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Tension enhances cell proliferation and collagen synthesis by upregulating expressions of integrin αvβ3 in human keloid-derived mesenchymal stem cells

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Abstract

Aims: Keloids are a dermal fibrotic disease whose etiology remains totally unknown and for which there is no successful treatment. Mechanical tension, in addition, is closely associated with the germination and development of keloids. In this study, we investigated the influence of human keloid-derived mesenchymal stem cells (KD-MSCs) on cell proliferation, collagen synthesis, and expressions of integrin αvβ3 under tension.

Main methods: KD-MSCs and human normal skin-derived mesenchymal stem cells (NS-MSCs) were isolated and cultured in stem cell medium with a gradual increase in the serum concentration. Cell proliferation and collagen synthesis were detected by Cell Counting Kit-8 (CCK-8) assay and hydroxyproline content analysis under tension respectively. We investigated the messenger RNA expressions of nine integrin subunits, including integrin units α2, α3, α5, αv, α8, α10, α11, β1, and β3, in KD-MSCs stimulated with tension. Identification of differentially expressed genes was performed by Western blot analysis and immunocytochemistry staining.

Key findings: We obtained high-purity KD-MSCs and NS-MSCs using the culture method of decreasing serum concentration gradient gradually. Furthermore, we found that tension enhances cell proliferation and collagen synthesis and promotes expressions of integrin αvβ3 in KD-MSCs. In addition, blocking experiments showed that increased integrin αvβ3 expression affects cell proliferation and collagen synthesis of KD-MSCs under tension.

Significance: Our results suggest that integrin αvβ3 receptor may be sensitive molecules of mechanical tension and could contribute to the occurrence and development of keloids. It could lead to novel targets for therapeutic intervention, treatment, and prevention of recurrence for keloid disorders.

Keywords: Mechanical tension; Keloid; Mesenchymal stem cells; Cell proliferation; Collagen synthesis; Integrin αvβ3

1. Introduction

Keloid disease, which is characterized by exaggerated response to injury and formation of excessive scar tissues, is a fibroproliferative cutaneous tumor of ill-defined pathogenesis characterized by clinical, behavioral, and histological heterogeneity[1, 2]. Its main features include excessive fibroblast proliferation and the overproduction of extracellular matrix (ECM) components such as collagen[3, 4]. Unlike hypertrophic scars, which may take years to form and tend to regress over time, keloid scars would appear many years later and extend beyond the site of injury[5]. Keloids can grow excessively and invade nearby normal skin, so the patient with keloid suffers from great physical and psychological pressure. However, most treatments for managing keloids, including surgery, drug therapy, radiotherapy, laser therapy, and cryotherapy, have very limited effectiveness, because keloids grow slowly but progressively and the recurrence rate is very high. Therefore, there is a pressing need to seek a new therapeutic regimen.

Mesenchymal stem cells (MSCs), which can be isolated from many different adult human tissues[6, 7], are generally defined as plastic-adherent cells with a fibroblast-like morphology and

the capacity for multipotent differentiation in vitro[8, 9]. Lately, many efforts have focused on the MSCs that have been postulated as a therapeutic agent to prevent fibrosis[10]. Recent studies have demonstrated that keloid-derived mesenchymal stem cells (KD-MSCs) maintain a relatively higher proliferative rate than their normal skin counterparts[11], but it is still unfamiliar how keloid pathogenesis is affected. Therefore, it is important to research the KD-MSCs and its contribution to the keloid therapeutic strategies.

Through clinical and histopathologic observation, we found that keloid usually occurs at sites that are frequently subjected to mechanical tension, such as anterior chest and scapular regions. Furthermore, data from basic and clinical studies have shown that the pathogenesis of keloid is associated with local mechanical tension[12-14]. The conductive process of tension is a mechanical stimulus transformed into biochemical signals and participated in cellular responses. Coincidentally, integrins comprise one of the most important families of mechanical receptors, which connect the ECM with intracellular actin cytoskeleton and thereby mechanically integrate the extracellular and intracellular compartments. Integrins are heterodimeric transmembrane receptors composed of subunits α and β at a ratio of 1:1. To date, a total of 18 α subunits and 8 β subunits have been identified, and these can form 24 functional heterodimers, which recognize a specific set of ECM ligands[15, 16]. They regulate many aspects of cell behavior, including providing positive or negative feedback on the synthesis of the extracellular proteins themselves. Therefore, faulty or absent integrins could be involved in the etiology of keloids. Previous studies found that mechanical tension and integrin α2β1 regulate fibroblast functions using a three-dimensional cultured dermal fibroblast[17]. However, the direct effects of pathologic mechanical tension on KD-MSCs are unclear.

In this study, we first examined cell proliferation and collagen synthesis in KD-MSCs under tension. On this basis we further detected the expressions of nine integrin subunits, including integrin units α2, α3, α5, αv, α8, α10, α11, β1, and β3 in KD-MSCs and investigated which subunits may be involved in cell proliferation and collagen synthesis. This research may improve our understanding of integrins working in complex biological systems on MSCs.

2. Materials and methods

2.1. Patients and sample collections

Three patients with keloids were enrolled in this study. The characteristics of the research subjects are shown in Table 1. Human keloid and surrounding unaffected skin tissue specimens were obtained from postsurgery materials in accordance with the Ethics Review Committee of Tangdu Hospital of Fourth Military Medical University authorized approval. Patients signed an informed consent form. None of the patients had received treatment for the keloids prior to surgical excision. Keloid diagnosis was performed on the basis of their clinical appearance, history, and anatomical location. All lesions analyzed in this study satisfied the histopathological criteria for keloids as described previously[18]. Human keloid and skin tissue samples were transported to the laboratory in ice-cold sodium chloride physiological solution.

Sample No.	Age (years)	Sex	Ethnicity	Biopsy site	Duration (years)	Any other disease
1	18	Female	Han people	Right shoulder	2	none
2	27	Male	Han people	Chest	1	none
3	31	Male	Hui nationality	Chest	2	none

Table 1. Characteristics of patients enrolled in this study.

2.2. Primary isolation and culture of KD-MSCs and NS-MSCs

MSCs expansion was performed as described elsewhere[19, 20]. The specimens were washed with cold phosphate buffered saline containing Ca^{2+}/Mg^{2+} ion free, 2% antibiotic mix (penicillin/streptomycin) and fungizone 2 μg/mL. This treatment was repeated three times in order to get rid of germs completely. The specimens were cut into cords 4 to 6 mm^2 in diameter and incubated in dispase II (neutral protease grade II, Roche) 0.6 U/mL for 2 h at 37°C. The epidermis was manually removed from tissue cords after dispase II incubation with tweezers. Dermis was cut into 1-mm^3 pieces following enzymatic digestion with 0.25 % trypsin (Hyclone) for 15 min at 37°C. Afterward, tissue pieces were dissociated by repeated pipetting into the pipette, passed through a 40-μm cell strainer to isolate cells, and centrifuged at 1,200 rpm for 5 min. The pellet was suspended in serum-free stem cell medium (A10332-01, Cell Therapy Systems STEMPRO MSC SFM CTS™, Gibco) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/mL penicillin G, and 100 mg/mL streptomycin respectively. Cells were then placed in 25-cm^2 flasks (Corning, MA) and incubated with 5 % CO_2 at 37°C. After 9 or 10 days, the serum-free stem cell medium containing 5% FBS is used to stimulate cells. Two weeks later, the serum-free stem cell medium containing 1% FBS was used. After 2 or 3 days, use the serum-free stem cell medium to stimulate cells until confluence. In primary culture, more MSCs can be obtained by changing half of the medium every 2 to 3 days. Unattached cells were discarded and adherent cells were retained. The cells were passaged when primary cultures grew to approximately 80% to 90 % confluency.

2.3. Flow cytometry analysis

Third-generation adherent MSCs were used. Trypsinized cells (1×10^6/tube) were washed with fluorescence-activated cell sorting (FACS) buffer (2 % FBS and 0.1 % NaN3 in PBS), stained with fluorescence-conjugated antibody (1:50 dilution) for 30 min on ice and then analyzed using a FACS Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences). Percent staining was defined as the percentage of cells in the gate (M1), which was set to exclude ~99% of isotype control cells. The expression index was calculated by using the formula: mean fluorescence × percentage of positively stained cells[21]. Dead cells were excluded by propidium iodide staining. Human monoclonal antibodies were used as follows: anti-CD29-PerCP, anti-CD44-FITC, anti-CD45-PE, and anti-CD73-APC (all purchased from eBioscience, San Diego, CA). Cell samples incubated in the absence of primary antibody were used as negative controls[22].

2.4. MSCs culture, mechanical tension treatment

Third passage of keloid and normal skin MSCs were divided into four groups: tensile KD-MSCs group, static KD-MSCs group, tensile NS-MSCs group, and static NS-MSCs group. Mechanical tension experiments were performed by seeding KD-MSCs and NS-MSCs onto Falcon Cell Culture Inserts (BD Falcon #353091, 6-well format, 23.1-mm diameter, 3.0-μm pore size, BD Biosciences) as described in detail by Kim et al[23] previously. The inserts are slid back into the wells of Falcon Companion TC Plate (BD Falcon #353502, 6 wells, BD Biosciences). MSCs and serum-free stem cell medium are added to the insert, and then MSCs are cultured for the whole night (referred to as day 0 samples). The next day, MSCs were harvested after this 24 h preculture and served as negative controls. Next, a 5-mm steel bead was placed in the dish beneath the center of the insert polyethylene terephthalate (PET) membrane, and approximately 1 kg of weight was applied to the lid of the insert to force the edge of the insert down onto the bottom of the dish. This produced distortion of the PET membrane, increasing its surface area and thereby mechanically stretching the MSCs and their ECM. The tension was continued for 2 weeks with 5 % CO_2 at 37°C. The serum-free stem cell medium was changed every 3 days and the MSCs in both culture conditions were collected at days 1, 7, and 14 for further analysis. Static MSCs culturing on the PET membrane was also used as controls. The slug above and each PET membrane insert were cut out, and total RNA and protein were extracted respectively.

2.5. CCK-8 assay

Following 24-h stimulation with mechanical tension, MSCs were seeded at 10^4 cells/well in 96-well plates with 100 μL culture medium. Cell proliferation was evaluated by water-soluble tetrazolium salt at the indicated time points using a Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Haimen, China). Briefly, 10 μL CCK-8 solution was added to each well and the cells were incubated at 37°C for 4 h, 5% CO_2. The absorbance at 450 nm was detected at 1, 4, 7, 10, and 14 days using a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Quantitative analysis of hydroxyproline content

After stimulation with mechanical tension, the hydroxyproline (Hyp) content of the cell lysis buffer and cell supernatants was quantified with the Hydroxyproline Assay Kit (A030-2, Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The samples were hydrolyzed with 6 mol/L HCl at 95°C for 20 min for oxidation of free hydroxyproline. Then, 1 mL hydroxyproline developer (β-dimethylaminobenzaldehyde solution) was added to the samples and standards. The absorbances were evaluated at 1, 4, 7, 10, and 14 days by spectrophotometry (Thermo Fisher Scientific) at 550 nm. In the end, the samples hydroxyproline content was calculated according to the standard curve constructed with serial concentrations of commercial hydroxyproline.

2.7. Immunohistochemical staining

Immunohistochemistry was performed using Horseradish Peroxidase Immunohistochemistry

Kit (Rabbit) (Sangon Biotech, Shanghai, China). Tensile and static KD-MSCs were fixed in 4% paraformaldehyde at room temperature for 15 min and washed with PBS, respectively. Endogenous peroxidase was inhibited using 3% H_2O_2 for 15 min. The cells were then incubated at 4°C overnight with a rabbit antibody against human collagen I (1:500; ab34710; Abcam, Cambridge, UK). PBS was used as a negative control. Cells were incubated with horseradish peroxidase-conjugated (HRP-conjugated) goat anti-rabbit IgG (1:800, Sangon Biotech, Shanghai, China) as the secondary antibody (60 min at 37°C). After being visualized with diaminobenzidine (DAB, Sangon Biotech, Shanghai, China), the cells were counterstained with hematoxylin. Samples were hydrated, washed, and analyzed using microscopy.

2.8. Quantitative real-time polymerase chain reaction

Total RNA was extracted from cultured MSCs by use of RNAiso Plus (Takara Bio Inc.). The relative quantity of messenger RNA (mRNA) was determined by real-time polymerase chain reaction (PCR). RNA concentration was quantified by spectrophotometry and real-time PCR was carried out using the PrimeScript™ Reverse Transcriptase Kit (Takara Bio Inc.). Real-time PCR was performed using the SYBR Premix Ex Taq™ II kit (Takara Bio Inc.). As an internal control, levels of β-actin were determined in parallel with the target genes. The primer sets for human integrin units α2, α3, α5, αv, α8, α10, α11, β1, β3, collagen I, collagen III, and β-actin are shown in Table 2. Reactions were carried out at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30s. The relative mRNA expression level was assessed by the $2^{-\Delta\Delta CT}$ method.

2.9. Western blot analysis

The MSCs were washed twice with ice-cold PBS and then harvested with RIPA Lysis Buffer I (Sangon Biotech, Shanghai, China) containing phosphatase and protease inhibitors. The protein concentration was quantified using a bicinchoninic acid protein assay kit (CoWin Biotech, Beijing, China). The proteins in the lysates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime) and were then transferred onto polyvinylidene difluoride membranes (BioRad), blocked for 1 hour with 5% non-fat milk in a PBS/0.5% Tween 20 at room temperature. This was then incubated at 4°C overnight with the primary antibodies rabbit anti-integrin α8, rabbit anti-integrin αv, rabbit anti-integrin β3, and rabbit anti-β-actin (all Cell Signaling Technology, USA); rabbit anti-collagen I and rabbit anti-collagen III (all Abcam, UK). After washing with Tris buffered saline with Tween, membranes were incubated with appropriate secondary antibodies for 2 h. Blots were developed by use of an enhanced chemiluminescence system (Roche Applied Science) to detect the expression quantity of each protein.

Gene	Forward (5'-3')	Reverse (5'-3')
α 2	TCAGGGCACTATCCGCACAA	TCCATAGCCATCCAAGGACCTC
α 3	GTGCTGGACCCTGCACTTTG	GTTCCCGGCACTCTGGTTGTA

α 5	CAAAGCCCTGAAGATGCCCTA	ATCCACAGTGGGACGCCATA
α v	AATGTAATGATGAGCTTGGTGGAGA	AGGTGACATTGAGATGGGTAGTGG
α 8	TGAGCCTGCAAATCAACATCAC	CCAGTTATGAATGGGCAGAACAA
α 10	CACTTGTGAGCAGCTACACTTCCA	TGTCCAAGGCAAAGGTCACAG
α 11	CACCACAGGGATGTGTTCAAGAG	GCCATCCAGGACAATGACGA
β 1	AAGCGAAGGCATCCCTGAAAG	TGCTGCATTCACAATGTCTACCAA
β 3	GAGGTCATCCCTGGCCTCAA	CTGGCAGGCACAGTCACAATC
collagen I	CATCTGGTGGTGAGACTTGC	TCCTGGTTTCTCCTTTGG
collagen III	GTCCCAGCGGTTCTCCA	CCCCGTGCTCCAGTGAT
β -actin	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

Table 2. Primers used for real-time polymerase chain reaction.

2.10. Inhibitory experiments

Integrin αvβ3 inhibitor Cyclo(-RGDfK)(S7834) was purchased from Selleck Chemicals (Texas, USA). Third passage of KD-MSCs were divided into four groups: static KD-MSCs group, tensile KD-MSCs group, Cyclo KD-MSCs group, and tensile-Cyclo KD-MSCs group. KD-MSCs were seeded in Falcon Cell Culture Inserts at a density of 1×10^5/well. After 48 h, cells were treated with tension or 20 μM Cyclo for 48 h at 37°C. Next, cell proliferation and collagen synthesis were detected by CCK-8 assay, Quantitative real-time PCR and Western blot analysis used the same methods previously mentioned, respectively.

2.11. Statistics

Results were expressed as the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance was determined using t-test and one-way analysis of variance by GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, Calif). A value of $P<0.05$ indicated a statistically significant difference.

3. Results

3.1. Primary isolation and characterization of KD-MSCs and NS-MSCs

To obtain purified MSCs, we isolated MSCs from keloid and matched peripheral normal skin tissues. The phenotype of cells was small round at preliminary stage (Fig. 1aa or be) by use of the stem cell medium containing 10% FBS, and the growth rate was slow. After 1 or 2 weeks of

incubation, the number of cells increases rapidly, and cells appeared to have diversified morphology, such as round, and spindle-shaped vertebra (Fig. 1ab or bf) using the stem cell medium with 5% FBS. After 2 weeks of culture, the cells showed homogeneous spindle-shaped morphology (Fig. 1ac or bg) in stem cell medium containing 1% FBS. A few days later, cells showed changed fibroblast-like morphology (Fig. 1ad or bh) in serum-free stem cell medium gradually, and cells grew so fast that they can double in 2 or 3 days.

Flow cytometric analysis of passage 3 MSCs from keloid and normal skin tissues showed positivity for CD29, CD44, and CD73 but negativity for CD45. The expression of KD-MSCs surface markers detected by flow cytometry was 99.99% for CD29, 99.70% for CD44, 0.69% for CD45, and 99.99% for CD73 (Fig. 1c); meanwhile, the expression of NS-MSCs surface markers measured was 99.60% for CD29, 99.63% for CD44, 0.52% for CD45, and 99.06% for CD73 (Fig. 1d), respectively. It indicated that MSCs were not contaminated by hematopoietic cell lineages.

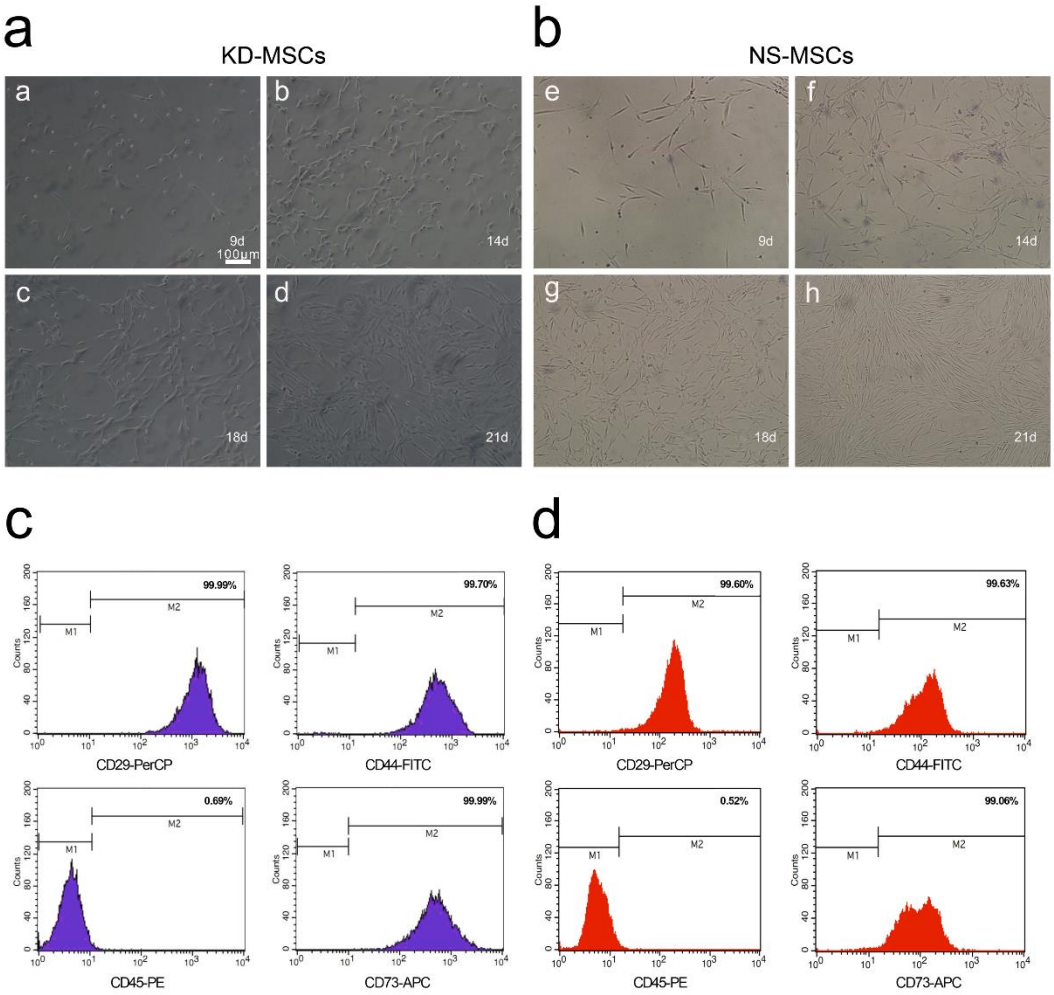

Fig.1. Morphological features and characterization of KD-MSCs and NS-MSCs: **a,** KD-MSCs or **b,** NS-MSCs. Cell morphology under cultured conditions of gradually decreased serum concentration. Primary MSCs were cultured using the stem cell medium with 10% FBS at 9 d (aa or be), 5%FBS at 14 d (ab or bf), 1%FBS at 18 d (ac or bg), and serum-free at 21 d (ad or bh)

respectively. **c** (KD-MSCs) or **d** (NS-MSCs), flow cytometric analysis of MSCs positive markers (CD29, CD44, and CD73) and negative markers (CD45). KD-MSCs: Keloid-derived mesenchymal stem cells, NS-MSCs normal skin-derived mesenchymal stem cells.

3.2. Mechanical tension increases cell proliferation in KD-MSCs

To better understand how the KD-MSCs proliferated under mechanical tension, we examined cell viability with a commercial CCK-8 proliferation kit at the indicated time points. Compared to the tensile and static groups, the cell proliferation was significantly increased in the tensile group at 1, 4, 7, 10, and 14 days ($P<0.01$; Fig. 2a). The results suggested that the mechanical tension enhances the proliferation of KD-MSCs.

3.3. Mechanical tension enhances collagen production in KD-MSCs

Hydroxyproline, a degradation product of collagen, represents an index of collagen secretion which, to an extent, can reflect cell secretion activity. Hydroxyproline content analysis was used to detect the amount of collagen in KD-MSCs under mechanical tension stimulating. As shown in Fig. 2b, compared to the static group, mechanical tension significantly increased the hydroxyproline content levels of the cell lysis buffer and cell supernatant in the tensile group at 1, 4, 7, 10, and 14 days ($P<0.01$).

We also tested the expression of collagen I and collagen III under mechanical tension. As shown in Fig 2c, the mRNA and protein levels of collagen I and collagen III in KD-MSCs were increased in response to mechanical tension. The effects of collagen I production of KD-MSCs under mechanical tension were further confirmed by immunohistochemistry. As shown in Fig. 3, KD-MSCs from the negative control group were stained negatively, whereas KD-MSCs in the mechanical tensile group were stained more intensely than those in the static cultured group.

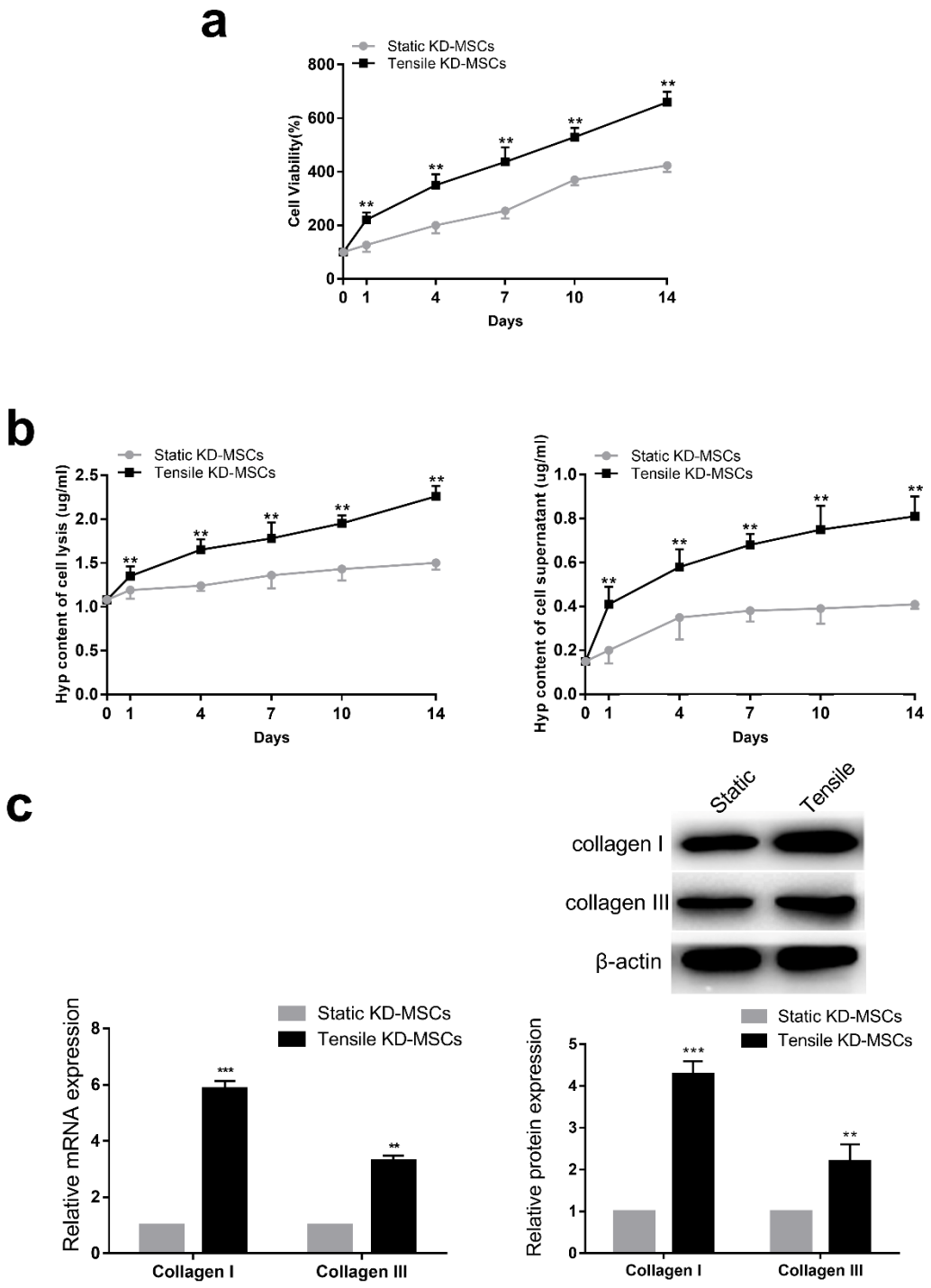

Fig.2. Mechanical tension increases cell proliferation and collagen synthesis in cultured KD-MSCs. **a** A Cell Counting Kit-8 assay was used to determine KD-MSCs viability for 1, 4, 7, 10, and 14 days. **b** Analysis of Hyp content in cell lysis buffer (*left*) and cell supernatant (*right*) for 1, 4, 7, 10, and 14 days. **c** The expression of collagen I and collagen III mRNA and proteins analyzed with real-time PCR and western blot under mechanical tension at 14 days (β-actin as the internal control). Results are presented as the mean ± SD, n =3. ***P* < 0.01, ****P* < 0.001.vs. static KD-MSCs at same time point. Hyp, hydroxyproline, KD-MSCs, keloid-derived

mesenchymal stem cells.

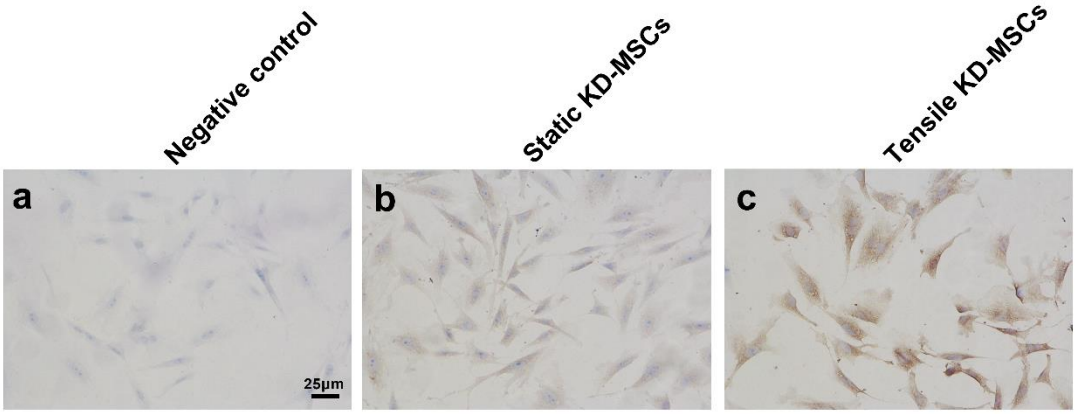

Fig.3. Immunohistochemical analysis of collagen I expression in tensile and static KD-MSCs. PBS was used as a negative control. KD-MSCs, keloid-derived mesenchymal stem cells.

3.4. Augmented expression of β3 integrin and decreased expression α8 integrin in KD-MSCs

The total RNA and protein were extracted respectively at 14 days. We investigated the mRNA and protein expressions of nine integrin subunits, including integrin units α2, α3, α5, αv, α8, α10, α11, β1, and β3, in KD-MSCs compared with NS-MSCs. Real-time PCR analysis indicated that the expression levels of α8 decreased obviously, and β3 increased significantly (Fig. 4a). Western blot analysis revealed that the changes in protein expression levels of integrin units α8 and β3 were consistent with those in their mRNA expression levels in both KD-MSCs and NS-MSCs (Fig. 4b). In addition, no significant difference was seen in mRNA expression between KD-MSCs and NS-MSCs for integrin units α2, α3, α5, αv, α10, α11, and β1.

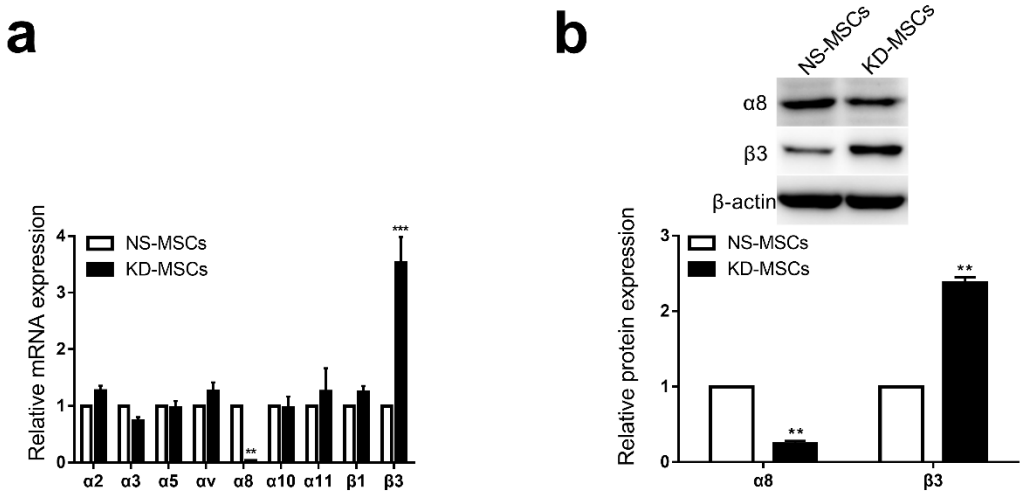

Fig.4. The expression of integrin genes and proteins in KD-MSCs and NS-MSCs. **a** Relative mRNA expression of integrin units α2, α3, α5, αv, α8, α10, α11, β1, and β3 measured by real-time polymerase chain reaction in KD-MSCs at 14 days (normalized by β-actin; relative to NS-MSCs). **b** Relative protein expression of integrin α8 and β3 analyzed with western blot in KD-MSCs at 14

days (β-actin as the internal control; relative to NS-MSCs). Results are presented as the mean ± SD, n =3. $^{**}P < 0.01$, $^{***}P < 0.001$. KD-MSCs, keloid-derived mesenchymal stem cells, NS-MSCs, normal skin-derived mesenchymal stem cells.

3.5. Mechanical tension promotes integrin αvβ3 expression in KD-MSCs

To determine whether integrins are involved in the mechanical tension of the KD-MSCs, we analyzed integrin expressions, including α2, α3, α5, αv, α8, α10, α11, β1, and β3 integrins, by real-time PCR and Western blotting at 14 days. There were no significant differences in α2, α3, α5, α8, α10, α11, and β1 integrin mRNA expressions, whereas αv and β3 integrin mRNA expressions were significantly increased in the mechanical tensile group of KD-MSCs, compared with the static group of KD-MSCs (Fig. 5a). The protein expression of αv and β3 integrin were observed to be higher in tensile KD-MSCs as compared to the static KD-MSCs (Fig. 5b).

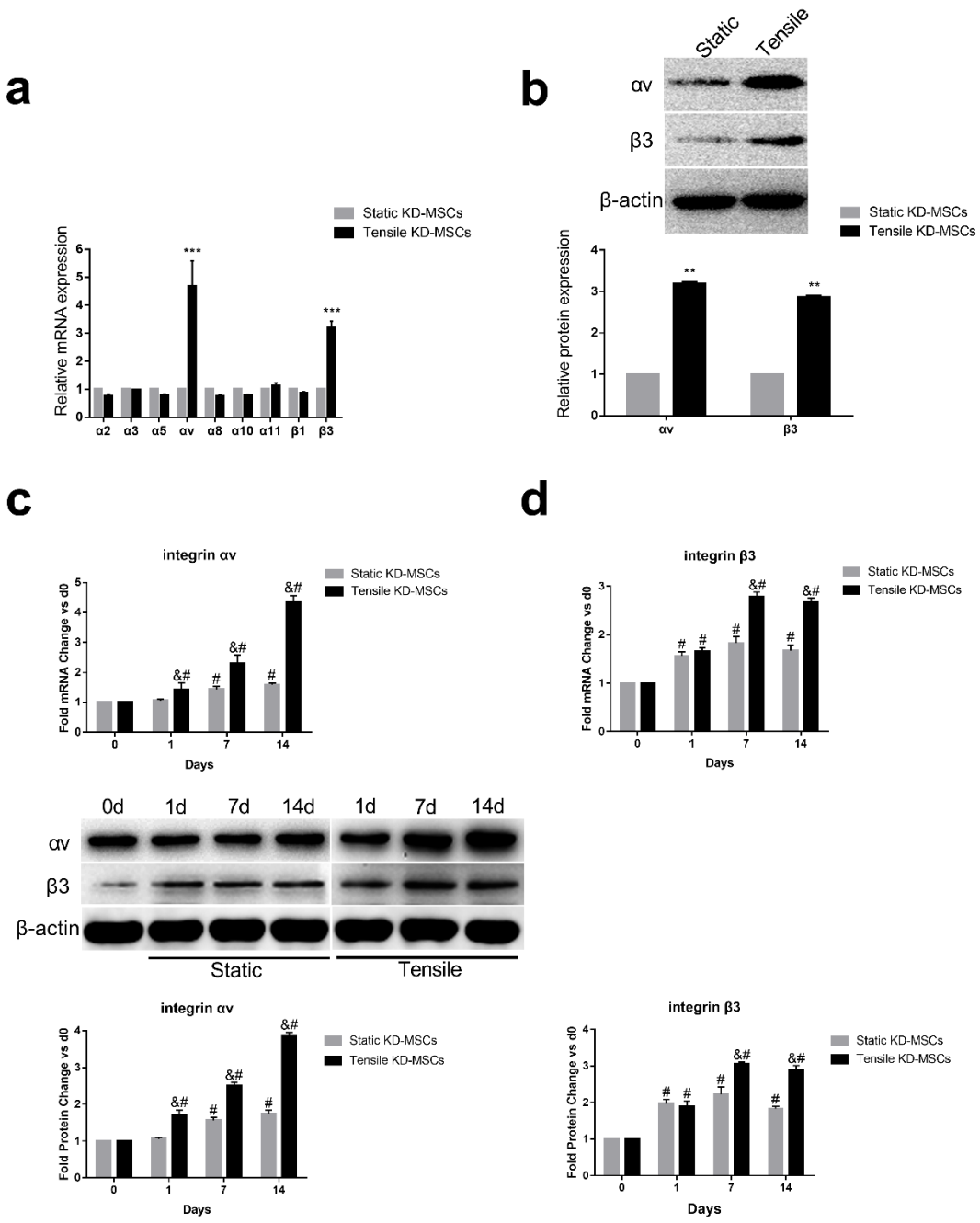

Fig.5. Effect of mechanical tension on integrin expression in KD-MSCs. **a** Real-time polymerase chain reaction (PCR) analysis of integrin units α2, α3, α5, αv, α8, α10, α11, β1, and β3 mRNA expression in KD-MSCs with tension for 14 days (normalized by β-actin; relative to static KD-MSCs). **b** Western blot analysis of integrin αv and β3 protein expression with tension for 14 days (β-actin as the internal control; relative to static KD-MSCs). **c d** The expression of integrin αv (c) and β3 (d) mRNA and proteins analyzed with real-time PCR and western blot under mechanical tension for 0, 1, 7, and 14 days (β-actin as the internal control). Results are presented as the mean ± SD, n =3. ***P* < 0.01, ****P* < 0.001. & Significance over static constructs at same time point. # Significance over day 0 for the same sample type. KD-MSCs, keloid-derived

mesenchymal stem cells.

Furthermore, we investigated dynamic changes in the expression of integrin αv and β3 for tensile KD-MSCs and static KD-MSCs. Significant differences were seen for integrin αv and β3 between tensile and static culture, particularly at later time points. Specifically, integrin αv mRNA and protein were significantly upregulated in tensile constructs on days 1, 7, and 14 as compared to static constructs at that time point. Upregulation of integrin αv mRNA and protein were also observed in both tensile and static samples at days 7 and 14 and in tensile samples on day 1 in comparison to day 0 (Fig. 5c). Integrin β3 mRNA and protein were significantly upregulated in tensile constructs on days 7 and 14 as compared to static constructs at that time point. Upregulation of integrin β3 mRNA and protein were also found in both tensile and static samples on days 1, 7, and 14 compared to day 0 (Fig. 5d).

3.6. No change in expression of integrin αvβ3 under mechanical tension in NS-MSCs

In the same way, we also detected the mRNA and protein levels of integrin αv and β3 in NS-MSCs stimulated by mechanical tension at 14 days. As shown in Fig. 6, there were no significant differences in mRNA and protein expression between the tensile group and the static group in NS-MSCs for integrin αvβ3.

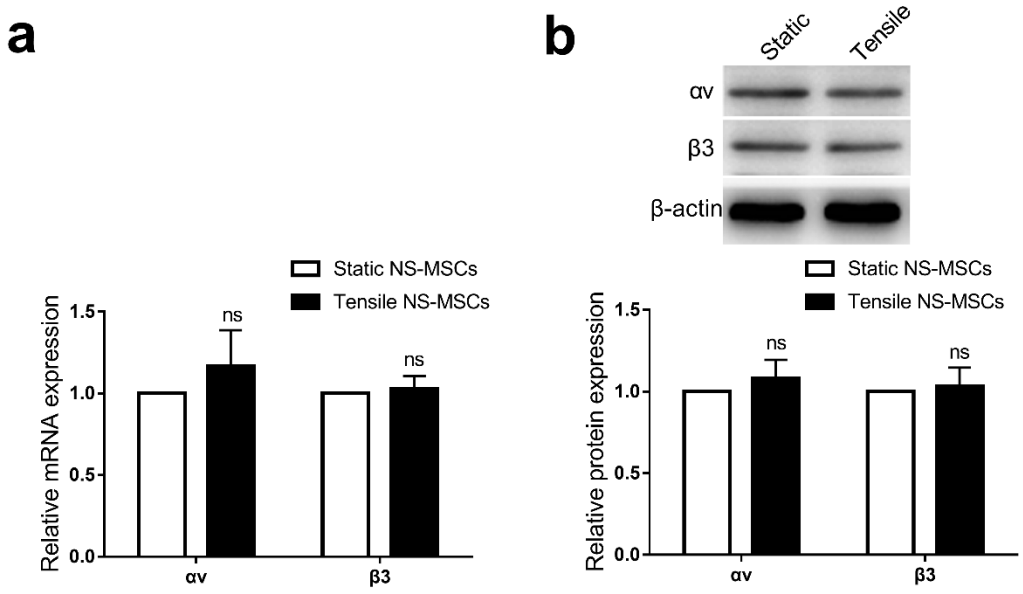

Fig.6. Effect of mechanical tension on integrin αv and β3 expression in NS-MSCs. **a** Relative mRNA expression of integrin αv and β3 measured by real-time PCR in NS-MSCs under mechanical tension at 14 days (normalized by β-actin; relative to static NS-MSCs). **b** Relative protein expression of integrin αv and β3 analyzed with western blot in NS-MSCs under mechanical tension at 14 days (β-actin as the internal control; relative to static NS-MSCs). Results are presented as the mean ± SD, n =3. ns, not significant. NS-MSCs, normal skin-derived mesenchymal stem cells.

3.7. Mechanical tension-mediated cell proliferation and collagen synthesis in KD-MSCs have decreased after inhibiting integrin αvβ3

According to previous experimental results, mechanical tension enhances cell proliferation and collagen synthesis and promotes expressions of integrin αvβ3 in KD-MSCs. Therefore, we investigated whether integrin αvβ3 played a role in cell proliferation and collagen synthesis. The experimental results indicated that treatment with Cyclo resulted in a dramatic decrease of KD-MSCs proliferation, collagen I, and collagen III for 48 h after either static culture or tensile culture. Notably, there were significant differences in cell proliferation and expression of collagen I and collagen III between the Cyclo group and tensile-Cyclo KD-MSCs group (Fig. 7). These data suggest that integrin αvβ3 are involved in mechanical tension-mediated cell proliferation and collagen synthesis, but not the only pathway to convert the change.

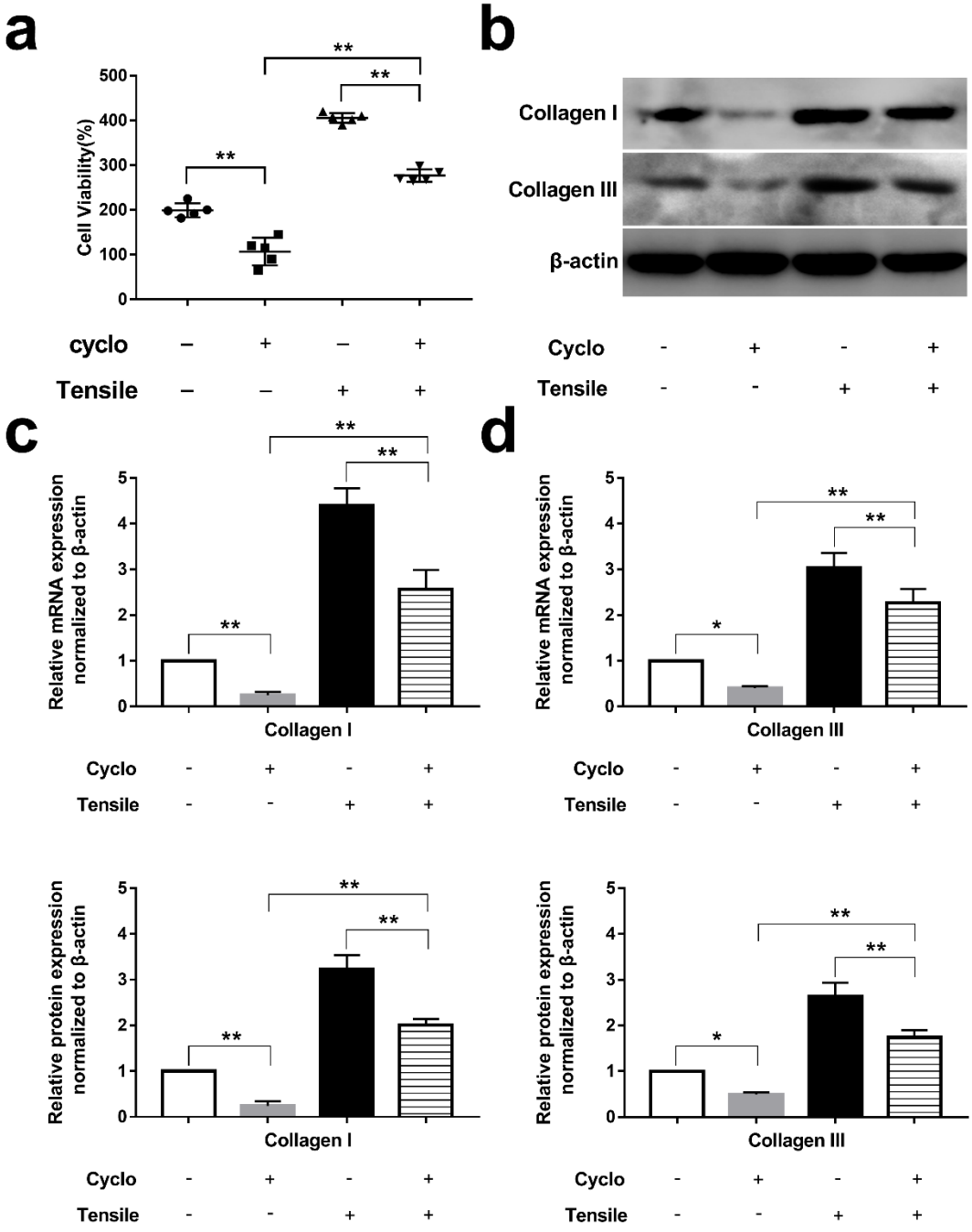

Fig.7. Effect of treatment with Cyclo on cell proliferation and collagen production in KD-MSCs after either static culture or tensile culture. **a** Cell proliferation of four groups in KD-MSCs detected by CCK-8 assay. **b c** Collagen I expression of four groups in KD-MSCs assessed by real-time PCR (*above*) and western blot (*below*). **b d** Collagen III expression of four groups in KD-MSCs assessed by real-time PCR (*above*) and western blot (*below*). Results are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01. KD-MSCs, keloid-derived mesenchymal stem cells.

4. Discussion

MSCs are multipotent cells derived from early developmental mesoderm, are self-renewing,

and migrate to sites of tissue injury[24]. They have the capacity to differentiate into osteocytes, adipocytes, and chondrocytes[25]. They are identified by their expression of mesenchymal markers, lack of hematopoietic markers, and adherence to plastic. Thus far, there is no uniform scheme for isolation and culture of MSCs *in vitro*. One of the major unsolved problems is the purity of MSC preparation for use in research and therapeutic purposes. In reality, because of a lack of specific MSC markers, the presence of contaminating mature stromal cells, such as fibroblasts, cannot be distinguished and quantified, particularly in a fresh preparation or even in an expanded culture of KD-MSCs. Therefore, it is very important to study the methods of culturing high-purity MSCs. Currently, there is no specific cell surface marker unique to MSCs and considerable variability in the cell surface marker expression profile has been observed between described MSC populations[26]. Some researchers in the world have isolated MSCs from keloids and normal skin tissues; hence, KD-MSCs used in this study express CD29, CD44, and CD73 and are negative for CD45 as described previously[11, 19, 27]. In this current research, the expression of KD-MSC surface markers detected was 99.99% for CD29, 99.7% for CD44, 0.69 % for CD45, and 99.96% for CD73; meanwhile, the expression of NS-MSCs surface markers tested was 99.60% for CD29, 99.63% for CD44, 0.52% for CD45, and 99.06% for CD73, respectively. These results indicated that we obtained high-purity MSCs from keloids using the culture method of decreasing serum concentration gradient gradually. The possible mechanism of action is that high concentrations of serum in the initial culture contribute to cell adhesion and proliferation; however, the growth of other cells was inhibited along with the decrease of serum concentration because of the possible lack of some nutrient elements. Thus, high-purity MSCs can be obtained from keloid and normal skin tissue. In addition, the growth rate of primary cultures of KD-MSCs was so slow that we considered our cell culture failed. At about 2 or 3 weeks, the MSCs can reach more than approximately 80 % confluence.

Keloids are pathologic scars that can cause serious functional and cosmetic problems for the patient, and to date, there are no good treatment options. Keloids are characterized by excessive production of ECM components including collagen I and collagen III[28]. Little is known about the molecular mechanisms that leads to keloid formation, and moreover, the lack of animal models have hampered the ability to study pathogenesis. The overproduction of collagen by proliferating fibroblasts is thought to be a major contributing factor. As discussed by Ogawa R, the mechanical force distribution around keloids revealed high skin tension at the keloid edges and lower tension at the keloid centers[12]. It also indicated that skin mechanical tension is closely associated with the pattern and degree of keloid growth. One of the major mechanisms for the signal transduction of mechanical forces is mediated by integrins. In this study, we first found that mechanical tension enhances cell proliferation and collagen synthesis in KD-MSCs. To further study this phenomenon, we focused on the integrins identified in studies on keloids and attempted to explore keloid pathogenesis by examining the expression differences of integrins in KD-MSCs. We assessed the expression patterns of α2, α3, α5, αv, α8, α10, α11, β1, and β3 integrins, and found that in

comparison with NS-MSCs, α8 integrin expression is downregulated and β3 integrin expression is upregulated in KD-MSCs. Whereas no marked differences were observed for integrin α8, integrin αv and β3 expression increased in KD-MSCs stimulated with mechanical tension.

In general, the integrin α8 subunit is known to dimerize exclusively with the integrin β1 subunit to form integrin α8β1 heterodimers[29], which is a receptor for fibronectin, vitronectin, tenascin C, osteopontin, nephronectin, and fibrillin-1[30, 31]. The integrin α8 subunit is expressed preferentially on mesenchymal cells but not on epithelial cells[32], and integrin α8β1 has an important role in lung and liver fibrosis[33]. Bouzeghrane et al[34] have reported that integrin α8β1 is upregulated in myofibroblasts of fibrotic and scarring myocardium in normal rat myocardial tissue. However, our studies revealed that integrin α8 expression is downregulated in KD-MSCs compared with NS-MSCs, and has no difference in KD-MSCs under tension. This result suggests that integrin α8 may be a negative feedback mechanism to regulate the fibrosis of keloids, however, it is not sensitive to tension.

As an important member of integrin, integrin αvβ3 plays an important role in regulating osteoclast[35], tumor proliferation[36], and angiogenesis[37]. In particular, αvβ3 integrin expression is frequently observed in aggressive tumors and has been shown to promote the adhesion, migration, and invasion of cancer cell. Montenegro et al[38] have reported that the αvβ3 integrin is the main receptor involved in cell directionality and its blockage may be an interesting alternative against metastasis. Integrin αvβ3 has received much attention as a potential antiangiogenic target because it is upregulated in tumor-associated blood vessels[39]. Keloids are defined as a dermal benign disorder and their behavior resembles that of tumor cells, with keloids exhibiting biological characteristics of tumor cells, including bioenergetics, hyperproliferation, and invasion[40]. Consistent with our findings, the tension of KD-MSCs induce upregulation of integrin αv and β3 expression; however, the expression of integrin αv and β3 was unchanged in NS-MSCs under mechanical tension. These reports and our results show that the expression of integrins αv and β3 are tissue specific between keloids and normal skin. It can also be shown that KD-MSCs are different from NS-MSCs. KD-MSCs are abnormal cell types highly sensitive to tension and may be associated with the pathogenesis of keloid. Notably, it is indicated that mechanical tension and integrin αvβ3 may play an important role in the occurrence and development of keloids. Interestingly, integrin β3 subunit expression is upregulated not only in KD-MSCs under mechanical tension but also in KD-MSCs with nonmechanical tension in comparison with NS-MSCs. This demonstrates integrin β3 is more sensitive to mechanical tension and can be used as target molecules of keloids for research. The latest research sets out to prove that intracellular tension differently affects the activation cycles of β1 and β3 integrins[41]. The evidence that changes in integrin gene expression translates into changes in collagen synthesis is entirely correlative. Urtasun R et al[42] have reported that recombinant osteopontin upregulated collagen I protein via integrin αvβ3 engagement in primary hepatic stellate cells. Last, a recent publication from Sun M et al[43] has demonstrated that an antibody blocking integrin α5

suppressed the expression of all osteoblast markers, including alpha-1 type I collagen, RUNX2, and BGLAP gene, in human mesenchymal stem cell. Our results suggested that cell proliferation and collagen synthesis was inhibited by Cyclo. It is proved that cell proliferation and collagen synthesis were regulated by integrin αvβ3 under mechanical tension in KD-MSCs. In particular, significant differences were seen for cell proliferation and collagen synthesis between the Cyclo group and the tensile-Cyclo KD-MSCs group. Conversely, it indicated that integrin αvβ3 is not the only signal transduction pathway implicated in cell proliferation and collagen synthesis under mechanical tension in KD-MSCs. Our work is similar to early work of Jumper et al[44], who performed large-scale studies by combining laser capture microdissection and transcriptomic array profiling of keloids versus normal surrounding tissue and a large number of high- or low-expression genes were found during the experiment. Interestingly, the αvβ3 integrin was not in the top 100 genes upexpressed or down- expressed. These results are different from our results, possibly because tension induces fibroblast differentiation through binding to αvβ3 integrin receptor in KD-MSCs. After completion of induction, the expression of αvβ3 integrin will gradually reduce to normal. Furthermore, a similar point has been made that a cancer-like stem cell population exists in keloids, and it provides a reasonable explanation for the persistent growth, recurrence and multidrug resistance characteristic of this disorder. Previous research found that TGF-β1/Smad pathway plays an important role in keloid formation[45, 46]. Our findings strongly implicate integrin αvβ3 in the pathogenesis of keloid. However, the effect of αvβ3 integrin in the TGF-β1/Smad pathway for keloids remains poorly understood. Many prior reports tend to claim that integrin αvβ3 is involved in the TGF-β1/Smad pathway in other diseases[47, 48]. Therefore, we hypothesized that mechanical tension mediated cell proliferation and collagen synthesis induced by integrin αvβ3 through the TGF-β1/Smad pathway for keloids. Obviously, additional studies would be required to research this hypothesis.

5. Conclusions

In conclusion, to the best of our knowledge, the findings of this study indicate for the first time that mechanical tension enhances cell proliferation and collagen synthesis and upregulates the expression of the integrin αvβ3 in KD-MSCs. Furthermore, we also demonstrate that mechanical tension upregulates cell proliferation and collagen synthesis via integrin αvβ3 in KD-MSCs. In summary, our findings may contribute to a better understanding of keloid pathogenesis and integrin αv or β3 may have potential as a target molecule for injection treatment of the early stage of keloids.

Abbreviations

MSCs: Mesenchymal stem cells; KD-MSCs: keloid-derived mesenchymal stem cells; NS-MSCs: Normal skin-derived mesenchymal stem cells; ECM: Extracellular matrix; PBS: Phosphate-buffered saline; FBS: Fetal bovine serum; FACS: Fluorescence-activated cell sorting; PerCP: Peridinin chlorophyll protein; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; APC: allophycocyanin; RT-PCR: Reverse-transcription polymerase chain reaction; CCK-8: Cell

Counting Kit-8; Hyp: Hydroxyproline; SD: Standard deviation; TGF: Transforming growth factor

Conflicts of interest statement

The authors declare that they have no conflict of interest.

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