Original Paper

Integrin $\beta 1$ Gene Therapy Enhances *in Vitro* Creation of Tissue-Engineered Cartilage Under Periodic Mechanical Stress

Wenwei Liang^a Chunhui Zhu^a Feng Liu^a Weiding Cui^a Qing Wang^a
Zhifeng Chen^a Qijia Zhou^b Shun Xu^a Chenjun Zhai^a Weimin Fan^a

^aDepartment of Orthopedics, The First Affiliated Hospital of Nanjing Medical University, Nanjing,

^bDepartment of Orthopedics, Funing People Affiliated Hospital of Nantong University, Yancheng, China

Key Words

Periodic mechanical stress • Integrin $\beta 1$ • Up-regulation • Chondrocyte proliferation • Matrix synthesis

Abstract

Background/Aims: Periodic mechanical stress activates integrin $\beta 1$ -initiated signal pathways to promote chondrocyte proliferation and matrix synthesis. Integrin $\beta 1$ overexpression has been demonstrated to play important roles in improving the activities and functions of several non-chondrocytic cell types. Therefore, in the current study, we evaluated the effects of integrin $\beta 1$ up-regulation on periodic mechanical stress-induced chondrocyte proliferation, matrix synthesis and ERK1/2 phosphorylation in chondrocyte monolayer culture, and evaluated the quality of tissue-engineered cartilage constructed *in vitro* under periodic mechanical stress combined with integrin $\beta 1$ up-regulation. **Methods and Results:** Our results revealed that under periodic mechanical stress, pre-treatment with integrin $\beta 1$ -wild type vector significantly enhanced chondrocyte proliferation and matrix synthesis and promoted ERK1/2 phosphorylation in comparison to mock transfectants. Furthermore, when chondrocytes were seeded in PLGA scaffolds, more accumulated GAG and type II collagen tissue were detected after Lv-integrin $\beta 1$ transfection compared with sham controls exposed to periodic mechanical stress. In contrast, in the Lv-shRNA-integrin $\beta 1$ group, the opposite results were observed. **Conclusion:** Our findings collectively suggest that in addition to periodic mechanical stress, integrin $\beta 1$ up-regulation in chondrocytes could further improve the quality of tissue-engineered cartilage.

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W. Liang and C. Zhu contributed equally to this work.

Weimin Fan

Department of Orthopedics, The First Affiliated Hospital of Nanjing Medical University,
300 Guang Zhou Road, Nanjing 210029, (China)
Tel. +86-25-83718836-6780, E-Mail liangwenwei321@sina.com

Introduction

Periodic mechanical stress has been shown to significantly enhance the quality of tissue-engineered cartilage [1-3]. Pingguan-Murphy and Nawi demonstrated that cyclic mechanical loading promoted glycosaminoglycan synthesis by bovine chondrocytes cultured in an agarose gel [4]. A study by Wang et al. revealed that under conditions of cyclic compression, the expression of type II collagen and aggrecan were significantly increased in rabbit chondrocytes [1-3]. The mechanisms by which chondrocytes sense and respond to periodic mechanical stress were also investigated. The MAPK signal cascade, especially ERK1/2 activity, has been proven to be associated with the biological effects induced by periodic mechanical stress in chondrocytes [5, 6]. Our previous studies demonstrated that periodic mechanical stress could activate three signaling pathways converging on ERK1/2: integrin $\beta 1$ -Src-Rac1-FAK-Tyr^{576/577}-ERK1/2, integrin $\beta 1$ -FAK-Tyr³⁹⁷-ERK1/2 and integrin $\beta 1$ -Src-PLC $\gamma 1$ -ERK1/2 to promote chondrocyte proliferation and matrix synthesis [7-10].

Integrin is the main receptor that plays an important role in mechanosensing and signal transduction [11-13]. Many studies have shown that integrin inhibition blocks the biological effects induced by mechanical stimulation in chondrocytes [14-16]. The work of Chowdhury et al. demonstrated that when integrin $\alpha 5 \beta 1$ was attenuated by GRGDSP in articular chondrocytes, dynamic compression-induced ³⁵SO₄ and [³H] thymidine were significantly reduced [17]. We previously observed that pretreatment of chondrocytes with blocking antibodies against integrin $\beta 1$ resulted in blockage of periodic mechanical stress-stimulated up-regulation of chondrocyte proliferation and matrix synthesis as well as ERK1/2 phosphorylation [9]. Furthermore, when subjected to numerous non-mechanical stimuli, integrin abolition also affected many cellular behaviors and functions in chondrocytes [18, 19].

As integrin $\beta 1$ inhibition markedly reduces the activities and functions of chondrocytes, this raised the possibility that integrin $\beta 1$ overexpression could elevate chondrocyte activity. Reviewing the past literature, we found no reports concerning the relationship between integrin $\beta 1$ up-regulation and cellular response of chondrocytes. However, in several non-chondrocytic cell types, integrin up-regulation resulted in a significant improvement in cellular activities. Liu et al. found that integrin $\beta 4$ overexpression was able to accelerate wound repair by promoting proliferation of airway epithelial cells [20]. Hu et al. discovered that when integrin $\alpha 2$ was overexpressed in hBMSCs from patients with senile osteoporosis, proliferation and osteogenic differentiation of hBMSCs were both significantly increased, accompanied by elevated ERK1/2 phosphorylation [21]. Furthermore, an earlier study in vascular smooth muscle cells revealed that either subjecting the cells to cyclic stretching or forcing expression of integrin $\alpha v \beta 3$ could abolish Ox-LDL-induced cytoskeleton disruption and apoptosis [22]. Thus, we speculated that in chondrocytes subjected to periodic mechanical stress, integrin $\beta 1$ over-expression would further promote chondrocyte proliferation and matrix synthesis accompanied by enhanced ERK1/2 phosphorylation.

Above all, the main aim of the present study was to explore whether, in chondrocytes under periodic mechanical stress, integrin $\beta 1$ -overexpression could promote formation of higher quality tissue-engineered cartilage. After integrin $\beta 1$ up-regulation, we measured chondrocyte proliferation and matrix synthesis in 2D culture and assessed the quality of cartilage tissue formation *in vitro* under conditions of periodic mechanical stress, with a view to seeking convincing methods for constructing better quality tissue-engineered cartilage in future.

Materials and Methods

Materials

Two-week-old Sprague-Dawley (SD) rats of either sex were provided by the Animal Center of Nanjing Medical University. Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering

Materials Co., Ltd (Zhejiang, China). DMEM-F12, trypsin, collagenase II, and type II collagen were obtained from Sigma (St Louis, MO). Cell Counting Kit-8 (CCK-8) was acquired from Beyotime Institute of Biotechnology (Jiangsu, China). Anti-integrin $\beta 1$ rabbit antibody (CST Co., Ltd, #4706S), anti-ERK1/2 rabbit antibody (CST Co., Ltd, #9102S), anti-phospho-ERK1/2 (Thr202/Tyr204) rabbit antibody (CST Co., Ltd, #9101S), and HRP-goat anti-rabbit IgG were supplied by CST Co., Ltd (USA). Anti-collagen II rabbit antibody (Abcam, ab34712) and anti-aggreCAN rabbit antibody (Abcam, ab36861) were supplied by Abcam (Cambridge, MA). HRP-conjugated GAPDH antibody was supplied by Kang Chen (Shanghai, China, KC-5G5). ECL was purchased from Thermo Scientific (Waltham, MA). RNAiso Plus, PrimeScript RT Reagent Kit and SYBR Premix Ex Taq II were acquired from TaKaRa (Shiga, Japan). Integrin $\beta 1$ shRNA lentiviral particles, control shRNA lentiviral particles and Polybrene, as well as the GV287 vector, were supplied by Genechem (Shanghai, China). Wild-type integrin $\beta 1$ cDNA was subcloned into GV287 to force the expression of integrin $\beta 1$; a GFP-containing blank vector (GV287) served as the control.

A cell culture incubator (Heraeus BB 5060), an air-tight cell culture device and reciprocating pressure pump, barrier type pressure transducer, and inverted microscope equipped with a camera system were acquired from Heraeus (Hanau, Germany), Taixing Experimental Instrument Factory (Jiangsu, China), Tianjin Plastics Research Institute (Tianjin, China) and Olympus (Tokyo, Japan), respectively.

Cell culture

Chondrocytes were harvested using the method described by Séguin and Bernier [23]. Briefly, under sterile conditions, cartilage derived from the limb joints of two-week-old SD rats was harvested and sliced into 1 mm³ pieces. Tissues were digested with 0.25% trypsin at 37°C for 0.5 h, followed by digestion with 0.2% collagenase II at 37°C for 4 h. Cells were harvested by filtering with a 200-mesh filter and cultured in DMEM-F12 medium supplemented with 10% FBS in an incubator at 37°C with 5% CO₂. The cell population was purified by repeated adherence, and morphology was examined under an inverted phase-contrast microscope following staining with collagen type II according to the conventional ABC method. Second-generation cells were seeded onto glass slides (25 mm × 25 mm) coated with type II collagen at a density of 10⁵ cells. Experiments were performed when cells reached 70–80% cell confluence.

Scaffold preparation

To promote cell attachment, the scaffolds (length of side 8 mm, height 2 mm polylactide (PLA)–polyglycolic acid (PGA) 9:1, porosity 90%; pore size 100 μ m) were pre-immersed in polylysine, and then sterilized with ethylene oxide, rinsed with phosphate-buffered saline (PBS), dried and stored in a desiccator before cell seeding.

In vitro construction of engineered cartilage

Rat chondrocytes at passage two were seeded into the scaffolds at a density of 5×10^7 cells/cm³, with a total of 6.4×10^6 cells per scaffold. To promote complete adhesion of cells to the scaffolds, the cell-scaffold constructs were then incubated at 37°C under 5% CO₂ for 4 h. Then, the constructs were covered with DMEM-F12 supplemented with 10% FBS and incubated at 37°C with 5% CO₂. The medium was replaced every 2 days and the constructs were cultured up to 6 weeks for further evaluation.

Construction of a periodic mechanical stress field

A periodic stress field encompassing the perfusion culture system with adjustable stress intensity and frequency was constructed by connecting a reciprocating intensifier pump to the air-tight cell culture device through a barrier-type pressure transducer, as described previously [7]. In an earlier study, we showed that rabbit chondrocytes subjected to stress varying from 0 to 200 kPa at 0.1 Hz yielded tissue-engineered cartilage of the best quality. Accordingly, a pressure range of 0–200 kPa and 0.1 Hz frequency were used in the current study.

Experimental groups

The experiment comprised two steps. In the first step, cells were pretreated with shRNA targeted to integrin $\beta 1$, wild-type integrin $\beta 1$ vector or GFP-vector (GV287). Cells were maintained under static conditions or periodic mechanical stress for 1 h prior to western blot analysis (ERK1/2 phosphorylation), 8 h prior to qPCR analysis (aggreCAN and type II collagen gene expression), and then subjected to mechanical stress for 8 h per day for 7 days prior to western blot analysis (aggreCAN and type II collagen protein

expression) and 3 days prior to direct cell counting and CCK-8 assay. All groups were incubated at 37°C under 5% CO₂.

In the second step, after pretreatment as described above, the different treatment groups of rat chondrocytes were seeded onto PLGA scaffolds and maintained under conditions of periodic mechanical stress for 6 weeks (8 h per day mechanical stress) prior to analysis of gross view and wet weight, GAG content, and histological and immunohistochemical assay. All groups of cells were incubated at 37°C under 5% CO₂.

Transfection analysis

The full length coding region of rat integrin $\beta 1$ cDNA (NM_017022) was inserted into a GV287 vector (Ubi-MCS-3FLAG-SV40-EGFP) to generate a GV287/integrin $\beta 1$ (+) overexpression vector, with transcription initiated by the CMV promoter. Chondrocytes transfected with mock control GV287 vector served as a control. The shRNA targeting integrin $\beta 1$ (GGGCTGAAGACTACCCTAT) and negative control sequences (TTCTCCGAACGTGTCACGT) were ligated into a GV118 vector (mU6-MCS-Ubi-EGFP), respectively. After the different fragments were combined with lentivirus vectors, they were transferred into 293T cells to package lentivirus particles.

Chondrocytes were divided into three treatment groups. Each group of cells was plated at a concentration of 10⁵ cells/well into six-well plates and allowed to adhere for one day before transfection. Next day the cells were transfected with Lv-integrin $\beta 1$ vector, Lv-shRNA-integrin $\beta 1$ vector or control Lv-GFP vector in serum-free medium. Twelve hours after transfection, the media were all changed to complete medium. Three days after transfection, GFP expression was detected by fluorescence microscopy, and the expression of integrin $\beta 1$ protein was assessed by western blot analysis.

Proliferation studies

Proliferation was assessed by two different methods, specifically, direct cell counting and the CCK-8 assay.

Direct cell counting

Cells were trypsinized and counted according to published protocols [24]. Second-generation chondrocytes were seeded onto glass slides (25 mm × 25 mm) coated with type II collagen at a density of 10⁵ cells and randomly divided into different groups, each comprising six glass slides. Experiments were performed when cells had reached approximately ~70–80% confluence. Chondrocytes were cultured for 3 days under non-pressure conditions or periodic mechanical stress for 8 h per day, prior to direct cell counting. Cells were trypsinized and counted, and the cell number was determined by counting cells from each glass slide independently. Experiments were repeated five times.

CCK-8 assay

Cell proliferation was determined using CCK-8 solution according to the manufacturer's (Dojindo Lab., Kumamoto, Japan) instructions [25]. Cells were added to 10 μ L of CCK-8 solution in each well of five 96-well plates (n = 5) and incubated for 4 h at 37°C. The absorbance of each well was determined at 450 nm using a microplate reader.

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted using RNAiso Plus and reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit, according to the manufacturer's protocol (Shiga, Japan). qPCR analysis was performed with the LightCycler System (Roche Diagnostics, Basel, Switzerland) using SYBR Premix Ex Taq II, as described previously [26]. The reaction was performed in a 20 μ L mixture containing 2 μ L cDNA. Each cDNA sample was amplified using specific primers (Table 1, TaKaRa) under the following cycling conditions: 30 s initial denaturation at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. Gene expression for AGC and Col2 was normalized against that for GAPDH.

Western blot analysis

Total protein was prepared and western blot analyses were performed as described previously [27]. Total protein was prepared using RIPA buffer, and the Bradford assay was employed to determine protein concentrations. Protein samples were resolved using SDS-PAGE, and transferred to nitrocellulose membranes. Following blocking for 1 h with 5% skimmed milk in TBST, membranes were incubated with

Table 1. Primer sequences and product sizes of real-time PCR. F: forward; R: reverse; AS: amplicon size; bp: base pairs; AT: annealing temperature

Genes	Sequence of primers (5'-3')	AS(bp)	AT(degrees)
Aggrecan	F GAAGTGATGCATGGCATTGAGG R ATGATGGCGCTGTTCTGAAGG	146	60
Type II collagen	F GAGGGCAACAGCAGGTTTAC R TGTGATCGGTACTCGATGATGG	95	60
GAPDH	F GGCACAGTCAAGGCTGAGAATG R ATGGTGGTGAAGACGCCAGTA	143	60

antibodies (1:1,000 dilutions for all antibodies) overnight at 4°C. Blots were incubated with horseradish peroxidase-conjugated secondary antibody at ambient temperature for 1 h, and colors were subsequently developed with ECL. Results were scanned using a gel imaging system (UVP LLC, Upland, CA) and measured using Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD).

Gross View and Wet Weight

The constructs harvested from Lv-integrin β 1, Lv-shRNA-integrin β 1 and Lv-GFP groups were continuously recorded by photography to evaluate macroscopic morphology. At the end of the 6-week culture period, differences in wet weight between specimens were assessed using an electronic balance.

GAG Content

After 6 weeks in culture, tissues from each group were collected and GAG content was recorded. The samples were rinsed with double-distilled H₂O, lyophilized for 12 h, then 1 mL cold H₂O was added and the solution was incubated at 4°C in a microcentrifuge tube overnight. After lysing by repeated cycles of freeze-thawing and sonication, samples were centrifuged at 8,000 rpm for 3 min. The supernatants were collected and 25 mL of the supernatant was mixed with 5 mL NaCl (2.3 M) and 200 mL 9-dimethylmethylene blue (DMMB) chloride solution (Sigma-Aldrich). Absorption at 520 nm was then measured using a spectrophotometer. A standard curve of known concentrations of chondroitin sulfate B (Sigma-Aldrich) was run concurrently and used to determine unknown sample concentrations.

Histological and immunohistochemical analysis

For histological examination, the engineered cartilage samples were washed with PBS and fixed in formalin. They were then embedded in paraffin and cut into 4- μ m thick sections. The sections were stained with hematoxylin-eosin (HE) and safranin-O. For immunohistochemical analysis of type II collagen, to block the endogenous peroxidase, the deparaffinized sections were incubated with H₂O₂ and methanol for 10 min and then incubated for 30 min with serum blocking solution. The sections were then incubated with the primary antibodies (Abcam, ab34712, 1:200 dilutions for all antibodies) for 1 h, followed by incubation with the horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies for 30 min. To visualize the bound antibodies, the sections were incubated with DAB for 10 min. All of the incubations were performed at room temperature.

Statistical analysis

Statistical analyses were performed using SPSS 14.0 software, and results are expressed as mean $\pm$ standard deviation. Student's unpaired *t*-test and one-way analysis of variance (ANOVA), followed by post-hoc Fisher's least significance difference (LSD) test, were used to determine statistical significance. A *P*-value < 0.05 was considered significant.

Results

Transfection of lentivirus vectors

To investigate the role of integrin β 1 up-regulation in periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis, we employed lentivirus vector Lv-integrin β 1 to up-regulate integrin β 1 expression and constructed lentivirus vector Lv-shRNA- integrin β 1 to suppress integrin β 1 expression, while the empty vector Lv-GFP

Fig. 1. The transfection efficiency of chondrocytes was estimated by light microscopy (a) and fluorescence microscopy (b). Comparing cells in bright field and fluorescent views of the same field suggested that the transfection efficiency was more than 80% (c).

Fig. 2. The effects of transfection with Lv-GFP, Lv-shRNA-integrin $\beta 1$ and Lv-integrin $\beta 1$ vectors on the relative protein expression of integrin $\beta 1$. Chondrocytes were pretreated with shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$) or non-targeting sequences (Lv-GFP) prior to lysis and western blotting for integrin $\beta 1$ proteins. Transfection with shRNA for integrin $\beta 1$ or wild-type integrin $\beta 1$ achieved ~60% reduction or ~50% increase in integrin $\beta 1$ protein levels, respectively ($n=5$, $p<0.05$ for each, One-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significance difference (LSD) test).

served as a control. Three days after transfection, we evaluated GFP expression in each group by fluorescence microscopy. More than 80% of chondrocytes were effectively transfected suggesting high transfection efficiency (Fig. 1).

Expression of integrin $\beta 1$ protein after transfection

The protein expression levels of integrin $\beta 1$ in each group were analyzed by Western blotting. We found that compared with the Lv-GFP group, integrin $\beta 1$ expression was significantly increased in the Lv-integrin $\beta 1$ group ($p<0.05$ for each, Fig. 2, $n=5$), while Lv-shRNA-integrin $\beta 1$ transfection markedly abolished integrin $\beta 1$ expression in the Lv-shRNA-integrin $\beta 1$ group ($p<0.05$ for each, Fig. 2, $n=5$).

Effect of integrin $\beta 1$ -upregulation on chondrocyte proliferation and matrix synthesis under periodic mechanical stress

After pretreatment with Lv-integrin $\beta 1$, Lv-shRNA-integrin $\beta 1$ and Lv-GFP plasmids, proliferation and matrix synthesis of chondrocytes exposed to periodic mechanical stress or

Fig. 3. Effects of inhibition or up-regulation of integrin $\beta 1$ on proliferation and gene expression of aggrecan and type II collagen in chondrocytes subjected to periodic mechanical stress or under static conditions. After pretreatment with control vehicle (Lv-GFP) or shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$), rat chondrocytes were cultured for 3 d under static conditions or conditions of periodic mechanical stress for 8 h per day prior to proliferation studies, or for 8 h with or without periodic mechanical stress prior to analysis of gene expression of aggrecan and type II collagen. Chondrocyte proliferation was analyzed using direct cell counting (a) and CCK-8 assay (b). Gene expression of aggrecan (c) and type II collagen (d) was analyzed using qPCR assay. Chondrocyte proliferation and gene expressions of aggrecan and type II collagen in Lv-integrin $\beta 1$ groups and Lv-shRNA-integrin $\beta 1$ groups were significantly increased and diminished, respectively, in comparison with Lv-GFP groups in response to periodic mechanical stress. ($n = 5$, $p < 0.05$ for each, analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Fisher's least significance difference (LSD) test). Under static conditions, there were no differences among these groups. ($n = 5$, $p > 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test).

under static conditions were examined. We found that stimulation by periodic mechanical stress strongly increased chondrocyte proliferation as well as aggrecan and type II collagen gene and protein expression in the Lv-integrin $\beta 1$ group compared to the Lv-GFP group ($p < 0.05$ for each, Fig. 3, 4, $n = 5$), while the Lv-shRNA-integrin $\beta 1$ group showed the opposite results ($p < 0.05$ for each, Fig. 3, 4, $n = 5$). However, when cultured under static conditions, chondrocyte proliferation and matrix synthesis were not affected by transfection with Lv-integrin $\beta 1$ or Lv-shRNA-integrin $\beta 1$ ($p > 0.05$ for each, Fig. 3, 4, $n = 5$).

Fig. 4. Effects of inhibition or up-regulation of integrin $\beta 1$ on protein expression of aggrecan and type II collagen in response to periodic mechanical stress or under static conditions. After pretreatment with control vehicle (Lv-GFP) or shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$), rat chondrocytes were cultured for 7 d under static conditions or conditions of periodic mechanical stress for 8 h per day prior to analysis of protein expression of aggrecan and type II collagen. Protein expression of aggrecan (a) and type II collagen (b) were analyzed using western blot assay. Protein expression of aggrecan and type II collagen in Lv-integrin $\beta 1$ groups and Lv-shRNA-integrin $\beta 1$ groups were significantly elevated and blocked, respectively, in comparison with Lv-GFP groups in response to periodic mechanical stress. ($n = 5$, $p < 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test). Under static conditions, there were no differences among these groups. ($n = 5$, $P > 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test).

Effects of integrin $\beta 1$ up-regulation on periodic mechanical stress-induced ERK1/2 phosphorylation

To clarify the specific role of integrin $\beta 1$ up-regulation in periodic mechanical stress-induced ERK1/2 phosphorylation, we transfected the cells with Lv-integrin $\beta 1$, Lv-shRNA-integrin $\beta 1$ or Lv-GFP plasmids. We observed that ERK1/2 activation in the Lv-shRNA-integrin

Fig. 5. Effects of inhibition or up-regulation of integrin $\beta 1$ on the expression and phosphorylation of ERK1/2 under conditions of periodic mechanical stress or static conditions. After pretreatment with control vehicle (Lv-GFP) or shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$), rat chondrocytes were cultured for 1 h under static conditions or conditions of periodic mechanical stress. The expression and phosphorylation levels of ERK1/2 were detected by western blotting. Optical density values are represented as a histogram ($n = 5$, $*$, $p < 0.05$ for each). The images shown are from a representative western blot. The phosphorylation levels of ERK1/2-Thr²⁰²/Tyr²⁰⁴ in Lv-integrin $\beta 1$ groups and Lv-shRNA-integrin $\beta 1$ groups were significantly increased and reduced, respectively, in comparison with Lv-GFP groups in response to periodic mechanical stress. ($n = 5$, $p < 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test). Under static conditions, there were no differences among these groups. ($n = 5$, $p > 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test).

$\beta 1$ group and the Lv-integrin $\beta 1$ group were relatively reduced or increased, respectively, compared with the Lv-GFP group, when exposed to periodic mechanical stress ($p < 0.05$ for each, Fig. 5, $n=5$), whereas stationary culture did not affect ERK1/2 activation ($p > 0.05$ for each, Fig. 5, $n=5$).

Gross view, wet weight and GAG content

After 6 weeks of culture under periodic mechanical stress, we recorded differences in the gross view and size, wet weight and GAG content of the *in vitro* constructed cartilage to evaluate the effects of Lv-integrin $\beta 1$, Lv-GFP or Lv-shRNA-integrin $\beta 1$ transfection on 3D cartilage formation. Our results showed that cartilage-like tissue was formed in all three groups, but a marked declining trend was observed in constructed cartilage size among Lv-integrin $\beta 1$, Lv-GFP and Lv-shRNA-integrin $\beta 1$ groups (Fig. 6a). The wet weight measurement showed an increase of wet weight from the Lv-shRNA-integrin $\beta 1$ group, Lv-GFP group, to the Lv-integrin $\beta 1$ group ($p < 0.05$ for each, Fig. 6b, $n=5$). GAG content measurement in the Lv-shRNA-integrin $\beta 1$, Lv-GFP and Lv-integrin $\beta 1$ groups was 2.99 ± 0.26 mg/g, 5.96 ± 0.35 mg/g and 9.13 ± 1.00 mg/g, respectively, further supporting these findings ($p < 0.05$ for each, Fig. 6c, $n=5$).

Histological and immunohistochemical staining

We further examined the engineered cell/scaffold constructs formed under periodic mechanical stress by staining with HE and Safranin-O as well as immunohistochemical staining with type II collagen. Typical cartilaginous metachromasia of the engineered tissues was observed. Safranin O staining reflected GAG content. Our results revealed that in response to periodic mechanical stress, more round chondrocyte-like cells with surrounding

Fig. 6. Effects of inhibition or up-regulation of integrin $\beta 1$ on gross view, wet weight and GAG content of *in vitro* engineered tissue under conditions of periodic mechanical stress. After pretreatment with control vehicle (Lv-GFP) or shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$), cell-scaffold constructs were cultured for 6 weeks under conditions of periodic mechanical stress prior to gross view (a), wet weight (b) and GAG content studies (c). Results of wet weight and GAG content are shown in the histogram. All three groups formed cartilage-like tissue, but a markedly declining trend was observed in the size of cartilage constructs among Lv-integrin $\beta 1$, Lv-GFP and Lv-shRNA-integrin $\beta 1$ groups.

Results of wet weight and GAG content studies (c). Results of wet weight and GAG content are shown in the histogram. All three groups formed cartilage-like tissue, but a markedly declining trend was observed in the size of cartilage constructs among Lv-integrin $\beta 1$, Lv-GFP and Lv-shRNA-integrin $\beta 1$ groups. ($n = 5, p < 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test). Wet weight and GAG content increased gradually from Lv-shRNA-integrin $\beta 1$, Lv-GFP to Lv-integrin $\beta 1$ groups. ($n = 5, p < 0.05$ for each, analyzed by one-way ANOVA followed by post-hoc Fisher's LSD test).

lacunae could be found in the Lv-integrin $\beta 1$ group compared to the Lv-GFP group while the Lv-shRNA-integrin $\beta 1$ group showed the opposite effect (Fig. 7a-c). Safranin O staining in the Lv-integrin $\beta 1$ group showed stronger staining than that in the Lv-GFP group, while the Lv-shRNA-integrin $\beta 1$ group appeared the most weakly stained under periodic mechanical stress (Fig. 7d-f). Immunohistochemical staining with type II collagen showed a similar pattern among these three groups (Fig. 7g-i).

Discussion

Periodic mechanical stress plays a pivotal role in cartilage tissue engineering, therefore exploring the signaling molecules involved in the chondrocytic mitogenic response to periodic mechanical stimulation could provide a theoretical basis for further enhancing the quality of tissue engineered cartilage. Integrins are known to be the most important mechanoreceptor in various cell types [11, 28, 29], and the results of our previous studies revealed that periodic mechanical stress-induced chondrocyte proliferation and matrix synthesis are partly mediated by the integrin $\beta 1$ -ERK1/2 signal cascade [7-10].

Previous studies regarding the roles of integrins in signaling pathways mainly involved detecting various cellular biological effects after integrin inhibition [30-32]. We have demonstrated that chondrocyte proliferation and matrix synthesis, as well as phosphorylation of ERK1/2, are all significantly reduced after integrin $\beta 1$ depletion [9]. However, up to now, no data have directly linked integrin $\beta 1$ up-regulation to cell behavior

Fig. 7. Effects of inhibition or up-regulation of integrin $\beta 1$ on histological and immunohistochemical staining of *in vitro* engineered tissue under conditions of periodic mechanical stress. After pretreatment with control vehicle (Lv-GFP) or shRNA targeted to integrin $\beta 1$ (Lv-shRNA-integrin $\beta 1$) or wild-type integrin $\beta 1$ (Lv-integrin $\beta 1$), cell-scaffold constructs were cultured for 6 weeks under conditions of periodic mechanical stress prior to hematoxylin-eosin (HE) staining (a-c), safranin-O staining (d-f) and immunohistochemical staining for collagen type II (g-i). Scale bar 25 μ m.

and function of chondrocytes. Based on our previous results, we evaluated the hypothesis that integrin $\beta 1$ up-regulation would improve the activities and functions of chondrocytes. In our study, chondrocyte proliferation, matrix synthesis and ERK1/2 phosphorylation levels were all markedly increased upon transfection with GFP-tagged integrin $\beta 1$ wild-type plasmid when exposed to periodic mechanical stress in chondrocyte monolayer culture. However, when cultured under static conditions, integrin $\beta 1$ up-regulation did not affect the cellular response or ERK1/2 phosphorylation in chondrocytes. These results indicate that in this context, periodic mechanical stress is the critical determinant in enhancing the quality of chondrogenesis, while integrin $\beta 1$ up-regulation is able to amplify this effect. Thus integrin $\beta 1$ up-regulation alone, without the addition of periodic mechanical stress, had no effect on the activities of chondrocytes. However, there have been contrasting findings, with some researchers reporting that under some non-mechanical stimuli, cell viability and function were also affected by integrin up-regulation in several cell types [21, 33]. For instance, an earlier study by Liu et al. showed that when integrin $\beta 4$ was up-regulated in airway epithelial cells, cell proliferation ability and anti-oxidation ability were significantly enhanced during wound repair [20]. Hayashido et al. found that once integrin αV was up-regulated, type I collagen could promote squamous cell carcinoma cell proliferation and elevate ERK1/2 phosphorylation levels [34]. These results indicate that in addition to their role in mechanosensing, integrins also possess many other functions. Indeed, integrins have also been shown to be adhesion receptors that mediate the interactions between cells and ECM to regulate various biological effects in a range of cell lineages [35, 36]. In any case, in our present study, the main role of integrin $\beta 1$ in chondrocytes was mechanosensing. Integrin $\beta 1$ up-regulation combined with periodic mechanical stress further promoted chondrocyte proliferation and matrix synthesis, possibly through enhancement of the number of mechanoreceptors, thus allowing the cells to receive more mechanical stimuli. To the best of our knowledge, this is the first study to strongly implicate integrin $\beta 1$ up-regulation in enhancing chondrocyte biological roles under a setting of periodic mechanical stress.

In order to determine whether integrin $\beta 1$ up-regulation could improve the quality of 3D tissue-engineered cartilage constructed *in vitro*, gross view and wet weight, GAG content, histological and immunohistochemical staining assays were carried out. As we had already shown that when integrin $\beta 1$ was up-regulated in chondrocyte monolayer culture, compared with mock transfectants, chondrocyte proliferation and matrix synthesis only increased under conditions of periodic mechanical stress, so we only evaluated the effects of integrin $\beta 1$ up-regulation on the quality of 3D tissue-engineered cartilage constructed *in vitro* in the setting of periodic mechanical stress. We observed that Lv-integrin $\beta 1$ transfection significantly promoted GAG and type II collagen synthesis, further confirming that integrin $\beta 1$ up-regulation can enhance the quality of tissue-engineered cartilage formed in 3D culture exposed to periodic mechanical stress.

Although the present study demonstrates for the first time that in addition to periodic mechanical stress, integrin $\beta 1$ up-regulation can further enhance the quality of tissue-engineered cartilage in both chondrocyte monolayer culture and three-dimensional culture, some limitations still exist. For example, the current investigations were only performed *in vitro*; the detailed and precise mechanism of integrin $\beta 1$ up-regulation in chondrocytes *in vivo* under periodic mechanical stress still awaits further investigation. Furthermore, the benefits of BMSCs in cartilage and bone repair were well proved, while we only focused on the roles of chondrocytes in the current investigations [37, 38].

In conclusion, compared with periodic mechanical stress alone, periodic mechanical stress plus integrin $\beta 1$ up-regulation can further promote chondrocyte proliferation and matrix synthesis and elevate ERK1/2 phosphorylation levels in chondrocyte monolayer culture, as well as promoting GAG and type II collagen accumulation in chondrocyte 3D culture. Briefly, in addition to periodic mechanical stress, integrin $\beta 1$ up-regulation could further enhance the quality of tissue-engineered cartilage.

Abbreviations

Lv (Lentivirus vector); GAG (glycosaminoglycan); ERK (extracellular signal-regulated kinase); DMSO (dimethyl sulfoxide); HRP (horseradish peroxidase); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); ECM (extracellular matrix).

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Disclosure Statement

The authors declare no competing interests.

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