Published in final edited form as:
Biomaterials. 2011 November ; 32(32): 8182–8189. doi:10.1016/j.biomaterials.2011.07.049.

Effects of Hypoxia and Scaffold Architecture on Rabbit Mesenchymal Stem Cell Differentiation towards a Nucleus Pulposus-like Phenotype

Ganjun Feng[a,b], **Xiaobing Jin**[b], **Jiang Hu**[b], **Haiyun Ma**[b], **Melanie J. Gupte**[c], **Hao Liu**[a,*], and **Peter X. Ma**[b,c,d,e,*]

[a]Department of Orthopedic Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China

[b]Department of Biologic and Materials Science, University of Michigan, Ann Arbor, MI 48109

[c]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109

[d]Macromolecular Science and Engineering Center, University of Michigan, Ann Arbor, MI 48109

[e]Department of Materials Science and Engineering, University of Michigan, Ann Arbor, MI 48109

Abstract

Nucleus pulposus grafts are needed for patients requiring replacement of their degenerated intervertebral discs. Bone marrow-derived mesenchymal stem cells (MSCs) are potential autologous stem cell source for the nucleus pulposus regeneration. One of the key issues of constructing functional nucleus pulposus using MSCs, however, is to differentiate MSCs into nucleus pulposus phenotype *in vitro* and to maintain their phenotypic stability *in vivo*. In this study, three-dimensional (3D) nanofibrous poly(L-lactide) (PLLA) scaffolds were seeded with multi-potent rabbit MSCs and the constructs were induced along nucleus pulposus development routes in a hypoxia chamber (2% O_2) in the presence of TGF-β1. It was found that nanofibrous scaffold could support the differentiation of rabbit MSCs towards a nucleus pulposus-like phenotype *in vitro*, as evidenced by upregulated expression of a few important nucleus pulposus-associated genes (aggrecan, type II collagen and Sox-9), abundant deposition of extracellular matrix (glycosaminoglycan (GAG) and type II collagen), and the continuous expression of the nucleus pulposus-specific marker, hypoxia-inducible factor (HIF)-1α. The subcutaneous implantation results confirmed that hypoxic induction before implantation could help the constructs to retain their phenotype and resist calcification *in vivo*. Therefore, the above data showed the promise of using 3D nanofibrous scaffolds in combination with TGF-β1 and hypoxic induction to regenerate functional nucleus pulposus grafts for intervertebral disc replacement.

Introduction

Low back pain is a frequent health problem that affects about 80% of the adult population at some point in their lives [1]. Intervertebral disc degeneration is the main cause of low back pain and is often associated with nucleus pulposus calcification [2, 3]. Current treatments, such as symptomatic treatment or surgical treatment, alleviate these symptoms in the short term but fail to address the underlying problem. Disc pathology treatment is now shifting toward disc regeneration. Although disc allografts have had moderate success in animal

*To whom correspondences should be addressed: Peter X. Ma, Ph.D., Richard H. Kingery Endowed Collegiate Professor, Department of Biologic and Materials Sciences The University of Michigan, 1011 North University Ave., Room 2211, Ann Arbor, MI 48109-1078, Tel: (734) 764-2209, Fax: (734) 647-2110, mapx@umich.edu.

models, availability of healthy human disc tissue is very limited. Tissue-engineered intervertebral disc grafts, resembling the natural intervertebral disc tissue structure and function, could potentially serve as an alternative source of grafts.

The native intervertebral disc is typically composed of two distinct anatomic regions, the annulus fibrosus and the nucleus pulposus [4]. The nucleus pulposus is composed of chondrocyte-like cells and embedded in a extracellular matrix containing mainly aggrecan and type II collagen, which provides elasticity and stability to the spine. Although nucleus pulposus cells or chondrocytes have been used to regenerate disc tissue in previous studies [5, 6], access to healthy nucleus pulposus cells or chondrocytes, especially autologous cells, is very limited in clinical settings. Bone marrow-derived mesenchymal stem cells (MSCs) are multi-potential stem cells that can differentiate along a number of lineages, including the chondrogenic lineage, potentially providing a suitable autologous stem cell source for nucleus pulposus tissue regeneration [7–10]. However, it is often observed that an inappropriate *in vivo* environment can alter the ultimate fate of implanted MSCs [11, 12]. This finding raises a concern over the feasibility of using MSCs to repair degenerated discs, where MSCs might tend to form ossified tissue after implantation. Therefore one of the key issues of constructing a functional nucleus pulposus with MSCs is to induce them into nucleus pulposus-like phenotype *in vitro* and maintain their phenotype after implantation. However, to date an MSC-based nucleus pulposus graft with the phenotypic stability has not been reported.

From previous studies, it is evident that the phenotypic stability of nucleus pulposus cells requires culture in a 3D format, where round cell morphology can be maintained [13–15]. As an artificial extracellular matrix, a good scaffolding material should mimic the advantageous features of the natural extracellular matrix [16]. Various hydrogel scaffolds have been widely used to support nucleus pulposus cells growth [17–19]. However, the low structural integrity of hydrogels may limit their application in disc regeneration. Previously we developed a 3D scaffold with highly interconnected macro-pores, nanofibrous matrix with a fiber diameter on the scale of 100 nm (similar to that of natural collagen fibers) and a high surface to volume ratio [20]. These physical characteristics are desired for constructing the nucleus pulposus tissue. On the other hand, the nucleus pulposus is a highly avascular tissue with no independent blood supply, low oxygen environment plays a vital role in maintaining or promoting the ideal nucleus pulposus phenotype [21]. We formed a hypothesis that, when combined with the low oxygen tension, the extracellular matrix-mimicking nanofibrous architecture could further mimic the physiological environment existing in the intervertebral disc, directing the differentiation of MSCs to a stable phenotype that is similar to native nucleus pulposus tissue. To test this hypothesis, rabbit MSCs were seeded on nanofibrous scaffolds, cultured in hypoxic environment to develop nucleus pulposus-like tissue, and then implanted into nude mice subcutaneously to examine phenotypic stability *in vivo*.

Materials and Methods

Fabrication of Nanofibrous PLLA matrices, smooth films and 3D scaffolds

Poly(L-lactide) (PLLA) with an inherent viscosity of approximately 1.6 dL/g was purchased from Boehringer Ingelheim (Ingelheim, Germany). Fabrication of thin nanofibrous matrices and smooth films has been previously described in details [22]. Briefly, the PLLA was dissolved in tetrahydrofuran (THF) (10% wt/v) at 60°C and cast into a pre-heated glass mold. The mold was quickly sealed using a cover glass. The PLLA solution was phase separated at −20°C for 2 hrs and then immersed into an ice/water mixture to exchange THF for 24 hrs. The matrix was washed with distilled water at room temperature for 24 hrs. The obtained thin sheets of nanofibrous matrices (thickness~40mm) were then vacuum-dried for

2 days. Smooth films were fabricated in a similar manner excluding the phase separation step. Instead, the solvent was evaporated at room temperature in a fume hood. The fabrication of the 3D nanofibrous scaffolds has also been previously described in details [22]. Briefly, PLLA/THF (10% wt/v) solution was cast into an assembled sugar template (formed from bound sugar spheres, 250–425 μm in diameter) under a mild vacuum, was phase separated at −20°C overnight, and then was immersed into cyclohexane to exchange THF for 2 days. The resulting composites were freeze-dried and the sugar spheres were leached out in distilled water and freeze-dried again to obtain highly porous scaffolds. The scaffolds were cut into circular discs with dimensions of 3.6 mm in diameter and 1 mm in thickness.

Cell culture and seeding

Totally nine 6-week old male rabbits were used in our experiments. Each time when we conducted an experiment with cells from one rabbit, we repeat the procedure with cells from 2 different rabbits. The data in our manuscript are representative of the triplicate samples. Rabbit bone marrow-derived MSCs were collected via aspiration from the femoral bone marrow using an 18-gauge syringe needle, collecting 10 ml of marrow blood into 1000 U of heparin [23]. The marrow blood was filtered through a cell strainer to exclude any fatty tissues and blood clots, and processed in a centrifuge at 600 g for 30 minutes. Rabbit MSCs were collected and cultured in 75-cm^2 flasks in low-glucose α-MEM (Gibco) containing 10% fetal bovine serum (Gibco) and antibiotics (penicillin G, 100 U/ml; streptomycin, 0.1 mg/ml) at 37°C under 5% CO_2. 3D nanofibrous PLLA scaffolds were pre-wetted by soaking in 70% ethanol for 30 min, washed three times with PBS for 30 min each, and twice in complete medium for 2 hrs each on an orbital shaker at 75 rpm. 2.5×10^5 cells were seeded onto each scaffold. After 2 hrs of initial seeding, cell-seeded scaffolds were cultured in chondrogenic medium supplemented with TGF-β1 (R&D Systems, Minneapolis, MN) at 0, 10, or 20 ng/mL in a hypoxia chamber (Stemcell technology, USA) at 2% O_2. Controls for the experiment were cell-scaffold constructs cultured in chondrogenic medium supplemented with corresponding doses of TGF-β1 at 20% O_2. The medium was changed twice a week.

Similar to treating 3D nanofibrous PLLA scaffolds, thin nanofibrous PLLA matrix and smooth films were also pre-wetted in 70% ethanol for 30 min, washed three times with PBS for 30 min each, and twice in complete medium for 1 hr each on an orbital shaker at 75 rpm. Cells were plated at a density of 10,000 cells/cm^2. After the initial 24 hrs of seeding and culture, the nanofibrous matrix and smooth films were transferred to the hypoxia chamber and the cell culture medium was replaced by chondrogenic medium supplemented with 10 ng/mL TGF-β1. Controls were maintained in the same medium but at 20% O_2. The medium was also changed twice a week.

RNA analysis

Cell-scaffold constructs were homogenized with a Tissue-Tearor. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and DNA was digested by RNase-free DNase set (Qiagen) according to the manufacturer's protocol. The cDNA was reversetranscribed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Real time PCR was done using TaqMan Universal PCR Master Mix (Applied Biosystems) and specific primers for Sox-9 (pre-designed, assay ID Oc04096872_m1), collagen type II (pre-designed, assay ID Oc03396134_m1), and aggrecan (primer sequence 5'-gctacggagacaaggatgagttc-3' and 5'-cgtaaaagacctcaccctccat). All RNA samples were adjusted to yield equal amplification of GAPDH as an internal standard.

Scanning electron microscopy (SEM)

Cell-scaffold constructs were rinsed in PBS, fixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight, post-fixed with 1% osmium tetroxide, and dehydrated in increasing concentrations of ethanol and hexamethyldisilizane (HMDS). The specimens were then sputter-coated with gold and observed under a scanning electron microscope (Philips XL30 FEG) at an accelerating voltage of 10 kV.

Histological and immunohistological analyses

Constructs were washed in PBS, fixed with 3.7% formaldehyde in PBS overnight, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at a thickness of 5 μm. For histological observation, sections were deparaffinized, rehydrated, and stained with H-E or Safranin O method. For immunohistochemical (IHC) staining for type I/II collagen, following deparaffinization and rehydration, slides were pretreated with pepsin solution (Fisher Scientific, USA) for 15 min, incubated with the primary antibody (goat type I collagen antibody of mouse type II collagen antibody, both from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilutions for 1 hr and prepared using a cell & tissue staining kit (R&D Systems Inc., Minneapolis, MN, USA). All sections were counterstained with hematoxylin. For immunofluorescent staining for HIF-1α, rehydrated sections were pretreated with pepsin solution for 15 min, incubated with HIF-1α antibody at 1:100 dilutions for 1 hr, rinsed with PBS three times for 5 min each, then incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibody at 1:100 dilutions for 1 hr. After rinsed for three times with PBS, sections were mounted using mounting medium containing 4'-6-diamidino-2-phenylindole (DAPI) and observed under a confocal fluorescence microscope. In order to quantify the cell number with positive immunofluorescent staining, at least four arbitrary view fields were chosen and the number of HIF-1α positive cells was recorded as a percentage of the total number of DAPI-stained cells.

Glycosaminoglycan (GAG) assay

Harvested constructs were washed with PBS, and digested with 200 μL papain solution (280 mg/mL in 50 mM sodium phosphate, pH 6.5, containing 5 mM N-acetyl cystein and 50 mM EDTA) for 24 hrs at 65°C. GAG content was determined by reaction with dimethylmethylene blue (DMMB) and measurement of optical density at 525 nm. Shark chondroitin 4-sulfate was used as the standard.

Subcutaneous implantation

Rabbit MSCs-scaffold constructs were induced by 20 ng/ml TGF-β1 under hypoxic or normoxic conditions for 3 weeks before implantation. The animal procedures were performed according to the protocol approved by the University of Michigan Committee on the Use and Care of Laboratory Animals. For the implantation experiments, 6–8 weeks old male nude mice (Charles River Laboratories, Wilmington, MA) were used. Surgery was performed under general inhalation anesthesia with isofluorane. Two midsagittal incisions were made on the dorsa and one subcutaneous pocket was created on each side of each incision using blunt dissection. One scaffold-cell construct with or without hypoxic induction was implanted subcutaneously into each pocket at random. Four samples were implanted for each group. After placement of implants, the incisions were closed with staples. At the end of 8 weeks of implantation period, the mice were euthanized and the implants were harvested for histological and immunohistochemical analyses.

Statistical analysis

Numerical data were reported as mean ±S.D. (n=3). The experiments were performed twice to ensure reproducibility. To test the significance of observed differences between the study groups, Student's t-test was applied. A value of $p < 0.05$ was considered to be statistically significant.

Results

Nucleus pulposus-like differentiation of rabbit MSCs on PLLA nanofibrous matrix

We first assessed the effect of nanofibrous architecture and hypoxia on the expression of genes that are associated with extracellular matrix production in nucleus pulposus (aggrecan, type II collagen and Sox-9). Rabbit MSCs were seeded on nanofibrous PLLA thin matrix and cultured under hypoxic conditions. As controls, rabbit MSCs were cultured on smooth PLLA films or/and under normoxia. After 2 weeks of culture, the expression levels of these genes were upregulated in rabbit MSCs cultured on nanofibrous matrix (Fig. 1A-C). In contrast, gene expression at lower levels was detected in cells cultured on smooth PLLA films. Furthermore, the effect of nanofibrous architecture on directing rabbit MSCs differentiation was synergically enhanced by low oxygen environment. To further test the hypothesis that rabbit MSCs under hypoxic conditions can achieve similar phenotype to that of nucleus pulposus cells, we examined the accumulation of HIF-1α, which is known as a phenotype marker continuously expressed in the nucleus pulposus cells. A significantly stronger fluorescent staining for HIF-1α was found in cells cultured on nanofibrous matrix under the hypoxic condition (Fig. 1D), indicating that hypoxia and nanofibrous architecture could also work in concert to upregulate HIF-1α expression.

In vitro culture of rabbit MSCs on 3D nanofibrous scaffolds

To develop nucleus pulposus-like tissue, rabbit MSCs were seeded on 3D nanofibrous scaffolds supplemented with varying doses of TGF-β1 in the low oxygen chamber. Cell-scaffold constructs were also cultured under normoxia as the controls. After 24 h of cell seeding, the cells aggregated inside the pores of the scaffolds (Fig. 2). After 3 weeks of culture, a TGF-β1 dose-dependent effect on the elevated expression of type II collagen, aggrecan and sox-9 genes was observed. Hypoxia also significantly upregulated the expression levels of these genes in the 3D scaffolds (Fig. 3A-C). Consistent with the result of gene expression, more abundant GAG (as shown by Safranin O staining) and type II collagen (as shown by immunohistochemical staining) amounts were detected in constructs cultured with 20 ng/ml TGF-β1 under hypoxia than under various control conditions (Fig. 4). The GAG content was also quantified to confirm this observation (Fig. 5). It was shown that both TGF-β1 and hypoxia increased GAG accumulation in the constructs. To further examine the phenotype of rabbit MSCs cultured on 3D scaffold, HIF-1α was stained using immunofluorescence method. Elevated expression of HIF-1α was noted in the constructs cultured under hypoxia compared with that cultured under normoxia (Fig. 6A-F). The percentage of HIF-1α positive cells under hypoxia was significantly higher than that of normoxia cultures (96.2±5.4% vs. 23.7±4.9%). However, no significant difference in HIF-1α expression was found between the constructs cultured with 10 ng/ml and 20 ng/ml of TGF-β1. Taken together, this data indicates that nucleus pulposus-like tissue can be achieved on 3D nanofibrous scaffold with a combination of hypoxia and TGF-β1.

Subcutaneous implantation of scaffold-cell constructs

To investigate the long-term stability of engineered nucleus pulposus-like tissue, the scaffold-cell constructs were implanted into nude mice subcutaneously after cultured *in vitro* under hypoxia or normoxia for 3 weeks. Because the *in vitro* result has demonstrated that

constructs cultured with higher dose of TGF-β1 could produce more extracellular matrix, we used 20 ng/ml TGF-β1 during the 3-week *in vitro* culture in both hypoxia and normoxia groups prior to implantation. After implantation for 8 weeks, the constructs were collected to examine the effect of the hypoxic induction prior to implantation on maintaining the phenotypic stability *in vivo*. The elevated expression of HIF-1α was retained in the implanted constructs that were pre-cultured under hypoxia (Fig. 6G-I), while the expression of HIF-1α was minimal in the constructs that were pre-cultured under normoxia (Fig. 6J-L). The percentage of HIF-1α positive cells in constructs with hypoxia induction was significantly higher than that with normoxia induction (94.7±6.5 vs. 9.1±3.2%). Consistently, strong positive staining for GAG (Fig. 7A) and type II collagen (Fig. 7C) was observed in the constructs that were pre-cultured under normoxia. These constructs were stained negative for type I collagen (Fig. 7E) or mineralization (Fig. 7G). In contrast, implanted constructs that were pre-cultured under normoxia were consisted of large areas of ossified tissue as stained positive using type I collagen antibody (Fig. 7F) and the von Kossa method (Fig. 7H), while they were stained weak for GAG (Fig. 7B) or type II collagen (Fig. 7D). These results indicated that hypoxic induction and the resultant HIF-1α expression might have plaid an important role in maintaining the stable phenotype of engineered nucleus pulposus-like tissue after subcutaneous implantation.

Discussion

The goal of the present investigation was to develop efficient methods to generate nucleus pulposus grafts with phenotypic stability. Our results clearly showed that, nanofibrous architecture and low oxygen tension could mimic the physiological environment in the intervertebral disc and thus provide necessary biochemical and physical cues to direct the differentiation of MSCs. When combined with hypoxia and TGF-β1, the nanofibrous scaffold was able to support nucleus pulposus-like tissue development and to retain their phenotype in a subcutaneous environment.

The controlled differentiation of MSCs is a necessary first step in using them as a cell source to engineer nucleus pulposus. Although researchers have not yet derived a list of specific gene expression profiles that fully characterize nucleus pulposus cells, they are normally described as “chondrocyte-like”, with characteristic markers of type II collagen, aggrecan and Sox-9. HIF-1α is a key transcription factor that is expressed in the nucleus pulposus cells and can be used as a phenotypic marker to distinguish nucleus pulposus cells from chondrocytes [24, 25]. Because collagen fibers are a main component of the extracellular matrix of nucleus pulposus and play an important role in the maintenance of cell and tissue function, we used nanofibrous architecture to mimic the structural feature of collagen fibers in this study. By combing the extracellular matrix-mimicking physical features with the physiological oxygen tension, we intended to further mimic the *in vivo* tissue-development environment in nucleus pulposus. It was found that nanofibrous architecture and hypoxia had a synergistic effect in inducing rabbit MSCs towards a phenotype similar to nucleus pulposus cells, suggesting the significant impact of environmental conditions that exist in the intervertebral disc on nucleus pulposus tissue regeneration.

Given the demonstrated superiority of the nanofibrous architecture in directing MSCs towards nucleus pulposus phenotype on the 2D substrate, porous 3D nanofibrous scaffolds were chosen to engineer nucleus pulposus tissue. Because the extracellular matrix in healthy nucleus pulposus is composed mainly of GAG and type II collagen, we examined the effect of different doses of TGF-β1 on GAG and type II collagen production. A significant effect of TGF-β1 on rabbit MSC differentiation was found either under normoxia or under hypoxia. Consistent with a previous study using a different system [26], the supplementation of TGF-β1 dramatically enhanced deposition of GAG and type II collagen, particularly with

the higher dose of 20 ng/mL TGF-β1. Consistent with the 2D culture study, the nucleus pulposus related genes (aggrecan, type II collagen and Sox-9) were also upregulated and the expression of HIF-1α at high levels was also detected under hypoxic conditions compared to that under the normoxic condition on 3D scaffolds. Furthermore, histological and immunohistochemical staining revealed that sulphate proteoglycan and type II collagen were significantly more abundant under hypoxic conditions than under the normoxic condition. It was previously reported that hypoxia might result in inhomogeneous extracellular matrix deposition within tissue-engineered constructs [27, 28]. However, we did not find an uneven tissue distribution in the 3D scaffolds under hypoxic conditions. This improvement could be partially attributed to the highly interconnected macro-pores and channels in our scaffolds, which likely have enhanced the mass transport conditions.

Calcification of intervertebral disc is associated with disease and aging, and is caused by inflammatory cytokines released in disc tissue that induce the differentiation of osteoprogenitor cells and promote angiogenesis, ultimately leading to ectopic calcification [2, 3]. This knowledge thus raises a concern over the potential clinical application of MSCs for nucleus pulposus repair. To reduce the risk of undesired tissue formation, prolonged *in vitro* induction is usually required to promote MSCs to a more differentiated stage and thus to retain more stable phenotype *in vivo*. In a previous study, stable engineered cartilage tissue is achieved by prolonging *in vitro* chondrogenic induction for 4 to 12 weeks [11]. However, this procedure is time consuming and the proper lineage commitment may not be guaranteed during culture. Another potential way is genetic manipulations before implantation. It has been reported that transfection with Sox-9 can facilitate differentiation of MSCs to nucleus pulposus-like cells with enhanced deposition of nucleus pulposus matrix [29]. However, the phenotypic stability of the construct was not examined *in vivo*. Furthermore, the safety concerns over genetic manipulations may limit its clinical application. Environmental cues could also affect the MSCs lineage commitment [30]. It has been recently recognized that hypoxia modulates endochondral bone formation and HIF-1α may serve as a regulator in preventing mineralization in the intervertebral disc [31, 32]. We therefore hypothesized that the cells on the scaffolds can be stably committed to the appropriate nucleus pulposus-like phenotype by priming under hypoxic conditions and become resistant to mineralization. Indeed, accompanied by a high level continuous expression of HIF-1α, abundant GAG and type II collagen were detected in the implanted cell-scaffold constructs that had been hypoxically primed. In contrast, the constructs that were not hypoxically primed tended to form ossified tissue associated with a lower level of HIF-1α expression. These results suggest that hypoxic priming may serve as an efficient way to achieve a stable and functional nucleus pulposus graft for intervertebral disc regeneration. Although the tissue generated in this study showed the promise to resist calcification for intervertebral disc regeneration, the subcutaneous implantation may not provide the same environment as that in the disc tissue. Studies on regenerative disc replacement in animal models are necessary to further test our hypothesis.

Conclusions

The experimental data presented above demonstrate that the nanofibrous architecture could work in concert with hypoxia to mimic physiological environment in nucleus pulposus and thus to direct the differentiation of MSCs towards the nucleus pulposus phenotype. The porous nanofibrous scaffolds, combined with TGF-β1 and hypoxic induction, were used to generate a nucleus pulposus-like tissue graft which resisted calcification in the subcutaneous environment. Thus, this model provides a very promising approach for nucleus pulposus regeneration.

Acknowledgments

The authors would like to acknowledge the financial support from the National Institutes of Health (Research Grants DE015384, and DE017689: PXM). GF was also supported by a fellowship from the China Scholarship Council.

References

1. Macfarlane GJ, Thomas E, Croft PR, Papageorgiou AC, Jayson MI, Silman AJ. Predictors of early improvement in low back pain amongst consulters to general practice: the influence of pre-morbid and episode-related factors. Pain. 1999; 80(1–2):113–119. [PubMed: 10204723]
2. Rutges JP, Duit RA, Kummer JA, Oner FC, van Rijen MH, Verbout AJ, et al. Hypertrophic differentiation and calcification during intervertebral disc degeneration. Osteoarthritis Cartilage. 2010; 18(11):1487–1495. [PubMed: 20723612]
3. Karamouzian S, Eskandary H, Faramarzee M, Saba M, Safizade H, Ghadipasha M, et al. Frequency of lumbar intervertebral disc calcification and angiogenesis, and their correlation with clinical, surgical, and magnetic resonance imaging findings. Spine. 2010; 35(8):881–886. [PubMed: 20354479]
4. Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine. 1995; 20(11): 1307–1314. [PubMed: 7660243]
5. Nishimura K, Mochida J. Percutaneous reinsertion of the nucleus pulposus. An experimental study. Spine. 1998; 23(14):1531–1538. discussion 1539. [PubMed: 9682309]
6. Okuma M, Mochida J, Nishimura K, Sakabe K, Seiki K. Reinsertion of stimulated nucleus pulposus cells retards intervertebral disc degeneration: an in vitro and in vivo experimental study. J Orthop Res. 2000; 18(6):988–997. [PubMed: 11192261]
7. Richardson SM, Walker RV, Parker S, Rhodes NP, Hunt JA, Freemont AJ, et al. Intervertebral disc cell-mediated mesenchymal stem cell differentiation. Stem Cells. 2006; 24(3):707–716. [PubMed: 16223853]
8. Steck E, Bertram H, Abel R, Chen B, Winter A, Richter W. Induction of intervertebral disc-like cells from adult mesenchymal stem cells. Stem Cells. 2005; 23(3):403–411. [PubMed: 15749935]
9. Richardson SM, Hughes N, Hunt JA, Freemont AJ, Hoyland JA. Human mesenchymal stem cell differentiation to NP-like cells in chitosan-glycerophosphate hydrogels. Biomaterials. 2008; 29(1): 85–93. [PubMed: 17920676]
10. Risbud MV, Albert TJ, Guttapalli A, Vresilovic EJ, Hillibrand AS, Vaccaro AR, et al. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. Spine. 2004; 29(23):2627–2632. [PubMed: 15564911]
11. Liu K, Zhou GD, Liu W, Zhang WJ, Cui L, Liu X, et al. The dependence of in vivo stable ectopic chondrogenesis by human mesenchymal stem cells on chondrogenic differentiation in vitro. Biomaterials. 2008; 29(14):2183–2192. [PubMed: 18289667]
12. Cui JH, Park SR, Park K, Choi BH, Min BH. Preconditioning of mesenchymal stem cells with low-intensity ultrasound for cartilage formation in vivo. Tissue Eng. 2007; 13(2):351–360. [PubMed: 17518569]
13. Gruber HE, Stasky AA, Hanley EN Jr. Characterization and phenotypic stability of humandisc cells in vitro. Matrix Biol. 1997; 16(5):285–288. [PubMed: 9501328]
14. Chou AI, Bansal A, Miller GJ, Nicoll SB. The effect of serial monolayer passaging on the collagen expression profile of outer and inner anulus fibrosus cells. Spine. 2006; 31(17):1875–1881. [PubMed: 16924203]
15. Reza AT, Nicoll SB. Serum-free, chemically defined medium with TGF-beta(3) enhances functional properties of nucleus pulposus cell-laden carboxymethylcellulose hydrogel constructs. Biotechnol Bioeng. 2010; 105(2):384–395. [PubMed: 19777586]
16. Ma PX. Biomimetic materials for tissue engineering. Adv Drug Deliv Rev. 2008; 60(2):184–198. [PubMed: 18045729]

17. Roughley P, Hoemann C, DesRosiers E, Mwale F, Antoniou J, Alini M. The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. Biomaterials. 2006; 27(3):388–396. [PubMed: 16125220]

18. Baer AE, Wang JY, Kraus VB, Setton LA. Collagen gene expression and mechanical properties of intervertebral disc cell-alginate cultures. J Orthop Res. 2001; 19(1):2–10. [PubMed: 11332616]

19. Joshi A, Fussell G, Thomas J, Hsuan A, Lowman A, Karduna A, et al. Functional compressive mechanics of a PVA/PVP nucleus pulposus replacement. Biomaterials. 2006; 27(2):176–184. [PubMed: 16115678]

20. Wei G, Ma PX. Macroporous and nanofibrous polymer scaffolds and polymer/bone-like apatite composite scaffolds generated by sugar spheres. J Biomed Mater Res A. 2006; 78(2):306–315. [PubMed: 16637043]

21. Mwale F, Roughley P, Antoniou J. Distinction between the extracellular matrix of the nucleus pulposus and hyaline cartilage: a requisite for tissue engineering of intervertebral disc. Eur Cell Mater. 2004; 8:58–63. discussion 63–54. [PubMed: 15602703]

22. Wei G, Ma PX. Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials. 2004; 25(19):4749–4757. [PubMed: 15120521]

23. Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K, et al. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: a potential therapeutic model for disc degeneration. Biomaterials. 2003; 24(20):3531–3541. [PubMed: 12809782]

24. Richardson SM, Knowles R, Tyler J, Mobasheri A, Hoyland JA. Expression of glucose transporters GLUT-1, GLUT-3, GLUT-9 and HIF-1alpha in normal and degenerate human intervertebral disc. Histochem Cell Biol. 2008; 129(4):503–511. [PubMed: 18172662]

25. Risbud MV, Guttapalli A, Stokes DG, Hawkins D, Danielson KG, Schaer TP, et al. Nucleus pulposus cells express HIF-1 alpha under normoxic culture conditions: a metabolic adaptation to the intervertebral disc microenvironment. J Cell Biochem. 2006; 98(1):152–159. [PubMed: 16408279]

26. Chen S, Emery SE, Pei M. Coculture of synovium-derived stem cells and nucleus pulposus cells in serum-free defined medium with supplementation of transforming growth factor-beta1: a potential application of tissue-specific stem cells in disc regeneration. Spine. 2009; 34(12):1272–1280. [PubMed: 19455002]

27. Malda J, Klein TJ, Upton Z. The roles of hypoxia in the in vitro engineering of tissues. Tissue Eng. 2007; 13(9):2153–2162. [PubMed: 17516855]

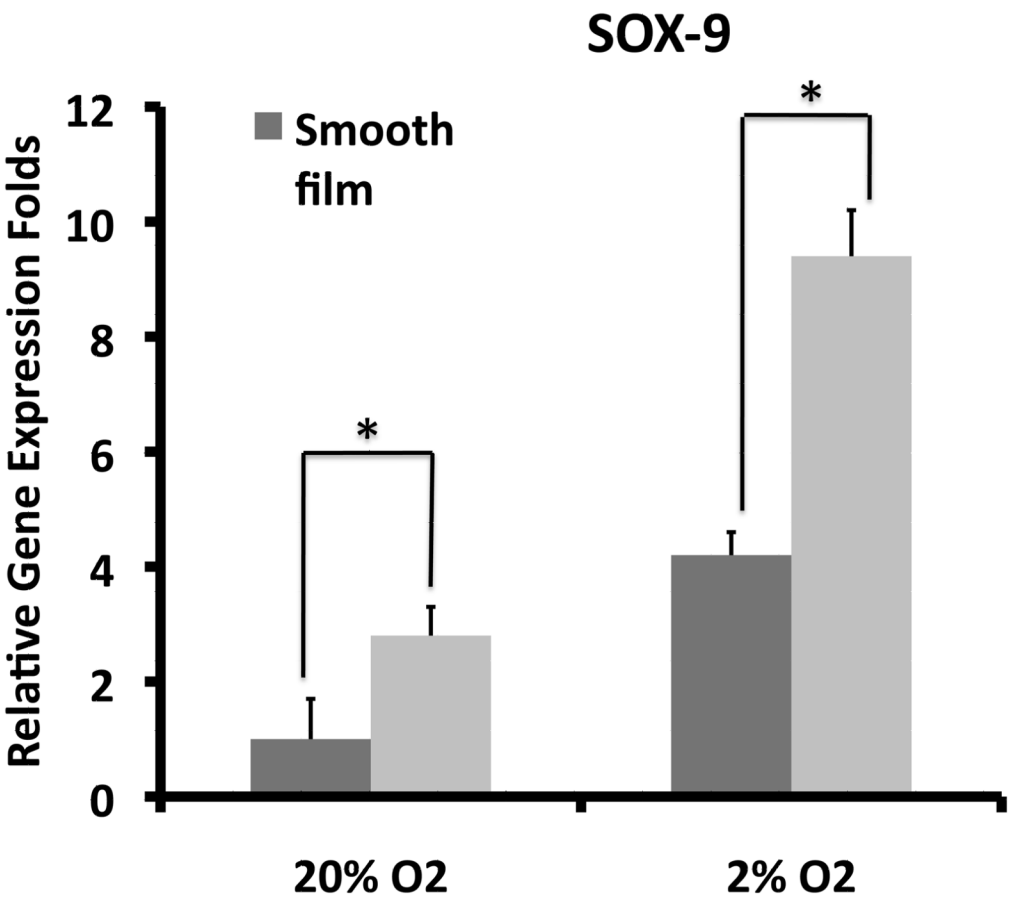
28. Volkmer E, Drosse I, Otto S, Stangelmayer A, Stengele M, Kallukalam BC, et al. Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. Tissue Eng Part A. 2008; 14(8): 1331–1340. [PubMed: 18601588]

29. Richardson SM, Curran JM, Chen R, Vaughan-Thomas A, Hunt JA, Freemont AJ, et al. The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-L-lactic acid (PLLA) scaffolds. Biomaterials. 2006; 27(22):4069–4078. [PubMed: 16569429]

30. Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol. 2001; 187(3):345–355. [PubMed: 11319758]

31. Araldi E, Schipani E. Hypoxia, HIFs and bone development. Bone. 2010; 47(2):190–196. [PubMed: 20444436]

32. Skubutyte R, Markova D, Freeman TA, Anderson DG, Dion AS, Williams CJ, et al. Hypoxia-inducible factor regulation of ANK expression in nucleus pulposus cells: possible implications in controlling dystrophic mineralization in the intervertebral disc. Arthritis Rheum. 2010; 62(9): 2707–2715. [PubMed: 20496369]



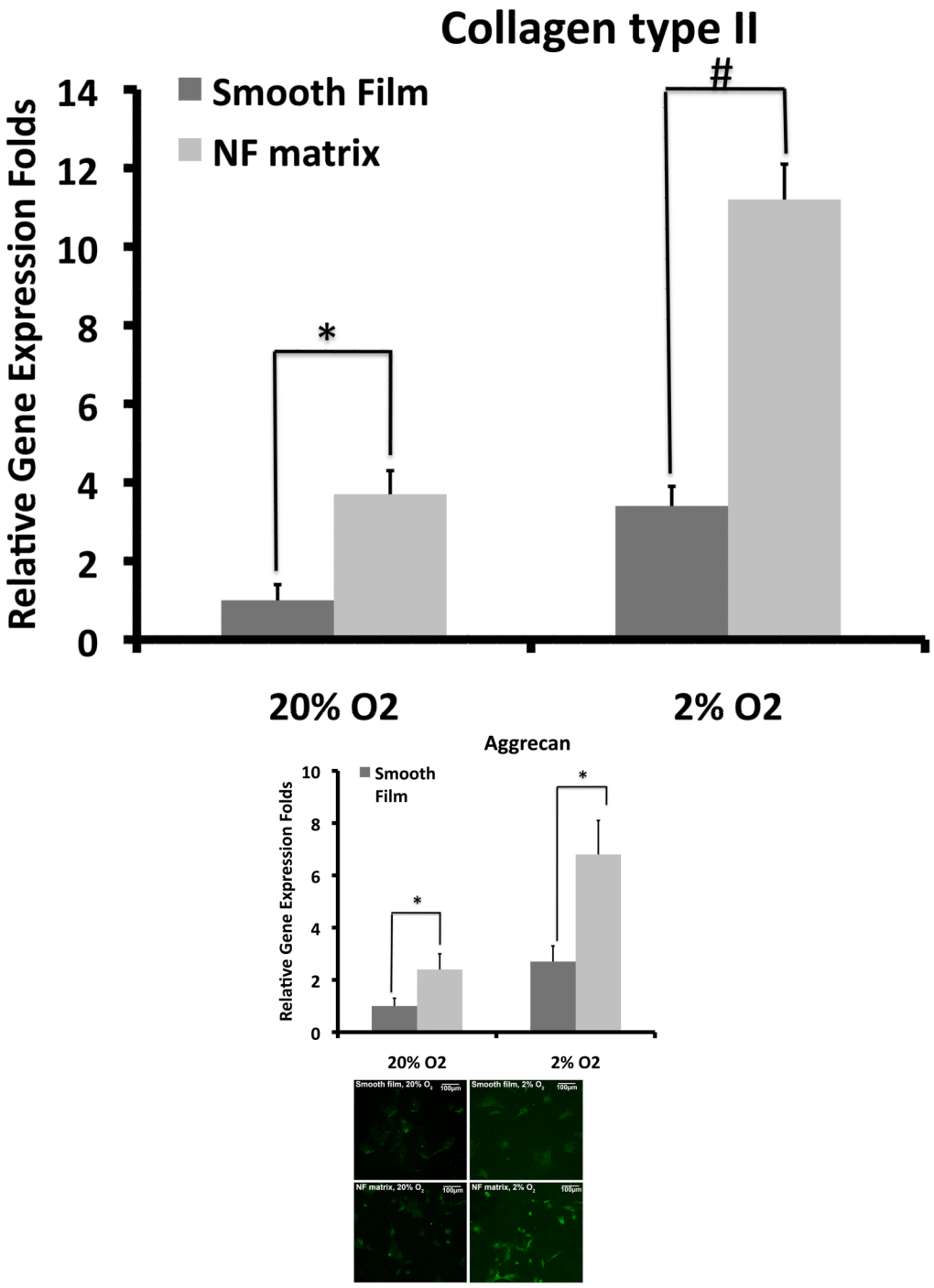
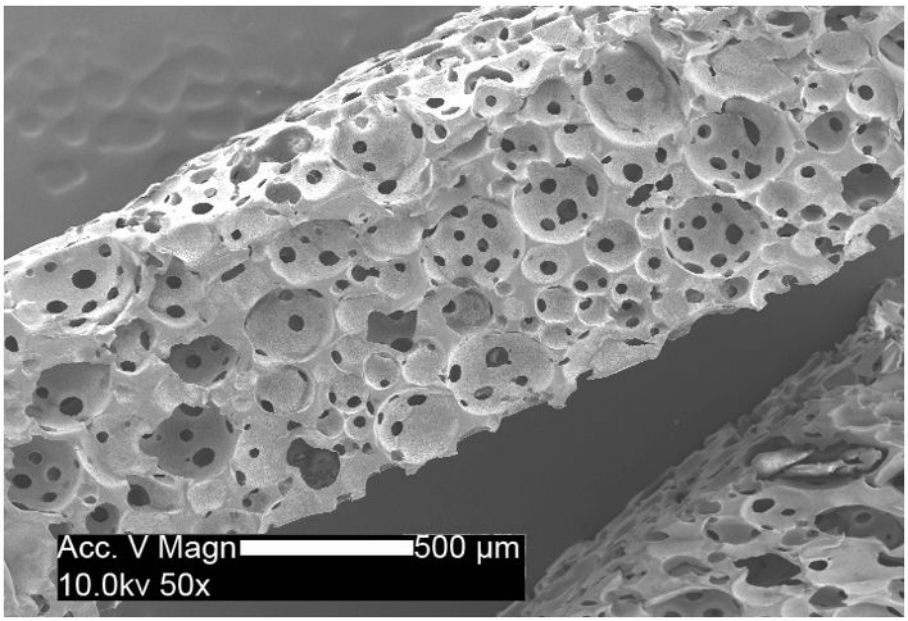
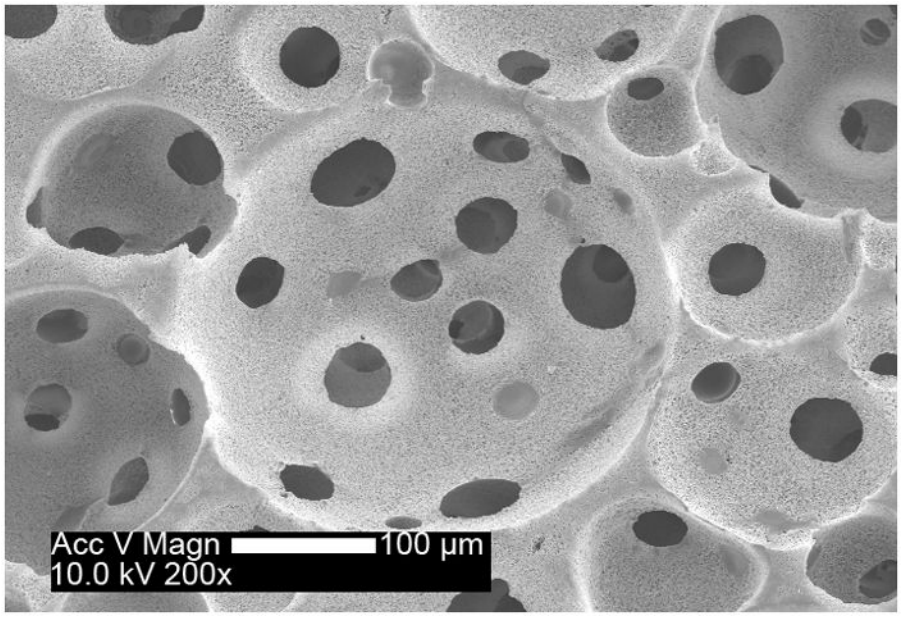


Figure 1.

Nanofibrous matrix and hypoxia synergistically induced nucleus pulposus-like differentiation of rabbit MSCs in the presence of TGF-β1 (10 ng/ml) for 2 weeks. Gene expression was examined using real time PCR. The expression of nucleus pulposus-associated genes (Sox-9, type II collagen and aggrecan) was significantly upregulated in cells on nanofibrous matrix than in those on smooth films (A–C). The expression levels of above genes were further upregulated by hypoxic environment. Relative gene expression folds on the Y-axis indicate the ratio (fold change) of the targeted gene expression level in

the experimental group over that of the reference group (smooth film, normoxia). The expression of HIF-1α (D) was visualized by confocal fluorescence microscope. The fluorescent signal intensity was significantly stronger on nanofibrous matrix compared with that on smooth films after hypoxic induction. Scale bar: 100 μm. *P<0.05, #P<0.01.





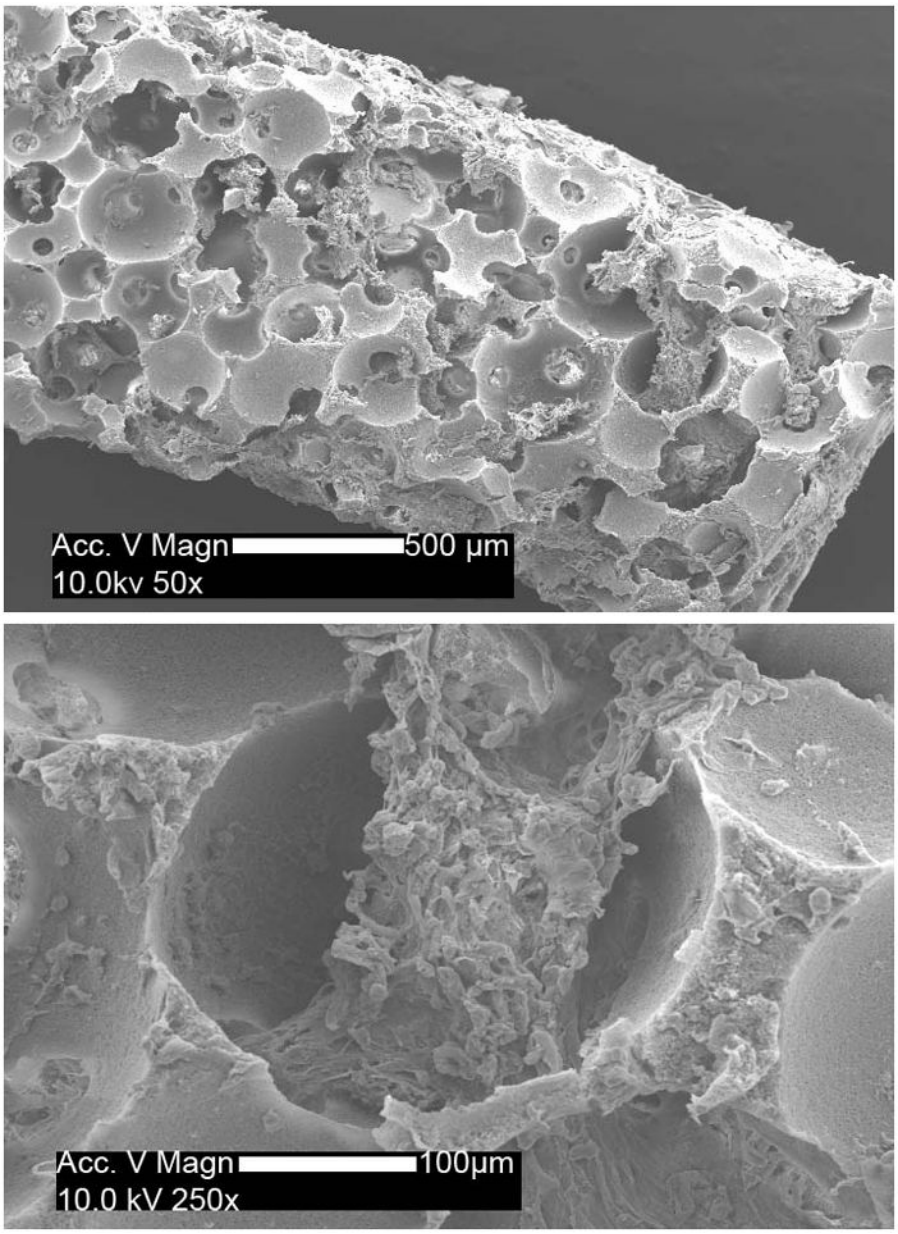


Figure 2.

SEM images of a nanofibrous PLLA scaffold and rabbit MSCs cultured on the scaffold: (A) A cross section of a blank scaffold at a low magnification; (B) The macro-pores and the interpore connections of the scaffold viewed at a higher magnification. The average diameter of nanofibers is on the scale of 100 nm as shown in our previous study [20]. However, the nanofibrous feature is not shown in these low magnification images. (C) An image of cells penetrated into the macro pores of a nanofibrous scaffold at a low magnification after 24 hrs of seeding and culture; and (D) The cell aggregates formed inside the pores of the nanofibrous scaffold viewed at a higher magnification.

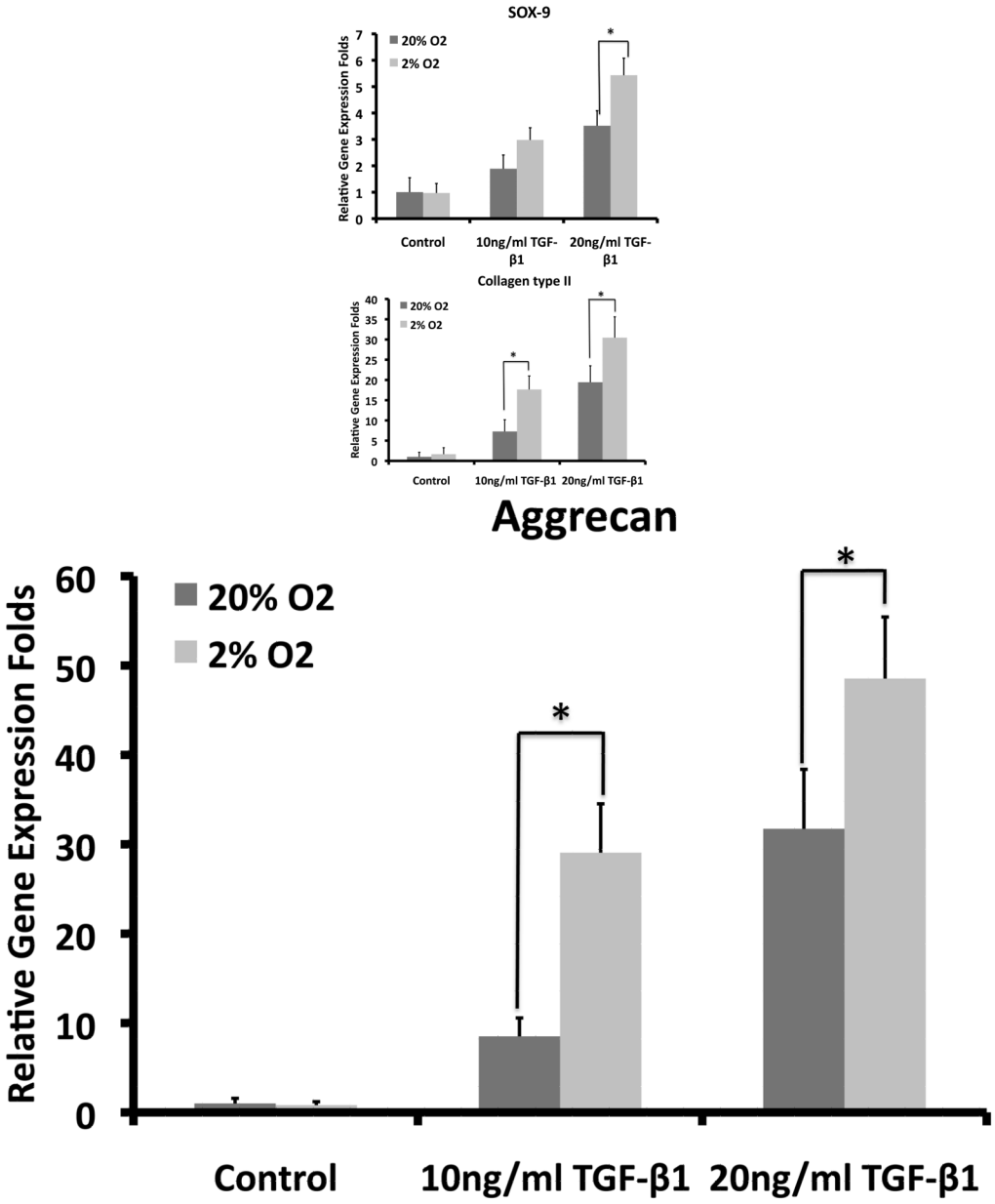


Figure 3.

Gene expression levels of cell-scaffold constructs cultured for 3 weeks with different doses of TGF-β1 supplementation under hypoxic or normoxic conditions: (A) Sox-9; (B) Type II collagen; and (C) Aggrecan. These genes were upregulated by both TGF-β1 and hypoxia. Relative gene expression folds on the Y-axis indicate the ratio (fold change) of targeted gene expression level in the experimental group over that of the reference group (0 ng/ml TGF-β1, normoxia). *P<0.05.

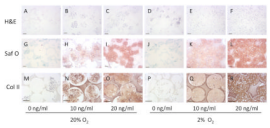


Figure 4.

Histological and immunohistochemical analyses of cell-scaffold constructs cultured with different doses of TGF-β1 supplementation under hypoxia or normoxia for 3 weeks: (A–F) H&E staining; (G–L) Safranin O staining; or (M–R) immunohistochemical staining for type II collagen. A stronger staining for GAG and type II collagen was found in constructs cultured with 20 ng/ml TGF-β1 under hypoxia than various control conditions. Scale bar: 100 μm.

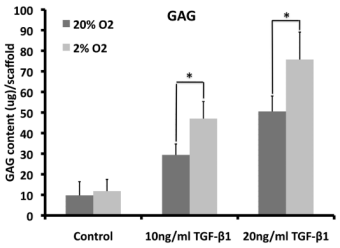


Figure 5.
GAG accumulation in cell-scaffold constructs cultured with different doses of TGF-β1 under hypoxia or normoxia for 3 weeks. The GAG content increased with the dose of TGF-β1 and the hypoxic condition. *P<0.05.

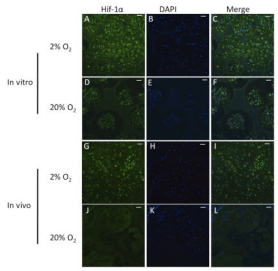


Figure 6.

Immunofluroscence staining for HIF-1α in cell-scaffold constructs after a 3-week *in vitro* culture and those with additional 8-week subcutaneous implantation: *In vitro*, strong positive staining for HIF-1α was observed in the constructs cultured under hypoxia (2% O_2) for 3 weeks; weak staining for HIF-1α was observed in the constructs cultured under normoxia (20% O_2) for 3 weeks.

In vivo (after 8 weeks of implantation), strong positive staining for HIF-1α was observed in constructs that had been pre-cultured under hypoxia for 3 weeks; weak staining for HIF-1α was observed in the constructs that had been pre-cultured under normoxia (20% O_2) for 3 weeks. Scale bars represent 50μm.

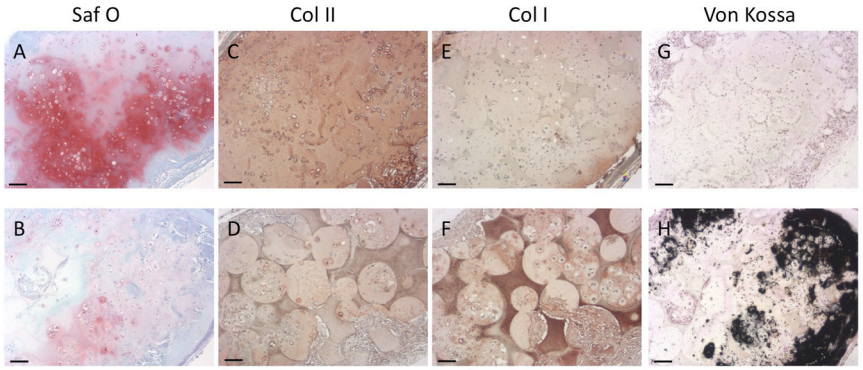


Figure 7.

Histological and immunohistochemical analyses of cell-scaffold constructs after 8 weeks of subcutaneous implantation, which were either pre-cultured under hypoxic conditions (A, C, E, G) or normoxic conditions (B, D, F, H) with 20 ng/ml TGF-β1 for 3 weeks before implantation. After 8 weeks of implantation, the constructs that had been pre-cultured under hypoxia were stained positive using Safranin O (A) and type II collagen antibody (C). These constructs were stained negative using type I collagen antibody (Fig. 7E) and von Kossa method (Fig. 7G). In contrast, the constructs that had been pre-cultured under normoxic conditions were stained weak using Safranin O method (Fig. 7B) and type II collagen antibody (Fig. 7D), while were stained positive using type I collagen antibody (Fig. 7F) and von Kossa method (Fig. 7H). Scale bar: 100 μm.