

SphK1 modulates cell migration and EMT-related marker expression by regulating the expression of p-FAK in colorectal cancer cells

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Abstract. Sphingosine kinase 1 (SphK1) plays an important role in colorectal carcinoma metastasis. However, whether SphK1 modulates epithelial-mesenchymal transition (EMT)-related marker expression and the underlying mechanisms remain unclear. In this study, in order to clarify this issue, we used various colorectal cancer (CRC) cell lines, Caco2, HT29, RKO and HCT116. Each of the cell lines was divided into 3 groups as follows: the control group, SKI-II (SphK1 inhibitor) group and PF-562271 [focal adhesion kinase (FAK) inhibitor] group. The migratory ability of the cells was examined by Transwell chamber assay. The mRNA and protein expression levels of SphK1, FAK (p-FAK), Slug, vimentin, N-cadherin and E-cadherin were detected by PCR and western blot analysis, respectively. The results revealed that the suppression of SphK1 reduced the cell migratory ability, and decreased the expression of Slug, vimentin and N-cadherin; however, the expression of E-cadherin was increased. Moreover, the inhibition of SphK1 reduced the expression of p-FAK. The inhibition of FAK (p-FAK) also decreased the cell migratory ability, and decreased the expression of Slug, vimentin and N-cadherin, whereas the expression of E-cadherin was increased. Thus, our data suggest that SphK1 modulates the expression of EMT-related markers and cell migration by regulating the expression of p-FAK in CRC cells. Thus, SphK1 may play a functional role in mediating the EMT process in CRC.

Introduction

Colorectal cancer (CRC) is a leading cause of cancer-related mortality (1). Metastasis is the most related cause of the majority of human cancer-related deaths (2). Metastasis is a complex process and many scientists have strived to understand the mechanisms behind this phenomenon over the years (3); however, the mechanisms responsible for the occurrence of metastasis remain to be fully elucidated. The treatment of colon cancer involves a comprehensive therapeutic approach (4). Thus, the more in depth understanding of the mechanisms responsible for CRC metastasis may provide valuable direction and an experimental basis for its treatment in future.

Epithelial-mesenchymal transition (EMT) had been highlighted as a process through which epithelial cells lose their characteristics and gain mesenchymal properties to be motile, playing a critical role in the metastasis of cancer cells (5,6). Among the EMT-related transcription factors, the Snail family of zing finger transcription factors is prominent, particularly Slug (Snail2) (7,8). Stimulating Slug expression has been shown to suppress E-cadherin and to concomitantly upregulate N-cadherin expression (9,10). Moreover, Slug induces vimentin expression (11). Therefore, Slug regulates E-cadherin and N-cadherin, and vimentin expression.

Sphingosine kinase 1 (SphK1) is an oncogenic enzyme which promotes the transformation, proliferation and angiogenesis of a number of human tumors (12,13). Recent studies have indicated that SphK1 is involved in regulating the NF- κ B pathways (14,15), AKT (16) and focal adhesion kinase (FAK) (17) in cancer. We wished to determine whether SphK1 potentially promotes the EMT process in CRC cells, as it has been previously shown to promote EMT in lung cancer cells (18).

To date, some researchers have discovered that FAK plays an important role in EMT and influences the expression of EMT-related makers (19-21). Thus, we hypothesized that SphK1 may affect metastasis, and the expression of Slug, E-cadherin, N-cadherin and vimentin via the FAK pathway in CRC cells. In order to confirm our hypothesis, in this study, we used various CRC cells, and we found indeed, that SphK1 modulates the expression of EMT-related markers by regulating the expression of p-FAK in CRC cells.

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Materials and methods

Cell lines and cell culture. The human colorectal carcinoma cell lines, Caco2 (Boster, Wuhan, China), HT29, RKO and HCT116 (R&S Biotechnology Co., Ltd., Shanghai, China) were routinely cultured in DMEM containing 100 ml/l fetal bovine serum (FBS; ExCell Bio, Shanghai, China) and incubated at 37°C with 5% CO₂.

Each of the cell lines was divided into 3 groups as follows: the control group (N group), the SKI-II (an inhibitor of SphK1) intervention group (SK group) and PF-562271 (an inhibitor of FAK) intervention group (PF group). The cells in the SK group were cultured for 24 h and then incubated with SKI-II (Selleck, Houston, TX, USA) at 20 μ M (22) for 48 h. The cells in the PF group were also cultured for 24 h and then incubated with PF-562271 (Selleck) 5 μ M (23) for 48 h.

Transwell chamber assay. The cells were cultured in serum-free medium to produce suspension, and the cell density was adjusted to 5×10^5 /ml. Subsequently, 200 μ l cell suspension were added to the upper chamber of the Transwell (Corning, Inc., Corning, NY, USA), while 600 μ l medium including 10% FBS were added to the bottom chamber, followed by incubation for a period of 24 h at 37°C. The assay was terminated as a result of the removal of the medium from the upper well and the filter was fixed with methanol for 10 min. The cells in the upper chamber were wiped off and the cells migrating to the lower side of the upper chamber were stained with 0.1% crystal violet (Beyotime, Shanghai, China) for 30 min. Random fields were scanned (6 fields/filter) under a fluorescent inverted phase contrast microscope (magnification, x200; TS100-F; Nikon, Tokyo, Japan) for the presence of the cells at the lower membrane side only.

RNA isolation, cDNA synthesis and quantitative PCR (qPCR). RNA isolation was performed using a RNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's instruction. cDNA synthesis was performed using a reverse transcription kit (Takara, Dalian, Japan). Fluorescence-based qPCR was performed using 2 μ l of cDNA and SYBR-Green (Takara) in a total volume of 20 μ l and using the ABI 7500 Real-time PCR system (Applied Biosystems, Rockford, IL, USA). The cycling parameters were: 95°C for 30 sec followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec, and a dissociation program that included 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec ramping up at 0.2°C/sec. Gene expression levels were determined with the $2^{-\Delta\Delta C_q}$ method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference and the non-stimulated condition was set to 1. The forward and reverse primers (Takara) of the genes are listed in Table I.

Western blot analysis. The cells were collected using cell scrapers and lysed with RIPA lysis buffer supplemented with 1% protease and 1% phosphatase inhibitor for 30 min following 2 washes with cold phosphate-buffered saline (PBS). The cells were subjected to centrifugation at 12,000 \times g/min for 15 min at 4°C to remove the cell debris. Protein concentrations were measured by bicinchoninic acid (BCA) assay (Solarbio, Beijing, China) according to the standard protocol.

Table I. The primers used in this study.

Gene name (protein name)	Sequence primers (5'→3')
GAPDH (GAPDH)	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA
SPHK1 (SphK1)	F: GGCTTCATTGCTGATGTGGA R: AGGAAGGTGCCCAGAGTGAA
FAK (FAK)	F: CAACCACCTGGGCCAGTATTATC R: CCATAGCAGGCCACATGCTTTA
SLUG (Slug)	F: TTTGCAAGATCTGCGGCAAG R: CTGCAAATGCTCTGTGTCAGTG
VIM (vimentin)	F: TGACATTGAGATTGCCACCTACAG R: TCAACCGTCTTAATCAGAAGTGTCC
CDH2 (N-cadherin)	F: AGCACAGTGGCCACCTACAAAG R: CAGCTCCTGGCCAGTTACA
CDH1 (E-cadherin)	F: GAGTGCCAACTGGACCATTTCAGTA R: AGTCACCCACCTCTAAGGCCATC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SPHK1, sphingosine kinase 1; FAK, focal adhesion kinase.

Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto 0.45 nitrocellulose membranes. The membranes were subsequently blocked in non-fat milk [dissolved in Tris-buffered saline with 5% Tween-20 (TBST)] buffer at room temperature for 30 min to block non-specific binding and incubated with antibodies diluted by western blot antibody diluents overnight at 4°C, and subsequently incubated with secondary fluorescent antibodies (IRDye® 680RD Goat anti-Rabbit 925-68071; C60329-11; dilution, 1:10,000; LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature following 3 washes with TBST. The membranes were scanned using an Odyssey infrared imaging system (LI-COR Biosciences). Relevant signal intensity was determined using LI-COR imaging software (LI-COR Biosciences). The strips were analyzed using Quantity One software (Bio-Rad, Shanghai, China). Antibodies against E-cadherin (3195S; 1:1,000) and p-FAK (Tyr397; 8556P; 1:1,000) were purchased from Cell Signaling Technology (CST, Beverly, MA, USA). Antibodies against SphK1 (10670-1-AP; 1:500), FAK (12636-1-AP; 1:1,000), Slug (12129-1-AP; 1:500), N-cadherin (66219-1-Ig; 1:500), vimentin (10366-1-AP; 1:500) and GAPDH (10494-1-AP; 1:1,000) were purchased from Proteintech Group (PTG, Rosemont, IL, USA).

Statistical analysis. Each experiment was repeated at least 3 times. Data were presented as the means \pm SD. The results of Transwell chamber assay and western blot analysis were analyzed using the t-test. The Mann-Whitney U test was used to analyze the results of PCR. Results were considered statistically significant at $P < 0.05$.

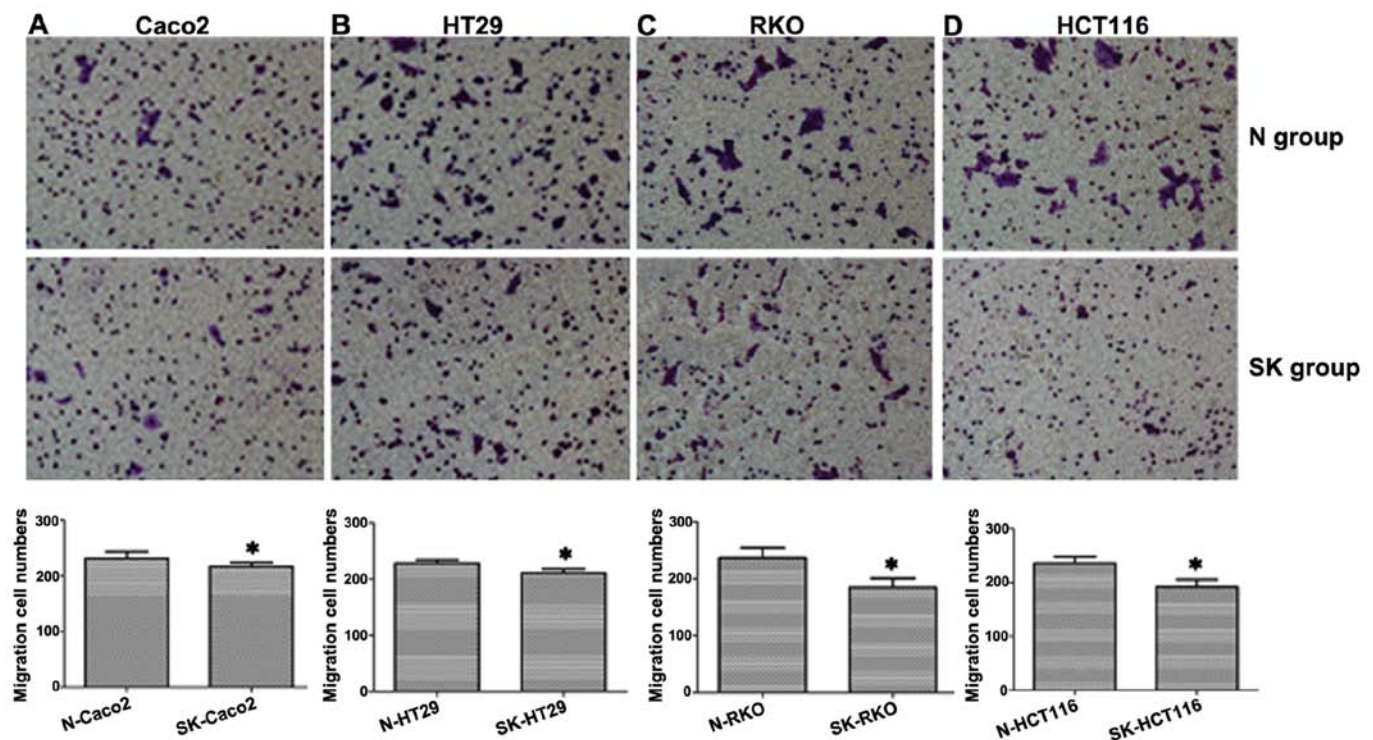


Figure 1. Sphingosine kinase 1 (SphK1) affects colorectal cancer cell migration. The suppression of SphK1 by SKI-II (20 μ M) for 48 h (SK group) significantly decreased the number of cells penetrating through the porous membrane. (A) Caco2 cell line. (B) HT29 cell line. (C) RKO cell line. (D) HCT116 cell line. The images are representative of cell penetration (crystal violet staining, magnification, x200) and the data are shown as the means \pm SD (* P <0.05 vs. N group).

Results

Suppression of SphK1 reduces the migratory ability of CRC cells. To examine the effect of SphK1 on the migration of human CRC cells, the cells were exposed to 20 μ M SKI-II for 48 h, in order to inhibit SphK1 expression. The migrating cells were observed under an inverted microscope. The results revealed that the number of migrating cells in the SK group was significantly lower than that of the cells in the N group in all 4 CRC cell lines (P <0.05; Fig. 1), indicating that SphK1 plays an important role in CRC cell migration.

mRNA expression of SphK1, Slug, vimentin, N-cadherin and E-cadherin detected by fluorescence-based qPCR. In order to investigate the signaling pathways involved in the regulatory effects of SphK1 on the migratory ability of CRC cells, some EMT-related markers were examined in the present study. EMT is known to play a critical role in the metastasis of cancer cells (24,25). The mRNA expression of EMT-related markers was detected, including the expression of the transcription factor, Slug (26), the mesenchymal cell markers, vimentin and N-cadherin, and the epithelial cell marker, E-cadherin (27-29). As shown in Fig. 2, the mRNA levels of Slug, vimentin and N-cadherin decreased, whereas that of E-cadherin increased (P <0.05). This indicated that the suppression of SphK1 suppressed Slug, vimentin and N-cadherin gene expression, while it promoted E-cadherin gene expression.

Protein expression of SphK1, Slug, vimentin, N-cadherin and E-cadherin detected by western blot analysis. The results

revealed that the expression of Slug, vimentin and N-cadherin decreased, whereas that of E-cadherin increased following the suppression of SphK1 (P <0.05; Figs. 3 and 4). These results were consistent with those obtained by PCR.

SphK1 is involved in modulating the expression of p-FAK. Compared to the N group, although in the SK group FAK mRNA and total FAK protein expression exhibited no significant difference (P >0.05; Fig. 5B and D), p-FAK protein expression was markedly decreased (P <0.05; Figs. 3 and 5E). This indicated that SphK1 regulated the expression of p-FAK, suggesting that SphK1 is involved in modulating the FAK pathway.

Inhibition of FAK affects the migratory ability, and the mRNA and protein expression of Slug, vimentin, N-cadherin and E-cadherin in CRC cells. To date, some researchers have demonstrated that FAK is involved in regulating the EMT process in cancer cells (30). Moreover, FAK affects the expression of Slug (31). In this study, our results revealed that the numbers of migrating cells in the PF group were lower than those in the N group in the Caco2, HT29, RKO and HCT116 cells (P <0.05; Fig. 1 and 6A). In addition, compared with the N group, the mRNA expression levels of FAK, Slug, vimentin and N-cadherin in the PF group were decreased, whereas the mRNA expression level of E-cadherin was increased (P <0.05; Fig. 6B). Moreover, the protein expression levels of FAK, p-FAK, Slug, vimentin and N-cadherin in the PF group were lower than the levels in the N group (P <0.05; Figs. 3 and 6C). Similar with the mRNA expression, the protein

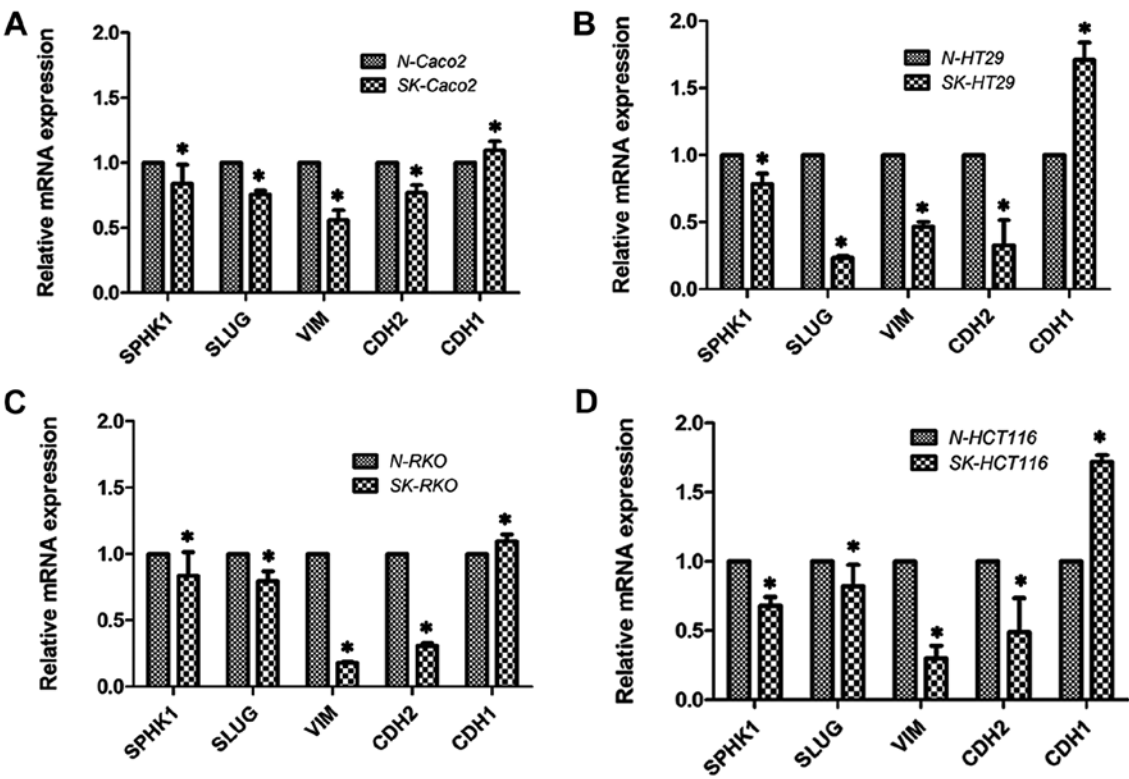


Figure 2. Suppression of sphingosine kinase 1 (SphK1) by SKI-II 20 μM for 48 h (SK group) significantly decreased the mRNA levels of SphK1 (SPHK1), Slug (SLUG), vimentin (VIM), N-cadherin (CDH2), while it increased E-cadherin (CDH1) expression. GAPDH was used as a reference and the unstimulated control (N group) was set to 1. (A) Caco2 cell line. (B) HT29 cell line. (C) RKO cell line. (D) HCT116 cell line. The data were analyzed using the Mann-Whitney U test and are shown as the means ± SD (*P<0.05 vs. N group).

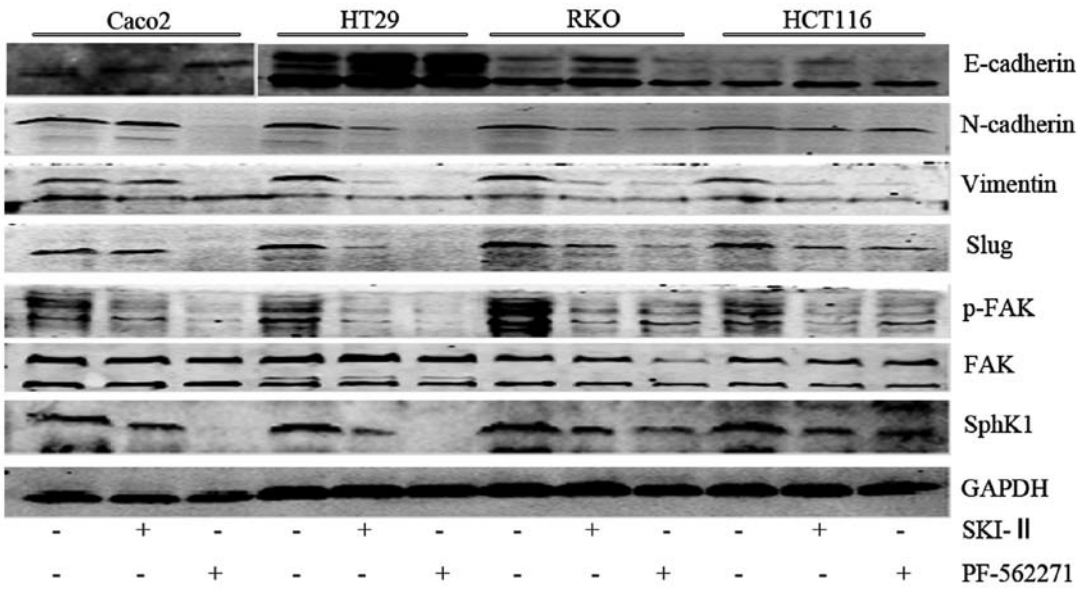


Figure 3. Protein expression of sphingosine kinase 1 (SphK1), FAK, p-FAK, Slug, vimentin, N-cadherin, E-cadherin in Caco2, HT29, RKO, HCT116 cells. '-' indicates culture without SKI-II or PF-562271, while '+' indicates treatment with SKI-II 20 μM for 48h or PF-562271 5 μM for 48 h.

expression of E-cadherin in the PF group was higher than that in the N group (P<0.05; Figs. 3 and 6C). Thus, the results revealed that FAK may affect the mRNA and protein expression levels of Slug, vimentin, N-cadherin and E-cadherin, as well as the cell migratory ability.

On the whole, according to our data, it can be suggested that SphK1 modulates the expression of Slug, E-cadherin, N-cadherin and vimentin, as well as CRC cell metastasis by regulating the expression of p-FAK in Caco2, HT29, RKO, HCT116 CRC cell lines.

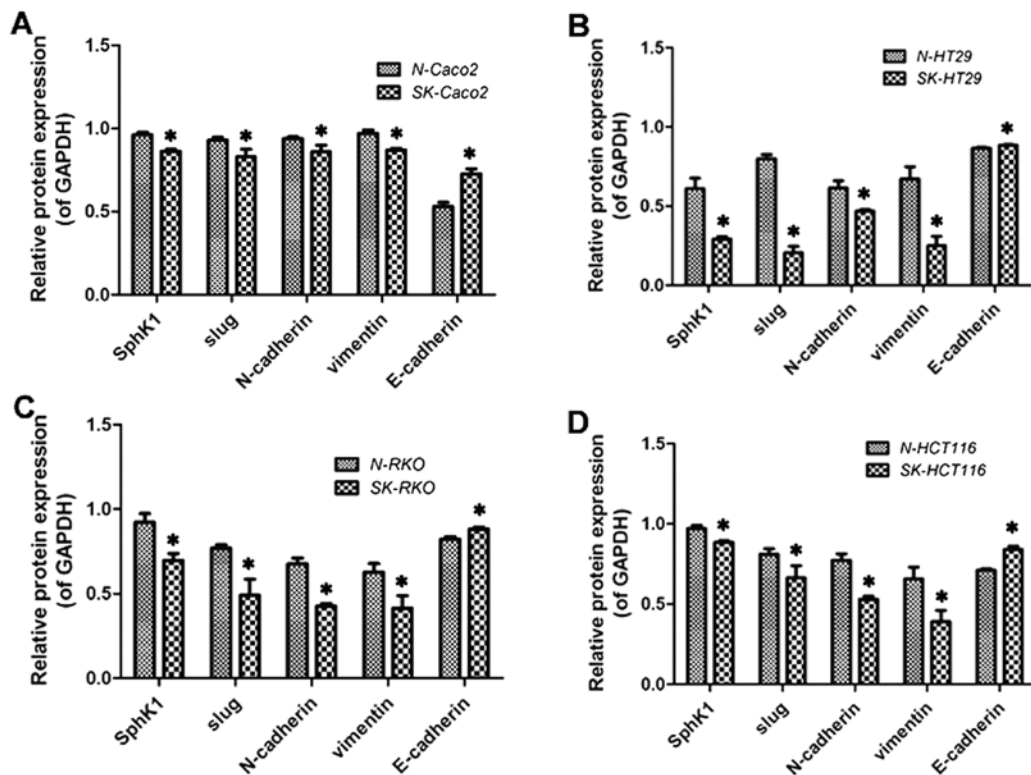


Figure 4. Relative protein expression levels of sphingosine kinase 1 (SphK1), Slug, vimentin, N-cadherin, E-cadherin. GAPDH was used as an internal control. (A) Caco2 cell line. (B) HT29 cell line. (C) RKO cell line. (D) HCT116 cell line. The data were analyzed by the t-test and are shown as the means \pm SD (*P<0.05, SK group vs. N group).

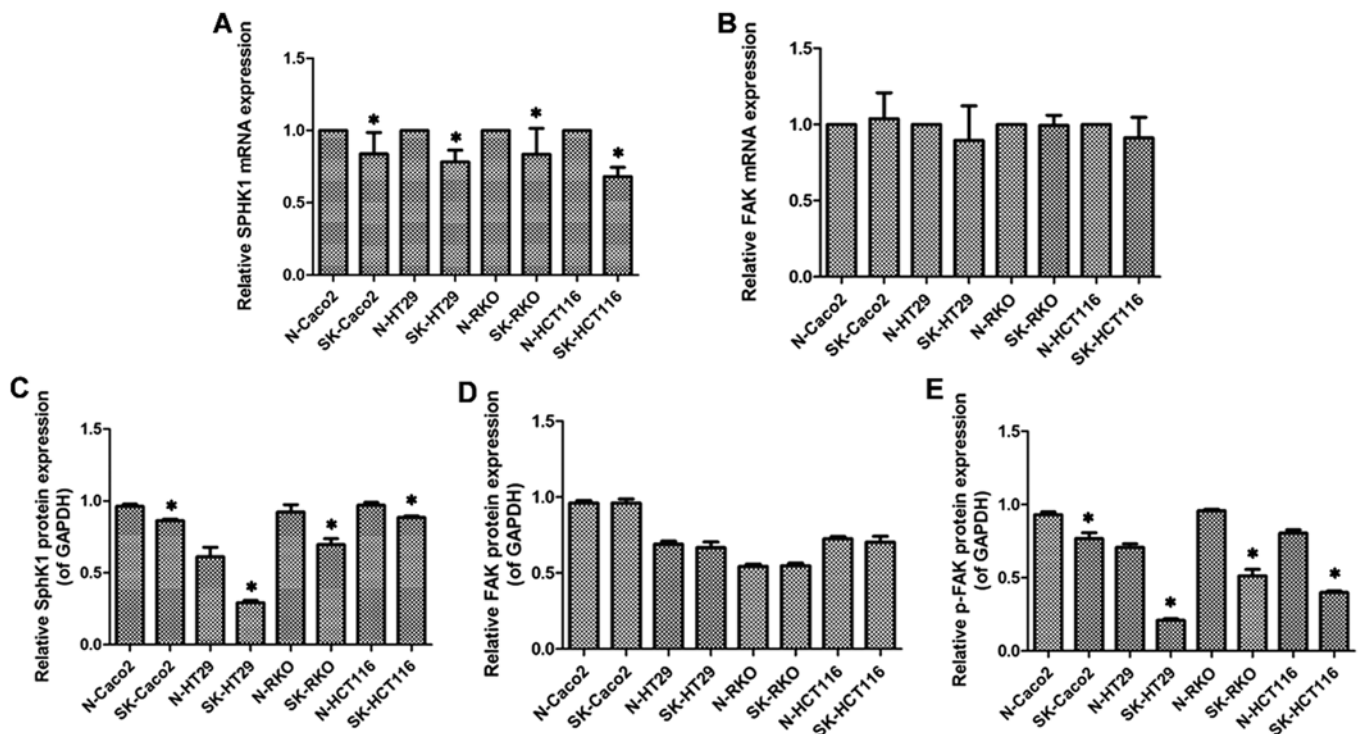


Figure 5. (A and B) The mRNA expression of sphingosine kinase 1 (SphK1) and FAK was detected by fluorescence-based quantitative PCR. GAPDH was used as a reference and the unstimulated control (N group) was set to 1. The data were analyzed using the Mann-Whitney U test. (C-E) Relative protein expression levels of SphK1, FAK, p-FAK. GAPDH was used as an internal control. The data were analyzed using the t-test. All data are shown as the means \pm SD (*P<0.05 vs. N group).

Discussion

SphK1 is a lipid kinase which catalyzes the phosphorylation of

sphingosine to S1P. There is evidence to indicate that SphK1 is an oncogenic enzyme, and its activation is closely associated with the transformation, proliferation and survival of tumor

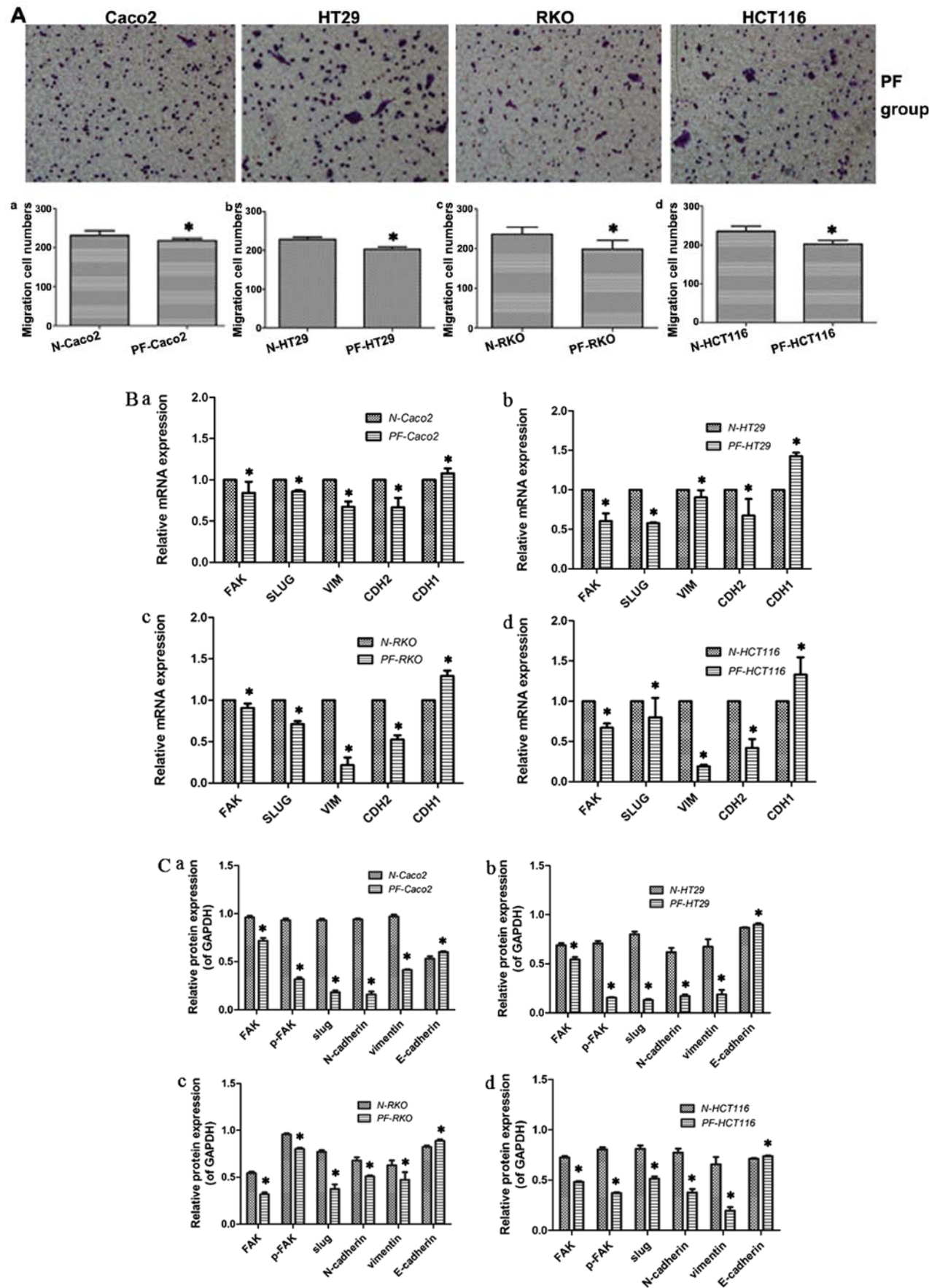


Figure 6. Effects of FAK inhibition of the expression of epithelial-mesenchymal transition (EMT)-related markers. PF group indicates the suppression of FAK by PF-562271 at 5 μ M for 48 h. (A) Cell migratory ability was examined by Transwell chamber assay (the N group is shown in Fig. 1). The data were analyzed using the t-test. (B) The mRNA expression of FAK and EMT-related markers was detected by fluorescence-based qPCR. GAPDH was used as a reference and the N group was set to 1. The PCR data were analyzed using the Mann-Whitney U test. (C) Relative protein expression levels of FAK, p-FAK and EMT-related markers. GAPDH was used as an internal control. The data were analyzed using the t-test. (a) Caco2 cell line. (b) HT29 cell line. (c) RKO cell line. (d) HCT116 cell line. All data are shown as the means \pm SD (* P <0.05, PF group vs. N group).

cells (32). In a previous study, SphK1 was shown to be overexpressed in colon cancer tissue compared with normal colonic tissue. In addition, the expression of SphK1 was found to correlate with the Dukes' stage, histological grading, lymphnode metastasis and distant metastasis. These data indicate that SphK1 may contribute to the metastasis and the malignant phenotype of colon cancer (17).

EMT has been confirmed to be an important step in metastasis and in the change to the malignant phenotype. After EMT has occurred in the cell, cell migratory ability increases, along with an increase in the expression of mesenchymal cell surface markers, and a decrease in the expression of epithelial cell surface markers and vice versa (33-35). Slug is one of the important transcription factors of EMT (26,36). E-cadherin is the molecular marker of epithelial cells (27-29). N-cadherin and vimentin are molecular markers of mesenchymal cells. In a word, they are all indispensable biological markers in the process of EMT. However, it is not yet clear whether SphK1 modulates the expression of EMT-related markers, though SphK1 may contribute to metastasis and to the change to the malignant phenotype.

A previous study demonstrated that the silencing of SphK1 decreased the expression of vimentin and enhanced the expression of E-cadherin in non-small cell lung cancer. Regretfully, Slug and N-cadherin expression was not detected (18). In the present study, the expression of Slug, N-cadherin and vimentin was decreased, whereas that of E-cadherin was increased following the inhibition of SphK1. These results indicate that SphK1 plays a role in regulating the EMT process. Moreover, the migratory ability of CRC cells was found to weaken following the inhibition of the expression of SphK1, which is consistent with the findings of other researchers (37). Thus, SphK1 may regulate cell migration and the EMT process in CRC cells.

However, it is also important to determine the potential mechanism behind the modulatory effects of SphK1 on the expression of EMT-related markers. It was previously found that the FAK pathway is involved in the SphK1-mediated acquisition of the malignant phenotype in colon cancer cells (17). FAK is a 125 kDa non-receptor protein tyrosine kinase that has been shown to be important in the tumorigenesis and development of human tumors (38). Studies have suggested that the FAK pathway positively participates in the EMT process, which is accompanied by a decrease in the expression of E-cadherin, and an increase in the expression of Slug, N-cadherin and vimentin (32,39,40). In our study, the expression of E-cadherin increased, while the expression of Slug, N-cadherin and vimentin decreased following the suppression of FAK. This study also confirmed that the inhibition of SphK1 suppressed the expression of p-FAK in the CRC cell lines, Caco2, HT29, RKO and HCT116, suggesting that SphK1 may be involved in the modulation of the FAK pathway in CRC cells. On the other hand, the results revealed that the inhibition of SphK1 and FAK expression using specific inhibitors had similar effects on the cell migratory ability and on the expression of EMT-related markers. This suggests that SphK1 modulates the EMT process and cell migration by regulating the expression of p-FAK in CRC cells.

In conclusion, in this study, we demonstrated that SphK1 modulated the expression of EMT-related markers and cell

migration by regulating the expression of p-FAK in the CRC cell lines, Caco2, HT29, RKO and HCT116. Our results provide evidence of the functional role of SphK1 in mediating the EMT process in colon cancer. Thus, SphK1 may be a promising therapeutic target in CRC.

Acknowledgements

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