Inhibitory effect of tranilast on the myofibroblast differentiation of rat mesenchymal stem cells induced by transforming growth factor-β1 *in vitro*

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Abstract. Mesenchymal stem cell (MSC) transplantation is able to attenuate organ fibrosis; however, increasing evidence has indicated that MSCs may be an important cell source of myofibroblasts, which are vital pathogenic cells in fibrotic diseases. The results of the present study revealed that co-culturing with exogenous transforming growth factor (TGF)-β1 can induce the transdifferentiation of cultured rat MSCs into myofibroblasts *in vitro*. Treatment of the MSCs with tranilast [N-(3',4'-dimethoxycinnamoyl)-anthranilic acid] attenuated this fibrotic process. Immunocytochemical staining, western blot analysis, reverse transcription-quantitative polymerase chain reaction analysis and cell viability assays were performed in order to evaluate the molecular mechanisms underlying the effects of tranilast on TGF-β1-mediated MSC-to-myofibroblast activation. The results demonstrated that TGF-β1 upregulated the expression of α-smooth muscle actin (α-SMA) and collagen type I, and increased the phosphorylation of mothers against decapentaplegic homolog 3 (Smad3) and extracellular signal-regulated kinase 1/2 (ERK1/2) in the rat MSCs; by contrast, tranilast pretreatment downregulated their expression. Furthermore, the proliferation of MSCs induced by TGF-β1 was decreased by pretreatment with tranilast. In conclusion, the results of the present study demonstrated that tranilast treatment markedly suppressed the TGF-β1-induced differentiation of cultured rat MSCs into myofibroblasts, potentially by inhibiting the Smad3 and ERK1/2 signaling pathways. Therefore, this may be a potential antifibrotic therapeutic strategy, serving as an adjuvant treatment following transplantation of MSCs.

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Introduction

Tissue and organ fibrosis are the major causes of disability and various life threatening diseases (1). Fibrotic diseases are typically characterized by a progressive, harmful cycle of abnormally high myofibroblast accumulation (2). Myofibroblasts express high levels of α-smooth muscle actin (α-SMA) under profibrogenic factor stimulation; however, their precise origin remains to be fully elucidated (1). It was widely reported that myofibroblasts may be derived from resident fibroblasts, epithelial-mesenchymal transition and fibrocytes in fibroproliferative disorders (3,4). However, studies using genetic lineage tracing technology have reported that mesenchymal stem cells (MSCs) and MSC-like cells may be involved in myofibroblast generation during the development of fibrosis (2,5,6).

Stem cell biology is becoming increasingly important for use as novel therapies for several incurable chronic diseases, including fibrotic disease; it has been reported that MSC transplantation may attenuate organ fibrosis (7). However, a number of previous studies have raised safety concerns regarding MSC transplantation, as intrahepatic injection of human bone marrow (BM)-MSCs in mice has been shown to contribute to myofibroblast formation, demonstrating that MSCs can give rise to myofibroblasts *in vivo* following injury (8,9). The information to date is controversial as different studies have demonstrated either the therapeutic or contributing effects of MSCs to organ fibrosis (2). LeBleu *et al* (5) reported that up to 35% of renal myofibroblasts are derived from BM-MSCs via the circulation. Tang *et al* (10) revealed that BM-MSCs are one of the major cell sources of myofibroblasts in the fibrotic lung; ~40% of α-SMA-positive cells were derived from BM-MSCs in the mouse lung following injury. Carlson *et al* (11) reported that MSCs were a source of scar forming myofibroblasts in heart fibrosis. Notably, a previous report indicated that MSC-like cells have been implicated in the myofibroblast formation of multiple organs during fibrosis development, and up to 37-60% of the myofibroblasts in different organs are derived

from glioma-associated oncogene homolog 1^+ MSC-like cells in mice (6). Preventing myofibroblast differentiation has been demonstrated to attenuate organ fibrosis (6,12). These results indicated that MSCs and MSC-like cells may be the major cellular origins of organ fibrosis, and demonstrated that these cells may be a relevant therapeutic target to prevent solid organ dysfunction following injury. Understanding the process and mechanism underlying MSC-to-myofibroblast activation (fibrogenesis) is of particular importance for the application of MSC therapies (13).

Transforming growth factor (TGF)-β1 is regarded as a key regulator of myofibroblast differentiation in fibrosis (14). TGF-β1 produces signals through the TGF-β type I and type II receptors, and activates the mothers against decapentaplegic (Smad) signaling pathway via the phosphorylation of Smad2 and Smad3 (15). In addition, previous studies have revealed that the TGF-β/Smad signaling pathway is closely controlled by mitogen-activated protein kinase (MAPK) signaling cascades, particularly extracellular signal-regulated kinase 1/2 (ERK1/2) (16). Previous studies have also reported that TGF-β1 induced the transition of fibroblasts from different sources to myofibroblasts *in vivo*; however, its effect on the differentiation of MSCs into myofibroblasts remains to be elucidated (16,17).

Tranilast [N-(3',4'-dimethoxycinnamoyl)-anthranilic acid] was originally developed as an antiallergic drug, used for treating asthma, autoimmune diseases, and inhibiting angiogenesis (18). Tranilast is also considered to be an antiproliferative drug; previous studies have investigated its applications against proliferative diseases, particularly against hypertrophic scars and keloids (19,20). Tranilast can reduce TGF-induced matrix production in different types of cells, and also attenuates pathological fibrosis in the kidneys and heart (21,22). Furthermore, in cultures of rat cardiac fibroblasts, tranilast has been shown to attenuate TGF-β1-stimulated fibrogenesis (23).

Previous studies have demonstrated that MSCs from tracheal aspirates of premature infants and human adipose-derived stem cells have the potential to differentiate into phenotypic myofibroblasts under TGF-β1 stimulation (24,25). Therefore, it was hypothesized that cultured rat MSCs may have the potential to differentiate into myofibroblasts when induced by TGF-β1, and tranilast may inhibit this process. The aim of the present study was to evaluate the following hypotheses: i) TGF-β1 induces the differentiation of cultured rat MSCs into myofibroblasts; ii) pretreating TGF-β1-induced cultured rat MSCs with tranilast prevents myofibroblast differentiation and thus, decreases collagen production; iii) tranilast inhibits TGF-β1-mediated differentiation by inhibiting the Smad3 and ERK1/2 signaling pathways; and iv) tranilast inhibits cell proliferation in TGF-β1-treated MSCs.

Materials and methods

Cell isolation and culture. A total of 10 Sprague-Dawley (SD) rats (specific pathogen free, male, 3-4 weeks old, 60-100 g) were purchased from a professional breeder (Hunan SJA Laboratory Animal Co., Ltd., Changsha, China). Animals were housed at 22±2˚C and 50±10% humidity, in a quiet environment with a 12/12 h light/dark cycle, and were fed standard feed and water *ad libitum*. MSCs were obtained from the femurs and tibias of the SD rats using an established protocol (26). The MSCs were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) and grown in plastic dishes to confluence. The animal experiments were approved by the Animal Ethics Committee and the Medical Ethics Committee of Hunan Normal University (Changsha, China). The adherent cells were expanded as monolayer cultures in a 5% CO_2/95% O_2 air atmosphere at 37˚C and the medium was replaced every 3 days. These primary cells were referred to as passage 0. The confluent cells were split with 0.25% trypsin and 0.01% EDTA at the ratio 1:2 or 1:3 every passage. When the MSCs reached 50-60% confluence, they were cultured in DMEM containing 0.5% FBS at 37˚C for 24 h, and the majority of cells were in the quiescent state. Following this, the cells were treated with or without tranilast (0.1, 1, and 10 μM; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 30 min at 37˚C and then exposed to TGF-β1 (10 ng/ml PeproTech, Inc., Rocky Hilly, NJ, USA). MSCs cultured in 10% FBS + DMEM alone at 37˚C were used as a control.

For the differentiation assays, MSCs expanded for passages 2-5 were cultured in differentiation media for 2-4 weeks and images from at least six randomly chosen microscopic fields were captured using an UOP DSZ2000 microscope (magnification, x200; Chongqing UOP Photoelectric Technology Co., Ltd., Chongqing, China). For adipogenic differentiation, MSCs were cultured in DMEM with 10% FBS, 1 mM dexamethasone, 0.5 mM isobutyl-1-methylxanthine, 100 mM indomethacin and 10 μg/ml insulin. After 14 days, cells were fixed with 4% formaldehyde at room temperature for 10 min and stained with 0.3% Oil Red O (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 10 min to visualize lipid droplets. Osteogenic differentiation was induced by DMEM with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid. After 28 days, the mineralization of the extracellular matrix was determined by 1% Alizarin Red (pH 4.2; Sigma-Aldrich; Merck KGaA) staining at 37˚C for 30 min. Chondrogenic differentiation was induced in pellet culture for 14-21 days and performed using SD rat MSC chondrogenic differentiation medium (Cyagen Biosciences, Santa Clara, CA, USA) according to the manufacturer's instructions, and chondrogenesis was evaluated by 1% Alcian blue staining for 30 min at room temperature.

Flow cytometric analysis of MSC surface markers. Cells were harvested using 0.25% trypsin solution, and following two washes in PBS, cells were single-stained with fluorescent-labeled antibodies against cluster of differentiation (CD)29 (cat no. 561796; 1:20), CD90 (cat no. 561973; 1:20), CD-11b/c (cat no. 554862; 1:20) and CD45 (cat no. 554878; 1:20; all BD Biosciences, San Jose, CA, USA) for 20 min at room temperature in the dark. Subsequently, cells were resuspended in 300 μl PBS and immediately analyzed using a flow cytometer (FC500; Beckman Coulter, Inc., Brea, CA, USA). Data analysis was performed using FACSDiva version 6.1.3 (BD Biosciences).

Immunocytochemical assay. The quiescent cells grown on a glass slide were divided into five groups: i) Control (culture in 10% FBS + DMEM); ii) TGF-β1 (10 ng/ml); iii) TGF-β1 + tranilast (0.1 μM); iv) TGF-β1 + tranilast (1 μM); and v) TGF-β1 + tranilast (10 μM). The MSCs were first either treated with or without tranilast for 30 min and then exposed to TGF-β1 for 24 h at 37˚C, with cells grown in 10% FBS + DMEM alone as a control. The cells were fixed with 4% paraformaldehyde for 15 min and permeabilized using 0.5% Triton X-100 for 5 min. The cells were then treated with the 2-step plus Poly-Horseradish Peroxidase Anti-Mouse/Rabbit Immunoglobulin G Detection System (OriGene Technologies, Inc., Beijing, China). Endogenous peroxide activity was quenched with 3% H_2O_2 for 10 min, and cells were washed with PBS. The cells were then incubated with a monoclonal antibody against α-SMA (1:500, cat no. ab124964; Abcam, Cambridge, UK) for 12 h at 4˚C. Following the addition of the 2-step agent, the cells were incubated with 3,3'-diaminobenzidine at room temperature for 1 min. The cell nuclei were stained with hematoxylin. Brown color staining was considered to indicate a positive result. Images were captured using a fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. Whole cell lysates were prepared in cell lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) supplemented with protease inhibitors (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min. The protein concentration of lysates was measured using a BCA protein assay (Beyotime Institute of Biotechnology, Shanghai, China). Equal quantities (25 μg) of protein were subjected to 10% SDS PAGE under reducing conditions and electrotransferred onto a nitrocellulose membrane. The membranes were blocked with 5% fat-free dry milk in TBS for 1 h at room temperature and incubated overnight at 4˚C with the following primary antibodies: Rabbit monoclonal antibody against α-SMA (1:1,000), mouse monoclonal antibody against collagen type I (1:1,000; cat no. sc-59772; Santa Cruz Biotechnology, Inc.), and rabbit polyclonal antibodies against Smad3 (1:500; cat no. YT4336; ImmunoWay Biotechnology Company, Plano, TX, USA), phosphorylated (p-)Smad3 (1:500; cat no. YP0585; ImmunoWay Biotechnology Company), ERK1/2 (1:500; cat no. YT1623; ImmunoWay Biotechnology Company), p-ERK1/2 (1:500; cat no. YP0100; ImmunoWay Biotechnology Company) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500; cat no. YT5052; ImmunoWay Biotechnology Company). Following washing three times with TBS-T, membranes were incubated for 2 h at 37˚C with horseradish peroxidase-conjugated goat anti-rabbit (1:1,000; cat no. A0208, Beyotime Institute of Biotechnology) or goat anti-mouse (1:1,000; cat no. A0216; Beyotime Institute of Biotechnology) immunoglobulin G secondary antibodies. The membranes were washed with PBS and were exposed to ECL reagent (Beyotime Institute of Biotechnology). The protein bands were quantified using Quantity One software (version 4.6.3; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized against the loading control, GAPDH.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The quiescent cells were divided into four groups: i) Control (10% FBS + DMEM); ii) tranilast (10 μM); iii) TGF-β1 (10 ng/ml); and iv) TGF-β1 + tranilast (10 μM). The cells were treated with or without tranilast for 30 min, followed by the addition of TGF-β1 for 24 h. Total RNA was isolated from cell cultures using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized using RNA (2 μg) with the RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The specific primer sequences (Invitrogen; Thermo Fisher Scientific, Inc.) used were as follows: α-SMA forward, 5'-TCCAGAGTCCAG CACAATACCAG-3' and reverse, 5'-AATGACCCAGATTAT GTTTGAGACC-3'; collagen type I forward, 5'-TGTTCG TGGTTCTCAGGGTAG-3' and reverse, 5'-TTGTCGTAG CAGGGTTCTTTC-3'; and actin forward, 5'-CATCCTGCG TCTGGACCTGG-3' and reverse, 5'-TAATGTCACGCACGA TTTCC-3'. The qPCR was performed using SYBRGreen PCR mix (ABI; Thermo Fisher Scientific, Inc.). The qPCR conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 55-59˚C (depending on the specific annealing temperatures of the primer used) for 50 sec. The data were analyzed using the $2^{-\Delta\Delta Cq}$ method (27) and normalized to actin.

Measurement of collagen contents. The collagen contents of the MSC cultures were measured using a hydroxyproline assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The cells were cultured to a quiescent state on 6-well plates, and were then divided into five groups and treated as described above. The cells were then exposed to TGF-β1 (10 ng/ml) for 48 h, using cells grown in 10% FBS-DMEM as the control. The protein contents of the cell lysates were measured using Coomassie reagent (Thermo Fisher Scientific, Inc.). Data were recorded as μg/mg protein, assuming that collagen contained an average of 13.5% hydroxyproline.

Cell viability assay. Cell viability was determined using a Cell Counting kit-8 assay (CCK-8; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The MSCs from passage three ($5x10^3$/100 μl) were seeded on 96-well plates and cultured at 37˚C in 5% CO_2 for 24 h, following which the cells were made quiescent for 24 h. The cells were treated as described above and then incubated for 24 h. Each well received 10 μl CCK-8 solution for 3 h. The optical density was measured at 450 nm using a microplate reader, with six samples for each group.

Statistical analysis. All experimental data are presented as the mean ± standard deviation of at least three independent experiments and were analyzed with SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA). Statistical analysis was performed using one-way analysis of variance and Student-Newman-Keuls post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of rat MSCs. Tri-lineage differentiation assays were performed; the rat MSCs exhibited a fibroblast-like

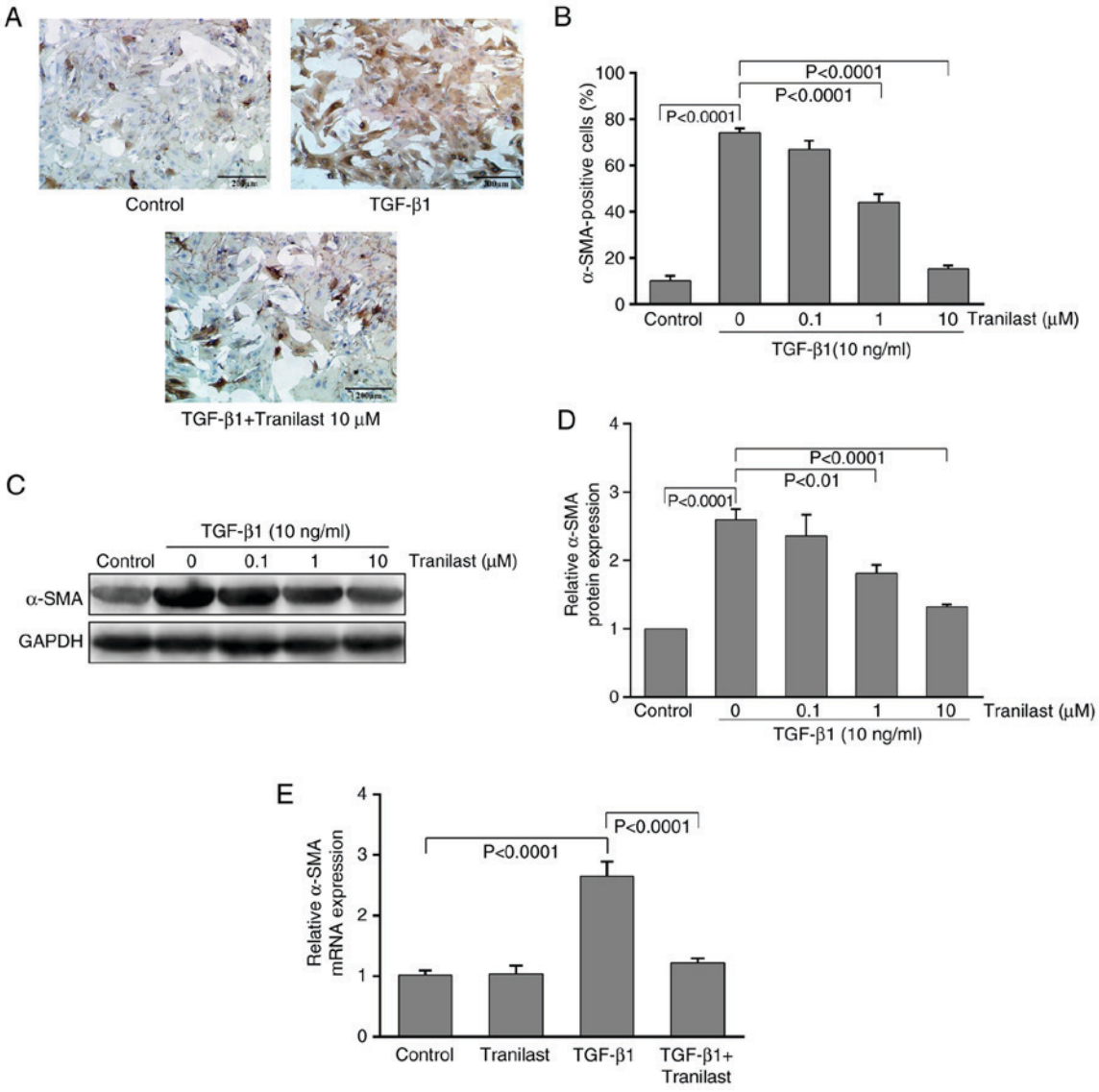

Figure 1. Tranilast inhibits the expression of α-SMA in TGF-β1-induced cultured rat MSCs. TGF-β1 upregulated the expression of α-SMA in MSCs to promote myofibroblast differentiation. By contrast, tranilast pretreatment prevented the expression of α-SMA. (A) Immunocytochemical staining assay of α-SMA (scale bars, 200 μm; magnification, x200). (B) Bar chart representing the quantitative data for α-SMA staining. (C) Western blot analysis and (D) statistical analysis of the protein expression of α-SMA. (E) mRNA expression of α-SMA was measured using reverse transcription-quantitative polymerase chain reaction analysis. n=3 experiments/group. MSCs, mesenchymal stem cells; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

morphology, and possessed osteogenic, adipogenic and chondrogenic differentiation capacity *in vitro*. In addition, flow cytometric analysis demonstrated that the majority of rat MSCs were positive for CD29 and CD90, but negative for CD45 and CD-11b/c. These assays confirmed the rat MSC differentiation potential as well as reliability of the isolation and culture method (data not shown).

Tranilast attenuates the TGF-β1-induced transformation of cultured rat MSCs to myofibroblasts. Immunochemical staining with α-SMA, a phenotypic marker of differentiated myofibroblasts, demonstrated that treatment with TGF-β1 increased the intensity of α-SMA staining in the MSCs; the control MSCs exhibited relatively few α-SMA-positive cells (10±2%). By contrast, >70% of the TGF-β1-treated MSCs were α-SMA-positive (Fig. 1A), indicating that the majority of MSCs differentiated into myofibroblasts following 24 h of treatment. Pretreatment with tranilast markedly inhibited these changes in the TGF-β1-induced MSCs; the number of α-SMA-positive cells was reduced in a dose-dependent manner, with significant inhibitory effects following treatment with 1 and 10 μM of tranilast (Fig. 1B).

The western blot analysis further confirmed the immunocytochemistry results, revealing that treatment of the cultured rat MSCs with TGF-β1 resulted in upregulation of the expression of α-SMA by 2.6-fold, compared with that in the control, and was significantly reduced by tranilast treatment (Fig. 1C and D). The mRNA levels of α-SMA were also evaluated; the RT-qPCR analysis revealed that the levels of α-SMA were significantly increased with TGF-β1 application, and this upregulation was significantly inhibited by pretreatment with tranilast (Fig. 1E). However, treatment with tranilast alone had no significant effect on the mRNA expression level of α-SMA.

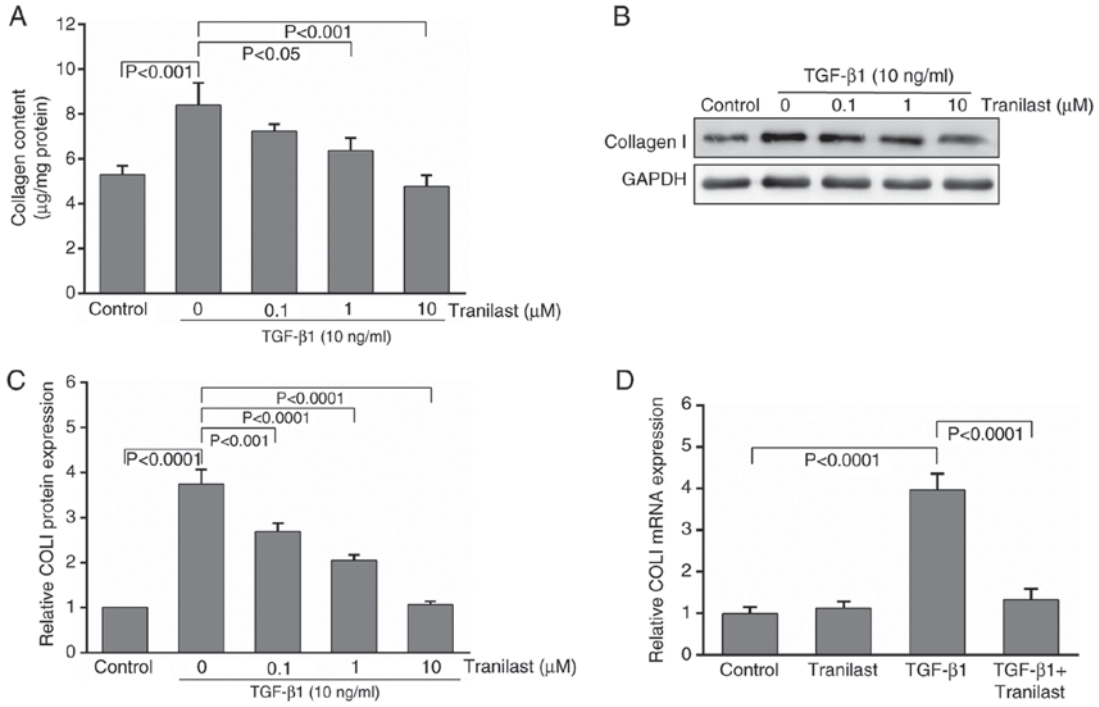

Figure 2. Tranilast attenuates collagen production in cultured rat mesenchymal stem cells induced by TGF-β1. Compared with control group, collagen content was significantly increased in the presence of TGF-β1, whereas tranilast pretreatment attenuated the expression of collagen. (A) Total collagen content measured using a hydroxyproline assay. (B) Western blot analysis and (C) statistical analysis of the protein expression of collagen type I. (D) mRNA expression of collagen type I was measured using reverse transcription-quantitative polymerase chain reaction analysis. n=3 experiments/group. TGF-β1, transforming growth factor β1; COL1, collagen type I; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Tranilast decreases collagen production in cultured rat MSCs induced by TGF-β1. To elucidate the effect of tranilast on extracellular matrix (ECM) synthesis in the TGF-β1-induced MSCs, the total collagen content was assessed using a hydroxyproline assay. Treatment with TGF-β1 for 48 h increased the production of collagen from control levels of 5.3±0.4 μg/mg protein to 8.4±1.1 μg/mg protein (P<0.01). Tranilast at 1 and 10 μM significantly decreased TGF-β1-stimulated collagen production; this effect was not evident at a lower concentration (0.1 μM, P>0.05; Fig. 2A).

The western blot analysis demonstrated that treatment of the cultured rat MSCs with TGF-β1 resulted in upregulation of the expression of collagen type I by 3.7-fold, compared with that in the control, and the expression was significantly decreased by pretreatment with tranilast (Fig. 2B and C).

Following induction by TGF-β1, the mRNA levels of collagen type I were significantly increased by 3.9-fold in the MSCs, compared with those in the control and tranilast-only treated groups. By contrast, this effect was markedly inhibited by pretreatment with 10 μM tranilast (Fig. 2D). Treatment with tranilast alone had no significant effect on the mRNA levels of collagen type I (P>0.05).

Tranilast inhibits the TGF-β1-induced phosphorylation of Smad3 and ERK1/2 in cultured rat MSCs. To determine whether TGF-β1 stimulates the transformation of cultured rat MSCs to myofibroblasts via the activation of Smad3 and ERK1/2, and whether tranilast has any effect on this process, the MSCs were pretreated with tranilast for 30 min and then incubated with TGF-β1 for 15-30 min. Western blot analysis was performed to detect pSmad3/pERK1/2 and total (t)-Smad3/tERK1/2. As presented in Fig. 3A-D, treatment with TGF-β1 significantly increased the phosphorylation of Smad3 and ERK1/2 compared with that in the control group; the phosphorylation was significantly inhibited by pretreatment with tranilast.

Tranilast inhibits the TGF-β1-induced proliferation of cultured rat MSCs. As presented in Fig. 4, TGF-β1 administration significantly increased the viability of the MSCs, as determined using the CCK-8 assay (P<0.01). Following pretreatment with tranilast, the TGF-β1-induced proliferation of the MSCs decreased in a dose-dependent manner and was significantly inhibited at 10 μM (P<0.01). The lower concentrations of tranilast (0.1 or 1 μM) did not have a significant effect on the cell viability (P>0.05).

Discussion

The present study revealed that, as expected, TGF-β1 induced cultured rat MSC differentiation into myofibroblasts and increased collagen production. Treating MSCs with tranilast resulted in the inhibition of TGF-β1-induced myofibroblast differentiation *in vitro*. It also decreased the expression of α-SMA and collagen type I in a dose-dependent manner, and suppressed the phosphorylation of Smad3 and ERK1/2 in the TGF-β1-induced MSCs. It was also observed that the administration of tranilast was able to inhibit the proliferation of TGF-β1-induced cultured rat MSCs.

Although a number of studies have revealed the beneficial effects of MSC therapy in treating several fibrotic diseases, certain cultured MSCs may have the potential to become

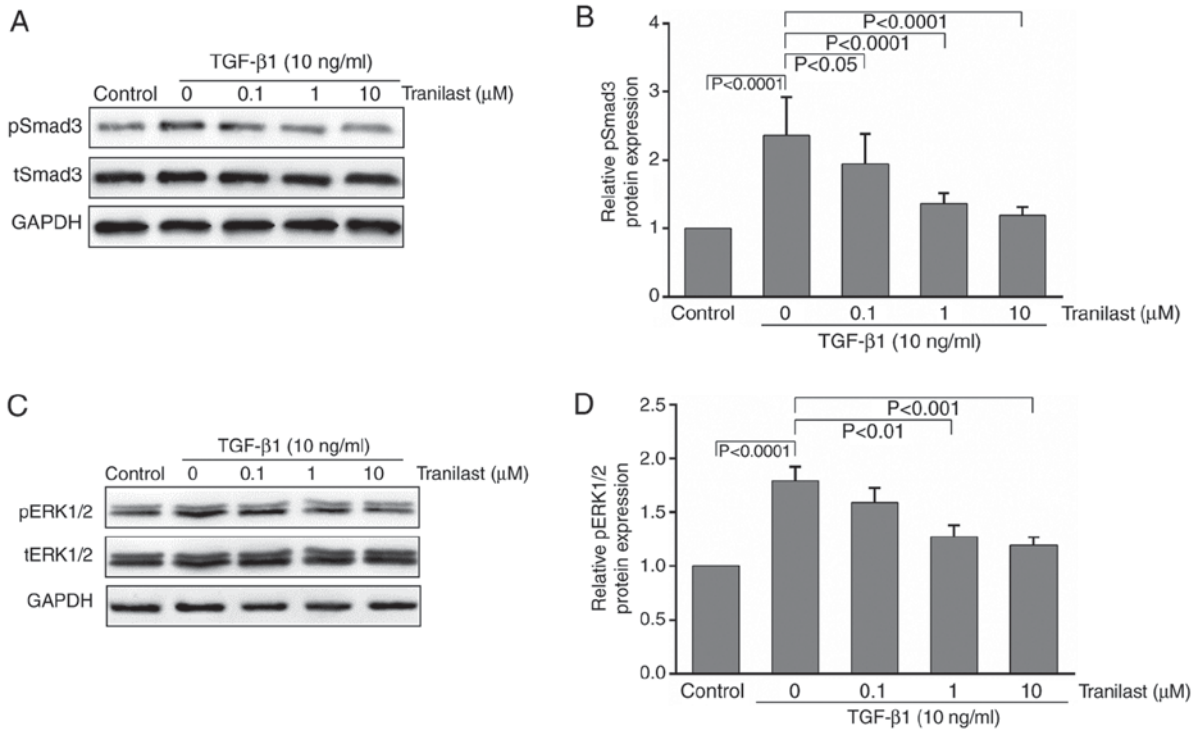

Figure 3. Tranilast inhibits the TGF-β1-induced phosphorylation of Smad3 and ERK1/2 in cultured rat mesenchymal stem cells. Total protein from whole cell lysates was extracted and western blot analysis was used to examine (A) pSmad3/t-Smad3 with (B) quantification; and (C) pERK1/2/t-ERK1/2 with (D) quantification. n=3 experiments/group. p, phosphorylated; t, total; ERK, extracellular signal-regulated kinase; Smad, mothers against decapentaplegic homolog; TGF-β1, transforming growth factor β1.

myofibroblasts that substantially contribute to organ fibrosis (2,5). Myofibroblasts are considered to be important pathogenic cells in all fibrotic diseases and TGF-β1 may be critical in the activation of fibrogenic myofibroblasts (28). Cultured BM-MSCs can give rise to myofibroblasts when transplanted into the murine liver (8,29). In addition, BM-MSCs may contribute to fibrosis by differentiating into tissue myofibroblasts in the lungs (30). Differentiated myofibroblasts are characterized by increased contractile capacity and an elevated production of ECM; the most frequently used molecular markers are α-SMA and collagen type I (3). Early reports indicated that α-SMA has a low baseline expression, which is characterized by disorganized intracellular α-SMA in cultured human cardiac fibroblasts and neonatal lung MSCs (16,24). The results of the present study showed that cultured rat MSCs became α-SMA-positive and contained well-organized α-SMA filaments when stimulated by TGF-β1. To the best of our knowledge, the results of the present study provide the first evidence that co-culturing with exogenous TGF-β1 can induce the transformation of cultured rat MSCs into myofibroblasts *in vitro*.

Previous studies have revealed that tranilast has therapeutic potential as an antifibrotic agent by inhibiting the proliferation or differentiation of fibroblasts and leading to the suppression of collagen synthesis (31,32). The results of the present study revealed that tranilast prevented the TGF-β1-induced myofibroblast differentiation of cultured rat MSCs, and inhibited the expression of α-SMA and collagen type I at the mRNA and protein levels. In addition, the mRNA expression levels of α-SMA and collagen type I remained stable following pretreatment with tranilast in the absence of exogenous TGF-β1. By contrast, these expression levels were significantly increased by TGF-β1, whereas pretreatment with tranilast markedly decreased this process. These results suggested that the action of tranilast is mainly associated with the TGF-β1 signaling pathway. The mechanism underlying the action of tranilast remains to be fully elucidated, however, its inhibitory effect on the activity of TGF-β has been demonstrated in a range of cells (18).

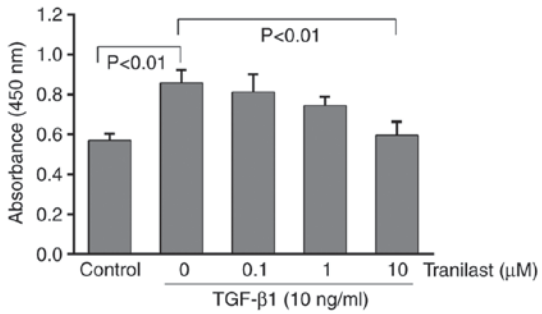

Figure 4. Tranilast decreases TGF-β1-induced proliferation in cultured rat MSCs. The MSCs were treated with TGF-β1 for 30 min in the presence or absence of tranilast. Cell proliferation was measured using a Cell Counting kit-8 assay. n=6/group. MSCs, mesenchymal stem cells; TGF-β1, transforming growth factor β1.

The major mode of tranilast efficacy appears to be via suppression of the expression and/or action of the TGF-β signaling pathway (18). It is hypothesized that TGF-β1 may be a key growth factor that mediates organ fibrosis and operates via TGF-β receptors and Smad2/3/4 transcription factors (33). Previous studies have revealed that Smad3, but not Smad2,

was essential in fibrosis and was observed to be required for myofibroblast generation (34). Smad3 has been implicated as a central mediator in the profibrotic effects of TGF-β1 (35). It has been demonstrated that the TGF-β1/Smad3 signaling pathway is activated in myofibroblast differentiation, and inhibiting its phosphorylation may attenuate myofibroblast differentiation and the fibrogenic response (36). In order to evaluate the possible signaling mechanism by which tranilast suppresses the generation of myofibroblasts, the effects of tranilast on the Smad3 and ERK1/2 signaling pathways in TGF-β1-stimulated MSCs were investigated. The results of the present study demonstrated that tranilast inhibited the phosphorylation of Smad3 in the TGF-β-stimulated MSCs, suggesting that it suppresses the transformation of MSCs to myofibroblasts and collagen synthesis partly via the Smad signaling pathway. In addition to the TGF-β/Smad signaling pathways, the ERK/MAPK signaling pathways, particularly ERK1/2, mediate the biological effects of TGF-β1 (16). In the present study, tranilast suppressed pERK1/2 in the TGF-β-stimulated MSCs, suggesting that its inhibitory effects on myofibroblast differentiation may be partially mediated through inhibition of the ERK/MAPK signaling pathway.

As demonstrated by the CCK-8 assays, the proliferation of cultured rat MSCs stimulated with TGF-β1 increased 1.5-fold, and tranilast inhibited the increase in a dose-dependent manner. These results indicated that one of the inhibitory mechanisms of collagen synthesis by tranilast in cultured rat MSCs may be involved in the inhibition of cell proliferation. The antiproliferative effect of tranilast appears to be involved in the inhibition of fibrosis. However, the detailed mechanisms through which tranilast attenuates the TGF-β1-induced proliferation and differentiation of cultured rat MSCs require further clarification. In addition, further investigations are required to assess whether tranilast has a cytotoxic effect.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time that in cultured rat MSCs that tranilast inhibited TGF-β1-induced myofibroblast differentiation and the Smad3/ERK1/2 signaling pathways. Tranilast also inhibited the proliferation of MSCs stimulated by TGF-β1. Tranilast has low adverse effects and is generally well tolerated by patients (18). Therefore, tranilast represents a potentially useful therapeutic agent in fibrotic diseases. Furthermore, tranilast has potential activity as an adjuvant treatment following the transplantation of MSCs for antifibrotic activity. Therefore, the present study demonstrated the role of tranilast in the differentiation of MSCs into myofibroblasts, and may provide further support for future clinical therapies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WT and BW conceived and designed the study. YZ, LT, JZ and LX performed the experiments. WT and BW wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Ethics Committee and the Medical Ethics Committee of Hunan Normal University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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