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MiR-145 regulates PAK4 via the MAPK pathway and exhibits an antitumor effect in human colon cells

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ABSTRACT

MicroRNAs (miRNAs) are regulators of numerous cellular events; accumulating evidence indicates that miRNAs play a key role in a wide range of biological functions, such as cellular proliferation, differentiation, and apoptosis in cancer. Down-regulated expression of miR-145 has been reported in colon cancer tissues and cell lines. The molecular mechanisms underlying miR-145 and the regulation of colon carcinogenesis remain unclear. In this study, we investigated the levels of miR-145 in human colon cancer cells using qRT-PCR and found markedly decreased levels compared to normal epithelial cells. We identified PAK4 as a novel target of miR-145 using informatics screening. Additionally, we demonstrated that miR-145 targets a putative binding site in the 3′UTR of PAK4 and that its abundance is inversely associated with miR-145 expression in colon cancer cells; we confirmed this relationship using the luciferase reporter assay. Furthermore, restoration of miR-145 by mimics in SW620 cells significantly attenuated cell growth in vitro, in accordance with the inhibitory effects induced by siRNA mediated knockdown of PAK4. Taken together, these findings demonstrate that miR-145 downregulates P-ERK expression by targeting PAK4 and leads to inhibition of tumor growth.

1. Introduction

Over one million new cases of colorectal cancer are diagnosed per year in the USA; it is the third leading cause of cancer-related death in both men and women, and is the most malignant of digestive system cancers [1]. Recent advances in treatments have decreased the death rate in most developed countries, but the colorectal cancer mortality rate remains at second place for women and third place for men [2]. Further elucidation of the molecular mechanisms underlying tumorigenesis in colon cancer is crucial for development of improved therapeutic approaches and to prolong patient survival.

MicroRNAs (miRNAs) are a diverse family of small RNA molecules of 20–22 nucleotides that are highly conserved evolutionarily; they are essential post-transcriptional regulatory molecules in various cellular functions. One gene can be repressed by multiple miRNAs, and one miRNA can repress multiple target genes, thus, complex regulatory feedback networks are formed [3]. Accumulating evidence indicates that miRNAs play pivotal roles in regulating various cellular functions, such as cell apoptosis, cell proliferation, neural development, and stem cell differentiation [4–6]. Altered miRNA expression has been previously demonstrated to regulate cell growth, apoptosis, migration, or invasion in colon carcinomas in various study cohorts [5,7–10]. It has been widely reported that miR-145 is down-regulated in many cancer types, including prostate cancer [11,12], bladder cancer [13], colon cancer [14–17], and ovarian cancer [18,19]; moreover, the genomic region encoding miR-145 has been shown to be located in a fragile site often deleted in cancer [20]. Thus, depending on the cellular context and target genes regulated, miR-145 potentially functions as a tumor suppressor.

P21-activated kinases (PAKs), a family of serine/threonine protein kinases that have been shown to be important regulators of cancer cell signaling networks, and are involved in cancer cell motility, survival, apoptosis, and metastasis [21–23]. Six mammalian isoforms of PAKs are classified into two groups, I and II; group I includes mammalian PAK1, PAK2, and PAK3, whereas group II includes PAK4, PAK5, and PAK6 (PAK4–6) [24]. Previous studies have confirmed that PAK4 is up-regulated in a variety of cancer cell lines, including breast, prostate, gall bladder and stomach [21,25] as well as in several primary tumors [23]. PAK4 is thought to regulate cell proliferation involving the c-Src/EGFR/cyclin D1 pathway, which is regulated by AKT [26,27]. However, prior to this study, no miRNA has been previously reported to target PAK4 in colorectal cancer.

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In this study, we demonstrated that miR-145 expression was markedly decreased in colorectal cancer cells, and its down-regulation was significantly associated with cell growth. Furthermore, we confirmed the direct regulatory relationship between miR-145 and PAK4 and demonstrated that miR-145 suppressed colon cell proliferation, in part by targeting PAK4. These results demonstrate, for the first time, that PAK4 is a direct target of miR-145 in the regulation of tumor growth in vitro.

2. Materials and methods

2.1. Cell culture and reagents

Five human colorectal cancer (CRC) cell lines, including LOVO, SW480, SW620 HT29, and HCT116, were purchased from American Type Culture Collection. Human normal epithelial cell line NCM460 cells were provided by INCELL Corp and cultured in M3A medium (INCELL) containing 10% fetal calf serum. SW620 and SW480 cells were grown in Dulbecco's Modified Eagle Medium (D-MEM) with high glucose (Hyclone). HCT116 cells were grown in McCoys 5A Media (Gibco), and LOVO and HT29 cells were grown in RPMI Media 1640 (Hyclone). All media were supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin G, and 100 mg/ml streptomycin, and cells were cultured at 37 °C in humidified atmosphere of 5% CO_2.

Antibody specific to PAK4 (ab62509) was purchased from Abcam, phospho-Raf1 (9427), phospho-MEK (2354), and GAPDH (HRP Conjugate, 3683) were obtained from Cell Signaling Technology. Antibodies specific to phospho-ERK (2219-1) and horseradish peroxidase-coupled anti-rabbit secondary antibodies (3053-1) were obtained from Epitomics.

2.2. RNA extraction and quantitative real time reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA, USA) and reverse-transcription into single-stranded cDNA was conducted using PrimeScript® RT Master Mix Perfect Real Time according to the manufacturer's recommendations. Next, cDNA was subjected to real time PCR (using Applied Biosystems 7900 HT Sequence Detection System) and SYBR-Green assay with gene-specific primers at a final concentration of 0.2 μM. The primer sequences and PCR conditions for detection were as follows: β-actin F 5′-AGTGTGACGTTGACATCCGT-3′, β-actin R 5′-GCAGCTCAGTAACAGTCCGC-3′, PAK4 F 5′-CCGCTCCTACCTGGACAAC-3′, and PAK4 R 5′-CTCCTCGTTCATCCTGGTGT-3′, respectively, and amplification was conducted at 95 °C for 15 s, then 60 °C for 60 s. All samples were analyzed in duplicate and data are expressed as the quantity of mRNA normalized to β-actin mRNA. Mature miR-145 levels were quantified using TaqManH microRNA Assay (4373133, Applied Biosystems). Quantification was normalized to U6 small nuclear noncoding RNA (RNU6) which served as an endogenous control (1973, Applied Biosystems). Data were collected during annealing steps and data were further analyzed by SDS2.3 (ABI). Data were processed using $2^{-\Delta\Delta}$CT method. All qPCR reactions were performed in triplicate and included no-cDNA control reactions.

2.3. Plasmid constructs and generation of stable cell lines

All constructs were validated by sequencing. In order to obtain stable expression of miR-145 in colon cancer cells, we used human genomic DNA to amplify human pri-miRNA with the following primers: miR-145 F 5′-AAAGAATTCGGAAGTTGCCAAACCCAGGC-3′, miR-145 R 5′-AAAGGATCCGTTCCCACATCCAGCCTCACA-3′; the PCR products were then inserted into pCDH-CMV-MCS-EF1-GFP lentiviral vector for packaging the lentivirus followed by transduction of colon cancer cell lines SW480 and HT-29. In short, pri-miRNA-145 plasmid DNA and the transfection complex DNAs were transfected into 293T cells by lip2000 according to the manufacturer's recommendations. The blank vector was used as a negative control. Lentivirus in the supernatant was collected and utilized to transduce SW480 and HT-29 cells. Stable cell lines were selected using GFP by flow cytometry.

A fragment of 3′UTR of PAK4 containing the putative miR-145 binding site was amplified from human cDNA using the following primers: PAK4-WT-UTR F 5′-AAACTCGAGGGCCCAGCGCCCTTCCCCT-3′ and PAK4-WT-UTR R 5′-AAAGCGGCCGCGCAGGGGTGGGCGGGCTG-3′; XhoI and NotI cutting sites were introduced into full length 3'UTR by PCR. The PCR product was subcloned into a psi-CHECK-2 vector (Promega) immediately downstream of the luciferase gene sequence. Point mutations in PAK4 genes were introduced using PCR-based site-directed mutagenesis.

2.4. Cell proliferation assay

In vitro growth of SW480 and HT29 cells was measured using the cell proliferation assay WST-1 (Roche). A total of 4000 cells with 90 μl culture media were seeded into each well of 96-well plates and transfected with control siRNA or PAK4 siRNA at a final concentration of 50 nM and then further incubated at 37 °C for 24, 48, 72, and 96 h respectively. Following the addition of 10 μl WST-1 reagent to each well at 2 h, the OD450 nm value in each well was determined using a microplate reader.

2.5. Cell transfection

Small interfering RNAs (siRNA) specific to human PAK4 (ATGGGATACTCGCTGTGGCdTdT) and control siRNA (UUCUCCGAACGUGUCACGUdTdT) were designed and synthesized. Transfection was performed using Lipofectamine 2000 (Invitrogen). Unless otherwise indicated, 50 nM of RNA duplex and 200 nM of miRNA inhibitor were used for each transfection.

2.6. Western blot analysis

Cells were lysed with lysis buffer [0.125 M Tris (pH 6.8) at 22 °C containing [1% Nonidet P-40 (vol/vol), 2 mM N-ethylmaleimide, 2 mM EDTA, 2 mM PMSF, 1 mM sodium orthovanadate, and 0.1 μM sodium okadate] and cleared by centrifugation at 4 °C. The protein concentrations were determined by BSA protein assay (Thermor). Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and hybridized with corresponding antibodies. The signals were visualized using a chemiluminescence HRP Substrate (Millipore) and autoradiography.

2.7. Luciferase reporter assay

HEK293 cells were seeded in 96-well clusters and co-transfected with 50 nM lenti-miR-145 and 10 ng of firefly luciferase reporter comprised of wild-type or mutant 3′-UTR of the target gene PAK4. To assess antagonist, HEK293 cells were transfected with 50 nM anti-miR-C or anti-miR-145; 48 h after transfection, luciferase activity was detected using a dual-luciferase reporter assay system (Promega, Madison, WI) and normalized to firefly luciferase activity.

2.8. Statistical analysis

All data from three independent experiments were expressed as mean ± SD and processed using SPSS 16.0 statistical software. The

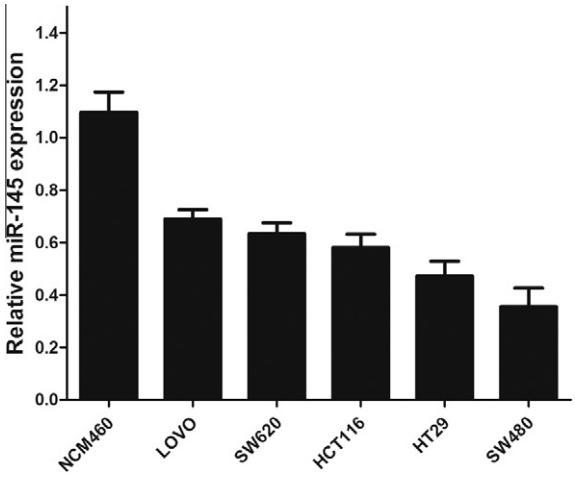

Fig. 1. Decreased expression of miR-145 in CRC cell lines. The expression of miR-145 was significantly decreased in five CRC cell lines compared with control normal colonic mucosa. Data are shown as the mean ± SD of three replicates.

differences among the groups in the cell proliferation assay were estimated using the Student's t-test or one-way ANOVA. A P-value of <0.05 was considered to indicate a statistically significant result.

3. Results

3.1. Lower levels of miR-145 are frequently detected in CRC cell lines

To determine whether miR-145 levels were downregulated in human colon cancer cells, we performed TaqMan real-time RT-PCR analysis to detect miR-145 expression levels in CRC cell lines. As shown in Fig. 1, miR-145 was significantly decreased in CRC cells compared with normal epithelial cell line NCM460; this finding is in concordance with previous studies in which miR-145 was found to be down-regulated in cancer cells.

3.2. MiR-145 directly targets PAK4

Next, we aimed to identify functionally relevant targets that could help further elucidate the role of miR-145 in cancer. We performed computational target prediction and hypothesized that PAK4, which exhibits a putative binding site of miR-145, is a target gene of miR-145. To confirm PAK4 as a target of miR-145, we cloned the 3'UTR of PAK4 mRNA containing the predicted conserved binding sites for miR-145 into psiCHECK2 downstream of a reporter gene; we also constructed its mutant version (PAK4-MUT-UTR) by mutagenesis of the binding site (Fig. 2A). We co-transfected pCDH-miR-145 or vector control and PAK4-WT-UTR or PAK4-MUT-UTR vector into HEK293 cells. The luciferase activity of miR-145 transfected cells was significantly reduced compared to vector control cells. However, miR-145-mediated repression of luciferase activity was abolished by the mutant putative binding site (Fig. 2B). In addition, inhibition of endogenous miR-145 in NCM460 cells by anti-miR-145 caused an increase in the luciferase activity of the wild-type reporter but not the mutant one (Fig. 2C). Next, we constructed stable SW480 and HT-29 cells that overexpessed miR-145. To verify the results of the gain-of-function study, we detected the expression levels of miR-145 in stable cell lines infected with the lentivirus carrying miR-145 (pCDH-miR-145) or the vector control (pCDH-V) using TaqMan. The expression levels of miR-145 were significantly increased in SW480-miR-145 and HT29-miR-145 cells (Fig. 2D), demonstrating that these stable cell lines successfully expressed miR-145. Moreover, the enhanced miR-145 expression in SW480 and HT29 cells significantly repressed PAK4 protein expression compared to vector control (Fig. 2E). These initial experiments suggest that miR-145 may

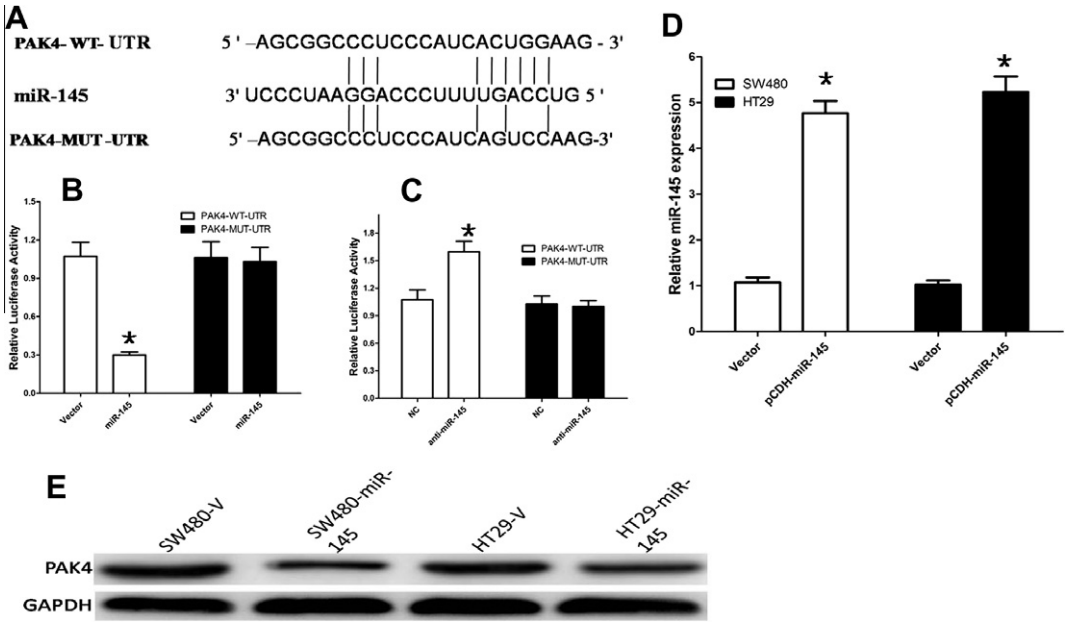

Fig. 2. MiR-145 directly binds at the PAK4 3'UTR region. (A) Construct sequence alignment of the miR-145 seed region and the predicted miR-145 binding site in PAK4 3′UTR (WT), and the mutant construct obtained by site-directed mutagenesis by changing three base pairs of miR-145 seed-sequence (MUT) and insertion into psiCHECK-2 vector. (B) The repression of luciferase activity by PAK4 3′UTR was dependent on miR-145 in 293T cells. Mutated PAK4 3′UTR eliminated miR-145 mediated repression of luciferase activity ($^*P < 0.05$). (C) NCM460 cells were co-transfected with anti-miR-C or anti-miR-145 and firefly luciferase reporter plasmid containing wild-type or mutant 3′UTR of putative target gene. The results indicated that downregulation of miR-145 significantly increased luciferase activity. Data shown are the mean ± SD of three replicates. $*$, $p < 0.05$ using a two-tailed t-test. (D) Cells stably expressing vector control (V) and miR-145 were subjected to TaqMan real-time RT-PCR analysis for miR-145 with U6 as a control. Results are presented as mean ± SD of three replicate experiments. (E) In comparison with vector controls, miR-145 stable overexpression by lentivirus inhibited PAK4 endogenous expression at the protein level.

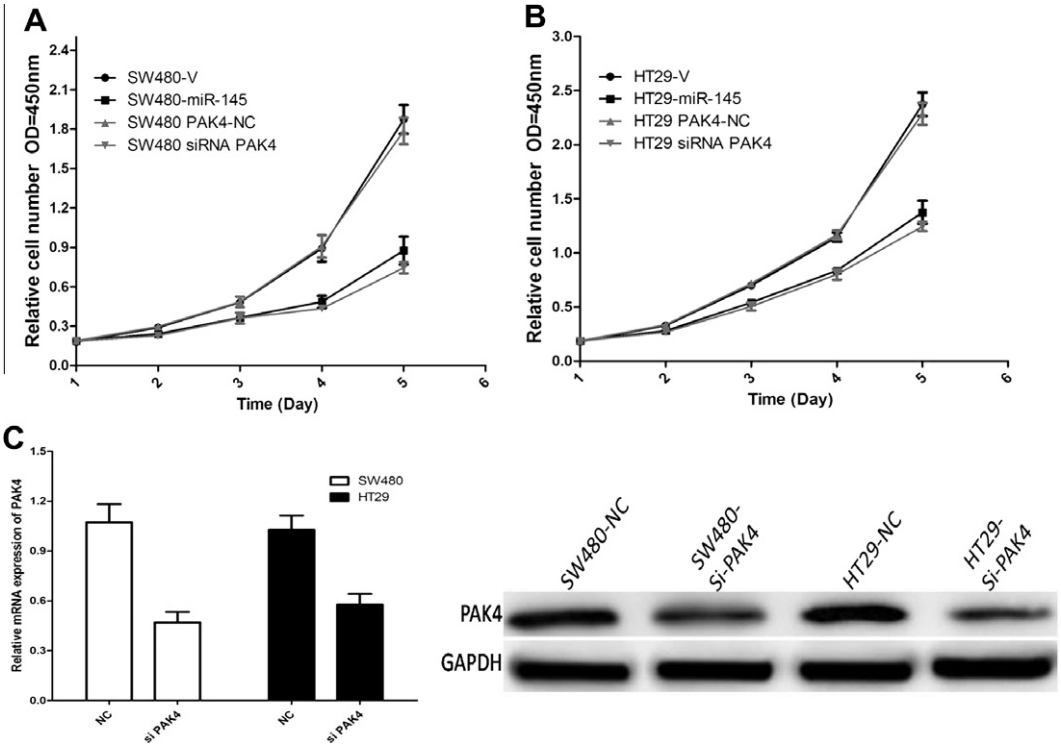

Fig. 3. CRC cell growth was effected by miR-145 and PAK4. (A and B) Ectopic expression of miR-145 by stable overexpression cells reduced proliferation in comparison with vector controls in SW480 and HT29 cells (*$P < 0.05$). Knockdown of PAK4 by siRNA was confirmed according to miR-145 overexpression. (C) Effective suppression of PAK4 protein expression by PAK4 siRNA. The mRNA and protein levels of PAK4 were analyzed by Western blot analysis and real-time PCR in cells transfected with NC and siPAK4.

negatively regulate the expression of PAK4 by directly targeting the 3′UTR of its mRNA.

3.3. MiR-145 function and CRC cell growth

To further explore the biological significance of miR-145 in CRC cells, we performed cell proliferation assays to assess SW480-miR-145 and SW480-miR-V. Overexpression of miR-145 markedly inhibited cell growth (Fig. 3A). The suppression of proliferation caused by miR-145 was reproducible in HT-29 cells (Fig. 3B). To clarify the relationship between miR-145 and the target gene PAK4, we knocked-down the PAK4 gene by siRNA transfection; this resulted in greatly reduced mRNA and protein PAK4 levels (Fig. 3C). In concordance with previous studies, silencing of PAK4 resulted in significant inhibition of cell growth (Fig. 3A and B). Taken together, these results indicated that PAK4 acted as a mediator of miR-145; however, other regulatory mechanisms are potentially involved.

3.4. MiR-145 potentially inhibits cell proliferation via the MAPK pathway and PAK4

Cell proliferation in response to mitogenic stimulation involves phosphorylated ERK1/ERK2. We investigated whether the Raf/MEK/ERK pathway was involved in the miR-145 antitumor effect by targeting PAK4. We found that specific transient Pak4 reduced P-RAF, P-MEK, and ERK1/2 activity, in concordance with published reports (Fig. 4A). Given the important roles of ERK1/2 in the induction of cell growth, we investigated whether miR-145 inhibited cell growth through the reduction of ERK1/2 expression by targeting PAK4. We detected the protein levels of RAF/MEK/ERK during miR-145 overexpression. Overexpression of miR-145 reduced the activation of the Raf/MEK/ERK pathway in SW480 and HT29 cells (Fig. 4B). Taken together, these findings indicate that miR-145 target PAK4 inhibits the PAK4/Raf/MEK/ERK pathway to suppress CRC pathogenesis.

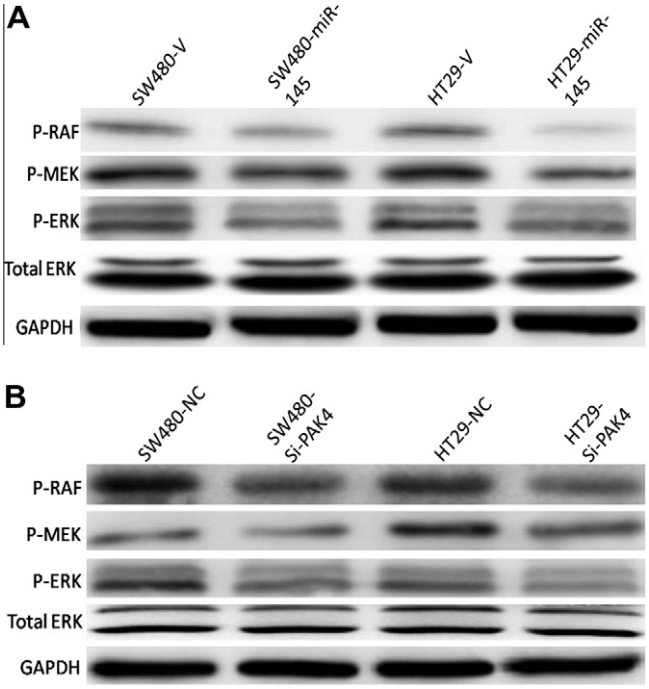

Fig. 4. Overexpression of miR-145 inhibited the Raf/MEK/ERK pathway. (A) Total cellular proteins were collected and subjected to Western blot analysis. Stable overexpression of miR-145 reduced the protein level of P-RAF, P-MEK and P-ERK. (B) The protein levels of the above study were also detected in SW480 and HT29 cells by siPAK4.

4. Discussion

Although dysregulation of miRNAs has been observed in various types of human cancer [28,29], the molecular mechanisms underlying miRNA modulation of carcinogenesis remain unclear. Recently, an important discovery demonstrated that miR-145 was involved in regulating cell reprogramming, neural development, cytoskeletal dynamics, and smooth muscle cell fate and plasticity [5,30,31]. Low-level expression of miR-145 was significantly associated with a more aggressive tumor phenotype, including colon cancer [14,15,17]. In addition, miR-145 has been reported to be involved in the death-promoting regulatory loop of p53 [15,32,33], indicating the key role of miR-145 in carcinogenesis and cancer development. In this study, our findings suggest that miR-145 plays a critical role in colon carcinoma.

We found that the expression level of miR-145 was commonly down-regulated in CRC cell lines compared to a normal epithelial cell line by quantitative PCR, in concordance with previous studies [17]. Because SW480 and HT29 cells exhibited lower miR-145 expression, we performed functional assays in these two cell lines. We constructed SW480 and HT29 stable cell lines that overexpressed miR-145, and found that miR-145 inhibited cell proliferation. Our results, taken together with previous studies, indicate that miR-145 plays a fundamental antitumor role as well as a role concerning the phenotype of cancer cells.

PAK4, a subfamily of serine/threonine kinases involved in cell motility and cytoskeletal dynamics, plays a fundamental role in oncogenic signaling pathways. Amplification of the PAK4 gene has been demonstrated in numerous human carcinomas [21,26,27]. The mechanisms of Pak4, involved in c-Src and Pak4 to MEK1/ERK1/2 to MMP2 signaling pathways, are linked to cell growth. To address the molecular mechanisms involved in miR-145-mediated effects, PAK4 was selected for further study because it was predicted to be one target of miR-145 by informatics screening. Our results demonstrated that miR-145 directly bound the specific complementary site of 3′-UTR of PAK4 according to the luciferase reporter study. Overexpression of miR-145 in human colon cancer cell lines SW480 and HT-29 dramatically decreased the mRNA and protein expression of PAK4. Knockdown of PAK4 by siRNA demonstrated suppressive effects on cellular growth identical to the gain-of-function study by miR-145 overexpression. Previous studies have demonstrated that the MAPK pathway participates in regulating cellular proliferation and that Pak4 can activate ERK by phosphorylating and activating Raf [34]. Taken together, these findings revealed that the inhibitory effect of PAK4 is similar to the overexpression of miR-145. This is the first study to show that PAK4 is negatively regulated by miR-145 at the post-transcriptional level by binding a specific target site within the 3′UTR of PAK4, and it suggests a potential molecular mechanism in which miR-145 participates in CRC aggressiveness.

In summary, our study demonstrates the dysregulation of miR-145 in CRC cells. We demonstrated that miR-145 plays an important role in inhibiting cell growth by directly targeting PAK4, a new target gene. These results indicated that miR-145 overexpression and inhibition of the PAK4/MAPK signaling pathway may potentially provide strategic therapeutic applications in colon cancer in the future.

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