

# ERK5 Transmits Shear Force Stimulation Signals of Chondrocyte Cultured in an Optimized in Vitro Shear Force Model

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## Research Article

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# Abstract

**Background:** In clinical practice, cartilage arthritis caused by cartilage tissue damage is difficult to treat due to the slow regeneration of cartilage tissue. In recent years, the orthopedic researches dedicate to promote the regeneration process. Recent reports reveal that appropriate shear force stimulation will exhibit an acceleration on proliferation and phenotypic differentiation of chondrocytes. At the same time, recent advances in 3D printing technology have made autologous cartilage tissue transplantation possible in clinical. Since the in vitro culture of autologous chondrocytes can provide a large amount of cell materials for clinical treatment, the optimization of in vitro culture conditions has become an urgent problem to be solved. For this reason, this study introduced an in vitro fluid shear force loading system and explored a set of optimal shear force parameters.

**Result:** On the basis of these parameters, the in vitro proliferation speed and phenotypic differentiation level of chondrocytes are better than that of static culture. In addition, we used RNA interference to reduce the expression of ERK5 and proved that ERK5 is a key factor for chondrocytes to transmit shear force stimulation signals.

**Conclusion:** These results have the opportunity to provide a theoretical basis for in vitro culture of chondrocytes and clinical treatment of cartilage injury.

## Introduction

Cartilage arthritis is caused by the destruction of articular cartilage and physical changes in the cartilage around the joint<sup>[1]</sup>. Since there are no nerves, blood vessels, and lymph vessels in the cartilage tissue of adults for nutrient transport, once cartilage tissue is damaged, its self-repair ability is insufficient and eventually the function of arthritis and joint are affected<sup>[2,3]</sup>. These pathological changes restrict the patient's movement and prolong the recovery time at the same time, bringing great pain to clinical patients. Therefore, the treatment of cartilage injury is an urgent need in orthopedics clinics.

Treatment of cartilage injury is a thorny issue. Until today, with highly developed technology, although many repair methods such as autologous chondrocyte transplantation and mosaic plasty have certain effects, large area cartilage defects in joints are still a challenging problem. The bottleneck is the regular regeneration of new cartilage tissue to replace the necrotic tissue<sup>[4]</sup>. Therefore, it is attractive to find a safe and effective method to promote cartilage cell proliferation and differentiation.

According to clinical experience, the mechanical environment of chondrocytes and their own mechanical properties are the key factors affecting articular cartilage<sup>[5]</sup>, and the physiological structure and normal function of articular cartilage require normal stress to maintain<sup>[6]</sup>. Histologically, physiological mechanical loads play an important role in regulating chondrocyte viability and extracellular matrix synthesis. Mechanical load exceeding the range of physiological tolerance will cause degenerative diseases of articular cartilage<sup>[7]</sup>, and excessive mechanical load will induce damage to the cell integrity,

which will affect the subsequent development of cartilage differentiation and degeneration<sup>[8]</sup>. Therefore, keeping chondrocytes in a suitable range of mechanical load will help chondrocytes grow in a favorable direction.

Previous studies have shown that cartilage repair is related to relative movement of cartilage joints. Akanji et al. found that NF-κB plays an active role in the mechanical and inflammatory pathways affecting chondrocyte metabolism<sup>[9]</sup>. Chowdhury et al. demonstrated dynamic pressure inhibits the release of NO and PGE2 from chondrocytes, which provide new treatment options ideas for the of inflammatory arthritis pain<sup>[10]</sup>. Our previous studies also proved that proper exercise can repair cartilage damage in animal models<sup>[11]</sup>. We speculate that the normal physiological process of cartilage cell synthesis and secretion of cartilage matrix precursor substances is regulated to a certain extent by the magnitude of stress. When chondrocytes lack sufficient stress stimulation, the synthesis and secretion functions will be declined, leading to growth inhibition and proliferation downgrading. Therefore, the study of mechanical loading promoting cartilage growth and differentiation will hopefully help find ways to promote cartilage proliferation and cartilage tissue repair.

The shear bioreactor is a culture device that applies various mechanical loads and biochemical stimuli. Its ultimate goal is to rebuild the physiological and chemical environment in the body in vitro and promote the growth and development of engineered cartilage tissue<sup>[12]</sup>. Studies have pointed out that the selection of seed cells in cartilage tissue engineering, the application of scaffold materials, the coupling of biochemical conditions, and changes in mechanical load will all affect the gene expression of cells and the content of collagen and PG in the extracellular matrix<sup>[2, 13-15]</sup>. As mentioned above, mechanical loads play an important role in this process; if there is no mechanical stimulation, cartilage morphology will be underdeveloped, so that it will face challenge to obtain engineered cartilage that is close to the mechanical properties of natural cartilage and is compatible with structure and function<sup>[12]</sup>.

Aiming at the role of mechanical loading in the construction of cartilage tissue engineering, in this study, we introduced a fluid shear loading system to confirm the optimal shearing environment for cartilage cell culture in vitro. On this basis, a set of intensity gradient and time gradient fluid shear forces were applied to mouse primary chondrocytes to study the relationship between cartilage proliferation, activation, differentiation and ERK pathway. The results show that ERK5 plays a decisive role in transmitting the signal of chondrocyte proliferation induced by shear stress, and helps improve the environment of chondrocyte culture in vitro in preclinical experiments.

## Result

# Primary chondrocytes possess higher proliferation activity under specific shear stress environment

The newly isolated and expanded rat primary chondrocytes for 2 generations were divided into 25 groups for orthogonal experiments, and five shear force gradients ranged from 0 dyn/cm<sup>2</sup> to 24 dyn/cm<sup>2</sup> orthogonally cross 5-time gradients ranged from 0 min to 90 min. The cells stimulated by shear force were transferred to T25 culture flask and cultured for 24h in 37°C 5% CO<sub>2</sub> environment, followed by measuring the proliferation activity via CCK-8 method. Cell proliferation capacity of each group was calibration with cell sample before the intervention of shear force (0 dyn/cm<sup>2</sup>, 0 min group). The results showed that, the proliferation activity of chondrocytes was the best after applying 12 dyn/cm<sup>2</sup> shear force to the chondrocytes for 45 min, compared with other concentration gradient groups (Fig. 1), and its proliferation rate was 53.5% higher than that of the group without shearing force. It is suggested that certain fluid shear stress treatment is helpful for the proliferation of chondrocytes in vitro.

## **Cartilage differentiation is improved under the shear force culture environment**

The cartilage differentiation biomarker expression of each chondrocyte group subjected to different shearing forces was determined in mRNA and protein level. The results show that in an ideal shear environment mentioned in previous section, the genes that are positively related to chondrocyte proliferation (such as ACAN and COL1A2) The transcription activity showed an up-regulation trend relative to the control group. The genes positively related to the promotion of cartilage differentiation, such as ERK5, FAK, Sox9, Runx2, PGE2, Col10a1, and PTGS2, showed up-regulation of mRNA level. The genes positively related to the promotion of cartilage differentiation, such as ERK5, FAK, Sox9, Runx2, PGE2, Col10a1, and PTGS2, also represented an up-regulation of transcription activity. In addition, the expression of RhoA, LIMk1 and Cofilin, which promote the formation of stress fibers and cytoskeleton also showed enhanced transcriptional activity.

The content of Col1A2, GAG, SOX9 in different groups of cells was determined by ELISA to separately compare the effects of different shear stress conditions on cartilage formation ability, proteoglycan metabolism ability in chondrocyte matrix and cartilage phenotype differentiation ability. Consistent with the protein quantification results, the change trend of Col1A2 and SOX9 determined by ELISA was consistent with qPCR and western blot. The content of GAG protein also showed a rising state, suggesting that chondrocytes treated by shear force are in a state of more active cartilage tissue formation.

## **ERK5 plays a key role in the process of shearing force promoting cartilage proliferation**

The expression of ERK5 was down-regulated by siRNAs targeting ERK5, and the mRNA (Fig. 4-A) and protein content (Fig. 4-B) of ERK5 was significantly reduced by qPCR and western blot verification. Subsequently, comparing chondrocytes with low expression of ERK5 and chondrocytes with normal

expression of ERK5, the results showed that after the chondrocytes were stimulated by shear stress, both groups were higher than those without shear stress stimulation. But the down-regulation of ERK5 significantly reduced the proliferation ability of chondrocytes. At the same time, even given the most appropriate shear force stimulation, the proliferation ability of the ERK5 down-regulation group was difficult to restore to the proliferation level of the un-down-regulated ERK5 cells. Moreover, after ERK5 is knocked down, the feedback of chondrocytes to shear force stimulation became sluggish (Fig. 4-C), suggesting that ERK5 is an important factor in transmit shear force signal transmitting of chondrocytes.

## Method

### Primary chondrocytes

Sprague-Dawley (SD) rats were purchased from the Model Animal Research Institute of Nanjing University and raised in the SPF Animal Center of Scientific Research Center. Animal experiment operations and animal welfare standards were approved by the Ethics Committee of the People's Hospital of Longhua District, Shenzhen. Before the experiment, 4 rats were randomly selected from 18 rats, and the cartilage tissue on the surface of the rat's femoral condyle was cut by sterile surgery, and the fragments were rinsed and trimmed in PBS containing penicillin sodium/streptomycin double antibody. The rat articular chondrocytes were extracted by a modified two-step enzymatic digestion method<sup>[16]</sup>, filtered with a 40  $\mu\text{m}$  cell strainer, and resuspended in a 10% fetal bovine serum high-glycemic DMEM. The medium was changed for the first time on the third day, and then every 2 days. The cells were passaged after the culture flask was 95% full, and the first passage chondrocytes were used as seed cells. When culturing the cells of subsequent generations, add chondrocyte induction medium (DMEM high glucose medium containing 2% fetal bovine serum, 1% penicillin-streptomycin solution, 1% ITS, 50 ml ascorbic acid, 100nmol/L ground Cells were resuspended with dexamethasone and 10ng/mL TGF- $\beta$ ), centrifuged and cultured in a 37°C, 5% CO<sub>2</sub> incubator. The medium was changed per 48h for 2 weeks.

### Shear force model establishment

In this study, the Allegro XRS 20 Bioreactor System (Pall Corporation, US) was used to give cells a quantitative shear force stimulation, and the assemble of equipment's component and the experimental operation methods refer to the official instructions. Briefly, the shear force was calculated according to the Poiseuille equation ( $s = 6Qu / wH^2$ )<sup>[17]</sup> to determine the strength of the fluid shear force used in this experiment. Among these parameters, Q represents the liquid flow rate,  $\eta$  represents the liquid viscosity coefficient ( $\eta_{\text{DMEM}}=0.03 \text{ dynes / cm}^2$  <sup>[18]</sup>), w represents the width of the fluid chamber (w=3.0 cm), and H represents the height of the fluid chamber (2.0 cm). During the experiment, the fluid shear stress was determined by adjusting the liquid flow in the formula. Establish 5 shear force gradients of 0 dyn/cm<sup>2</sup>, 6 dyn/cm<sup>2</sup>, 12 dyn/cm<sup>2</sup>, 18 dyn/cm<sup>2</sup>, 24 dyn/cm<sup>2</sup>, and duration gradients of 0 min, 30 min, 45 min, 60 min, and 90 min, orthogonally. Each gradient was used to stimulate 10 thousand chondrocytes separately

(repeat experiment n=3). The cells stimulated by shearing force were transferred to T25 culture flask and cultured in 37°C 5% CO<sub>2</sub> environment for 24h.

## Cell proliferation curve

The cell proliferation ability was determined by the Cell Counting Kit-8 (CCK-8, GlpBio Technology, USA). The cell collection, measurement and data analysis methods were carried out with reference to the standard instructions. Briefly, remove 100  $\mu$ L of cell suspension to 96 well plate, and add 10  $\mu$ L CCK-8 solution. Incubate the culture plate in an incubator for 2 hours, and measure the absorbance at 450 nm using a microplate reader. Calculate the increase in cell number based on the previously established standard proliferation curve. All data were calibrated with the proliferation capacity of the 0 dyn/cm<sup>2</sup>, 90 min group to obtain the relative proliferation percentage.

## RT-qPCR and Western Blot

The expression of genes related to cartilage proliferation and development was determined by reverse transcription-qPCR and Western Blot. The cells were collected by centrifugation, the supernatant was removed, and the total RNA was extracted using the TRIzol Total RNA Extraction Kit (BioTeke, China), and the Advantage RT-for-PCR Kit (TAKARA, Japan) was used to reverse transcription of RNA into cDNA. Subsequently, quantitative PCR was performed using 12 pairs of primers (Table 1) shown in Table 1 with Premix Ex Taq (TAKARA, Japan), according to PCR temperature in official instruction and annealing temperature corresponding to each pair of primers. PCR reaction was conducted and fluorescence signal was collected with a Thermal Cycler Dice Real Time System III (TaKaRa, Japan), while GAPDH was used as an internal reference for the experiment and the delta-delta CT ( $\Delta\Delta$ Ct) of each sample were calculated<sup>[19]</sup>.

Table 1  
The primer pairs used in qPCR

Gene ID	Ref.Accession	Primers	Product length
ACAN	L07049.1	F: 5'- CAGATGGCACCTCCGATAC -3' R: 5'- GACACACCTCGGAAGCAGAA -3'	151 bp
COL1A2	NM_007743.3	F: 5'- CCCAGAGTGGAACAGCGATT -3' R: 5'- TTTTGGAGCAGCCATCGACT -3'	449 bp
PTGS2	NM_011198.4	F: 5'- CATCCCCTTCCTGCGAAGTT -3' R: 5'- CATGGGAGTTGGGCAGTCAT -3'	178 bp
Sox9	FJ790141.1	F: 5'- CCACCATCTGCATCCCTACC -3' R: 5'- GGTCAACTGAAGCGGAGTGA -3'	983 bp
Runx2	DQ458792.1	F: 5'- AAGCCACAGTGGTAGGCAGT -3' R: 5'- GCCACTTGGGGAGGATTTGT -3'	604 bp
PGE2	BC004846.1	F: 5'- AACTTTGCCACCCAACGACC -3' R: 5'- GGAGTTCTGATGGTCAGCGT -3'	451 bp
col10a1	NM_009925.4	F: 5'- AAGGCCATGAATGACCAGGG -3' R: 5'- TGTTCCGGTACACGTTGGGAG -3'	183 bp
RhoA	JN971019.1	F: 5'- CTTGCTAGCCCCAAGACACA -3' R: 5'- CTGGAATGCCATGGTTCCT -3'	973 bp
LIMk1	NM_010717.3	F: 5'- TCGGCCTCTCTCTTATCCA -3' R: 5'- ACAAGATGAGGCACCCAGAC -3'	375 bp
Cofilin	D00472.1	F: 5'- TGCACCCTCAAGAGCAAAA -3' R: 5'- ATACGGAGTAGGGGTGTCGTT -3'	375 bp
ERK5	AB019373.1	F: 5'- GCGCATTAAAGGAGGCCATTG -3' R: 5'- AGGAGTACTAGTGGGCTGGG -3'	682 bp
FAK	NM_007982.2	F: 5'- AAAATCCAGCCAGCTCCTCC -3' R: 5'- CACGGGCTACAGAGGCTAAG -3'	435 bp

Another set of harvested cells were subjected to western blot analysis for quantification of ERK5, PGE2, COX-2 and phosphorylated ERK5 (pERK5). The primary antibodies were Anti-ERK5 antibody (ab40809, Abcam, USA), Anti-PGE2 antibody (ab2318, Abcam, USA), Anti-COX2 antibody (ab15191, Abcam, USA) and Anti-GAPDH antibody (ab8245, Abcam, USA). After each incubation step, the NC films were washed with TBST (pH=7.0, 0.5% Tween-20) for 30 min, and incubated with the enhanced HRP-DAB substrate color development kit PA110 (Beyotime Biotechnology, China) for 1min. Images were captured with an Alphamager HP imaging system (ProteinSimple LLC., USA). The relative quantification of each sample in the western blot was normalized to that of GAPDH.

## Enzyme linked immunosorbent assay (ELISA)

The cell mass was dissociated by trypsinization, and the suspended cells were collected by centrifugation. The total cell density was counted by a Countstar BioTech cell counter (Countstar, China). Take 20 thousand cells and add 200  $\mu$ L RIPA cell lysate (Beyotime, China) for cell lysis. Marker proteins related to cartilage differentiation (Col1A2, GAG, SOX9) was quantitative determined through ELISA. Rat collagen type I  $\alpha$ 2 (Col1A2) was tested with Rat COL1A2 ELISA detection kit (JL34211-48T, JomIn Bio, China). The GAG protein content in the cell samples was detected by the rat glycosaminoglycan (GAG) ELISA detection kit (SenBeiJia Biological Technology, China). SOX-9 ELISA kit (BNCC, China) was used for the quantitative experiment of SOX-9 protein. The pipetting, color development and data conversion of the kit were performed according to the kit instructions and standard curves.

## siRNA oligos and transfection

The expression level of ERK5 protein is knocked down by RNA interference (RNAi). Three small interfering RNAs (siRNA) were designed for the 573th bp, 648th bp, and 789th bp sites of Rat ERK5 mRNA through the online software Ambion siRNA target finder ([www.ambion.com](http://www.ambion.com)), and synthesized chemically from Sangon Biotech, China. 25 pmol of each siRNA oligo was transfected to 10 thousand chondrocytes pre-cultured in 6 well plate with Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher, USA) according to user manual. A set of mimic siRNA (Invitrogen Silencer Negative Control No. 1 siRNA, Thermo Fisher, USA) that does not affect the expression of ERK5 was set up as a negative control.

## Statistical analysis

All data in the experiment were recorded in the format of mean  $\pm$  standard deviation (SD). The statistical analysis software is Origin 2018 (OriginLab Corporation, Massachusetts, USA). One-way analysis of variance (ANOVA) is used to compare whether there is a difference between multiple groups; \*P <0.05 indicates a statistical difference, \*\*P <0.01 indicates a Very obvious difference. In terms of experimental parallel testing, rat primary chondrocytes were derived from 4 parallel experimental animals and used

after mixing. In the grouping of shear force model and cell experiment, each group set up 3 parallel groups (n=3); CCK-8 cell count, qPCR, Western Blot and ELISA set up 5 parallel groups (n=5).

## Discussion

Cartilage is an important tissue that guarantees the normal function of joints. However, due to the special structure of cartilage, cartilage pathological damage is difficult to cure clinically. There is no innervation, blood vessels, and lymphatic vessels in articular cartilage tissue, which causes Most of its substitute material exchange comes from joint fluid. On the other hand, joint movement makes the joint fluid flow continuously, which benefits the material exchange of cartilage cells<sup>[20]</sup>. At the same time, mechanical load stimulation is also an important factor to promote the proliferation and differentiation of chondrocytes. At the same time, mechanical load stimulation is also an important factor to promote the proliferation and differentiation of chondrocytes. According to reports <sup>[21]</sup>, one of the keys in cartilage tissue engineering is to find the appropriate mechanical load, which can simulate joint movement, generate appropriate and coupled mechanical loads and biochemical stimulation to the cartilage, and promote the functional construction of engineered cartilage. For this reason, the cultivation method of using bioreactors as shear force providers has gradually been used widely in orthopedics researches<sup>[22]</sup>. With the help of researches on shear force, the treatment of cartilage damage is gradually developing in the direction of precise treatment based on the specific condition of the patient.

In the classic application, the bioreactor can not only apply appropriate mechanical load to the engineered cartilage, but also have monitoring and detection technology for studying the biological response of tissue mechanics, and determine the behavior and state of cells under different culture conditions through the development and changes of tissue structure. In order to obtain an ideal shear force culture environment, it is first necessary to clarify the effects of different mechanical loads on cartilage tissue and find the appropriate mechanical conditions. Therefore, this study started with the optimum shear force for culturing primary chondrocytes in vitro, and determined the optimum shear force and duration by using orthogonal experiments.

ERK5 (Mapk7, Mitogen-activated protein kinase 7) belongs to the MAPK signaling pathway. It plays a role in various cellular processes of chondrocytes such as proliferation, differentiation and cell survival. Upon activation, it translocates to the nucleus and phosphorylates various downstream targets. Yang et. al reported conditional ablation of MAPK7 expression in chondrocytes impairs endochondral bone formation in limbs and adaptation of chondrocytes to hypoxia<sup>[23]</sup>. The embryo development evaluation of Li et. al showed that ERK5 deficiency exhibit embryonic lethality in mice through bone dysplasia<sup>[24]</sup>, indicating ERK5 contributes to the development and maintenance of cartilage tissue. At the same time, our previous research revealed that chondrocytes have phosphorylation of ERK5 protein after applying fluid shear force. The results of this study showed that the expression of ERK5 was reduced by RNAi, the acceleration of proliferation caused by the shearing force is slowed down, and the level of cartilage

differentiation is reduced, suggesting that ERK5 plays an influential role in the process of chondrocyte shearing force stimulation.

It may be assumed that, based on the most suitable shear force environment, supplemented by appropriate nutrients and inducing factors, cartilage can proliferate rapidly in a three-dimensional environment. It will be possible to use 3D culture and 3D printing technology to assemble engineered cartilage from autologous sources, which will be a potential clinical treatment plan.

## **Conclusion**

In this study, a set of shear force parameters suitable for in vitro culture of rat chondrocytes was established through the shear force loading system. After a set of optimal shearing environment interventions, the proliferation and differentiation of chondrocytes was significantly improved. This study also proves that the signal transmission of cell processing shear force depends on the realization of ERK5. These findings may help optimize the in vitro culture of chondrocytes, and hopefully provide hints for the treatment of clinical cartilage trauma.

## **Declarations**

### **Ethics approval and consent to participate:**

We got the approval of the Ethics Committee of Shenzhen Longhua District Central Hospital. We confirm that all methods were carried out in accordance with relevant guidelines and regulations and the study was carried out in compliance with the ARRIVE guidelines.

### **Consent for publication:**

The written consent to publish this information was obtained from all authors.

### **Availability of data and material:**

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

### **Competing interests:**

NO.

### **Funding:**

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## Authors' contributions:

JQS directed the research and accomplished the manuscript. JQS, DGH and XBW conducted the surgery; XFD performed literature retrieval. All authors have read and approved the manuscript.

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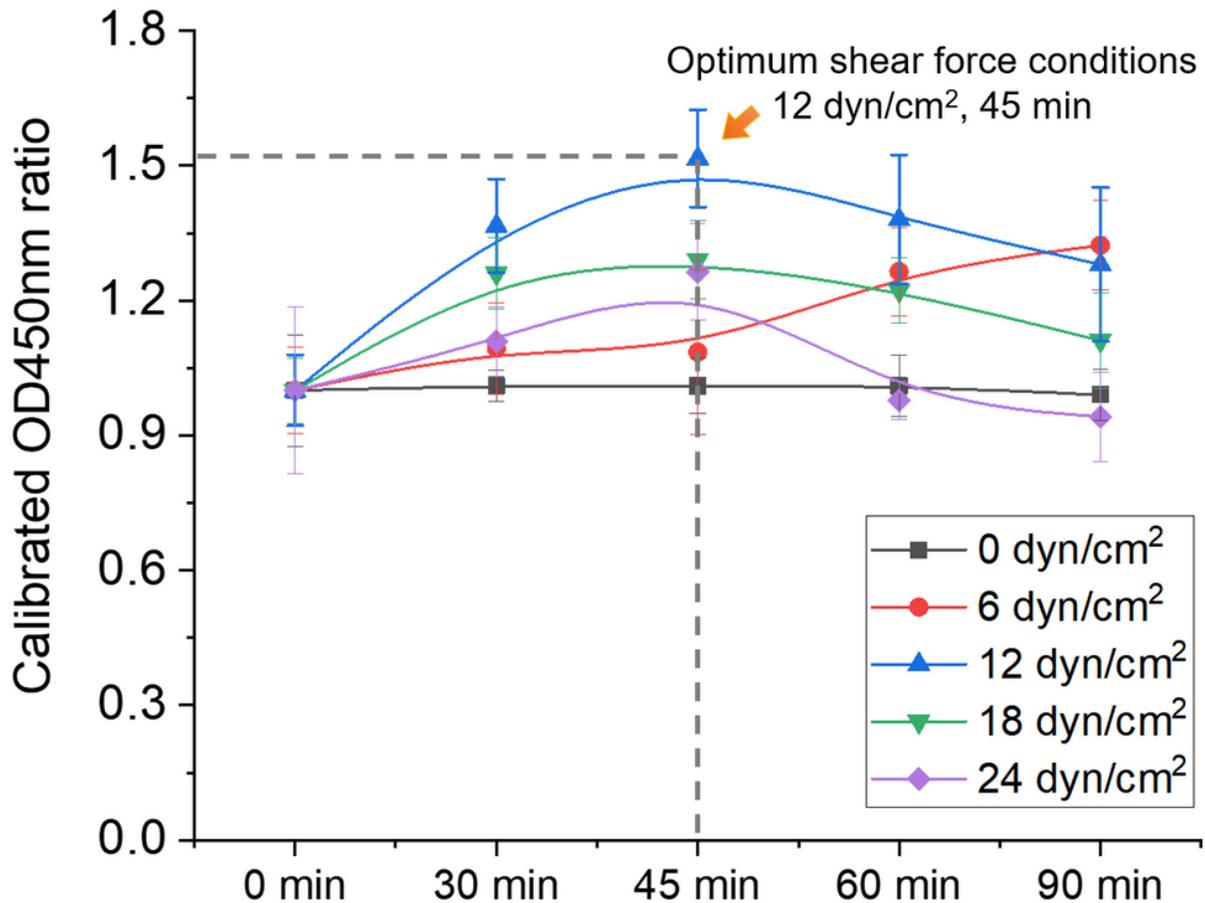
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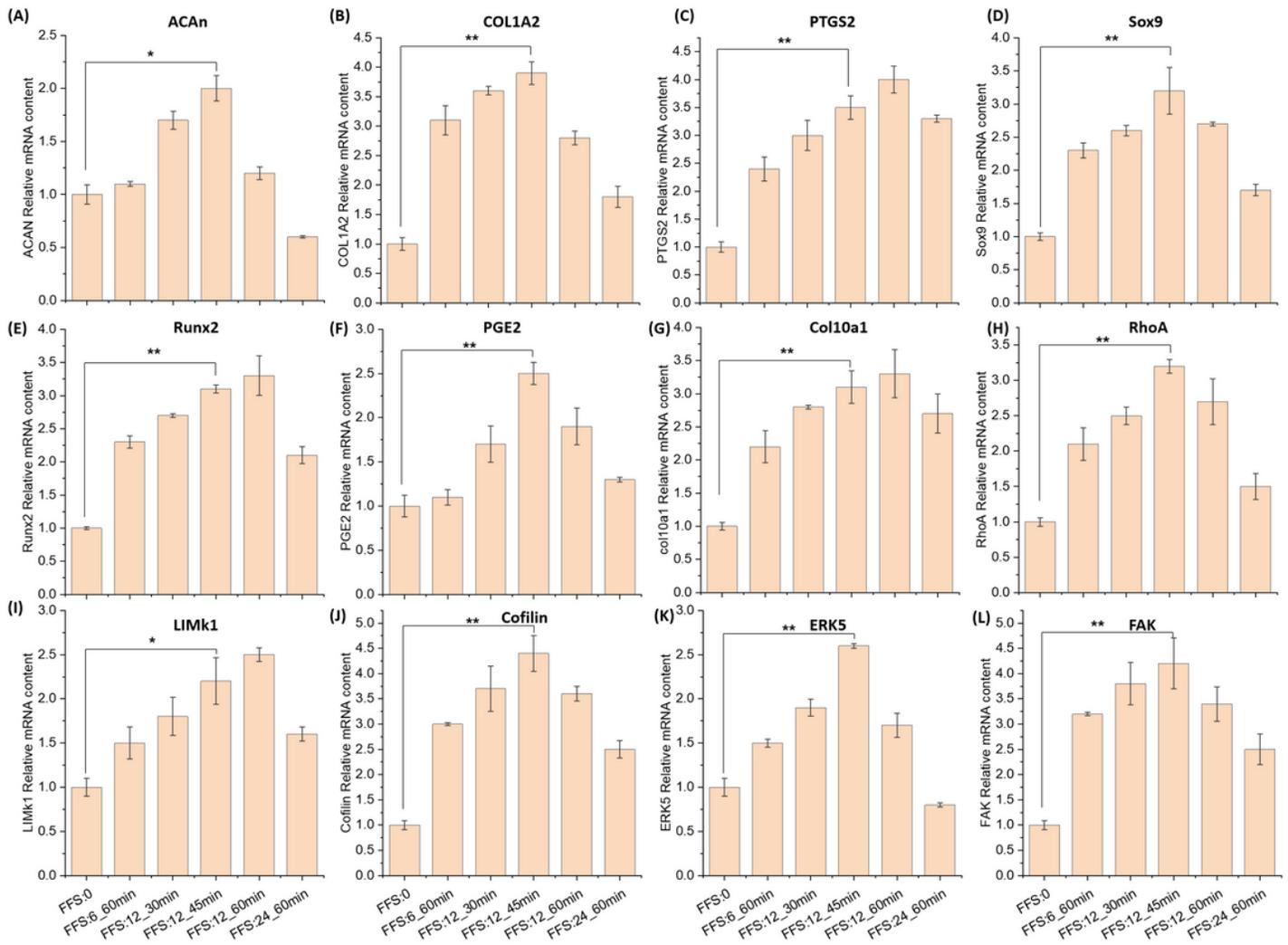
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## Figures



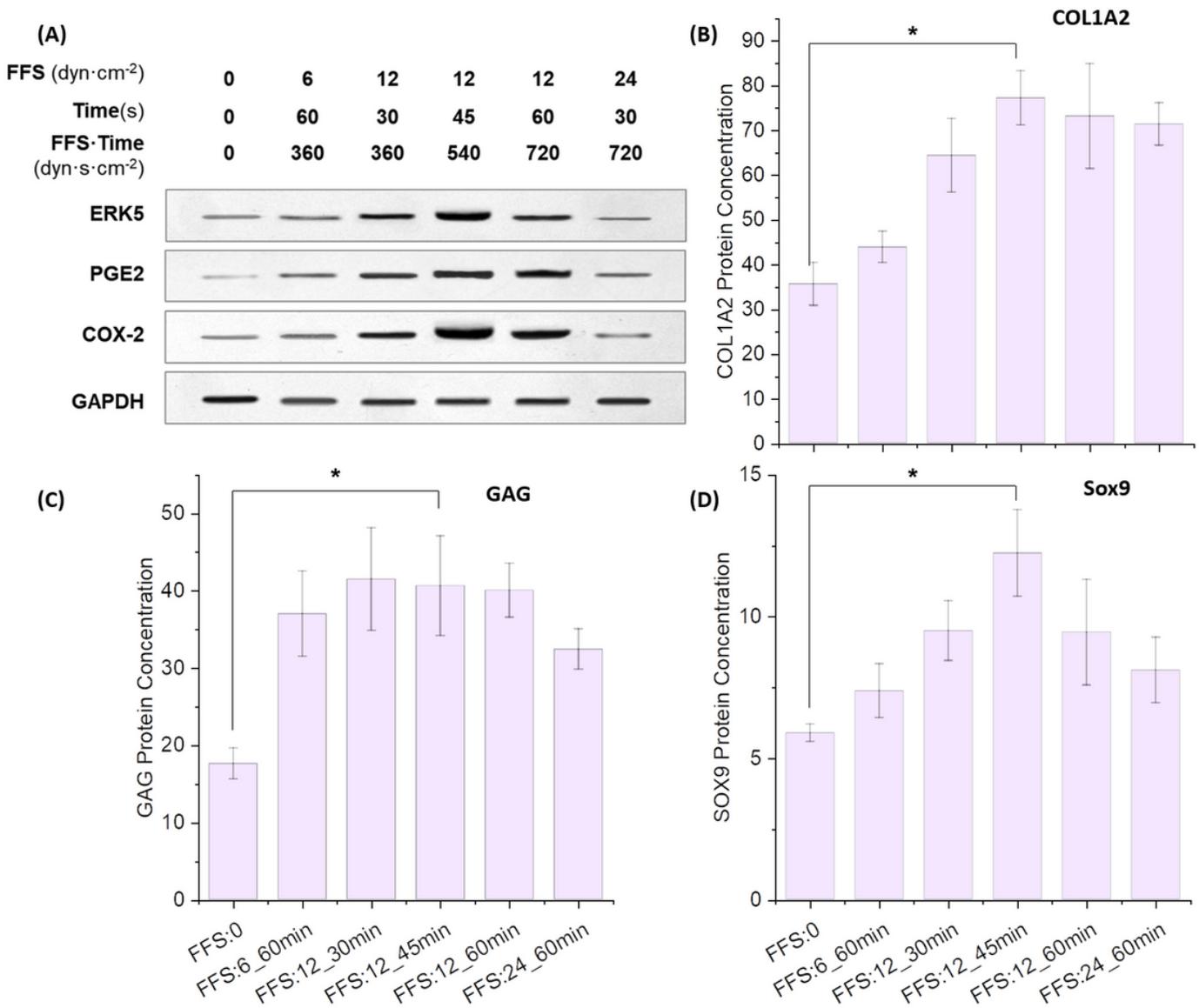
**Figure 1**

Comparison in the proliferation ability of chondrocytes under different shear forces. Figure 1 Comment: The proliferation activity of cartilage after the intervention of different shearing forces was determined with CCK-8 method. Primary chondrocytes of the same origin were treated with orthogonal test containing five shears (0 dyn/cm<sup>2</sup>, 6 dyn/cm<sup>2</sup>, 12 dyn/cm<sup>2</sup>, 18 dyn/cm<sup>2</sup>, 24 dyn/cm<sup>2</sup>) and five duration (0 min, 30 min, 45 min, 60 min and 90 min). The proliferation activity curve was drawn, while the number of cells before the experiment was used as a reference. (N=3)



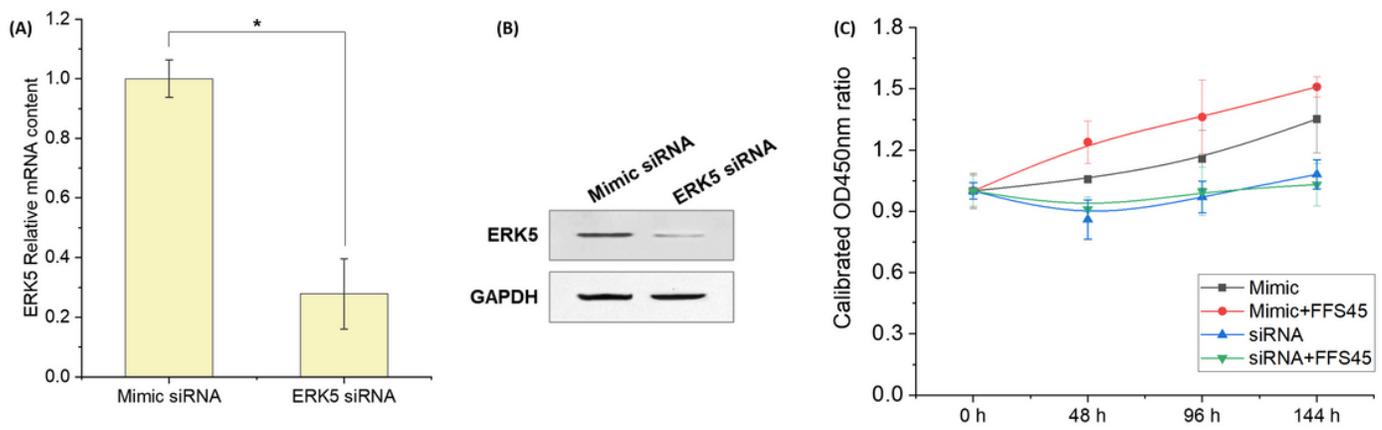
**Figure 2**

Changes in mRNA content of chondrocyte markers after intervention in different shear stress environments Figure 2 Comment: Determine the mRNA level of chondrocyte proliferation and differentiation-related proteins after the intervention of different shear stress environments by reverse transcriptional qPCR method. \*p<0.05, extremely significant difference: \*\*p<0.01. (N=5)



**Figure 3**

Changes in protein content of chondrocyte protein marker after intervention of different shear stress environments Figure 3 Comment: (A) The protein content of three related genes were measured by western blotting. (B-D) The content of Col1A2, GAG, and SOX9 protein in the cells was determined by ELISA. \* $p < 0.05$ , extremely significant difference: \*\* $p < 0.01$ . (N=5)



**Figure 4**

ERK5 knocked-down blocked the proliferation of chondrocytes and reduced its sensitivity to shear force stimulation Figure 4 Comment: (A) the mRNA content of ERK5 and (B) the intracellular protein content decreased significantly after downregulation by siRNAs. (C) At the same time, the cell proliferation ability decreases, and the recovery is not obvious after the intervention of shear force. Significant difference:  $*p < 0.05$ . (N=5)