TGF-β1-Sp1 signaling pathway regulates the expression of collagen I in guinea pig scleral fibroblasts

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Abstract

Background

TGF-β1(Transforming growth factor beta 1)has been shown to be involved in scleral remodeling in myopia; however, it is not fully clear how this pathway regulates collagen I expression.This aim to study the effect of TGF-β1-Sp1(specificity protein 1)signaling pathway on the expression of collagen I in guinea pig scleral fibroblasts.

Methods

A primary cell culture was prepared from guinea pig scleral tissue. The obtained fibroblasts were divided into four groups, which were given the following treatments: no treatment, TGF-β1, TGF-β1 + Sp1 inhibitor, and TGF-β1 + TGF-β1 inhibitor. The mRNA and protein expression of collagen I and Sp1 was examined by immunohistochemistry, quantitative real-time PCR and western blotting.

Results

Compared with the control group, the protein and mRNA expression levels of Sp1 and collagen I were increased in the TGF-β1 group ($P < 0.05$), whereas they were decreased in the TGF-β1 + TGF-β1 inhibitor group ($P < 0.05$). The expression levels of these genes in the TGF-β1 + Sp1 inhibitor group were weaker than in the control, but stronger than those in the TGF-β1 + TGF-β1 inhibitor group ($P < 0.05$).

Conclusions

To a certain extent, TGF-β1 can mediate the expression of type I collagen through the Sp1 signaling pathway.

Introduction

In the last 50 years, the prevalence of myopia has significantly increased. According to statistics, nearly 28.3% of the world's population suffers from myopia, and the prevalence rate has an obvious upward trend. It is expected that by 2050, the total number of people suffering from myopia will account for more than half of the world's population [1]. Myopia affects the visual quality of patients. Increased severity of myopia is accompanied by complications such as retinal detachment, subretinal neovascularization, cataract, and glaucoma, which can result in serious decline of vision and irreversible damage. With the development of molecular biology, more and more bioactive molecules have been found to play an important role in scleral remodeling in myopia. Among them, transforming growth factor beta 1 (TGF-β1) has been confirmed to be involved in this process [2]. The transcription factor, specificity protein 1 (Sp1), is downstream of TGF-β1 and it targets and regulates the synthesis and degradation of collagen I protein

[3–4]. However, whether Sp1 is expressed in scleral fibroblasts and its relationship with the synthesis and degradation of TGF-β1 and collagen I protein in scleral fibroblasts have not been reported. In this study, guinea pig scleral fibroblasts were cultured *in vitro* by tissue cultivation. After treatment, the effect of the TGF-β1-Sp1 pathway on collagen I expression was examined by western blottingimmunohistochemistry and qRT-PCR. The purpose of this study was to understand the role of the TGF-β1-Sp1 signaling pathway in scleral remodeling *in vitro*, and to further confirm that this pathway regulates the expression of collagen I.

Materials And Methods

Subjects

Ten healthy newborn tricolor guinea pigs (provided by the animal experimental center of the First Affiliated Hospital of the University of Science and Technology of China) were randomly selected at about 7 days of age, weighing 100–140 g. All animal experiments and procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision research. The study was examined and approved by the Experimental Animal Ethics Committee of Anhui Medical University.

Main reagents: Dulbecco's Modified Eagle Medium (DMEM) culture medium, fetal bovine serum (GIBCO, Carlsbad, CA, USA); TGF-β1 (Sigma, St. Louis, MO, USA); TGF-β1 inhibitor (SB-431542), Sp1 inhibitor (Plicamycin; MedChemExpress, Monmouth Junction, NJ, USA); Sp1 antibody (1: 150; 21962-1-AP), collagen I antibody (1: 150; 14695-1-AP; a polyclonal antibody.Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China); immunohistochemical secondary antibody universal PV6000 (Beijing Zhongshan golden bridge Co., Ltd., Beijing, China);vimentin (Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China).

Guinea pig scleral fibroblasts culture

First, the eyes of guinea pigs were removed under strict aseptic conditions and operation. The eyes were rinsed with D-Hank solution at 4°C. The cornea was removed along the incision of the corneal margin and the lens, and the vitreous, retina, and pigment epithelium-choroid tissues were also removed. The posterior sclera tissue was separated and cut into 1 mm^3 tissue blocks and inoculated on the wall of a 25-cm culture bottle. The tissue blocks were placed with a spacing of 0.3–0.5 cm in a 37°C and 5% CO_2 incubator. After 2 h, DMEM containing 15% fetal bovine serum and double resistance solution was added. Contact the medium with the tissue mass. The medium was replaced every 3–5 days. When the cells reached 80% confluence and showed fusion, they were digested with 0.25% trypsin. Depending on the number of primary cells, 2–3 times of the culture medium volume was added. The cell suspension was placed in two or three bottles for subculture. Three generations of cells were divided into four groups. TGF-β1 was added to the first group at a final concentration of 10 ng/mL(According to pre-experimental results), the second group was treated with TGF-β1 + 100 ng/mL TGF-β1 inhibitor, the third group was

treated with TGF-β1 + 100 ng/mL Sp1 inhibitor, and the fourth group was a blank control group(the vehicle is added only).

Immunohistochemistry: Cells from the four groups were placed on slides,and continue to be cultured immediately for 2 d, washed with phosphate-buffered saline (PBS) three times for 2 min, and then fixed with glacial acetone for 10 min. Then, they were washed with PBS twice for 2 min, followed by incubation in formaldehyde containing 3% H_2O_2 for 30 min at room temperature to block endogenous enzymes. Trypsin (0.1%) was added for 5 min at room temperature, and then the primary antibody (1:50 dilution) was added at 4°C overnight. After rewarming the samples, the secondary antibody was added for 30 min. Next, the samples were washed with PBS three times for 2 min, and then DAB was used for color development and hematoxylin staining. The expression of Sp1, collagen I protein and vimentin was observed.

Quantitative PCR (qPCR): According to the instructions, total RNA was extracted from the cells and put into 0.2 mL PCR tubes (without RNase). Total RNA(2 μg) and 10 μmol/L Oligo (dT) were added to 0.2 mL of RNase-free water and heated at 65°C for 5 min, and then placed in an ice bath for 3 min.Reverse transcription was performed in a tube containing 4.0 μL of 5× reaction buffer, 2 μL of 10 mmol/L dNTP mix, 1 μL of RibolockTM RNase inhibitor, and 1 μL of Revert Aid TM M-MuLV reverse transcriptase at 42℃ for 60min and 70℃ for 5min. cDNA was extracted and stored at -80℃ until PCR analysis.PCR amplification was carried out with cDNA as the template. PCR reaction conditions: denaturation at 95°C for 5 min, and then 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, followed by a final extension at 72°C for 10 min and 4°C for 10 min. Electrophoresis was used to examine the PCR products, which were photographed by a gel imaging system. To ensure accuracy, all experiments were conducted at least three times.Table 1 demonstrates nucleotide sequences of primers used in the experiments and β-actin serves as an internal reference primer.

Table 1
Primer sequence,annealing temperature and predicted product size

Gene	Forward primer	Reverse primer	Tm(℃)	Size(bp)
β-actin	GCTCTATCCTGGCCTCACTC	GGGTGAGGGACTTCCTGTAA	55	400
Collagen I	ACAAGCGATTACACACCCAA	TTAGTTTCCTGCCTCTGCCT	55	239
Sp1	CTCAAAGGAACAGAGTGGCA	GAGCTGGGAGTCAAGGTAGC	55	486

Protein expression detection: Total protein was extracted by adding lysis buffer to the tissues. The protein concentration of the scleral tissues was measured by the BCA method, and the expression levels of Sp1 and collagen I in scleral tissues were measured by western blotting. Anti-Sp1 and anti-collagen I antibodies were used as the primary antibodies, and β-actin was used as the internal reference. Quantity

One software was used to conduct strip gray analysis: the relative expression level of Sp1 protein = the gray value of Sp1 protein band / β-actin band; the relative expression level of collagen I protein = the gray value of collagen I protein band / β-actin band.

Scoring systems: A double blind method was used to examine and score the slides with immunohistochemically stained cells. The positive products of Sp1 and collagen I were brown-yellow fine granules after immunohistochemical staining. Using a semi-quantitative standard, 10 high-power visual fields were randomly observed in each case and evaluated by calculating the number of positive cells and the staining depth of the recorded cells: ≤ 10% positive cells = 0 points, 11–25% positive cells = 1 point, 26–50% positive cells = 2 points, 51–75% positive cells = 3 points, ≥ 76% positive cells = 4 points. According to the staining intensity of positive cells: light yellow without positive staining or uniform background = 0 points, light brown yellow = 1 point, light brown = 2 points and brown = 3 points. Finally, the two integrals were multiplied: 0 points is (−), 1–4 points is (+), 5–8 points is moderately positive (++) and 9–12 points is (+++). The results were examined twice, and those with inconsistent scores were observed and confirmed again.

Statistical analysis

SPSS 17.0 software was used for statistical analysis. The data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to analyze the samples that had more than two groups, and Dunnett's multiple comparisons test was performed with the control groups. $P < 0.05$ was considered statistically significant. GraphPad Prism 5 was used to draw the statistical quantitative graph.

Results

Cell morphology

After 4–5 days of tissue block culture, some cells crawled out of the tissue block and began to adhere to the wall. The cells were transparent, spindle shaped, with full, flat, and long protrusions. After three generations, the cells were transparent, spindle shaped, uniform in size and arranged in irregular palisades (Fig. 1A).

Cell identification

High vimentin expression was observed in the cytoplasm of fibroblasts, as shown in Fig. 1B.

Sp1 and collagen I protein expression

Sp1 and collagen I protein expression was observed in each group (Fig. 2). The expression of Sp1 in the TGF-β1 group was stronger than that in the control group (Fig. 2, $P < 0.004$). However, the TGF-β1 inhibitor suppressed this expression to a level below that of the control group ($P = 0.0000$). In the TGF-β1 + Sp1

inhibitor group, Sp1 expression was stronger than that in the TGF-β1 + TGF-β1 inhibitor group, but weaker than that in the control group ($P < 0.001$). The color rendering of collagen I protein in the cytoplasm of the TGF-β1 group was stronger than that in the control group ($P < 0.05$). In contrast, in the TGF-β1 + TGF-β1 inhibitor group it was weaker than that in the control group ($P < 0.001$). Although collagen I protein expression in the TGF-β1 + Sp1 inhibitor group was stronger than that in the TGF-β1 + TGF-β1 inhibitor group, it was weaker than that in the control group ($P < 0.05$; Fig. 2).

Sp1 and collagen I mRNA expression

Sp1 and *collagen I* mRNA was expressed in all the guinea pig scleral fibroblast groups (Fig. 3). The *Sp1* mRNA expression in the TGF-β1 group was stronger than that in the control group ($P < 0.001$), whereas in the TGF-β1 + TGF-β1 inhibitor group it was weaker than that in the control group ($P < 0.05$). Although the *Sp1* mRNA expression in the TGF-β1 + Sp1 inhibitor group was stronger than that in the TGF-β1 + TGF-β1 inhibitor group, it was weaker than that in the control group ($P < 0.05$). Similarly, the *collagen I* mRNA expression in the TGF-β1 group was stronger than that in the control group ($P < 0.002$), whereas in the TGF-β1 + TGF-β1 inhibitor group it was weaker than that in the control group ($P < 0.05$).Finally, the *collagen I* mRNA expression in the TGF-β1 + Sp1 inhibitor group was stronger than that in the TGF-β1 + TGF-β1 inhibitor group, but weaker than that in the control group ($P < 0.05$).

Discussion

The mechanism of myopia may be that the retina is affected by the change in the visual environment, which produces signal transmission across the retina and choroid.The increase in the axial length is the main reason for changes in the progress of myopia [5]. Current refractive theories suggest that the sclera plays an important role in controlling the size of the human eye and the development of myopia. As myopia progresses, the sclera thins to increase its malleability [6]. At present, form deprivation is an effective method for establishing animal models of myopia and is widely used in myopia research [7–8]. The retina is affected by changes in the visual environment, producing signals that travel across the retina and choroid. These signals can stimulate scleral remodeling, affect the increased degradation and decreased synthesis of sclera collagen, cause progressive thinning of sclera, decrease the resistance to mechanical deformation caused by intraocular pressure, and accelerate the growth of the eyeball, resulting in the occurrence of myopia [9]. Collagenous fibers are the most abundant in the sclera, accounting for 90% of the dry weight of the sclera, of which collagen I protein fiber accounts for more than 75%; the expression of collagen I is significantly decreased in scleral remodeling in myopia [10–11]. TGF-β1 is a polypeptide growth factor, which exists in many kinds of cells and tissues. It has been suggested that it is an important transcription factor in scleral remodeling, and its expression in the sclera promotes the expression of collagen I protein [12]. The current study showed that the protein and mRNA expression of collagen I in the TGF-β1 group was enhanced compared with the blank control group, whereas in the TGF-β1 + TGF-β1 group it was weakened compared with the blank control group, suggesting that there was a positive correlation between TGF-β1 and collagen I in guinea pig scleral fibroblasts.

The transcription factor, Sp1, is expressed in all mammalian cells. It regulates many cell processes, including the cell cycle, proliferation, growth, metabolism, and apoptosis [13–14]. Martin *et al.* [15] have shown that TGF-β1 can bind to Sp1 and activate it. Several studies have shown that Sp1 can promote the expression of matrix metalloproteinase (MMP) 2 in cells. Furthermore, the increased activity of MMP2 and the decreased activity of MMP inhibitor can lead to the production of scleral extracellular matrix, thus promoting scleral remodeling[16–18]. In liver and heart fibrosis, the TGF-β1-Sp1 pathway facilitates the synthesis of collagen I [19]. This signaling pathway was found to play a role in the regulation of fibrosis [20]. It has also been shown that at a certain concentration TGF-β1 can stimulate Sp1 activation and promote collagen I expression [21]. In the current study, the mRNA and protein expression of Sp1 positively correlated with the mRNA and protein expression of collagen I, indicating that the changes in Sp1 and collagen I in guinea pig scleral fibroblasts tend to be consistent. Our results suggest that TGF-β1may regulates the expression synthesis and degradation of collagen I protein through Sp1 in a certain extent.

Conclusions

In this study, the changes in Sp1 and collagen I in guinea pig scleral fibroblasts correlated, possibly because of other TGF-β1 signaling pathways that regulate the synthesis of collagen I; however, the specific pathways remain to be revealed, and thus further research is needed.

In conclusion, we found that Sp1 is expressed in guinea pig scleral fibroblasts, and that TGF-β1 regulates the expression of Sp1 and collagen I. Furthermore, we speculate that Sp1 has a certain relationship with collagen I, and then may be able to regulate the scleral remodeling during myopia development. but this relationship requires further research.

Declarations

Availability of data and materials

The datasets generated and analysed during the study are not publicly available but are available from the corresponding author on reasonable request.

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Contributions

Chong-Hui Ying and Xia-Wei Liu contributed equally to this work. Xia-Wei Liu,Chong-Hui Ying and Chu Zhang: contributions to design and conduct the work. Zi-Cheng Zhu designed and directed the study. All authors discussed the results and commented on the manuscript.

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Ethics declarations

Ethics approval and consent to participate

All animal experiments and procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision research. The study was examined and approved by Experimental Animal Ethics Committee of Anhui Medical University,NO.LLSC20150115. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Consent for publication

Not applicable.

Disclosure Statement

The authors state that they have nothing to disclose.

Competing interests

The authors declare that they have no competing interests.

Additional information

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Figures

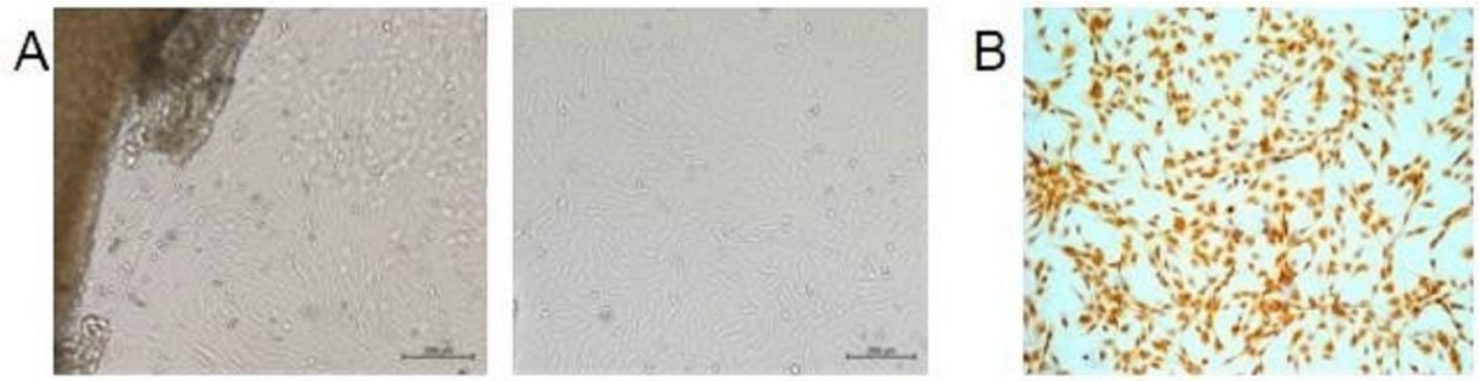

Figure 1

A: Primary guinea pig scleral fibroblasts in culture (left) and after three generations (right). B: Immunofluorescence was used to detect vimentin positivity and tan staining was observed in the cytoplasm. Scale bars: 200 μm.

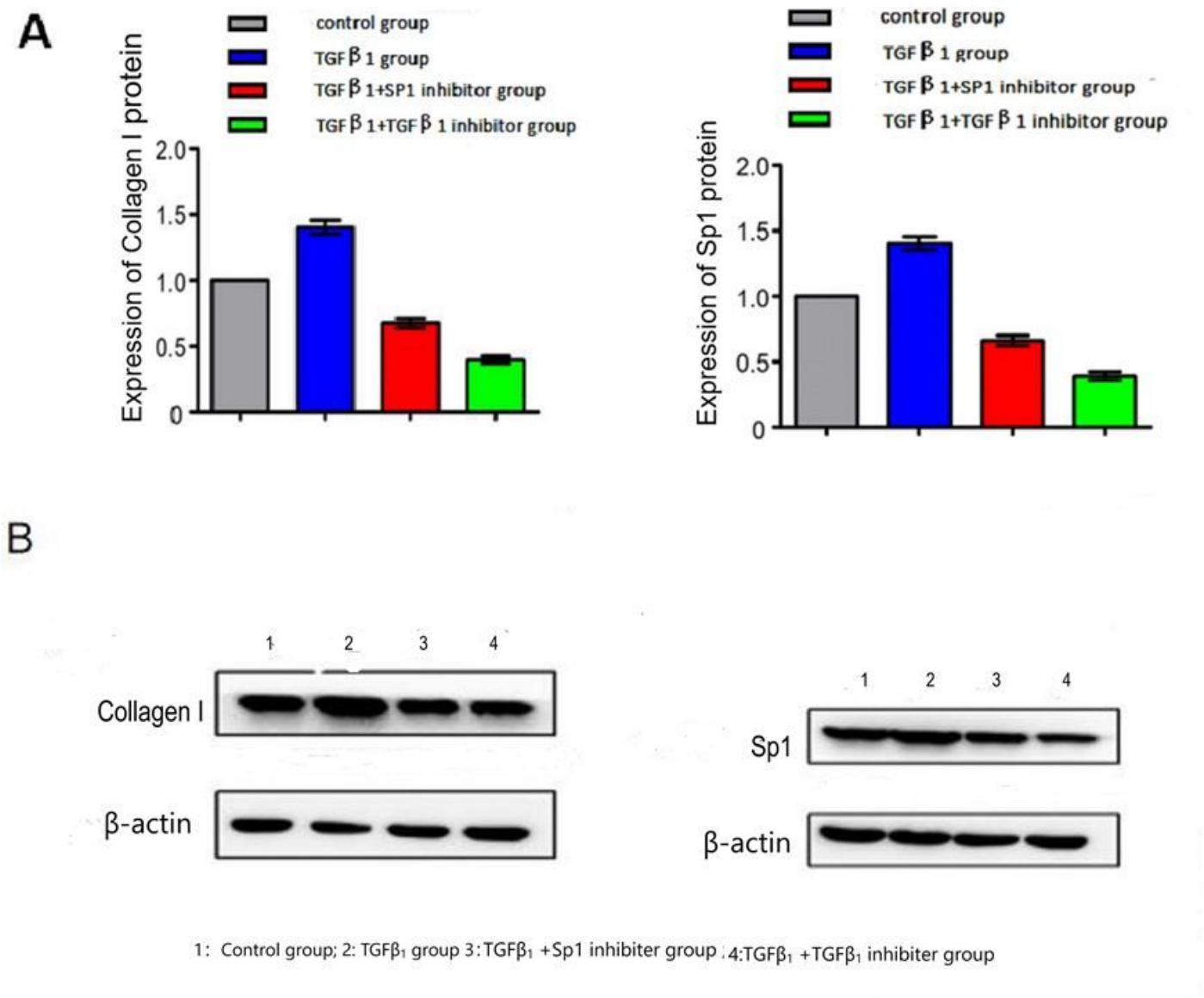

Figure 2

Sp1 and collagen I protein expression was increased in the TGFb1 group($p<0.005$,compared with the control group).Sp1 inhibitor decreased these expression($p<0.001$,compared with the control group).

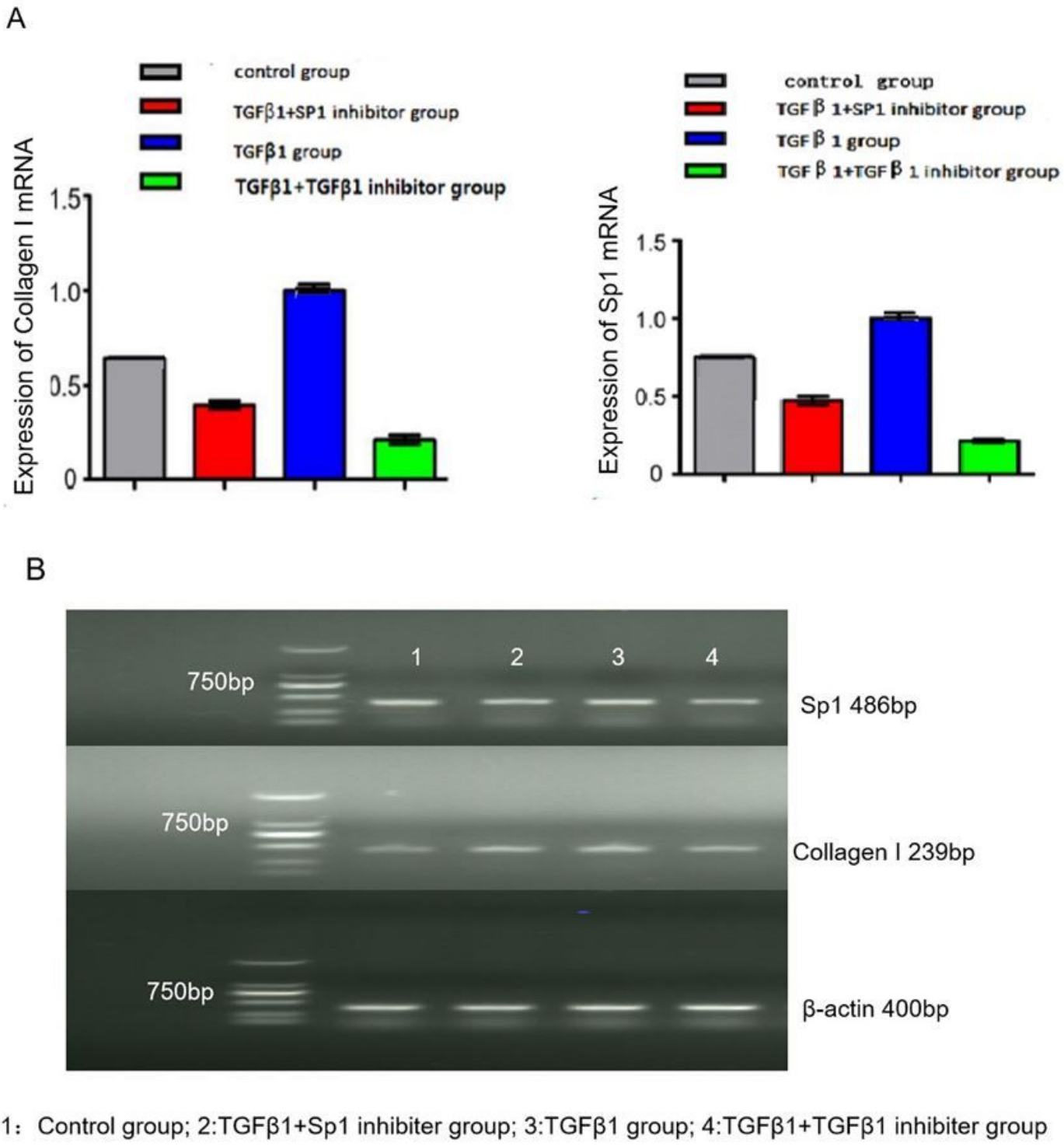

Figure 3

TGF-b1 increased the expression of SpP1 and collagen I mRNA in the TGF-b1 group($p<0.001$,compared with the control group). The Sp1 inhibitor decreased these expression($p<0.05$,compared with the control group).