

Role of the ClC-2 Chloride Channel in TGF- β 1-induced Proliferation, Collagen Synthesis, and Collagen Gel Contraction Mediated by Human Conjunctival Fibroblasts

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Abstract

Background: Excessive scar tissue can reduce postoperative survival of filtering blebs in patients with glaucoma. Previous studies have highlighted the role of chloride channels in wound healing, whereas the role of chloride channels in the formation of follicular scar has not been studied.

Objectives: To investigate the effects of the ClC-2 chloride channel on scar formation of filtering blebs after glaucoma filtering surgery.

Methods: We inhibited ClC-2 chloride channels of Human Conjunctival Fibroblasts (HConFs) by transfecting HConFs with ClC-2 siRNA. Then cell proliferation, cycle and collagen synthesis of HConFs were measured. ClC-2 siRNA-transfected HConFs were cultured in type I collagen gels in the presence of transforming growth factor (TGF)- β 1. Collagen gel contraction was evaluated based on the gel area. The expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in HConFs were assessed by western blotting and q-PCR.

Results: TGF- β 1 induced cell proliferation, cell cycle progression, collagen synthesis, and collagen gel contraction in HConFs. TGF- β 1 increased MMP-2 and MMP-9 levels but inhibited the expression of TIMPs. ClC-2 siRNA transfection inhibited TGF- β 1-induced cell proliferation, cell cycle progression, collagen synthesis, and collagen gel contraction, mediated by HConFs. TGF- β 1-induced increases in MMP-2 and MMP-9 were also inhibited by NPPB and ClC-2 siRNA transfection, but TIMP expression was increased by ClC-2 siRNA transfection.

Conclusions: These findings demonstrate that ClC-2 gene knockout inhibited TGF- β 1-induced cell proliferation, collagen synthesis, and collagen gel contraction of HConFs by attenuating MMP-2 and MMP-9 production and by stimulating TIMP-1 production.

Keywords: ClC-2 chloride channel; conjunctival fibroblasts; TGF- β 1; wound heal

Background:

Excessive filtering bleb scar formation after glaucoma filtration surgery is the most frequent cause of surgical failure. Human conjunctival fibroblasts (HConFs) are key cells involved in the subconjunctival wound healing response, which involves the activation, proliferation, and migration of local fibroblasts as well as the synthesis of extracellular matrix components and its subsequent contraction [1, 2]. Excessive deposition of matrix components and contraction of the subconjunctival tissue at the wound site, resulting in destruction of the normal tissue architecture, determine scar formation and surgery failure.

TGF- β is secreted during the early stages of scar formation by inflammatory cells and acts as a chemoattractant to promote fibroblast transformation to myofibroblasts, which synthesize extracellular matrix [3, 4]. Accumulation of ECM structural proteins increases bleb capsule fibrosis. Matrix metalloproteinases (MMPs) constitute a large family of enzymes that remodel ECM by degrading ECM

molecules during the wound-healing process. The action of MMPs is regulated by a group of endogenous tissue inhibitors of metalloproteinases (TIMPs)[5]. Imbalance between MMPs and TIMPs may lead to excessive degradation or increased accumulation of the ECM, resulting in excessive wound healing.

Chloride channels (ClCs) are a type of permeable channel protein for chloride ions or other anions on the cell membrane, and ClC proteins are encoded by genes of the ClC family[6]. ClC-2 is a member of the ClC voltage-gated chloride channel superfamily and is ubiquitously expressed in organs act as a regulator of cell volume and control the response to swelling and post-synaptic responses to GABA and glycine [7, 8]. In the prevention of hyperplasia and remodeling of cerebrovascular smooth muscle cells ClC-2 plays an important role by inhibiting cell proliferation and migration [9]. ClC-2 also modulates wound epithelialization by regulating the migratory ability of rat keratinocytes [10]. In this study, the effects of the ClC-2 chloride channel on cell proliferation, collagen synthesis, collagen contraction, and MMP and TIMP production by HConFs were evaluated.

Materials And Methods

Cell Culture

HConFs were obtained from ScienCell Research Laboratories (San Diego, CA, USA), where they were isolated from human conjunctiva. HConFs were characterized by their spindle morphology and immunoreactivity with antibodies to fibronectin. Cells were grown at 37 °C in a humidified incubator with 5% CO₂ in fibroblast medium (ScienCell Research Laboratories) containing 2% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and fibroblast growth supplement (undisclosed formulation).

Transfection of HConFs with siRNA

To knockdown ClC-2 expression, we obtained commercially available stealth siRNA duplex oligoribonucleotides targeting the human ClC-2 gene. The sequence was 5'-UCCUCAUGAGGAAACGCCUGCUCUU-3', and its corresponding complementary strand was 5'-AAGAGCAGGCGUUUCCUCAUGAGGA-3'. The negative control consisted of a non-silencing scrambled sequence that did not recognize any known sequence available in the GenBank. To examine the uptake of ClC-2 siRNA by HConFs, siRNA was labeled with Alexa Fluor 488 (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) for determination of siRNA transfection efficiency. HConFs were transfected with oligonucleotides in the presence of Lipofectamine 2000 (Invitrogen Life Technologies, Inc.) in Opti-MEM1 (Invitrogen Life Technologies, Inc.) for 4 h at 37 °C. After removing the transfection mixture, the cells were further incubated for 24 h before experiments under normal growth conditions. The expression of ClC-2 was detected by western blotting and RT-qPCR analysis.

Cell Proliferation Assay (CCK-8 Assay)

HConFs were plated at a density of 5,000 cells/well in a 96-well plate. After treatment, 10 µL of CCK-8 solution (BestBio, Jiangsu, China) was added to each well, followed by incubation for another 3 h at

37 °C. Absorbance at 450 nm was measured using an automated microplate reader (Model 3001 – 1387; Thermo Fisher, Waltham, MA, USA). Each group was provided with 6 duplicate holes, and the experiment was repeated 3 times.

Cell Cycle Analysis

Cell cycle status was assessed by flow cytometry using a Cell Cycle and Apoptosis Analysis Kit (Beyotime, Jiangsu, China). Briefly, cells were grown in 100-mm plates with 10% FBS for 24 h. After treating the cells with media containing different reagents for 48 h, they were collected and fixed in 75% ethanol for 24 h at 4 °C. After rinsing cells with PBS, the cells were stained with PI buffer (containing 500 µL staining buffer, 25 µL of 20 × propidium iodide, and 10 µL of 50 × RNase). The cell cycle distribution was assessed by flow cytometry (CYTOMICS FV 500, Beckman Coulter, Brea, CA, USA).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from HConFs at 60–80% confluency with RNAiso Plus (Takara Bio Inc., Shiga, Japan), and 500 ng RNA was used to synthesize single-stranded cDNA with the RT for PCR Kit (Takara Bio Inc.). Quantitative real-time PCR was performed using methods similar to those previously described[11]. Oligonucleotide primers for the collagen III, MMP2, MMP9, TIMP1, and GAPDH genes were designed using the Primer3 program (<http://frodo.wi.mit.edu/primer3/>), and sequences are listed in Table 1.

Table 1
Oligonucleotide primers for genes in quantitative real-time RT-PCR

Human genes	Sequences	Product size (kb)
collagen III	Forward: (5'–3') TCAGGGTGTCAAGGGTGAA	130
	Reverse:(5'–3') AGGGTTTCCATCTCTTCCA	
MMP2	Forward: (5'–3') TATGGCTTCTGCCCTGAGAC	142
	Reverse:(5'–3') CACACCACATCTTTCCGTCA	
MMP9	Forward: (5'–3') AGTCCACCCTTGTGCTCTTC	117
	Reverse:(5'–3') ACTCTCCACGCATCTCTGC	
TIMP1	Forward: (5'–3') CTGTTGTTGCTGTGGCTGAT	130
	Reverse:(5'–3') TCTGGTTGACTTCTGGTGTCC	
GAPDH	Forward: (5'–3') CAGGAGGCATTGCTGATGAT	126
	Reverse:(5'–3') CAGGAGGCATTGCTGATGAT	

Western Blotting Analysis

HConFs were washed with pre-cooled PBS three times and lysed with RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF]. The total protein was quantified with the Enhanced BCA Protein Assay Kit (Beyotime). Protein (20 µg per sample) was separated by SDS-PAGE and transferred to PVDF membranes, which were then blocked with blocking solution for 1 h at 37 °C. The membranes were incubated with rabbit polyclonal anti-CIC-2 (Abcam), mouse monoclonal anti-collagen I (Abcam), mouse monoclonal anti-collagen III (Abcam), rabbit polyclonal anti-MMP2 (Abcam), rabbit polyclonal anti-MMP9 (Abcam), rabbit polyclonal anti-TIMP1 (Abcam), and/or mouse monoclonal anti-β-actin (Beyotime) antibodies overnight at 4 °C. Following two washes with TBST, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Beyotime) or anti-mouse IgG secondary antibody (Beyotime) for 1 h at room temperature. Final detection was accomplished with enhanced chemiluminescence western blotting reagents (Beyotime). Band density was analyzed using ImageJ (NIH, Bethesda, MD, USA).

Assay of Collagen Gel Contraction

The collagen gel contraction assay was performed according to previously described methods [9], with some modifications. HConFs were harvested by treatment with trypsin-EDTA, washed twice with serum-free MEM, and resuspended in the same medium. Briefly, collagen I (from a rat tail) stock solution (5 mg/mL) (Solarbio, Beijing, China), 0.1 M NaOH, 10 × concentrated PBS, and the cell suspension were mixed on ice at a volume ratio of 200:12:23:765 (final concentration of type I collagen, 1 mg/mL; final cell density, 2×10^5 /mL). A portion (0.5 mL) of the mixture was added to each well of the 24-well culture plate and allowed to solidify by incubation at 37 °C under 5% CO₂ for 20 min. The collagen gels were freed from the sides of the wells using a 10-µL tip, and serum-free MEM (0.5 mL) containing TGF-β1 (10 ng/mL) was then added to the top of each gel. The gel area was examined at 48 h using ImageJ. For normalization, the area of the collagen gel containing untreated HConFs was set to 100%, and the fold changes in the area for each treatment group were determined. Four gels were assayed for each experimental condition, and all experiments were repeated four times.

Data Analyses

Results are expressed as means ± standard error (number of observations), and differences among groups were evaluated using analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$. All experiments were repeated four times.

Results

Effect of CIC-2 Chloride channel on TGF-β1-Induced HConF proliferation

As shown in Fig. 1, TGF-β1 (10 ng/mL) increased HConF proliferation by 28.66% ($p < 0.05$ vs. control) after 48 h of stimulation. HConF transfected with CIC-2 siRNA in the presence of TGF-β1 showed a 39.20% reduction in cell viability compared to that of TGF-β1-treated negative siRNA control cells. Non-

transfected and mutant siRNA-transfected cells had similar viabilities. These results demonstrate that knockout of CIC-2 inhibits TGF-β1-induced HConF cell proliferation.

Effect of CIC-2 Chloride channel on TGF-β1-Induced HConF cell cycle

There were statistically significant differences in the proportion of cells in each cell cycle among the four groups(G0/G1 phase:F = 237.08, P = 0.00; S phase:F = 11.07, P = 0.00; G2/M phase: F = 228.09, P = 0.00). Compared with the control group, the proportion of cells in G1 phase was significantly reduced in the TGF- β1 treatment group, while the proportion of cells in S phase and G2/M phase was increased, with statistically significant differences TGF-β1 promoted cell cycle progression by advancing cells from the G0/G1 to S and G2/M phases, but knockout of CIC-2 inhibited TGF-β1-induced cell cycle progression by arresting cells in the G0/G1 phases (Fig. 2, Table 2).

Table 2
Cell cycle distribution of HconF in each group(mean ± SD, %)

Group	Sample size	Cell distribution in different cell cycle		
		G0/G1 phase	S phase	G2/M phase
Control	4	64.43 ± 3.71	8.37 ± 3.02	27.20 ± 1.92
TGF-β1	4	54.60 ± 2.19	10.40 ± 2.19	35.00 ± 2.56
TGF-β1 + siRNA-NC	4	52.40 ± 3.14	10.31 ± 3.02	37.32 ± 3.23
TGF-β1 + siRNA-CLC-2	4	71.80 ± 3.16	8.51 ± 3.12	19.70 ± 3.18
F value		237.08	11.07	228.09
P value		0.00	0.00	0.00

Effect of CIC-2 Chloride channel on TGF-β1-Induced Collagen Gel Contraction Mediated by HConFs

As shown in Fig. 3, exposure to TGF-β1 stimulated HConF-mediated collagen contraction of the free-floating three-dimensional collagen gel and resulted in a significant reduction in the gel area as compared to that of controls (p < 0.01). The gel area of TGF-β1 + CIC-2 siRNA group was reduced compared with the TGF-β1 group and TGF-β1-treated negative siRNA control group, The TGF-β1-induced contraction of HConF cells was inhibited by the siRNA-mediated downregulation of CIC-2.

Effect of CIC-2 Chloride channel on TGF-β1-Induced Collagen Synthesis

TGF-β1 induced high levels of collagen III expression at the protein and mRNA levels in HConFs compared to levels in control cells. The knockout of CIC-2 significantly inhibited the TGF-β1-induced expression of collagen III. The expression levels of collagen III were similar in TGF-β1-treated non-transfected cells and TGF-β1-treated negative siRNA control cells. (Fig. 4)

Effect of ClC-2 Chloride channel on TGF- β 1-Induced MMP/TIMP Expression

TGF- β 1 increased the protein and mRNA expression levels of MMP-2 and MMP-9, which negatively regulate collagen synthesis and deposition. TGF- β 1 resulted in lower expression of TIMP1, a positive regulator that promotes collagen expression, at the protein and mRNA levels in HConFs than in control cells. Levels of MMP-2 and MMP-9 were lower in the ClC-2 siRNA-transfected group than in the non-transfected and mutant siRNA-transfected groups in the presence of TGF- β 1. Conversely, the expression of TIMP1 was higher in the ClC-2 siRNA-transfected group than in the non-transfected and mutant siRNA-transfected groups in the presence of TGF- β 1 (Fig. 5,6).

Discussion

In the present study, we induced a series of conjunctival scarring responses with TGF- β 1. Subsequently, the effects of ClC-2 chloride channel on HConF scarring responses were measured. We found that ClC-2 siRNA transfection inhibited TGF- β 1-induced HConF proliferation, collagen synthesis, and collagen gel contraction. Exaggerated wound contraction induced by elevated TGF- β levels has become a therapeutic target in fibrosis [12]. A study by Arslan et al. [13] showed that filtration surgeries increased TGF- β expression. Similar to our results, Liu et al. [14] found that TGF- β -induced collagen gel contraction was mediated by human Tenon fibroblasts.

Chloride channels are expressed in nearly all eukaryotic cells and play an important role in cell proliferation, apoptosis, and migration [15]. The regulatory effect of ClC chloride channels on cell proliferation has been shown in many studies[16]. A study by Warsi et al. [17] showed that downregulation of the chloride channel ClC-2 by Janus kinase 3 influenced cell proliferation and apoptosis. In osteosarcoma cells, ClC-3 silencing inhibited cell proliferation and migration via the AKT/GSK3 β signaling pathway [18]. Li et al. [19] found that overexpression of the chloride intracellular channel 5 (CLIC5) inhibited proliferation of C2C12 cells and drove more C2C12 cells into the G0/G1 phase. We previously blocked the chloride channel of ARPE19 in human adult RPE cells using NPPB and tamoxifen (TAM) and found that this inhibited proliferation by arresting cells in the G0/G1 phase[20]. ClC-2, a ubiquitously expressed member of the superfamily of ClC transporters and channels, has been confirmed to be expressed in human conjunctival fibroblasts [11]. Our result showed that ClC-2 siRNA transfection inhibited TGF- β 1-induced HConF cell proliferation by arresting cells in the G0/G1 phase.

Recent studies have highlighted the role of chloride channels in wound healing. Fuchigami et al. [21] showed that the chloride channel blocker NPPB reduced epidermal wound closure by blocking the ClC-3 chloride channel. Further, local enhancement of ClC-3 expression at the leading edge of the wounded epidermis was specific to closing wounds, suggesting that the intracellular translocation of ClC-3 was involved in wound closure. Schiller et al. [22] found that CFTR chloride channels positively regulated wound healing in cell culture models of the airway surface epithelium. Thus, inhibition of conjunctival fibroblast proliferation is the main strategy for treatment of anti-glaucoma filtration scarring.

Cell-mediated tissue contraction is a complex process that involves various cellular activities, including cell migration and reorganization of the ECM, and MMPs have been implicated in wound contraction and matrix reorganization [23]. Collagen contraction involves the cell-directed reorganization of collagen fibrils and plays a significant role in wound healing. In the present study, a three-dimensional model of wound healing that closely mimics the *in vivo* situation was established. We found that TGF- β 1 promoted HConF collagen synthesis and collagen gel contraction. CIC-2 gene knockout inhibited TGF- β 1-induced HConF collagen synthesis and collagen gel contraction. Multiple studies have shown that chloride channels are involved in collagen synthesis [24–26]. Qi et al. [24] showed that the chloride channel blocker DIDS inhibited the collagen release of HFL1 fibroblasts. Downregulation of CLIC4 also reduced TGF- β 1-induced expression of ECM components in primary fibroblasts [25]. In the development of atrial fibrillation, CLIC 1, 4, and 5 played an important role by interacting with collagen IV[26, 27].

Inhibition of MMPs has been shown to limit subconjunctival scarring after experimental glaucoma filtration surgery in rabbits [27]. The balance between MMPs and TIMPs in the ECM is important for regulation of the ECM and wound healing [23]. The ratio of MMPs to TIMPs is an important factor in various biological activities, including cell migration, angiogenesis, and remodeling of the ECM [28]. Du and Yang [21] found that CIC-3 knockout attenuated osteosarcoma cell migration via downregulation of MMP-2 and MMP-9. In the present study, TGF- β 1 induced expression of MMP-2 and MMP-9 but inhibited TIMP-1 expression in HConFs. TGF- β 1-induced expression of MMP-2 and MMP-9 was inhibited by CIC-2 siRNA transfection in HConFs. CIC-2 siRNA transfection increased TIMP-1 expression in the presence of TGF- β 1.

Consistent with our results, Guan et al. [29] found that MMP-1, MMP-2, and MMP-3 levels were increased by TGF- β 1, while TGF- β 1 inhibited TIMP-1 expression. They also found knockdown of CIC-3 expression inhibited MMP-9 expression in ectopic endometrial cells. Wang et al.[30] silenced the CIC-3 gene and found that MMP-3 and MMP-9 levels were reduced. Our results showed that the CIC-2 knockout decreased TGF- β 1-induced proliferation, collagen synthesis, and contractility of HConFs cultured in a three-dimensional collagen gel, in part by blocking MMP production. Further *in vivo* studies are needed to confirm the preventive effect of CIC-2 chloride channels on filtering bleb scarring after filtration surgery.

Conclusions:

These findings demonstrate that knockdown of CIC-2 expression inhibited TGF- β 1-induced cell proliferation, collagen synthesis, and collagen gel contraction of HConFs by attenuating MMP-2 and MMP-9 production and by stimulating TIMP-1 production. CIC-2 chloride channels may therefore prove to be a new control target to inhibit scar formation of filtering blebs.

Abbreviations

HConFs

Human Conjunctival Fibroblasts

MMPs
matrix metalloproteinases
TIMPs
metalloproteinases
CICs
Chloride channels

Declarations

Ethics approval and consent to participate

This article has been reviewed and approved by the ethical committee of Yanbian University Affiliated Hospita.

Consents to publish

Not applicable

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lixia Sun writed the article; Renzhe Cui and Huan Meng guided the experiment; Xin Liu and Xiwen Liu conducted the experiment; Kun Liu and Liang Jia analysed the data; Yan Lu and Yonghu Chi revised the article; Yajuan Zheng designed the work. All authors read and approved the final manuscript.

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Figures

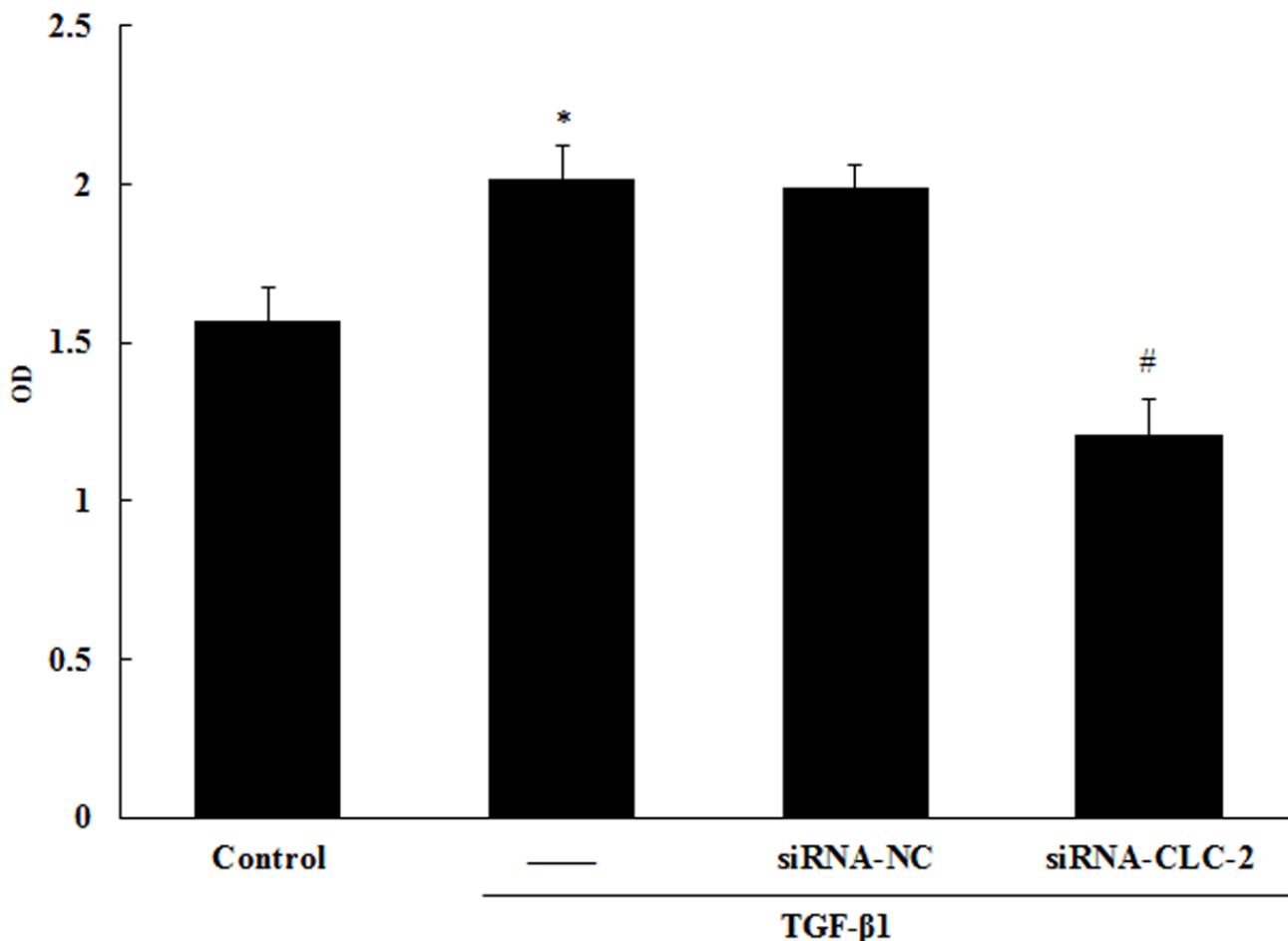


Figure 1

Effect of CIC-2 siRNA on transforming growth factor (TGF)- β 1-induced Human conjunctival fibroblasts (HConF) cell proliferation. Relative cell counts were determined using the CCK-8 assay and results are expressed as the optical density (OD) values. Results are presented as means \pm SD (n = 4); *p < 0.05, vs. control; #p < 0.05, vs. TGF- β 1 (ANOVA).

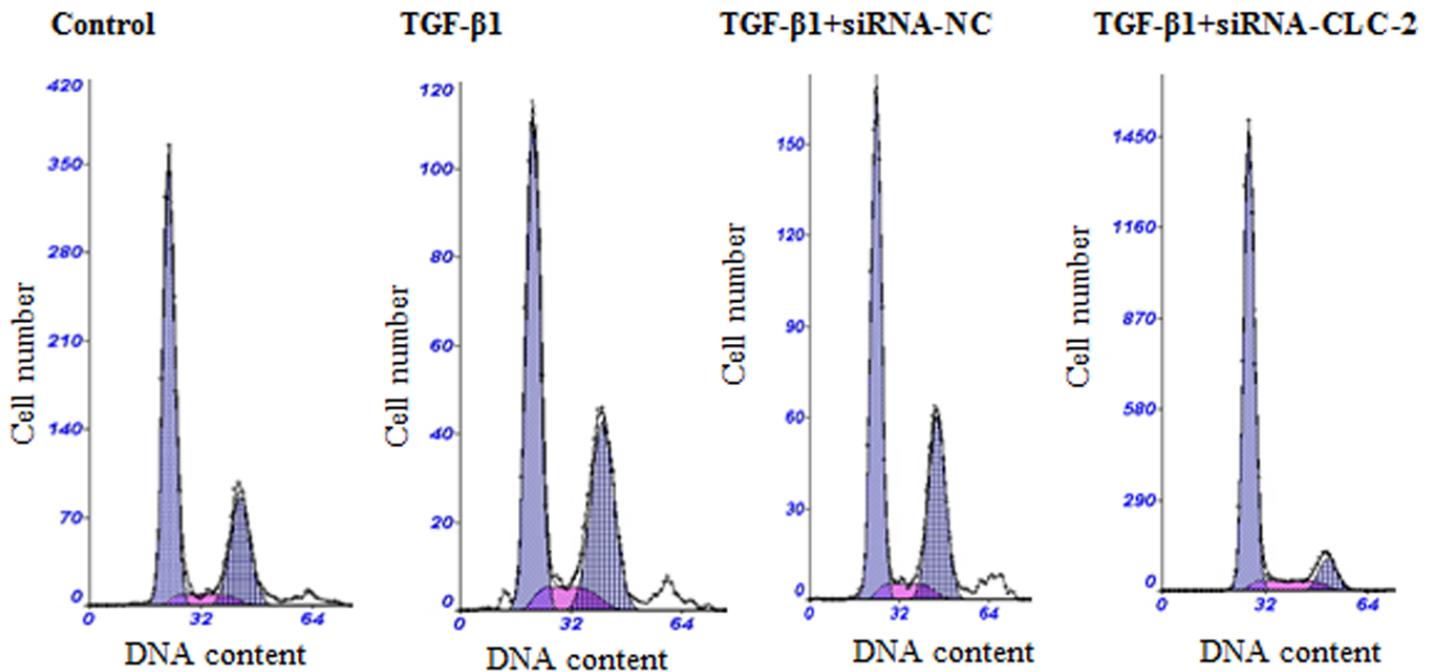


Figure 2

Effects of CIC-2 siRNA on HConF cell cycle progression, as determined by flow cytometry. Typical cell cycle distribution and quantitative distribution of cells in different phases for various treatments.

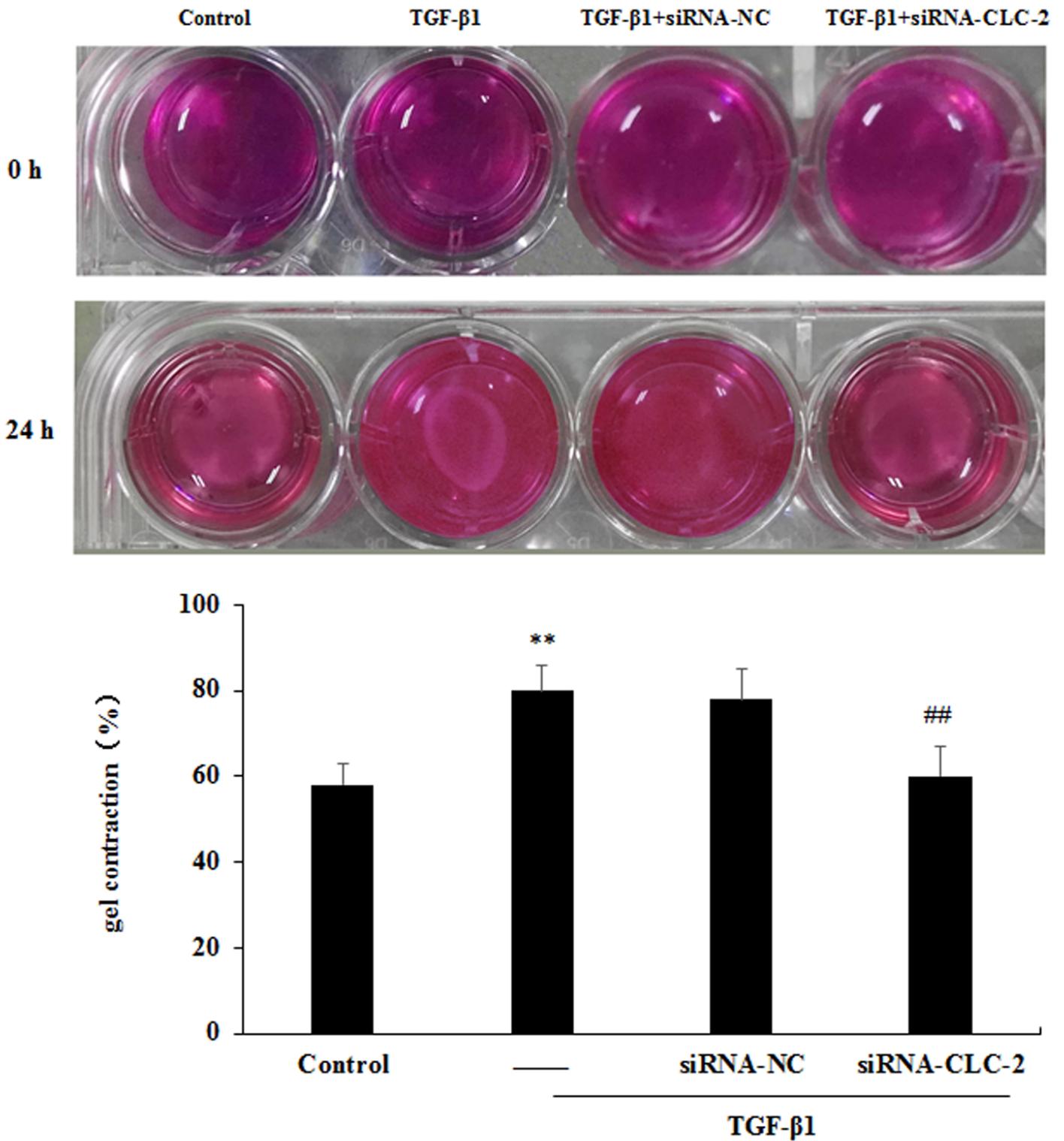


Figure 3

Effect of CLC-2 siRNA on TGF- β 1-induced collagen gel contraction mediated by 3D-cultured HConFs. The extent of gel contraction was determined. Results are presented as means \pm SD (n = 4), **p < 0.01 vs. control; ##p < 0.01 vs. TGF- β 1 (ANOVA).

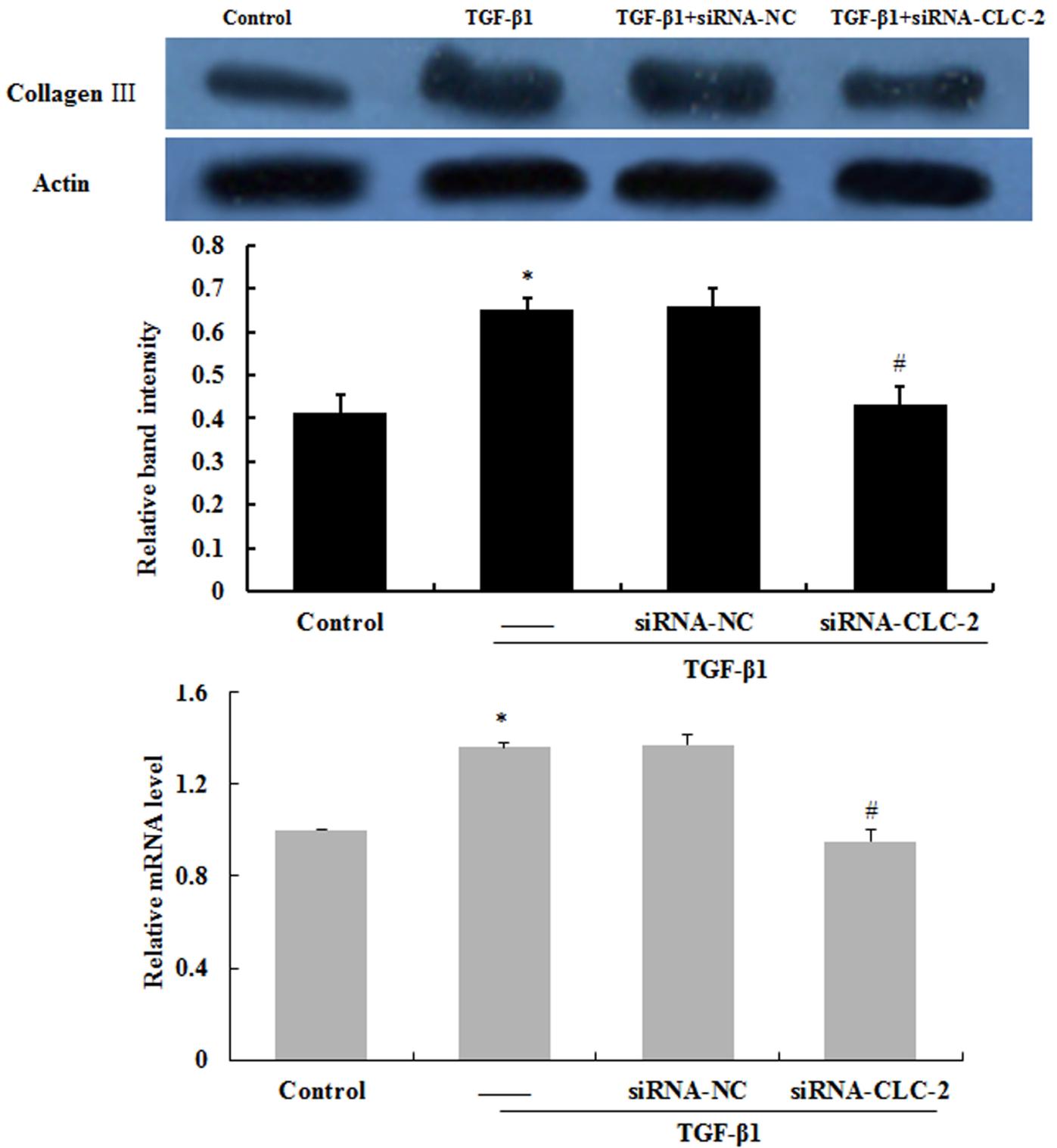


Figure 4

Protein and mRNA expression of collagen III in Human conjunctival fibroblasts (HConF) after different treatments as determined by western blotting. Results are presented as means \pm SD (n = 4); *p < 0.05, vs. control; #p < 0.05, vs. TGF-β1.

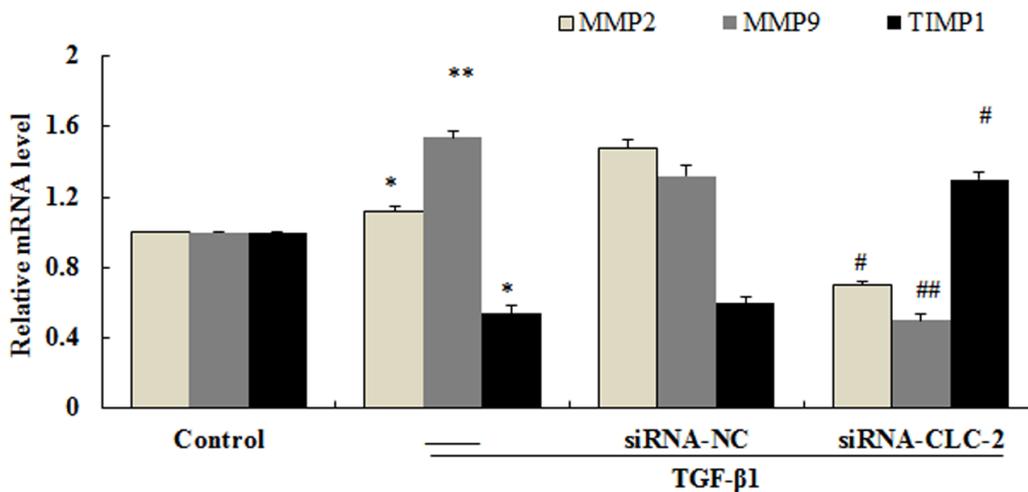
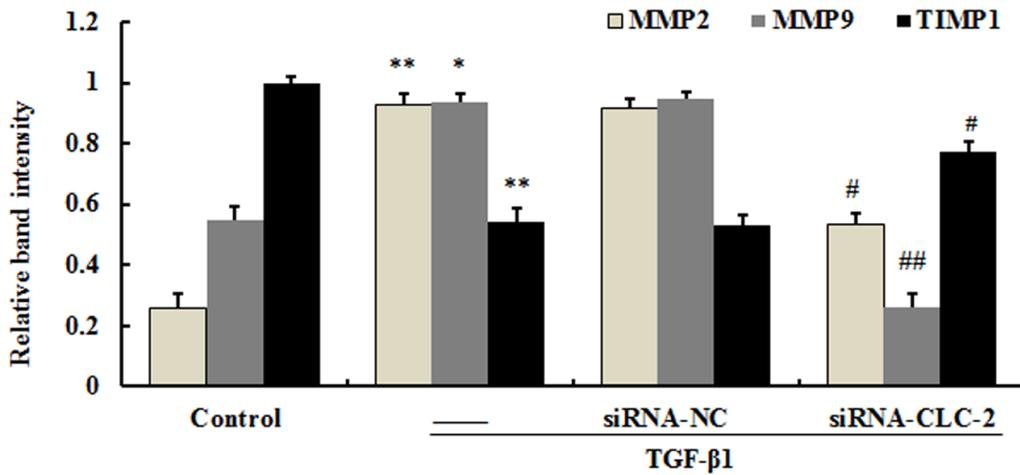
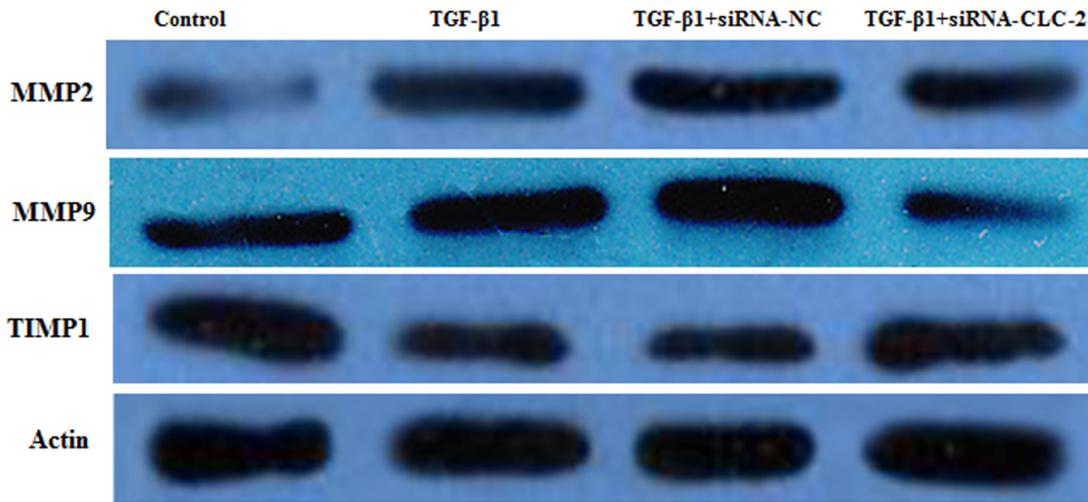


Figure 5

Protein and mRNA expression of MMP-2, MMP-9, and TIMP1 in Human conjunctival fibroblasts (HConF) after different treatments as determined by western blotting. Results are presented as means \pm SD (n = 4); *p < 0.05, **p < 0.01 vs. control; #p < 0.05, ##p < 0.01 vs. TGF-β1.