

miR-145 suppresses colorectal cancer cell migration and invasion by targeting an ETS-related gene

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Abstract. MicroRNA-145 (miR-145) has been demonstrated to be downregulated in various cancer types including colorectal cancer (CRC). However, the function of miR-145 in CRC has not been clearly elucidated. In this study, we examined miR-145 expression by quantitative real-time PCR (qRT-PCR) in CRC cell lines as well as tumors and corresponding normal mucosa, and the results were correlated to the clinicopathological parameters. In addition, using computational algorithms we investigated putative miR-145 targets. The role of miR-145 was further examined in studies *in vitro*. In our study miR-145 was significantly decreased in CRC tissues and cell lines compared with non-cancerous colorectal mucosa, especially lymph node or distance metastasis cases. Based on computational algorithms, we assumed that ERG was directly modulated by miR-145 in colorectal cancer cells. For the first time, we demonstrated that ERG was highly expressed in CRC tissues compared with normal ones by qRT-PCR. The inverse correlation between the expression of miR-145 and ERG was observed in CRC tissues. Dual-Luciferase assays demonstrated the direct interaction between miR-145 and 3'-UTR of ERG mRNA. Ectopic expression of miR-145 suppressed the proliferation and invasion ability of colorectal cancer cells, while ERG knockdown partially restored the tumor suppressive effect of miR-145. These results suggested that miR-145 might act as a tumor suppressor during the process of CRC malignant transformation by interacting with ERG.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the third-leading cause of cancer death in the United States (1). Although most CRC patients undergo surgical resection of tumors, regional and distant metastases strongly impact 5-year survival rates (2). Unfortunately, treatments of patients with metastatic CRC are not very efficient. Therefore, it is of great importance to further explore the underlying molecular mechanisms of CRC progression in order to develop new therapeutic strategies, especially for patients with metastatic CRC.

Erythroblast transformation-specific (ETS)-related gene (ERG) belongs to the ETS transcription factor family, and plays an important physiological role in hematopoiesis (3), angiogenesis (4), and bone development (5). In addition, aberrant ERG expression has been observed in solid tumors and leukemia (6,7). Furthermore, ERG gene fusion proteins such as transmembrane protease, serine 2 (TMPRSS2)-ERG have been associated with poor prognosis in prostate cancer (8). In prostate cancer, ectopic ERG expression may induce acquired invasive traits and endothelial mesenchymal transition (EMT) (9). Although an increasing number of studies indicate that ectopic ERG expression is involved in the pathogenesis of various solid tumors and hemopathies, further studies are still required to fully elucidate the underlying biological mechanisms of the ERG action in tumorigenesis.

microRNAs (miRNA or miR) are single strand non-coding RNAs composed of 21-24 nucleotides, which exert their function by interacting with the complementary sites in the 3' untranslated region (UTR) of target mRNAs. miRNAs regulate multiple cellular functions such as cell proliferation, apoptosis and differentiation (10). In addition, it has been shown that aberrant miRNA expression results in ectopic expression of different gene products, which may consequently lead to carcinogenesis and tumor progression (11).

miR-145 was first reported to be consistently decreased in precancerous colorectal lesions as well as in different stages of colorectal cancer tissue samples compared to normal colorectal mucosa (12). Since then, increasing evidence has shown that ERG expression is reduced in different types of cancer, supporting the idea that miR-145 acts as a tumor

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suppressor in breast cancer (13), prostate cancer (14) and neuroblastoma (15). In addition, it has been shown that ectopic expression of miR-145 influences cell cycle distribution, invasion and differentiation level of tumor cells in CRC (16). Contrary to these reports, Arndt *et al* showed that miR-145 may have an oncogenic role in CRC by suppressing E-cadherin expression and promoting anchorage-independent growth *in vitro* (17). Therefore, depending on the cell lines and cancer types, miR-145 may have either a tumor suppressive or an oncogenic effect. Hence, the exact miR-145 function and regulation in CRC is still unknown and demands further investigation.

Therefore, we undertook to further investigate the role of miR-145 in tumorigenesis as well as its relationship to ERG in clinical samples of colorectal tumors and CRC cell lines *in vitro*.

Materials and methods

Clinical specimens. In this study, 48 pairs of colorectal tumors and corresponding normal mucous tissue (5 cm away from the cancer lesions) were collected from colorectal cancer patients who underwent colorectal resection at the Third Affiliated Hospital of Guangzhou Medical University. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80°C until further use. The pathological diagnosis of CRC specimens and confirmation of the adjacent normal intestinal mucosa were performed by at least two pathologists. The TNM classification was performed according to the National Comprehensive Cancer Network (NCCN) guideline (18). None of the patients received pre-operative chemotherapy or radiotherapy. Patients with other malignancies were excluded. The Clinical Research Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University approved the research protocols. Written informed consents were obtained from all patients.

Cell lines and treatment. Human embryonic kidney cell line HEK-293T and five human CRC cell lines (RKO, DLD-1, HCT-116, SW620 and SW480) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin, and cultured in humidified incubator at 37°C and supplemented with 5% CO₂.

Total RNA isolation and quantitative real-time PCR. Total RNA from clinical specimens and CRC cell lines was isolated with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) was used to measure RNA concentration. Next, reverse transcription reactions were performed with PrimeScript RT Reagent kit (Takara, Dalian, China), and quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix DimerEraser (Takara) and the Step One Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). For miR-145 detection, miRNA-specific primers were

purchased from Shanghai Generay Biotechnology Co., Ltd. (Shanghai, China), and the relative miR-145 expression level was normalized to U6 expression. ERG expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primer sequences were as follows: miR-145 reverse transcription, 5'-TGGTGTCTGGAGTCG-3'; miR-145 sense, 5'-ACACTCCAGCTGGGGTCCAGT TTTCCCAGGAA-3' and antisense, 5'-CTCAACTGGTGT CGTGGAGTCGGCAATTCAGTTGAGAGGGAT-3'; ERG forward, 5'-ATCGTGCCAGCAGATCCTAC-3' and reverse, 5'-CCTTGGTCATCTTGCACAGTTC-3'; GAPDH forward, 5'-CGCTGAGTACGTCGTGGAGTC-3' and reverse, 5'-GCT GATGATCTTGAGGCTGTTGTC-3'. The 2^{-ΔΔCt} method was used for relative quantification. All reactions were performed in triplicate.

Western blot analysis. Proteins from tissue samples and CRC cell lines were extracted with lysis buffer and protein concentration was determined by Enhanced BCA Protein Assay kit (both from Beyotime Institute of Biotechnology, Haimen, China). Protein lysate was separated on 10% SDS-PAGE gels, transferred onto a PVDF membrane (Millipore, Billerica, MA, USA), and blocked with 5% non-fat dried milk. Next, membranes were incubated with antibodies against ERG (1:2,000) (ab133264; Abcam, Cambridge, MA, USA) or GAPDH (1:5,000) (ProteinTech, Chicago, IL, USA) overnight at 4°C, washed with Tris-buffered saline with Tween (TBST) four times, and incubated with horse raddish peroxidase (HRP) labeled anti-rabbit secondary antibody (1:5,000) for 1 h at room temperature. Enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA) was used to visualize protein bands according to the manufacturer's protocol. The relative density of the western blot bands was measured by ImageJ (19) and normalized to sample T1.

Oligonucleotide transfection. For the oligonucleotide transfection experiments miR-145 mimics, miR-145 inhibitors and corresponding normal controls were purchased from RiboBio (Guangzhou, China). ERG siRNAs and non-targeting siRNA were obtained from GenePharma (Shanghai, China). siRNA sense and antisense sequences were as follows: 5'-GACGUCAACAUCUUGUUAUTT-3' and 5'-GACGUC ACAUCUUGUUAUTT-3'. Oligonucleotide transfection was performed according to the manufacturer's protocol using Lipofectamine 2000 (Invitrogen Life Technologies). In brief, 50 nmol/l of miR-145 mimics or 100 nmol/l of miR-145 inhibitors were used in transfections. For knockdown of endogenous ERG 100 nmol/l of ERG siRNA was used.

Luciferase reporter assay. We used Targetscan (<http://www.targetscan.org>) for the prediction of putative miR-145 targets. Using this analysis the ERG (NG_029732) 3'-UTR region was predicted to have putative binding site complementary to the seed region of miR-145. In order to construct the plasmid containing this site, 1.5 kb sequences of the ERG 3'-UTR region were amplified from human genomic DNA, and inserted into restriction sites (*SgfI* and *NotI*) of Dual-Luciferase reporter psiCHECK2 plasmid (Promega, Madison, WI, USA) and this plasmid was denoted as psiCHECK2-WT. The Dual-Luciferase reporter plasmid containing a mutated

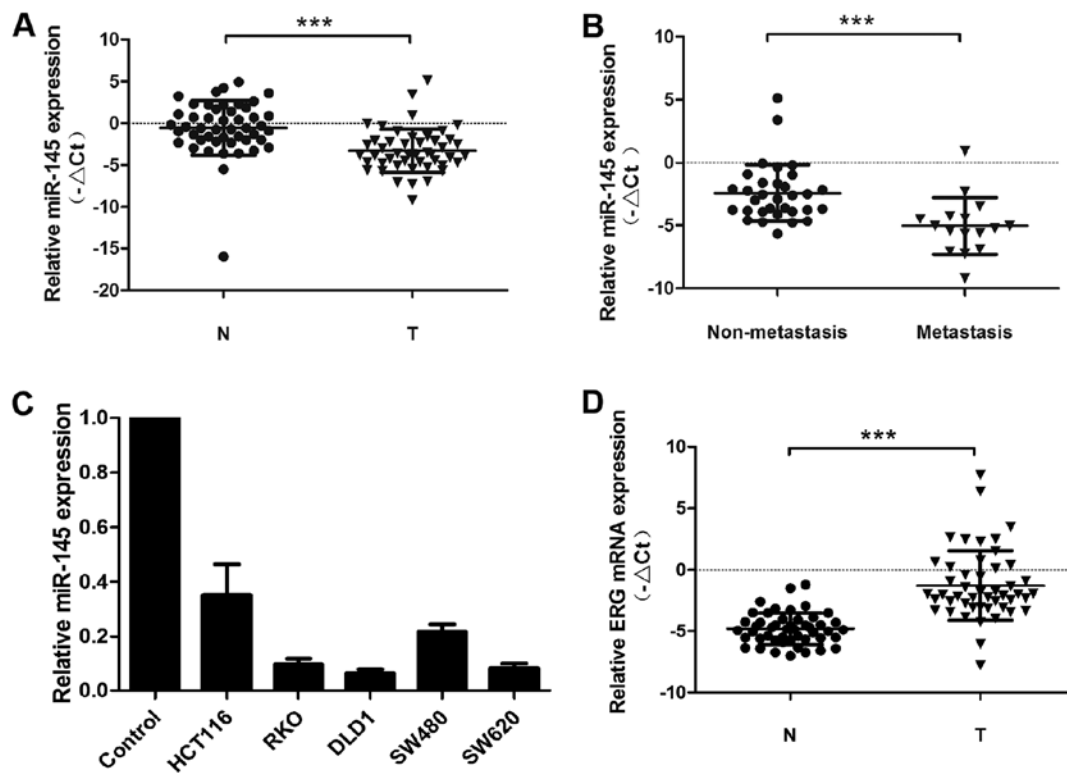


Figure 1. Downregulation of miR-145 expression in CRC cell lines and tissues. (A) qRT-PCR analysis of miR-145 expression in 48 pairs of CRC tissue and corresponding adjacent non-cancerous mucous tissue. ΔC_t represents miR-145 expression normalized to U6. (B) miR-145 expression in primary CRC of patients with or without lymph node metastasis. (C) miR-145 expression in five human colorectal cancer cell lines compared to adjacent non-cancerous mucous tissue. (D) ERG expression in CRC tissue and adjacent non-cancerous colorectal mucosa. ***P<0.001. N, adjacent non-cancer mucosa; T, colorectal tumor tissue.

miR-145 binding site in the ERG 3'-UTR region was also constructed and denoted as psiCHECK2-MUT. The primers used for these constructs were as follows: psiCHECK2-WT forward, 5'-CGCGCGATCGCAGACCTGGCGGAGGCTTTTC-3' and reverse, 5'-ATAAGAATGCGGCCGCGGCTCTCCCTTGCACAAGTTC-3'; psiCHECK2-MUT forward, 5'-TCTTTGTTTGTCAAATGAAAATTTTCCAGTTTTGTCTGATATTTAAGAGAAACATT-3' and reverse, 5'-AATGTTTCTCTTAAATATCAGACAAAACACTGGAA AAAATTTTCATTTGACAAACAAAGA-3'. For Dual-Luciferase assays, 50 nM of miR-145 mimics or 100 nM of miR-145 inhibitor were co-transfected with 0.5 μ g of psiCHECK2-WT or psiCHECK2-MUT using Lipofectamine 2000 (Invitrogen Life Technologies). The renilla luciferase signal and the firefly luciferase signal were consecutively detected according to the manufacturer's protocols for Dual-Luciferase Reporter assay system (E1910) (Promega). The firefly luciferase signal was used to normalize the renilla luciferase signal.

Scratch wound healing assay. For the scratch wound healing assay, cells (1×10^6) were seeded homogeneously on 6-well plates and cultured for 24 h to form a monolayer. Next, monolayers were scratched carefully with a sterile plastic 200 μ l pipette tip. The floating cell debris was washed with DMEM. At 0 and 24 h after scratch wound formation, images were obtained using an inverted microscope (Nikon, Tokyo, Japan) at a magnification of 200x and measured by Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Invasion assay. For invasion assays, Falcon inserts were used with Falcon Companion Tissue Culture Plates (Corning, Inc., Corning, NY, USA) (24 wells). The Falcon Cell Culture inserts with 8- μ m pore membranes were coated with Matrigel (BD Biosciences, San Jose, CA, USA) and placed in a 37°C incubator for 3 h to solidify. Homogeneous single cell suspensions with serum-free medium were added to the upper chamber at a total of 5×10^5 cells per well. Medium containing 10% FBS was added to the lower chambers and served as a chemoattractant. After 24 h, cells that remained on the upper surface of the membrane were carefully removed by a cotton swab, and cells which invaded the pores and adhered to the lower surface of the membranes were fixed with methanol and stained with hematoxylin. Stained cells were observed and counted (five random $\times 100$ magnification fields per well) under an inverted microscope. Each experiment was performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS 18.0 (IBM SPSS, Chicago, IL, USA). Results of all experiments are presented as mean \pm SD. Student's t-test was used to compare miRNA expression in clinical tissue samples and CRC cell lines. Pearson's correlation coefficient was used to measure the correlation between miR-145 and ERG expression. P<0.05 was considered statistically significant.

Results

miR-145 is decreased in colorectal cancer cell lines and clinical samples. In this study we examined miR-145 expres-

Table I. miR-145 expression and clinicopathological parameters of CRC patients and their tumors.

Clinicopathological parameters	No. of patients (N=48)	miR-145 expression		P-value
		Low	High	
Age (years)				
≤65	13	8	5	0.0569
>65	35	17	18	
Gender				
Female	19	9	10	0.6675
Male	29	16	13	
LN involvement				
No	32	9	23	<0.0001 ^a
Yes	16	16	0	
Distant metastasis				
No	36	13	23	<0.0001 ^a
Yes	12	12	0	
TNM stage				
I, II	29	6	23	<0.0001 ^a
III, IV	19	19	0	

^aStatistically significant. miR-145, microRNA-145; CRC, colorectal cancer; LN, lymph node.

sion in 48 pairs of CRC tissues and adjacent non-cancerous mucosa samples by qRT-PCR (Fig. 1A). In this sample set, miR-145 expression was decreased in most of the CRC samples compared to non-cancerous adjacent mucous tissue. Moreover, even greater miR-145 downregulation was detected in CRC tumors obtained from patients with lymph node metastasis compared to those without lymph node metastasis (Fig. 1B). miR-145 expression in colorectal cancer cell lines (RKO, DLD-1, HCT-116, SW620 and SW480) was also decreased compared to adjacent non-cancerous mucous tissue (Fig. 1C). In contrast to miR-145 expression, ERG expression levels were upregulated in most CRC tissues compared to adjacent normal mucosa (Fig. 1D). In addition, we examined the relationship between miR-145 expression and clinicopathological features of CRC patients, including gender, age, lymph node metastasis, distant metastasis and TNM stage (Table I). The mean value of relative miR-145 expression (-2.7566) was used as a cut-off value for the division of CRC patients into miR-145 low or high expression groups, accordingly. Statistical analysis revealed that low miR-145 expression correlated with poor outcome indicators including lymph node metastasis ($P<0.0001$), distant metastasis ($P<0.0001$) and TNM stage ($P<0.0001$).

miR-145 suppresses migration and invasion ability of human CRC cells. We examined miR-145 in human CRC cell lines using the wound healing and Transwell assays. Transient transfection of miR-145 mimic, miR-145 inhibitor and their corresponding control RNAs was performed in CRC cell lines RKO and SW480. The wound healing assay revealed that upregulation of miR-145 using the miR-145 mimic transfection suppressed the migration rate of CRC cells compared to control cells (Fig. 2A), while miR-145 knockdown in miR-145

inhibitor transfection accelerated the migration rate compared to the control cells (Fig. 3A). Matrigel Transwell assays showed similar results. miR-145 overexpression in RKO and SW480 significantly suppressed the invasion ability of the cells (Fig. 2B), while the miR-145 knockdown increased the number of invaded cells (Fig. 3B). Collectively these results indicate a possible tumor suppressive role of miR-145 in CRC migration and invasion.

ERG is targeted by miR-145 in CRC cells. As miRNA exerts its function by interacting with the 3'-UTR of its target genes, bioinformatic algorithms were applied for target prediction. ERG was predicted to be a potential target of miR-145 (Fig. 4A). Based on this prediction, western blot analysis was performed to investigate the effects of ectopic expression of miR-145 in CRC cells. In our study miR-145 upregulation suppressed ERG protein expression in human CRC cell lines, RKO and SW480, while miR-145 knockdown resulted in increased ERG protein expression (Fig. 4B). Results of Dual-Luciferase reporter assays in HEK-293T cells revealed that miR-145 mimic transfection reduced the luciferase activity, while mutation in the predicted targeting region abrogated this suppressive effect (Fig. 4C). These results indicate that the interaction between miR-145 and ERG depended on the miRNA recognition element in the 3'-UTR region. Furthermore, miR-145 and ERG protein expression in eight colorectal cancer tissue samples was examined both by qRT-PCR and western blot analysis, respectively, and an inverse correlation was observed between miR-145 and ERG expression (Fig. 4D).

Downregulation of ERG partially reverses the effect of miR-145 knockdown in CRC cells. To further investigate

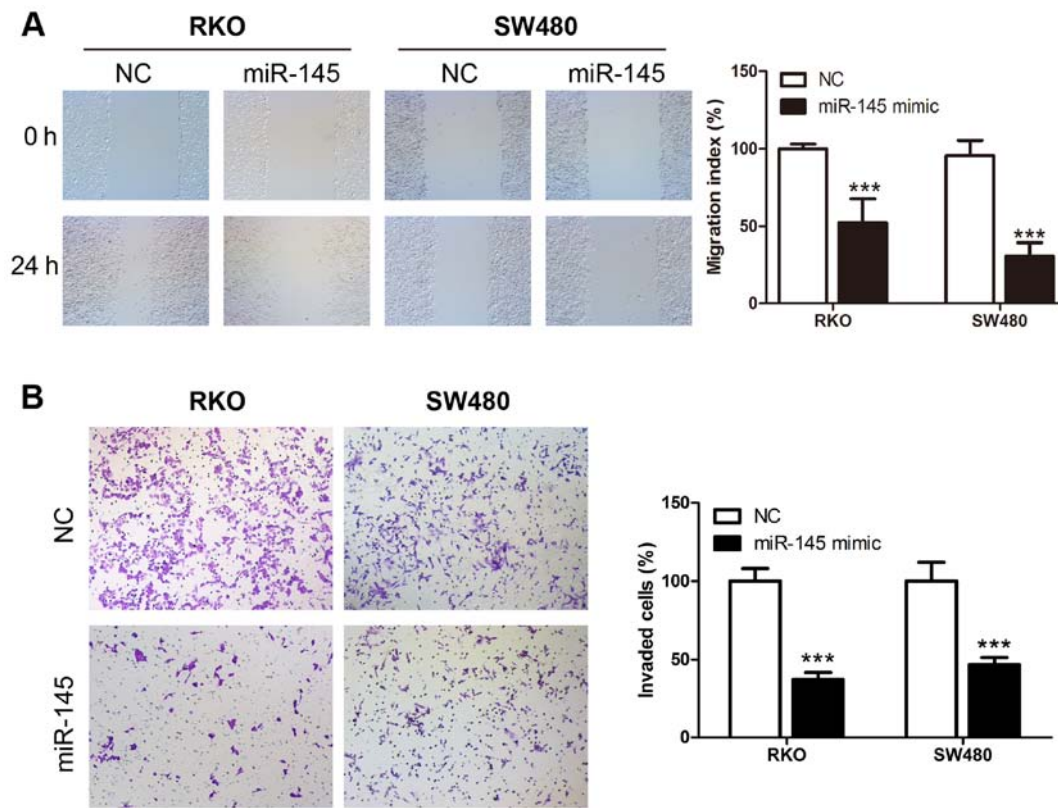


Figure 2. miR-145 suppressed migration and invasion of colorectal cancer cells. (A) Scratch wound healing assay revealed that miR-145 mimic transfection significantly reduced the horizontal migration ability of CRC cells (RKO and SW480) compared to the mimic control transfected cells. (B) Matrigel Transwell assay revealed that miR-145 mimic transfection significantly reduced the number of invaded cells compared to the mimic control transfected cells. *** $P < 0.001$.

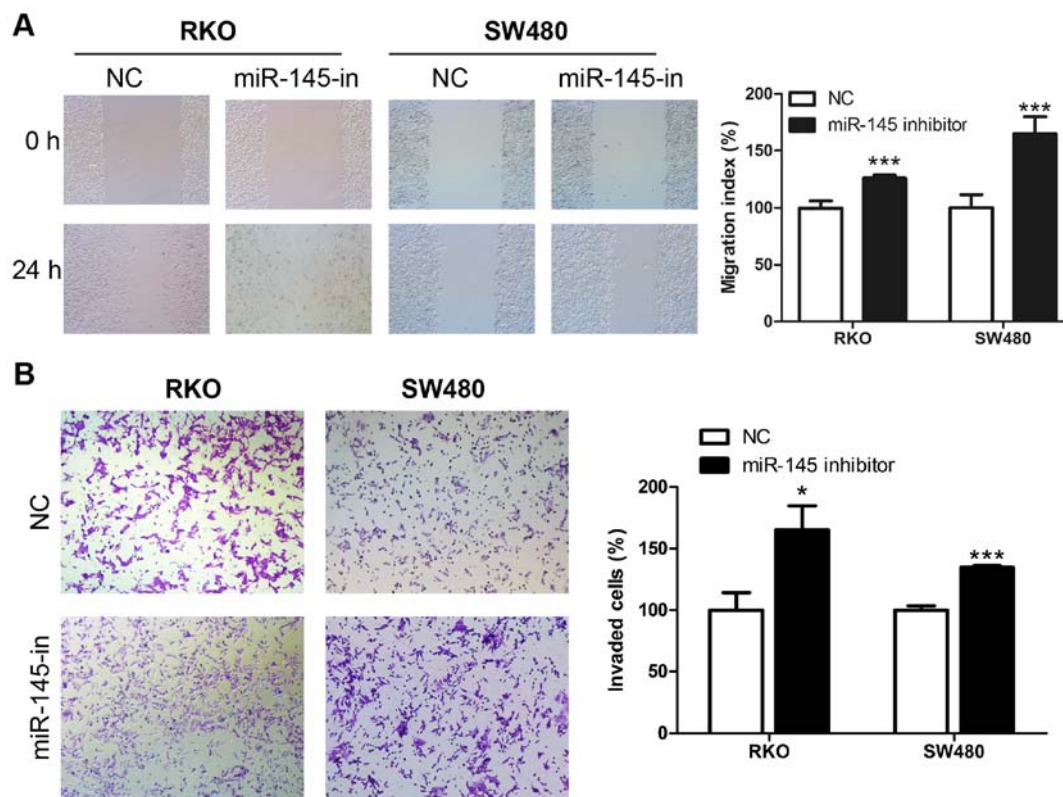


Figure 3. Downregulation of miR-145 promoted migration and invasion of colorectal cancer cells. (A) Scratch wound healing assay revealed that miR-145 inhibitor transfection significantly accelerated wound healing in CRC cells (RKO and SW480) compared to the inhibitor control transfected cells. (B) Matrigel Transwell assay revealed that miR-145 inhibitor transfection significantly increased the number of invaded cells compared to the inhibitor control transfected cells. * $P < 0.05$, *** $P < 0.001$.

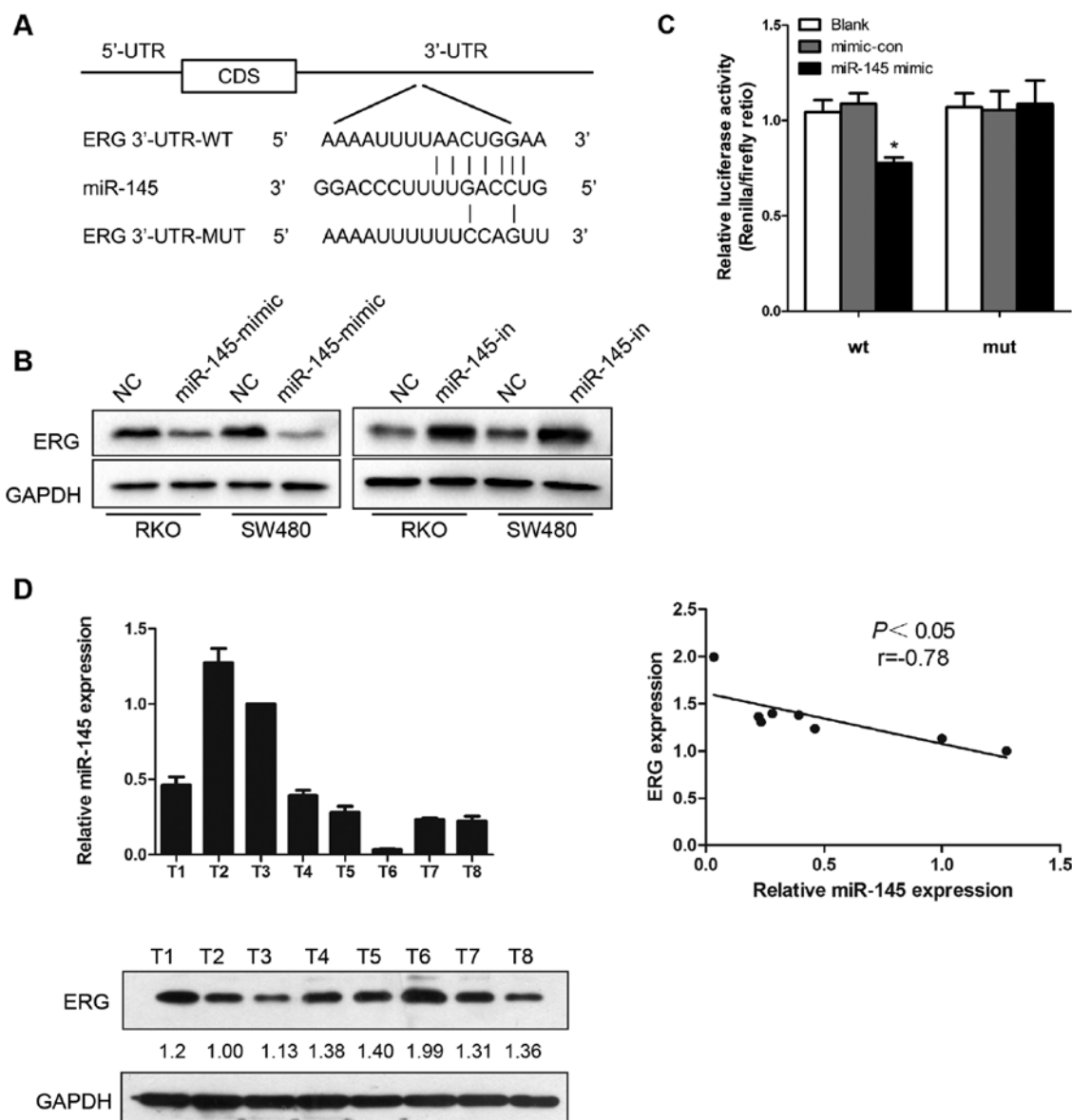


Figure 4. miR-145 downregulates ERG expression through post-transcriptional regulation in CRC cells. (A) Schematic representation of the putative miR-145 target site in the ERG 3'-UTR. The original or mutant binding site of miR-145 in the 3'-UTR of ERG was inserted into the Dual-Luciferase plasmid. (B) Western blot analysis indicated that miR-145 mimic transfection suppressed ERG protein levels, while anti-miR-145 inhibitor transfection generated the opposite effect, compared to their negative controls, respectively. (C) Transfection of miR-145 mimics in HEK-293T cells resulted in decreased luciferase activity of the reporter plasmid containing the wild-type ERG 3'-UTR compared to cells transfected with mimic control. This effect was abrogated by the mutation of the complementary site in the 3'-UTR of ERG mRNA. * $P < 0.05$. (D) The relative expression of miR-145 represents $2^{-\Delta\Delta C_t}$ normalized to sample T3. ERG expression is presented as the ratio of ERG/GAPDH normalized to sample T2, in which ERG expression was the lowest. The ERG protein level in eight CRC tissues showed an inverse correlation with miR-145 ($P < 0.05$, $r = -0.78$).

whether miR-145 exerts its function in CRC cells through ERG regulation, siRNA targeting ERG was synthesized and transfected into CRC cell lines RKO and SW480. In our experiments, siRNA transfection downregulated the ERG protein level (Fig. 5A). Moreover, miR-145 inhibitor transfection increased ERG levels, while miR-145 inhibitor and ERG siRNA co-transfection partially recovered the ERG expression compared to cells transfected only with ERG siRNA (Fig. 5A). In the invasion experiments Matrigel Transwell assay revealed that ERG siRNA suppressed the invasiveness of RKO and SW480. In our study ERG siRNA transfection decreased the number of invaded cells compared to the negative control, while transfection with miR-145 inhibitors increased the number of

invading cells. Co-transfection of miR-145 inhibitor and ERG siRNA recovered, to some degree, the invasive properties of RKO cells promoted by the miR-145 inhibitor (Fig. 5B).

Discussion

Over the past few decades, research efforts and improved understanding of the carcinogenesis and progression of colorectal cancer have contributed to its better diagnosis opening new avenues for targeted therapy. However, the efficacy of targeted therapy remains unsatisfactory due to our incomplete understanding of the tumor pathology and cell signaling pathways (20).

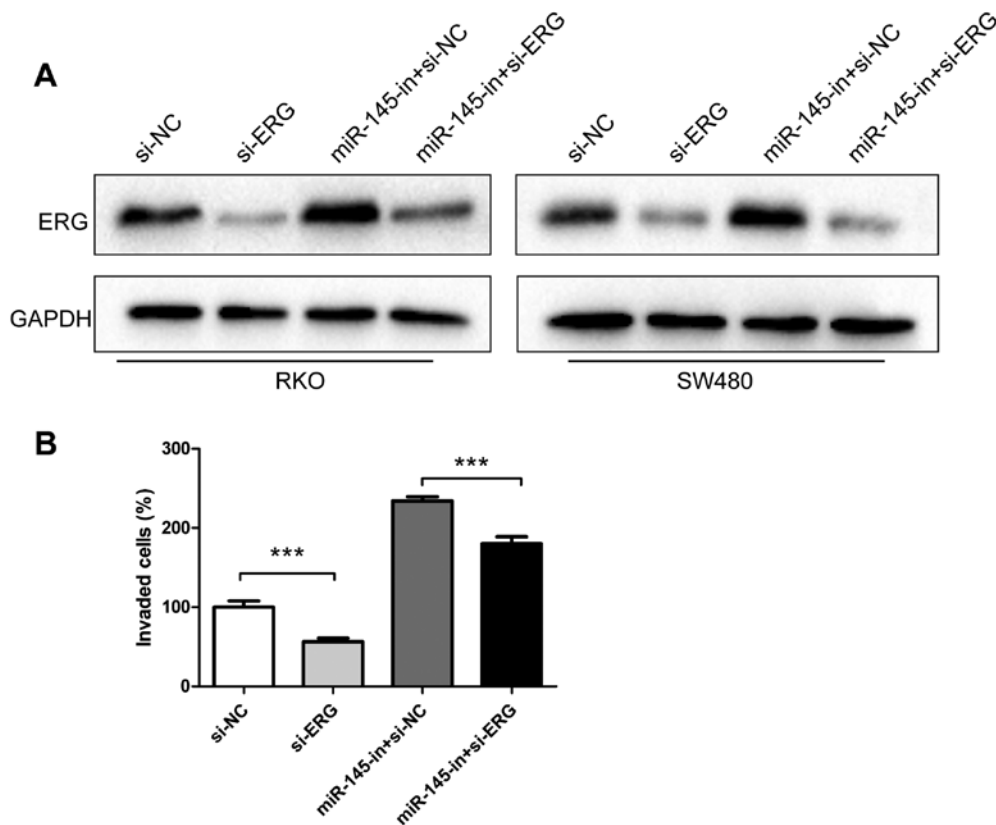


Figure 5. Inhibition of ERG expression by ERG siRNA partially reverses the effect of miR-145 knockdown in CRC cells. (A) Western blot analysis revealed that transfection of ERG siRNA into RKO cells resulted in decreased ERG expression compared to the cells transfected with scrambled siRNA. These effects of siRNA were attenuated by anti-miR-145 inhibitor transfection. (B) Transwell assays revealed that ERG siRNA transfection suppressed the invasiveness of RKO and SW480 cells. Co-transfection of ERG siRNA and anti-miR-145 inhibitors abrogated the suppressive effect caused by ERG knockdown. ***P<0.001.

Previous studies have shown that miR-145 is commonly downregulated in various types of cancer, including CRC, which indicated the possible role of miR-145 in carcinogenesis and tumor progression (21). With regards to its expression, miR-145 was found to be persistently decreased in adenoma and colorectal neoplasms (12). In addition, miR-145 downregulation was reported in ovarian cancer (22), cervical cancer (23), lung cancer (24), and gastric cancer (25). Previous functional studies have shown that miR-145 transfection represses CRC cell proliferation, invasion and metastasis by directly suppressing fascin-1 (26) and paxillin (27). In addition, miR-145 transfection negatively regulated IGF-1R expression and suppressed proliferation of Caco-2 cells (28). Furthermore, other recent studies reported that miR-145 could suppress RAD18 expression and consequently enhance DNA damage in CRC cells after 5-FU treatment, therefore implying that there is a drug-resistance reversal effect of miR-145 (29). Moreover, upregulation of miR-145 increased the sensitivity of drug-resistant Colo205 cells to vemurafenib both *in vitro* and *in vivo* (30). In a study by Pagliuca *et al*, resaturation of miR-145 and miR-143 in colon cancer cells showed that by targeting CD44, KLF5, KRAS and BRAF, miR-145 and miR-143 there was a resultant coordinated decrease in proliferation, migration and chemoresistance of colon cancer cells (31). Therefore, downregulation of miR-145 might play an important role in the tumorigenesis and progression of colorectal cancer and further studies on

miR-145 in cancer might bring new avenues for miR-145 targeted therapy.

Our previous study (32) examined the miRNA expression profiles of 31 pairs of CRC tissues and adjacent non-cancerous tissues and miR-145 was downregulated in most of the clinical samples. Thus, we further explored the role of miR-145 in colorectal tumorigenesis. Consistent with previous studies and our earlier findings (32), miR-145 expression was downregulated in both clinical CRC samples, as well as in cell lines, compared to normal colorectal tissue samples. Moreover, miR-145 expression in primary tumors taken from patients with lymph node metastasis was significantly lower than in tumors of patients without lymph node metastasis. This finding indicates a potential tumor-suppressive role of miR-145 in tumor metastasis. Moreover, miR-145 transfection in RKO and SW480 CRC cell lines significantly reduced cell migration and invasiveness. Contrary to these findings, miR-145 downregulation via the miR-145 inhibitor promoted migration and invasion of CRC cells compared to untreated cells.

In contrast to a majority of studies, a few studies have proposed that miR-145 is CRC oncogenic. For instance, Yuan *et al* reported that miR-145 promoted HCT-8 colon cancer cell metastasis by stabilizing Hsp-27, a protein associated with metastasis (33). In another study by Arndt *et al* miR-145 promoted cell proliferation and anchorage-independent growth by downregulating E-cadherin in the colorectal

cancer cell line SW620 (17). The fact that a specific miRNA can regulate various target genes may explain its dual and even contradictory influence in tumor initiation and progression. Indeed, diverse cellular contexts, tumor types and target gene sequence variations may explain why a specific miRNA might have diverse functions in tumor initiation and progression (34,35). Based on the present results and our previous findings (36) we propose that miR-145 acts as a tumor suppressor during the progression of CRC.

ERG is one of the transcription factors belonging to the ETS family, which is one of the largest families of transcriptional regulators with diverse functions and activities (37). All members of the ETS family functionally regulate carcinogenesis-related processes such as cell proliferation, angiogenesis, apoptosis and metastasis (38,39). Previous studies have demonstrated that ETS genes function as proto-oncogenes in colon cancer, and ETS-1 and -2 expression was associated with lymph node metastasis and advanced tumor grade in colon cancer (40). Ectopic expression of ERG was demonstrated in other tumors, such as acute myeloid leukemia and Ewing's sarcoma (41). Furthermore, invasive breast cancer mRNA expression datasets reflected a general ERG-driven pattern of malignancy (39). Emerging evidence suggest that ERG overexpression is involved in oncogenesis and progression of various cancer types; however, little is known about the regulation of ERG in CRC carcinogenesis. Interestingly, other members of the ETS family have been reported to be associated with tumor grade and metastasis status in colon cancer (42). Furthermore, aberrant expression of ERG and ETS-2 were associated with the development of cervical carcinoma (43). In addition, Scheble *et al* suggested that the TMPRSS2-ERG gene fusion, which can lead to overexpression of ERG, is specific for prostate cancer, and no such gene fusion was observed in CRC (44). Recent studies identified various miRNAs that can regulate ETS. For example, miR-196a and b act as ERG regulators in acute leukemia (45). miR-145 directly targets ERG in prostate cancer and suppresses proliferation of prostate cancer cells (46). Furthermore, miR-145 inhibits expression of ETS-1 in gastric cancer cells and exerts its suppressive effect towards invasion, metastasis and angiogenesis by regulating the genes downstream of ETS-1, such as matrix metalloproteinase-1 and -9 (47). miR-139 suppresses ETS-1 expression in CRC cells and inhibits cell proliferation and G1/S phase cell cycle transition (48). Thus, based on these findings we hypothesized that some regulatory mechanisms other than transcriptional regulation might contribute to the dysregulation of ERG observed in various cancer types including CRC. Our current results showed that ERG was upregulated in CRC cancer specimens compared to corresponding adjacent normal tissue. Based on the computational algorithm analysis, we speculated that miR-145 directly regulates ERG through interaction with the 3'-UTR of ERG mRNA. Our results confirmed our hypothesis since the downregulation of miR-145 increased ERG expression, while the restoration of miR-145 resulted in decreased ERG protein expression in CRC cells. Dual-Luciferase reporter assay suggested that miR-145-mediated suppression of ERG is dependent on the 3'-UTR of ERG mRNA. miR-145 expression inversely correlated with ERG protein levels, further confirming the negative

regulation of ERG by miR-145. Moreover, ERG knockdown suppressed the invasion of CRC cells *in vitro*, demonstrating that ERG may serve as a proto-oncogene in CRC. Furthermore, co-transfection of ERG siRNA and miR-145 inhibitor showed that ERG downregulation partially, but not completely, reversed the tumor promoting effect caused by the miR-145 inhibitor. Taken together, these results indicate that ERG acts as a proto-oncogene in CRC cells.

Although it is not yet clear whether ERG is subjected to transcriptional regulation in CRC, this current study indicates that there is post-transcriptional regulation of ERG by miR-145 in CRC. Previous studies have shown that miR-145 inhibits colon cancer cell growth by targeting Friend leukemia virus integration-1 (FLI-1), another member of the ETS family of transcription factors (49). Furthermore, Ban *et al* reported feedback regulation between EWS-FLI-1 and Hsa-miR-145 in Ewing's sarcoma (50). Therefore, along with our results, it is reasonable to speculate that miR-145 may regulate different members of the ETS transcription factor family and consequently modulate diverse pathological processes in CRC. Further studies are needed to understand the roles of miR-145 and ERG in CRC metastasis, which might provide us with a novel therapy for the treatment of colorectal cancer.

In conclusion, our results show that miR-145 is persistently downregulated during CRC progression. In addition, miR-145 might suppress CRC cell proliferation and invasion through direct repression of the proto-oncogene ERG. Therefore, restoration of miR-145 and suppression of ERG might be utilized as a potential new therapeutic strategy in colorectal cancer in the future.

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