criteria were formulated by two pathologists in our hospital. Each experiment was repeated at least 3 times.

Statistical analysis

All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). Using GraphPad Prism 8.0 software (Graph-Pad) Analyze the statistical results. Quantitative data were compared using a Two-tailed t test (paired or unpaired), qualitative data were evaluated by an x^2 test, and linear regression was used for correlation analysis of the two genes. $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

Result

MiR-125b-5p inhibits metastasis and proliferation of colorectal cancer

The recent literatures have provided compelling evidence that the decreased expression of miR-125b-5p plays an integral role in the advancement of oncogenic processes [23, 24]. Analysis of data derived from The Cancer Genome Atlas (TCGA) database revealed a significant reduction in miR-125b-5p levels within colorectal cancer tissues as compared to healthy intestinal tissues (**Figure 1A**). In a comparative analysis between normal intestinal epithelial cells and colorectal cancer cells, the expression level of miR-125b-5p was found to be significantly down-regulated in colorectal cancer cells (**Figure 1B**). To illustrate the specific role of miR-125b-5p in colorectal cancer cells, we conducted an experiment utilizing HCT-8 cells. These cells were transfected with either miR-125b-5p mimics or inhibitors, leading to an elevation or suppression of miR-125b-5p levels respectively (**Figure 1C**). Cell proliferation was inhibited in the miR-125b-5p mimics group and promoted in the miR-125b-5p inhibitor group (**Figure 1D**). Moreover, overexpression of miR-125b-5p could inhibit the migration and invasion of HCT-8 cells (**Figure 1E**, **1F**). Results demonstrated that transfection with miR-125b-5p mimics led to an increase in the EMT marker protein E-cadherin (E-cad), while simultaneously resulting in a decrease in

N-cadherin (N-cad) and SNAIL (**Figure 1G**, **1H**). Conversely, in the group treated with the miR-125b-5p inhibitor, the trend was distinctly inverted, leading to the promotion of metastatic capacity in HCT-8 cells (**Figure 1I**, **1J**). The EMT marker proteins E-cad was decreased, N-cad and SNAIL were increased (**Figure 1K**, **1L**). The similar results were detected in RKO cells (Figure S1). These results suggest that miR-125b-5p suppresses metastasis and proliferation of colorectal cancer and is under-expressed in colorectal cancer.

MiR-125b-5p targets TRAF6 and reduces its expression

Previous researches have shown a strong correlation between TRAF6 and the metastatic progression of colorectal cancer [25, 26]. Potential targets of miR-125b-5p were predicted using Targetsscan, miRBD, miRWalk and starBase databases, and TRAF6 was identified as their common intersection (**Figure 2A**). In order to determine whether miR-125b-5p directly interacted with TRAF6, the dual luciferase reporting assay was performed. Our results revealed that the fluorescence signal of the miR-125b-5p mimics-Wt (wild type) group was notably diminished. However, this reduction was effectively rescued in the miR-125b-5p mimics-Mut (mutant) group (**Figure 2B**, **2C**). We observed that the expression of TRAF6 was effectively inhibited in those with high miR-125b-5p expression. Conversely, cell lines with low miR-125b-5p expression exhibited a promotion of TRAF6 expression (**Figure 2D**, **2E**). These data demonstrated that miR-125b-5p directly engaged with TRAF6, leading to an inhibition of its expression. Furthermore, our experiments involving miR-125b-5p mimics revealed a reduction in both TRAF6 mRNA and protein levels, displaying a distinct time-dependent relationship (**Figure 2F**, **2G**). Previous literature has elucidated that the AKT/GSK-3β signaling pathway is a critical downstream mediator of TRAF6 [27, 28]. WB analysis revealed that both p-AKT and p-GSK-3β were down-regulated in the group treated with miR-125b-5p mimics (as shown in **Figure 2H**). Conversely, an increase in these phosphorylation levels was observed in the group treated with the miR-125b-5p inhibitor (**Figure 2I**). These results suggest that miR-125b-5p directly targets TRAF6 and reduces its downstream signal pathway.
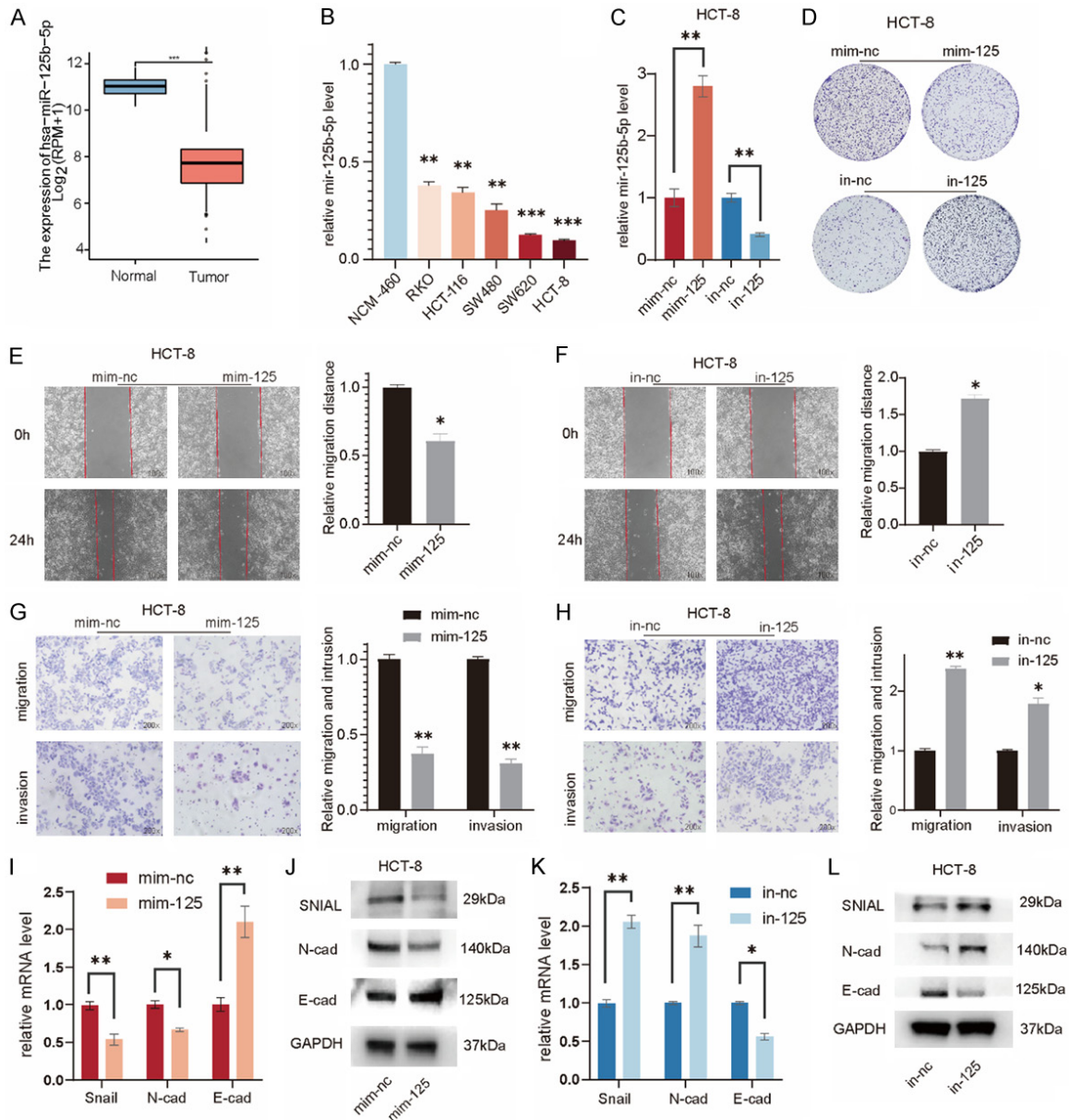
Figure 1. MiR-125b-5p suppressed metastasis and proliferation of colorectal cancer and was under-expressed in colorectal cancer. A. TCGA bioinformatics analysis showed that the expression of miR-125b-5p gene was relatively low in tumor tissues. B. The expression of miR-125b-5p in normal colorectal epithelial cell line (NCM460) and five colorectal cancer cell lines (HCT-8, HCT-116, SW480, SW620, and RKO) was analyzed by qRT-PCR. C. HCT-8 cells were transfected with 50 nM miR-125b-5p mimics or miR-125b-5p inhibitors. D. Colony formation assay revealed that miR-125b-5p inhibited colony formation ability. E, F. Scratch wound healing assay and trans-well assay showed that miR-125b-5p inhibited the metastasis of HCT-8 cell line. G, H. The expression of E-cadherin (E-cad), N-cadherin (N-cad) and SNAIL after overexpression of miR-125b-5p was detected by qRT-PCR and western blotting (WB). I, J. Scratch wound healing assay and trans-well assay were performed to analyzed cells migration and invasion. K, L. WB and qRT-PCR indicated that low expression of miR-125b-5p promoted EMT in HCT-8 cell line. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). P < 0.05 was considered statistically significant (*, P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

TRAF6 activates AKT/GSK-3β pathway to promote colorectal cancer cell metastasis

TRAF6 was detected to be highly expressed in colorectal cancer cell lines, contrasting with its notably reduced expression in normal intestinal epithelial cells (**Figure 3A**). TRAF6 was knocked down in HCT-116 cells by using siRNA and over-expressed in HCT-8 cells with pcDNA OE-TRAF6, respectively (**Figure 3B**). Overexpression of
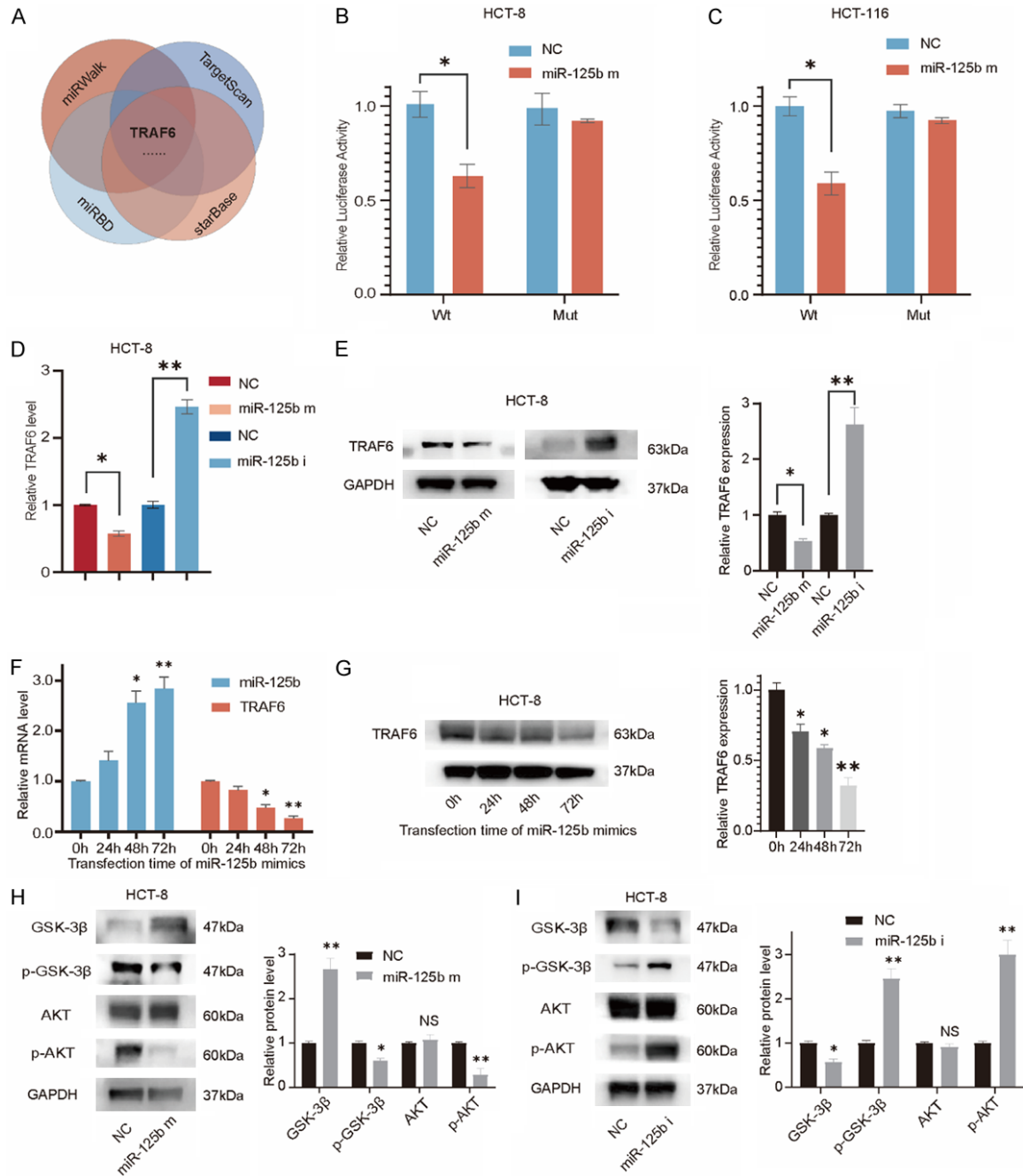
Figure 2. MiR-125b-5p targeted TRAF6 and reduced its expression in a time-dependent manner. A. Public database searches by TargetScan, miRWark, miRBD, and starBase predicted TRAF6 as a target of miR-125-5p. B, C. Dual luciferase reporter assay was performed to detect the binding of miR-125b-5p and TRAF6 mRNA 3'-UTR. D, E. WB and qRT-PCR indicated that miR-125b-5p suppressed the expression of TRAF6 in HCT-8 cell line. F, G. WB and qRT-PCR showed that miR-125b-5p mimics reduced the expression of TRAF6 in a time-dependent manner. H, I. WB showed that miR-125-5p inhibited AKT/GSK-3β pathway. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). P < 0.05 was considered statistically significant (*, P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

TRAF6 (OE-TRAF6) was observed to promote cell proliferation, while inhibition via small interfering RNA targeted at TRAF6 (si-TRAF6) effectively inhibited cell proliferation (**Figure 3C**). The migration and invasion ability of HCT-8 cells with TRAF6 over-expression were in-
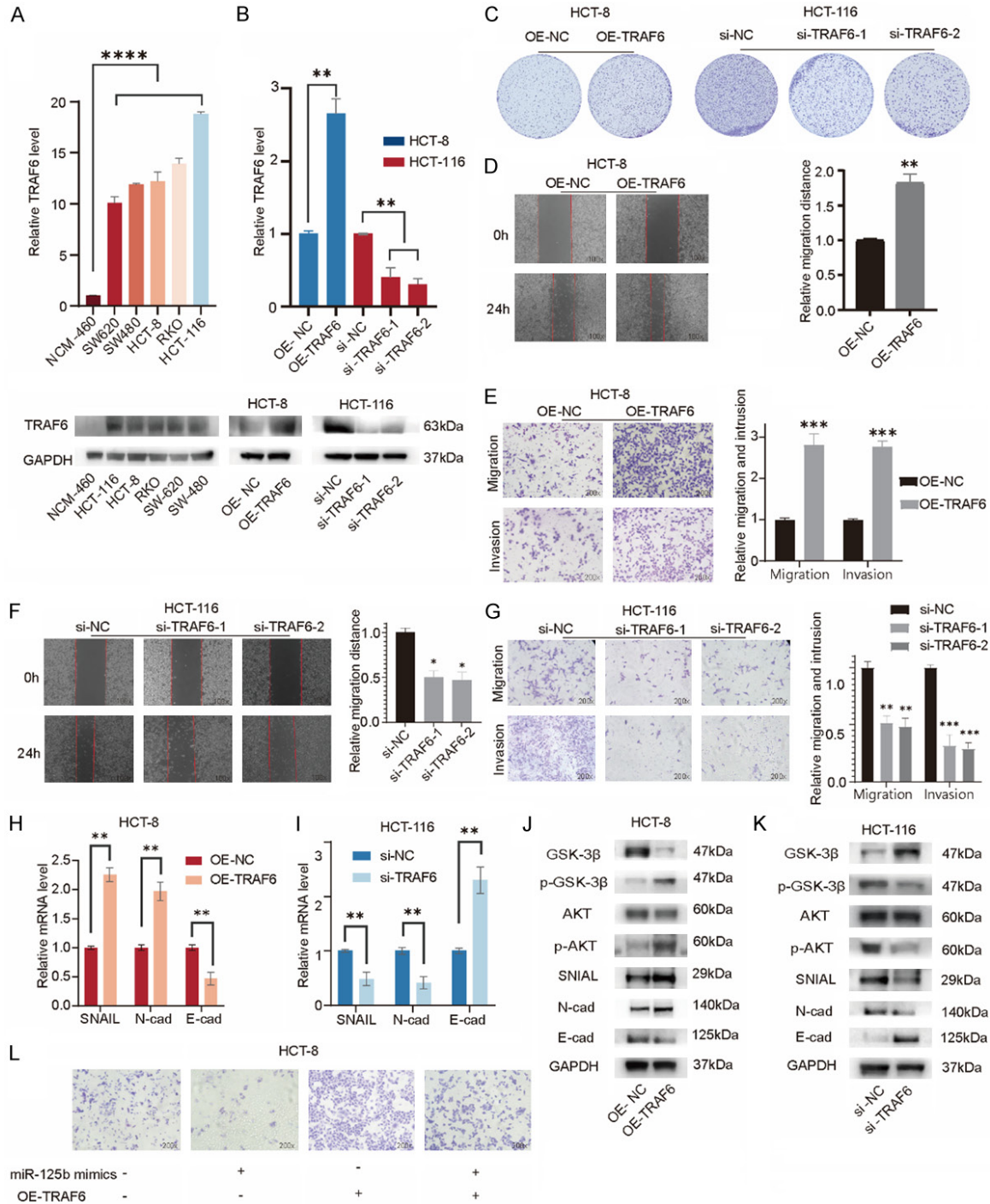
Figure 3. TRAF6 promoted colorectal cancer cell metastasis through activating AKT/GSK-3β/SNAIL pathway. A. WB and qRT-PCR showed that the expression of TRAF6 in five colorectal cancer cell lines was higher than that in normal intestinal epithelial cell lines. B. HCT-8 cell were transfected with OE-TRAF6 and HCT-116 cell were transfected with siRNAs. The TRAF6 level was analyzed by qRT-PCR and WB. C. The proliferation ability of the cells was detected by clonal formation assay. D-G. TRAF6 promoted cell migration and invasion in scratch wound healing assay and trans-well assay. H, I. SNAIL, E-cad and N-cad were detected by qRT-PCR. J, K. WB showed that TRAF6 promoted EMT through AKT/GSK-3β pathway. L. Trans-well migration assay indicated that co-transfection of miR-125b-5p inhibitor and OE-TRAF6 abrogated the migration ability of HCT-8 cell line. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). P < 0.05 was considered statistically significant (*, P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

creased (**Figure 3D**, **3E**). Upon the selective knockdown of TRAF6 utilizing small interfering RNAs (siRNAs), we observed a significant decrease in the migratory and invasive capabilities of HCT-116 cells (**Figure 3F**, **3G**). Moreover, mRNA level of E-cad was down-regulated while SNAIL and N-cad were up-regulated after transfection with OE-TRAF6 (**Figure 3H**). The opposite results were observed after being transfected with si-TRAF6 (**Figure 3I**). Additionally, the expression of E-cad decreased while the expression of SNAIL, N-cad, p-AKT and p-GSK-3β increased after transfection with OE-TRAF6 (**Figure 3J**). The targeted transfection with small interfering RNA against TRAF6 (si-TRAF6) yielded an opposite result (**Figure 3K**). The collective findings strongly suggest that TRAF6 promotes colorectal cancer metastasis by activating the AKT/GSK-3β/SNAIL signaling pathway. Furthermore, a co-transfection experiment involving miR-125b-5p and TRAF6 was conducted to delve deeper into their interaction. As depicted in **Figure 3L**, the increase in miR-125b-5p expression significantly inhibited the migration of HCT-8 cells. Intriguingly, this inhibitory effect could be antagonized by the overexpression of TRAF6.

Resveratrol promotes the stability of miR-125b-5p and inhibited the TRAF6/AKT/GSK-3β pathway

Resveratrol has been used in clinical studies for the prevention and treatment of colorectal cancer [29], but its specific mechanism has not been clarified. We treated three colorectal cancer cell lines with different concentrations of resveratrol for 48 h and measured their cell viability. There was no significant cytotoxic effect after treating cells with resveratrol at concentrations < 10 µM. Therefore, 10 µM resveratrol was used in the subsequent experiments (**Figure 4A**). Elevated miR-125b-5p was detected in all three colorectal cancer cell lines after resveratrol administration (**Figure 4B**). It was also found that resveratrol up-regulated miR-125b-5p in a dose/time-dependent manner (**Figure 4C**, **4D**). In the presence of actinomycin D, a typical inhibitor of RNA synthesis, miR-125b-5p degraded rapidly. Concurrent administration of actinomycin D and resveratrol led to an increase in the stability of miR-125b-5p (**Figure 4E**). Additionally, resveratrol down-regulated the mRNA and protein level of TRAF6 in a dose-dependent manner, resulting in a decrease of p-GSK-3β and p-AKT (**Figure 4F**, **4G**). These results suggest that resveratrol could promote the stability of miR-125b-5p and inhibit the TRAF6/AKT/GSK-3β pathway.

Down-regulated miR-125b-5p disrupts the inhibitory effect of resveratrol on colorectal cancer metastasis

To further investigate the correlation between resveratrol and miR-125b-5p, miR-125b-5p inhibitor was transfected into HCT-8 cell line and then resveratrol was administered. Compared with the NC group, the Res group inhibited colorectal cancer cell migration. However, the inhibitory effect was impeded when co-administered with miR-125b-5p inhibitor (**Figure 5A**). Similar results were obtained in trans-well migration and invasion experiments (**Figure 5B**). To further investigate its downstream mechanisms, we evaluated the expression of AKT/GSK-3β/SANIL pathway related molecules. TRAF6, p-GSK-3β, p-AKT, N-cad decreased, while E-cad increased in the Res group. In contrast, when comparing the Res group with the group co-treated with a miR-125b-5p inhibitor and Resveratrol (miR-125b-5p i+Res) group, we observed an increase in TRAF6, p-GSK-3β, p-AKT, and N-cad, coupled with a decrease in E-cad (**Figure 5C**, **5D**). These findings provide evidence that low expression of miR-125b-5p could antagonize the inhibitory effect of Resveratrol on colorectal cancer metastasis.

Overexpression of TRAF6 antagonizes the inhibitory effect of resveratrol on colorectal cancer metastasis

To further investigate the association between Resveratrol and TRAF6, OE-TRAF6 was transfected into HCT-8 cells, followed by the addition of Resveratrol. Migration was decreased in HCT-8 cells by resveratrol treatment. However, the inhibitory effect was prevented by the up-regulation of TRAF6 (**Figure 6A**). Similar results were found in the trans-well migration and invasion experiments (**Figure 6B**). Resveratrol could down-regulate TRAF-6 and EMT, which could be suppressed by overexpression of TRAF6 (**Figure 6C**, **6D**). Our findings suggest that TRAF6 might function as a key regulator in the resveratrol-induced inhibitory effect on colorectal cancer metastasis.
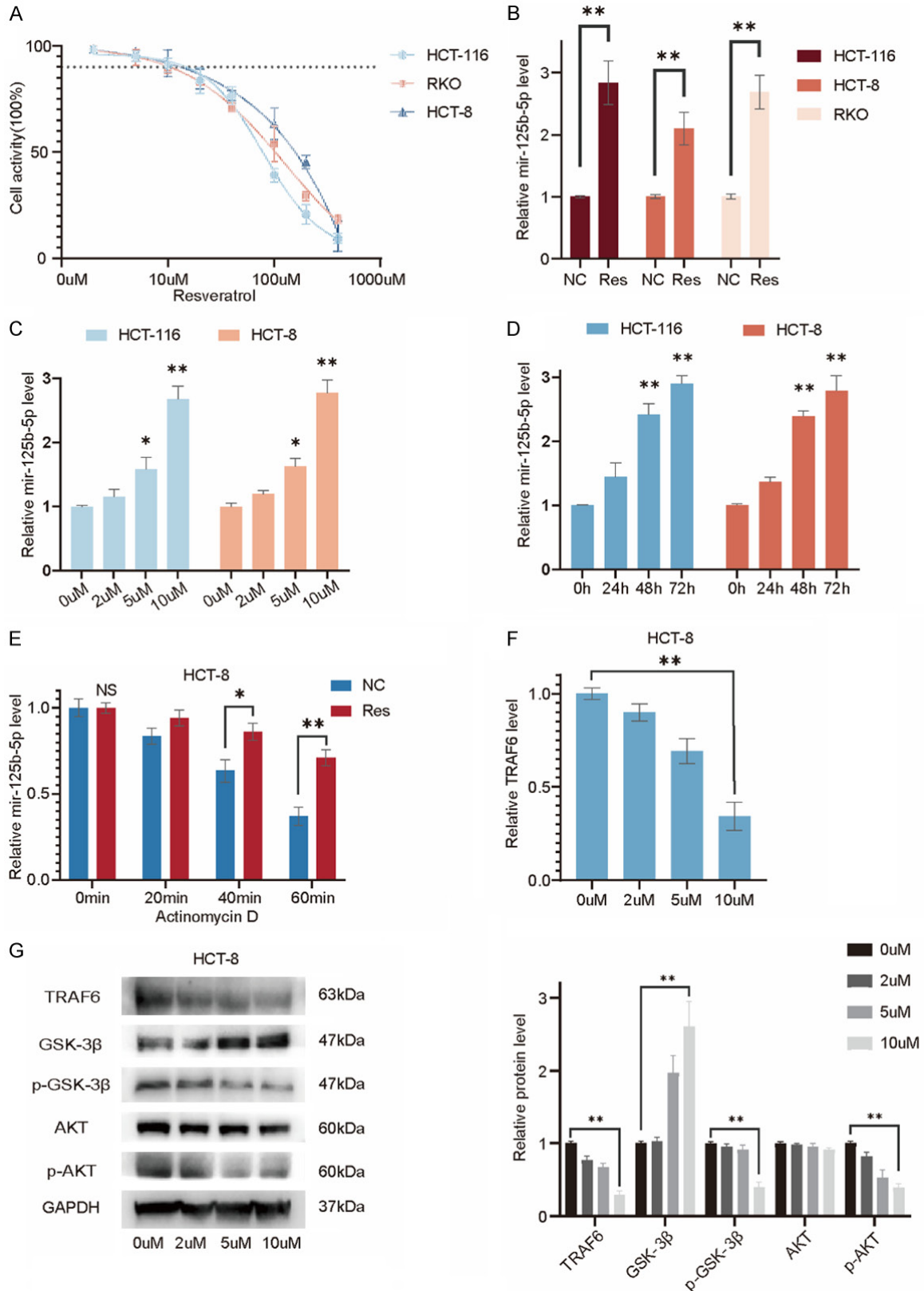
Figure 4. Resveratrol could promote the stability of miR-125b-5p and inhibited the TRAF6/AKT/GSK-3β pathway. A. Cell Counting Kit-8 assay was performed to investigate the cytotoxic effects of resveratrol on HCT-8, HCT-116 and RKO cell lines. B. The expression of miR-125b-5p was detected by qRT-PCR after resveratrol treatment. C, D.

qRT-PCR results showed that resveratrol increased the expression of miR-125b-5p in a dose- and time-dependent manner. E. Decay of miR-125-5p was monitored in HCT-8 cell co-treated with Actinomycin D and resveratrol. F. The expression of TRAF6 was detected after resveratrol treatment with different concentration. G. WB showed that resveratrol inhibited the TRAF6/AKT/GSK-3β pathway in a dose-dependent manner. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).
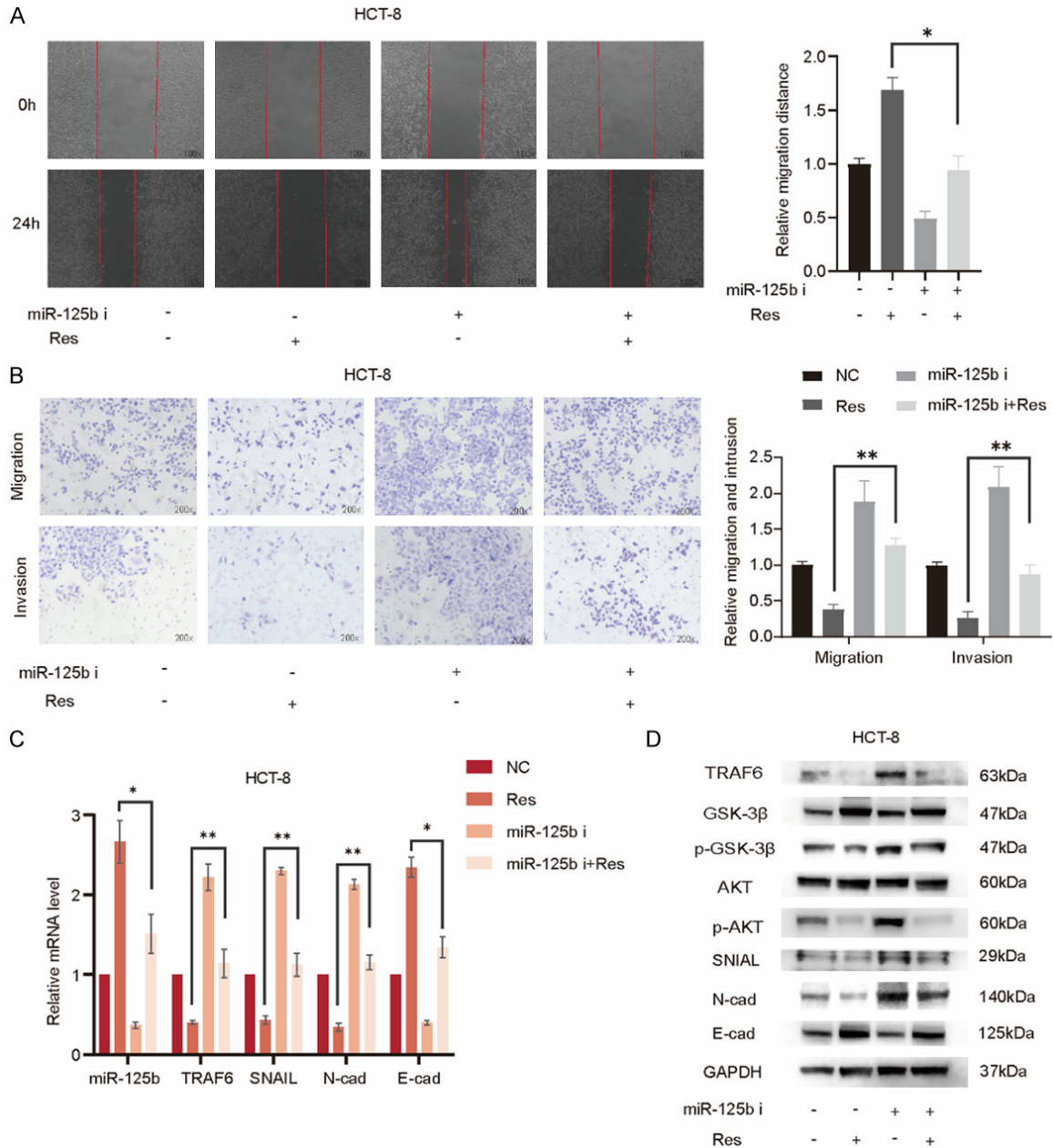

Figure 5. Low expressed miR-125b-5p could antagonize the inhibitory effect of resveratrol on colorectal cancer metastasis. A. The scratch wound healing assay revealed that the Res group inhibited the migration of colorectal cancer cells, and the inhibitory effect was impeded by co-administrating with miR-125b-5p inhibitor. B. The ability of migration and invasion of cells was detected by trans-well migration and invasion assay. C, D. HCT-8 cells were treated with miR-125-5p inhibitor, resveratrol or their combination. The expression of RNA or protein was analyzed by qRT-PCR or WB, respectively. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).
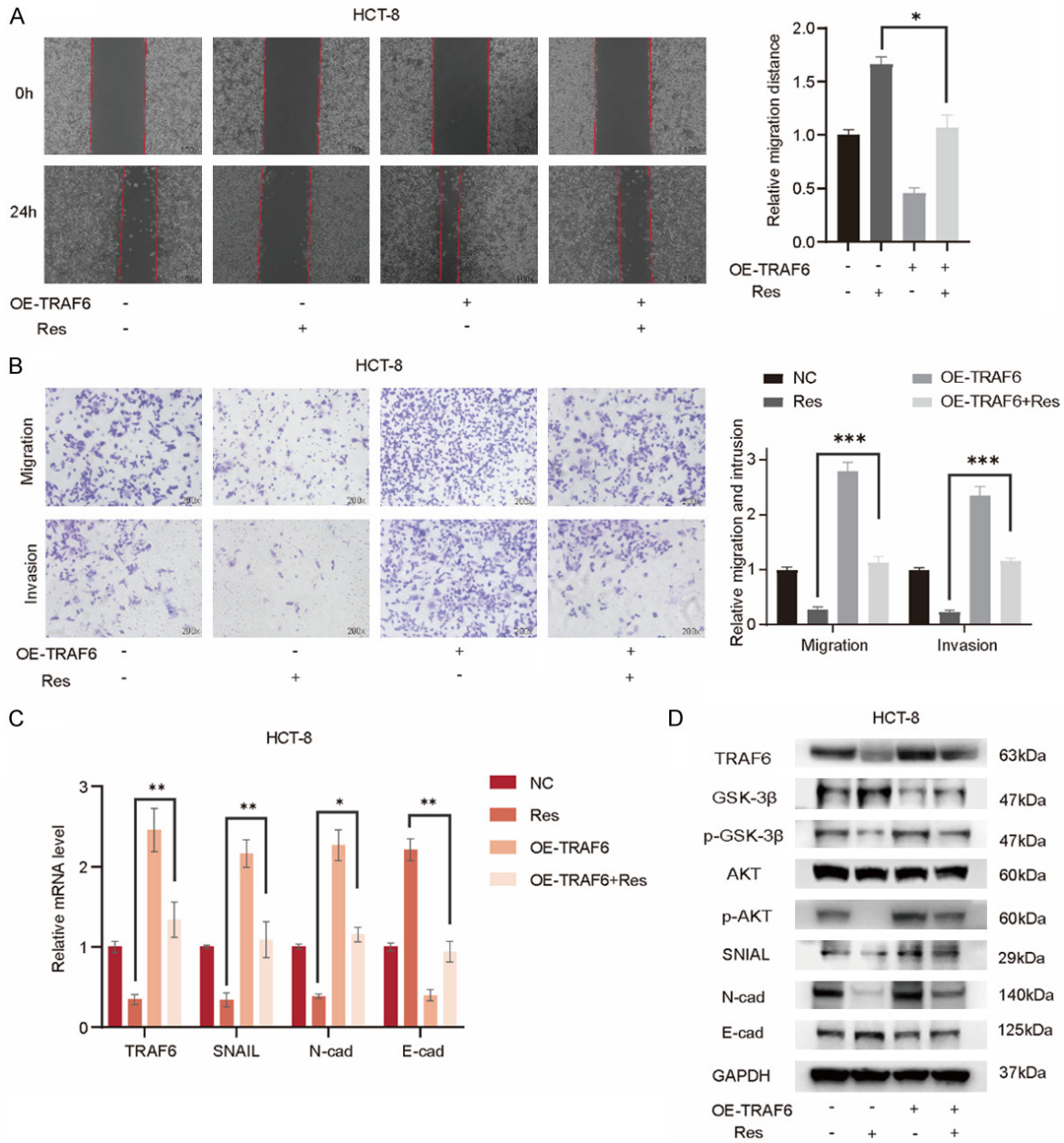
Figure 6. Overexpression of TRAF6 antagonized the inhibitory effect of resveratrol on colorectal cancer metastasis. A. The scratch wound healing assay revealed that the Res group inhibited the migration of colorectal cancer cells, and the inhibitory effect was impeded by co-administrating with OE-TRAF6. B. The ability of migration and invasion of cells was detected by trans-well migration and invasion assay. C, D. HCT-8 cells were treated with OE-TRAF6, resveratrol or their combination. The expression of RNA or protein was analyzed by qRT-PCR or WB, respectively. All experiments were independently repeated at least three times, and the data were expressed as mean ± standard deviation (SD). P < 0.05 was considered statistically significant (*, P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

Resveratrol antagonizes the promotion effect of low expressed miR-125b-5p on colorectal cancer metastasis in vivo

In order to study the effect of resveratrol on metastasis of colorectal cancer cells in vivo, the models of lung metastasis were constructed and evaluated by using live fluorescence imaging. The total flux of the miR-125b-5p inhibitor group was higher than that of the NC group, which indicated that low expressed miR-125b-5p could promote metastasis in vivo.
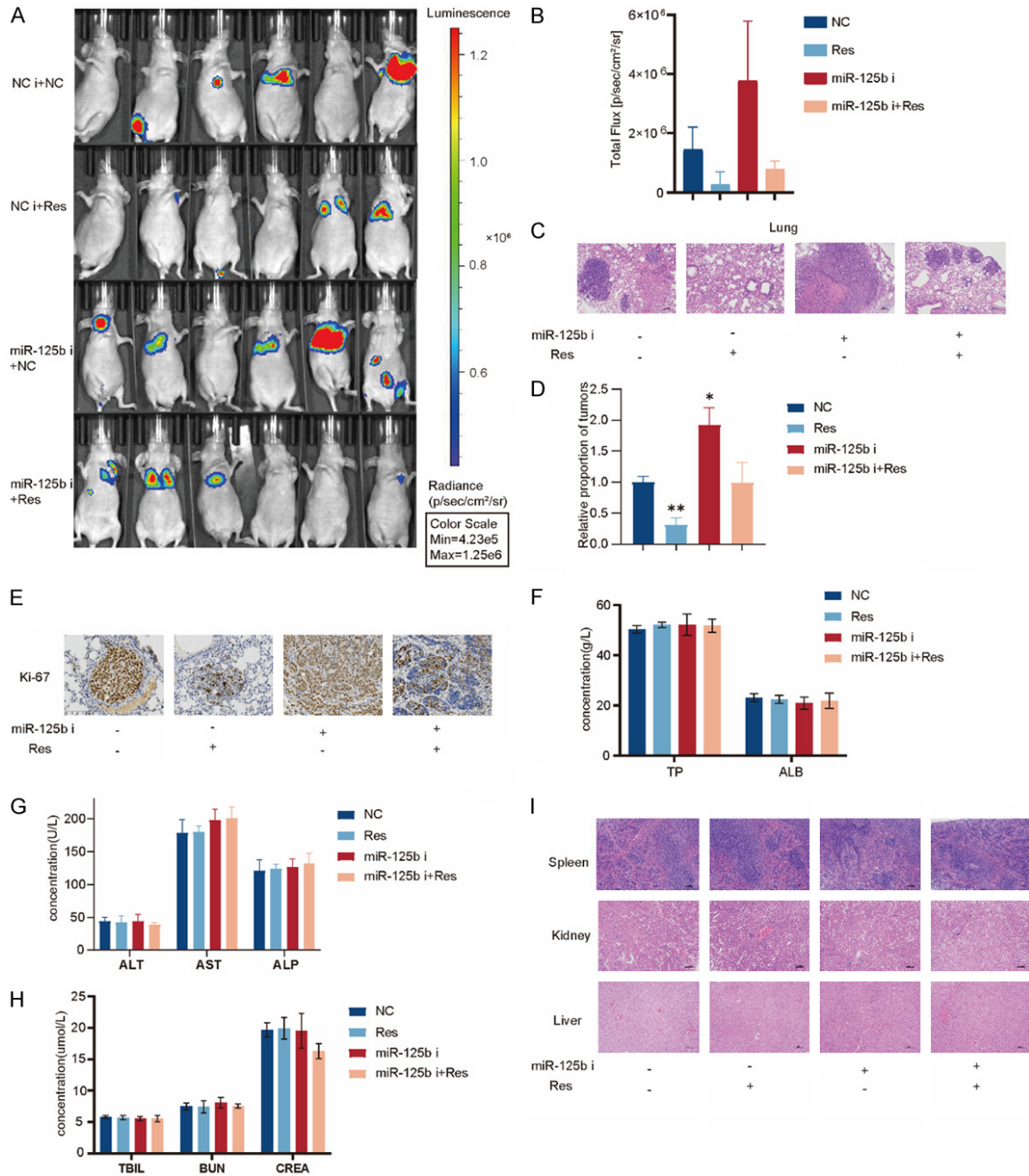
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Figure 7. Resveratrol antagonized the promotion effect of low expressed miR-125b-5p on colorectal cancer metastasis in vivo. A. Live fluorescence imaging revealing lung metastasis of colorectal cancer in mice administered different treatments. B. Total fluxes of mouse fluorescence signals were analyzed among different groups. C. H&E staining revealed histopathological changes of tumor foci in lung tissue. D. The relative proportion of tumors in lung tissue was calculated after H&E staining. E. Immunohistochemical staining assays were conducted to evaluate the expression of Ki-67 in mouse tumor tissues. F-H. Liver and kidney function indexes were detected in mouse serum, and there was no significant difference among all groups. I. H&E staining showed no significant changes in liver, kidney and spleen morphology of the four groups. $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

Compared with miR-125b-5p inhibitor group, the total flux of miR-125b-5p inhibitor+Res group was much lower (**Figure 7A**, **7B**). H&E

staining of lung tissue was undertaken, and the resulting images were observed under 100× magnification. A comparative analysis revealed

distinct variations among the groups. Specifically, the proportion of metastatic tumors in the lung was found to be lower in the Res group when compared with the NC group. Conversely, the proportion was higher in the group treated with the miR-125b-5p inhibitor. Interestingly, when comparing the miR-125b-5p inhibitor group with the combination treatment of miR-125b-5p inhibitor+Res group. The latter group exhibited a significant reduction in metastatic tumors (**Figure 7C**, **7D**). These results revealed that resveratrol could antagonize the promotional effect of low-expressed miR-125b-5p on colorectal cancer metastasis in vivo. Compared with the NC group, the expression of Ki-67 in Res group was down-regulated but up-regulated in miR-125b-5p inhibitor group. Furthermore, resveratrol could antagonize the increase of Ki-67 induced by miR-125b-5p inhibitor (**Figure 7E**). Urea nitrogen (BUN), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), direct bilirubin (DBIL), total bilirubin (TBIL), albumin (ALB), total protein (TP) and uric acid (CREA) were detected to assess the damage to liver and kidney (**Figure 7F-H**). There was no significant difference among all groups. Furthermore, no significant changes in morphology of liver, kidney and spleen were observed in the four groups of mice (**Figure 7I**).

Discussion

Colorectal cancer represents a form of malignant neoplasm characterized by a high incidence and mortality rate. Metastasis remains a significant challenge in the clinical management of colorectal cancer and is a principal factor underlying treatment failure [1]. The complexity of colorectal cancer's underlying molecular mechanisms, its propensity for metastasis, and the heterogeneity of its clinical presentation call for a nuanced understanding and tailored therapeutic approaches. The present study's exploration into the interactions between Resveratrol, miR-125b-5p, and associated molecular pathways provides critical insights into potential targets and interventions to counteract metastasis. Our study showed that miR-125b-5p is notably down-regulated in colorectal cancer, a finding that may have significant therapeutic implications. Further investigation within this research has shown that the up-regulation of miR-125b-5p has the potential

to inhibit both migration and invasion of cancer cells. Furthermore, our findings have identified that resveratrol possesses the capability to significantly inhibit the migration and invasion of colorectal cancer cells. Intriguingly, this inhibitory effect was found to be antagonized by either the knockdown of miR-125b-5p or the overexpression of TNF receptor associated factor 6 (TRAF6). The low expression of miR-125b-5p has been found to promote colorectal cancer metastasis in vivo, an effect that our study showed could be inhibited by resveratrol. These findings illuminate the miR-125b-5p/TRAF6 signaling axis as a novel molecular mechanism underlying colorectal cancer metastasis, marking it as a potential therapeutic target. Resveratrol could inhibit colorectal cancer metastasis by regulating miR-125b-5p/TRAF6 pathway and might improve the prognosis of colorectal cancer patients.

Resveratrol (3,4', 5-trimethylol stilbene) is a natural plant antitoxin polyphenol with a wide range of biological and pharmacological properties, as well as anti-cancer properties [30]. It has also been reported to interfere with apoptosis, autophagy and necrotic apoptosis [31], which inhibited cancer growth and metastasis without treatment-related side effects on normal organs [32]. By inhibiting the expression of RYR2, resveratrol suppresses the growth of pancreatic cancer (24). Resveratrol induces autophagy and apoptosis of non-small cell lung cancer cells through NGFR-AMPK-mTOR pathway [33]. In colorectal cancer, resveratrol inhibits the proliferation through Hippo/YAP pathway [34]. In our study, colorectal cells were treated with 10 μM resveratrol which imposed no significantly cytotoxic effect. Additionally, Many studies have shown that natural extracts like quercetin [35] and resveratrol [36] have regulatory effects on many miRNAs. For example, resveratrol can inhibit microRNA-21 to inhibit tumor growth [37]; Resveratrol can improve nutritional steatohepatitis through the Mir-599/PXR pathway [38]; At the same time, resveratrol can regulate miR-671-5p in A549 cells, thereby regulating STOML2/PINK1/parkin axle-mediated autophagy signaling pathway to enhance paclitaxel sensitivity [39]. However, no relevant studies have shown that resveratrol has a direct correlation with miR-125b-5p. Our research showed that resveratrol could impede the migration and invasion of colorectal cancer
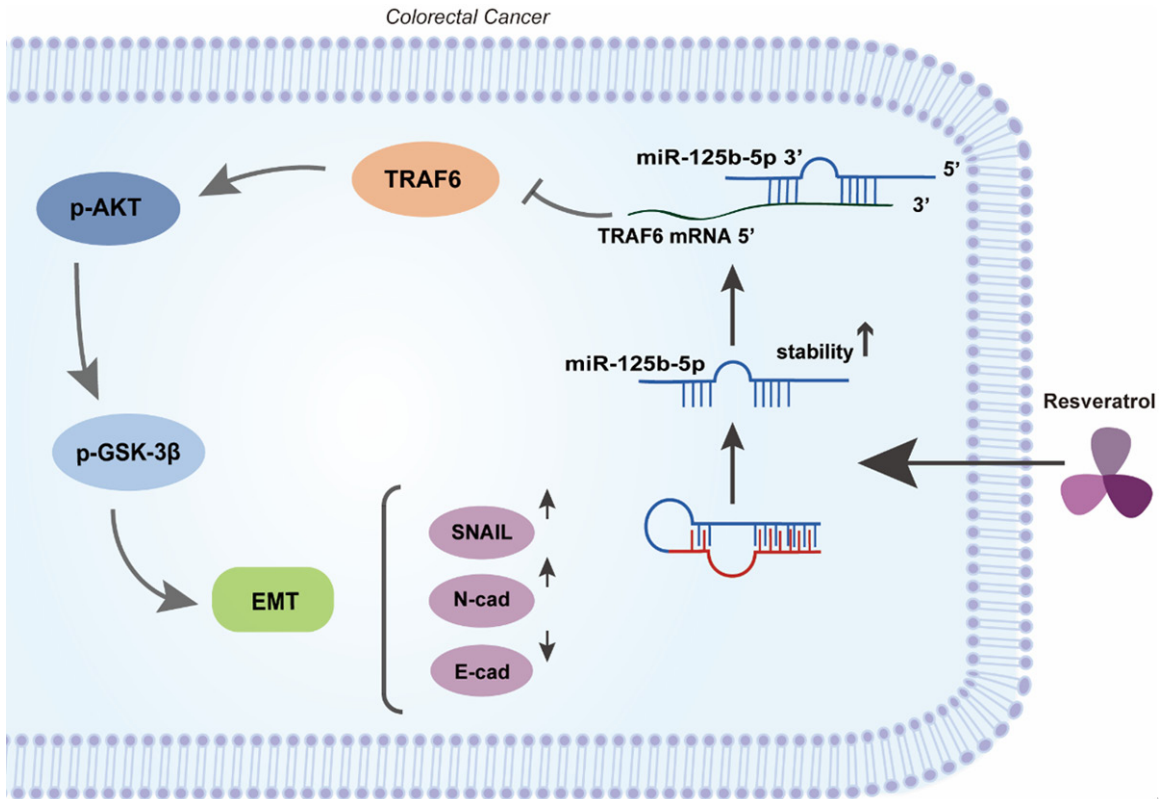
Figure 8. Resveratrol suppresses the metastasis of colorectal cancer by regulating miR-125b/TRAF6/GSK-3β/AKT/SNAIL pathway.

cells by significantly increasing the stability of miR-125b-5p and inhibiting TRAF6-induced signal pathway. Both down-regulated miR-125b-5p and overexpression of TRAF6 could disrupt the inhibitory effect of resveratrol on colorectal cancer metastasis. In the model of colorectal cancer lung metastasis, data also demonstrated that resveratrol could inhibit colorectal cancer metastasis in vivo. These data suggest that resveratrol is an effective drug for metastatic colorectal cancer patients. Even so, a complex question remains unresolved within the context of our study. Specifically, the regulatory effect of resveratrol on miR-125b-5p, whether it occurs at the pri-miRNA pre- or post-transcriptional levels, is yet to be clarified. Further studies are needed to clarify the specific mechanism.

MiR-125b-5p serve as a potential diagnostic or prognostic biomarker and regulates the proliferation, migration, and invasion of cancer cells [40]. Abnormal expression of miR-125b-5p could lead to disease onset and progression. MiR-125b-5p could inhibit the proliferation and

invasion of colorectal cancer cell by targeting TAZ and VEGF [41]. Our study demonstrated that miR-125b-5p is expressed at low levels in both colorectal cancer tissues and associated cell lines. It has an inhibitory effect on colorectal cancer metastasis. In addition, resveratrol could up-regulate miR-125b-5p by increasing its stability. The down-regulation of miR-125b-5p, as evidenced in our study, has been found to promote colorectal cancer growth and metastasis in vivo, a phenomenon that could be antagonized by the introduction of resveratrol. These results demonstrated that miR-125b-5p plays an anti-tumor role in colorectal cancer. Up-regulation of miR-125b-5p by resveratrol might improve the survival rate of patients with colorectal cancer metastasis.

Furthermore, our data revealed that TRAF6 was up-regulated in colorectal cancer cells and had a promoting effect on the metastasis of colorectal cancer. TRAF6 can be directly regulated by MicroRNAs. MiR-146b-5p/TRAF6 regulates the occurrence and progression of pancreatic cancer [42], Likewise, miR-589-5p,

miR-643 and miR-124 have also been reported to down-regulate the expression of TRAF6 [43-45]. Our study had identified have identified miR-125b-5p as a direct regulator of TRAF6, a finding that adds substantial depth to our understanding of the molecular mechanisms underpinning tumorigenesis and progression. We also observed that miR-125b-5p mimics were able to reduce TRAF6 mRNA and protein levels in a time-dependent manner. This specific function of TRAF6 has been illuminated by the work of Feng et al., who demonstrated that EGFR phosphorylation of DCBLD2 recruits TRAF6 and stimulates AKT-promoted tumorigenesis [46]. Also, TRAF6 has been reported as a direct E3 ligase for AKT, essential for AKT ubiquitination and activation [47]. In our study, overexpression of TRAF6 promoted the phosphorylation of AKT and activated AKT/ GSK-3β pathway to promote colorectal cancer cell metastasis. Furthermore, resveratrol could inhibit the expression of TRAF6 and GSK-3β/ AKT signaling pathway. Down-regulation of TRAF6 by resveratrol has provided a novel strategy for preventing colorectal cancer metastasis.

In this study, we have delineated the miR-125b-5p/TRAF6 signaling axis as an unprecedented molecular mechanism instrumental in colorectal cancer metastasis (**Figure 8**), and thereby identified it as a potential therapeutic target. Our results reveal that resveratrol, a natural compound with proven medicinal properties, serves to impede colorectal cancer metastasis through its regulation of the miR-125b-5p/ TRAF6 signaling pathway.

Acknowledgements

Disclosure of conflict of interest

None.

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Table S1. The primers used in this study

| Primer	Sequence (5'->3')
Actin F	TCCCTGGAGAAGAGCTACGA
Actin R	AGCACTGTGTTGGCGTACAG
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
miR-125 F	GGTGTCCGAGGTATTCGCACT
miR-125 R	TCCCTGAGACCCTTTAACCTGTG
TRAF6 F	GCCCATGCCGTATGAAGAGA
TRAF6 R	ACTGAATGTGCAGGGGACTG
SNAIL F	TACAAAACCCACGCAGACA
SNAIL R	ACCCCACATCCTTCTCACTG
E-Cadherin F	GCCATGGACTCCCATTTCCA
E-Cadherin R	ACAACGAAGATGGCCCTGAG
N-Cadherin F	TTGTACATGCAGTGTCCATGCT
N-Cadherin R	ACGATTTCAAGAGATCAGGAGC
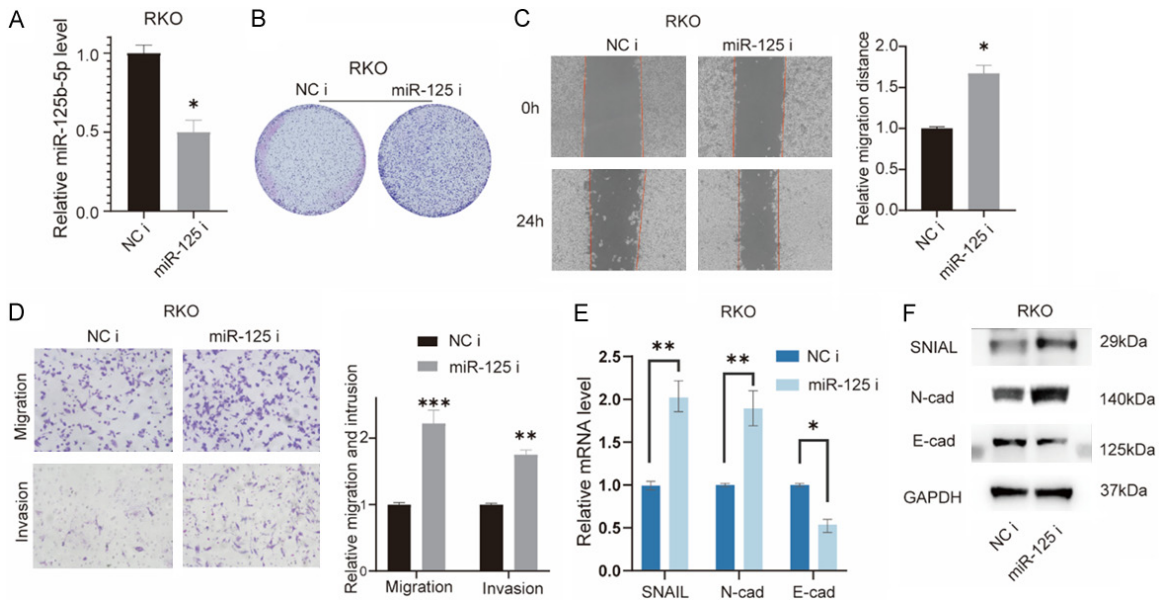
Figure S1. Low expression of miR-125b-5p promoted metastasis and proliferation of RKO cells. (A) RKO cells were transfected with 50 nM miR-125b-5p inhibitors. MiR-125b-5p levels were detected using qRT-PCR. (B) Colony formation ability was analyzed by colony formation assay. (C, D) Scratch wound healing assay and trans-well assay showed that miR-125b-5p inhibited the metastasis of RKO cells. (E, F) WB and qRT-PCR indicated that low expression of miR-125b-5p promoted EMT in RKO cells.