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SMAD-4 Cooperates with TGF-β to Enhance PTEN Expression Upon The Inhibition of RAS/ERK Pathway in Gastric Carcinoma Cells*

MATLOOB Ammar[1)**], PAN Li-Na[2)**], PAN Hong[1)], LU Jun[1)], HUANG Bai-Qu[1)***]
([1)] *Institute of Genetics and Cytology, Northeast Normal University, Changchun* 130024, China;
[2)] *College of Life Science, Tianjin Normal University, Tianjin* 300387, China)

Abstract SMAD-4 plays an important role in tumor suppression though controversies still exist regarding its behavior in carcinogenesis and its relationship with the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is regarded as a key controller of cell cycle progression and cell growth. The role of SMAD-4 on PTEN expression was investigated through TGF-β signaling in 293T cells and in malignant gastric carcinoma MGC-803 cells. Results showed that SMAD-4 and TGF-β enhanced the expression of PTEN in 293T cells, while they suppressed PTEN expression in MGC-803 cells. However, this suppression was relieved upon the inhibition of RAS/ERK pathway. Moreover, the maximum expression of PTEN was achieved by the cooperation of SMAD-4 with TGF-β when SMAD-4 was translocated into the nucleus. Enhancement of early apoptosis of about three folds was achieved with this cooperation, compared with the action of TGF-β alone in MGC-803 cells. These findings shed light on the role of SMAD-4 as a co-Smad protein in TGF-β protein-signaling and in PTEN regulation.

Key words SMAD-4, TGF-β1, PTEN, RAS/ERK
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Transforming growth factor-β (TGF-β) was so named because it was originally found to assist in inducing malignant transformation in cultured, nonmalignant fibroblasts, and it functions as a growth inhibitory factor for epithelial cells[1]. TGF-β plays a beneficial role as a guardian to maintain cellular order against carcinogenesis, though it is also a major parameter for cancer metastasis [1]. Deregulation of TGF-β signaling has been reported in many human diseases [2–3]. Moreover, TGF-β has anti-proliferation effects in a variety of cell types that undergo tumor progression and development when its signaling pathway is inactivated[4].

SMAD proteins are the principal transducers of signals of TGF-β[5]. TGF-β binds to the homo-dimers of the TGF-β type Ⅱ receptor (TβRⅡ), which recruits and activates the homo-dimers of TGF-β type Ⅰ receptor (TβRⅠ). Activated TβRⅠ phosphorylates SMAD-2 or SMAD-3[6–7], which hetero-dimerizes with SMAD-4[8–9].

Mutations of the tumor suppressor SMAD-4, which is the only known co-Smad protein in TGF-β protein-signaling [10], are found in various epithelial tumors, including pancreatic carcinoma [11], esophageal carcinoma [12], colorectal carcinoma [13], renal cell carcinoma[14], and breast carcinoma[15–16]. However, the expression level and function of SMAD-4 in various cancer cells have not been extensively studied.

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**These authors contributed equally to this work.
***Corresponding author.
Tel: 86-431-85099798, Fax: 86-431-85099768
E-mail: huangbq705@nenu.edu.cn

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RAS proteins are small G-proteins that cycle between active (RAS-GTP) and inactive (RAS-GDP) forms, in response to stimulation from a cell surface receptor such as EGFR (the epidermal growth factor receptor). The RAS gene can harbor oncogenic mutations that result in a constitutively activated protein, independent of EGFR ligand binding. RAS mutations are found in approximately 30% ~ 50% of colorectal cancer tumors and are common in other tumor types [17-18]. RAS is also mutated in more than 90% of pancreatic cancers but can also be found in benign pancreatic lesions [19]. PTEN (phosphatase and tensin homolog deleted on chromosome 10) was first identified as a tumor suppressor gene mutated in brain, breast and prostate tumors [20-21]. PTEN acts as a lipid phosphatase that removes the phosphate group from phosphatidylinositol 3,4,5-triphosphate(PIP3) and directly antagonizes the action of the phosphatidylinositol 3-kinases (PI3K)[22]. Thus, PTEN functions as a critical negative regulator of the ubiquitous PI3K pathway [23]. The lipid phosphatase activity of PTEN has been shown to be essential for its tumor suppressor function in many assays[24].

Loss or reduced expression of PTEN protein occurs commonly in tumorigenesis and in progression of gastric carcinoma. It was also suggested that PTEN could be a target marker for pathological behavior of gastric carcinoma [25]. Promoter analysis indicated that SMAD-4 is a potential transcriptional factor of PTEN. Previous reports described that the expression of *smad-4* was lost frequently in gastric cancer; but mutations in PTEN were not found in precancerous lesions and early stages of gastric cancer [26]. In human pancreatic adenocarcinoma cells, the expression of PTEN was down-regulated by TGF-β, while this could be reversed by PD98059 (a MEK inhibitor that blocks RAS/ERK pathway)[27]. However, the effects of TGF-β and RAS/MEK/ERK pathway in PTEN expression are unclear. The purpose of this study was to investigate the diverse function and behavior of SMAD-4 in normal 293T cells and in malignant gastric carcinoma cells (MGC-803), as well as to study its role in PTEN regulation, with the hope that some useful information in understanding the etiology of gastric cancer can be obtained.

1. 1 Materials and methods

1.1 Reagents

The MEK inhibitor, PD98059 was purchased from Sigma (USA). The substance was diluted in dimethyl sulfoxide and was used at a concentration of 50 μmol/L. TGF-β was purchased from R&D Systems China, Co. Ltd, and a stock solution was prepared by adding 200 μl of 4 mmol/L HCl/0.1% BSA to the lyophilized TGF-β1. The stock solution was diluted using Iscove's Modified Dulbecco's Medium (IMDM) to a concentration of 1 mg/L, from which different concentrations were prepared.

1.2 Cell culture

The 293T human embryonic kidney epithelial and the MGC-803 malignant gastric carcinoma cells were purchased from the Institute of Cell Biology (Shanghai, China). Cells were cultured in IMDM medium supplemented with 10% FBS (fetal bovine serum), 100 U/ml penicillin and 100 mg/L streptomycin, and were kept in a humidified atmosphere of 5% CO_2.

1.3 Plasmids

SMAD-4 expression plasmid was a gift from Dr. Rik Derynck (University of California, San Francisco), and has been maintained in this laboratory. The SMAD-4 RNA interference plasmid was constructed using the pSilencer4.1-CMV vector according to the manufacturer instructions. The forward sequence was 5′ GGAAUUGAUCUCUCAGGAUTT 3′ and the reverse was 5′ AUCCUGAGAGAUCAAUUCCTT 3′. The human PTEN gene promoter luciferase reporter construct was obtained by subcloning the 1 978 bp genomic DNA region upstream of the human PTEN gene into the pGL-3basic-luc vector.

1.4 Transient transfection of the cells

Cells were cultured in 24-well or 6-well plates for 18 h before transfection. 293T cells were transfected using the standard calcium chloride method, while the MGC-803 cells were transfected using the Lipofectamine (Invitrogen) method following the instructions of the manufacturer.

1.5 Luciferase reporter assay

After transfection, 293T cells were cultured for 30 h before harvested, washed with phosphate-buffered saline (PBS) and lysed in 30 μl lysis buffer. Reporter gene expression was measured and quantified using a dual Luciferase Reporter Assay System (Promega, Madison). Relative luciferase activity was analyzed by using a Turner Designs TD20/20 Luminometer (Sunnyvale). The firefly luciferase activity was normalized to the activity of the Renilla luciferase control. Extracts from 3 independent transfection experiments were assayed in triplicate. The results are shown as $\bar{x} \pm s$.

1.6 Chromatin immunoprecipitation (ChIP)

ChIP assays were carried out using a kit supplied by Upstate Biotechnology (Lake Placid, NY) following the manufacturer's protocol. Cells were plated at a density of 1×10^5/ml in 6-well plates before they were transfected with SMAD-4 expression plasmid in the presence of TGF-β for 24 h. The transfected cells were cross-linked with 2% formaldehyde for 10 min at 37℃, and then lysed in SDS lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris, pH 8.1) with protease inhibitor. The sonicated lysates were processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate Biotechnology). The SMAD-4 monoclonal antibody was purchased from Abcam, UK. Immunoprecipitated chromatin was analyzed by using PCR at 3 different regions of the PTEN promoter; the primer pairs were: 5′ GGTCACCTGGTCCTTTTCA 3′/ 5′ TGCCTGGGGCTTGCTC 3′ for Region Ⅰ, 5′ GAGGCCGAGGCTTAGCTCGTTATCC 3′/ 5′ CGCATAAAGAGTCCCGCCACATCAC 3′ for Region Ⅱ, and 5′ AGGCAGCCGTTCGGAGGATTATTCG 3′/ 5′ GTCTGGGAGCCTGTGGCTGAAGAAA 3′ for Region Ⅲ.

1.7 Total RNA isolation and RT-PCR

Total RNA was extracted and reverse-transcribed to cDNA using the RNA extraction and RT Systems supplied by Promega. The resultant cDNA was diluted 5-fold with RNase-free water. PCR was conducted to estimate the amounts of expressed PTEN and SMAD-4, which were standardized with β-actin. The primers were: 5′ GCGTGCAGATAATGAC 3′ and 5′ GATTTGACGGCTCCT 3′ for PTEN gene, and 5′ AACGTTAGCTGTTGTTTTTCAC 3′ and 5′ AGAGTATGTGAAGAGATGGAG 3′ for SMAD-4 gene.

1.8 Preparation of nuclear and cytoplasmic fractions and Western blotting

To prepare the cytoplasmic and nuclear fractions, cells were scraped in 1.5 ml of hypotonic lysis buffer consisting of 10 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.9), 2.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 40 mmol/L NaF, 4 mmol/L β-glycerophosphate, 0.2% nonidet P-40, 0.5 mmol/L phenylmethylsulphonyl fluoride, 0.5 mmol/L benzamidine HCl and 2 mg/L each aprotinin and leupeptin. The cell suspension was kept on ice for 20 min and inverted several times. The nuclear fraction was isolated by centrifugation of the suspension at 3 500 *g* for 5 min, followed by washing in 0.5 ml of hypotonic buffer. The supernatant of cytoplasmic fraction was added with NaCl to a final concentration of 0.14 mol/L and clarified by 10 min centrifugation at 10 000 *g*. Nuclear proteins were extracted from nuclear fraction by lysis in 0.3 ml hypertonic lysis buffer(50 mmol/L Tris-HCl, 0.14 mol/L NaCl, 0.25 mol/L KCl, 1 mmol/L ethyleneglycol-bis-aminoethylether-tetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 mmol/L dithiothreitol, plus protease and phosphatase inhibitors), followed by a 1 h rotation at 4℃, and clarified by 10 min centrifugation at 10 000 *g*. 50 μg of cytoplasmic and 30 μg of nuclear proteins were analyzed by Western blotting with specific antibodies, respectively[28].

Total cell lysates were prepared by washing the cells 3 times with cold phosphate buffered saline (PBS) before lysed using total lysis buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.8, 1 mmol/L ethylenediaminetetraacetic acid, 0.5% Triton X-100, 1 mmol/L sodium orthovanadate) containing protease inhibitors (1 mg/L leupeptin and 100 mg/L phenylmethylsulfonyl fluoride). Cells were then incubated at 4℃ for 30 min with constant shaking, then scraped into microcentrifuge tubes and centrifuged at 12 000 *g* for 15 min to remove insoluble materials. The protein contents in each sample were determined using Western blot. For Western blot analysis, the lysates were separated on SDS-PAGE in 12% gels and then transferred to nitrocellulose membranes and immuno-detected with rabbit polyclonal antibodies against PTEN, Erk1/2 and pErk1/2, and mouse polyclonal antibodies against β-actin and SMAD-4 (Santa Cruz Biotechnology, Santa Cruz).

1.9 Cell apoptosis and flow cytometry

Cells were seeded in 6-well plates and treated with PD98059 and TGF-β. After transfection of the cells with SMAD-4, they were collected and washed with PBS, and then resuspended in 1 ml of PBS containing 1 mg FITC-Annexin. After incubation for 10 min in dark, 0.1 ml of PI solution was added prior to analysis to give a final concentration of 1 g/L. The samples were finally analyzed by flow cytometry.

1.10 Data analysis

Student's *t* test was used to calculate the statistical significance of the promoter activity and cell apoptosis. The significance level was set as $^*P < 0.05$ and $^{**}P < 0.01$.

2 Results and discussion

2.1 SMAD-4 up-regulated PTEN in 293T cells

It was reported that SMAD-4 may be translocated into the nucleus and bind to genes' promoters to regulate their expression[10,29]. By using the dual luciferase reporter assay, we tested the PTEN promoter activity in the presence of SMAD-4 protein in 293T cells. The results showed that the promoter activity of PTEN exhibited a significant increase of about 2.7 folds when 293T cells were transfected with SMAD-4 expression plasmid, compared with the empty control vector of pcDNA3.1 (Figure 1a).

We then examined the effects of both TGF-β1 and SMAD-4 on the expression of PTEN in 293T cells. Transfection of the cells with SMAD-4 resulted in a modulate PTEN overexpression at both mRNA and protein levels, compared with that in cells transfected with the empty vector; but this increment was smaller than that in cells treated with TGF-β1 alone (Figure 1b). Meanwhile, the maximal expression of PTEN was observed in cells simultaneously treated with TGF-β1 and transfected with SMAD-4(Figure 1b).

Also, knockdown of SMAD-4 expression using specific siRNA in 293T cells treated with TGF-β1 resulted in down-regulation of PTEN expression at both mRNA and protein levels (Figure 1c). This indicated that SMAD-4 acted as a positive modulator of PTEN expression.

Next, chromatin immunoprecipitation (ChIP) assays were conducted to detect the potential SMAD-4 binding sites on PTEN promoter. The ChIP results revealed a positive interaction between the SMAD-4 protein and the 3 sites at PTEN promoter with variable binding affinities (Figure 1d).

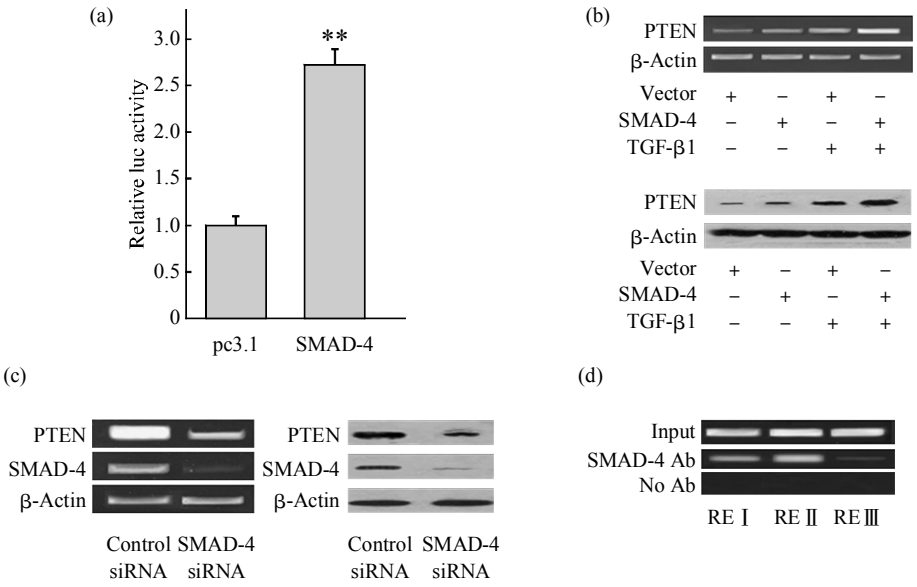

Fig. 1 SMAD-4 up-regulated PTEN in 293T cells

(a) 293T cells were transfected with SMAD-4 expression plasmid; cells were collected 30 h after transfection and the Luciferase reporter activity of PTEN promoter was measured. (b) 293T cells were transfected with SMAD-4 expression plasmid in the presence or absence of (10 μg/L) of TGF-β1 for 24 h in growth medium; the mRNA level of PTEN was estimated by RT-PCR, and PTEN protein was determined by Western blotting. (c) RT-PCR detection for PTEN mRNA and Western blotting for PTEN protein after transfection with small interfering RNA targeting SMAD4. (d) 293T cells were transfected with SMAD-4 plasmid in the presence of 10 μg/L of TGF-β1 for 24 h, and ChIP assays were preformed by anti-SMAD-4 antibody.

2.2 SMAD-4 suppressed PTEN expression in gastric carcinoma MGC-803 cells

The above results intrigued us to investigate the function and behavior of SMAD-4 in regulating PTEN in cancer cells. The gastric carcinoma MGC-803 cell line was chosen for this study, because PTEN was not mutated in gastric carcinoma[25-26], and the MGC-803 cells have the same epithelial origin as 293T cells.

By using the similar experimental approaches, we showed that transfection of MGC-803 cells with SMAD-4 plasmid in the presence or absence of TGF-β1 down- regulated PTEN expression (Figure 2).

Moreover, a lower PTEN expression level was observed when the cells were transfected with SMAD-4 in the presence of TGF-β1 (Figure 2). Apparently, these results were contrary to that observed in 293T cells, where SMAD-4 promoted PTEN expression (Figure 1b).

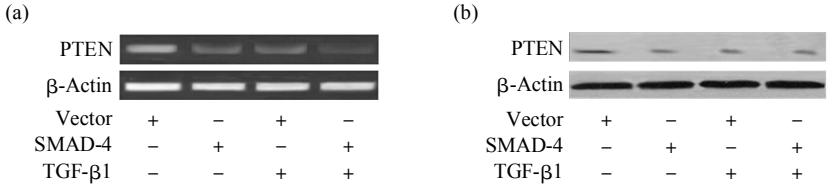

Fig. 2 SMAD-4 suppressed PTEN expression in gastric carcinoma MGC-803 cells

(a) MGC-803 cells was treated with 10 μg/L of TGF-β1 for 24 h in growth medium upon the transfection with SMAD-4 plasmids, and the PTEN mRNA level was estimated by RT-PCR. (b) MGC-803 cells was treated with 10 μg/L of TGF-β1 for 24 h in growth medium upon the transfection with SMAD-4 plasmids, then Western blotting was used to measure the PTEN protein.

2.3 Active RAS/ERK pathway was required for SMAD-4 function in MGC-803 cells

Mutations of the RAS oncogene have been implicated in many epithelial derived cancers. RAS mutation was involved in carcinogenesis of sporadic colorectal cancer and in most gastric cancers, while different mutation types of RAS may occur in the development of gastric cancer at different stages [18]. Moreover, the resistance of gastric cancer patients to EGFR inhibitors may be induced by RAS mutation[30-31].

To investigate the roles of RAS and RAS/MEK/ERK signaling in SMAD-4 function in gastric cancer, we treated MGC-803 cells with MEK inhibitor PD98059 and transfected the cells with SMAD-4 plasmid in the presence or absence of TGF-β1. Results obtained from RT-PCR and Western blotting showed that the PTEN mRNA and protein expression levels were apparently up-regulated(Figure 3a, b), implicating that the function of SMAD-4 to stimulate PTEN

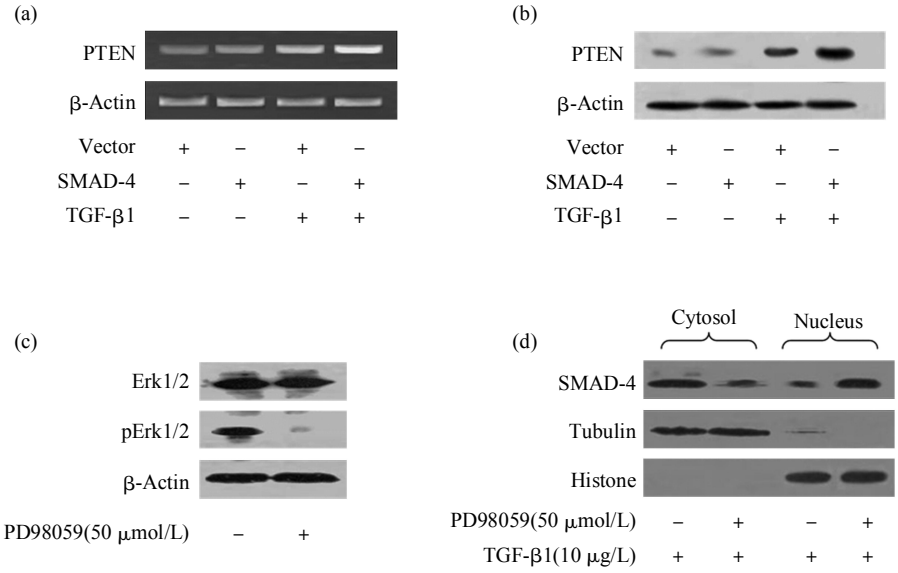

Fig. 3 Active RAS/ERK pathway was required for SMAD-4 function in MGC-803 cells

(a) MGC-803 cells was treated with MEK inhibitor of PD98059 after transfected with SMAD-4 plasmids in the presence or absence of 10 μg/L of TGF-β1 for 24 h in growth medium; RT-PCR was used to examine the PTEN mRNA level, and (b) Western blotting for estimating the PTEN protein. (c) MGC-803 cells were treated with 50 μmol/L PD98059 for 48 h, and Erk1/2 and pErk1/2 were tested by Western blotting. (d) MGC-803 cells were transfected with SMAD-4 and treated with 10 μg/L TGF-β1 in the presence of 50 μmol/L PD98059, and the cytoplasmic and nuclear fractions were collected for Western blotting.

expression was restored when PD98059 blocked the RAS/MEK/ERK pathway. The inhibitory effect of PD98059 was determined by measuring the ERK1/2 and phosphor-ERK1/2 (pERK1/2) proteins, which indicated that RAS/MEK/ERK signaling was interfered since little pERK1/2 protein was detected upon PD98059 treatment (Figure 3c).

Furthermore, we studied the SMAD-4 function in relation to its cellular locations, by checking the abundance of SMAD-4 protein in cytosol and nuclear fractions of MGC-803 cells, respectively. And the results revealed that SMAD-4 was translocated into nucleus when the cells were treated with the MEK inhibitor PD98059 (Figure 3d). These results suggested that TGF-β1 and SMAD-4 up-regulated PTEN expression in cooperation as a result of the inhibition of RAS/MEK/ERK by PD98059.

Finally, we measured the changes of apoptosis rate in MGC-803 cells upon SMAD-4 transfection in the presence of TGF-β and MEK inhibitor PD98059, by using flow cytometry. The results showed that the early apoptosis increased by as much as nearly 3 folds in cells transfected SMAD-4, compared with that in cells transfected with empty vector(Figure 4, and Table 1). This is consistent with the above results that the SMAD-4 co-acted with TGF-β to enhance PTEN expression in MGC-803 cells treated with inhibitor PD98059.

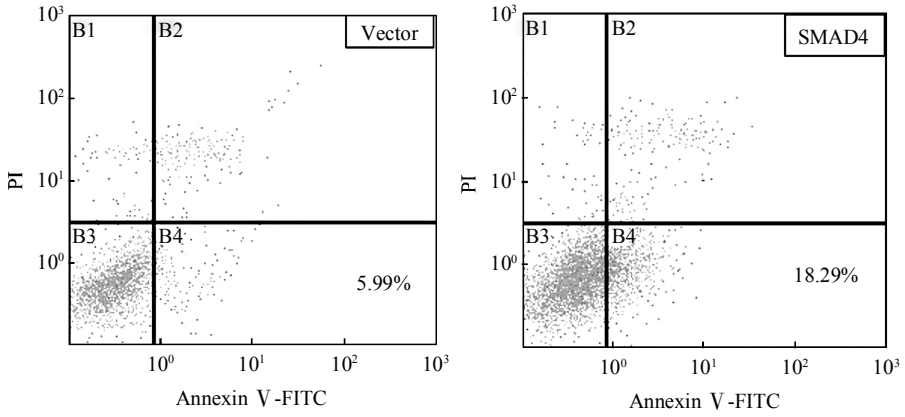

Fig. 4 The apoptosis rate was significantly higher in MGC-803 cells treated with SMAD-4

MGC-803 cells were transfected with empty vector or SMAD-4, treated with 10 μg/L TGF-β1 and 50 μmol/L PD98059, and the apoptosis was evaluated by flow cytometry analysis. Two representative flow cytometric graphics are shown.

Table 1 The apoptosis rate was significantly higher in MGC-803 cells treated with SMAD-4

	Apoptosis rate
Vector	(6.02 ± 0.45)%
SMAD-4	(18.36 ± 0.89)%

*$P < 0.05$. (MGC-803 cells were transfected with empty vector or SMAD-4, treated with 10 μg/L TGF-β1 and 50 μmol/L PD98059, and the apoptosis was evaluated by flow cytometry analysis.)

3 Conclusion

Data presented in this report describe the differential functions of SMAD-4 in regulating PTEN expression in normal 293T cells and in MGC-803 gastric cancer cells. Specifically, SMAD-4 up-regulates PTEN expression in 293T cells while it suppressed PTEN expression in malignant gastric carcinoma cells, probably due to the prevention of the translocation of SMAD-4 into nucleus through the active action of RAS oncogene. However, when the RAS/MEK/ERK signaling pathway was blocked by the inhibitor PD98059, the ability of SMAD-4 to stimulate PTEN expression was restored, and this resulted in a significant increase in early apoptosis. Moreover, a cooperative action of SMAD-4 and TGF-β brought about the maximum PTEN expression. These results provides a new dimension in understanding the roles of SMAD-4 in carcinogenesis, especially its role in regulating PTEN in relation to cell cycle and growth control in gastric cancer cells.

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胃癌细胞中 SMAD-4 与 TGF-β 通过抑制 RAS/ERK 途径促进 PTEN 表达 *

MATLOOB Ammar[1)**] 潘丽娜[2)**] 潘 虹[1)] 陆 军[1)] 黄百渠[1)***]

([1)] 东北师范大学遗传与细胞研究所，长春 130024；[2)] 天津师范大学生命科学学院，天津 300387)

摘要 SMAD-4 在肿瘤抑制方面有重要作用，但它在肿瘤发生中的作用及其与细胞周期进程中的一种关键调控因子——PTEN(phosphatase and tensin homolog deleted on chromosome 10)的关系仍存在争议．分别在人胚肾细胞(293T)及人胃癌细胞(MGC-803)中研究 SMAD-4 及 TGF-β 信号通路对 PTEN 基因表达的影响．结果发现，在 293T 细胞中，SMAD-4 与 TGF-β 促进 PTEN 表达，而 MGC-803 细胞中，SMAD-4 与 TGF-β 抑制 PTEN 转录．进一步研究发现，胃癌细胞中，SMAD-4 与转化生长因子 β(TGF-β)对 PTEN 的抑制可被 PD98059(MEK 抑制剂)解除．此外，SMAD-4 的核转移也明显促进 PTEN 表达，并且 PD98059 存在下，SMAD-4 与 TGF-β 协同刺激可促进胃癌细胞凋亡．综上，实验发现，SMAD-4 作为一种 co-Smad 蛋白，通过 TGF-β 信号途径影响 PTEN 表达．

关键词 SMAD-4，转化生长因子 β1(TGF-β1)， PTEN， RAS/ERK

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** 共同第一作者.

*** 通讯联系人.

Tel: 0431-85099798, Fax: 0431-85099768, E-mail: huangbq705@nenu.edu.cn

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