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Down regulation of lncRNA MEG3 promotes colorectal adenocarcinoma cell proliferation and inhibits the apoptosis by up-regulating TGF-β1 and its downstream sphingosine kinase 1

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Abstract. – OBJECTIVE: LncRNA MEG3 is involved in the pathogenesis of several types of cancers. While its participation and function network in colorectal (CR) adenocarcinoma, which is one of the most common malignancies, still hasn't been well studied. Therefore, our study aimed to investigate the role of MEG3 in colorectal adenocarcinoma and to explore the possibly related mechanisms.

PATIENTS AND METHODS: Tumor tissues and adjacent healthy tissues were collected from colorectal adenocarcinoma patients. Blood samples were collected from both colorectal adenocarcinoma patients and healthy controls to prepare serum sample. Expression of MEG3 in those tissues was detected by qRT-PCR. MEG3 knockdown and sphingosine kinase 1 (SPHK1) overexpression colorectal adenocarcinoma cell lines were established. Its effects on cell proliferation and apoptosis were investigated by CCK-8 assay and MTT assay, respectively. Effects of MEG3 overexpression on TGF-β1 and SPHK1 were investigated by Western blot.

RESULTS: MEG3 expression level was decreased in tumor tissues than that in adjacent healthy tissues. Serum level of MEG3 was lower in cancer patients than that in healthy controls, and the serum level decreased with the increased stage of primary tumor. Serum TGF-β1 can be used to predict colorectal adenocarcinoma and its prognosis accurately. MEG3 knockdown and SPHK1 overexpression promoted tumor cell proliferation, but inhibited cell apoptosis. MEG3 knockdown also increased the expression level of TGF-β1 and SPHK1. Treatment with TGF-β1 inhibitor reduced the expression level of SPHK1 but showed no significant effects on MEG3. SPHK1 overexpression showed no significant effects on MEG3 and TGF-β1 expression.

CONCLUSIONS: Downregulation of lncRNA MEG3 can promote colorectal adenocarcinoma cell proliferation and inhibit the apoptosis by up-regulating TGF-β1 and its downstream sphingosine kinase 1.

Key Words:
Colorectal adenocarcinoma, MEG3, TGF-β1, SPHK1.

Introduction

Colorectal (CR) adenocarcinoma is a type of malignancy that develops from the tissues of large bowel. It has been recognized as the third most common cancer in men and the second most common cancer in women world widely[1]. In spite of the achievements that have been made in the prevention and treatment of colorectal adenocarcinoma, this disease is still the 4th most common cause of cancer-related death. It causes more than 600,000 deaths and affects more than 70,000 men and 60,000 women at the same time every year[2]. Treatment of colorectal adenocarcinoma is challenged by the unclear pathogenesis, which may be a major cause of impropriate treatment and post-treatment recurrences. Activation of several oncogenes and loss of functions regarding certain tumor suppressor genes has been proved to be involved in the progression of colorectal adenocarcinoma[3]. They were also involved in the formation of resistance to chemical drugs during long-term treatment[4]. Besides protein-coding messenger RNAs (mRNAs), human genome also encodes a large set of noncoding RNAs (ncRNAs). Within the ncRNAs, long non-coding RNA is a subset with length longer than 200 nt and have regulatory functions in both normal physiological processes and the occurrence, development, and progression of various human diseases[5,6]. LncRNA MEG3 is usually downregulated in the development of human cancers. The reduced expression level of MEG3 is closely correlated with disease progression and poor prognosis[7]. However, the

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participation of MEG3 and function network in colorectal adenocarcinoma still hasn't been well studied. Therefore our study aimed to investigate the role of MEG3 in CR adenocarcinoma, and to explore the possible mechanism.

Patients and Methods

Patients

A total of 84 patients with CR adenocarcinoma were enrolled in PLA Army General Hospital from July 2010 to July 2012. Those patients included 45 males and 39 females, and the age ranged from 24 years to 77 years, with an average age of 49 ± 7.4 years. All patients were diagnosed by pathological and imaging examinations. Patients with other colon diseases and mental disorders were excluded. Primary tumors were classified into different stages using the following criteria: tumor invades submucosa, T1; tumor invades muscularis propria, T2; tumor invades into the pericolorectal tissues through the muscularis propria, T3; Tumor invades the visceral peritoneum or invades or adheres to an adjacent structure or organ T4. There were 14 cases of T1, 18 cases of T2, 24 cases of T3 and 28 cases of T4. Tumor tissues and adjacent healthy tissues were collected during surgery and were confirmed by pathological examinations. A total of 34 healthy people with similar age and gender distributions were also enrolled at the same time to serve as controls. The Ethics Committee of our hospital approved this study, and all participants signed informed consent.

Cell Lines and Cell Culture

Human colorectal adenocarcinoma cell lines Hs 698.T (American Indian), Hs 255.T (Caucasian) and SNU-C1 (Asian) were obtained from ATCC (Manassas, VA, USA). Cells were cultured under the conditions that described in the instructions of ATCC. Cells were collected during logarithmic growth phase for subsequent experiments.

Construction of MEG3 Knockdown and SPHK1 Overexpression Cell Lines

MEG3 siRNA silencing cell lines were constructed using commercial MEG3 siRNA (cat. no. 4392420; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Silencer™ Select Negative Control No. 1 siRNA (cat. no. 4390843; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The SPHK1 expression vector was constructed by inserting full-length MEG3 cDNA (V0728, GeneCopoeia, Rockville, MD, USA) into pIRSE2-EGFP vector (Clontech, Palo Alto, CA, USA). Cells were cultured overnight in Eagle's Minimum Essential Medium (ATCC, Manassas, VA, USA) containing 10% fetal bovine serum (FBS) (FBS was not added in case of drug treatment) to reach 80-90% confluence. Empty vector without MEG3 cDNA was used as negative control. Lipofectamine 2000 reagent (cat. no. 11668-019; Invitrogen, Carlsbad, CA, USA; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transfect 40 nM siRNA and 10 nM vector into 4×10^5 cells. After that, cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, South Logan, UT, USA) at 37°C for 48 h prior to subsequent experimentation.

Preparation of Serum Samples

Fasting blood (15 ml) was obtained from each participant in the morning of the day just after admission. Serum was separated from the blood by keeping the blood at room temperature for 3 h, followed by centrifugation at 1875 rpm for 15 min. Serum samples were stored at -80ºC before use.

Cell Proliferation Assay

The cell suspension was prepared using cells that collected during the logarithmic growth phase. Then 100 µl of diluted cell suspension was added into 96-well plate with $4x10^4$ cells for each well. 10 uL CCK-8 solution was added into each well 24, 48, 72, and 96 h later. After cell culture at 37°C for another 4 h, OD values at 450 nm were measured using Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific, Hampton, NY, USA). This experiment was performed for three times, and three replicate wells were set for each treatment to calculate the mean value.

MTT Assay

The cell suspension was diluted in culture medium containing 10 mM tetraethylammonium (TEA) to make a final cell density of $5x10^4$ cells /ml, and 100 µl cell suspension containing $6x10^3$ cells was added into each well of 96-well plate. Cells were cultured at 37ºC for 6 h, and 10 µl of MTT was then added into each well. Cells were cultured for another 4 h, and the absorbance at 570 nm was measured using a Fish-

erbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific, Hampton, NY, USA). Cell apoptosis was normalized to control cells.

Real-Time Quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Tumor tissues and adjacent healthy tissues were ground in liquid nitrogen before the addition of TRIzol. RNA quality was tested using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA), and only the ones with an A260/A280 ratio between 1.8 and 2.0 were subjected to reverse transcription to synthesize cDNA. PCR reaction system was prepared using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cDNA. Following primers were used in PCR reactions: 5'-ATCATCCGTCCACCTCCTTGTCTTC-3' (forward) and 5'-GTATGAGCATAGCAAAGGTCAGGGC-3' (reverse) for MEG3; 5'-AGGCTGAAATCTCCTTCACGC-3' (forward) and reverse 5'-GTCTCCAGACATGACCACCAG3' (reverse) for SPHK1; 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for β-actin. PCR reaction conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 12 s and 60°C for 38 s. Ct values were processed using the $2^{-\Delta\Delta CT}$ method, and relative expression levels of MEG3 and SPHK1 were normalized to endogenous control β-actin.

Western Blot

Total protein was extracted using RIPA solution (Thermo Fisher Scientific, Township, NJ, USA), and quantified by BCA method. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 20 μg protein from each sample, followed by transmembrane to PVDF membrane. Blocking was performed by incubating the membranes with 5% skimmed milk for 1 h at room temperature. Then, membranes were incubated with primary antibodies including rabbit anti-TGF-β1 (1:2000, ab92486, Abcam, Cambridge, MA, USA), anti-SPHK1 (1:2000, 46719, Abcam, Cambridge, MA, USA), and anti-GAPDH primary antibody (1:1000, ab8245, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, membranes were washed with PBS and further incubated with anti-rabbit IgG-HRP secondary antibody (1:1000, MBS435036, MyBioSource, San Diego, CA, USA) at room temperature for 4 h. ECL (Sigma-Aldrich, St. Louis, MO, USA) was added, and MYECL™ Imager (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the signal. Image J software was used to normalize relative expression level of each protein to endogenous control GAPDH.

Statistical Analysis

SPSS19.0 (IBM, Armonk, NY, USA) was used to conduct statistical analysis. Count data were analyzed by the x^2-test. Measurement data were expressed as (`x±s), and comparisons between two groups were performed by the *t*-test, and analysis of variance and LSD test were used for comparisons among multiple groups. $p < 0.05$ indicated a difference with statistically significant.

Results

Expression of lncRNA MEG3 in Tumor Tissues and Adjacent Healthy Tissues of Patients with Colorectal Adenocarcinoma

Expression of MEG3 in tumor tissues and adjacent healthy tissues of 84 patients with colorectal adenocarcinoma was detected by qRT-PCR. As shown in Figure 1, the expression of MEG3 was significantly downregulated in tumor tissues compared with adjacent healthy tissues in 69 out of 84 patients ($p < 0.05$). Expression of MEG3 was significantly upregulated in tumor tissues compared with adjacent healthy tissues in only 5 patients ($p < 0.05$). No significant difference in expression level of MEG3 was found between tumor tissues and adjacent healthy tissues in 10 patients ($p > 0.05$). These results suggest that downregulation of MEG3 is very likely to be involved in the pathogenesis of colorectal adenocarcinoma

Expression of MEG3 in Serum of Healthy Controls and Patients with Different Stages of Colorectal Adenocarcinoma

Expression of MEG3 in serum of healthy controls and patients with different stages of colorectal adenocarcinoma was detected by qRT-PCR and compared among different groups of patients. As shown in Figure 2, the expression level of MEG2 in serum was downregulated in patients with different stages of colorectal adenocarcinoma compared with healthy controls. Also, the expression level of MEG2 decreased with the in-

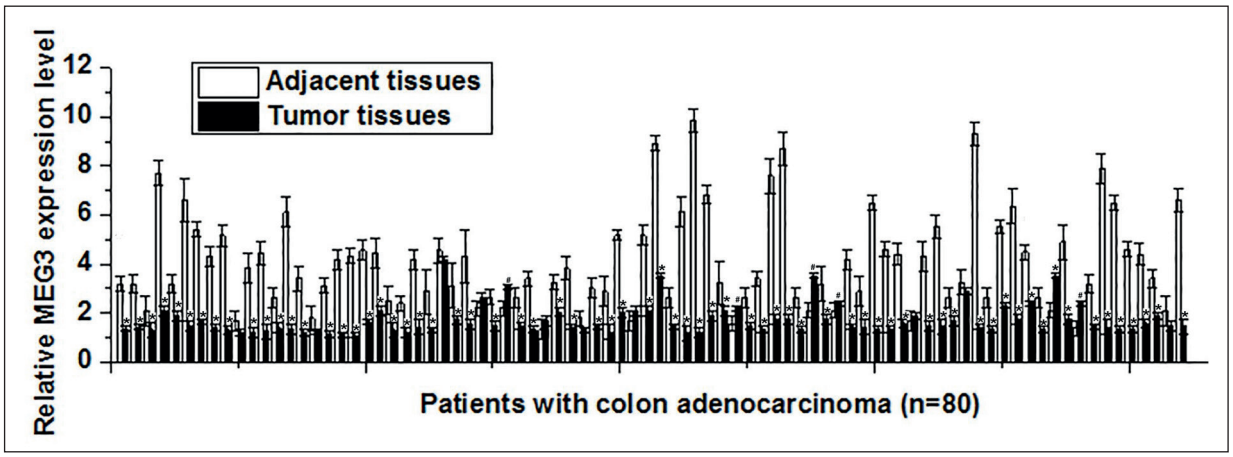

Figure 1. Expression of lncRNA MEG3 in tumor tissues and adjacent healthy tissues of patients with CR adenocarcinoma. Notes: *Compared with adjacent healthy tissue, $p < 0.05$; #Compared with tumor tissue, $p < 0.05$.

creased primary tumor stage (T1-T4). These data suggest that downregulation of MALAT1 expression is involved in the progression of colorectal adenocarcinoma.

Diagnostic and Prognostic Value of serum MEG3 for Colorectal Adenocarcinoma

ROC curve analysis showed that the area under the curve (AUC) of serum MEG3 in the detection of CR adenocarcinoma was 0.9384 with 95 interval of 0.8984 to 0.9785 ($p < 0.0001$, Figure 3a). According to the median serum level of MEG3, patients were divided into high MEG3 expression group and low MEG3 expression group. Survival curves of those two groups of patients during the 5-years follow-up were plotted using the Kaplan-Meier method and compared by log-rank *t*-test. As shown in Figure 3b, the overall survival rate of patients with low expression level of MEG3 was significantly lower than that of patients with high expression level of MEG3. These data suggest that serum MEG3 may serve as a biomarker for the diagnosis and prognosis of CR adenocarcinoma.

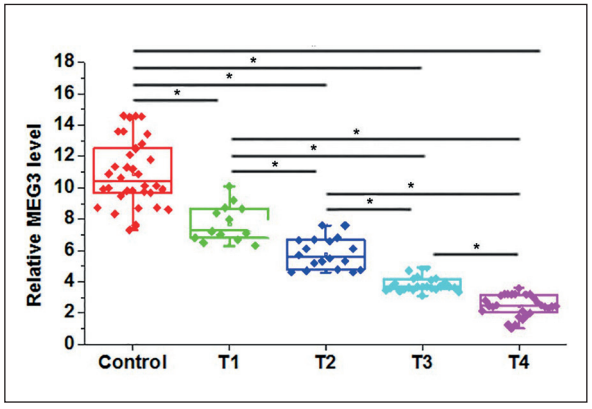

Figure 2. Expression of MEG3 in serum of healthy controls and patients with different stages of colorectal adenocarcinoma. Notes: *$p < 0.05$.

MEG3 Knockdown, SPHK1 Overexpression, and TGF-β1 Treatment Promoted Tumor Cell Proliferation and Inhibited Cell Apoptosis to Different Extents

MEG3 knockdown and SPHK1 overexpression cell lines were confirmed by measuring the expression level of MEG3 and SPHK1 by qRT-PCR (data not shown). As shown in Figure 4a, MEG3 knockdown, SPHK1 overexpression, and TGF-β1 treatment all promoted tumor cell proliferation, and SPHK1 overexpression showed the strongest enhancing effect, followed by TGF-β1 treatment, and the enhancing effect of MEG3 knockdown was the weakest. The MEG3 knockdown, SPHK1 overexpression, and TGF-β1 treatment also inhibited tumor cell apoptosis, and SPHK1 overexpression showed the strongest inhibitory effect, followed by TGF-β1 treatment, and the inhibitory effect of MEG3 knockdown was the weakest (Figure 4b).

Possible Interactions between MEG3, SPHK1, and TGF-β1

As shown in Figure 5a, MEG3 knockdown significantly increased the expression level of both SPHK1 and TGF-β1 in all the three cell lines ($p < 0.05$). Treatment with TGF-β1 showed

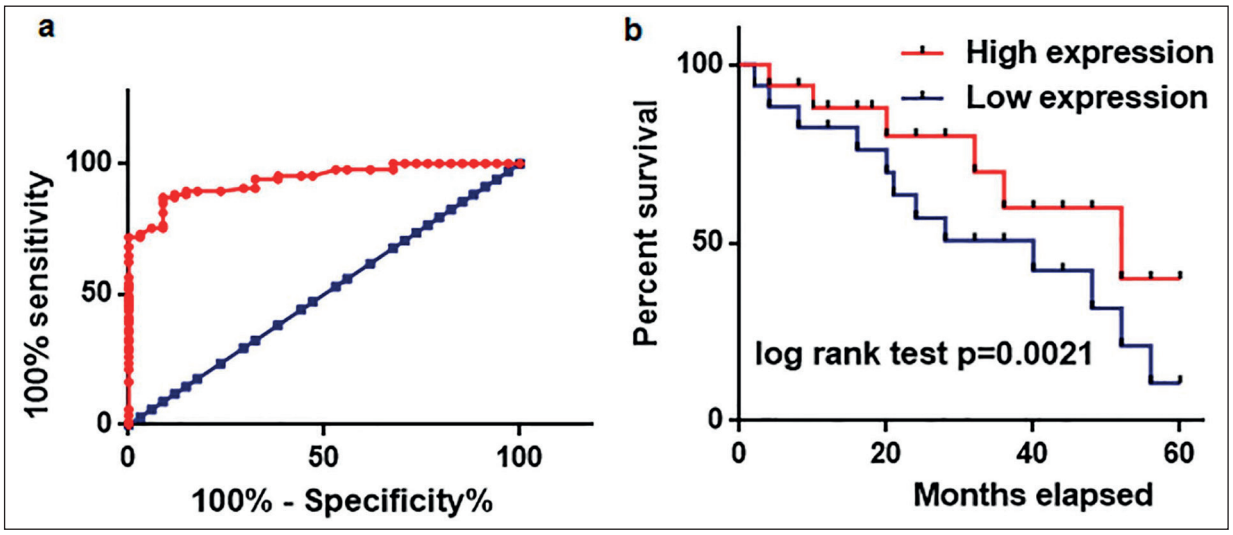

Figure 3. Diagnostic and prognostic value of serum MEG3 level for *t* CR adenocarcinoma. **a,** Diagnostic values of serum MEG3 for colorectal adenocarcinoma analyzed by ROC curve analysis; **b,** Comparison of survival curves of patients with high and low serum level of CR adenocarcinoma.

no significant effects on MEG3 expression but significantly promoted the expression of SPHK1 ($p < 0.05$, Figure 5b). SPHK1 overexpression showed no significant effects on the expression of MEG3 and SPHK1 ($p > 0.05$, Figure 5c). These data suggest that MEG3 is in the upstream of TGF-β1, and SPHK1 is in the downstream of TGF-β1.

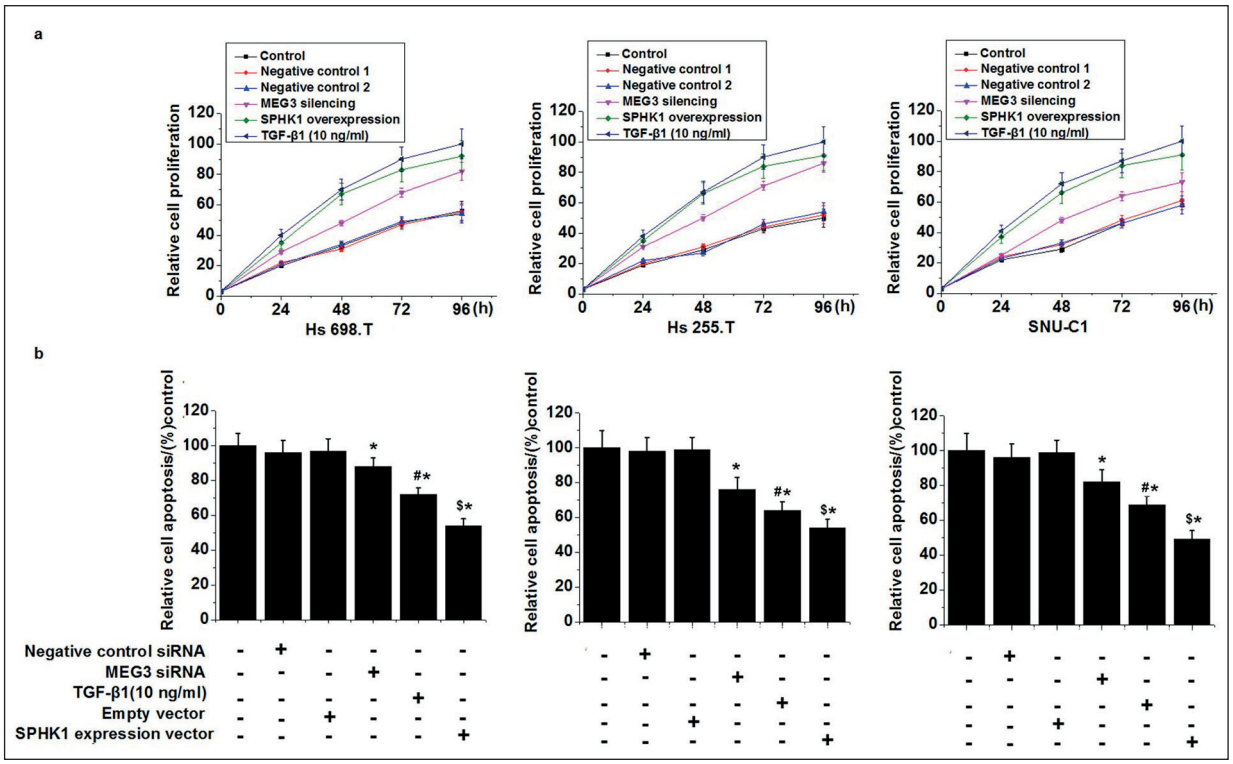

Figure 4. MEG3 knockdown, SPHK1 overexpression and TGF-β1 treatment promoted tumor cell proliferation and inhibited cell apoptosis to different extents. **a,** Cell proliferation of different cell lines with different treatment; **b,** Cell apoptosis of different cell lines with different treatment. Notes: *Compared with control, $p < 0.05$; #Compared with MEG3 knockdown, $p < 0.05$; $ compared with TGF-β1 treatment, $p < 0.05$.

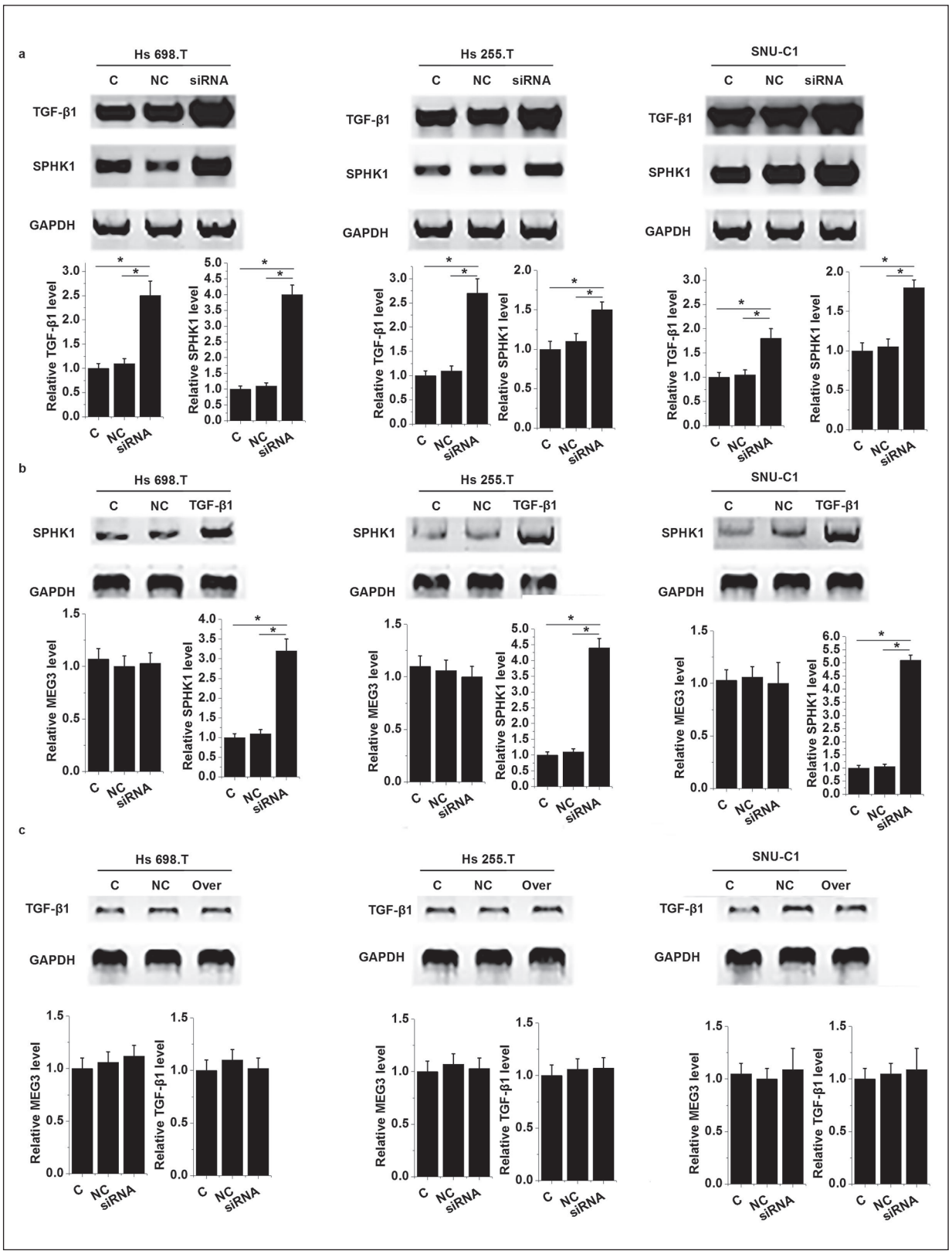

Figure 5. Possible interactions between MEG3, SPHK1 and TGF-β1. **a,** Effects of MEG3 knockdown on SPHK1 and TGF-β1; **b,** Effects of TGF-β1 treatment on MEG3 and SPHK; **c,** Effects on SPHK1 overexpression on MEG3 and SPHK1. Notes: *$p < 0.05$.

Discussion

LncRNA MEG3 plays a role as tumor suppressor gene in the development of various types of cancers and usually shows a decreased expression level. In the study of gastric cancer, Sun et al[8] found that the expression level of MEG3 was significantly lower in tumor tissues than that in adjacent healthy tissues, and the reduced expression level of MEG3 was significantly correlated with the progression of this disease and the poor survival of the patients. The MEG3 expression is also downregulated in the development of cervical cancer, and an increased expression level of MEG3 may inhibit tumor growth and metastasis[9]. The development of colorectal cancer is also accompanied with the downregulation of MEG3[7]. Consistent with previous studies, in our study, expression of MEG3 was significantly downregulated in tumor tissues compared with adjacent healthy tissues in 69 out of 84 patients, and expression of MEG3 was significantly upregulated in tumor tissues compared with adjacent healthy tissues in only 5 patients. In addition, the expression level of MEG2 in serum was downregulated in patients with different stages of CR adenocarcinoma compared with healthy controls, and expression level of MEG2 decreased with the increased primary tumor stage. These results demonstrate that downregulation of MEG3 is very likely to be involved in the development and progression of CR adenocarcinoma.

Most patients with colorectal adenocarcinoma are diagnosed in advanced stages, and most of them miss the best time point for surgical resection, which is the only radical treatment for this disease[10]. In addition, existing of local or distant invasion and metastasis in patients in advanced stages can usually cause postoperative recurrence, which is also a major cause of poor survival even after appropriate treatment[11]. Therefore, early diagnosis and treatment is currently the key to improve the survival of patients with colorectal adenocarcinoma. The occurrence, development, and progression of human diseases are always accompanied by changes of substances in blood, and the detection of certain substances in blood may provide guidance for the diagnosis and prognosis of certain diseases[12,13]. In this study, ROC curve analysis showed that serum MEG3 can be used to predict colorectal adenocarcinoma accurately. In addition, the overall survival rate of patients with low expression level of MEG3 was significantly lower than that of patients with high expression level of MEG3. These data suggest that serum MEG may serve as an accurate and reliable diagnostic and prognostic biomarker for colorectal adenocarcinoma. It has been reported that lncRNA MEG3 can regulate the expression of TGF-β1[14], and SPHK1 has been proved to be essential for the signal transduction of TGF-β pathway[15]. In this study, MEG3 knockdown, SPHK1 overexpression, and TGF-β1 treatment all promoted tumor cell proliferation, and inhibited cell apoptosis. In addition, MEG3 knockdown increased the expression level of both SPHK1 and TGF-β1, and treatment with TGF-β1 showed no significant effects on MEG3 expression but significantly promoted the expression of SPHK1, and SPHK1 overexpression showed no significant effects on expression of MEG3 and SPHK1. These data indicate that MEG3 is in the upstream of TGF-β1, and SPHK1 is in the downstream of TGF-β1 as well as in the MEG3-mediated regulation of colorectal adenocarcinoma proliferation and apoptosis. It's worthy to note that the three colorectal adenocarcinoma cell lines that used in this study, including Hs 698.T (American Indian), Hs 255.T (Caucasian) and SNU-C1 (Asian) had different ethnic backgrounds. It is known that ethnic difference can affect the pathogenesis of certain types of cancers. Therefore, our study excluded the ethnic differences and obtained solid data.

Conclusions

We observed that MEG3 expression level was lower in tumor tissues than that in adjacent healthy tissues. Serum level of MEG3 was lower in cancer patients than that in healthy controls, and it decreased with the increase of primary tumor stage. Serum TGF-β1 can be used as an accurate diagnostic and prognostic marker for colorectal adenocarcinoma. MEG3 knockdown and SPHK1 overexpression promoted tumor cell proliferation and inhibited cell apoptosis. MEG3 knockdown also increased the expression level of TGF-β1 and SPHK1. Treatment with TGF-β1 inhibitor reduced the expression level of SPHK1 but showed no significant effects on MEG3. SPHK1 overexpression showed no significant effects on MEG3 and TGF-β1 expression. Therefore, we concluded that downregulation of lncRNA MEG3 can promote colorectal adenocarcinoma cell proliferation and inhibit apoptosis by up-regulating TGF-β1 and its downstream sphingosine kinase 1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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