Chondrogenic differentiation of neonatal human dermal fibroblasts encapsulated in alginate beads with hydrostatic compression under hypoxic conditions in the presence of bone morphogenetic protein-2

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Abstract: In the present work, neonatal human dermal fibroblasts (nHDFs) were evaluated as a potential cell source for intervertebral disc repair. Chondrogenic differentiation of nHDFs was studied in the presence or absence of hydrostatic compression under normal and hypoxic conditions. In addition, the potentially synergistic effects of mechanical stimulation and bone morphogenetic protein (BMP)-2 on the chondrogenic differentiation of nHDFs were assessed. Mechanical stimulation was applied to the cells encapsulated in alginate beads using a custom-built bioreactor system for either a 1- or 3-week period at a frequency of 1 Hz for 4 h/day. In general, after 21 days of culture, high cell viability was observed for all the groups, with the exception of the groups exposed to intermittent mechanical stimulation for 3 weeks. Long-term intermittent application of mechanical stimulation under low O_2 conditions resulted in elevated collagen biosynthesis rate from day 0. Inclusion of BMP-2 for this group improved the chondrogenic differentiation of nHDFs, as indicated by elevated aggrecan gene expression and an increased collagen production rate compared to the day 0 group. Thus, the combination of hypoxia, BMP-2 supplementation, and long-term intermittent application of dynamic hydrostatic pressure was found to be a potent promoter of the chondrogenic differentiation of nHDFs. © 2011 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A: 000–000, 2011.

Key Words: hydrostatic compression, BMP-2, chondrogenic differentiation, dermal fibroblasts, alginate

INTRODUCTION

Degeneration of the intervertebral disc (IVD) is one of the leading causes of lower back pain, and the resulting disability evokes major socioeconomic challenges.[1,2] Hence, IVD regeneration and repair constitute an important area of investigation for the tissue engineering community. The IVD comprises three specific biologic parts: the endplates, the annulus fibrosus (AF), and the nucleus pulposus (NP), where the gelatinous NP core is surrounded by the AF region containing circumferentially oriented collagen fibers. The endplates form the superior and inferior surfaces of the IVD. The IVDs are avascular, which results in a limited ability for self-repair. For structural and functional restoration of damaged/degenerated IVDs, cell transplantation approaches have been widely investigated in the past for cell-mediated tissue repair. Specifically, autologous cells from a number of sources have been investigated for IVD repair purposes, such as IVD-derived cells,[3,4] chondrocytes from articular cartilage,[5] bone marrow-derived stem cells,[6–8] and adipose-derived mesenchymal stem cells.[9] Among other possible cell sources for IVD repair and regeneration are dermal fibroblasts, a relatively less-explored alternative cell-source for cartilage tissue engineering.[10–12] A tissue regeneration strategy leveraging dermal fibroblasts presents several potential advantages, including the following: (1) these cells can be easily isolated in a noninvasive and patient-specific manner, and (2) there is a relatively abundant supply.

Load-bearing cartilaginous tissues, such as intervertebral discs, naturally experience deformational compression during joint loading, which translates to hydrostatic compression due to the pressurization of the fluid phase in the biphasic tissue. Both deformational and hydrostatic pressures affect the metabolic activities of chondrocytes and their biosynthesis via mechanotransduction (see reviews[13,14]). Therefore, the role of mechanical stimulation as a chondrogenic signal has been widely studied in the cartilage tissue engineering field. Specifically, a number of studies have reported complex effects of mechanical stimulation via hydrostatic pressure on the overall cellular performance (i.e., gene expression and/or biosynthesis), where the profile of loading (static vs. dynamic; magnitude, duration and frequency of loading) has been found to be a highly important determinant (comprehensively reviewed previously[13]). Usually, dynamic hydrostatic pressure has been applied with amplitudes in the range of 3–18 MPa and frequencies in the range of 0.25–1 Hz, corresponding to the physiological levels.[13–17] In addition to its role as a mechanoregulator for chondrocytes, dynamic hydrostatic pressure has also been employed as a chondrogenic differentiation signal/stimulator *in vitro*, such as for bone marrow-derived

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stem cells,[18–21] embryonic fibroblasts,[22] and dedifferentiated chondrocytes,[23,24] a feature that may be harnessed for the chondrogenic differentiation of dermal fibroblasts.

Another critical element of the physiological cartilage environment is hypoxia. Cartilage is devoid of blood vessels and intrinsically experiences low oxygen tension. Typically, oxygen tension in the articular cartilages varies in the range of 1–7%.[25] A hypoxic environment (5% O_2) has been found to act as a potent chondro-promoter, leading to an improvement in the biosynthetic activity of the chondrocytes[26] and promoting mesenchymal stem cell differentiation[27] and redifferentiation of dedifferentiated chondrocytes.[28] Interestingly, hypoxia (2% O_2) has been found to influence human dermal fibroblasts grown in monolayers by up-regulating the synthesis of transforming growth factor (TGF)-β1,[29] which is a known chondrogenic factor. A hypoxic environment (5% O_2) was also found to improve the chondroinduction of human dermal fibroblasts by demineralized bone matrix.[30]

Growth factors form another potential set of signals that can be employed to guide cell differentiation *in vitro*. Many members of the TGF-β superfamily are known potent chondrogenic stimulators,[31] of which TGF-β1, TGF-β3, and bone morphogenetic protein (BMP)-2 are among the most commonly used growth factors for chondrogenesis.

A three-dimensional (3-D) culture environment, where the cells can maintain their spherical morphology, is known to be important for the culture of chondrocytes. In the absence of such an environment, chondrocytes lose their phenotype and dedifferentiate to fibroblast-like cells.[32] In this regard, alginate provides a natural material for 3-D encapsulation of cells. Alginate is soluble in aqueous solutions, and can be polymerized into a hydrogel by ionic crosslinking in the presence of divalent calcium ions. Alginate bead culture has been shown to support chondroinduction previously.[23,24,28,33]

Prior approaches explored for the chondrogenic differentiation of dermal fibroblasts include high density micromass culture in the presence of lactic acid,[12] culture in the presence of demineralized bone powder/matrix,[11,34] and culture of insulin-like growth factor (IGF)-1 pre-treated fibroblasts on an aggrecan substrate.[10] Building upon the existing knowledge of the role of dynamic hydrostatic pressure and hypoxia in the chondroinduction and maintenance of the chondrogenic phenotype for numerous cell types, combination of these signals may provide a biomimetic strategy for the chondrogenic differentiation of HDFs. In this work, chondrogenic differentiation of neonatal human dermal fibroblasts (nHDFs) was attempted in 3-D alginate beads using two primary potential chondrogenic signals in isolation or conjugation: (1) dynamic hydrostatic pressure and (2) hypoxia. A full factorial design was employed, where biomechanical stimulation was applied intermittently for either a short-term (1 week) or a long-term (3 week) duration (static culture served as a control environment), and hypoxic culture was performed with 5% O_2 (normoxia at 20% O_2 served as a control condition). Chondrogenic differentiation was also evaluated in the presence of simultaneous biomechanical and biochemical stimulation under hypoxic/normoxic conditions, where biochemical stimulation was applied by supplementing the culture medium with BMP-2. The following questions were specifically raised: (1) whether dermal fibroblasts can maintain viability and undergo chondrogenic differentiation in a 3-D environment using dynamic hydrostatic pressure and/or hypoxia as chondrogenic signals and (2) whether medium supplementation with BMP-2 in combination with mechanical stimulation promotes chondrogenic differentiation in an additive/synergistic manner. We hypothesized that a combination of hypoxia and long-term intermittent application of dynamic hydrostatic pressure, conditions that mimic the physiological cartilage environment, would enable nHDFs to undergo chondrogenic differentiation while maintaining high viability. Moreover, we hypothesized that, due to the addition of growth factor stimulation, simultaneous application of dynamic hydrostatic pressure along with BMP-2 stimulation would enhance chondrogenic differentiation.

MATERIALS AND METHODS

Experimental design

In this study, the effects of biomechanical stimulation alone and in combination were evaluated on chondrogenic differentiation of nHDFs under normoxia (20% O_2) and hypoxia (5% O_2). nHDF culture was performed for a 3 week duration using alginate bead encapsulation as a 3-D cell culture environment. A schematic depiction of the study design is shown in Figure 1. Specifically, intermittent biomechanical stimulation (4 h/day) was applied at three levels: 1 week duration, 3 week duration (referred to as short-term and long-term durations, respectively), and no stimulation (static, control). In order to observe the combined effects of biomechanical and biochemical stimulations, beads from specific groups were cultured in medium supplemented with BMP-2 during the mechanical stimulation phase. In addition, effects of normoxia and hypoxia were assessed by performing the bead culture for all the groups in two different culture environments differing in oxygen tension: 20% O_2 and 5% O_2 (referred to as high O_2 and low O_2, respectively).

Cell expansion

nHDFs isolated from human foreskin were purchased from Invitrogen Life Technologies (Carlsbad, CA; catalog C-004-5C, lot 747436). The cells were plated for expansion in monolayer and incubated at 37°C in 5% CO_2 with media replacement every other day. The culture medium for cell expansion and maintenance consisted of Dulbecco's modified eagle medium (DMEM, high glucose), 1% penicillin-streptomycin (PS), 25 m*M* HEPES, (all from Invitrogen Life Technologies), and 10% fetal bovine serum (FBS, Gemini, Calabasas, CA).[10,30] At about 90% confluence, the cells were trypsinized and replated. nHDFs were passaged twice before encapsulation in alginate beads.

Alginate bead preparation

Alginate solution (2.0% w/v) was prepared by mixing alginic acid sodium salt from brown algae (medium

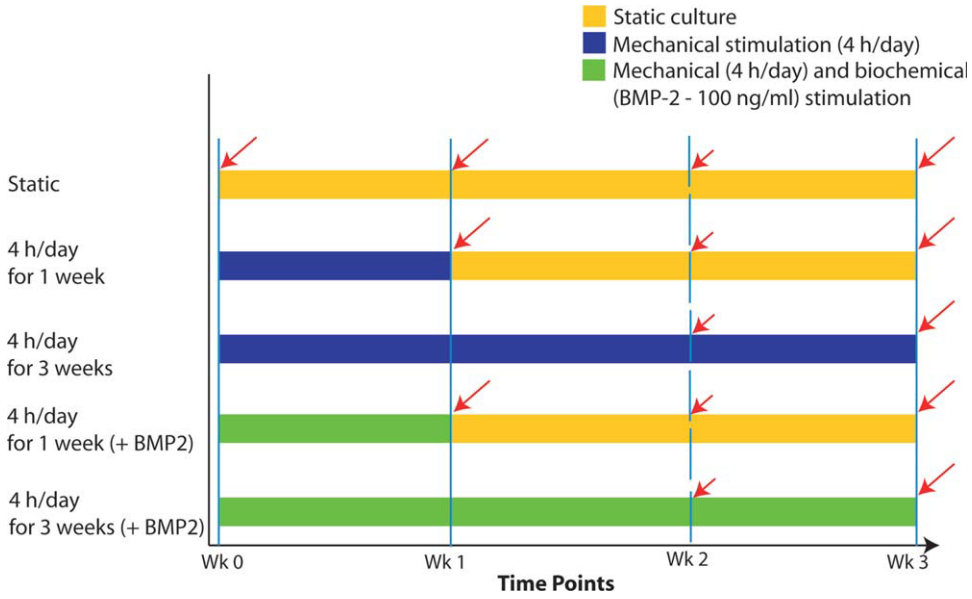

FIGURE 1. A schematic representation of the study design. Mechanical stimulation was applied at three levels: static (no stimulation), short term (1 week), and long term (3 weeks). Additionally, to assess the combined effects of biomechanical and biochemical stimulation, beads from certain groups were cultured in the presence of BMP-2 during the mechanical stimulation phase. All the groups shown here were cultured in one of two different environments—normoxic (20% O_2) or hypoxic (5% O_2). In the scheme, long arrows indicate the common time points of analysis. Short arrows (at week 2) indicate an additional time point used for the determination of matrix synthesis rates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

viscosity, Sigma-Aldrich, St. Louis, MO), 0.025*M* HEPES, and 0.15*M* NaCl in deionized water (pH 7.4), which was mildly heated on a hot plate to dissolve the alginate and then sterile filtered. Before cell encapsulation, the temperature of the alginate solution was equilibrated to 37°C. A cell suspension in alginate was prepared by suspending the nHDFs in the alginate solution at a density of $\sim 10^7$ cells/mL, which was then injected into a 102 m*M* $CaCl_2$ solution (Sigma-Aldrich) using a 20-gauge needle. Alginate beads, ~2–3 mm in diameter (~250,000 cells per bead), formed instantaneously and were incubated for 15 min in the $CaCl_2$ solution under stirring. The alginate beads were rinsed twice with PBS and once with DMEM, and then placed into 6-well plates with 0.5 mL medium/bead. nHDFs encapsulated in the alginate beads were cultured in DMEM, 1% PS, 25 m*M* HEPES, 1% nonessential amino acids (NEAA), 0.4 m*M* L-proline (all from Invitrogen Life Technologies, Carlsbad, CA), 10% FBS and 50 μg mL^{-1} L-ascorbic acid (Sigma). Beads were equilibrated for 2 days under static culture conditions. Following this bead consolidation period, marked as day 0, beads were allotted to specific groups and cultured for 3 weeks according to the aforementioned experimental design (Fig. 1).

Biomechanical stimulation system

A custom hydrostatic pressure bioreactor was used for the biomechanical stimulation of the beads (Fig. 2). The bioreactor consisted of a pressure-rated vessel (volume 600 mL, pressure rating 2000 psi; Parr Instruments, Moline, IL) connected to a medium-duty hydraulic cylinder-piston assembly (35-mm bore; PHD, Inc., Fort Wayne, IN) via a pressure-rated nylon hose. Using custom-designed mounts, the cylinder was mounted in a servohydraulic testing machine (MTS 858 Mini Bionix, MTS Systems Corp., Minneapolis, MN) hosting a 10 kN load cell. The operating temperature of the pressure vessel was regulated to physiological levels (37.0 ± 0.5°C) by submerging the vessel in a water bath. The temperature inside the vessel was monitored by a bench-top temperature controller (EW-02110-82, Cole Palmer, Inc., Vernon Hills, IL) using a thermocouple installed in the vessel, which was corrected to the desired operating temperature by altering the temperature of the water bath via a commercial immersion heater. To achieve the desired levels of hydrostatic pressure, the cylinder, pressure vessel, and pressure hose were fully charged with water before beginning the operation.

Bead culture

Static bead culture was performed in 6-well plates in two environments of different oxygen tensions: high O_2 and low O_2. Hypoxia was attained in a HeraCell 150 tri-gas incubator, which maintained the oxygen tension at a 5% level. Beads exposed to hydrostatic pressure were cultured as schematically shown in Figure 3. Briefly, beads undergoing biomechanical stimulation from each group were heat-sealed in separate sterilized polyethylene/nylon bags (FoodSaver®, Tilia, Inc., San Francisco, CA) with approximately 0.5 mL medium/bead.[35] Bags were then loaded in the pressure vessel, and a sinusoidal hydrostatic pressure profile was applied under force-control mode between the levels of 0.3 and 5 MPa at a frequency of 1 Hz [Fig. 2(B)] for 4 h/day. Pressure was approximated as the force applied by the MTS divided by the cross-sectional area of the cylinder.[22,35] The temperature was consistently maintained at

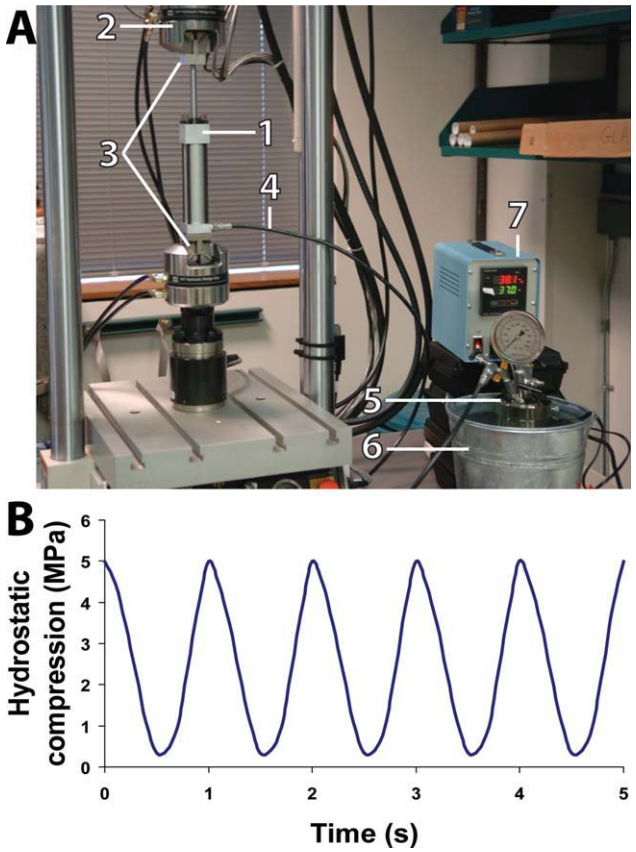

FIGURE 2. (A) An image of the hydrostatic compression bioreactor set-up; (1) A cylinder-piston assembly, (2) MTS 858 Mini Bionix Servohydraulic Testing Machine, (3) two stainless steel mounts, (4) a pressure-rated nylon hose, (5) a high pressure vessel, (6) water bath, (7) a bench-top temperature controller. (B) Applied hydrostatic compression profile having a sinusoidal pressure variation from 5 to 0.3 MPa at a frequency of 1 Hz. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

37.0 ± 0.5°C throughout the duration of mechanical stimulation. During the dynamic pressurization phase, oxygen tension was not controlled in the bags and may be approximated to the ambient normoxic conditions. Following the 4 h stimulation period, the beads were unpacked from the bags and returned to static culture in normal or hypoxic conditions for the remaining period each day, and the medium was replaced every other day. Beads undergoing simultaneous biomechanical and biochemical stimulation were cultured in a similar manner with the only difference being that the culture medium was supplemented with 100 ng/mL of recombinant human BMP-2 (355-BM, R&D System, Minneapolis, MN). At each time-point, beads from specific groups were collected for construct analysis.

Cell viability assessment

At days 0, 2, 4, 7, and 21, nHDF viability was evaluated by staining the beads using a LIVE/DEAD reagent (2 μM calcein AM, 4 μM ethidium homodimer-1; Molecular Probes) followed by 30 min incubation at room temperature, before being subjected to confocal microscopy (Zeiss LSM 510, Thornwood, NY).[36] From each group, one bead was scanned along the depth, and images were acquired from a 200 μm deep section at 10 μm intervals. Percent viability was quantified from the images acquired from sequential scaffold volumes of 900 × 900 × 50 μm along the depth using NIH ImageJ ($n = 4$). Briefly, individual live/dead scans were converted to binary images, which were then segmented using an adaptive 3-D threshold tool (base threshold 127, mask diameter 3 pixels, local weight 5%) of MacBiophotonics ImageJ (MBF ImageJ, freeware). The segmentation protocol was used for the demarcation of cells present on the plane of imaging from those present in the adjacent planes, which considerably reduced the possibility of repeated counting of a single cell. Following the segmentation, cell counts were estimated using automatic particle count, and percent viability was obtained from the ratio of live cells to total cells.

Real time RT-PCR

Samples from each group were subjected to RT-PCR analysis at different time points. Briefly, cells were isolated from the alginate as described above. The cell pellet was then transferred into RNA lysis buffer, and the cell suspension was homogenized and purified using a Qiagen shredder column.

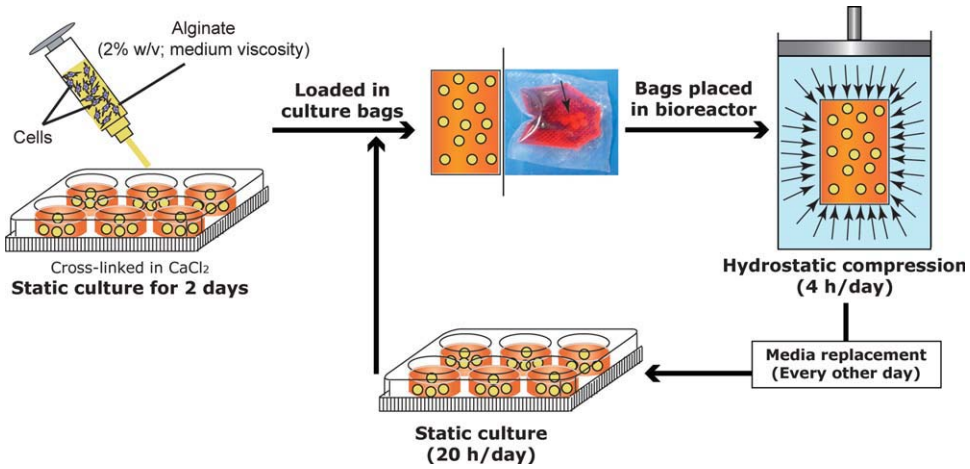

FIGURE 3. A schematic diagram displaying the bead culture procedure for the beads exposed to hydrostatic compression. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Total RNA extraction was then performed using an RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Using the RNA samples, cDNA was synthesized via reverse-transcription using Oligo dT primers (Promega) and superscript III transcriptase (Invitrogen). The cDNA samples were then subjected to real time PCR (ABI Biomed 7300 Real-Time PCR System) in order to determine the expressions of collagen type II, aggrecan, and collagen type I genes, as described previously.[37] Gene expressions were determined by the $2^{-(\Delta\Delta C(t))}$ method using the glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) gene as the house keeping gene[38] and expressed as the fold ratio compared with baseline gene expression of the day 0 group.[37] The sequence of primers for GAPDH, type I collagen, type II collagen, and aggrecan were as follows: GAPDH gene: 5′-TGGGCTACACTGAGCACCAG-3′, 5′-CAGCGTCAAAGGTGGAGG AG-3′ (BC029618); collagen type I gene: 5′-ATGCCTGGTGAA CGTGGT-3′, 5′-AGGAGAGCCATCAGCACCT-3′; collagen type II gene: 5′-GAGACAGCATGACGCCGAG-3′, 5′-GCGGATGCTCTCAA TCTGGT-3′ (BC007252); aggrecan gene: 5′-AGCCTGCGCTCC AATGACT-3′, 5′-TGGAACACGATGCCTTTCAC-3′ (NM_013227) (gene bank account numbers indicated in parentheses, where applicable).[33,39]

Determination of biosynthesis rates using radiolabeling

Radiolabeled [^{3}H]-proline and [^{35}S]-sulfate incorporation rates were used as a measure of collagen and sulfated glycosaminoglycan (sGAG) synthesis rates, respectively, as previously described.[40,41] On days 0, 7, 14, and 21 of culture, beads from each group were transferred to 24-well plates, and 1 mL of radiolabeled medium was added to each bead in separate wells. The radiolabeled medium consisted of proline-free culture medium (bead culture medium without the L-proline) supplemented with 10 μCi/mL [^{3}H]-proline and 5 μCi/mL [^{35}S]-sulfate (both from PerkinElmer, Waltham, MA). Following a 24 h static incubation period in respective oxygen tension conditions, the radiolabeled medium was removed, and beads were stored at −4°C until the completion of the final time-point. Subsequently, the beads were washed three times over a 1 h period in PBS supplemented with 1 m*M* unlabeled-proline and -sulfate,[41] which were then digested individually in 1 mL of a papain solution (120 μg/mL) overnight at 65°C.[42] A 100 μL aliquot of the digest was used for DNA content determination using a PicoGreen assay kit (Molecular Probes). Remaining digests were transferred to Mini Poly-Q vials (Beckman Coulter, Brea, CA) with 5 mL of a scintillation fluid, and [^{3}H] and [^{35}S] counts per minute (cpm) were measured using a liquid scintillation counter (LS-6500; Beckman Coulter, Fullerton, CA).

Histology and immunohistochemistry

Alcian blue staining of the beads was performed according to a critical electrolyte concentration technique for selective staining of GAGs, as described previously.[43] Briefly, beads were stained for 72 h in a solution of 0.05% alcian blue 8GX containing 25 mM sodium acetate, 2.5% glutaraldehyde, and 0.4*M* $MgCl_2$ (pH adjusted to 1.5) (all reagents from Sigma). Following the staining, beads were sequentially washed and dehydrated with 3% acetic acid, 3% acetic acid in 25% ethanol (v/v), 3% acetic acid in 50% ethanol, and 3% acetic acid in 75% ethanol (for simultaneous sequential dehydration). Beads were then embedded in Tissue-Tek cryo-OCT Compound (Fisher Scientific). Sections of 10 μm thickness were then prepared from the beads using a cryostat (Microm HM 500, Ramsey, MN) and were then mounted onto Superfrost Plus slides (Fisher Scientific) and imaged using a light microscope (Nikon Eclipse E600, Melville, NY) with a video camera attachment (Sony DXC950P, New York, NY).

Immunohistochemistry was performed to qualitatively identify the localization of major extracellular matrix (ECM) components (collagen type II, aggrecan and collagen type I).[42] Briefly, beads were fixed, dehydrated, and embedded in paraffin, from which sections of 5 μm thickness were prepared using a microtome and mounted on Superfrost Plus slides (Fisher Scientific). For immunostaining, sections were deparaffinized and blocked for endogeneous peroxidase activity with 3% H_2O_2 in methanol for 10 min. The sections were then blocked in 3% rabbit serum for 30 min and incubated with a primary antibody overnight at 4°C. The primary antibodies used in this study included goat polyclonal immunoglobulin (IgG) anti-collagen type I, goat polyclonal IgG anti-collagen type II, goat polyclonal IgG anti-aggrecan (all at 1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing the slides, the sections were incubated with a streptavidin-linked anti-goat IgG secondary antibody (VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingame, CA) for 30 min, after which the sections were incubated with avidin-biotinylated enzyme complex (ABC complex; Vector Laboratories) for 30 min. Finally, ImmPACT DAB substrate (Vector Laboratories) was applied on sections for 5–6 min. Slides prepared by the omission of primary antibody incubation served as the negative controls.

Statistics

Data (% cell viability, gene expression fold ratio, matrix synthesis rate and DNA content) obtained for different groups at the specified time points are reported as means ± standard deviations ($n = 4$). Repetitive ANOVA was used to determine the possible statistical differences between the groups, which was followed by a Tukey's *post hoc* test when significance was detected ($p < 0.05$). Definitions of statistical significance symbols used in Figures 4–6 are provided in Table I.

RESULTS

For the purpose of brevity, the nomenclature that has been used in the remainder of this manuscript identifies different culture groups using the levels of four specified variables, which are the duration of culture (time point), oxygen tension level (high or low), mechanical stimulation level (static, short-term, or long-term), and presence/absence of BMP-2 during the mechanical stimulation phase (+/−).

nHDF viability was characterized using the Live/Dead assay on days 0, 2, 4, 7, and 21, where the objective was to

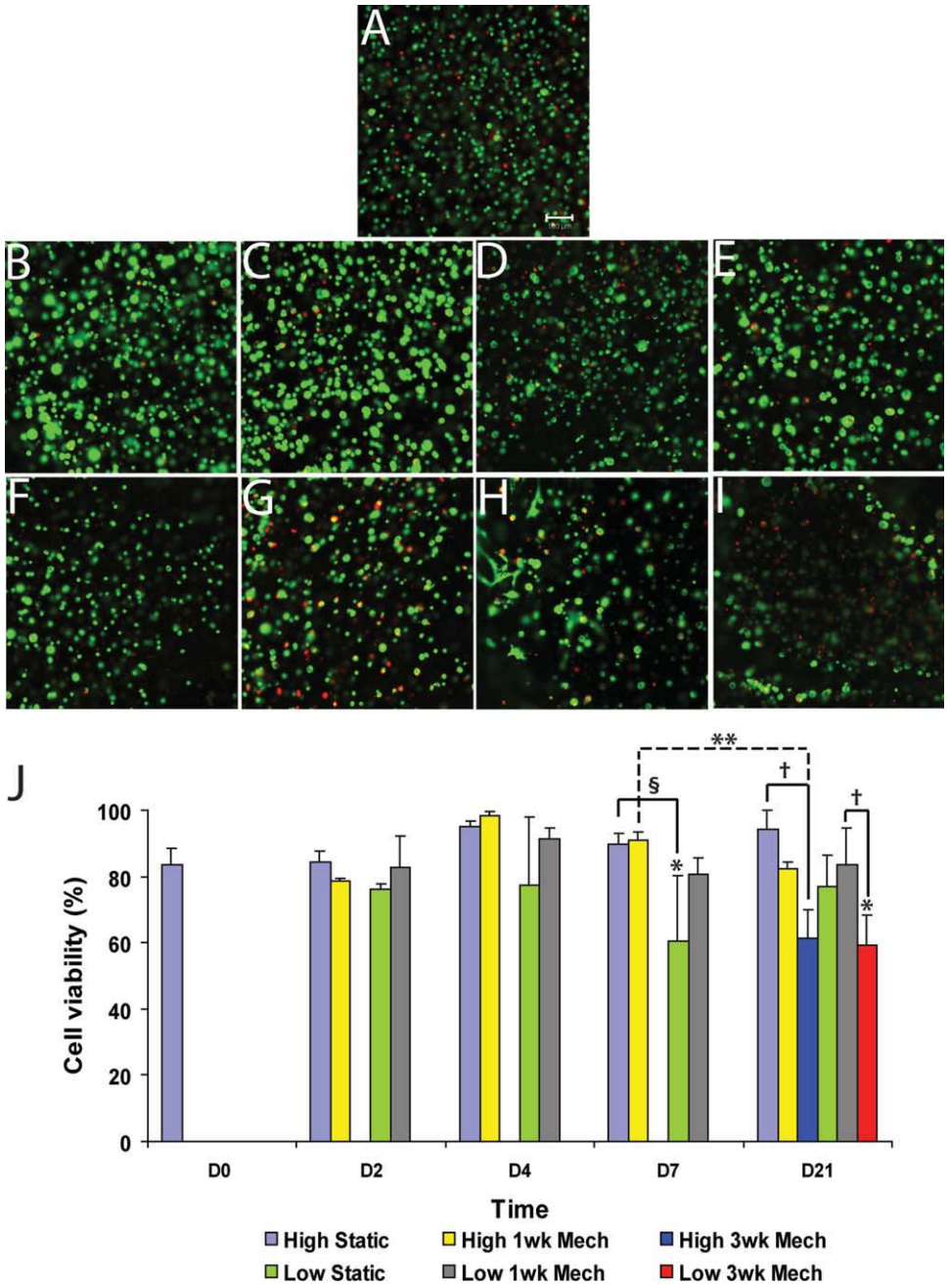

FIGURE 4. Representative fluorescence micrographs of Live/Dead dye-stained constructs, assorted by time point/oxygen tension/hydrostatic compression level(BMP-2 level); (A) Day 0, (B) Day 7/high O_2/static(−), (C) Day 7/high O_2/short-term(−), (D) Day 7/low O_2/static(−), (E) Day 7/low O_2/short-term(−), (F) Day 21/high O_2/static(−), (G) Day 21/high O_2/long-term(−), (H) Day 21/low O_2/static(−), (I) Day 21/low O_2/long-term(−). Here, high O_2 and low O_2 indicate 20% and 5% oxygen tension, respectively. Short-term and long-term are levels of intermittent hydrostatic compression, corresponding to 1-week stimulation and 3-week stimulation, respectively. In the images, green corresponds to live cells, and red corresponds to dead cells. Scale bar represents 100 μm and applies to A–I. (J) Cell viability (%) of the constructs quantified using Live/Dead image analysis. Live and dead cell counts were estimated from the images acquired from four equal scaffold volumes of 900 × 900 × 50 μm. Error bars represent means ± standard deviation ($n = 4$). Statistical significance symbols are defined in Table I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

evaluate the effects of mechanical stimulation and oxygen tension on the overall cell viability. Representative fluorescence micrographs of Live/Dead dye-stained constructs from the selected time point/oxygen tension/mechanical stimulation groups are shown in Figure 4. As indicated by the representative images in Figure 4(A–I), cells were homogeneously distributed in the beads in all culture conditions at all times. nHDFs were mostly constricted to a rounded morphology in the beads with some cells forming small clusters, with the only exception of the D21/low/static(−) group [Fig. 4(H)] where some cells demonstrated spindle-shaped fibroblast-like morphology. Quantification of

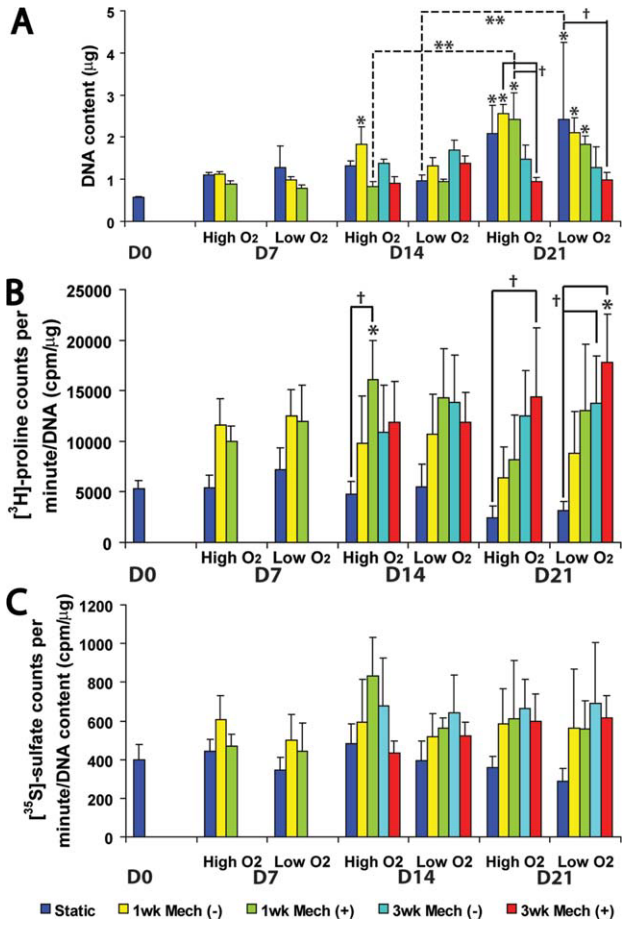

FIGURE 5. (A) DNA content. (B) [^{3}H]-proline incorporation rate normalized to DNA content. (C) [^{35}S]-sulfate incorporation rate normalized to DNA content. Error bars represent means ± standard deviation ($n = 4$). Statistical significance symbols are defined in Table I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

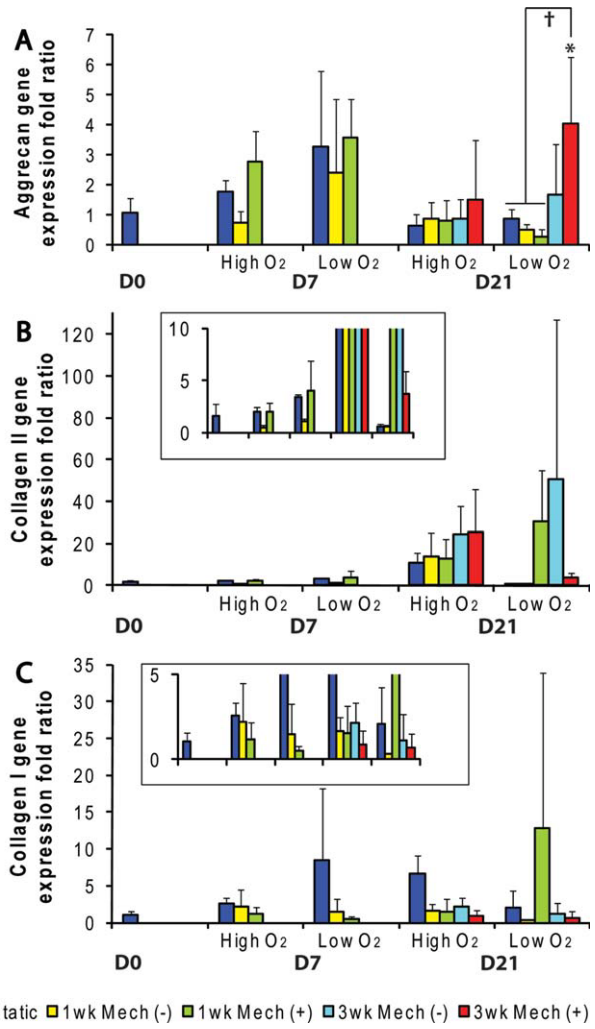

FIGURE 6. Quantitative gene expression fold ratio for aggrecan (A), collagen type II (B) and collagen type I (C). GAPDH was used as the house-keeping gene. Error bars represent means ± standard deviation ($n = 4$). Statistical significance symbols are defined in Table I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cell viability using image analysis revealed that percent viability of cells for all groups showed no significant difference compared to day 0 until day 7, where the D7/low/static(−) group showed a significantly decreased cell viability compared to the day 0 group and the corresponding high O_2 group (the D7/high/static(−) group). At day 21, cell viability for the groups exposed to the long-term intermittent mechanical stimulation under high O_2 conditions (the D21/high/long-term(−) groups) was found to be significantly lower compared to the corresponding groups cultured in static conditions (the D21/high/static(−) group). Moreover, the reductions in percent cell viability for the D21/high/long-term(−) was significant compared to the D7/high/short-term(−) group (the previous corresponding time point). At day 21, cell viability for the groups exposed to the long-term intermittent mechanical stimulation under low O_2 conditions (the D21/low/long-term(−) group) was found to be significantly lower compared to the day 0 group and the corresponding short-term mechanical stimulation group (the D21/low/short-term(−) group).

Cellularity of the constructs, as measured by quantification of the DNA content on days 0, 7, 14, and 21, is shown in Figure 5. The trends in cellularity appeared similar to those observed for cell viability. Specifically, no significant difference in the DNA content was seen for all the groups compared to the day 0 group until day 14. Among the day 14 groups, only the D14/high/short-term(−) group had a significant increase in the DNA content compared to the day 0 control group. By day 21, all static and short-term intermittent mechanical stimulation groups showed a significant increase in the DNA content compared to the day 0 group; only the groups exposed to the long-term intermittent mechanical stimulation (the D21/high or low/long-term(+/−) groups) did not demonstrate such an increase. In particular, the D21/high/long-term(+) group had a significantly lower amount of DNA than the corresponding short-term mechanical stimulation groups (the D21/

TABLE I. Definition of Statistical Significance Symbols

Symbol	Statistical significance ($p < 0.05$)
*	A significant difference compared to day 0 group
†	A significant difference compared to other mechanical stimulation/BMP-2 treatment groups of same O_2 treatment at the same time point
**	A significant difference compared to the same mechanical stimulation/BMP-2/O_2 treatment group at a previous time point
§	A significant difference compared to the same mechanical stimulation/BMP-2 treatment group at the same time point with different O_2 treatment

high/short-term(+/−)), and the D21/low/long-term(+) group showed a significantly lower DNA content than the corresponding static group (the D21/low/static(−) group).

Gene expression for aggrecan, collagen type I, and collagen type II was quantified at days 0, 7, and 21 using real-time RT-PCR (Fig. 6). As shown in Figure 6(A), for the aggrecan gene-expression, the D21/low/long-term(+) group (the group exposed to long-term intermittent mechanical stimulation and cultured under low O_2 conditions in the presence of BMP-2) demonstrated a significantly higher level of aggrecan gene expression compared to the day 0 group and the corresponding groups cultured under static or short-term intermittently mechanically stimulated conditions (the D21/low/static(−) and D21/low/short-term(−/+) groups). For collagen type II gene expression, the differences in the expression levels among the groups were found not to be statistically significant [Fig. 6(B)]. Collagen type I gene expression levels were generally found to be higher for the statically cultured groups compared to the groups that were exposed to mechanical stimulation [Fig. 6(C)]. However, the differences in the expression levels were not statistically significant.

Effects of culture conditions on biosynthesis rates were identified at different time points. Radiolabeled [^{3}H]-proline and [^{35}S]-sulfate incorporation rates normalized to the DNA content were quantified at days 0, 7, 14 and 21, which were used as a measure of collagen and sGAG biosynthesis rates (Fig. 5). The rate of collagen synthesis at day 21 for the D21/high or low/long-term(+) groups (the groups cultured under long-term intermittent mechanical stimulation in the presence of BMP-2 under high or low O_2 conditions) was found to be significantly higher (greater than 5.5 fold) than for the corresponding static controls [Fig. 5(B)]. Specifically, the D21/low/long-term(+) group had the highest rate of collagen synthesis, which was also significantly higher (~3.4 fold) compared to the day 0 control group. In addition, the D21/low/long-term(−) group showed a significantly higher collagen synthesis rate (~4.4 fold) compared to the corresponding static control. For the rate of sGAG synthesis, no significant differences were observed among the groups [Fig. 5(C)]. However, the groups exposed to intermittent hydrostatic pressure (short- or long-term) generally demonstrated a higher rate of sGAG synthesis compared to the corresponding static culture groups at each time point.

Intact beads were stained with alcian blue, which binds to glycosaminoglycans, and then cryosectioned (Fig. 7). A representative image of a section from the D21/low/long-term(+) group displays a pericellular matrix around the cells, which was stained with a dark blue color, while alginate alone (used as the negative control) was mildly stained with a light blue color (not shown). Immunohistochemical staining was performed for collagen type I, collagen type II, and aggrecan, and representative images obtained from the groups cultured under low O_2 condition for 21 days are shown in Figure 8. In general, all the cells appeared round in shape, corresponding to a chondrocyte-like phenotype. However, the cells at the periphery showed a dense pericellular matrix, while the cells closer to the center did not display this feature. As seen in the images, the sections stained

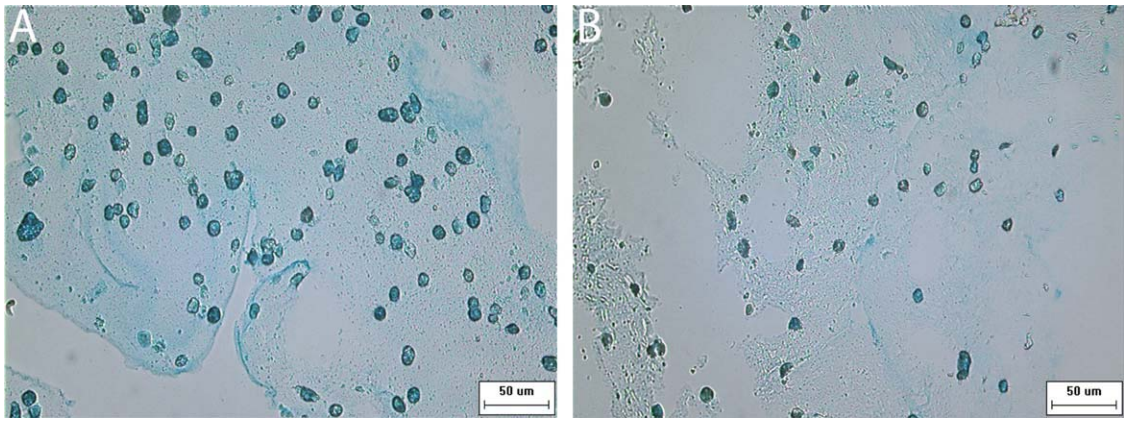

FIGURE 7. (A) A representative image of an alcian blue-stained section, showing the presence of glycosaminoglycans in the pericellular region (dark blue). The section shown here was prepared from a bead cultured for 3 weeks with exposure to long-term (3-week) intermittent mechanical stimulation under 5% oxygen tension in the presence of BMP-2 (the D21/low/long-term(+) group). Alginate stained with a light blue color (background). (B) Corresponding week 0 control group. Scale bars represent 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

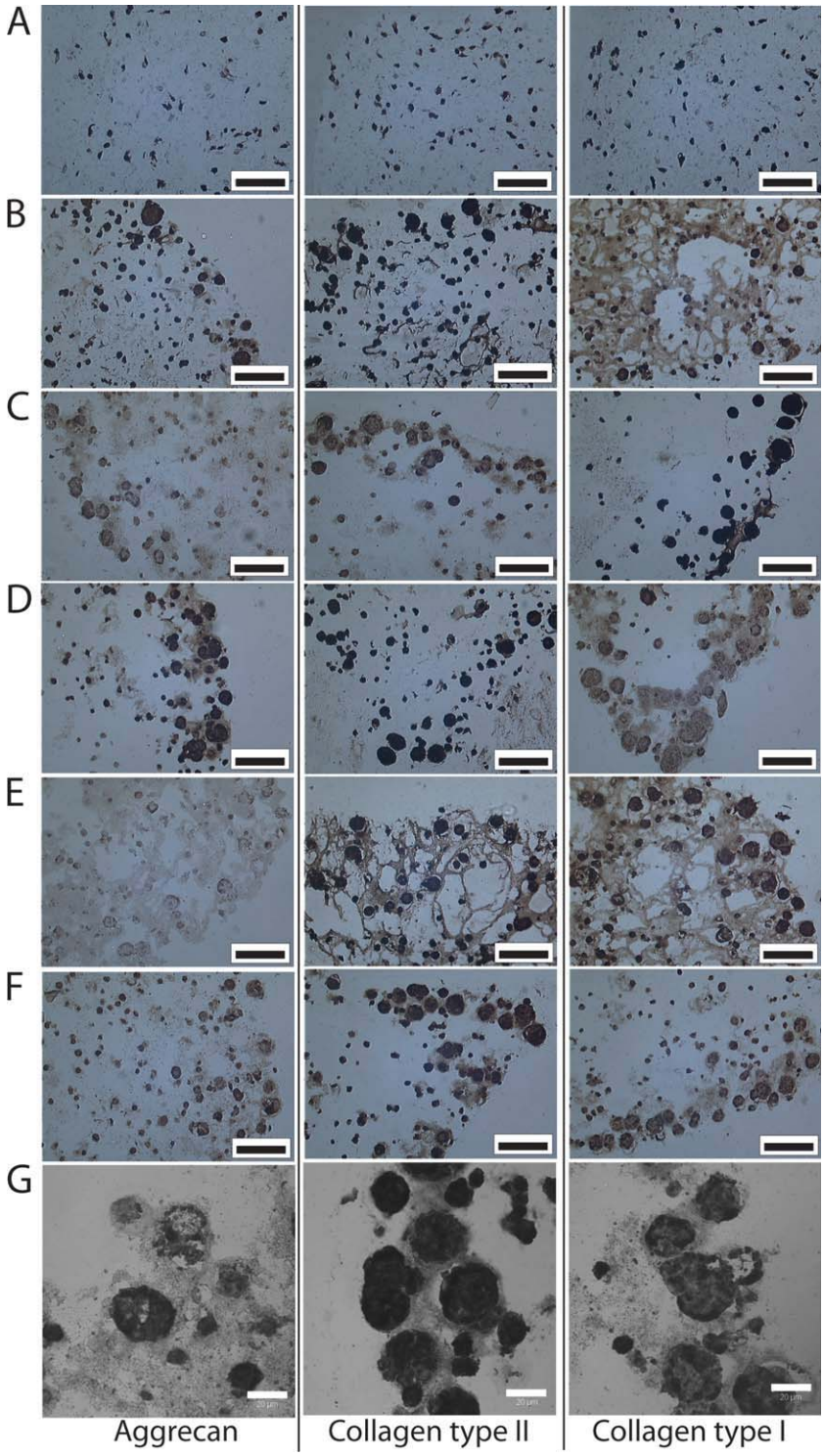

FIGURE 8. (A–F) Representative images of immunohistochemically-stained sections, assorted by time point/oxygen tension/hydrostatic compression level (BMP-2 level); (A) Day 0, (B) Day 21/low O_2/static(−), (C) Day 21/low O_2/short-term(−), (D) Day 21/low O_2/short-term(+), (E) Day 21/low O_2/long-term(−), (F) Day 21/low O_2/ long-term(+). Immunohistochemistry demonstrated the presence of aggrecan (left column), collagen type II (middle column), and collagen type I (right column) in the constructs. Scale bars represent 100 μm (A–F). (G) Representative bright field micrographs of cells at the periphery of the beads, magnified from panel F. These images were captured from the sections prepared from the Day 21/low O_2/long-term(+) group. Scale bars represent 20 μm (G). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

positive for collagen type II, aggrecan, as well as collagen type I.

DISCUSSION

In the current work, nHDF culture was performed in alginate beads using intermittent hydrostatic pressure (short-term or long-term with static culture as a control), hypoxia (with normoxia as a control), and BMP-2 growth factor supplementation as chondrogenic stimuli. Specifically, the study investigated: (1) cell viability and chondrogenic differentiation of the cells using dynamic hydrostatic pressure and/or hypoxia as chondrogenic signals and (2) effects of combined biochemical (BMP-2) and biomechanical stimulations on chondrogenic differentiation in hypoxic/normoxic conditions.

While there are numerous publications exploring the effects of hydrostatic pressure alone on different cell types, only a limited number of studies have identified the effects of combined signal delivery using hypoxia or growth factor stimulation as an additional signal.[21,23,44,45] Simultaneous application of the specific signals used in this study have heretofore never been tested for the chondrogenic differentiation of nHDFs. The three aforementioned chondrogenic signals differ from each other in the mechanism of regulating gene expression and cellular activity. BMP-2 is a known chemotransducer, which physically binds to cell surface receptors and initiates a complex signaling pathway. In contrast, hydrostatic pressure induces a state of stress on the cells with negligible deformational strain[46,47] and results in mechanotransduction, likely by changing the ion transportation across the cell boundary and intracellular ion concentrations.[13] Chronic hypoxia acts in yet another way, where changes in the microenvironment of the cells trigger an immediate homeostatic response and a late response through alterations in the expression levels of hypoxia-inducible transcription factors, regulating a number of other genes and growth factors.[29,48,49] Given these differences in the signaling mechanisms, the effects of combinations of these signals on the chondrogenic differentiation of nHDFs was studied with the hypothesis that conditions that mimic the physiological cartilage environment (long-term dynamic hydrostatic pressure, hypoxia, and presence of BMP-2) would be superior for the chondrogenic differentiation of nHDFs.

The specific profile of intermittent hydrostatic pressure (magnitude $\sim$5 MPa, frequency 1 Hz) used in this study was shown previously to be beneficial in the chondrogenic differentiation of MSCs in a 3-D environment (duration 4 h/day for 7 days),[21] and also for a murine embryonic fibroblast cell line in a 2-D environment (duration 2 h/day for 3 days).[22] As mentioned earlier, an oxygen tension of 5% was selected for the present study because it has been commonly used as a hypoxic environment in many cartilage tissue engineering investigations.[26,28,30] Moreover, a BMP-2 concentration of 100 ng/mL was selected based on previous reports that demonstrated positive effects of BMP-2 at the chosen concentration for the chondrogenic differentiation of murine embryonic fibroblast cells and up-regulation of chondrogenic gene expression in human chondrocytes.[50,51] While a wide variety of scaffold materials have been explored for cartilage tissue engineering,[52] alginate was selected as the matrix for the present study to mitigate potential influences of cell adhesion to the scaffold material, as the hydrophilicity of alginate is known to deter protein adhesion[53] and consequent protein-mediated cell adhesion.[53–55] The cells were encapsulated in beads of alginate, given the simplicity of the fabrication/encapsulation procedure and in order to facilitate the handling of the constructs throughout the study.

Cell viability

Cell viability was examined at different time points during the culture under various combinations of dynamic hydrostatic pressure and oxygen tensions, as shown in Figure 4. As such, no adverse effects of short-term intermittent hydrostatic compression (1 week exposure or less) were seen on cell viability. However, exposure to long-term intermittent hydrostatic compression did lead to a significant reduction in cell viability irrespective of the oxygen tension in the culture environment, where the D21/high/long-term($-$) and D21/low/long-term($-$) groups demonstrated a significantly lower cell viability compared with the corresponding static group (the D21/high/static($-$) group) and the day 0 group, respectively. A very similar trend was seen in the cellularity, as shown in Figure 5(A). Until day 14, no significant difference in the DNA content was seen with short-term intermittent hydrostatic compression compared to the day 0 group. However, at day 21, both static and short-term intermittent hydrostatic compression groups demonstrated a significant increase in the DNA content compared to day 0, whereas the long-term intermittent hydrostatic compression groups (the D21/high or low/long-term($+/-$) groups) did not demonstrate such cellular proliferation. Also, hypoxia had no significant effect on cellularity. These observations suggest that the long-term intermittent hydrostatic compression profile used in this study leads to a reduction in cell viability, thus affecting cellularity. In addition, a lower cellularity for long-term intermittent hydrostatic compression groups compared to the corresponding static and short-term intermittent hydrostatic compression groups at day 21 may also be indicative of an increase in biosynthetic activities, as cellular proliferation and matrix production are usually inversely related phenomena. Moreover, an additional contributing reason for this reduction in cellularity for the long-term intermittent hydrostatic compression groups compared to the corresponding static and short-term intermittent hydrostatic compression groups could be compromised alginate bead integrity during extensive culture in mechanically stimulated conditions. It is known that the alginate network changes as a function of culture duration (i.e., number of media changes) due to a slow depletion of Ca^{2+} ions from the alginate beads to the cell culture media via ion exchange.[56] In the case of long-term intermittent hydrostatic compression, it is likely that the overall loss of Ca^{2+} ions may have increased due to convection.

Effects of mechanical stimulation and hypoxia on the chondrogenic differentiation of nHDFs

Chondrogenic differentiation of nHDFs was analyzed at different time points during the culture under different combinations of dynamic hydrostatic pressure and oxygen tension. The different groups were compared for the relative extent of chondrogenic differentiation quantitatively using gene expression data and biosynthesis activity.

nHDFs primarily express collagen type I gene, whereas chondrocytes express collagen type II and aggrecan genes.[12,57] Under different combinations of hydrostatic compression and oxygen tension (in the absence of BMP-2), no statistically significant differences were observed in aggrecan, collagen type I, and collagen type II gene expression compared to the day 0 group (Fig. 6). However, continued application of intermittent hydrostatic compression was generally seen to result in suppressed levels of collagen type I gene expression compared to the corresponding static culture groups, as seen with the D7/high or low/short-term(−) and D21/high or low/long-term(−) groups [Fig. 6(C)]. In terms of biosynthesis activity, a common trend observed was a lower rate of matrix (GAG or collagen) production normalized to the DNA content for the statically cultured groups compared to groups exposed to intermittent hydrostatic compression (Fig. 5). The differences in GAG production rates among the groups were found to be statistically insignificant [Fig. 5(C)]. However, the collagen production rate at day 21 was significantly enhanced with long-term (3-week) intermittent hydrostatic compression under low O_2 conditions (the D21/low/long-term(−) group) compared to the corresponding group cultured in a static environment [Fig. 5(B)], indicating that long-term mechanical stimulation was a significant stimulator of collagen biosynthesis in low O_2 conditions. Alcian blue staining confirmed a dense pericellular region, which stained positive for glycosaminoglycans (Fig. 7). As seen by the immunohistochemical staining (Fig. 8), all three extracellular matrix components investigated, namely collagen type I, collagen type II, and aggrecan were identified in the beads qualitatively. Given the hydrophilicity of alginate and consequent lack of cell adhesion to the material,[53–55] cells commonly form aggregates when encapsulated in alginate,[55,58] and the presence of the extracellular matrix components was generally observed qualitatively in the micrographs surrounding clusters of cells (Fig. 8).

These evaluations suggest a positive effect of long-term intermittent hydrostatic compression under low O_2 conditions on collagen production rate at day 21 compared to the day 0 group, while aggrecan gene expression and GAG production rates were not significantly affected under any combination of hydrostatic compression and oxygen tension (in the absence of BMP-2).

Effects of simultaneous biomechanical and biochemical stimulation on chondrogenic differentiation of nHDFs

Effects of inclusion of BMP-2 during the mechanical stimulation phase on the chondrogenic differentiation of nHDFs were assessed at days 0, 7, and 21. While gene expression of collagen type I and collagen type II demonstrated no significant change due to BMP-2 supplementation compared to the day 0 group, aggrecan gene expression was significantly elevated at day 21 for the constructs cultured under long-term intermittent hydrostatic compression in hypoxic conditions (the D21/low/long-term(+) group) compared to the day 0 group and the corresponding groups cultured under static or short-term hydrostatic compression conditions (Fig. 6). For the biosynthesis activity, the collagen production rate at day 21 was found to be maximum for the D21/low/long-term(+) group, significantly greater than the rates observed for the day 0 group and the corresponding group cultured in static conditions [Fig. 5(B)]. However, inclusion of BMP-2 did not affect the GAG synthesis rate [Fig. 5(C)].

Overall, the D21/low/long-term(+) group demonstrated an elevation in aggrecan gene expression compared to the day 0 group and the highest collagen production rate among all groups, which suggests a positive effect of BMP-2 incorporation. However, no significant differences were observed between D21/low/long-term(+) and D21/low/long-term(−) groups.

In this study, the combination of the application of hypoxia, long-term intermittent mechanical stimulation, and BMP-2 supplementation were found to positively contribute to chondrogenic differentiation of nHDFs. One of the limitations of the bead culture protocol used in this study was the absence of maintenance of continuous hypoxia throughout culture for the low O_2 group, since the beads were exposed to prolonged periods of normoxic conditions during the times of mechanical stimulation. In this regard, maintenance of continuous hypoxia through a modified bioreactor design with O_2 monitoring may further improve the outcomes. In addition, since hydrostatic pressure was one of the primary chondrogenic signals in the present study design, a general cell culture medium with FBS was utilized in this study to eliminate the possibility of media-induced chondrogenic differentiation. An alternate differentiation strategy could be to use a serum-free defined chondrogenic medium to induce chondrogenic differentiation of nHDFs before or during the hydrostatic compression phase, as has been done previously with BMSCs in a successful manner.[18,21] Moreover, alternate intermittent hydrostatic compression profiles can also be investigated to optimize chondrogenic differentiation. Further investigation in these directions may provide an improved hydrostatic compression-assisted chondrogenic differentiation strategy for nHDFs.

This chondrogenic differentiation strategy may be suitable in approaches for the repair of the gel-like NP core of the IVD, where autologous nHDFs encapsulated in a degradable hydrogel structure and exposed for a brief period to the combination of hydrostatic compression, hypoxia, and BMP-2 can be directly transplanted *in vivo* following nucleus aspiration/removal. It can be expected that the presence of physiological mechanical stimulation, hypoxia, and endogenous growth factors, which are inherently present in the *in vivo* IVD environment, will substitute for the need for a long-term *in vitro* culture. In addition, the presence of the

3-D hydrogel environment *in vivo* should support the retention of the chondrogenic phenotype of the nHDFs without the risk of dedifferentiation. Follow-up animal work will be required to test this hypothesis and validate the results obtained *in vitro*.

CONCLUSIONS

In the present work, chondrogenic differentiation of neonatal human dermal fibroblasts (nHDFs) was achieved in 3-D alginate beads using long-term (3 weeks) intermittent mechanical stimulation and hypoxia. A positive effect of long-term intermittent mechanical stimulation under low O_2 conditions was observed, as reflected by a significant increase in collagen production rate from day 0. However, both aggrecan gene expression and GAG synthesis rates were unaffected under various combinations of hydrostatic compression and oxygen tension. Addition of biochemical stimulation (BMP-2 supplementation) during the long-term intermittent mechanical stimulation phase under hypoxic conditions improved the chondrogenic differentiation, as indicated by elevated aggrecan gene expression and an improved collagen production rate. Thus, the combination of long-term dynamic hydrostatic pressure, hypoxia, and BMP-2 was found to be the most potent of all the potential chondrogenic signals examined for the chondrogenic differentiation of nHDFs.

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