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Dynamic compression combined with exogenous SOX-9 promotes chondrogenesis of adipose-derived mesenchymal stem cells in PLGA scaffold

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Abstract. – OBJECTIVE: Mechanical stimuli play a crucial role in cartilage repair and regeneration. Dynamic compression, as a physical stimulus, has been demonstrated to be an important factor in regulating the proliferation and differentiation of adipose-derived mesenchymal stem cells (ADSCs). However, the interaction of mechanical stimuli and chondrogenesis regulator on the chondrocyte phenotype and differentiation of ADSCs remains unknown. In the present study, we investigated the effects of dynamic compression combined with exogenous SOX-9 on chondrogenesis of ADSCs in a three-dimensional porous polylactic-co-glycolic acid (PLGA) scaffold.

MATERIALS AND METHODS: The morphology of ADSCs on the scaffolds was examined using scanning electron microscopy (SEM). The proliferation of ADSCs was evaluated by MTT assay. The expression of cartilage-specific genes in early chondrogenic differentiation was assessed by real-time PCR.

RESULTS: Our results indicated that the combination of dynamic compression with exogenous SOX-9 induces the expression of chondrogenic genes and promotes the proliferation of ADSCs.

CONCLUSIONS: Therefore, compression and SOX-9 have positive effects on chondrogenesis process of ADSCs, which may benefit articular cartilage regeneration.

Key Words:
Dynamic compression, SOX-9, Adipose-derived mesenchymal stem cells, Chondrogenesis.

Introduction

Mechanical compression is an important physical stimulus. Appropriate pressure load helps to overcome deficient culture conditions in traditional cartilage tissue engineering, maintain cell viability, promote cell proliferation as well as extracellular matrix (ECM) production, and improve the mechanical property of cell. Dynamic compression is reported to act as a key factor in regulating the proliferation and differentiation of adipose-derived mesenchymal stem cells (ADSCs). Appropriate compressive stress promotes the chondrogenic differentiation of bone marrow-derived stem cells (BMDCs) concomitant with elevated gene expression of aggrecan and collagen II[1,2].

Chondrocyte is one of cell types in articular cartilage tissue and is essential for cartilage formation and functionality. It arises from mesenchymal stem cells (MSCs) and is regulated by a series of cytokine and transcription factors, including the transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF) and insulin-like growth factor-1 (IGF-1)[3]. SOX-9 (SRY-related high-mobility group box gene-9) is also a transcription factor belonging to the SOX (SRY-related high-mobility group box gene) protein family, which is essential for chondrogenesis and has been termed a "master regulator" of the chondrocyte phenotype[4,5]. Sox-9 is expressed in all chondroprogenitor cells, predominantly in mesenchymal condensations and cartilage[6]. In addition, Sox-9 is able to prevent chondrocyte hypertrophy and exert re-differentiated effects on osteoarthritic chondrocytes that have been dedifferentiated[7].

However, the interaction of mechanical stimuli and chondrogenesis regulator-SOX 9 on the chondrocyte phenotype and differentiation of ADSCs remains unknown. In this study, we investigated the effects of dynamic compression combined with exogenous SOX-9 on chondrogenesis of ADSCs in a 3D porous PLGA scaffold.

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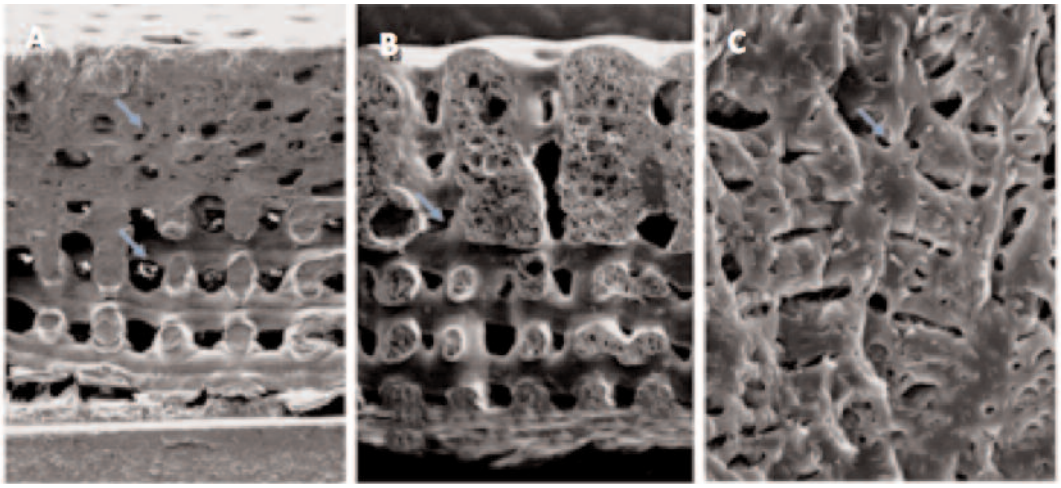

Figure 1. SEM images of PLGA scaffolds (×1000). ***A,*** PLGA gradual porous composite scaffolds; ***B,*** PLGA uniform porous composite scaffolds; ***C,*** 5 μm mutual unicom microtubules (×1000).

Materials and Methods

ADSCs Separation, Culture and Sox-9 Gene Transfection

ADSCs from Sprague Dawley rats (4 weeks and 200 g) were separated and cultured as previously described[8]. Briefly, the harvested cells were re-suspended and cultured in DMEM-F12 with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified 5% CO_2 incubator. The medium was changed 2 days after the seeding of the ADSCs and then subsequently every 2 days. Non-adherent cells were removed by changing the culture medium after 5 d days of incubation.

The third passage of cells were transfected with adenovirus containing either green fluorescent protein (Adv-GFP) or both Sox-9 and GFP (Adv-Sox-9-GFP). The transfected cells were then cultured overnight at a multiplicity of infection (MOI) of 150 plaque-forming units (PFU)/cell. Western blotting was performed to confirm Sox-9 expression.

PLGA Scaffolds

Porous PLGA gradual and uniform composite scaffolds were manufactured by Tsinghua University, China. They were prefabricated using low-temperature rapid prototyping technology so that the process of chondrogenesis could be observed within the scaffolds. PLGA was molded at low temperature and then recombinant human collagen II was added to form an active scaffold. Gradual PLGA composite (70% porosity) had a unique structure at each level. The superficial layer was developed with 55% porosity and an aperture (Figure 1A). Tiny channels were achieved between pores through 5 μm microtubules (Figure 1C). Uniform composite (85% porosity, aperture 100-300 μm) also had the same mutual unicom (Figure 1B and C).

Cell Seeding on Scaffold

ADSCs at 4th passage were digested and their densities were adjusted to 5×10^7 cells/ml. 250 μl of cell suspension was seeded on each scaffold and plated in 6-well plates. After 4h incubation, the cell-scaffold composites were immersed in chondrogenic culture medium and cultured for 2 days before being exposed to dynamic compression.

These transfected ADSCs were divided into 4 groups: control (Adv-GFP cells on uniform scaffold), Sox-9 (Adv-Sox-9-GFP cells on uniform scaffold), gradual (Adv-GFP cells on gradual scaffold), and Sox-9 gradual (Adv-Sox-9-GFP cells on gradual scaffold) group.

Scanning Electron Microscopy (SEM)

The morphology of ADSCs on the scaffolds and the deposited ECM within the scaffolds were examined by SEM. After 7-day culture, the cell-scaffold composites were washed with ice-cold phosphate buffered saline (PBS) and fixed with 2.5% glutaraldehyde for 30 min at 4°C. The fixed cell-scaffold composites were then dehydrated in a series of ethanol solutions with increasing ethanol concentrations (50%-100%). Following

CO_2 critical point drying, the growth status and secretion of ECM were observed by SEM (SSX550, Shimadzu, Japan).

Cell Proliferation Assay

Cell-scaffold composites were cut into small fragments. 1ml of MTT (BioSharp, Carlsbad, CA, USA) was added into each well, and the plates were then kept for 4 h at 37°C. Thereafter, 1 ml of dimethyl sulfoxide was added into each well. The fragments were ground and extensively washed by repeated pipetting. The solutions were centrifuged at 12,000 g for 10 min. Then, the optical density (OD) of the culture broth at 490 nm was measured on a microplate reader (BioTek, Winooski, VT, USA).

Real-time PCR Analysis

Cell-scaffold composites were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to isolate total RNA. One microliter (0.5 μg/μl) of RNA was used to synthesize cDNA with the RevertAid First-Strand cDNA Synthesis Kit (Takara, Kyoto, Japan) according to the manufacturer's instruction. Regular PCR was performed using a DNA Engine (ABI 9700) with first strand cDNA as template. The cycling process included 2 minutes polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and then 55°C for 60 s. The primers for HIF-1 α and six chondrocyte-specific genes were listed in Table I. GAPDH was used as an internal control to normalize the data thus to determine the relative expression of the target genes. The condition of reaction was set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression was represented using the $2^{-\varnothing\varnothing Ct}$ method.

Compressive Mechanical Properties

Compressive mechanical properties of the cell-scaffold composites were assessed using a compression bioreactor (BioDynamicTM ELF5110, Bose, MN, USA). Mechanical testing was conducted in uniaxial, unconfined compression while the sample was submerged in culture media at 37°C. The applied force and the resulting deformation were collected at a frequency of 0.1 Hz using WinTest 4.0 data acquisition software through a Bose PCI serial digital controller (Bose Biodynamic Test Instrument, Minnetonka, MN, USA). The samples were first preloaded to 1 g, which was defined as the zero strain state. The samples were then subjected to sequential step compressions of 5% strain to a maximum of 10% strain. At each step, the resulting force decay was recorded until equilibrium was reached. Equilibrium was defined as a change in force less than 0.2 g/min. The equilibrium stress, defined as the equilibrium force normalized to the cross-sectional area of the sample, was plotted as a function of the applied strain. The equilibrium modulus was determined from the numerical derivative of the equilibrium stress-strain curve and also plotted as a function of the applied strain.

Computer Modeling Force Analysis

The computer modeling force analysis of the scaffolds was performed using MIDAS software (MIDAS Information Technology, V7.8.0 Release No. 1). This software was used to reconstruct 3D scaffolds according to the manufacturing operation of the uniform porous and gradient porous scaffolds and to simulate the stress distribution of the various scaffolds under uniaxial compression.

Statistical Analysis

The experimental data were presented as the mean ± standard deviation (x±s). Differences between sample means were evaluated using a two-factor ANOVA and followed by a two-tailed Student-Newman-Keuls multiple comparison test using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was set a statistically significant difference.

Results

Exogenous SOX-9 Expression

Exogenous SOX-9 was determined by examining GFP-positive ADSCs under a fluorescence microscope at 3, 7 and 14 days after transfection. The percentages of GFP-positive cells were 69.3±7.2%, 81.3±3.8% and 94.8±2.4%, respectively (Figure 2). Sox-9 protein level was evaluated by western blot after incubation in a chondrogenic medium. As seen from Figure 3, Adv-Sox-9-GFP transfected-cells expressed maximal Sox-9 at day 14.

SEM Analysis of ADSCs Morphology on the Scaffolds

Cell morphology on the scaffolds was observed by SEM. After 7-day culture under dynamic compression, cells in Sox-9 gradual group grew in

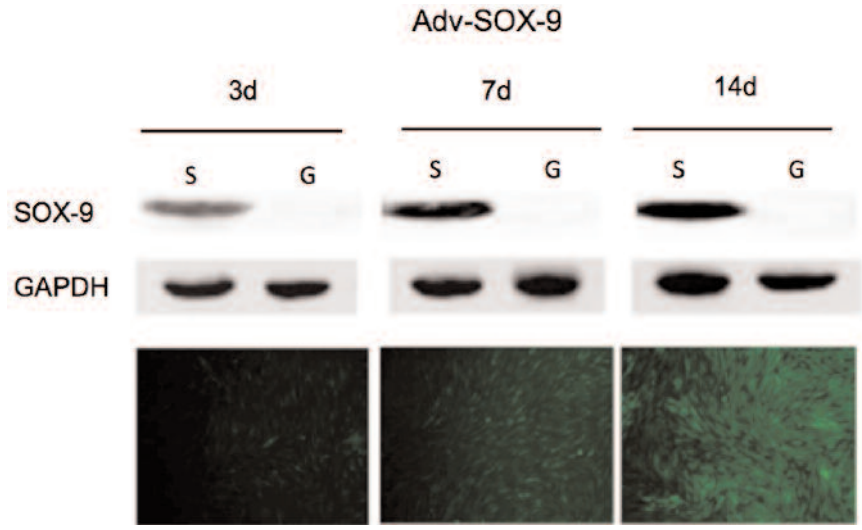

Figure 2. Fluorescence microscope examination showed cell transduction efficiency at day 3, 7 and 14 after transfection. Sox-9 protein expression was evaluated by western blot at day 3, 7 and 14 after transduction."S" represents Adv-SOX-9-GFP and "G" represents Adv-GFP.

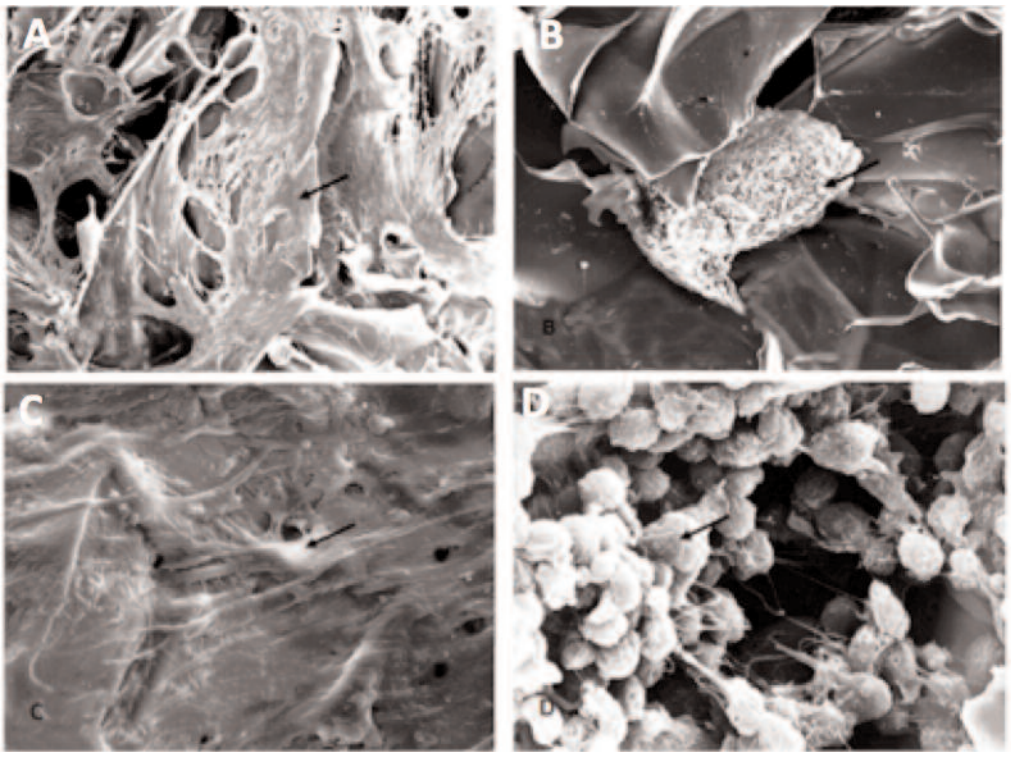

Figure 3. SEM images of cell morphology (×1000). Section of cell-scaffold composite cultured for 7 days. ***A,*** control group; ***B,*** SOX-9 group; ***C,*** gradual group; ***D,*** SOX-9 gradual group.

multilayer. These cells seemed rounded and embedded in sufficient ECM, making the structure of the scaffold nearly unobservable (Figure 3D). In contrast, cell-scaffold composites in the control group had dull and rough surfaces with poor flexibility, and the cells looked flat and less dense (Figure 3A). Cells in Sox-9 group were rounded but with less ECM (Figure 3B). In gradual group, the cells were highly confluent with ECM but less rounded. Notably, cells in Sox-9 gradual group

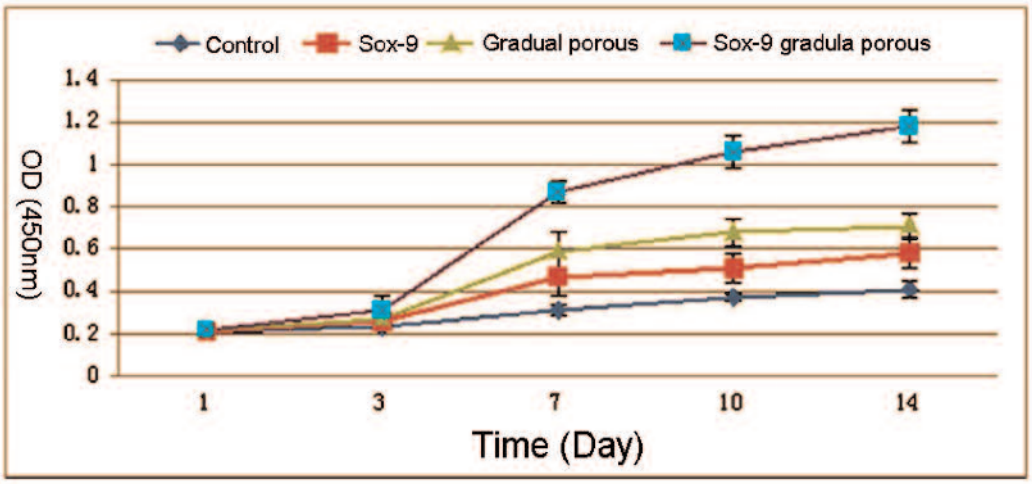

Figure 4. ADSC proliferation at different time points. The proliferation of ADSCs in each group was assessed by MTT assay.

had the highest proliferation rate and they had more ECM production than other groups.

Cell Proliferation

The proliferation of ADSCs in each group was shown in Figure 4. Differences in the proliferation rate were observed. The proliferation rate of cells in Sox-9 gradual group was significantly higher than that in other groups (p <0.05). This result suggested that exogenous Sox-9 promotes cell proliferation in a rational bionic environment.

ECM Deposition Onto the Cell-Scaffold Composite After 14-Day Culture

Compared with the control group, the production of proteoglycan and collagen by cells on gradual scaffolds significantly increased (Figure 5A). The amount of proteoglycan and collagen was the highest in Sox-9 gradual group. In addition, exogenous Sox-9 and gradual scaffolds promoted DNA synthesis (Figure 5B). These results were consistent with the results of cell proliferation. We normalized ECM content to the total DNA content. The normalized data demonstrated that the ECM content (either proteoglycan or collagen) in Sox-9 gradual group was higher than that in any other group (p <0.01, Figure 5C).

The Expression of Chondrogenic Related Genes

Real-time PCR analysis showed that the expression of chondrogenic genes was markedly up-regulated, except the type X collagen (p <0.01). Figure 5D showed that the expressions of PRG4, PTHrp, type II collagen, aggrecan, Sox-9 and HIF-1α in Sox-9 gradual group were significantly higher than those in Sox-9 group after 14 days culture (p <0.01).

Mechanical Properties of Tissues Formed on the Scaffold

The mechanical properties of the tissues formed on the scaffold were significantly improved at day 14 of cyclic compressive stimulation (Figure 5E). Tissues cultured in SOX-9 gradual group displayed an increase of load beating capacity (equilibrium stress at 10% strain) (control group: 2.011±0.235 KPa; SOX-9 group: 8.431±0.967 KPa; gradual group: 18.43±2.401kPa; SOX-9 gradual group: 29.13±3.669 KPa; n=6, p <0.01).

Computer Modeling Force Analysis

Both the top and bottom surfaces of each scaffold are subject to the largest force, and the force decreases progressively with depth (Figure 6). Compared with the uniform scaffold, the force exerted on the gradual scaffold decreases more slowly. The minimal stress levels approach the undersurface side, signifying that the gradual scaffold mildly absorbs the stress. The large area of low stress in the uniform porous scaffold may cause poor force distribution.

Discussion

Articular cartilage is stratified into zones delineated by characteristic changes in cellular, matrix, and nutritive components[9]. As a result, biochemical and biomechanical properties vary greatly between the different zones, giving the

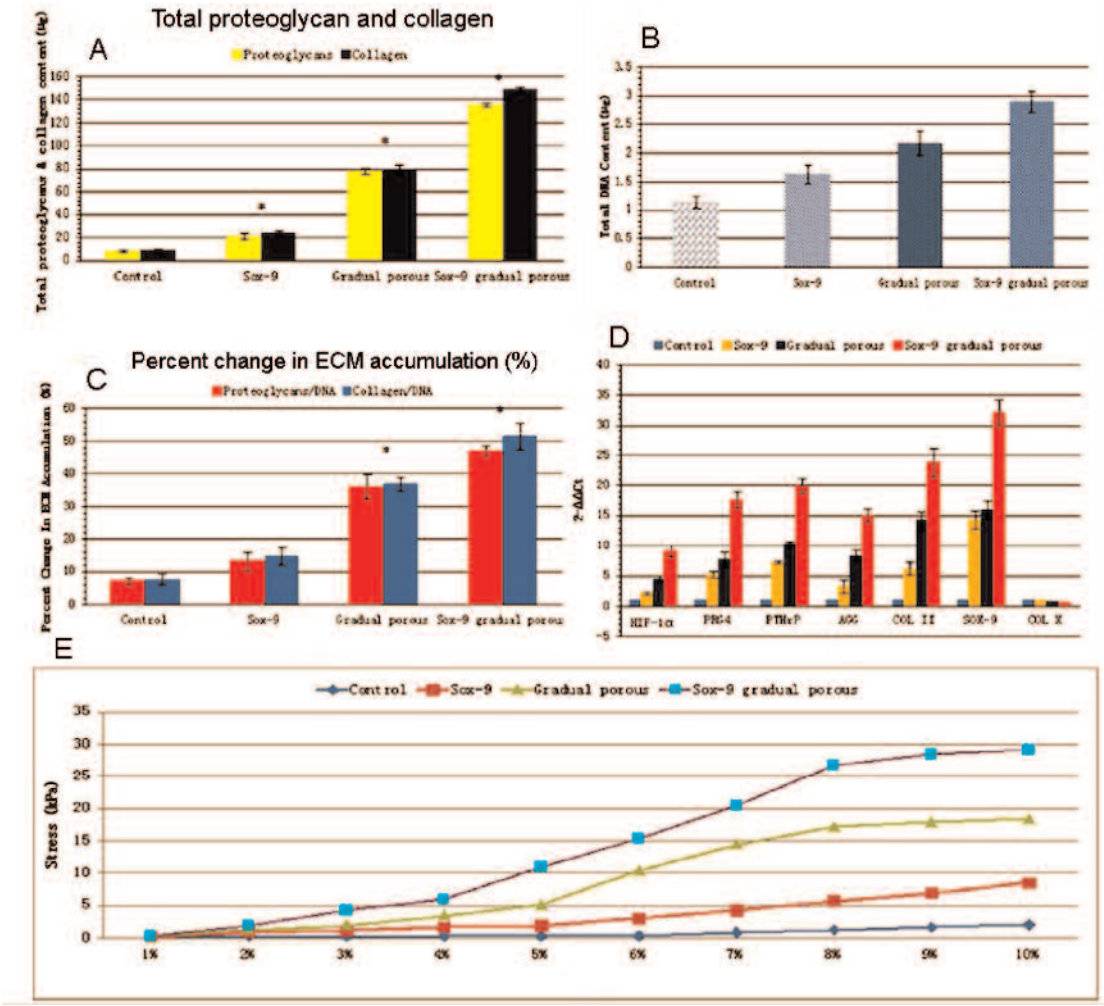

Figure 5. ***A,*** Total amounts of proteoglycan and collagen deposited in cell-scaffold composite after 7 days of culture. ***B,*** Total DNA content of the cell-scaffold composite. ***C,*** The proteoglycan and collagen normalized to total DNA of the cell-scaffold composite after 7 days of culture. ***D,*** The expression of HIF-1α, COL II, aggrecan, PTHrP, PRG4, SOX-9, and COL X. ***E,*** Stress versus strain profile for PLGA scaffolds.

tissue its unique structure and, thus, the ability to cope with extreme loading[10]. Due to the susceptibility of cartilage to damage and its poor reparative response, even small lesions can produce significant amounts of pain, joint stiffness, immobility, and, over time, an increased risk of osteoarthritis. One possible explanation for this effect is that the de novo tissue lacks the zonal organization critical for normal cartilage function[10].

Chondrocytes from different zones have distinct morphologies and protein expression. Therefore, engineering tissue with a zonal structure requires a thorough understanding of the *in vitro* behavior of zonal chondrocytes as well as a set of suitable markers that define their different phenotypes. The flattened cells of the superficial zone have been characterized by expression of specific proteins, such as proteoglycan 4 (PRG4)[11]. The more spherical and randomly distributed chondrocytes from the deeper layers have been found to specifically express osteogenic markers, e.g., type X collagen and PTHrP[12], Which were often used as an indicator of hypertrophy, and usually confined to the calcified, deeper regions of normal articular cartilage[13].

Wang et al[14] reported that compression at a frequency of 0.001-1 Hz could effectively stimulate cell proliferation and ECM synthesis, and a higher compression load was also beneficial for the construction of tissue-engineered cartilage. However, Stephanie et al[15] found that a sustained compression load could only stimulate the ana-

bolic activity of the cell, whereas intermittent stress stimulation could up-regulate both the anabolic and catabolic activities of the cell. Thus, intermittent mechanical compression is more beneficial for cell metabolism. Previous studies suggested that dynamic compression enhanced chondrogenic-related gene expression in ADSCs and promoted the aggregation of ECM.

Sox-9 is expressed in all chondroprogenitor cells, predominantly in mesenchymal condensations and cartilage[6]. However, the interaction of dynamic compression combined with SOX-9 on the chondrocyte phenotype and differentiation of ADSCs remains unclear.

Therefore, in this study, we investigated the effects of dynamic compression combined with exogenous SOX-9 on chondrogenesis of ADSCs. We found that dynamic compression and exogenous SOX-9 promotes chondrogenesis of ADSCs in a gradual porous PLGA scaffold.

We noticed that the combination of exogenous Sox-9 with the gradual PLGA composite scaffold benefited the metabolism and distribution of cells and synergistically upregulating HIF-1α expression of ADSCs. We assumed that the increased HIF-1α promoted the chondrogenic differentiation of ADSCs. HIF-1α might play a crucial role in the regulation of chondrogenic differentiation and proliferation of ADSCs. Here, a question was raised. What is the proper pore density to maintain HIF-1 α expression within the physiological range? The limitation of our study is the lack of *in vivo* animal experiments. These need to be further investigated.

Conclusions

We successfully constructed ADSCs that stably expressed Sox-9 and cultured Sox-9-ADSCs on 3D gradual porous PLGA composite scaffolds. ADSCs cultured on the 3D gradual porous PLGA composite scaffolds significantly enhanced the biological effects of Sox-9. In addi-

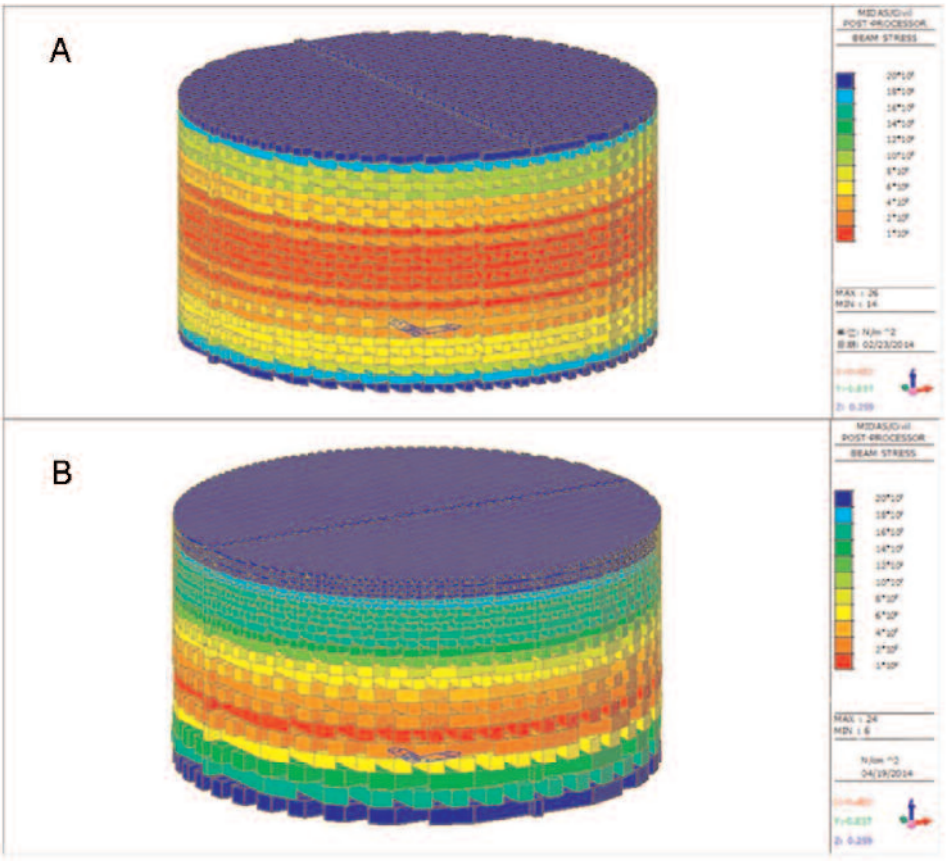

Figure 6. Computer modeling force analysis of PLGA scaffolds. Different force distribution in the uniform/porous scaffold was shown.

tion, a combination of exogenous Sox-9 with the gradual porous PLGA composite scaffold benefited the metabolism and distribution of the cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Huang CY, Hagar KL, Frost LE, Sun Y, Cheung HS. Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. Stem Cells 2004; 22: 313-323.
2) Meyer EG, Buckley CT, Steward AJ, Kelly DJ. The effect of cyclic hydrostatic pressure on the functional development of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells. J Mech Behav Biomed Mater 2011; 4: 1257-1265.
3) Lin Z, Willers C, Xu J, Zheng MH. The chondrocyte: biology and clinical application. Tissue Eng 2006; 12: 1971-1984.
4) Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. Nat Genet 1995; 9: 15-20.
5) Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Sox9 is required for cartilage formation. Nat Genet 1999; 22: 85-89.
6) Ikeda T, Kawaguchi H, Kamekura S, Ogata N, Mori Y, Nakamura K, Ikegawa S, Chung UI. Distinct roles of Sox5, Sox6, and Sox9 in different stages of chondrogenic differentiation. J Bone Miner Metab 2005; 23: 337-340.
7) Cucchiarini M, Thurn T, Weimer A, Kohn D, Terwilliger EF, Madry H. Restoration of the extracellular matrix in human osteoarthritic articular cartilage by overexpression of the transcription factor SOX9. Arthritis Rheum 2007; 56: 158-167.
8) Dai J, Wang H, Liu G, Xu Z, Li F, Fang H. Dynamic compression and co-culture with nucleus pulposus cells promotes proliferation and differentiation of adipose-derived mesenchymal stem cells. J Biomech 2014; 47: 966-972.
9) Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect 1998; 47: 477-486.
10) Klein TJ, Malda J, Sah RL, Hutmacher DW. Tissue engineering of articular cartilage with biomimetic zones. Tissue Eng Part B Rev 2009; 15: 143-157.
11) Schumacher BL, Block JA, Schmid TM, Aydelotte MB, Kuettner KE. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. Arch Biochem Biophys 1994; 311: 144-152.
12) Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001; 268: 189-200.
13) von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K, Stöss H. Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 1992; 35: 806-811.
14) Wang PY, Chow HH, Tsai WB, Fang HW. Modulation of gene expression of rabbit chondrocytes by dynamic compression in polyurethane scaffolds with collagen gel encapsulation. J Biomater Appl 2009; 23: 347-366.
15) Bryant SJ, Nicodemus GD, Villanueva I. Designing 3D photopolymer hydrogels to regulate biomechanical cues and tissue growth for cartilage tissue engineering. Pharm Res 2008; 25: 2379-2386.