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Basic Study

Total flavone of *Abelmoschus manihot* suppresses epithelial-mesenchymal transition *via* interfering transforming growth factor- β 1 signaling in Crohn's disease intestinal fibrosis

Bo-Lin Yang, Ping Zhu, You-Ran Li, Min-Min Xu, Hao Wang, Li-Chao Qiao, Hai-Xia Xu, Hong-Jin Chen

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Abstract

AIM

To explore the role and mechanism of total flavone of *Abelmoschus manihot* (TFA) on epithelial-mesenchymal transition (EMT) progress of Crohn's disease (CD) intestinal fibrosis.

METHODS

First, CCK-8 assay was performed to assess TFA on the viability of intestinal epithelial (IEC-6) cells and select the optimal concentrations of TFA for our further studies. Then cell morphology, wound healing and transwell assays were performed to examine the effect of TFA on morphology, migration and invasion of IEC-6 cells treated with TGF- β 1. In addition, immunofluorescence, real-time PCR analysis (qRT-PCR) and western blotting assays were carried out to detect the impact of TFA on EMT progress. Moreover, western blotting assay was performed to evaluate the function of TFA on the Smad and MAPK signaling pathways. Further, the role of co-treatment of TFA and si-Smad or MAPK inhibitors has been examined by qRT-PCR, western blotting, morphology, wound healing and

transwell assays.

RESULTS

In this study, TFA promoted transforming growth factor- β 1 (TGF- β 1)-induced (IEC-6) morphological change, migration and invasion, and increased the expression of epithelial markers and reduced the levels of mesenchymal markers, along with the inactivation of Smad and MAPK signaling pathways. Moreover, we revealed that si-Smad and MAPK inhibitors effectively attenuated TGF- β 1-induced EMT in IEC-6 cells. Importantly, co-treatment of TFA and si-Smad or MAPK inhibitors had better inhibitory effects on TGF- β 1-induced EMT in IEC-6 cells than either one of them.

CONCLUSION

These findings could provide new insight into the molecular mechanisms of TFA on TGF- β 1-induced EMT in IEC-6 cells and TFA is expected to advance as a new therapy to treat CD intestinal fibrosis.

Key words: Crohn's disease; Intestinal fibrosis; Epithelial-to-mesenchymal transition; Total flavone of *Abelmoschus manihot*; Transforming growth factor- β 1/Smad signaling; Transforming growth factor- β 1/non-Smad signaling

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Core tip: Regulating transforming growth factor- β (TGF- β) and its downstream signaling pathways, mediating the epithelial-mesenchymal transition (EMT) process and restoring the biological function of abnormally activated intestinal fibroblasts, may be an important way to seek drug therapy for Crohn's disease (CD) intestinal fibrosis. Total flavone of *Abelmoschus manihot* (TFA) can inhibit TGF- β 1-induced morphological change, migration, invasion of rat intestinal epithelial cells, and promote induction of EMT partially by inhibiting TGF- β 1-activated Smad and non-Smad signaling pathways. Therefore, TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

Yang BL, Zhu P, Li YR, Xu MM, Wang H, Qiao LC, Xu HX, Chen HJ. Total flavone of *Abelmoschus manihot* suppresses epithelial-mesenchymal transition *via* interfering transforming growth factor- β 1 signaling in Crohn's disease intestinal fibrosis. *World J Gastroenterol* 2018; 24(30): 3414-3425 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i30/3414.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i30.3414>

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammation of the gut, which causes significant impairment of quality of life with a rising incidence and prevalence during recent decades^[1,2]. Although the clinical manifestations and

pathologic progress of CD are different, fibrosis of intestinal organization and strictures induced by transmural inflammation will eventually cause intestinal obstruction, which is the characteristic clinical manifestation^[3-5]. In addition, more than 1/3 of CD patients need at least one intestinal operation in their lives, while 70% of the CD patients with fibrosis strictures need partial resection of the intestinal tract within 10 years of disease progression, and 70%-90% patients will have a recurrence of anastomotic strictures and over 50% patients will form new strictures^[6,7]. Moreover, a large number of clinical and experimental results have confirmed that the main drugs for treatment of CD, such as glucocorticoids, immune agents and biological agents, can effectively inhibit intestinal inflammation, but do not have positive activity in preventing the further progress of intestinal fibrosis^[8,9]. Thus, there is still a lack of drugs that can effectively inhibit or reverse CD intestinal fibrosis.

The process of intestinal fibrosis in CD patients involves a variety of cells and multiple molecular signaling pathways^[10,11]. Due to the continuous role of chronic intestinal inflammation, activated T and B cells will produce large amounts of pro-inflammatory cytokines and pro-fibrogenic factors, and induce fibroblast, epithelial cells, endothelial cells and stellate cells to migrate, proliferate, activate and differentiate into myofibroblasts, which finally results in excessive proliferation of myofibroblasts and excessive deposition of extracellular matrix (ECM), leading to the formation of intestinal fibrosis^[12-14]. Studies have shown that even if inflammation of the intestinal tract is effectively controlled, the process of fibrosis will continue and eventually lead to intestinal stenosis^[15]. Epithelial to mesenchymal transition (EMT) plays an important role in the activation of fibroblasts^[16]. Epithelial cells will lose epithelial polarity and epithelial phenotype contacted with basement membrane and produce fibroblasts to repair tissue injury caused by trauma and inflammatory reactions through the EMT progress^[17]. In physiological states, when the inflammatory reaction is relieved, the transformation process stops spontaneously. However, in the case of continuous activation of the inflammatory reaction, the EMT process will also continue to exist, and eventually cause organ fibrosis. Under pathophysiologic conditions, when the inflammatory reaction is relieved, the transformation process will stop spontaneously. However, in the case of continuous activation of inflammatory response, the EMT process will also exist continuously, and eventually cause organ fibrosis^[18,19]. Nowadays, although the role and regulation mechanism of EMT in CD intestinal fibrosis has not been fully understood, the transforming growth factor- β (TGF- β)/Smad/MAPK signaling pathway has been confirmed to play an important role in regulating EMT in organs such as lung, liver, kidney and so on^[20-22]. Therefore, studying the role of EMT in the formation of intestinal fibrosis based on the TGF- β /Smad/MAPK signaling pathway, may provide a new target for the treatment of CD intestinal fibrosis.

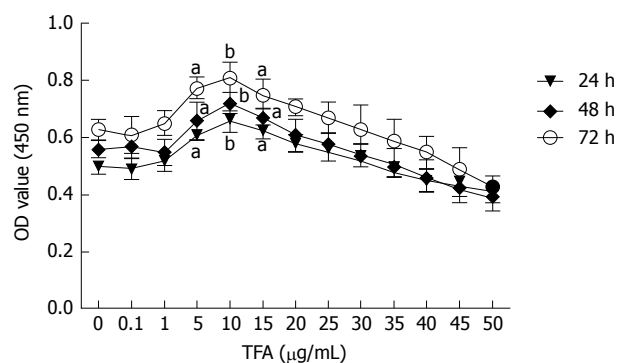


Figure 1 Effect of total flavone of *Abelmoschus manihot* at different concentrations on IEC-6 cell viability. IEC-6 cell viability after treatment with TFA at increasing concentrations was examined by CCK-8 at 24, 48 and 72 h. The results were expressed as the mean \pm SD of three independent experiments, and each was performed in triplicate. ^a $P < 0.05$, ^b $P < 0.01$ compared with that of non-TFA treated group. TFA: Total flavone of *Abelmoschus manihot*.

Total flavone of *Abelmoschus manihot* L. Medic (TFA), as the main components of the water extract of traditional Chinese medicine *Abelmoschus manihot* (L.), has been reported to play an important role in the improvement of renal inflammation, nephrotic syndrome, purpura nephritis, IgA nephropathy, membranous nephropathy and diabetic nephropathy (DN) effectively in the clinical trial^[23-26]. Further, our previous research has shown that TFA could significantly inhibit the release of the intestinal inflammatory cytokines tumor necrosis factor (TNF)- α and interferon (IFN)- γ in a CD rat model induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS), improve the model animal survival rate, and effectively improve intestinal inflammation. Moreover, immunohistochemical staining showed that the expression of TGF- β , alpha-smooth muscle actin (α -SMA) and matrix metalloproteinase-2 (MMP-2) were obviously reduced after intervention of TFA. Further, Masson staining confirmed that collagen fibrils of the mucosa and lamina propria of the colon were markedly decreased, indicating that TFA had positive inhibitory effects on CD intestinal fibrosis, which may be related to the EMT mechanism mediated by TGF- β . Thus, we hypothesized that TFA could inhibit or reverse the CD intestinal fibrosis *via* regulation of EMT based on TGF- β and its downstream Smad and MAPK signaling pathways. Therefore, this study was designed to focus on the influence of EMT on CD intestinal fibrosis and to further explore the role and mechanism of TFA on the progress of CD intestinal fibrosis.

MATERIALS AND METHODS

Preparation of TFA

Abelmoschus Manihot L. Medic was collected from Jiangyan of Jiangsu province, China. TFA was extracted from the flowers of *Abelmoschus Manihot* by the Jiangsu Province Hospital of TCM, Nanjing, China. A total of 500 g of *Abelmoschus Manihot* flowers was immersed in 8000 mL 75% ethanol for 1 h. The mixture was refluxed

for 1 h at 90 °C and filtered by analytical filter paper. The extracts were evaporated by rotary evaporation under vacuum at 60 °C^[27]. For cell experiments, TFA was dissolved in dimethyl sulfoxide.

Cell culture

Rat intestinal epithelial (IEC-6) cells were obtained from the American Type Culture Collection (Manassas, VA, United States). Cells were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, United States) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, United States), 2 mmol/L GlutaMAX-I (Invitrogen, CA, United States), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) in 5% CO₂ atmosphere at 37 °C.

TFA working concentration

The concentration of TFA for further study was evaluated based on cell viability. Cell viability was determined by Cell Counting Kit-8 assay (CCK-8, Sigma Chemical Co, St Louis, MO, United States). Briefly, IEC-6 cells with a density of 1×10^4 cells/well in 100 µL of complete culture medium were seeded in 96-well plates. After culturing for 24 h, the medium was replaced with serum-free media or serum-free media containing TFA at concentrations ranging from 0.1 µg/mL to 50 µg/mL, and incubated in a humidified incubator at 37 °C for 24, 48 or 72 h. After incubation, 10 µL CCK-8 was added to each well for 2 h at 37 °C. The optical density (OD) was recorded at 450 nm using a microplate reader (Dojindo Molecular Technology, Rockville, MD, United States).

Based on the results in Figure 1A, 5, 10, and 15 µg/mL TFA represented the optimal working concentrations and were used in our subsequent experiments.

Gene silencing with siRNAs

Cells were transfected with non-targeting negative control siRNA (Dharmacon, Lafayette, CO, United States) or Smad2/3 (Dharmacon, Lafayette, CO, United States) using LipoRNAiMax according to the manufacturer's protocol. The cells were maintained for 72 h and then subjected to protein extraction. The antisense and sense oligo template sequences for these siRNAs were: Smad2 siRNA antisense: AAGAGGAGTGCGCTTATATTACCTGTCTC, sense: AATAATATAAGCGCACTCCTCCCTGTCTC; Smad3 siRNA antisense: AATATCCAGAAACCCACCCCTGTCTC, sense: AAGGGTGGGGTT TCTGGAATACCTGTCTC.

Wound healing assay

IEC-6 cells were seeded into 6-well plates at a density of 6×10^5 cells per well, and were cultured in fresh culture media to full confluence. After that, we created a wound using a plastic scraper. After being washed with PBS, the medium was replaced with 10 ng/mL TGF- β ^[14] and TFA, and incubated at 37 °C for 48 h. The cell migration images were photographed at 0 and 48 h following scraping. Three to four different fields were visualized and photographed under a microscope (Nikon, Tokyo,

Japan).

Trans-well invasion assay

The cell invasion assay was performed using Trans-well chambers (8 μ m pore-size, Corning, United States). Matrigel was purchased from BD Biosciences and stored at -20°C . After thawing at 4°C overnight, the matrigel was diluted in serum-free medium, and 30 μ L of the diluted matrigel were evenly inoculated into the upper chamber to form a gel at 37°C . Cells (1×10^5) suspended in 300 μ L of serum-free medium were seeded into the upper compartments and treated with 10 ng/mL TGF- β 1 and TFA for 48 h, and the lower compartments were filled with 600 μ L of medium with 20% FBS. After incubation, the non-invasive cells were removed from the upper surface of the membrane by scrubbing. The cells that invaded to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained in 10% crystal violet. Cells were counted under a microscope (Olympus, Tokyo, Japan).

Real-time PCR analysis (qRT-PCR)

IEC-6 cells were incubated in a 6-well plate with a density of 6×10^5 cell per well. When cells reached 60% confluence, 10 ng/mL TGF- β 1 and TFA (0, 5, 10 and 15 μ g/mL) were added to the plates and incubated for 48 h. Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions and reversed transcribed into cDNA using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, United States). The qRT-PCR reactions were performed using the Fast Start Universal SYBR Green Master (Rox) (Roche Applied Science) on a 7500 Real-time system (Applied Biosystems, United States) according to the manufacturer's protocols. Primer sequences used were designed as follows: E-cadherin forward, 5'-GAGGTCTACATTCCTGGTG-3', E-cadherin reverse, 5'-TCTGTAGACATTTGAATCGG-3', ZO-1 forward, 5'-CCATCTTGGACCGATTGCTG-3', ZO-1 reverse, TAATGCCCCGAGCTCCGATG-3', Vimentin forward, 5'-CCGACACTCCT ACAAGATTTAGA-3', Vimentin reverse, 5'-CAAAGATTTATTGAAGCAGAACC-3', N-cadherin forward, 5'-ATCCTACTGGACGGTTTCG-3', N-cadherin reverse, 5'-TTGGCTAATGGCACTTGA-3', GAPDH forward, 5'-GGACCTGACCTGCCGTCTAG-3', GAPDH reverse, 5'-GTAGCCCAGGATG CCCTTGA-3'. GAPDH was used as an internal standard.

Western blotting assay

IEC-6 cells were plated into 6-well plates at a density of 3×10^5 cells/mL. When cells reached 60% confluence, 10 ng/mL TGF- β 1 and TFA (0, 5, 10 and 15 μ g/mL) were added to the plates and incubated for 48 h. Each concentration group was performed in triplicate. Protein lysates were prepared using RIPA lysis buffer. Lysates were then subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with non-fat milk, blots

were incubated overnight at 4°C with the indicated antibodies. After incubation for 2 h, membranes were washed and incubated for 2 h at room temperature with corresponding secondary antibodies. For protein detection, membranes were developed with SuperSignal west femto maximum sensitivity substrate (Pierce, Rockford, IL, United States) and the Gel-Pro Analyzer 6.0 software was applied for image analysis. E-cadherin (1:500), ZO-1 (1:500), Vimentin (1:500), N-cadherin (1:500), p-ERK (1:500), ERK (1:500), p-p38 (1:500), p38 (1:500), p-JNK (1:500), JNK (1:500) and GAPDH (1:1000) antibodies were purchased from Sigma-Aldrich, and anti-mouse secondary antibodies were obtained from Proteintech (Chicago, IL, United States).

Immunofluorescence assay

IEC-6 cells were plated into 6-well plates at a density of 3×10^5 cells/mL. When cells reached 60% confluence, 10 ng/mL TGF- β 1 and TFA (0, 5, 10 and 15 μ g/mL) were added to the plates and incubated for 48 h. After that, IEC-6 cells were fixed with 4% paraformaldehyde. After blocking cells with 3% BSA for 2 h at room temperature, the cells were incubated with the anti-E-cadherin (1:50) antibody or anti-Vimentin (1:50) antibody at room temperature for 2 h. After washing three times with PBS, the second antibody conjugated with FITC was incubated on these cells for 1 h at room temperature. The nuclei were counterstained with DAPI for 5 min. Cells were imaged using a Nikon Eclipse TE2000-U fluorescence microscope.

Statistical analysis

Graph Pad Prism 5.0 statistical software was utilized to analyze the above experimental data. Measurement data were represented as $\bar{X} \pm \text{SD}$ ($n = 3$); One-way analysis of variance was applied to compare differences between multiple groups. When only two groups were compared, Student's *t*-test was conducted. A value of $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Effect of TFA on IEC-6 cell viability

In order to observe the effect of TFA on the EMT progress of IEC-6 cells mediated by TGF- β 1, a CCK-8 assay was performed to assess the effect of TFA on the viability of IEC-6 cells and to select the optimal concentrations of TFA for our further studies. IEC-6 cells were treated with increasing concentrations of TFA ranging from 0.1 to 50 μ g/mL at different time points (24, 48 and 72 h). From the results of Figure 1, we found that TFA had the positive activities on viability of IEC-6 cells with the concentrations of 5, 10 and 15 μ g/mL compared to the control group (TFA 0 μ g/mL). Further, the TFA-treated group at 10 μ g/mL displayed the maximum proliferative rate at 24, 48 and 72 h. Therefore, 5, 10 and 15 μ g/mL TFA were chosen to be the optimal concentrations for our further studies.

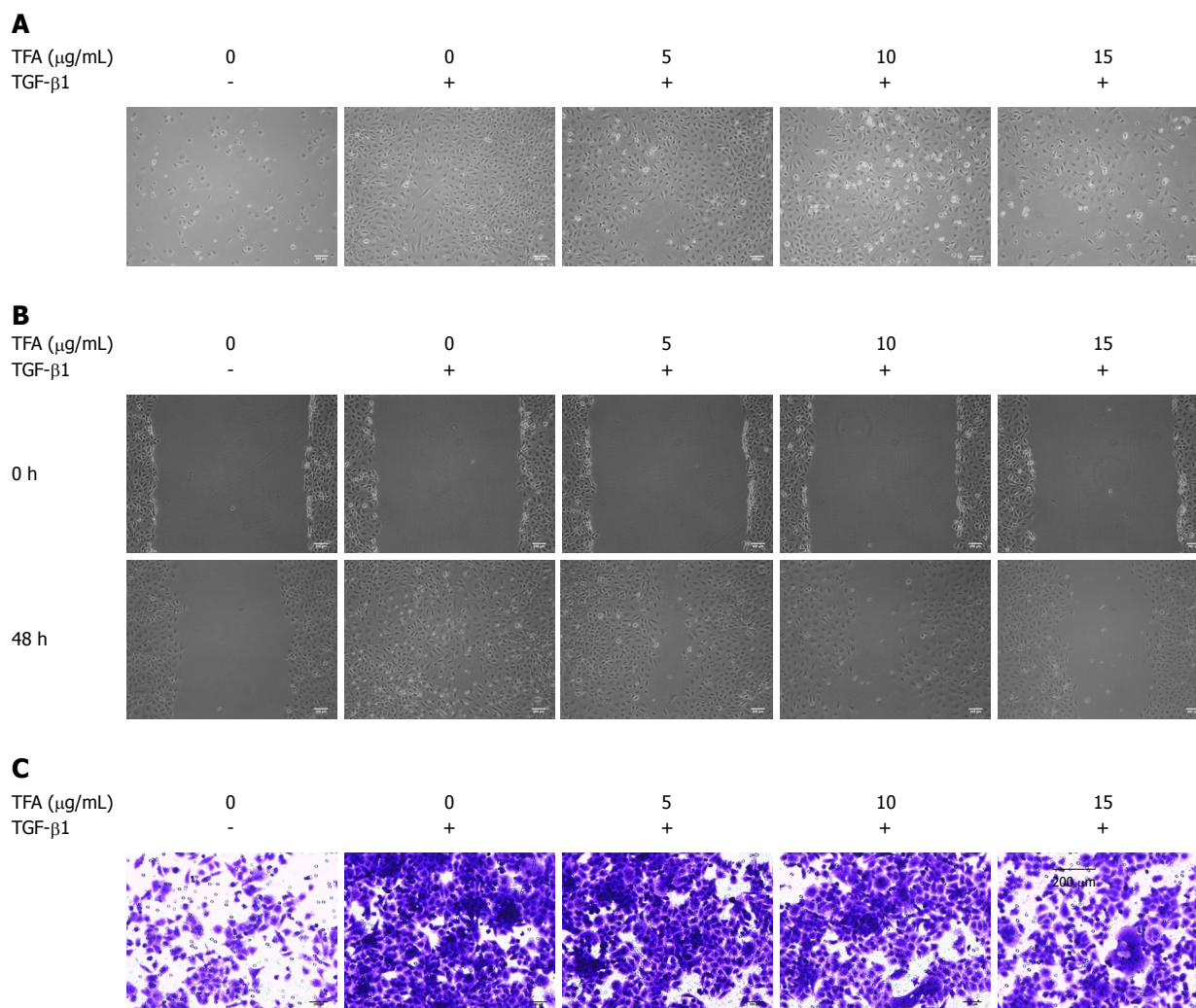


Figure 2 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1 induced migration and invasion of IEC-6 cells. A: The inhibitory effect of TFA (5, 10 and 15 μg/mL) on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B: The representative image of the wound healing assay; C: The representative image of transwell assay. The tendency is concomitant with the wound healing assay. TGF-β1 induced the migration and invasion of IEC-6 cells, whereas TFA suppressed this phenomenon. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

TFA inhibited TGF-β1 induced migration and invasion of IEC-6 cells

The EMT process is characterized by alteration of cell morphology, migration and invasion capacity, as well as epithelial and mesenchymal markers expression. In the morphology assay, 10 ng/mL TGF-β1 in the medium for 48 h led to significant morphological changes from cuboidal epithelial cells to fibroblast-like spindle-shaped cells when compared with the control group. Interestingly, TFA effectively suppressed TGF-β1-induced morphological changes of IEC-6 cells in a dose dependent manner (Figure 2A). Moreover, for wound healing assays, 10 ng/mL TGF-β1 could promote the migration of IEC-6 cells compared with the control group, while TFA obviously reduced the TGF-β1-induced migration of IEC-6 cells in a dose dependent manner (Figure 2B). In addition, we examined the effects of TFA on TGF-β1-induced invasion, and we found that 10 ng/mL TGF-β1 in the medium for 48 h induced IEC-6 cells across the membrane, and addition of 5, 10 and 15 μg/mL TFA

decreased the number of IEC-6 cells that passed through the Matrigel (Figure 2C). The results indicated that TFA could significantly suppress the morphological change, migration and invasion of IEC-6 cells induced by TGF-β1.

TFA inhibited TGF-β1-induced EMT

To examine the alterations of EMT markers, as shown in Figure 3A, the expression of epithelial marker E-cadherin was decreased along with the increasing expression of the mesenchymal marker Vimentin after treatment with 10 ng/mL TGF-β1. However, TFA effectively inhibited this induction, especially at higher concentrations. Besides, western blotting and qRT-PCR were carried out to examine the alterations of EMT markers, and we found that the protein levels of epithelial markers, including E-cadherin and ZO-1, were markedly increased by TFA treatment. On the contrary, TFA treatment obviously decreased the expression levels of mesenchymal proteins, including Vimentin and N-cadherin (Figure 3B). Likewise, similar changes were observed for mRNA

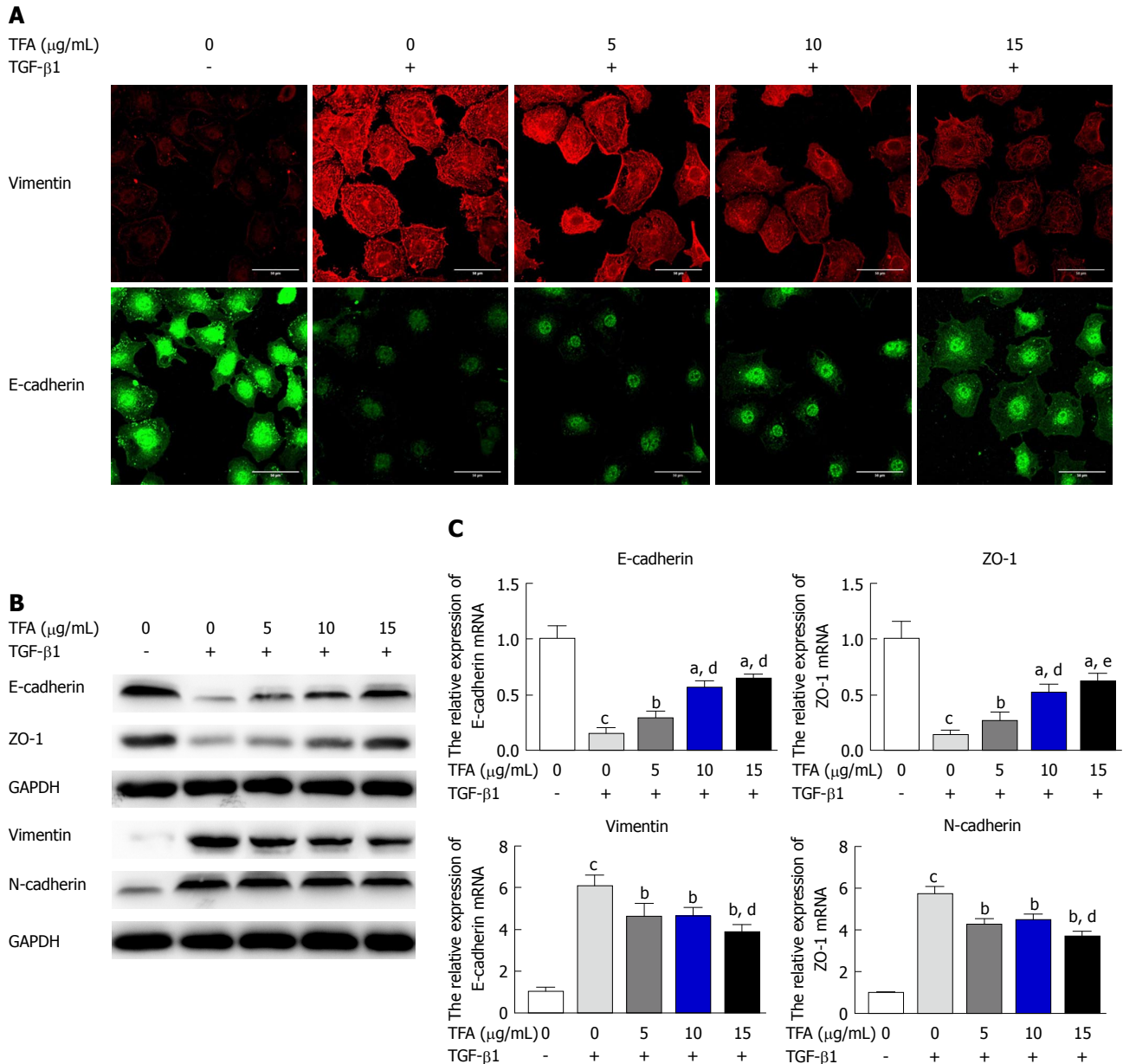


Figure 3 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor- β 1-induced epithelial-mesenchymal transition. A and B: The EMT-related label proteins were measured by immunocytochemistry (A) and western blotting (B) assays when IEC-6 cells were treated with TGF- β 1 (10 ng/mL) alone or in combination with TFA (5, 10 and 15 $\mu\text{g/mL}$) for 48 h; C: The inhibitory effect of TFA (5, 10 and 15 $\mu\text{g/mL}$) on TGF- β 1-induced EMT-related mRNAs was detected by qRT-PCR assay. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ compared with the non-TFA treated group, ^d $P < 0.05$, ^e $P < 0.01$ compared with the TGF- β 1 (10 ng/mL) treated group. EMT: Epithelial-mesenchymal transition; TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

expression of epithelial and mesenchymal markers in TFA-treated TGF- β 1-induced IEC-6 cells (Figure 3C). Together, these results demonstrated that TFA could attenuate TGF- β 1-induced EMT of IEC-6 cells.

TFA inhibited TGF- β 1-induced activation of the Smad signaling pathway

The activation of phosphorylated Smad2/3 plays an important role in the TGF- β 1-mediated EMT progress^[28]. The status of the Smad pathways was determined in TGF- β 1-treated IEC-6 cells following TFA treatment with the concentrations of 5, 10 and 15 $\mu\text{g/mL}$ for 48 h, and the results have shown that TFA remarkably inhibited

the level of phosphorylated Smad 2/3 in a dose dependent manner (Figure 4A). Next, we examined the role of Smad in TGF- β 1-mediated IEC-6 cells. In IEC-6 cells, western blotting analysis demonstrated that Smad2/3 expression was inhibited after transfection of si-Smad2/3 (Figure 4B). Moreover, our results revealed that si-Smad2/3 could diminish TGF- β 1-triggered EMT in IEC-6 cells and inhibit the TGF- β 1-elicited changes in the expression of EMT markers, as measured by western blotting and qRT-PCR assays (Figure 4B and C). Besides, si-Smad2/3 suppressed TGF- β 1-mediated mesenchymal-like morphological changes in IEC-6 cells as shown in Figure 5A. Wound healing and trans-well

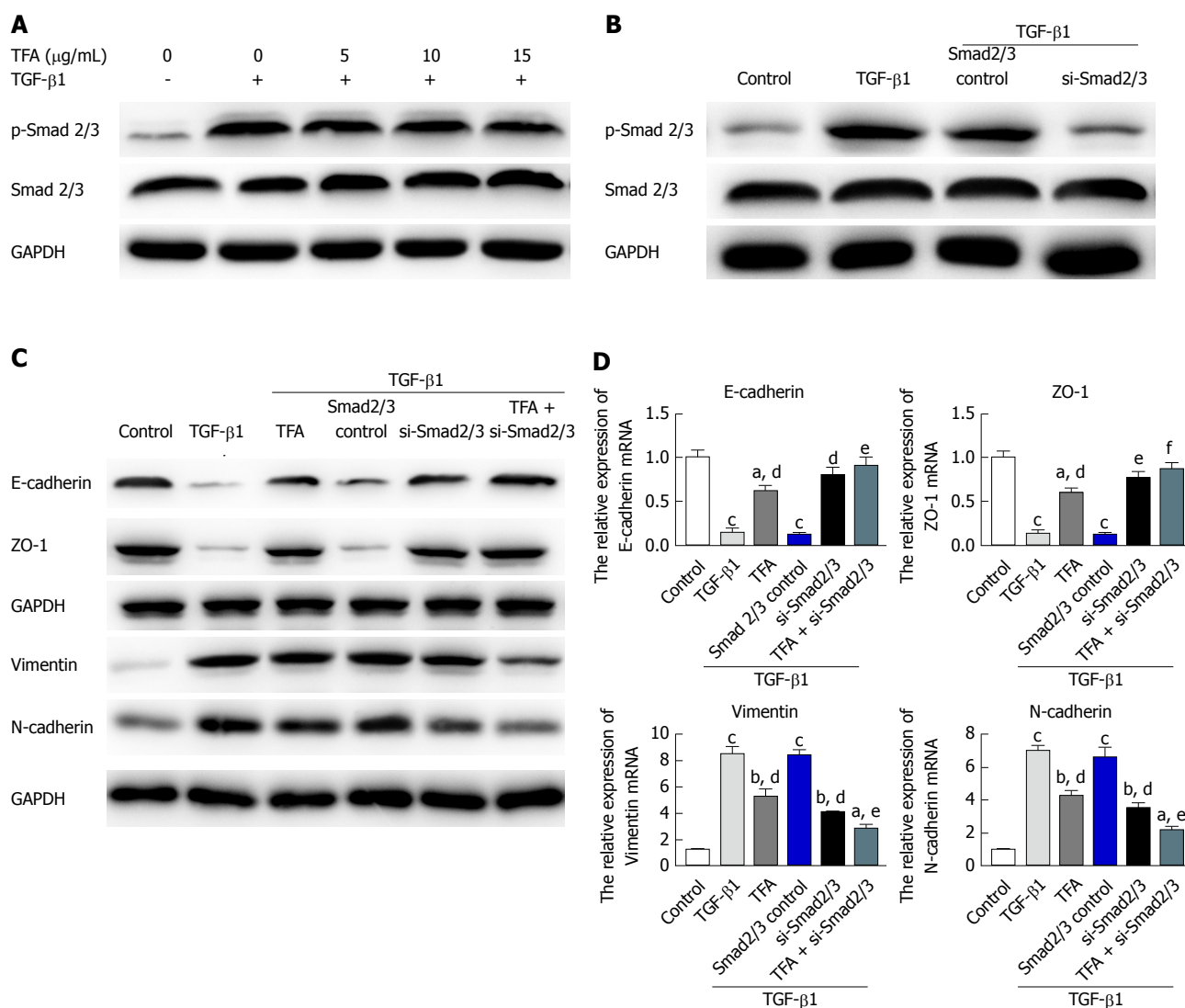


Figure 4 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1-induced activation of the Smad signaling pathway. A: The effects of TFA on TGF-β1-induced activation of Smad signaling were evaluated by western blotting; B: The expression of Smad signaling was examined by western blotting after transfection of si-Smad2/3; C and D: The effect of si-Smad or TFA combined with si-Smad2/3 on TGF-β1-induced activation of Smad signaling was evaluated by western blotting (C) and qRT-PCR (D) assays. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 compared with that of non-TFA treated group, ^d*P* < 0.01, ^e*P* < 0.001 compared with that of TGF-β1 (10 ng/mL) treated group. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

assays also showed that si-Smad2/3 suppressed the migration and invasion capacities of IEC-6 cells triggered by TGF-β1 (Figure 5B and C). Furthermore, 15 μg/mL TFA combined with si-Smad2/3 could further restrain TGF-β1-mediated EMT progress in IEC-6 cells. These results suggested that TFA might reverse EMT induced by TGF-β1 *via* Smad inactivation in IEC-6 cells.

TFA inhibited TGF-β1-induced activation of the non-Smad signaling pathway

In addition to the Smad2/3 signaling pathway, TGF-β has been reported to activate other signaling molecules, such as MAPKs, which include extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK)^[29]. To explore the involvement of non-Smad signaling in the anti TGF-β1-induced EMT activity of TFA, we tested whether TFA could inhibit the TGF-β1-induced

activation of p38, JNK and ERK1/2 by western blotting, and we found that TGF-β1 could significantly activate the expressions of p-p38, p-JNK and p-ERK1/2, while TFA at the concentrations of 5, 10 and 15 μg/mL obviously suppressed the TGF-β1-induced increase in p-p38, p-JNK and p-ERK1/2 levels in IEC-6 cells in a dose dependent manner (Figure 6A). Thus, in order to further explore the role of MAPKs in TGF-β1-treated IEC-6 cells, ERK1/2 inhibitor (PD98059, 5 μmol/L), p38 inhibitor (SB203580, 5 μmol/L) and JNK inhibitor (SP600125, 2 μmol/L) were used to treat IEC-6 cells mediated by TGF-β1. From the results in Figure 6A, we found that PD98059, SB203580 and SP600125 could significantly inhibit the activation of p-p38, p-JNK and p-ERK, respectively. Besides, PD98059, SB203580 and SP600125 could promote the changes in EMT markers by western blotting and qRT-PCR assays (Figure 6B and C). In addition, PD98059, SB203580 and SP600125 treatment inhibited TGF-β1-induced men-

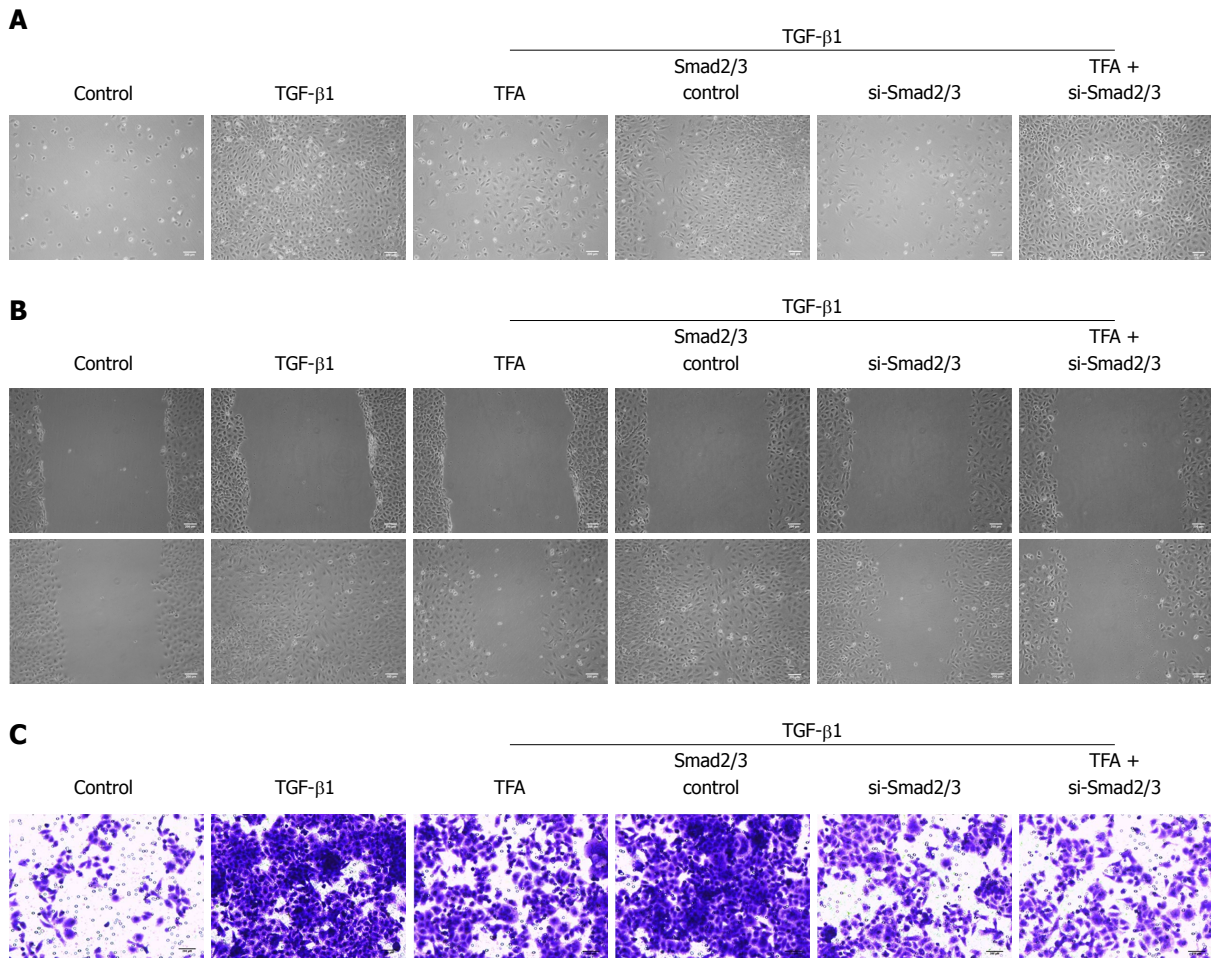


Figure 5 Total flavone of *Abelmoschus manihot* (TFA) combined with si-Smad2/3 had synergistic role in inhibiting transforming growth factor-β1-induced migration and invasion of IEC-6 cells. A: The inhibitory effect of si-Smad or TFA (15 μg/mL) combined with si-Smad2/3 on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B and C: The migration and invasion abilities of si-Smad or TFA (15 μg/mL) combined with si-Smad2/3 were examined by wound healing (B) and transwell (C) assays. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.α

senchymal-like morphological changes in IEC-6 cells, as shown in Figure 7A. Moreover, PD98059, SB203580 and SP600125 inhibited the migration and invasion of IEC-6 cells mediated by TGF-β1 *via* wound healing and transwell assays (Figure 7B and C). Importantly, co-treatment of TFA with PD98059, SB203580 or SP600125 had better inhibitory effects on TGF-β1-induced EMT in IEC-6 cells than either one of them. Taken together, these results indicated that TFA might exert its role in reversing TGF-β1-mediated EMT *via* non-Smad inactivation in IEC-6 cells.

DISCUSSION

CD intestinal fibrosis is characterized by activation of myofibroblasts, resulting in abnormal ECM deposition, which eventually leads to tissue stiffness and progressive intestinal dysfunction^[30]. In addition to interstitial cells such as fibroblasts and smooth muscle cells, the differentiation of epithelial cells through EMT is a major source of intestinal fibrotic cells^[18]. EMT refers to the transformation of epithelial cells with polar cells to the interstitial cells under a specific physiological and

pathological condition. The most important features of EMT are the loss of epithelial cell phenotype and the acquisition of interstitial properties, as reflected in the down-regulation of E-cadherin and ZO-1, resulting in loss of adhesion between cells or between cells and matrix, over-expression of N-cadherin and vimentin, leading to the acquisition of migration and invasion, and over-expression of EMT transcription factors^[31,32]. Moreover, EMT plays a key role in tissue formation, organ fibrosis and so on. Through EMT, epithelial cells lose cell polarity and epithelial phenotypes, such as connection to the basement membrane, while acquiring interstitial cell phenotypes with the functions of fibroblasts and myofibroblasts^[33-35]. Moreover, in the case of continuous activation of inflammatory response, the EMT process will also continuously exist and eventually cause organ fibrosis. Therefore, it will have a positive significance to search for a method for the treatment of CD intestinal fibrosis.

Our previous studies have found that TFA, a main component of the water extract of traditional Chinese medicine, could reduce the expression of TGF-β, α-SMA and MMP-2 and decrease collagen fibrils, which indi-

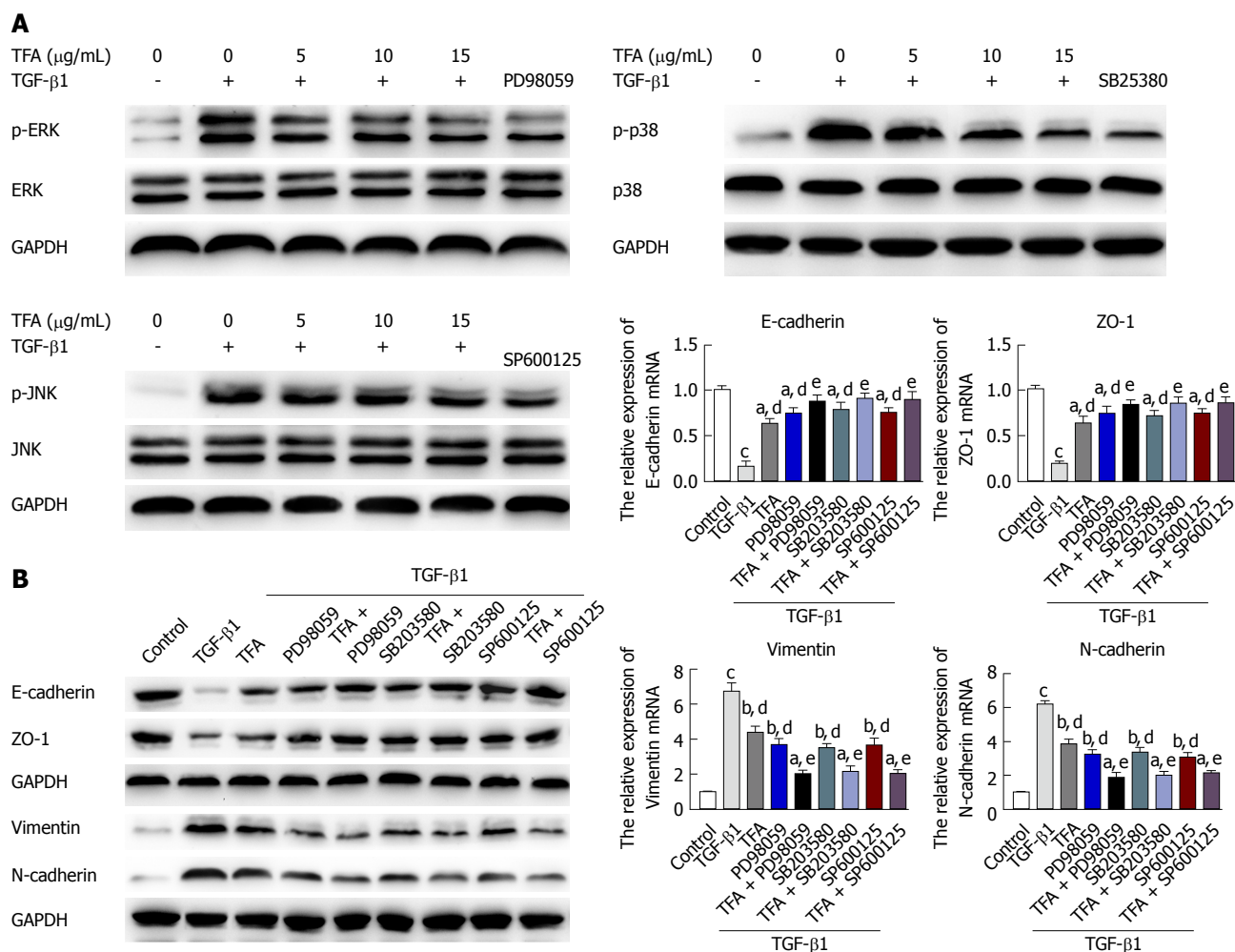


Figure 6 Total flavone of *Abelmoschus manihot* inhibited transforming growth factor-β1-induced activation of MAPK signaling pathway. A: The effect of TFA on TGF-β1-induced activation of MAPK signaling was evaluated by western blotting; B: The expression of MAPK signaling was examined by western blotting after transfection with MAPK inhibitors; B and C: The effects of MAPK inhibitors or TFA combined with MAPK inhibitors on TGF-β1-induced activation of Smad signaling were evaluated by western blotting (B) and qRT-PCR (C) assays. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 compared with that of non-TFA treated group, ^d*P* < 0.05, ^e*P* < 0.01 compared with that of TGF-β1 (10 ng/mL) treated group. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

cated that TFA may inhibit or reverse EMT during CD intestinal fibrosis. Firstly, we used TGF-β1 to induce EMT of IEC-6 cells and chose the optimal concentrations of TFA to disrupt TGF-β1 activity in IEC-6 cells. From the results, we knew that TGF-β1 could obviously promote the progression of EMT, such as changing morphology from cuboidal epithelial cells to fibroblast-like spindle-shaped cells, and enhancing the abilities of migration and invasion. Despite this, TFA treatment could restrain the changes caused by TGF-β1 in a dose dependent manner.

Effective inhibition of CD intestinal inflammation does not play a positive role in preventing intestinal fibrosis and inhibiting the pro-fibrosis signaling pathway, resulting in persistence of the process of tissue fibrosis^[36,37]. Therefore, it is necessary to further study how to block the fibrotic signaling pathway and promote ECM decomposition to inhibit or reverse the process of fibrosis. TGF-β is the strongest inducer, playing an important role in EMT^[38]. The Smad signaling pathway induced by TGF-β can promote the expression of ECM and inhibit the transcriptional activity of matrix degradation

genes, resulting in ECM deposition in interstitial cells and leading to fibrosis^[39]. Actually, in our experiments, we found that 10 ng/mL TGF-β1 could significantly inhibit the expression of p-Smad2/3, while TFA remarkably reversed the phenomenon in a dose dependent manner in TGF-β1-treated IEC-6 cells. Blocking TGF-β/Smad signaling has become a hot spot to inhibit or even reverse fibrosis. It has been reported that silencing Smad 2/3 could block Smad signaling and reduce collagen synthesis and proliferation of fibroblasts^[40,41]. We silenced the expression of p-Smad2/3 and explored the role of Smad2/3 in TGF-β1-treated IEC-6 cells. We found that silencing Smad2/3 could effectively improve the EMT progression of IEC-6 cells induced by TGF-β1 no matter morphology, migration, invasion or EMT markers. Interestingly, when TFA was combined with si-Smad2/3, it had better inhibitory activities on EMT progression than either one of them alone, which suggested that TFA might exert its effects in reversing TGF-β1-mediated EMT via Smad inactivation in IEC-6 cells.

Recent researches have shown that the MAPK path-

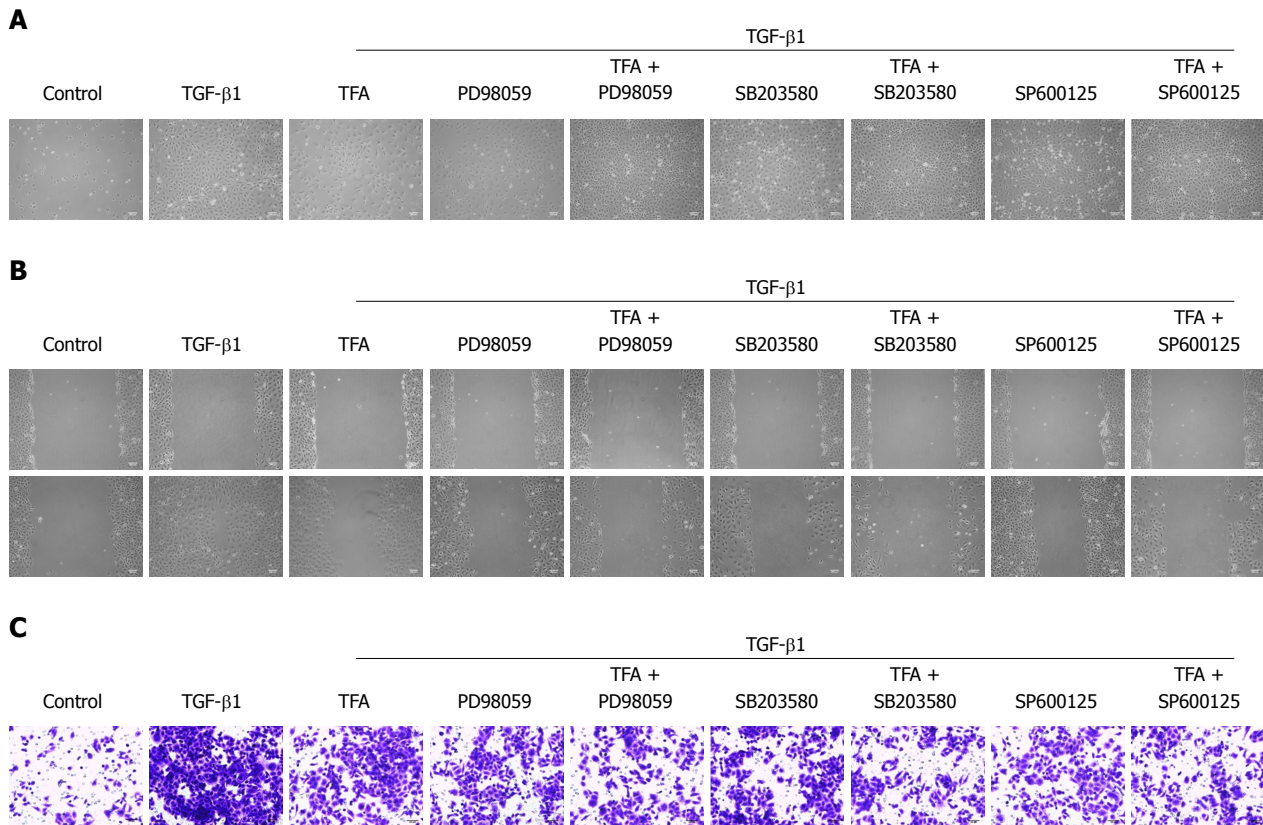


Figure 7 Total flavone of *Abelmoschus manihot* combined with MAPK inhibitors had synergistic role in inhibiting transforming growth factor-β1-induced migration and invasion of IEC-6 cells. A: The inhibitory effect of MAPK inhibitors or TFA (15 μg/mL) combined with MAPK inhibitors on TGF-β1-induced morphological changes at 48 h was observed by phase contrast microscopy; B and C: The migration and invasion abilities of MAPK inhibitors or TFA (15 μg/mL) combined with MAPK inhibitors were examined by wound healing (B) and transwell (C) assays. TFA: Total flavone of *Abelmoschus manihot*; TGF: Transforming growth factor.

way, including p38, JNK and ERK1/2, is closely related to the regulation of TGF-β signaling pathway and fibrosis^[42]. TGF-β can not only carry out signal transduction through TGF-β/Smad, but can also activate p38, JNK and ERK signaling pathways, which indicates that regulation of MAPK signaling pathways can affect the TGF-β/Smad signal transduction pathway and play a role in inhibiting fibrosis^[43,44]. In this experiment, we found that 10 ng/mL TGF-β1 could significantly inhibit the expression of p-p38, p-JNK and p-ERK1/2, while TFA obviously suppressed the levels in a dose dependent manner in TGF-β1-treated IEC-6 cells. In addition, we also investigated the role of MAPK in TGF-β1-mediated EMT of IEC-6 cells. We used an ERK1/2 inhibitor (PD98059), p38 inhibitor (SB203580) and JNK inhibitor (SP600125) to inhibit the expression of p-p38, p-JNK and p-ERK1/2 in IEC-6 cells, respectively. We found that PD98059, SB203580 and SP600125 treatment could have positive inhibitory effects on EMT progress, similar to the si-Smad. Moreover, co-treatment of TFA combined with PD98059, SB203580 or SP600125 had better positive activities on inhibiting EMT progress in TGF-β1-mediated IEC-6 cells than either one of them alone, indicating that TFA might exert its role in reversing EMT induced by TGF-β1 *via* MAPK inactivation in IEC-6 cells.

Stated thus, regulating TGF-β and its downstream

Smad and MAPK signaling pathways, mediating the EMT process and restoring the biological function of abnormally activated intestinal fibroblasts, may be an important way to seek drug therapy for CD intestinal fibrosis. TFA is able to inhibit TGF-β1-induced morphological change, migration, invasion of IEC-6 cells. TFA promoted the induction of EMT partly by inhibiting TGF-β1-activated Smad signaling pathway and non-Smad signaling pathway. To conclude, TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

ARTICLE HIGHLIGHTS

Research background

Epithelial-mesenchymal transition (EMT) is a crucial process in Crohn's disease (CD) intestinal fibrosis. Total flavone of *Abelmoschus manihot* (TFA) has been found as an effective component to reduce CD intestinal fibrosis *in vivo*. However, the role and mechanism of TFA on EMT progress of CD intestinal fibrosis have not been understood yet.

Research motivation

EMT is a crucial process in CD intestinal fibrosis. TFA has been found as an effective component to reduce CD intestinal fibrosis *in vivo*. However, the role and mechanism of TFA on EMT progress of CD intestinal fibrosis have not been understood yet. In the present study, we performed CCK-8, morphology, wound

healing, transwell, qRT-PCR, western blotting and immunofluorescence assays to explore the role and the underlying mechanisms of TFA on CD intestinal fibrosis, and the results indicated that TFA was expected to advance as a new therapy to treat CD intestinal fibrosis.

Research objectives

To explore the role and mechanism of TFA on EMT progress of CD intestinal fibrosis.

Research methods

First, a CCK-8 assay was performed to assess the effect of TFA on the viability of IEC-6 cells and to select the optimal concentrations of TFA for our further studies. Then cell morphology, wound healing and transwell assays were performed to examine the effect of TFA on morphology, migration and invasion of IEC-6 cells treated with transforming growth factor- β 1 (TGF- β 1). In addition, immunofluorescence, qRT-PCR and western blotting assays were carried out to detect the impact of TFA on EMT progress. Moreover, western blotting assay was performed to evaluate the function of TFA on the Smad and MAPK signaling pathways. Further, the role of co-treatment of TFA and si-Smad or MAPK inhibitors was examined by qRT-PCR, western blotting, morphology, wound healing and transwell assays.

Research results

In this study, TFA promoted TGF- β 1-induced IEC-6 cell morphological change, migration and invasion, and increased the expression of epithelial markers and reduced the levels of mesenchymal markers, along with the inactivation of Smad and MAPK signaling pathways. Moreover, we revealed that si-Smad and MAPK inhibitors effectively attenuated TGF- β 1-induced EMT in IEC-6 cells. Importantly, co-treatment of TFA and si-Smad or MAPK inhibitors had better inhibitory effects on TGF- β 1-induced EMT in IEC-6 cells than either one of them alone.

Research conclusions

These findings could provide new insight into the molecular mechanisms of TFA on TGF- β 1-induced EMT in IEC-6 cells, and TFA is expected to advance as a new therapy to treat CD intestinal fibrosis.

Research perspectives

TFA promoted the induction of EMT partly by inhibiting TGF- β 1-activated Smad signaling pathway and non-Smad signaling pathway. TFA is expected to advance as a new therapy to treat CD intestinal fibrosis, and its continued advancement may open the door to a new class of treatment for CD intestinal fibrosis.

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