Effects of Mechanical Compression on Cell Morphology and Function in Human Corneal Fibroblasts

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Abstract

Purpose: To explore the effect of mechanical compression (similar to that induced by eye rubbing) on cell morphology, proliferation, apoptosis, and extracellular matrix synthesis and degradation in human corneal fibroblasts.

Materials and methods: Human corneal fibroblasts were isolated from corneal lenticule tissue of 10 patients after small incision lenticule extraction surgery. A system was established to mechanically compress corneal fibroblasts with pressure ranging from 0 to 524 Pa. Morphological changes, cell proliferation, apoptosis, and corneal matrix synthesis and degradation were examined using microscopy imaging, bromodeoxyuridine staining, flow cytometry, and qPCR analysis in human corneal fibroblasts after mechanical compression.

Results: Human corneal fibroblasts showed short and thick cytoplasmic extensions, as well as a relatively low aspect ratio, suggesting significant morphological alterations caused by high levels of compressive stress. Mechanical compression inhibited cell proliferation and promoted cell apoptosis. Furthermore, compressive stress led to significant elevation in the expression of genes related to extracellular matrix degradation (matrix metalloproteinases *MMP1* and *MMP9*) as early as 6 h after compression and moderate changes in the expression of tissue inhibitors of metalloproteinases. In addition, the mRNA expression levels of *COL1A1*, lumican, and vimentin were reduced 24 and 48 h after mechanical compression.

Conclusions: Mechanical compression alters cell morphology, inhibits proliferation, induces apoptosis, upregulates genes related to extracellular matrix

degradation, and downregulates corneal structural genes in human corneal fibroblasts. This study provides evidence that compressive stress significantly influences corneal keratocytes. Our findings suggest that this mechanical effect may be related to keratoconus associated with chronic eye rubbing.

Keywords: mechanical compression; eye rubbing; keratoconus; corneal fibroblasts; extracellular matrix

Introduction

Keratoconus (KC) is a progressive corneal ectasia characterized by a cone-like deformation due to local stromal thinning and weakening of the cornea [1]. Many studies propose that the etiology of KC is a multifactorial pathological process involving both genetic and environmental factors [2-4]. Recent studies suggest that chronic and abnormal eye rubbing might play a crucial role in the development and/or progression of KC [2, 3, 5-7]. Multiple uni- or bilateral KC cases are closely correlated with vigorous eye rubbing [8, 9]. Approximately half of the patients with KC have been found to have eye rubbing habits, and case-control studies have provided more convincing evidence that eye rubbing serves as a risk factor for KC in both univariate and multivariate analyses [10-13]. However, how eye rubbing promotes KC is not yet known.

It is crucial to unravel the mechanism underlying the influence of eye rubbing on KC progression. Multiple biochemical factors have been identified, including inflammation events in the corneal epithelial and stromal layers, abnormal keratocyte functions, altered enzymatic activities, and loss of collagen and other extracellular matrix (ECM) components [14]. The changes in cellular functions of keratocytes and primary stromal cell type are heavily involved in the development and progression of KC [15]. The reduction of keratocyte density in KC patients indicates the imbalance of keratocyte apoptosis and proliferation. The gradual loss of keratocytes and the associated reduction in the production of collagen and proteoglycans may contribute to the loss of stromal tissue [16-20]. The activation of keratocytes during corneal wound healing induces the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) to support the remodeling of the damaged stroma. Abnormal levels of MMPs/TIMPs in the corneal tissue and tears of KC patients has also been reported [21, 22].

Aside from these biochemical factors, mechanical cues critically influence various cellular functions and thus play important roles in a wide range of physiological and pathological activities [23]. Living cells sense and respond to various mechanical signals through mechanotransduction [24], including ECM stiffness [25], fluid shear stress [26], mechanical stretch [27], and compressive stress [28, 29]. Mechanical stretch modulates the function of corneal fibroblasts (CFs), which are isolated and cultured keratocytes. Low-magnitude cyclic equibiaxial stretching alone decreases the production of MMP2 and membrane type 1 MMP and increases the production of TIMP2 in rabbit CFs, whereas high-magnitude stretching increases the protein expression of MMP2 and MMP9 [30]. The progression of KC is closely related to eye rubbing, in which corneal keratocytes experience considerable levels of compressive stress. However, the influence of the eye rubbing-mediated compression on the functions of CFs and KC progression remains unclear.

In this study, we established an *in vitro* compression model to apply pressure ranging from 0 to 524 Pa to human CFs, mimicking the compression force of eye rubbing. In human CFs, we report a change in cell morphology, inhibition of proliferation, and promotion of apoptosis after compression. In addition, we found a significant alteration in the expression of ECM degradation and structural genes when CFs were exposed to mechanical compression.

Materials and methods

Human corneal fibroblasts isolation and culture

Human corneal stromal tissue was obtained from lenticule leftovers from small incision lenticule extraction (SMILE) surgery at Tianjin Eye Hospital, Tianjin Medical University

(Tianjin, China) in September 2018. Ten patients (male: 5, female: 5, age: 20-40 years) had donated 20 lenticules after undergoing SMILE surgery (stromal lenticule diameter of 6.0 mm, thickness ranging from 80-120 μm). The lenticules were immersed in phosphate-buffered saline (PBS) immediately after extraction. The tissue was subsequently cut into several pieces and was digested for 6 h at 37°C into single cells with Dulbecco's Modified Eagle Medium (DMEM)/F12 medium containing 2 mg/ml of collagenase I (Sigma-Aldrich). Cells were cultured in DMEM/F12 containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. The purified CFs were confirmed by positive immunostaining for vimentin. Cells in passages 3-5 were used for the following experiments.

The study was approved by the Ethics Committee of Tianjin Eye Hospital, Tianjin Medical University, and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from each patient to use their tissue for cell culture and subsequent experiments.

In vitro *mechanical compression device*

The mechanical compression device was designed, as seen in Figure 1, based on a compression model previously introduced by Tse et al [31]. When the cell density reaches 80-90%, a piston of specified weight applies a constant force to a 2% agarose disk (diameter: 20 mm, weight: 0.8 g) in contact with cells growing in a 12-well plate (surface area: 3.5 cm^2). The area of the piston surface (diameter: 20 mm) was 3.14 cm^2, thus approximately 90% of the cells were loaded with pressure. The total weight on the cells was calculated with both the agar and piston weight. Both the applied weight and the stress translating to cells are listed in Table 1. Since the current literature lacks information on the magnitude of pressure applied on the cornea during eye rubbing, we referred to the study of Tse et al, which reported that breast cancer cells could sustain a

solid pressure of around 5 mmHg (667 Pa) [31]. Our preliminary study confirmed that CFs could survive for more than 48 h under around 774 Pa. We arranged the weight to produce stress ranging from 0 to 524 Pa, and the cells were compressed for 6, 8, 24, and 48 h in this study.

Morphological analysis

After 24 h of compression, the cells on the coverslip were fixed with 4% paraformaldehyde (PFA) for 30 min and then incubated with 0.5% Triton-X 100 in PBS for 5 min. Phalloidin (Solarbio, CA1610, 1:200) was diluted in PBS and applied to the coverslip overnight at 4°C. After rinsing, the coverslip was mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The aspect ratio of the cells was determined using ImageJ (National Institutes of Health, Bethesda, MD, USA).

qPCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's instructions. cDNA synthesis was performed with All-in-One cDNA Synthesis Supermix (Bimake, Shanghai, China). The qPCR was performed using the 7500 Real-Time PCR System, and the cDNA was amplified with 2× SYBR Green qPCR Master Mix (Bimake). Relative mRNA levels of each sample were determined by the $2^{-\Delta\Delta Ct}$ method with the housekeeping gene GAPDH. Primers are listed in Supplemental Table 1.

Cell proliferation assay

Before the mechanical stress stimulation, the culture medium was refreshed with new medium containing 10 μM bromodeoxyuridine (BrdU). Following 8 h of stimulation, the

cells were fixed with 4% PFA, then incubated with 2 N HCl solution for 30 min, and subsequently incubated with 0.1 N sodium borate for 20 min at room temperature. Then, the cells containing BrdU were immunostained following the procedure mentioned above using the primary antibody anti-BrdU [BU1/75 (ICR1)] (1:250, Abcam) and the secondary antibody rabbit anti-rat IgG (Alexa Fluor 488). The cell proliferation state was expressed as the ratio BrdU-positive cell number/DAPI-positive cell number.

Flow cytometry assay

After 8 h of mechanical compression, cells were harvested and collected for flow cytometry. The Annexin V-FITC/PI Cell Apoptosis Detection Kit (TransGen) was used to double-stain the cells with FITC-annexin-V and PI following the manufacturer's instruction. Cell apoptosis was assessed using a CytoFLEX flow cytometer (Beckman Coulter), and the results were analyzed using CytExpert2.3 software (Beckman Coulter). Cells were classified as dead cells, living cells, early apoptotic cells, and late apoptotic cells.

Statistical analysis

All independent experiments were performed at least in triplicate. Data are presented as the mean ± standard deviation (SD). Statistical comparisons were made using Student's t-test for the determination of P-values. P-values of <0.05 were considered statistically significant.

Results

Mechanical compression induces morphological changes in human corneal fibroblasts

To investigate the response of human CFs to mechanical compression, a system for mechanical compression was developed as introduced in the study by Tse et al [31]. Human CFs were exposed to compression force generated by a weighted piston via contacting an agarose disk to spread the pressure evenly (Figure 1a). Since the current literature lacks information on the magnitude of pressure applied to the cornea during eye rubbing, we referred to the study by Tse et al [31] to apply stress ranging from 0 to 524 Pa (Table 1). To evaluate the cell morphology more precisely, we performed immunocytochemistry with phalloidin to observe F-actin distribution and cell shape 24 h after compression at 0, 25, 150, and 524 Pa (Figure 1b). CFs in the 0 Pa group, consistent with the typical morphology of fibroblasts, were long spindle-shaped cells with a high cell density and tight cell-to-cell adhesions. Similarly, cells showed elongated cell processes with long cytoplasmic extensions and a high aspect ratio when exposed to relatively low loading pressure (25 Pa). However, with increasing pressure (150 and 524 Pa), CFs gradually presented with shorter and thicker cytoplasmic extensions and a decreased aspect ratio (Figure 1b, c). These results indicate that continuous high-magnitude compression pressure could directly affect cell morphology.

Mechanical compression inhibits cell proliferation and promotes cell apoptosis

Since a gradual loss of keratocytes was observed in KC corneal stromal tissue with disease progression [14], we aimed to evaluate the effect of mechanical compression on cell proliferation and apoptosis. Cell proliferation was examined using BrdU labeling during mechanical compression for 8 h, as the cell cycle of fibroblasts is around 8-12 h. The

results showed that there was no significant difference in the percentage of BrdU-positive CFs between 0 Pa (47.15 ± 5.08%) and 25 Pa (46.84 ± 6.81%) after 8 h of mechanical compression (Figure 2a, b). When the compressive stress increased to 150 and 524 Pa, the proportion of BrdU-positive cells was significantly decreased to 36.55 ± 5.14% (P<0.05 vs. 0 Pa) and 37.42 ± 2.39% (P<0.05 vs. 0 Pa), respectively, suggesting that mechanical compression suppresses cell proliferation in human CFs. However, there was no difference in cell proliferation when CFs were compressed with 0 and 25 Pa. To examine the effect of compressive stress, cell apoptosis was tested via flow cytometry after 8 h mechanical compression (Figure 2c). The 150-Pa group showed an increase in early apoptotic cells with annexin-V^+ PI^- (Figure 2c) compared to the 0-Pa and 25-Pa groups, and 524 Pa resulted in an increase in late apoptotic cells with increased annexin-V^+ PI^+ compared to the 0-Pa and 25-Pa groups (Figure 2c). These results suggest that mechanical compression inhibits cell proliferation and facilitates apoptosis of human CFs.

Effects of mechanical compression on the expression of MMPs/TIMPs and structural proteins

The production and degradation of the ECM are crucial for corneal transparency and integrity. We next investigated the influence of mechanical compression on the mRNA expression levels of structural proteins and the genes related to ECM degradation (MMPs: ECM enzymes for degradation; TIMPs: MMP inhibitors) in human CFs. We examined the immediate response of mRNA expression after compression for 6 h, whereas 24 and 48 h were selected to observe the following response at relatively late stages. The results show that the expression levels of *MMP1* and *MMP9* genes were increased dramatically after 6 h compression under various compressive stress levels compared to control cells (Figure 3a, b), whereas this effect was diminished for *MMP9* after 48 h of mechanical compression (Figure 3b). Interestingly, *MMP1* and *MMP9* were also upregulated when

only the agar membrane (25 Pa group) was applied without additional loading. This effect may be explained by the compression by the agar membrane and/or the interaction between agar gels and CFs. TIMPs have the potential to inhibit the functions of MMPs. To explore the effect of mechanical compression on TIMPs, human CFs were compressed under various stress levels for different times (6, 24, and 48 h). The expression levels of *TIMP1*, *TIMP2*, and *TIMP3* were significantly reduced after 6 and 48 h of mechanical compression (Supplemental Figure 1), which may partially explain the upregulation of MMPs in response to compressive stress.

We further explored the influence of mechanical compression on structural proteins in both cells and ECM. When CFs were compressed only for 6 h, there was no significant alteration in the expression of collagen, type I, alpha 1 (*COL1A1*), lumican, and vimentin (Figure 4). After 24 h of mechanical compression, *COL1A1* expression was dramatically reduced in cells loaded with various weights but not in cells exposed only to agar without additional loading (Figure 4a). The downregulation of *COL1A1* was dependent on the magnitude of compressive stress. When the cells were compressed for 48 h, the expression levels of *COL1A1*, lumican, and vimentin were considerably decreased in human CFs compared to control cells (Figure 4). There were no significant differences between the group exposed to only the agar membrane and the group exposed to both agar and weights, as well as among the groups exposed to different weight levels. Taken together, mechanical compression upregulates MMPs, downregulates TIMPs, and suppresses the expression of genes related to corneal structural proteins and ECM components.

Discussion

Since chronic vigorous eye rubbing shows an epidemiological association with KC, the question arises about whether and how mechanical force contributes to the pathogenesis

of KC [6]. Previously, the effect of cyclic stretching on CFs was demonstrated [30]. Given the complexity of eye rubbing forces, we considered it necessary to evaluate also other forms of forces, such as compressive forces. To the best of our knowledge, the present study is the first attempt to explore the response of human CFs to compression. We established a mechanical compression loading system based on the work of Tse et al [31]. Hafezi et al reported three types of eye rubbing (fingertip, knuckle, or fingernail) in KC patients with direct forces ranging from 2.6 ± 3.3 N to 9.6 ± 6.3 N, but the exact pressure level remains unclear [32]. A force gradient ranging from 0 to 1 kPa was applied in our preliminary study demonstrating that CFs can survive compression of less than 524 Pa for 48 h of stimulation. It is obvious that the current mechanical compression model is still unable to exactly mimic the eye rubbing situation *in vivo*, but it can be utilized to study the effect of compression force on cells *in vitro*.

Mechanical cues may induce changes in cell morphology. Petroll et al reported that CFs actively respond to local matrix stress alterations to maintain tensional homeostasis, which may be mediated by Rho or Rac [33]. Cyclic equibiaxial stretching can also increase the contractility of F-actin fibers [34]. Our study showed cell morphological alterations, with shorter and thicker cytoplasmic protrusions and enlarged cell nuclei when CFs were subjected to compression. Compressive forces may result in deformation and reorientation of the nucleus in endothelial cells and fibroblasts, exerted by actin filaments [35, 36]. Clinical confocal images showed the presence of irregularities in KC keratocyte nuclei [37-39]. Although not identical with clinical findings, our present study provides a novel angle in that the nuclear deformation in KC might be caused by mechanical cues from eye rubbing. In addition, alterations in keratocyte cell densities were identified in different stages of KC, suggesting a gradual loss of keratocytes based on the severity of the disease [37, 39, 40]. Such cell loss could be related to the release of

factors that cause the apoptosis of keratocytes [18]. Combined with our result that compression force inhibits proliferation and promotes apoptosis of CFs, we postulate that the excessive compression force from eye rubbing possibly contributes to the deterioration of the disease through the modulation of proliferation and apoptosis.

Abnormal synthesis and degradation of the ECM were also identified in patients with KC. Chaerkady et al found a widespread decrease in ECM components including collagen I, III, V, XII, lumican, and keratocan using proteome analysis of corneal tissue from KC patients [20]. Our results showed that mechanical compression inhibited the expression of *COL1A1*, lumican, and vimentin. The reduction in these structural proteins might play a role in the remodeling of the stromal structure in KC by influencing the composition and alignment of collagen fibers. Extensive evidence has indicated that the expression levels of MMPs, particularly MMP1 and MMP9, are elevated in the corneal epithelium and tears of KC patients, implicating dysregulated proteolysis in KC [41-43]. We found that CFs rapidly responded to mechanical compression with an elevation in *MMP1* and *MMP9* levels and moderate changes in TIMP expression, indicating that compression stress may disrupt the balance between MMPs and TIMPs. Liu et al found that small-magnitude stretching (5%) promotes corneal stromal synthetic events, whereas large-magnitude stretching (15%) promotes corneal stromal degradation by modulating the balance between MMPs and TIMPs through the ERK signaling pathway [30]. In KC, the disturbed balance between MMPs and TIMPs leads to the abnormal production and degradation of ECM [22]. Taken together, we hypothesize that mechanical compression may disturb the balance between synthesis and degradation of ECM components to modulate the corneal stromal remodeling, suggesting a possible disease mechanism in KC cases associated with chronic eye rubbing.

We must point out several limitations in our study. The current study only focused on the response of relatively healthy CFs; thus, whether fibroblasts from KC patients are more sensitive to such compression stimulation remains unclear. In addition, our investigation only provides some preliminary data on how mechanical compression affects the biological function of CFs. The specific mechanotransduction signaling involved in this process needs to be addressed. Furthermore, compared to relatively new 3D environments, traditional 2D cultures cannot maintain the keratocyte phenotype well, and the cells behave in a more fibroblastic way. The current model only provides compression pressure with a constant value for a specific period, whereas eye rubbing applies compression over short periods (usually a few seconds), and the magnitude would vary over time. Thus, further studies should consider investigating the role of mechanical compression on human CFs *in vivo*.

In summary, human CFs respond to mechanical compression with cell morphology alterations, inhibition of proliferation, and activation of apoptosis, as well as the regulation of genes related to ECM production and degradation. These findings enable further investigations on the specific mechanism of compression force in KC associated with chronic eye rubbing and provide a novel perspective to better understand the pathogenesis of KC.

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Declaration of interest

The authors declare no conflict of interest.

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Figures

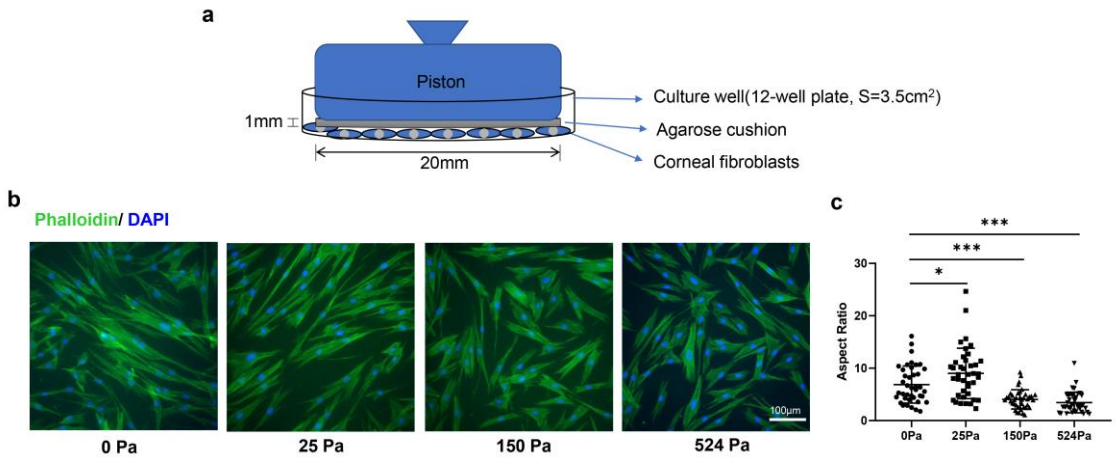

Figure 1. Cell morphological changes after mechanical compression in human corneal fibroblasts (CFs). (a) Schematic diagram of the compression device. A piston filled with an adjustable weight applies a constant force to an agarose cushion disk (thickness: 1 mm, diameter: 20 mm) in contact with a monolayer of CFs growing on the cell culture well (surface area: 3.5 cm^2). (b) Representative immunocytochemistry for phalloidin (green) and nuclei (blue) after human CFs had been exposed to 0, 25, 150, and 524 Pa mechanical compression for 24 h (scale bar: 100 μm). (c) Comparison of the aspect ratios of cells exposed to 0, 25, 150, and 524 Pa compression (n=3; **P*<0.05, ****P*<0.001 vs. 0 Pa).

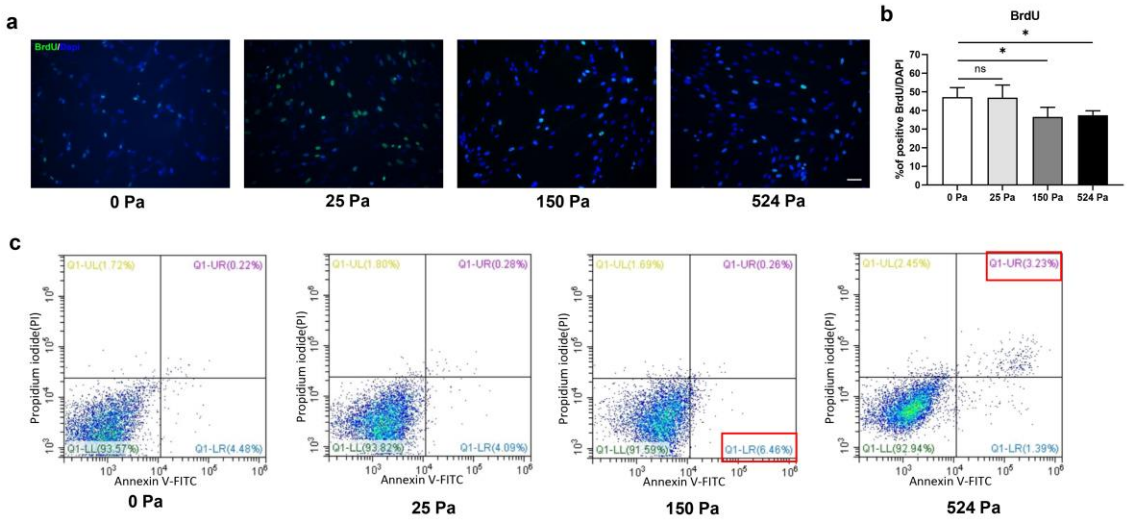

Figure 2. Mechanical compression inhibits cell proliferation and promotes cell apoptosis of human corneal fibroblasts (CFs). Human CFs were subjected to compressive pressure of 0, 25, 150, and 524 Pa for 8 h. (a) Representative bromodeoxyuridine (BrdU) immunofluorescent staining image of human CFs with and without mechanical compression (green, BrdU; blue, 4',6-diamidino-2-phenylindole [DAPI]; scale bar: 100 μm). (b) Percentage of BrdU-positive cells (n=3; **P*<0.05 vs. 0 Pa). (c) Cells were stained with annexin-V and PI after compression, for flow cytometry to examine cell apoptosis.

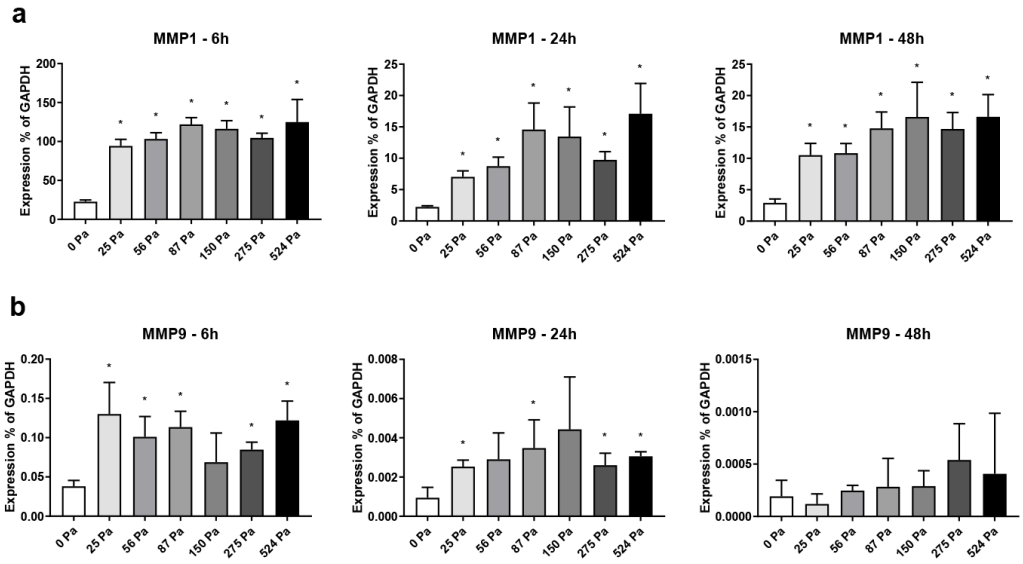

Figure 3. Mechanical compression induces the expression of matrix metalloproteinases (MMPs). Human corneal fibroblasts (CFs) were subjected to various levels of mechanical compression for 6, 24, and 48 h. The mRNA expression levels of *MMP1* (a) and *MMP9* (b) are shown for different pressure groups (n=3; **P*<0.05 vs. 0 Pa).

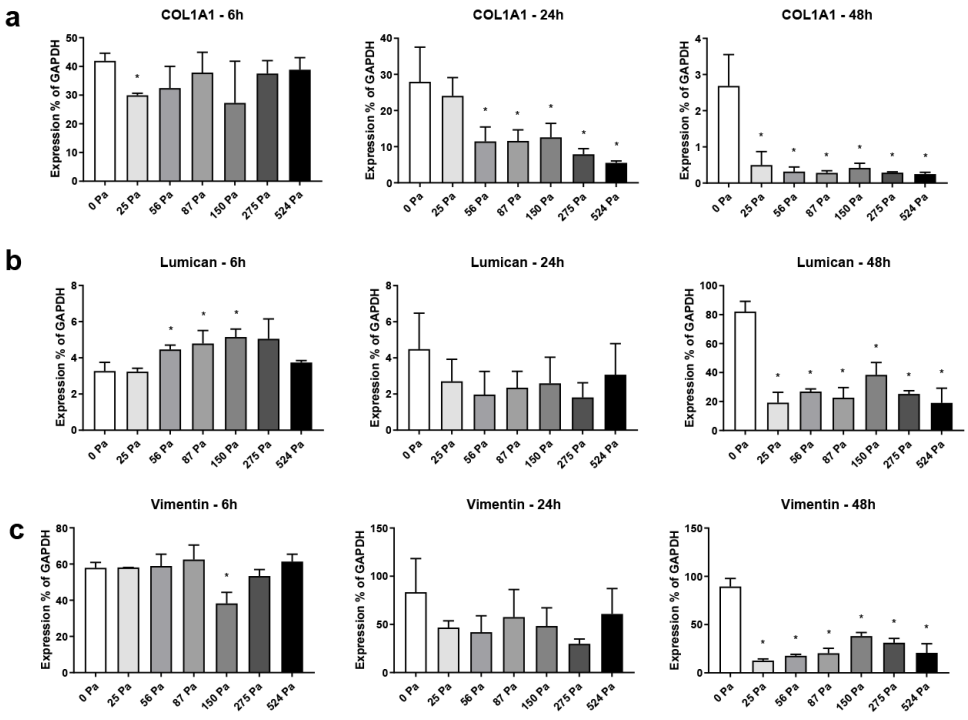

Figure 4. Mechanical compression suppresses the expression of genes related to corneal structural components. Human corneal fibroblasts (CFs) were subjected to various levels of mechanical compression for 6, 24, and 48 h. The mRNA expression levels of *COL1A1* (a), lumican (b), and vimentin (c) are shown for different pressure groups (n=3; **P*<0.05 vs. 0 Pa).