

# TGF- $\beta$ 1/PTEN/PI3K signaling plays a critical role in the anti-proliferation effect of tetrandrine in human colon cancer cells

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**Abstract.** The diagnosis and treatment for colon cancer have been greatly developed, but the prognosis remains unsatisfactory. There is still a great clinical need to explore new efficacious drugs for colon cancer treatment. Tetrandrine (Tet) is a bis-benzylisoquinoline alkaloid. It has been shown that Tet may be a potential candidate for cancer treatment, but the explicit mechanism underlying this activity remains unclear. In this study, we investigated the anticancer activity of Tet in human colon cancer cells and dissected the possible mechanism. With cell viability assay and flow cytometry analysis, we confirmed that Tet can effectively inhibit the proliferation and induce apoptosis in HCT116 cells. Mechanically, we found that Tet greatly increases the mRNA and protein level of TGF- $\beta$ 1 in HCT116 cells. Exogenous TGF- $\beta$ 1 enhances the anti-proliferation and apoptosis inducing effect of Tet in HCT116 cells, which has been partly reversed by TGF- $\beta$ 1 inhibitor. Tet decreases the phosphorylation of Akt1/2/3 in HCT116 cells. This effect can be enhanced by exogenous TGF- $\beta$ 1, but partly reversed by TGF- $\beta$ 1 inhibitor. Tet exhibits no effect on total level of PTEN, but decreases the phosphorylation of PTEN; exogenous TGF- $\beta$ 1 enhances the effect of Tet on decreasing the phosphorylation of PTEN, which was partly reversed by TGF- $\beta$ 1 inhibitor. Our findings suggested that Tet may be a promising candidate for colon cancer treatment, and the

anticancer activity may be mediated by inactivating PI3K/Akt signaling through upregulating TGF- $\beta$ 1 to decrease the phosphorylation of PTEN.

## Introduction

Colon cancer is one of the most common malignancies. In the last decades, the diagnosis and treatment for colon cancer have been improved greatly, especially with the screening at the early stage (1,2). However, the prognosis is still far from satisfactory and the incidence of colon cancer is increasing, even though the targeted therapy agents have been introduced to clinic (3). The challenges for the treatment of colon cancer include the serious adverse effects of the traditional chemotherapy and the metastasis to disseminate the cancer cells to other organs or tissues. Therefore, there is still a great clinical need to provide new effective reagents for ameliorating the treatment of colon cancer.

The natural products or their derivate is one of the important sources for anticancer drugs (4). Multiple drugs from this source have been used for cancer treatment for a few decades, such as camptothecin (5), paclitaxel (6) and vincristine (7). Tetrandrine (Tet), an alkaloid of bis-benzylisoquinoline, originates from the dried root of herbal medicine *Stephania tetrandra* S. Moore (Chinese herb *hang fang ji*) (8). Tet possesses various pharmacological effects, so it can be used for anti-inflammatory, immunosuppression and anti-hypertension (9-11). Tet has essential anticancer effects in prostate cancer, lung cancer, bladder cancer, gastric and colon cancer (8,12-15). It has been reported that several signaling pathways or critical factors have been involved in this activity, such as inhibition of PI3K/Akt, ERK and induction of p53 (16-18). Our previous study indicated that Tet can inhibit Wnt/ $\beta$ -catenin signaling in human colon cancer cells (14,19). However, the detail molecular mechanism remains unclear.

The transforming growth factor  $\beta$  (TGF- $\beta$ ) is a superfamily, which plays an important role in regulating many

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cellular physiological processes, such as proliferation, differentiation, apoptosis and other functions (20). TGF- $\beta$  functions as ligand binding to the type II receptor, which recruits and phosphorylates the type I receptor. The phosphorylated type I receptor then phosphorylates the receptor-regulated SMADs (R-SMADs), and then the R-Smads binds with the coSmad (SMAD4).

The R-Smad/coSmad complexes translocate to the nucleus where they act as transcriptional factors to regulate the expression of downstream targets (20). Therefore, the appropriate TGF- $\beta$  signaling is critical for the maintenance of homeostasis (21). Three sub-types of TGF- $\beta$  are found in this family, including TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. In reference to cancer, all the three isoforms of TGF- $\beta$  are involved in cancer progression (22-24). For colon cancer, the dysfunction or loss of TGF- $\beta$  signaling will promote progression of cancer (25,26). Although TGF- $\beta$ 1 has been demonstrated to be associated with colon cancer (27), its role in colon cancer remains controversial. It can promote or suppress the growth of colon cancer cells. These may be dependent on the microenvironment, or have genetic defects (28). Evidence indicated that Tet may be a powerful anticancer agent for colon cancer treatment (14,19). It has been reported that Tet can inhibit the transcription of TGF- $\beta$ 1 to suppress the reproductive activity of cells (29). To date, it remains unclear whether TGF- $\beta$ 1 is associated with the anticancer activity of Tet in colon cancer.

In this investigation, we studied the effect of Tet on TGF- $\beta$ 1 in HCT116 cells, to evaluate the effect of TGF- $\beta$ 1 on the anticancer activity of Tet. We found that Tet can substantially upregulate TGF- $\beta$ 1 in HCT116 cells, and TGF- $\beta$ 1 may mediate the anti-proliferation activity of Tet through inactivating PI3K/Akt signaling partly.

## Materials and methods

**Chemicals and drug preparations.** Tetrandrine (Tet) was from Hao-xuan Bio-tech (Xi'an, China). The HCT116 cells was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). LY294002 and LY364947 was from Targetmol Co., Ltd. (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin at 37°C and 5% CO<sub>2</sub>.

**Recombinant adenoviral constructs for TGF- $\beta$ 1, GFP and siRNA for PTEN.** The recombinant adenoviral vectors were constructed following the AdEasy system (30). Briefly, the coding sequence (CDS) of human TGF- $\beta$ 1 and green fluorescent protein (GFP) were amplified, the siRNA fragments for PTEN knockdown was synthesized commercially. These fragments were cloned into the shuttle vector pAdTrace, respectively. Then, the shuttle vectors were linearized and transfected into HEK293 cells for the package of the recombinant adenoviruses, which were designated as AdTGF- $\beta$ 1 and AdsiPTEN. The recombinant adenoviruses mediated the over-expression were tagged with green GFP or red fluorescent protein (RFP) for knockdown to track the viruses, and the

recombinant adenovirus expressing GFP (AdGFP) only was used as vehicle control.

**Cell viability assay.** The cell viability was determined with Cell Counting Kit-8 (CCK-8). In brief, HCT116 cells were seeded in 96-well plates with a density of  $3 \times 10^3$  cells/well. Then, the cells were treated with different concentrations of Tet, recombinant adenovirus or DMSO for 24, 48 and 72 h. At the scheduled time-point, 10  $\mu$ l of CCK-8 was added into each well and incubated for 4 h. The absorbance was determined at 450 nm with a microplate reader. Each test was conducted in triplicate.

**Clonogenic assay.** The clonogenic assay was introduced to determine the ability of cells to undergo unlimited division and form colonies in a given population. Briefly, cells were pre-treated with different concentrations of Tet for 24 h, and then re-plated the cells to 12-well plates with a density of 2000 or 200 cells per well. Cells were maintained without Tet treatment up to 14 days until the colonies were formed. Finally, the plates were gently washed with PBS and incubated with 0.25% crystal violet formalin solution at room temperature for 20 min, and finally washed with tap water and air-dried. The assay was conducted in triplicate independently.

**Flow cytometric analysis for cell cycle and apoptosis.** Cells were seeded in 6-well plates and treated with different concentrations of Tet for 48 h. For cell cycle analysis, cells were harvested and washed with phosphate buffered saline (PBS, 4°C), fixed with cold (4°C) 70% ethanol, washed with 50% and 30% ethanol, and PBS. Finally, cells were stained with 1 ml of propidium iodide (PI, 20 mg/ml) containing RNase (1 mg/ml) in PBS for 30 min, followed by flow cytometry analysis. For apoptosis analysis, cells were harvested and washed with PBS (4°C), followed by incubating with Annexin V-EGFP (#KGA104, KeyGen Biotech, Nanjing, China) and PI. Finally, the cells were analyzed with fluorescence activated cell sorting (FACS). Each assay was done in triplicate.

**Reverse transcription (RT) and real-time polymerase chain reaction (PCR) analysis.** The cells were plated in T25 flask and treated with different concentration of Tet. At the scheduled time-point, total RNA were extracted with TRIzol reagent (Invitrogen), and followed by RT reaction to generate cDNA. Then, the cDNA products were used as templates for real-time PCR to detect the expression level of target genes. The data of each sample were normalized with the corresponding expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for this investigation are presented in Table I.

**Western blot assay.** Sub-confluent HCT116 cells were seeded in 6-well plates, and then treated with different concentrations of Tet and/or combined with corresponding recombinant adenovirus. At the scheduled time point, cell lysates were collected and boiled for 10 min. All samples were subjected to electrophoresis with SDS-PAGE and transferred to polyvinylidene fluoride membranes, blotted with primary antibodies and corresponding secondary antibodies conjugated with horseradish peroxidase successively. Finally, the target bands were

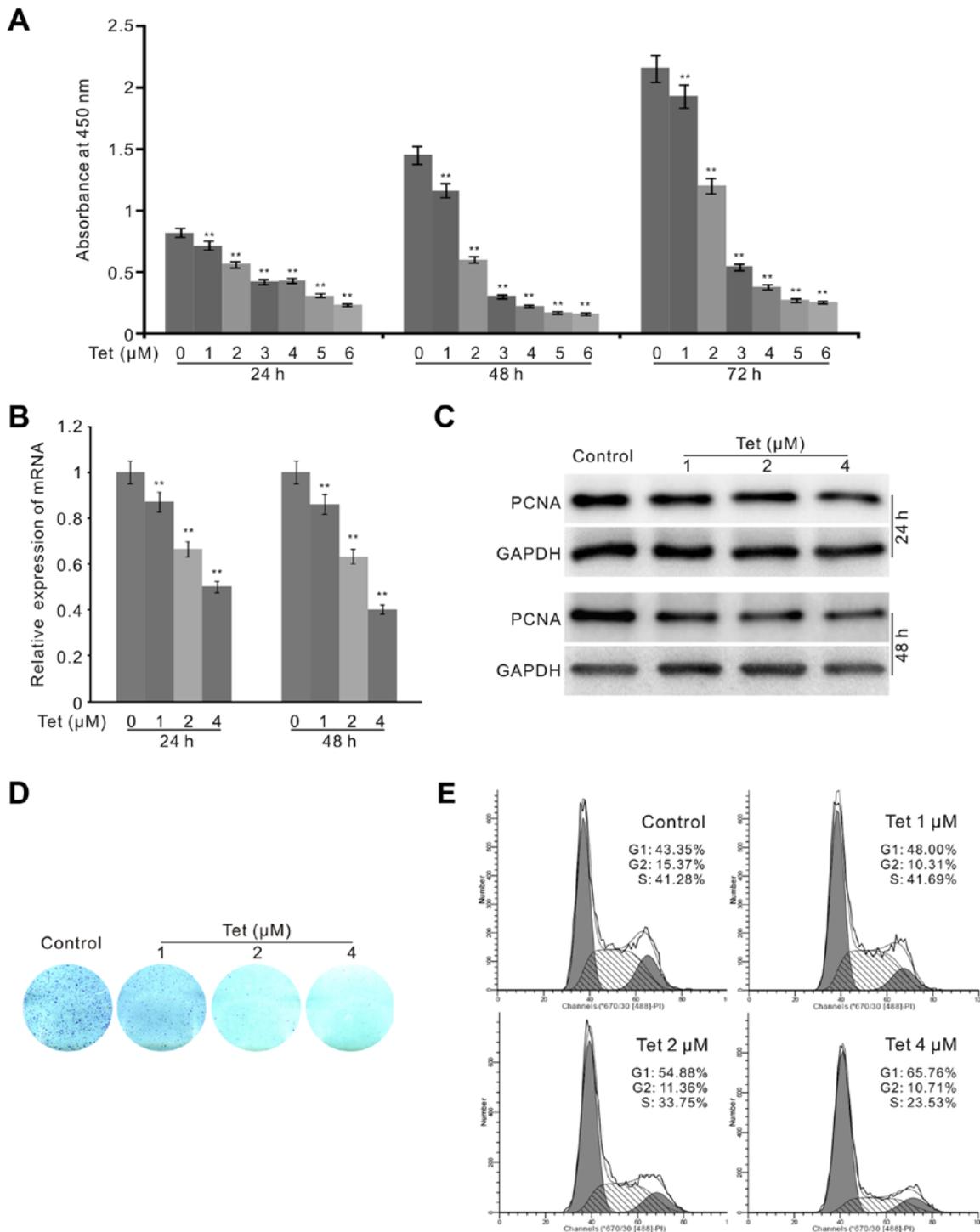


Figure 1. Effects of Tet on the proliferation of HCT116 cells. (A) CCK-8 assay results show the effect of Tet on the proliferation of HCT116 cells (\*\*P<0.01 vs. control). (B) Real-time PCR analysis results show the effect of Tet on the mRNA expression of PCNA in HCT116 cells (\*\*P<0.01 vs. control). (C) Western blot assay results show the effect of Tet on the protein level of PCNA. (D) Crystal violet staining of clonogenic formation results show the effect of Tet on the proliferation ability of HCT116 cells. (E) The results of flow cytometry analysis show the effect of Tet on the cell cycle arrest in HCT116 cells.

developed with SuperSignal West Femto Substrate (#34095, Thermo Scientific, Rockford, IL, USA). Each assay was done in triplicate.

**Immunocytochemical staining.** Cells were plated into 48-well plates and treated as the experimental design. At the scheduled time point, cells were fixed with cold (4°C) methanol for 15 min, and washed with cold PBS and permeabilized with

0.5% Triton X-100. Cells were blocked with 5% BSA at room temperature for 1 h, followed by incubation with p-Akt1/2/3, or PTEN antibody, the corresponding IgG were used as control, followed by incubation with rhodamine conjugated corresponding secondary antibodies for 30 min. Finally, cells were stained with DAPI (1 μg/ml). The fluorescence images were recorded under an inverted microscope. Each assay was done in triplicate.

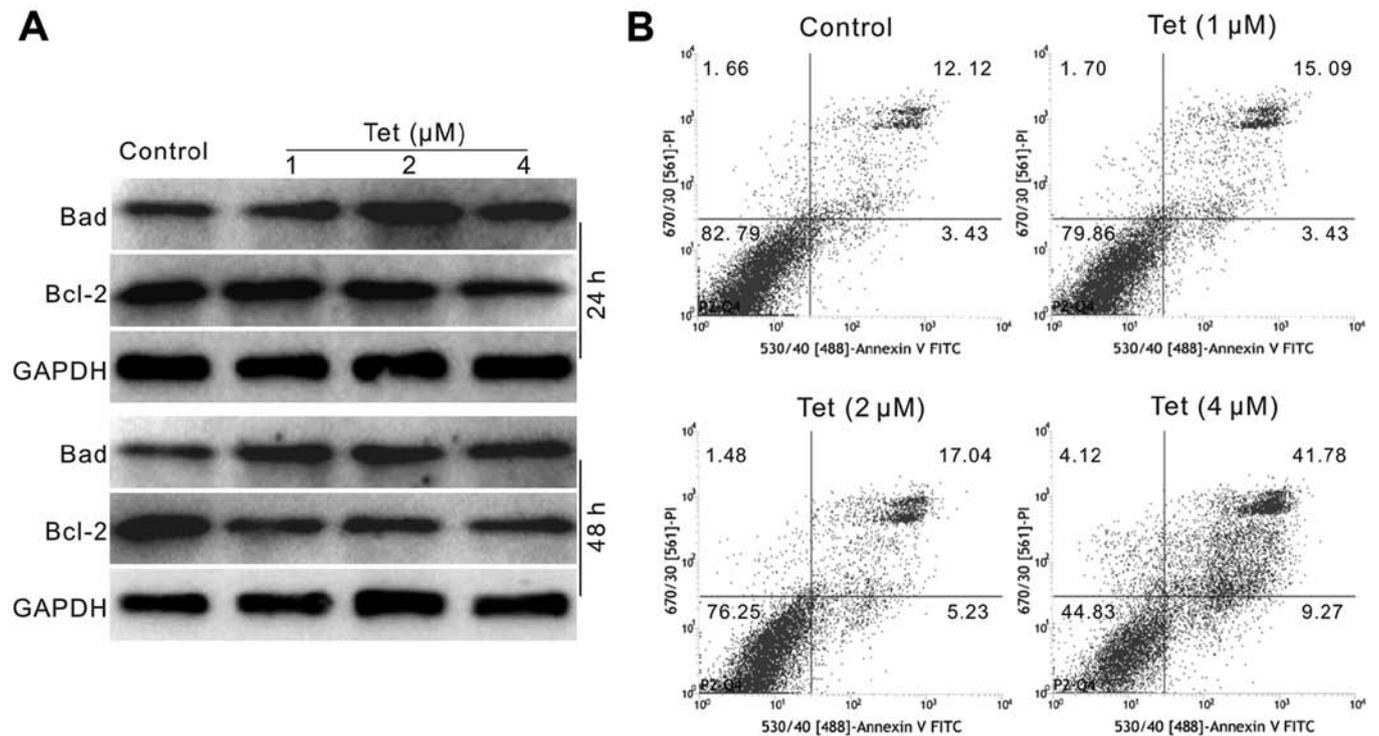


Figure 2. Effects of Tet on the apoptosis of HCT116 cells. (A) Western blot assay results show the effect of Tet on the protein level of Bad and Bcl-2 in HCT116 cells. (B) Flow cytometry analysis results show the effect of Tet on apoptosis in HCT116 cells.

*Statistical analysis.* Microsoft Excel was employed to calculate the standard deviations. The differences were analyzed using the Student's t-test.

## Results

*Effects of Tet on the proliferation of HCT116 cells.* Tet has been shown to have powerful anticancer activity in various types of cancer cells. In this study, we tested the function of Tet in human colon cancer cell line HCT116. The CCK-8 assay results revealed that Tet can inhibit the proliferation of HCT116 cells concentration-dependently (Fig. 1A). The PCR assay results showed that the expression of PCNA was decreased substantially by the treatment of Tet (Fig. 1B), and western blot analysis results recaptured the same effect of Tet on PCNA in HCT116 cells (Fig. 1C). The colony formation assays demonstrated that the proliferation ability of HCT116 cells was greatly reduced by Tet (Fig. 1D). Finally, we introduced flow cytometry analysis to determine the effect of Tet on the cell cycle. The results showed that Tet arrested the cell cycle at G1 phase and obviously reduced the percentage of S phase in HCT116 cells (Fig. 1E). These data suggested that Tet can effectively suppress the proliferation of HCT116 cells.

*Effects of Tet on apoptosis in HCT116 cells.* As apoptosis-inducing is one of the important properties of drugs for chemotherapy to show the anticancer activity, we next investigated the effect of Tet on the apoptosis in HCT116 cells. Western blot assay results showed that Tet decreases the level of Bcl-2, but increases the level of Bad in HCT116 cells (Fig. 2A). The flow cytometric analysis results showed that Tet

increases the percentage of apoptotic cells in HCT116 cells (Fig. 2B). These results suggested that Tet may be an effective apoptosis inducer for cancer cells.

### *Effects of Tet on the expression of TGF- $\beta$ 1 in HCT116 cells.*

The above data demonstrated that Tet can effectively suppress the growth and induce apoptosis in HCT116 cells, but the specific molecular mechanisms corresponding to these effects remains unclear. TGF- $\beta$  has been reported to be involved in the cause of colon cancer (24,26). Therefore, TGF- $\beta$  may be one of the potential candidate targets for colon cancer treatment, although its role in colon cancer remains unclear. Hence, we detected the endogenous expression of the three sub-types of TGF- $\beta$  in the available colon cancer cell lines and FHC cells. PCR results showed that the expression level of TGF- $\beta$ 1 is higher than that of TGF- $\beta$ 2 and TGF- $\beta$ 3 in these cells (Fig. 3A). As reports showed that TGF- $\beta$ 1 and TGF- $\beta$ 3 were related with colon cancer (24,27), we checked the endogenous protein level of these two types of TGF- $\beta$ , the results showed that both the protein of TGF- $\beta$ 1 and TGF- $\beta$ 3 are detectable in these cells, and the level of TGF- $\beta$ 1 in FHC cells is much lower than that of TGF- $\beta$ 3. However, the protein level of TGF- $\beta$ 1 in cancer cells is much higher than that of FHC cells (Fig. 3B). These data may imply that TGF- $\beta$ 1 is more associated with colon cancer cells. Hence, we focus on TGF- $\beta$ 1 in the following experiments. Firstly, we checked the effect of Tet on the mRNA expression of TGF- $\beta$ 1 in HCT116 cells. The results showed that Tet greatly increases the expression of TGF- $\beta$ 1 (Fig. 3C). Western blot assay confirmed that Tet increases the level of TGF- $\beta$ 1 obviously in HCT116 cells (Fig. 3D). These data implied that TGF- $\beta$ 1 may be involved in the anticancer activity of Tet in HCT116 cells.

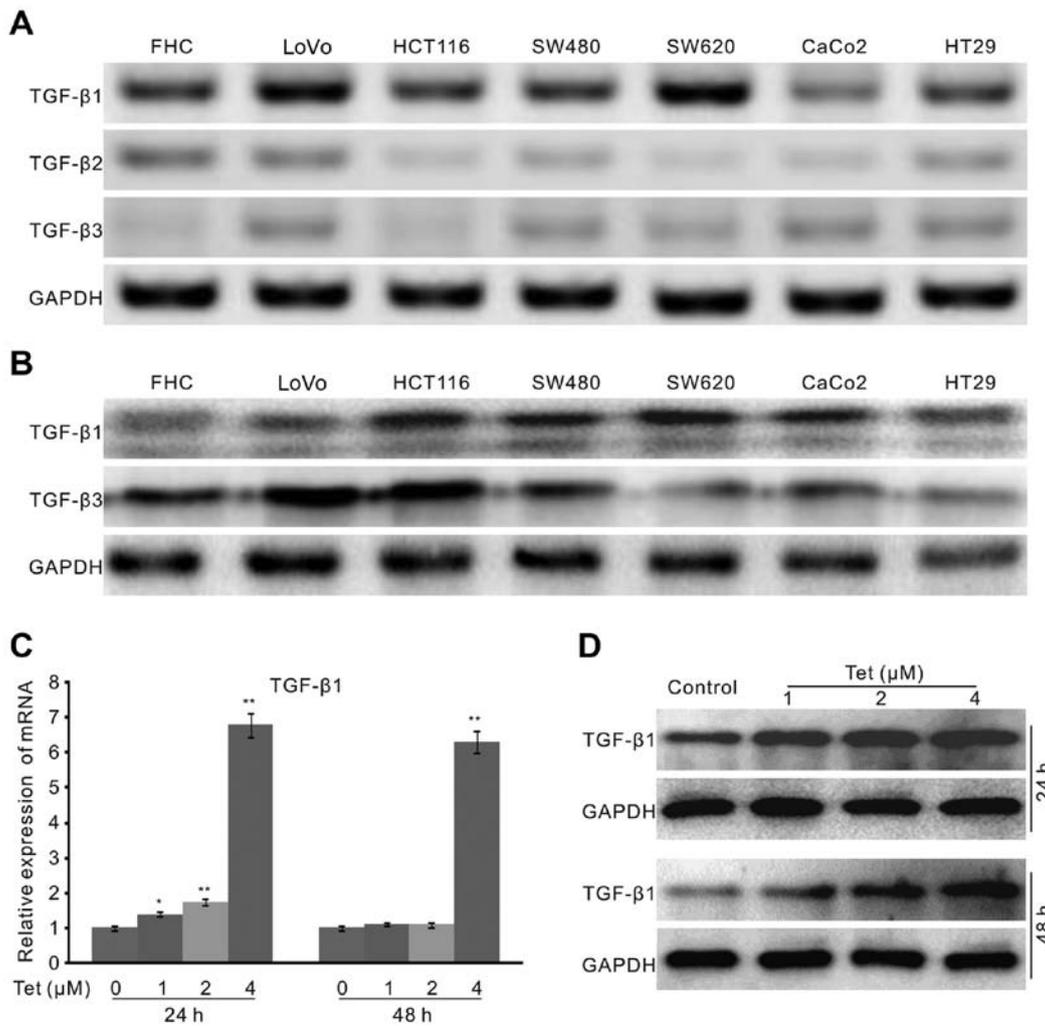


Figure 3. Effects of Tet on the expression of TGF-β in HCT116 cells. (A) PCR results show the endogenous expression level of the three isoforms of TGF-β in the available colon cancer cell lines and FHC cells. GAPDH was used as loading control. (B) Western blot assay results show the endogenous protein level of TGF-β1 and TGF-β3. (C) Real-time PCR results show the effect of Tet on the mRNA expression level of TGF-β1 in HCT116 cells (\*P<0.05 vs. control; \*\*P<0.01 vs. control). (D) Western blot assay results show the effect of Tet on the protein level of TGF-β1 in HCT116 cells.

Table I. The primers used for PCR assay.

Gene	Primer sequence (5'-3')
GAPDH	F: CAACGAATTTGGCTACAGCA R: AGGGGAGATTCAAGTGTGGTG
PCNA	F: GGCTCTAGCCTGACAAATGC R: GCCTCCAACACCTTCTTGAG
TGF-β1	F: CCCACAACGAAATCTATGACAA R: AAGATAACCACTCTGGCGAGTC
TGF-β2	F: ACTACGCCAAGGAGTTTACAA R: TCTGAACTCTGCTTTCACCAA
TGF-β3	F: TGTTAGAGGAAGGCTGAACTC R: ATGAGCAAATCCAACCTCAGAT
PTEN	F: TAAAGGCACAAGAGGCCCTA R: CGCCACTGAACATTGGAATA

F, forward; R, reverse.

*Effects of TGF-β1 on the anti-proliferation and apoptosis-inducing effect of Tet in HCT116 cells.* As Tet greatly increases the level of TGF-β1 in colon cancer cells and the endogenous level of TGF-β1 in colon cancer cells is much higher than that of FHC cells, we next investigated the effect of TGF-β1 on the anticancer activity of Tet in HCT116 cells. We introduced the recombinant adenovirus to mediate the exogenous expression of TGF-β1, and specific inhibitor to block the TGF-β1 signaling. The results showed that the exogenous TGF-β1 partly reduces the survival and apparently potentiates the anti-proliferation effect Tet in HCT116 cells, while the TGF-β1 specific inhibitor increases the proliferation and partly reverses the anti-proliferation effect of Tet in HCT116 cells (Fig. 4A). Moreover, the exogenous TGF-β1 increases the level of Tet-induced Bad and decreases the level of Bcl-2 induced by Tet (Fig. 4B). The TGF-β1 inhibitor apparently decreases the level of Tet-induced Bad, and increases the level of Bcl-2 which was decreased by Tet in HCT116 cells (Fig. 4C). These results suggested that TGF-β1 may play an important role in mediating the anticancer activity of Tet in HCT116 cells, although the explicit mechanism underlying this effect remains unknown.

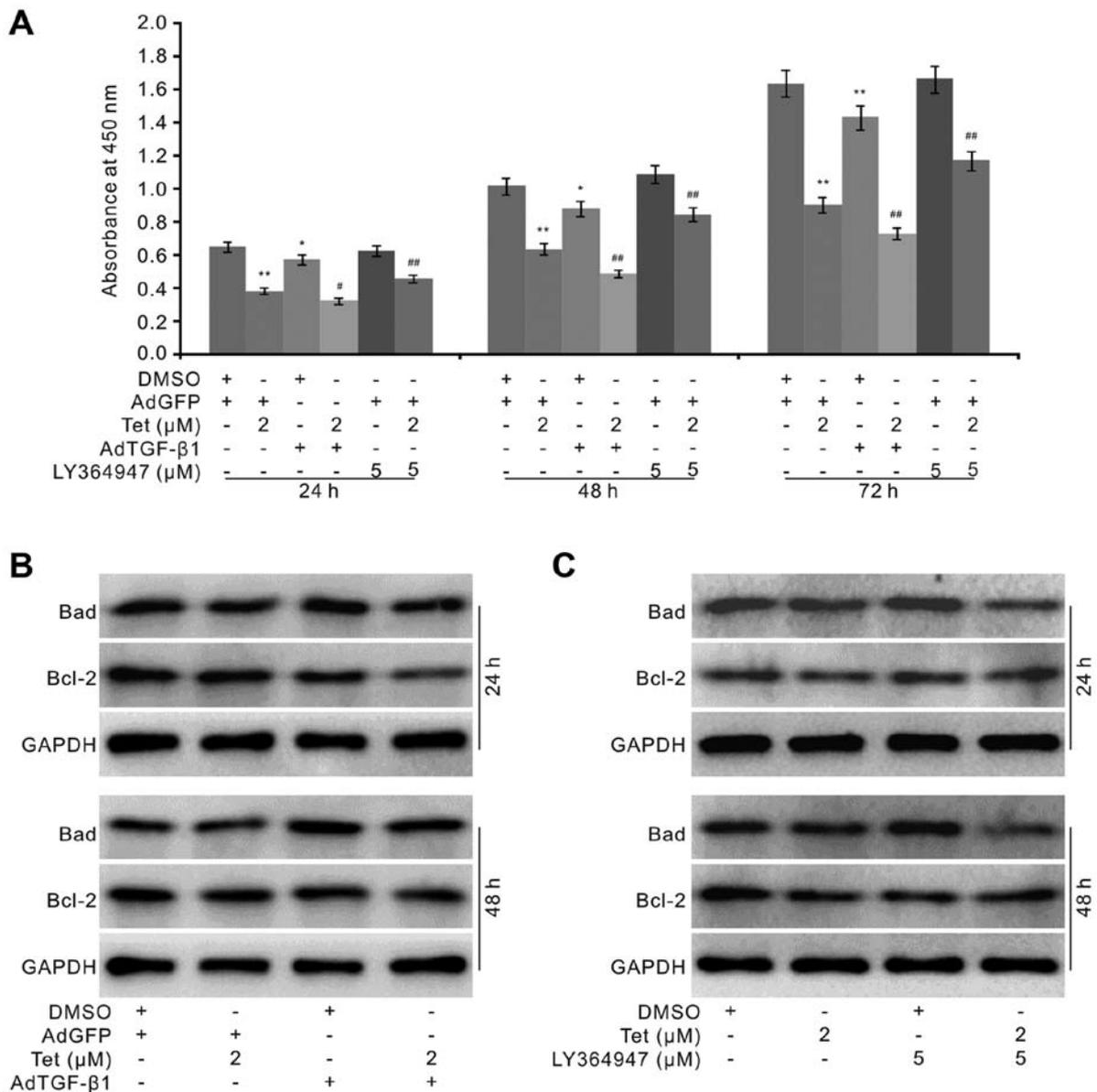


Figure 4. Effects of TGF- $\beta$ 1 on the anticancer activity of Tet in HCT116 cells. (A) CCK-8 assay results show the effect of TGF- $\beta$ 1 on the anti-proliferation effect of Tet in HCT116 cells (LY364947, the inhibitor of TGF- $\beta$ 1. \* $P$ <0.05 vs. control; \*\* $P$ <0.01 vs. control; # $P$ <0.05 vs. Tet treated group, ## $P$ <0.01 vs. Tet treated group). (B) Western blot assay results show the effect of exogenous expression of TGF- $\beta$ 1 on the level of Bad and Bcl-2 affected by Tet in HCT116 cells. GAPDH was used as loading control. (C) Western blot assay results show the effect of TGF- $\beta$ 1 inhibitor on the level of Bad and Bcl-2 affected by Tet in HCT116 cells. GAPDH was used as loading control.

*Effects of TGF- $\beta$ 1 on the PI3K/Akt signaling in HCT116 cells.* TGF- $\beta$ 1 ligand binding with type II receptor activate TGF- $\beta$  signaling, but our pilot tests demonstrated that Tet can not increase the phosphorylation of Smad2/3 (data are not shown). Hence, TGF- $\beta$ 1 may mediate the anticancer effect of Tet through non-canonical TGF- $\beta$  signaling, such as PI3K/Akt pathway. PI3K/Akt signaling is one of the essential pathways for the regulation of cell survival and differentiation, and drugs have been developed and targeted on this signaling to inhibit the proliferation of cancer cells. It has been reported that Tet can induce cell cycle arrest through inhibiting the PI3K/Akt pathway in HT29 cells (31). However, it is unclear whether the anti-proliferation effect of Tet in HCT116 cells can also be mediated through downregulating this pathway. Hence, we determined the effect of Tet on PI3K/Akt pathway

in HCT116 cells. Western blot analysis showed that Tet markedly reduces the level of phosphorylated Akt1/2/3 (p-Akt1/2/3), although no obvious effect was seen on the total level of Akt1/2 (Fig. 5A). The CCK-8 assay results showed that PI3K inhibitor markedly potentiates the anti-proliferation effect of Tet in HCT116 cells (Fig. 5B). These data indicated that the anti-proliferation effect of Tet in HCT116 cells may be mediated through the inactivation of PI3K/Akt signaling, but how Tet suppresses this pathway remains unclear. As Tet substantially increases the level of TGF- $\beta$ 1 in HCT116 cells, we speculated that the inactivation of PI3K/Akt signaling by Tet may be associated with the Tet-induced increase of TGF- $\beta$ 1 in HCT116 cells.

Western blot assay showed that exogenous TGF- $\beta$ 1 promotes the effect of Tet by decreasing the level of p-Akt1/2/3

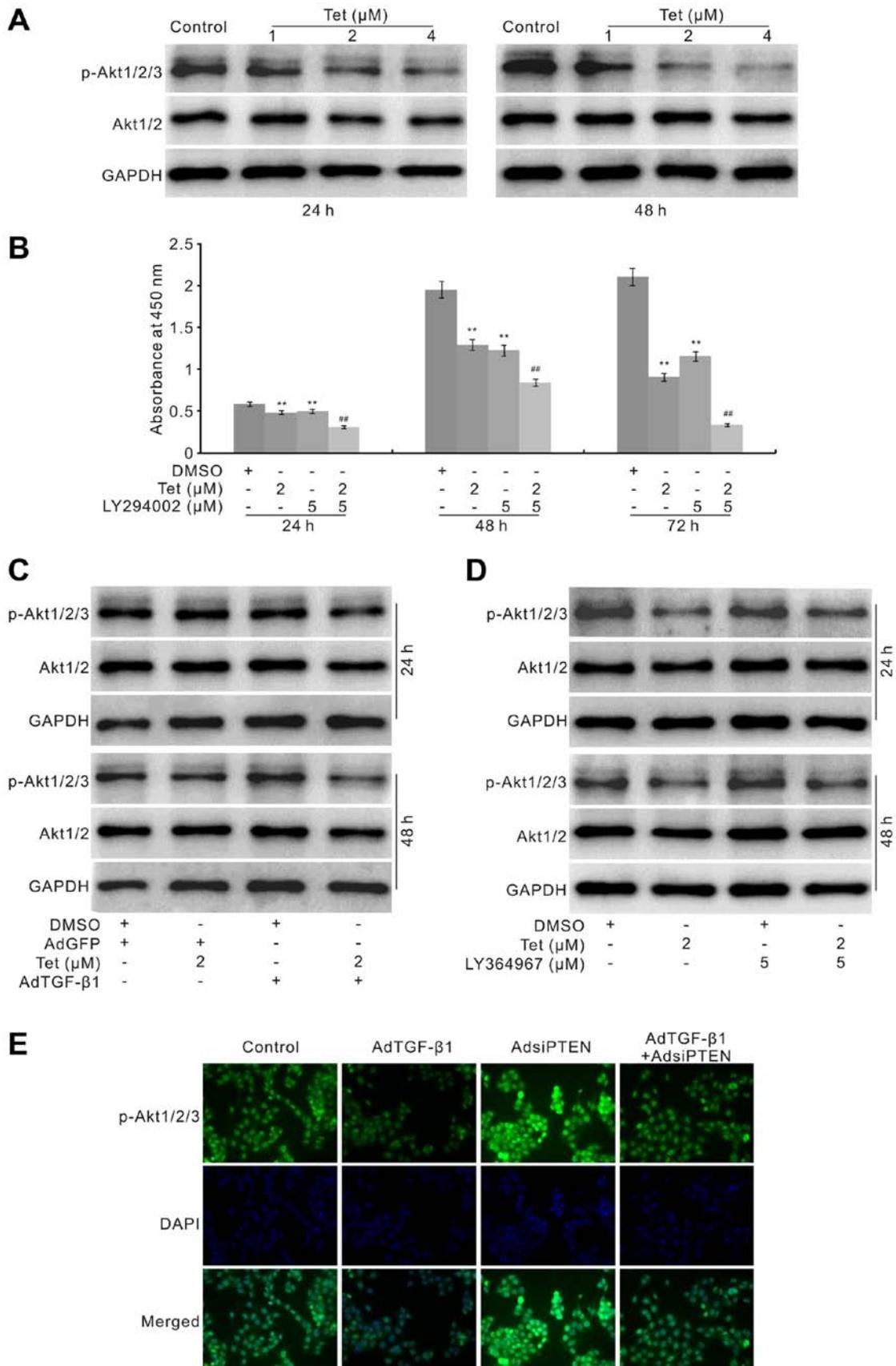


Figure 5. Effects of Tet and TGF-β1 on the activation of PI3K/Akt pathway in HCT116 cells. (A) Western blot assay shows the effect of Tet on the total level of Akt1/2 and phosphorylated Akt1/2/3 (p-Akt1/2/3) in HCT116 cells. GAPDH was used as loading control. (B) CCK-8 assay results show the effect of PI3K inhibitor on the anti-proliferation effect of Tet in HCT116 cells (LY294002, the inhibitor for PI3K. \*\*P<0.01 vs. control; ##P<0.01 vs. Tet treated group). (C) Western blot assay results show the effect of exogenous expression of TGF-β1 on the level of Akt1/2 and p-Akt1/2/3 affected by Tet in HCT116 cells. GAPDH was used as loading control. (D) Western blot assay results show the effect of TGF-β1 inhibitor on the level of Akt1/2 and p-Akt1/2/3 affected by Tet in HCT116 cells. GAPDH was used as loading control. LY364947, the inhibitor for TGF-β1. (E) Immunofluorescent stain results show the effect of exogenous expression of TGF-β1 and/or knockdown of PTEN on the level of p-Akt1/2/3 in HCT116 cells.

but no obvious effect on the level of total Akt1/2 (Fig. 5C). The TGF- $\beta$ 1 inhibitor increases the level of p-Akt1/2/3 and partly reverses the Tet-induced decrease of p-Akt1/2/3 (Fig. 5D). In order to confirm the effect of TGF- $\beta$ 1 on the p-Akt1/2/3 in HCT116 cells, we constructed the recombinant adenovirus for siRNA fragments of PTEN, a well-known negative regulator for PI3K/Akt signaling. The immunofluorescence staining results showed that knockdown of PTEN greatly increases the level of p-Akt1/2/3, but this effect can be thoroughly eliminated by the exogenous TGF- $\beta$ 1 in HCT116 cells (Fig. 5E). All these data suggested that the anti-proliferation effect of Tet in HCT116 cells may be mediated by inactivating PI3K/Akt signaling through upregulating the expression of TGF- $\beta$ 1.

*Effects of TGF- $\beta$ 1 on the PTEN affected by Tet in HCT116 cells.* PI3K/Akt signaling is finely regulated by various factors, one of which is PTEN. PTEN dephosphorylates PIP3 to form PIP2, which makes the inactivation of PI3K/Akt signaling. The above mentioned data showed that Tet decreases the level of p-Akt1/2/3 in HCT116 cells, which may be mediated by upregulating TGF- $\beta$ 1. As PTEN is a critical negative regulator for PI3K/Akt signaling, it implied that the effect TGF- $\beta$ 1 on PI3K/Akt signaling may result from the upregulation of PTEN in HCT116 cells. With this hypothesis, we determined the effect of TGF- $\beta$ 1 on PTEN and phosphorylated PTEN (p-PTEN). The PCR assay results showed that Tet suppresses the mRNA expression of PTEN in HCT116 cells (Fig. 6A). However, western blot analysis results showed that Tet exerts no substantial effect on the total protein level of PTEN, but decreases the level of p-PTEN (Fig. 6B). The immunofluorescence staining results confirmed that Tet exerts no obvious effect on the level of PTEN in HCT116 cells (Fig. 6C). Further analysis showed that exogenous TGF- $\beta$ 1 can partly decrease the level of p-PTEN and synergistically reduces the level of p-PTEN induced by Tet in HCT116 cells (Fig. 6D). However, the TGF- $\beta$ 1 inhibitor increases the level of p-PTEN and partly reverses the Tet-induced decrease of p-PTEN (Fig. 6E). These data indicated that the upregulation of TGF- $\beta$ 1 may mediate the anticancer effect of Tet in HCT116 cells through partly suppressing the phosphorylation of PTEN.

## Discussion

Colon cancer is one of the leading malignancies in gastrointestinal system and the unsatisfactory prognosis need to be greatly ameliorated. Tet has been demonstrated as a powerful reagent with anticancer activity. We investigated the anticancer activity of Tet in HCT116 cells and found that the effect of Tet in colon cancer cells may be mediated by upregulating TGF- $\beta$ 1 to inactivate the PI3K/Akt signaling through partly decreasing the phosphorylation of PTEN.

The treatment of colon cancer includes chemotherapy, and surgery. The prognosis remains undesirable even though the targeted therapy has been introduced to the clinic (3). The challenge for treatment of cancer includes the serious side effects of the traditional chemotherapy drugs, metastasis and the drug resistance of cancer cells. There is a great clinical need to explore new effective drugs for the treatment of colon cancer. Natural products or their derivatives is one of the essential resources for anticancer drugs. With regard to

colon cancer, camptothecin or its derivative irinotecan have been clinically used for decades (32,33). Tet is a plant-derived bisbenzylisoquinoline alkaloid, mostly extracted from the root of medicine herb *Stephania tetrandra* S Moore (8).

It is a well-know calcium blocker and possesses multi-pharmacological activities, so Tet can be used widely for the treatment of hypertension, asthma, tuberculosis, dysentery and hyperglycemia (34). Increasing evidence supports that Tet is also a potent anticancer reagent, it exhibits efficacious anticancer activity to many kinds of cancer cells, such as prostate cancer, lung cancer, bladder cancer, gastric and colon cancer (8,12-15). Our previous study also suggested that Tet may be a chemotherapy and/or chemoprevention drug for colon cancer (14,19). Consequently, Tet may be a promising anticancer candidate agent for colon cancer treatment. In terms of the mechanism, various targets have been reported to be related with the anticancer activity of Tet, such as the inhibition of Wnt/ $\beta$ -catenin signaling, PI3K/Akt signaling, ERK and inducing p53 (16-19). So far, the specific molecular mechanism remains unclear, and further thorough investigation need to be carried out for unveiling this process.

TGF- $\beta$  super-family includes secreted proteins, such as TGF- $\beta$ , bone morphogenetic proteins (BMPs), activins, and Nodal (35). These proteins act as ligand to bind with TGF- $\beta$  type II receptor, and followed by the activation of TGF- $\beta$  type I receptor. Finally, it interacts with different Smads to regulate multiple downstream targets, which are involved in a plethora of physiological processes. Therefore, the aberrant TGF- $\beta$  signaling has been referred to in many diseases (36,37). For TGF- $\beta$ , there are three isoforms, including TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3, and they are all expressed in mammalian cells. The role of TGF- $\beta$  is still unclear in tumor initiation and progression, as it can act as a tumor suppressor or promoter (36). The dual role of TGF- $\beta$  may be context and stage of cancer-dependent. In epithelial cells, TGF- $\beta$  usually functions as a tumor suppressor though inhibiting proliferation and inducing apoptosis; in other tissues, TGF- $\beta$  may promote the progression of cancer, such as increasing the invasion and metastasis of tumor cells (38).

TGF- $\beta$  may also suppress the tumor at the early stage and act as a promoter at the late stage (39). In term of colon cancer, TGF- $\beta$ 1 has been reported to increase the progression of colon cancer by upregulating the expression of Human Cripto-1 (CR-1) (40), and promoting the migration of cancer cells (41). It remains unknown whether TGF- $\beta$ 1 can also exert anti-proliferation activity in colon cancer cells. Our previous study demonstrated the potential anti-proliferation effect of Tet in colon cancer (14,19). It has been reported that Tet can decrease the mRNA level of TGF- $\beta$ 1 in the nitrofen-induced congenital diaphragmatic hernia (42), but its effect on the expression of TGF- $\beta$ 1 in cancer cells remains unclear.

In this study, we found that the isoforms of TGF- $\beta$  all are detectable in the colon cancer and FHC cells. However, the protein level of TGF- $\beta$ 1 in FHC cells is much lower than any of the cancer cell lines. For TGF- $\beta$ 3, its level in FHC cells are higher than that of other cancer cell lines. These data suggested that TGF- $\beta$ 1 and TGF- $\beta$ 3 may function differently for colon cancer. With PCR and western blot analysis we found that Tet can obviously increase the expression of TGF- $\beta$ 1 in HCT116

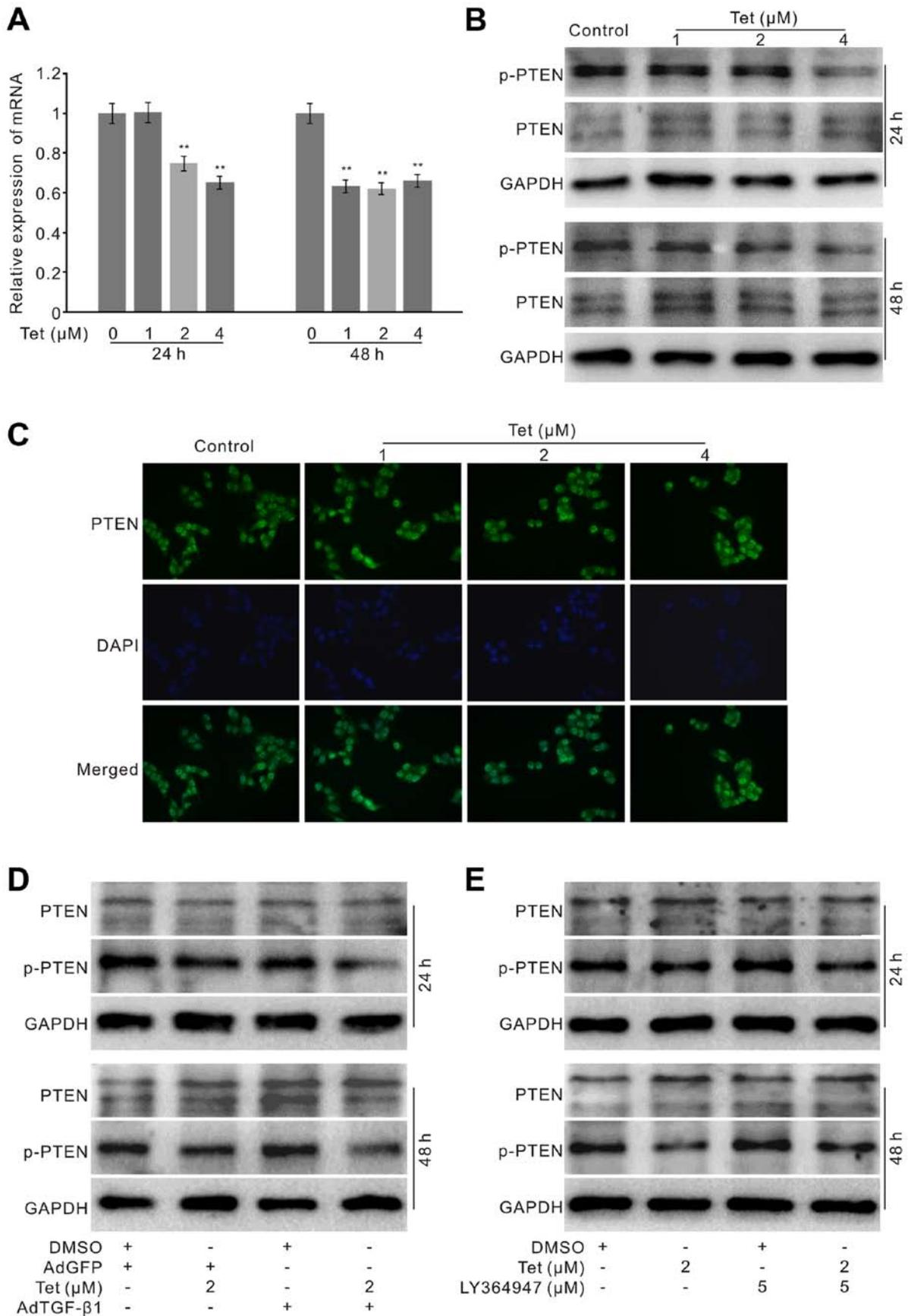


Figure 6. Effects of TGF-β1 on the PTEN affected by Tet in HCT116 cells. (A) Real-time PCR assay results show the effect of Tet on the mRNA expression of PTEN in HCT116 cells (\*\*P<0.01 vs. control). (B) Western blot assay results show the effect of Tet on the total level of PTEN and phosphorylated PTEN (p-PTEN) in HCT116 cells. GAPDH was used as loading control. (C) Immunofluorescent stain results show the effect of Tet on the level of PTEN in HCT116 cells. (D) Western blot assay shows the effect of exogenous expression of TGF-β1 on the level of PTEN and p-PTEN affected by Tet in HCT116 cells. GAPDH was used as loading control. (E) Western blot assay results show the effect of TGF-β1 inhibitor on the level of PTEN and p-PTEN affected by Tet in HCT116 cells. GAPDH was used as loading control (LY364947, the inhibitor for TGF-β1).

cells. Hence, we speculated that the anti-proliferation activity of Tet in HCT116 cells may be associated with the upregulation of TGF- $\beta$ 1. With further analysis, we found that exogenous expression of TGF- $\beta$ 1 enhances the anti-proliferation effect of Tet, and TGF- $\beta$ 1 inhibitor partly reverses the Tet-induced suppression of proliferation. Exogenous expression of TGF- $\beta$ 1 enhances the effect of Tet on the increase of Bad and reduces Bcl-2. On the contrary, TGF- $\beta$ 1 inhibitor decreases the effect of Tet on increasing the level of Bad and partly reverses the Tet-induced decrease of Bcl-2 in HCT116 cells. Herein, our data suggested that the anti-proliferation activity of Tet may be partly mediated by upregulating TGF- $\beta$ 1 in HCT116 cells.

One of the important functions of TGF- $\beta$  is to regulate cell proliferation, apoptosis and differentiation through the canonical TGF- $\beta$  pathway, by binding with their receptor, or the non-canonical TGF- $\beta$  pathway, such as Wnt/ $\beta$ -catenin and PI3K/Akt signaling. TGF- $\beta$  prevents osteogenic differentiation by inhibiting BMP and Wnt/ $\beta$ -catenin signaling (43). TGF- $\beta$  has also been reported to activate PI3K/Akt signaling to promote the migration and invasion of prostate cancer cells, and the differentiation of radial glia into astrocyte (44,45). Our results demonstrated that Tet can not increase the phosphorylation of Smad2/3, instead of reducing it (data are not shown). Thus, TGF- $\beta$ 1 may mediate the anti-proliferation effect of Tet through non-canonical TGF- $\beta$  signaling pathway. It was reported that Tet can inactivate PI3K/Akt signaling to induce cell cycle arrest and apoptosis in HT29 cells (31).

Our results indicated that Tet can also decrease the phosphorylation of Akt1/2/3 (p-Akt1/2/3) in HCT116 cells, although it did not exert any obvious effect on the total level of Akt1/2. Thus, it is possible for Tet to inactivate PI3K/Akt signaling through the upregulation of TGF- $\beta$ 1 in HCT116 cells. Further analysis revealed that exogenous expression of TGF- $\beta$ 1 potentiates the inhibitory effect of Tet on the level of p-Akt1/2/3, while TGF- $\beta$ 1 inhibitor partly reverses the Tet-induced decrease of p-Akt1/2/3 in HCT 116 cells. Our data also showed that exogenous expression of TGF- $\beta$ 1 substantially diminishes the level of p-Akt1/2/3 induced by knockdown of the phosphatase and tensin homolog deleted on chromosome ten (PTEN) in HCT116 cells. Therefore, the evidence suggested that Tet may inhibit the phosphorylation of Akt1/2/3 through upregulating TGF- $\beta$ 1 in colon cancer cells.

PTEN is a well-known tumor suppressor, as phosphatase negatively regulates the phosphatidylinositol-3-kinase (PI3K) signaling pathway by catalyzing phosphatidylinositol 3,4,5-triphosphate (PIP3) dephosphorylates to PI-4,5-bisphosphate (PIP2). The PI3K/Akt signaling is critical for cell survival and differentiation. Accordingly, the dysfunction of PTEN will make the PI3K/Akt signaling over-activated and the cell growth out of control. Therefore, it is also considered as the potential target for cancer treatment (46). Various factors may lead to the function loss of PTEN, such as inherited mutations, epigenetic and/or transcriptional silence, and post-transcriptional modification (47). In colon cancer, the dysfunction of PTEN, may result from genetic and/or epigenetic changes, and is one of the main causes for the initiation and progression of cancer (48). PTEN may be used as a prognostic and predictive factor for colon cancer, and a target for the treatment of colon cancer. In this study,

we demonstrated that Tet decreases the phosphorylation of Akt1/2/3 in HCT116 cells from upregulating TGF- $\beta$ 1. Thus, we speculated that this effect of TGF- $\beta$ 1 on the phosphorylation of Akt1/2/3 may result from the regulation of PTEN. With PCR, we found that Tet decreases the mRNA expression of PTEN rather than increasing it. However, western blot assay results showed that Tet substantially decreases the phosphorylation level of PTEN, although has no obvious effect on the total level of PTEN. Exogenous expression of TGF- $\beta$ 1 increases the inhibitory effect of Tet on the phosphorylation of PTEN (p-PTEN), but the TGF- $\beta$ 1 inhibitor promotes the phosphorylation of PTEN and partly reverses the Tet-induced decrease of p-PTEN. However, how TGF- $\beta$ 1 regulates the phosphorylation of PTEN need to be further elucidated.

Taken together, our data strongly suggested that Tet may be a potential candidate for colon cancer treatment. The anti-cancer activity of Tet in HCT116 cells may be partly mediated by upregulating the expression of TGF- $\beta$ 1, which may inactivate the PI3K/Akt signaling through partly decreasing the phosphorylation of PTEN.

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