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Temporal and Spatial Changes in Cartilage-Matrix-Specific Gene Expression in Mesenchymal Stem Cells in Response to Dynamic Compression

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Various forms of mechanical stimulation have been shown to enhance chondrogenesis of mesenchymal stem cells (MSCs). However, the response of MSCs undergoing chondrogenesis to such signals has been shown to depend on the temporal application of loading. The objective of this study was to determine the effect of dynamic compression on cartilage-matrix-specific gene expression and to relate this response to the local biochemical environment and cell phenotype at the time of loading. At 0, 7, 14, and 21 days extracellular matrix (ECM) deposition within MSC-seeded agarose hydrogels due to transforming growth factor- β 3 stimulation was determined biochemically and histologically, and then reverse transcription-polymerase chain reaction was used to examine the effects of dynamic compression on cartilage-matrix-specific gene expression. The results of these experiments show that the local environment in the core of the constructs is more favorable for chondrogenesis in comparison to the annulus, as evident from both ECM synthesis and gene expression. Additionally, we found that the response of the cells to mechanical stimulus varied with both the spatial region within the constructs and the temporal application of loading. Dynamic compression applied at day 21 was found to enhance levels of cartilage matrix gene expression following a peak in expression levels at day 14 in free swelling constructs, suggesting that mechanical signals play a key role in the maintenance of a chondrogenic phenotype. The application of mechanical stimulus to enhance cartilage ECM synthesis may be an important tool in regenerative medicine-based cartilage repair. The results of this study suggest that a chondrogenic phenotype and/or a well-developed pericellular matrix must first be established before dynamic compression can have a positive effect on cartilage-matrix-specific gene expression.

Introduction

BONE MARROW-DERIVED mesenchymal stem cells (MSCs) are an attractive cell source for cartilage tissue engineering due to both their ease of isolation and expansion, and their ability to undergo chondrogenic differentiation in three-dimensional culture.¹⁻⁴ Chondrogenic differentiation of MSCs is often regulated through cytokines and growth factors, although it is still unclear if such factors alone can produce tissue-engineered cartilage with properties comparable to native articular cartilage.⁵⁻¹¹ Alternatively, MSCs have been shown to be sensitive to mechanical stimulus. This sensitivity is evident *in vivo* during MSC fate decisions in development and response to injury,¹²⁻¹⁵ and is also being explored *in vitro* to enhance MSC-based tissue engineering through the use of bioreactors.^{1,5,10,16-20} An understanding of

the factors that determine the response of MSCs to mechanical stimulus is consequently key to advancing our understanding of stem cell fate decisions *in vivo* and enabling the use of mechanical signaling to enhance MSC-based tissue engineering therapies.

Investigating the response of MSCs to extrinsically generated force, even in well-characterized bioreactor systems, is complicated by the fact that chondrogenic differentiation and extracellular matrix (ECM) accumulation vary both temporally and spatially within cell-seeded constructs. Previous studies in our laboratory have shown that sulfated glycosaminoglycan (sGAG) content is higher in the core of agarose constructs than in the annular region.^{16,21-23} These differences have been hypothesized to be a result of higher levels of matrix synthesis in response to the lower oxygen tension in the core of the constructs.^{16,23-25} These differences in ECM

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accumulation will alter the spatial patterns of mechanical signals during loading and may also be indicative of spatial differences in chondrogenic differentiation. Both of these factors would be expected to have an effect on the response of MSCs to mechanical stimulus. These factors may explain the observation that dynamic compression can negatively affect cartilage-matrix synthesis when applied at the onset of chondrogenic differentiation, whereas a more positive response was observed when the application of mechanical stimulus was delayed.^{5,16,26} For example, Mouw *et al.*²⁶ observed that dynamic compression did not produce increases in gene expression or matrix synthesis unless the application of loading was delayed until day 16. Taken together, the results of these studies suggest that mechanical signals may play a role not only in the initiation of chondrogenesis, but perhaps also in the maintenance of this phenotype.

To properly address such questions involves investigating the relationship between the response of MSCs to mechanical signals and the temporal and spatial changes in MSC phenotype and biochemical composition of the local ECM. Therefore, the goal of this study was to determine the effect of dynamic compression on cartilage-matrix-specific gene expression and to relate this response to the local biochemical environment and cell phenotype at the time of loading. The specific objectives of this study were to (1) investigate temporal changes in gene expression and matrix accumulation as MSCs undergo transforming growth factor (TGF)- β 3-induced differentiation in agarose hydrogels and (2) quantify changes in gene expression due to the application of dynamic compression at discrete times during this process.

Materials and Methods

Cell isolation and preparation of agarose gel constructs

MSCs were isolated from bone marrow harvested from porcine femora (3 donors, 4 months old, ~50 kg). A protocol adapted from a method developed for human tissue was used to isolate MSCs from the bone marrow.²⁷ Once isolated, the MSCs were plated on T-175 flasks (Sarstedt) at a density of 5×10^3 cells/cm² in high-glucose Dulbecco's modified Eagle's medium GlutaMAX™ (hgDMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (all GIBCO, Biosciences). MSCs were subcultured at a ratio of 1:3 following colony formation and expanded to passage 3 in a humidified atmosphere at 37°C and 5% CO₂. After isolation and expansion to P3, MSCs from the three donors were pooled, then suspended in hgDMEM and mixed with 4% agarose (Type VII; Sigma-Aldrich) in phosphate-buffered saline at a 1:1 ratio. This produced a 2% agarose gel containing 15×10^6 cells/mL. The gel was cast in a stainless steel mold to produce a 3-mm-thick agarose sheet, from which cylindrical constructs ($\varnothing 5 \times 3$ mm, 8.75×10^5 cells) were created using a biopsy punch. Agarose gel constructs were maintained in chemically defined basal media (CDM), which consisted of hgDMEM supplemented with 100 U/mL penicillin/streptomycin, 100 μ g/mL sodium pyruvate, 40 μ g/mL L-proline, 1.5 mg/mL bovine serum albumin, 4.7 μ g/mL linoleic acid, 1 \times insulin-transferrin-selenium, 50 μ g/mL L-ascorbic acid-2-phosphate, 100 nM dexamethasone (all Sigma-Aldrich), and 10 ng/mL of TGF- β 3 (ProSpec-Tany TechnoGene Ltd). The medium was changed every 3–4 days. Agarose gel constructs were cultured for 60 h in TGF-

β 3-supplemented CDM before the start of the experiment (defined as day 0).

Dynamic loading protocol

To investigate the effects of loading at different time points, samples were subjected to dynamic loading at day 0, 7, 14, or 21. Loaded samples were subjected to 10% strain at a frequency of 1 Hz for 1 h only in a dynamic compression bioreactor and harvested for reverse transcription-polymerase chain reaction (RT-PCR) analysis directly after loading.^{16,22} No further loading was applied to these constructs. A pre-load of 0.01 N was applied to prevent lift-off during the loading session due to viscoelastic creep. The control group was incubated in a free swelling (FS) environment.

Quantitative biochemical analysis

FS samples were assessed at days 0, 7, 14, and 21. Sample cores ($\varnothing 3$ mm) and annuli were separated using a biopsy punch, weighed, and frozen (-85°C) for analysis at a later date. Samples were digested with 125 μ g/mL papain in 0.1 M sodium acetate, 5 mM L-cystine HCl, and 0.05 M EDTA (all Sigma-Aldrich), pH 6.0, at 60°C under constant rotation for 18 h. Cell number was evaluated using the Hoechst 33258 DNA assay, which fluorescently labels double-stranded DNA (Sigma-Aldrich), according to a previously published protocol and using calf thymus DNA (Sigma-Aldrich) as a standard.²⁸ sGAG content was measured using the dimethylmethylene blue dye-binding assay (Blyscan; Biocolor Ltd.) using shark-derived chondroitin sulfate (Sigma-Aldrich) as a standard. Collagen content was determined through measuring the hydroxyproline content. Samples were hydrolyzed at 110°C for 18 h in 38% HCL and assayed using a chloramine-T assay²⁹ and assuming a hydroxyproline:collagen ratio of 1:7.69.³⁰ All assays were carried out with a minimum of three samples analyzed in triplicate for each group at every time point, and all the biochemical data were normalized to the wet weight of the constructs.

RNA isolation and real-time PCR

RT-PCR was used to determine the relative expression of aggrecan, collagen type I, and collagen type II, with *GAPDH* as the house-keeping reference gene. Loaded groups were harvested directly after the application of loading, with the FS control samples harvested at the same time point. As described previously, samples were separated into core and annular regions using a biopsy punch and frozen (-85°C) for analysis at a later date. RNA was extracted from the agarose constructs by homogenizing samples in 1 mL of QIAzol reagent (Qiagen), followed by chloroform extraction. After chloroform extraction the RNA was precipitated in isopropanol with 5 μ L glycogen (Ambion). The samples were centrifuged and the supernatant removed before re-suspending the RNA pellets in 40 μ L RNase-free water. RNA yield and purity were assessed using a nanodrop spectrophotometer (ND-1000; Thermo Scientific), with 260/280 ratios of >1.8 for all samples. RT-PCR was carried out using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) and an Applied Biosystems 7500 real-time PCR machine (Applied Biosystems). Primers for type I collagen, type II collagen, aggrecan, and *GAPDH* are shown in Table 1.^{31,32} RT-PCR data were

TABLE 1. PRIMERS USED IN REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION EXPERIMENTS

Gene	Primer sequence (5'-3')	Gene ID
GAPDH	F-GCTTTGCCCGCGATCTAATGTT R-GCCAAATCCGTTCACTCCGACCT	AF017079
Collagen I	F-CCAAGAGGAGGGCCAAGAAGAAG R-GGGGCAGACGGGGCAGCACTC	AF201723
Collagen II	F-CTGGAGCTCCTGGCCTCGTG R-CAGATGCGCCTTTGGGACCAT	AF201724
Aggrecan	F-TGCAGGTGACCATGGCC R-GGGCGGTAATGGAACAAC	AF201722

analyzed using the $2^{-\Delta\Delta C_T}$ method as described previously, with the FS core samples at day 0 used as the calibrator.³³

Histology

As with the biochemical analysis, samples were assessed at days 0, 7, 14, and 21. At each time point, two samples per group were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin using an automatic tissue processor (ASP300; Leica). All samples were sectioned perpendicular to the disc face at a thickness of 5 μ m throughout the depth of the sample using a rotary microtome (Leica microtome; Leica). Samples were stained histologically with 1% alcian blue 8GX solution (Sigma-Aldrich) for sGAG, and picrosirius red for collagen. Types I and II collagen were identified through immunohistochemistry.²¹ Negative and positive controls of porcine ligament and cartilage were included.

Statistical analysis

Results are expressed as mean \pm standard deviation. Two-way analysis of variance (ANOVA), with time and location as the independent factors, followed by pairwise multiple

comparison procedures (Holm-Sidak test) were used to evaluate the results from the quantitative biochemical assays. Three-way ANOVA, with time, location, and loading as the independent factors, followed by pairwise multiple comparison procedures (Holm-Sidak test) were used to evaluate the differences in gene expression within the constructs following the application of dynamic compression. Statistical significance was declared at $p \leq 0.05$.

Results

DNA content and biochemical composition in FS conditions

In general, DNA content within the agarose constructs was stable over the 21-day culture period; however, a significant reduction in cell number was observed between days 14 and 21 (Fig. 1A, $p < 0.05$). As expected, both sGAG and collagen content were found to increase with time in FS conditions (Fig. 1B–E). Significant increases in sGAG content were observed in both core and annular regions between days 7–14 and 14–21 (Fig. 1B and C, $p < 0.05$). Additionally, sGAG content in the core was significantly higher than the

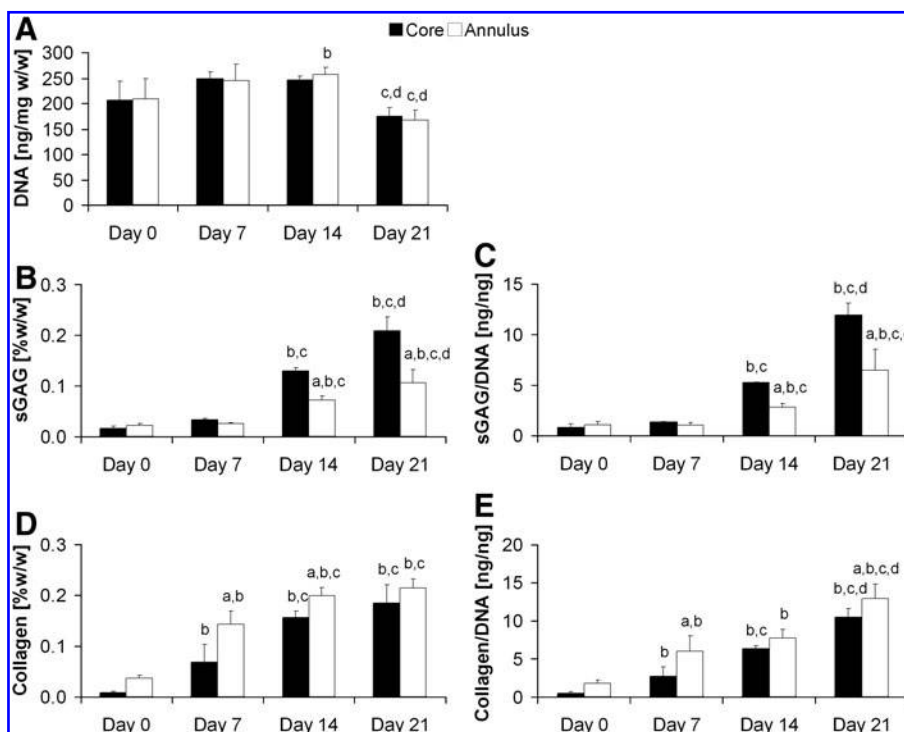


FIG. 1. DNA content and biochemical composition of agarose constructs cultured in free swelling conditions over 21 days. Day 0 is defined as 60 h postagarose gel encapsulation. (A) DNA content normalized to construct mass; (B) sGAG content normalized to construct mass; (C) sGAG content normalized to DNA content; (D) collagen content normalized to construct mass; and (E) collagen content normalized to DNA content. $n = 3$ samples per group per timepoint, ^a $p < 0.05$ versus core at the corresponding time point; ^b $p < 0.05$ versus corresponding region at day 0; ^c $p < 0.05$ versus corresponding region at day 7; ^d $p < 0.05$ versus corresponding region at day 14. sGAG, sulfated glycosaminoglycan.

annular region, with 1.8- and 2-fold differences at days 14 and 21, respectively (Fig. 1B, $p < 0.05$). Similar temporal increases in collagen content were observed, with significant increases in collagen content between days 0–7 and 7–14 (Fig. 1D and E). Counter to the trends observed in the spatial distribution of sGAGs, collagen content was found to be greater in the annular region of the agarose constructs (Fig. 1D, $p < 0.05$). However, while this difference was significant at days 7 and 14, no significant difference in collagen content was observed at day 21 (Fig. 1D). Significant interactions between time and location were reported in the two-way ANOVA for sGAG content but not collagen content.

Histological analysis of ECM deposition and distribution

Histological analysis of ECM deposition was found to agree with the biochemical data (Fig. 2). Staining for both sGAG and collagen can be seen to increase in strength from 0 to 21 days. The spatial differences in ECM deposition can also be observed in the 10× images, where sGAG staining can be seen to be stronger in the core of the samples, whereas collagen stains stronger in the annular region. The development of a pericellular matrix (PCM) was also evident. Strong staining for a sGAG PCM can be seen at day 14 and becomes more evenly distributed by day 21, whereas a collagen PCM is evident as early as day 7. At day 21, collagen I staining appeared more intense in the annular region of the construct, whereas greater staining for type II collagen was observed in the extracellular space within the core of the constructs (Fig. 3).

Chondrogenic gene expression in FS conditions

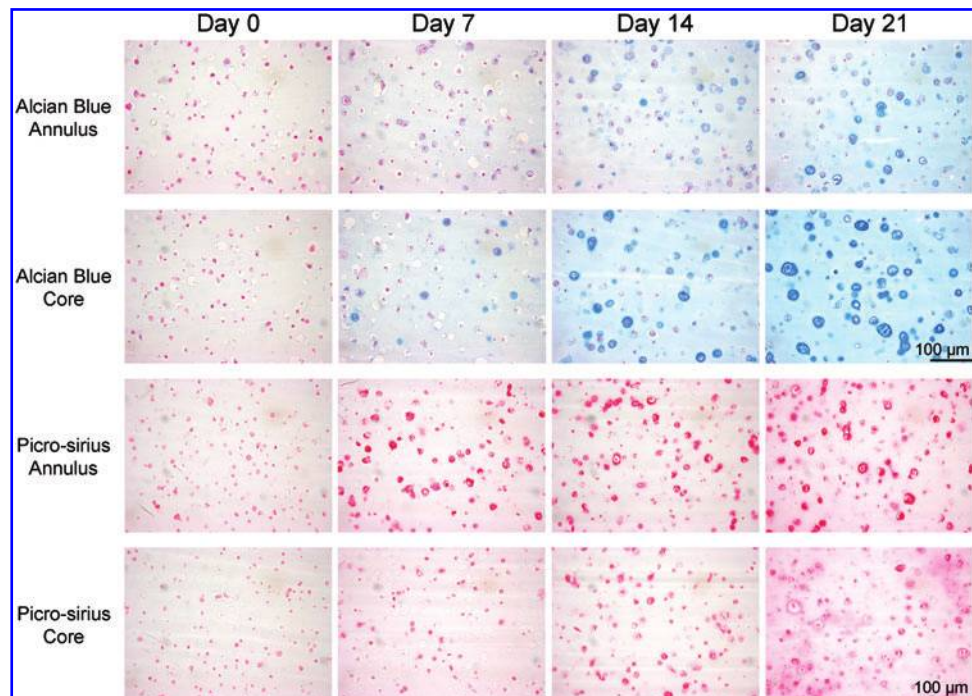
Aggrecan and collagen type II expression can be seen to change over time with peak expression for both genes at day 14 (Fig. 4A, B). Aggrecan expression was found to peak at day 14 with a 31-fold increase in expression relative to day 0;

this peak was only observed in the core of the samples and was significantly different from expression in the annulus (Fig. 4A). Collagen type II expression followed a similar trend with an earlier 12-fold increase in expression at day 7, followed by a 444-fold increase in expression at day 14, both of these increases in expression occurred exclusively in the core of the samples (Fig. 4B). No significant changes in collagen type I expression were observed from day 0 levels (Fig. 4C). Significant interactions between time and location were reported in the two-way ANOVA for aggrecan and collagen type II expression but not collagen type I expression.

Chondrogenic gene expression in response to dynamic compression

Dynamic compression was found to have a significant effect on aggrecan and collagen type II expression, which was dependant on the time frame of the application of loading and the spatial region within the agarose constructs (Figs. 5 and 6). Aggrecan expression in the core of the constructs was found to significantly increase in comparison to nonloaded controls following the application of loading at days 7 and 21, whereas a decrease in expression was observed at day 14. In the annular region a significant increase in expression was observed following the application of loading at days 14 and 21. Similarly, collagen type II expression in the core of the constructs was found to increase following loading at days 7 and 21, whereas a reduction in expression was observed at day 14. In the annular region, collagen type II expression increased in response to loading at days 14 and 21. Dynamic compression was found to have little effect on the expression of collagen type I (Figs. 5 and 6). Significant interactions between all factors (time, location, and loading) were reported in the three-way ANOVA for aggrecan and collagen type II expression but not collagen type I expression.

FIG. 2. Histological analysis of free swelling agarose constructs showing the deposition and distribution of extracellular matrix at days 0, 7, 14, and 21. Day 0 is defined as 60 h postagarose gel encapsulation. Alcian blue stains for sulfated proteoglycans and picrosirius red stains for collagen. 10× images were taken of appropriate zones with the aim of emphasizing pericellular matrix development. Color images available online at www.liebertonline.com/tea



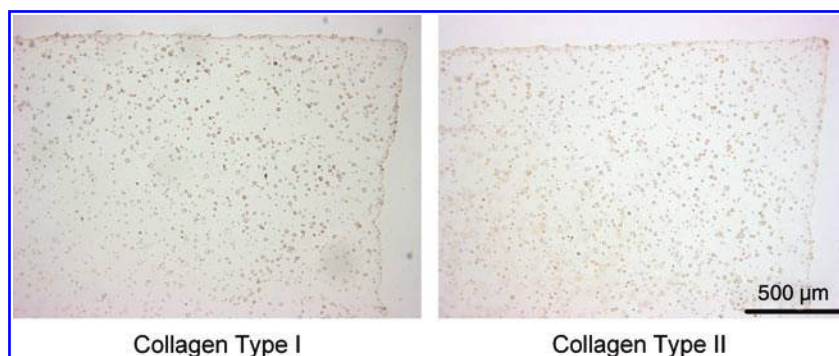


FIG. 3. Immunohistological analysis of free swelling agarose constructs showing the deposition and distribution of collagen type I and II at day 21. Color images available online at www.liebertonline.com/tea

Discussion

Dynamic compression has been previously shown to regulate chondrogenesis of MSCs within agarose constructs.^{1,10,16,20,22,26} However, the response of MSCs to such a mechanical stimulus has been shown to vary with the time

frame of application.^{16,26} With this in mind, we sought to determine the effect of dynamic compression on cartilage-matrix-specific gene expression and to relate this response to the local biochemical environment and cell phenotype at the time of loading. At 0, 7, 14, and 21 days, ECM deposition within the constructs was determined biochemically and histologically and RT-PCR was used to examine the effects of dynamic compression on cartilage-matrix-specific gene expression. The results of these experiments show that the local environment in the core of the constructs is more favorable for chondrogenesis in comparison to the annulus, as evident from both ECM synthesis and gene expression. Additionally, we found that the response of the cells to mechanical stimulus varied with both the spatial region within the constructs and the temporal application of loading. That is not to say that the specific time of loading itself is critical, but rather spatial differences in phenotype and/or PCM accumulation with time are responsible for changes in cartilage-matrix-specific gene expression in response to loading. This may explain the different response to loading in the core and annulus of the constructs observed at earlier time points. The altered mechanical environment within the annular region of dynamically compressed agarose hydrogels, which consists of higher levels of fluid flow, may also be partially determining these spatial findings.

The authors chose to pool MSCs from multiple donors rather than investigate the response of MSCs isolated from individual donors to dynamic compression. This approach provides information on the mean response of a sample of a given population to a stimulus, in this case mechanical loading. The advantage of this approach is that other sources of experimental variability that would have to be considered when analyzing cells from multiple donors separately are inherently minimized as the constructs generated from pooled MSCs are maintained in identical conditions. The disadvantage of this approach is that it provides no information on donor variability in the response to loading. Therefore, it is possible, for example, that only a subset of the donors utilized in this study are responsive to dynamic compression. The results of studies that adopt this approach must be interpreted in this light.

Biochemical analysis of the samples shows a steady increase in sGAGs and collagen accumulation in the constructs over the 21-day incubation period (Figs. 1–3). The rate of sGAG and collagen synthesis increases between day 7 and 21, which correlates with the peaks found in the analysis of gene expression (Fig. 4). Previous studies have also investigated temporal changes in cartilage-matrix-specific gene

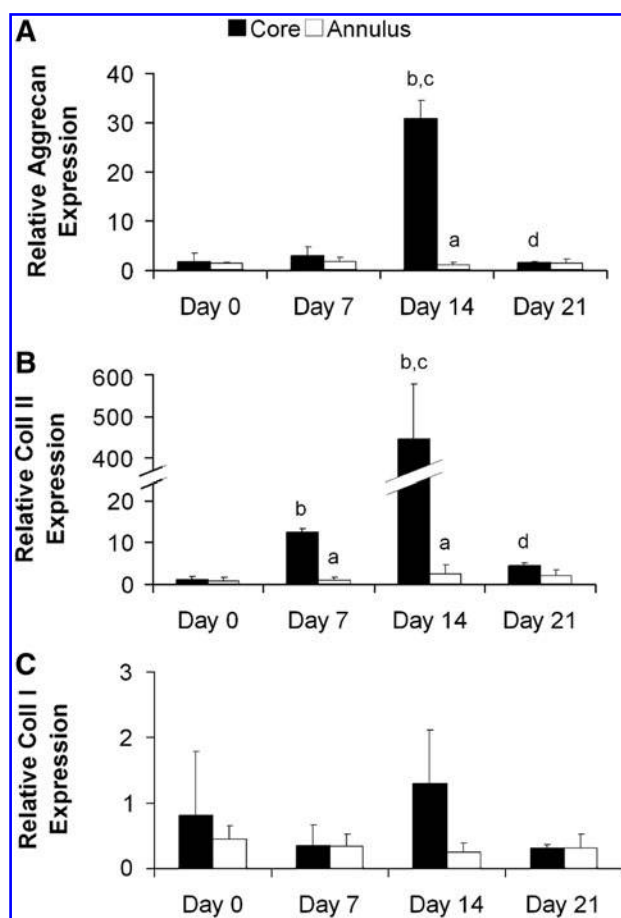


FIG. 4. Cartilage-matrix-specific gene expression within agarose constructs cultured in free swelling conditions over 21 days. Expression is normalized relative to core expression at day 0. Day 0 is defined as 60 h after agarose gel encapsulation. (A) Aggrecan expression; (B) collagen type II expression; and (C) collagen type I expression. $n=3$ samples per group per timepoint, ^a $p < 0.05$ versus core at the corresponding time point; ^b $p < 0.05$ versus corresponding region at day 0; ^c $p < 0.05$ versus corresponding region at day 7; ^d $p < 0.05$ versus corresponding region at day 14.

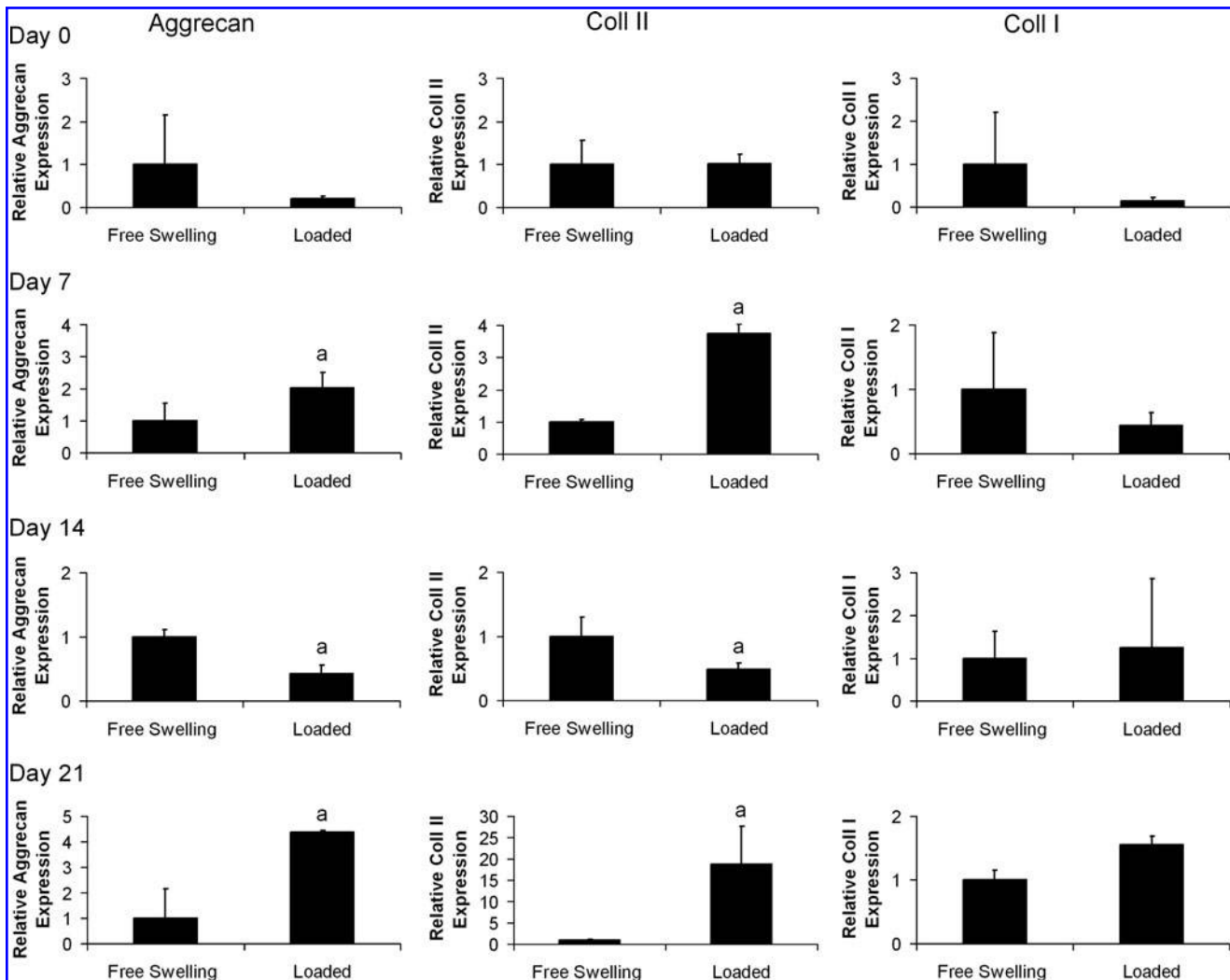


FIG. 5. The changes in core cartilage-matrix-specific gene expression in response to dynamic compression at days 0, 7, 14, and 21. Day 0 is defined as 60 h postagarose gel encapsulation. Relative gene expression is normalized to expression in free swelling core samples at each time point. $n=3$ samples per group per timepoint, ^a $p<0.05$ versus free swelling core group.

expression of MSCs encapsulated in hydrogels.^{26,34} Campbell *et al.*³⁴ found that both aggrecan and collagen type II expression within alginate constructs increased from day 0 to 10, which was the last time point investigated in that study. Mouw *et al.*²⁶ observed that within agarose constructs aggrecan expression increased from day 0 to 16, whereas collagen type II expression peaked at day 8 and maintained similar levels of expression until day 16. We found that aggrecan and collagen type II expression increases from day 0 to a peak at day 14 followed by a reduction in expression at day 21 (Fig. 4). The differences in the time points used in each study and the discrete temporal nature of gene expression make comparisons difficult, but the general trend of peaks in cartilage-matrix-specific gene expression at day 8–14 is in agreement. Additionally, as distinct spatial areas of the constructs were analyzed in our study, we were able to detect changes in the smaller core region that may have been obscured in an analysis of a homogenized sample. The levels of expression of aggrecan and collagen type II, particularly in the annular region, appear to be lower than what would be expected following culture in TGF- β 3-supplemented media

(Fig. 4). However, the RT-PCR data are normalized to day 0 of the experiment, which in this study is defined as 60 h postagarose encapsulation and TGF- β 3 supplementation. As a result, the gene expression data are normalized to a time point when expression of cartilage-matrix-specific genes would be expected to be elevated relative to pre-TGF- β 3 supplementation values, explaining why the levels of gene expression at days 7, 14, and 21 may appear to be lower than expected. Normalization to gene expression in MSCs before TGF- β 3 supplementation may have led to higher relative expression values, but this limitation does not affect our analysis of the changes in expression due to loading nor the temporal changes in FS conditions.

As observed in earlier studies,^{16,23} we found significant differences in the accumulation of sGAG within the core and annular regions of MSC-seeded hydrogels. It has been previously hypothesized that this is due to chondrogenic stimulus provided by lower oxygen tension in the core of the constructs.^{23–25,35,36} Additionally, Thorpe *et al.*¹⁶ demonstrated that sGAGs are secreted from the constructs, further complicating the analysis of spatial sGAG accumulation by

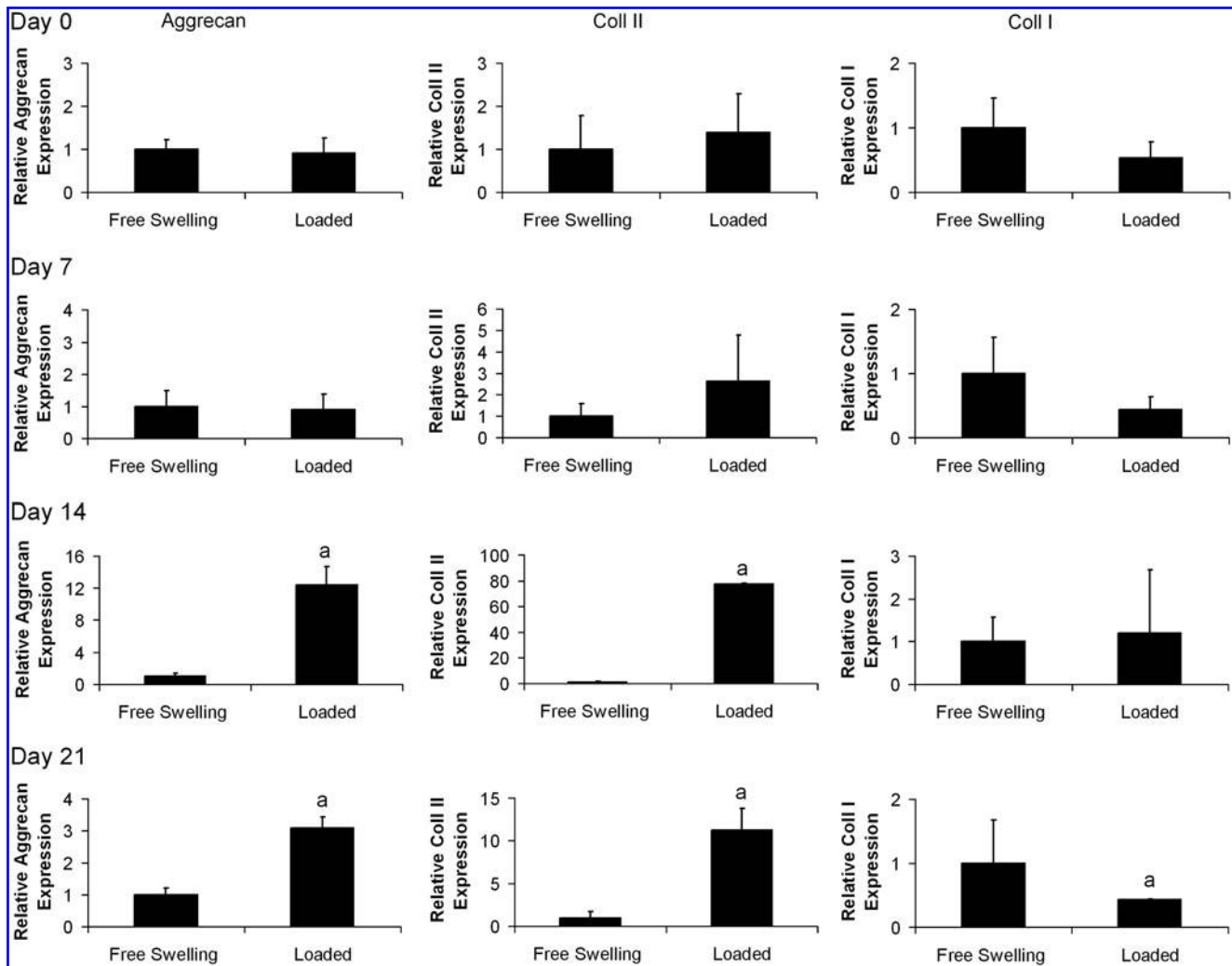


FIG. 6. The changes in annular cartilage-matrix-specific gene expression in response to dynamic compression at days 0, 7, 14, and 21. Day 0 is defined as 60 h postagarose gel encapsulation. Relative gene expression is normalized to expression in free swelling core samples at each time point. $n=3$ samples per group per timepoint, ^a $p<0.05$ versus free swelling group.

raising the possibility that lower levels of accumulation in the annular regions are simply due to greater diffusion of matrix components into the surrounding media. However in this study, spatial analysis of gene expression shows that aggrecan expression is significantly higher in the core of the constructs (Fig. 4A), suggesting that the local microenvironment is providing a stimulus that is responsible for enhanced cartilage-matrix-specific gene expression within the core, resulting in increased sGAG accumulation. Conversely, it was observed that in spite of collagen type II expression following the same trends as aggrecan expression, collagen accumulation was initially higher in the annulus of the constructs before being similar in both regions by day 21 (Fig. 1D, E). However, immunohistochemistry shows that a significant portion of the collagen accumulated in the annular region is type I collagen (Fig. 3).

The constructs used in this study were analyzed directly after one loading session; therefore, any differences in matrix synthesis relative to the FS samples at the same time point would be too small to measure using the biochemical assays or histological techniques implemented in this study. The

effects of several loading parameters need to be determined before an optimal loading regime can be designed to enhance MSC cartilage matrix synthesis. This study therefore aimed to use RT-PCR, as a more sensitive measure, to identify how dynamic compression influences cartilage-matrix-specific gene expression both spatially and temporally. The data gained from such analysis may be useful in designing further studies seeking to optimize a loading regime to enhance matrix synthesis for cartilage tissue engineering applications.

Our results show that expression of cartilage-matrix-specific genes is increased by dynamic compression initiated at later time points, which suggests that mechanical stimulus can help support or maintain a chondrogenic phenotype. Furthermore, we found that the cells were not initially sensitive to stimulus, and became responsive to loading at different time points depending on the spatial region of the constructs (Figs. 5 and 6). It has been postulated that the establishment of a chondrogenic phenotype and development of a PCM before the initiation of loading may be a crucial determinant of MSC response to this stimulus.^{37,38} At day 7, a small response to loading was observed in the core of the construct, which was

not observed in the annular region until day 14. This response to loading can be correlated with an earlier pericellular staining for sGAG in the core of the construct (Fig. 2). Interestingly, PCM staining for sGAG was similar in the core of the construct at day 7 and in the annulus at day 14, suggesting a role for the PCM in determining the response of MSCs to loading. Additionally, the local osmotic environment (tonicity), which is related to both the PCM and ECM, has been shown to regulate chondrocyte cartilage-matrix-specific gene expression and matrix production^{39–43} and may be further regulated by the application of dynamic compression.

There was one noted exception to this general response to loading. It was observed that dynamic compression can downregulate expression when basal levels are naturally elevated in FS conditions (day 14 core levels, see Fig. 4), as evidenced by the lower levels of aggrecan and collagen type II expression in the cores of loaded constructs at day 14 (Fig. 5). It is possible that the application of loading when levels of cartilage-matrix-specific gene expression are already high in FS conditions (30–450-fold increase in comparison to day 0, Fig. 4) may result in overstimulation, causing a reduction of gene transcription through receptor-based regulatory mechanisms. This reduction in gene expression at this time point correlates with previous studies demonstrating that dynamic compression applied within 14 days of TGF- β 3 supplementation can inhibit cartilage-specific ECM accumulation in MSC-seeded agarose hydrogels.^{5,22,26} At day 21, a well-developed PCM was observed around MSCs in both the core and annulus of the constructs. At this time point, loading enhanced cartilage-matrix-specific gene expression in both regions of the construct. These changes in expression following loading are modest in comparison to the peak in expression found at day 14 (Fig. 4A, B). However, as a significant reduction in expression follows this peak in FS conditions, the effect of loading after this time point may be significant as a mechanism for sustaining enhanced levels of cartilage-matrix-specific gene expression.

Conclusions

The application of mechanical stimulus to enhance cartilage ECM synthesis may be an important tool in regenerative medicine-based cartilage repair. Our results suggest that a chondrogenic phenotype and/or the establishment of a cartilage-specific PCM must first be established before mechanical signals can have a positive effect on cartilage-matrix-specific gene expression. Further, this study provides evidence that mechanical stimulus may play a key role in the maintenance of a chondrogenic phenotype.

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Disclosure Statement

No competing financial interests exist.

References

- Huang, C.Y., Hagar, K.L., Frost, L.E., Sun, Y., and Cheung, H.S. Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. *Stem Cells* **22**, 313, 2004.
- Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* **9**, 641, 1991.
- Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
- Tuan, R.S., Boland, G., and Tuli, R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* **5**, 32, 2003.
- Huang, A.H., Farrell, M.J., Kim, M., and Mauck, R.L. Long-term dynamic loading improves the mechanical properties of chondrogenic mesenchymal stem cell-laden hydrogel. *Eur Cell Mater* **19**, 72, 2010.
- Erickson, I.E., Huang, A.H., Chung, C., Li, R.T., Burdick, J.A., and Mauck, R.L. Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels. *Tissue Eng Part A* **15**, 1041, 2009.
- Kopesky, P.W., Lee, H.Y., Vanderploeg, E.J., Kisiday, J.D., Frisbie, D.D., Plaas, A.H., Ortiz, C., and Grodzinsky, A.J. Adult equine bone marrow stromal cells produce a cartilage-like ECM mechanically superior to animal-matched adult chondrocytes. *Matrix Biol* **29**, 427, 2010.
- Vinardell, T., Thorpe, S.D., Buckley, C.T., and Kelly, D.J. Chondrogenesis and integration of mesenchymal stem cells within an *in vitro* cartilage defect repair model. *Ann Biomed Eng* **37**, 2556, 2009.
- Sheehy, E.J., Buckley, C.T., and Kelly, D.J. Chondrocytes and bone marrow derived mesenchymal stem cells undergoing chondrogenesis in agarose hydrogels of solid and channelled architectures respond differentially to dynamic culture conditions. *J Tissue Eng Regen Med* 2011 [Epub ahead of print]; doi: 10.1002/term.385.
- Mauck, R.L., Byers, B.A., Yuan, X., and Tuan, R.S. Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic loading. *Biomech Model Mechanobiol* **6**, 113, 2007.
- Babalola, O., and Bonassar, L. Effects of seeding density on proteoglycan assembly of passaged mesenchymal stem cells. *Cell Mol Bioeng* **3**, 197, 2010.
- Mikic, B., Isenstein, A.L., and Chhabra, A. Mechanical modulation of cartilage structure and function during embryogenesis in the chick. *Ann Biomed Eng* **32**, 18, 2004.
- Nowlan, N.C., Bourdon, C., Dumas, G., Tajbakhsh, S., Prendergast, P.J., and Murphy, P. Developing bones are differentially affected by compromised skeletal muscle formation. *Bone* **46**, 1275, 2010.
- Mikic, B., Johnson, T.L., Chhabra, A.B., Schalet, B.J., Wong, M., and Hunziker, E.B. Differential effects of embryonic immobilization on the development of fibrocartilaginous skeletal elements. *J Rehabil Res Dev* **37**, 127, 2000.
- Chen, X., Macica, C.M., Nasiri, A., and Broadus, A.E. Regulation of articular chondrocyte proliferation and differentiation by indian hedgehog and parathyroid hormone-related protein in mice. *Arthritis Rheum* **58**, 3788, 2008.
- Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A., and Kelly, D.J. The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF-beta3 induced chondrogenic differentiation. *Ann Biomed Eng* **38**, 2896, 2010.
- Kelly, D.J., and Jacobs, C.R. The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchy-

- mal stem cells. *Birth Defects Res C Embryo Today* **90**, 75, 2010.
18. Li, Z., Yao, S.-J., Alini, M., and Stoddart, M.J. Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites is modulated by frequency and amplitude of dynamic compression and shear stress. *Tissue Eng Part A* **16**, 575, 2010.
 19. McMahon, L.A., Reid, A.J., Campbell, V.A., and Prendergast, P.J. Regulatory effects of mechanical strain on the chondrogenic differentiation of MSCs in a collagen-GAG scaffold: experimental and computational analysis. *Ann Biomed Eng* **36**, 185, 2008.
 20. Kisiday, J.D., Frisbie, D.D., McIlwraith, C.W., and Grodzinsky, A.J. Dynamic compression stimulates proteoglycan synthesis by mesenchymal stem cells in the absence of chondrogenic cytokines. *Tissue Eng Part A* **15**, 2817, 2009.
 21. Buckley, C.T., Vinardell, T., Thorpe, S.D., Haugh, M.G., Jones, E., McGonagle, D., and Kelly, D.J. Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells. *J Biomech* **43**, 920, 2010.
 22. Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A., and Kelly, D.J. Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells. *Biochem Biophys Res Commun* **377**, 458, 2008.
 23. Meyer, E.G., Buckley, C.T., Thorpe, S.D., and Kelly, D.J. Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. *J Biomech* **43**, 2516, 2010.
 24. Guaccio, A., Borselli, C., Oliviero, O., and Netti, P.A. Oxygen consumption of chondrocytes in agarose and collagen gels: a comparative analysis. *Biomaterials* **29**, 1484, 2008.
 25. Buckley, C.T., Vinardell, T., and Kelly, D.J. Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes. *Osteoarthritis Cartilage* **18**, 1345, 2010.
 26. Mouw, J.K., Connelly, J.T., Wilson, C.G., Michael, K.E., and Levenston, M.E. Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. *Stem Cells* **25**, 655, 2007.
 27. Lennon, D.P., and Caplan, A.I. Isolation of human marrow-derived mesenchymal stem cells. *Exp Hematol* **34**, 1604, 2006.
 28. Kim, Y.J., Sah, R.L., Doong, J.Y., and Grodzinsky, A.J. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* **174**, 168, 1988.
 29. Kafienah, W., and Sims, T.J. Biochemical methods for the analysis of tissue-engineered cartilage. *Methods Mol Biol* **238**, 217, 2004.
 30. Ignat'eva, N., Danilov, N., Averkiev, S., Obrezkova, M., Lunin, V., and Sobol', E. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. *J Anal Chem* **62**, 51, 2007.
 31. Kumar, B.M., Yoo, J.G., Ock, S.A., Kim, J.G., Song, H.J., Kang, E.J., Cho, S.K., Lee, S.L., Cho, J.H., Balasubramanian, S., and Rho, G.J. *In vitro* differentiation of mesenchymal progenitor cells derived from porcine umbilical cord blood. *Mol Cells* **24**, 343, 2007.
 32. Zou, L., Zou, X., Chen, L., Li, H., Mygind, T., Kassem, M., and Bunger, C. Multilineage differentiation of porcine bone marrow stromal cells associated with specific gene expression pattern. *J Orthop Res* **26**, 56, 2008.
 33. Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402, 2001.
 34. Campbell, J.J., Lee, D.A., and Bader, D.L. Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells. *Biorheology* **43**, 455, 2006.
 35. Lewis, M.C., MacArthur, B.D., Malda, J., Pettet, G., and Please, C.P. Heterogeneous proliferation within engineered cartilaginous tissue: the role of oxygen tension. *Biotechnol Bioeng* **91**, 607, 2005.
 36. Zhou, S., Cui, Z., and Urban, J.P.G. Nutrient gradients in engineered cartilage: Metabolic kinetics measurement and mass transfer modeling. *Biotechnol Bioeng* **101**, 408, 2008.
 37. Huang, A.H., Farrell, M.J., and Mauck, R.L. Mechanics and mechanobiology of mesenchymal stem cell-based engineered cartilage. *J Biomech* **43**, 128, 2010.
 38. Vigfúsdóttir, Á., Pasrija, C., Thakore, P., Schmidt, R., and Hsieh, A. Role of pericellular matrix in mesenchymal stem cell deformation during chondrogenic differentiation. *Cell Mol Bioeng* **3**, 387, 2010.
 39. van der Windt, A.E., Haak, E., Das, R.H., Kops, N., Welting, T.J., Caron, M.M., van Til, N.P., Verhaar, J.A., Weinans, H., and Jahr, H. Physiological tonicity improves human chondrogenic marker expression through nuclear factor of activated T-cells 5 *in vitro*. *Arthritis Res Ther* **12**, R100, 2010.
 40. Urban, J.P., Hall, A.C., and Gehl, K.A. Regulation of matrix synthesis rates by the ionic and osmotic environment of articular chondrocytes. *J Cell Physiol* **154**, 262, 1993.
 41. Palmer, G.D., Chao Ph, P.H., Raia, F., Mauck, R.L., Valhmu, W.B., and Hung, C.T. Time-dependent aggrecan gene expression of articular chondrocytes in response to hyperosmotic loading. *Osteoarthritis Cartilage* **9**, 761, 2001.
 42. Hung, C.T., LeRoux, M.A., Palmer, G.D., Chao, P.H., Lo, S., and Valhmu, W.B. Disparate aggrecan gene expression in chondrocytes subjected to hypotonic and hypertonic loading in 2D and 3D culture. *Biorheology* **40**, 61, 2003.
 43. Negoro, K., Kobayashi, S., Takeno, K., Uchida, K., and Baba, H. Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three-dimensional culture system. *Clin Exp Rheumatol* **26**, 534, 2008.

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