

FIBRONECTIN- AND COLLAGEN-MIMETIC LIGANDS REGULATE BONE MARROW STROMAL CELL CHONDROGENESIS IN THREE-DIMENSIONAL HYDROGELS

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Abstract

Modification of tissue engineering scaffolds with bioactive molecules is a potential strategy for modulating cell behavior and guiding tissue regeneration. While adhesion to RGD peptides has been shown to inhibit *in vitro* chondrogenesis, the effects of extracellular matrix (ECM)-mimetic ligands with complex secondary and tertiary structures are unknown. This study aimed to determine whether collagen- and fibronectin-mimetic ligands would retain biologic functionality in three-dimensional (3D) hydrogels, whether different ECM-mimetic ligands differentially influence *in vitro* chondrogenesis, and if effects of ligands on differentiation depend on soluble biochemical stimuli. A linear RGD peptide, a recombinant fibronectin fragment containing the seven to ten Type III repeats (FnIII7-10) and a triple helical, collagen mimetic peptide with the GFOGER motif were covalently coupled to agarose gels using the sulfo-SANPAH crosslinker, and bone marrow stromal cells (BMSCs) were cultured within the 3D hydrogels. The ligands retained biologic functionality within the agarose gels and promoted density-dependent BMSC spreading. Interactions with all adhesive ligands inhibited stimulation by chondrogenic factors of collagen Type II and aggrecan mRNA levels and deposition of sulfated glycosaminoglycans. In medium containing fetal bovine serum, interactions with the GFOGER peptide enhanced mRNA expression of the osteogenic gene osteocalcin whereas FnIII7-10 inhibited osteocalcin expression. In conclusion, modification of agarose hydrogels with ECM-mimetic ligands can influence the differentiation of BMSCs in a manner that depends strongly on the presence and nature of soluble biochemical stimuli.

Keywords: Chondrogenesis, mesenchymal progenitors, extracellular matrix, fibronectin, collagen, tissue engineering, microenvironment.

Introduction

Mesenchymal stem cells (MSCs) are an attractive cell source for tissue engineering and cell-based therapies given their multilineage differentiation potential and ability to be expanded in culture (Caplan, 1991; Pittenger *et al.*, 1999). The repair of damaged articular cartilage is one particular application that would be well suited for MSCs as a result of the limited capacity for cartilage regeneration and the rapid dedifferentiation of chondrocytes during monolayer expansion (Benya and Shaffer, 1982). Many populations of mesenchymal progenitors such as bone marrow stromal cells (BMSCs) display chondrogenic-like differentiation in three-dimensional (3D) culture environments and in the presence of transforming growth factor- β (TGF- β) family growth factors (Johnstone *et al.*, 1998; Caterson *et al.*, 2001; Bosnakovski *et al.*, 2004). However, recent studies have shown that even over extended culture periods, engineered cartilage constructs derived from BMSCs have a distinct composition and inferior mechanical properties compared with those derived from native articular chondrocytes (Mauck *et al.*, 2006; Connelly *et al.*, 2008b). It is therefore likely that additional signals are required for complete chondrogenic differentiation and the development of a functional tissue replacement.

Interactions between cells and the extracellular matrix (ECM) provide important cues for the differentiation and development of many tissues, including cartilage. For example, integrin-mediated adhesion to fibronectin is required for precartilage condensation of limb bud cells (Bang *et al.*, 2000), and the presence of specific fibronectin isoforms influences the extent of condensation (White *et al.*, 2003). Interactions with Type II collagen can also enhance the *in vitro* chondrogenesis of BMSCs (Bosnakovski *et al.*, 2006). Although much work is still required to fully understand the complex roles of cell-matrix interactions in chondrogenesis, it is clear that the ECM provides key signals for guiding this process.

A potential strategy for controlling cell-matrix interactions *in vitro* is to engineer synthetic matrices to present specific ligands in a precise manner. The incorporation of peptides containing the integrin adhesive sequence Arginine-Glycine-Aspartic acid (RGD) into non-adhesive hydrogels was reported to enhance MSC survival and osteogenic differentiation (Shin *et al.*, 2005). However, studies in our laboratory suggest that alginate and agarose hydrogels functionalized with RGD peptides inhibit BMSC chondrogenesis (Connelly *et al.*,

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2007; Connelly *et al.*, 2008a). In addition to modulating integrin-mediated adhesion, engineered matrices also provide a means for introducing small molecules (Benoit *et al.*, 2008) or larger moieties such as chondroitin sulfate (Varghese *et al.*, 2008) that can enhance *in vitro* chondrogenesis. Although interactions with the RGD motif inhibit BMSC chondrogenesis, interactions with adhesive sequences that mimic other ECM proteins and target a distinct set of integrin receptors may induce different responses. For example, $\alpha 5\beta 1$ binding to fibronectin requires a synergy site containing the “PHSRN” sequence in addition to RGD (Aota *et al.*, 1994), and $\alpha 2\beta 1$ binds to the “GFOGER” motif within fibrillar collagens (Reyes and Garcia, 2003). However, the effects of ECM-mimetic ligands with complex secondary and tertiary structures on chondrogenesis are unknown. The goals of this study were to determine whether fibronectin- and collagen-mimetic ligands, targeting the $\alpha 5\beta 1$ integrins and $\alpha 2\beta 1$ integrins, respectively, retain biologic functionality when conjugated to 3D agarose hydrogels, whether interactions with different ECM-mimetic ligands differentially influence BMSC chondrogenesis in 3D hydrogels, and whether the responses to such engineered matrices depend on the nature of soluble biochemical stimuli presented through the culture medium.

Materials and Methods

Materials

The synthetic peptides GRGDSP (RGD) and the non-adhesive control GRGESP (RGE) were obtained from Bachem (King of Prussia, PA, USA), and the collagen mimetic peptide GGYGGGPC[GPP]5GFOGER[GPP]5GPC (GFOGER) was prepared by the Emory University Microchemical Facility. The sulfo-SANPAH crosslinker, EZ-link Maleimide-PEG-Biotin kit, and Ultralink Immobilized Avidin were from Pierce (Rockford, IL, USA). Seaprep agarose was from Cambrex (Frederick, MD, USA). Immature bovine hind limbs were from Research 87 (Marlborough, MA, USA). Recombinant human TGF- $\beta 1$ was from R&D Systems (Minneapolis, MN, USA), and basic-fibroblast growth factor (bFGF) was from Peprotech (Rocky Hill, NJ, USA). Dexamethasone, 1,9 dimethyl methylene blue (DMMB), agarase, and Hoechst dye 33258 were from Sigma Aldrich (St Louis, MO, USA). The ITS+ premix and Proteinase K were from BD Biosciences (San Jose, CA, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle medium (DMEM), antibiotic/antimycotic, trypsin, nonessential amino acids (NEAA), and phosphate buffered saline (PBS) were from Invitrogen (Carlsbad, CA, USA). The radiolabel precursor ^{35}S -sodium sulfate was from MP Biomedicals (Irvine, CA, USA). The ^3H -proline and ECF substrate were purchased from GE Healthcare (Piscataway, NJ, USA). The anti-vinculin and anti-biotin antibodies were from Sigma-Aldrich, and the FITC anti-mouse antibody was from Abcam (Cambridge, UK). AlexaFluor 594-conjugated phalloidin was from Molecular Probes (Eugene, OR, USA). The RNeasy mini kit was from Qiagen (Valencia, CA,

USA), and the AMV reverse transcriptase kit was from Promega (Madison, WI, USA). The Sybr Green master mix was from Applied Biosystems (Foster City, CA, USA), and the primers were from Invitrogen.

Preparation of ligand-modified agarose

A monobiotinylated fibronectin fragment containing the seventh to tenth Type III repeats (FnIII7-10) of human fibronectin was expressed in JM109 cells and purified by affinity chromatography as previously described (Petrie *et al.*, 2006). A biotin tag was coupled to the cysteines in the GFOGER peptide using the EZ-link Maleimide-PEG-Biotin kit according to the manufacturer's instructions. The synthetic peptides and fibronectin fragment were then conjugated to agarose hydrogels with the heterobifunctional sulfo-SANPAH crosslinker (Dodla and Bellamkonda, 2006). Briefly, the primary amines on the ligands were reacted with the NHS-ester group of the sulfo-SANPAH in PBS at room temperature for 4 h in the dark with a 10-fold molar excess of the crosslinker. Seaprep agarose solutions were prepared in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, autoclaved, and cooled to 37 °C. One part peptide/sulfo-SANPAH solution was combined with three parts 4 % agarose and mixed thoroughly to yield a 3 % agarose solution. The mixture was exposed to 365 nm ultraviolet light for 3 min to activate the photoreactive groups of the sulfo-SANPAH and conjugate the peptide to CH groups in the agarose. The agarose was allowed to gel at 4 °C for 20 min and washed four times with fivefold excess PBS over 3 days to remove the unbound peptide and crosslinker. Controls were prepared as stated previously but without addition of the sulfo-SANPAH. Final ligand densities in the agarose gels were determined by dot blot detection of biotinylated ligands (Fig. 1). Gels were digested with agarase (4 U/gel) at 45 °C for 4 h and blotted onto nitrocellulose ($n = 3/\text{condition}$). Membranes were probed with alkaline phosphatase-conjugated anti-biotin antibodies and developed with the ECF substrate and Fuji Image Analyzer (Fuji, Tokyo, Japan). For an input concentration of 8 μM (400 $\mu\text{g}/\text{mL}$) FnIII7-10, the conjugation efficiency was approximately 30 %, resulting in a final density of 2.4 μM (Fig. 1C). For an input density of 37 μM (200 $\mu\text{g}/\text{mL}$) GFOGER, the conjugation efficiency was approximately 20 %, resulting in a final density of 7.4 μM (Fig. 1C). Low levels (less than 5 %) of the ligands were present in gels without the sulfo-SANPAH crosslinker. For reference, the conjugation efficiencies for the fibronectin- and collagen-mimetic ligands were slightly higher than those previously reported for laminin-1 (Dodla and Bellamkonda, 2006).

Cell seeding and gel culture

Bone marrow was harvested from the tibiae and femora of an immature calf as previously described (Connelly *et al.*, 2007). The adherent BMSCs were expanded three times in low-glucose DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 ng/mL bFGF. The 3 % modified agarose gels were melted at 45 °C and cooled to 37 °C. BMSCs were then seeded into cylindrical (4 mm diameter) gels by resuspending the cells in the melted agarose at a density of 10e6 cells/mL and casting the cell suspension into

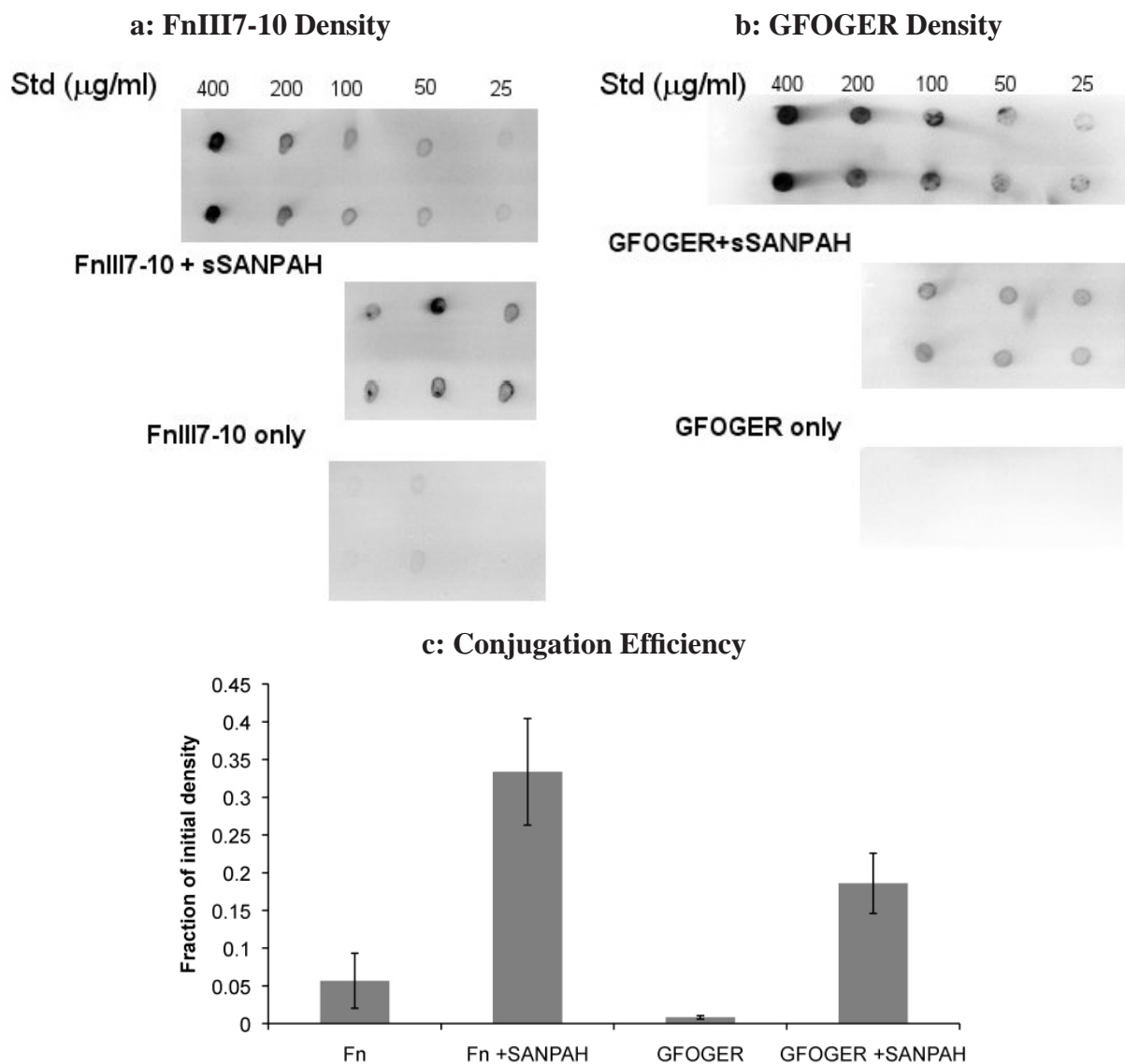


Fig. 1. Conjugation of FnIII7-10 and GFOGER ligands to agarose hydrogels. (a) FnIII7-10 and (b) GFOGER densities in the agarose gels were determined by dot blot detection of the biotin tag after agarase digestion. The initial concentration of FnIII7-10 and GFOGER were 400 $\mu\text{g/ml}$ (8 mM) and 200 $\mu\text{g/ml}$ (37 mM), respectively, and the standards were diluted from the ligand stocks. Control gels were prepared with the same initial ligand density but without the sulfo-SANPAH crosslinker. (c) Conjugation efficiency for the FnIII7-10 and GFOGER modified gels was quantified by measuring the dot blot intensities relative to the initial densities. For the concentrations examined, there was a linear relationship between biotin intensity and ligand concentration. Data represent the mean \pm SEM, $n = 3$ gels per condition.

custom molds. The gels were cooled at 4 $^{\circ}\text{C}$ for 30 min and transferred to fresh culture medium. The basal, chemically defined medium consisted of high-glucose DMEM, 1 % ITS+ Premix, 1 % NEAA, 1 % antibiotic/antimycotic, and 50 $\mu\text{g/ml}$ ascorbate. The chondrogenic medium was basal medium supplemented with 10 ng/ml TGF- β 1 and 100 nM dexamethasone. The FBS-supplemented medium consisted of DMEM, 10 % FBS, 1 % NEAA, 1 % antibiotic/antimycotic, and 50 $\mu\text{g/ml}$ ascorbate. BMSC seeded gels were cultured up to 8 days under the specified conditions and media were changed every 2 days.

Ligand functionality

To examine the functionality of the ligands in the 3D hydrogels, BMSCs were seeded into agarose gels

functionalized with the RGD peptide (0, 50, and 100 μM), FnIII7-10 fragment (0, 0.5, and 1 μM), GFOGER peptide (0, 3.75, 15 μM), or nonadhesive RGE peptide (100 μM). Cells in gels were cultured for 24 h in basal medium and examined for changes in cell morphology. Agarose gels were fixed with 10 % neutral-buffered formalin for 30 min at 4 $^{\circ}\text{C}$ and rinsed in PBS. Portions of the fixed gels were blocked with 5 % FBS and permeabilized with 1 % Triton-X100. Vinculin localization was examined by staining with the anti-vinculin antibody (10 $\mu\text{g/ml}$) for 90 min at room temperature and secondary detection was performed with the anti-mouse FITC-labeled antibody (10 $\mu\text{g/ml}$) for 90 min at room temperature. The F-actin cytoskeleton was visualized by staining with AlexaFluor594-phalloidin for 90 min at room temperature,

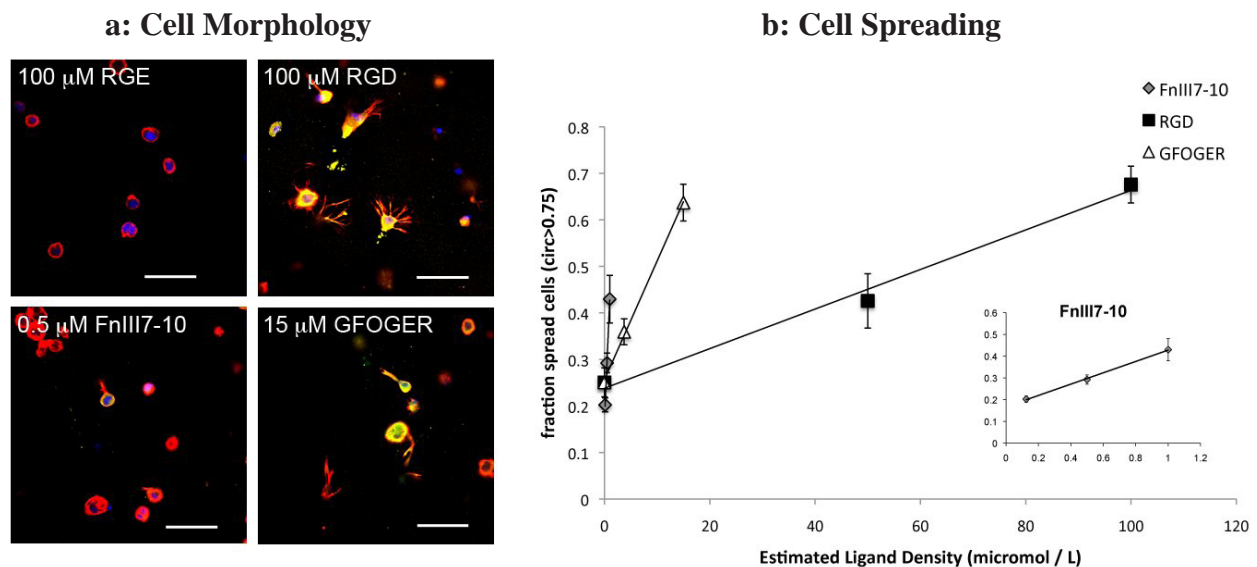


Fig. 2. Effects of ligand-modified agarose on bone marrow stromal cell (BMSC) morphology. **(a)** BMSC morphology was examined 24 h after seeding into functionalized gels by immunofluorescence detection of the F-actin cytoskeleton (red), vinculin (green), and DNA (blue). Agarose gels were modified with 100 μM RGE, 100 μM RGD, 0.5 μM FnIII7-10, and 15 μM GFOGER (final densities). Scale bars are 50 μm . **(b)** Three-dimensional cell morphology was quantified by measuring the fraction of spread cells (circularity greater than 0.75) over a range of ligand densities for RGD, FnIII7-10 (inset), and GFOGER. Data represent the mean \pm SEM, $n = 6$ (Original magnification, 10 \times).

and DNA was labeled with Hoechst dye. The agarose gels were imaged with a Zeiss 510 laser scanning confocal microscope (Zeiss, Heidelberg, Germany). Fluorescence images of the F-actin cytoskeleton from two different gels (three images per gel) were analyzed with Scion Image software (Frederick, MD, USA). The area (A) and perimeter (P) for each individual cell were used to calculate the circularity ($\text{circularity} = 4\pi A/P^2$) as a quantitative measure of cell spreading with a lower circularity indicating a greater degree of spreading (Connelly *et al.*, 2008a).

Effects of ligands on BMSC differentiation

To investigate the effects of these ligand-functionalized gels on chondrogenic differentiation, BMSCs were first seeded into RGE (100 μM), RGD (100 μM), FnIII7-10 (1 μM), or GFOGER (15 μM) modified agarose, cultured for 8 days in basal or chondrogenic medium, and examined for changes in matrix synthesis rates and accumulation ($n = 5/\text{group}$). To determine the lineage-specific effects of the gels, BMSCs were again seeded into RGE, RGD, FnIII7-10, or GFOGER modified agarose with the same ligand densities as in the chondrogenic experiment, cultured for 8 days in chondrogenic or serum supplemented medium, and examined for changes in chondrocytic and osteoblastic gene expression ($n = 4/\text{group}$).

During the final 24 h of culture, 5 $\mu\text{Ci}/\text{mL}$ ^{35}S -sodium sulfate and 10 $\mu\text{Ci}/\text{mL}$ ^3H -proline were included in the culture medium to measure sulfated glycosaminoglycan (sGAG) and protein synthesis, respectively. At the end of the culture period, radiolabel incorporation was quenched with four sequential 30 min washes in PBS plus 0.8 mM sodium sulfate and 1.6 mM L-proline at 4 $^\circ\text{C}$. The agarose gel constructs were weighed wet,

lyophilized, and sequentially digested with Proteinase K (1 mg/80 mg sample) at 60 $^\circ\text{C}$ overnight and agarase (4 U/construct) at 45 $^\circ\text{C}$ for 4 h. Radiolabel contents were measured using a liquid scintillation counter. The sGAG contents were measured using the 1,9-dimethylmethylene blue assay (Farndale *et al.*, 1982), and the DNA contents were measured using the Hoechst dye assay (Kim *et al.*, 1988). Total collagen content was measured using the hydroxyproline assay (Woessner, 1961).

RNA was isolated from the agarose gels using the Tri-spin method (Chomczynski and Sacchi, 1987). Gels were immediately dissociated in lysis buffer containing 2-mercaptoethanol. RNA was extracted from the gel using the Trizol reagent and chloroform and precipitated with 100% isopropanol. The RNA was further purified using the Qiagen RNeasy kit according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed to cDNA using the Promega AMV reverse transcriptase kit. Gene expression was measured by real-time reverse transcription-polymerase chain reaction (PCR) using the SybrGreen master mix and custom primers for bovine collagen II, aggrecan, collagen I (Brodtkin *et al.*, 2004), and osteocalcin (Bosnakovski *et al.*, 2005). The PCR reactions and detection were performed with an ABI Prism 7700 (Applied Biosystems) and the transcript number relative to total RNA was calculated based on standards of known concentrations run on the same plate.

Data analysis

All data are presented as the mean \pm SEM (standard error of the mean) of representative experiments. Gene expression levels were transformed by optimal Box-Cox transformation to improve normality (Sokal and Rohlf, 1995). The effects of ligands in the presence or absence

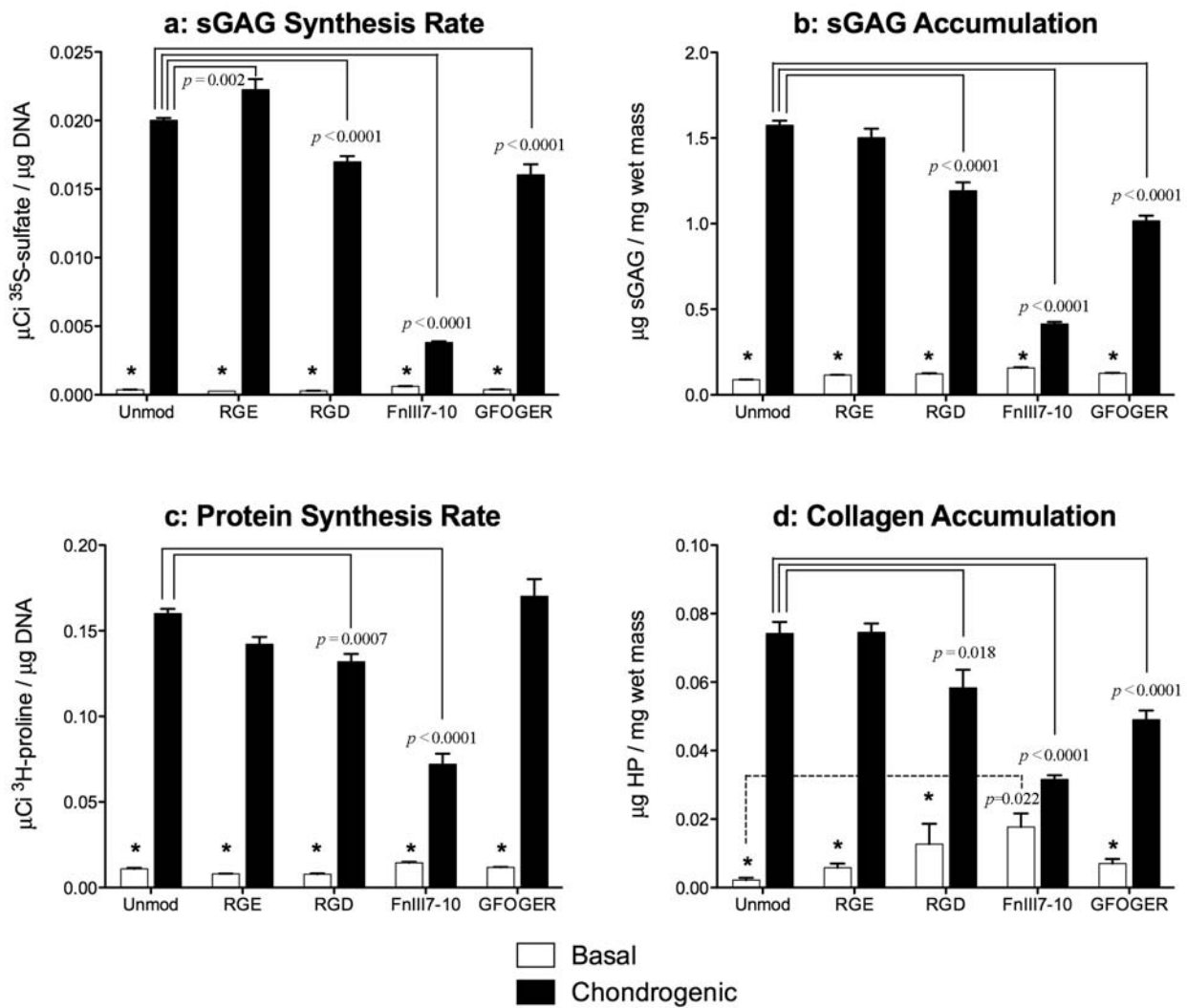


Fig. 3. Regulation of matrix accumulation by ligand-modified agarose over 8 days of culture in basal or chondrogenic medium (10 ng/mL TGF-β1 and 100 nM dexamethasone). **(a)** Proteoglycan synthesis as measured by incorporation of ³⁵S-sulfate over the final 24 h of culture (Day 7-8) for unmodified and functionalized agarose with 100 μM RGE, 100 μM RGD, 1 μM FnIII7-10, or 15 μM GFOGER final ligand densities. **(b)** Total sGAG accumulation in the gels after the 8-day culture period as measured by the DMMB assay. **(c)** Protein synthesis as measured by incorporation of ³H-proline over the final 24 h of culture; and **(d)** total collagen accumulation as measured by assaying for hydroxyproline (HP) content. Data represent the mean ± SEM, n = 4-6 gels/condition. Significance vs. chondrogenic for a given gel is indicated by * (p < 0.0001) or + (p < 0.001).

of chondrogenic supplements on matrix synthesis rates and sGAG accumulation were examined using two factor (ligand, medium) general linear models with Tukey’s test for pairwise comparisons. The effects of ligands on chondrocytic and osteoblastic gene expression in chondrogenic or serum-supplemented medium were examined using two factor (ligand, medium) general linear models with Tukey’s test for pairwise comparisons. Significance was at p < 0.05.

Results

Integrin adhesive ligands alter BMSC morphology in 3D agarose gels

Like the linear RGD peptide, the recombinant fibronectin fragment FnIII7-10 and collagen-mimetic peptide

containing the GFOGER sequence retained biologic functionality after conjugation to the agarose. After 24 h, BMSCs in the non-adhesive RGE hydrogels maintained a rounded morphology. In contrast, cells in hydrogels conjugated with RGD, FnIII7-10, and GFOGER demonstrated marked changes in morphology characterized by large F-actin projections and positive staining for vinculin (Fig. 2A), consistent with integrin-mediated adhesion to the hydrogel. For each of the adhesive molecules, there was a monotonic increase in the number of spread cells with increasing ligand density (Fig. 2B). On a molar basis, FNIII7-10 was the most effective at inducing cell spreading, whereas RGD was the least effective. Similar changes in cell morphology at 24 h were observed for 100 μM RGD, 1 μM FnIII7-10, and 15 μM GFOGER.

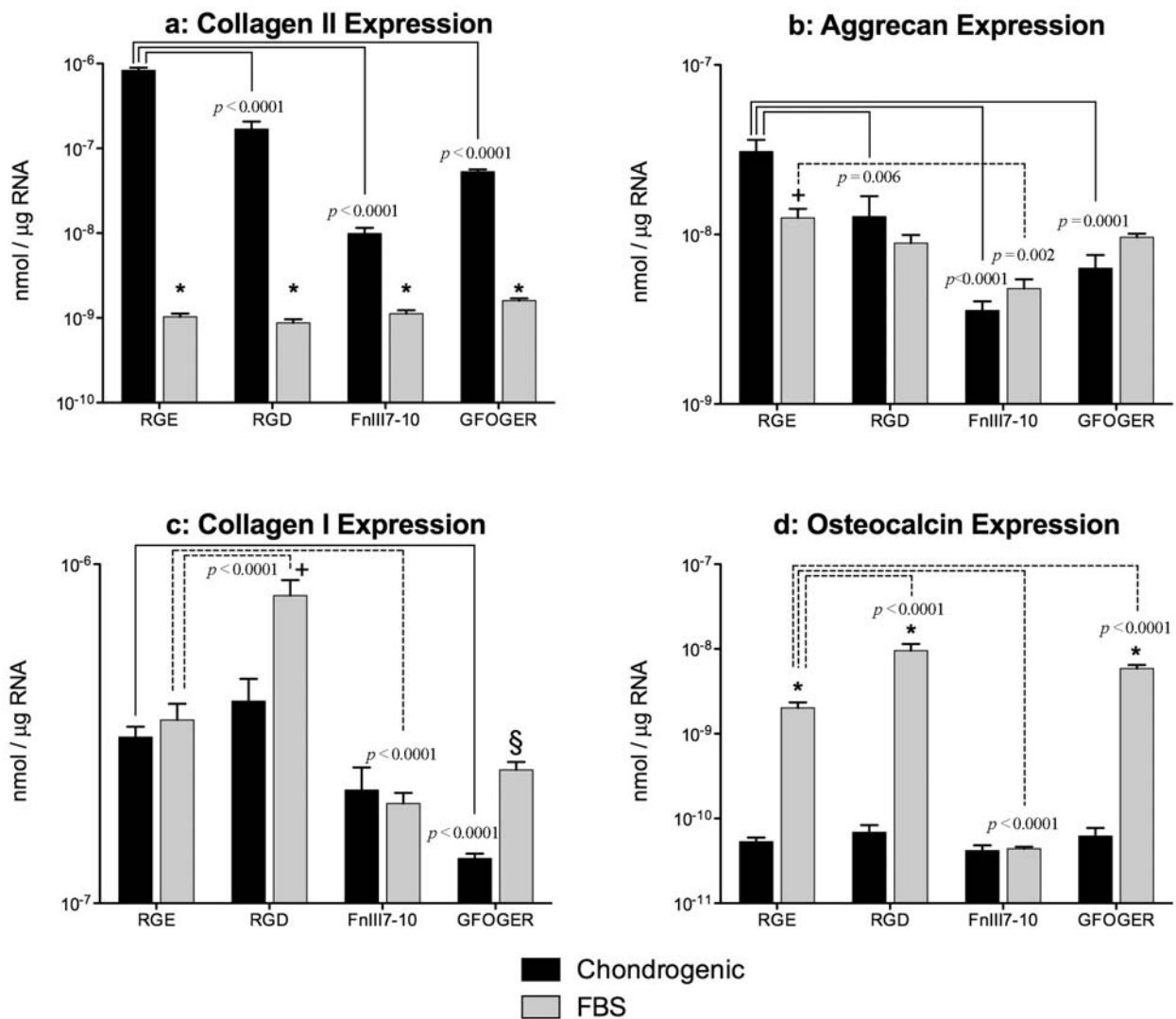


Fig. 4. Effects of ligand modified agarose on lineage-specific gene expression. Levels of mRNA for the chondrocytic genes (a) Type II collagen and (b) aggrecan were measured by real-time polymerase chain reaction after 8 days of culture in RGE, RGD, FnIII7-10, or GFOGER modified agarose. Gels were cultured in chondrogenic medium or basal medium supplemented with 10 % FBS. Expression of (c) Type I collagen and the osteogenic marker (d) osteocalcin were also measured at day 8. Data represent the mean ± SEM, *n* = 4-6 gels/condition, and are presented on a logarithmic scale. Significance vs. chondrogenic for a given gel is indicated by * (*p* < 0.0001), + (*p* < 0.001) or § (*p* < 0.05).

Interactions with FnIII7-10 and GFOGER inhibit matrix production during BMSC chondrogenesis

At concentrations inducing comparable degrees of cell spreading at 24 h, all three adhesive ligands inhibited stimulation of ECM production by chondrogenic medium. Compared to basal medium, chondrogenic medium stimulated sGAG synthesis on day 8 (as measured by ³⁵S-sulfate incorporation) and sGAG accumulation within the agarose gels over the 8 day culture (both *p* < 0.0001). Compared with unmodified gels, these responses were both inhibited by the presence of RGD, FnIII7-10 or GFOGER (all *p* < 0.0001) (Fig. 3A,B). The chondrogenic medium also stimulated overall protein synthesis on day 8 (as measured by ³H-proline incorporation) and collagen accumulation over the 8 day culture (both *p* < 0.0001). Compared with unmodified gels, interactions with RGD (*p* = 0.0007) or FnIII7-10 (*p* < 0.0001) inhibited protein

synthesis, and interaction with RGD (*p* = 0.018), FnIII7-10 (*p* < 0.0001), or GFOGER (*p* < 0.0001) inhibited collagen accumulation (Fig. 3C,D). The RGE peptide did not substantially alter matrix synthesis rates or accumulation, as only the sGAG synthesis rate was significantly different from unmodified gels (*p* = 0.002). The presence of FnIII7-10 substantially increased (*p* = 0.022) collagen deposition in basal medium (Fig. 3D).

Interactions with functionalized hydrogels influence lineage-specific gene expression in a context dependent manner

The effects of the adhesive ligands on BMSC gene expression differed depending on the biochemical cues present in the culture medium. After 8 days of culture in the presence of the chondrogenic stimuli TGF-β1 and dexamethasone, interactions with RGD, FnIII7-10

or GFOGER all inhibited mRNA levels (Fig. 4A-B) of the chondrocytic genes Type II collagen (all $p < 0.0001$) and aggrecan ($p = 0.0064$, $p < 0.0001$ and $p = 0.0001$, respectively). In contrast, expression of collagen II was substantially lower after 8 days of culture in 10 % FBS-supplemented medium than in chondrogenic medium for all gel groups (all $p < 0.0001$) and was not significantly affected by the presence of any of the adhesive ligands. In serum-supplemented medium, expression of aggrecan was significantly lower than in chondrogenic medium for RGE-modified gels only ($p = 0.009$), and was inhibited by interactions with FnIII7-10 ($p = 0.0018$). Type I collagen expression (Fig. 4C) did not significantly differ between chondrogenic and serum-supplemented medium in gels modified with RGE or FnIII7-10, but was significantly greater in serum-supplemented medium in gels modified with RGD ($p = 0.002$) or GFOGER ($p = 0.025$). Relative to RGE-modified gels, collagen I expression was stimulated only by interactions with RGD in serum-supplemented medium ($p < 0.0001$), and was inhibited by interactions with FnIII7-10 in serum-supplemented medium and GFOGER in chondrogenic medium (both $p < 0.0001$). Expression of the osteogenic marker osteocalcin (Fig. 4D) was substantially higher in serum-supplemented medium compared with the chondrogenic medium in gels modified with RGE, RGD or GFOGER (all $p < 0.0001$), and did not significantly vary among ligands in chondrogenic medium. In serum-supplemented medium, osteocalcin expression was enhanced by RGD ($p < 0.0001$) or GFOGER ($p = 0.0011$) interactions and inhibited by FnIII7-10 interactions ($p < 0.0001$).

Discussion

Our data demonstrate that ECM-mimetic ligands with complex secondary and tertiary structures can be coupled to agarose hydrogels and retain their biologic activity. Similar to short, RGD containing oligopeptides (Connelly *et al.*, 2008a), GFOGER and FnIII7-10 promoted density-dependent changes in the three-dimensional morphology of BMSCs. On stimulation with the chondrogenic factors TGF- β 1 and dexamethasone, the presence of each of the adhesive ligands inhibited matrix synthesis and chondrocytic gene expression relative to non-adhesive controls. Several studies found that adhesion to short RGD peptides inhibits the chondrogenesis of BMSCs (Connelly *et al.*, 2007; Salinas and Anseth, 2008) and that this effect is mediated by the actin cytoskeleton (Connelly *et al.*, 2008a). Our data provide additional support for the role of the cytoskeleton and suggest that cell shape, more so than the specific integrin ligand, is a key regulator of chondrogenesis. Similarly, surface chemistry has less of an effect on human MSC chondrogenesis than overall morphology and cell aggregation (Phillips *et al.*, 2010).

In contrast, the RGD and GFOGER ligands increased expression of osteocalcin, an osteoblastic gene, in the presence of 10 % FBS, whereas the FnIII7-10 inhibited this response. Taken together, these findings indicate that the specific responses of BMSCs to cues supplied

by engineered three-dimensional matrices depend on the biochemical environment. Although cell shape and cytoskeletal tension also control osteogenic *versus* adipogenic differentiation (McBeath *et al.*, 2004; Engler *et al.*, 2006), our findings and others further suggest that osteogenic differentiation may depend on the specific ECM and integrins involved (Keselowsky *et al.*, 2005). It is interesting to note that the RGD peptide, but not GFOGER or FnIII7-10, increased collagen I expression in the serum supplemented medium. These results raise the intriguing possibility that different components of the ECM may regulate different aspects of osteoblastic differentiation.

When coupled to biomaterial surfaces, the FnIII7-10 and GFOGER ligands enhance the osteoblastic differentiation of rat MSCs *in vitro* and the osseointegration of titanium screws *in vivo* (Reyes *et al.*, 2007; Petrie *et al.*, 2008). Although the effects of the GFOGER modified gels on osteocalcin expression in the present study are consistent with these findings, the inhibitory effects of FnIII7-10 appear somewhat contradictory. Such differences may reflect variations in the specific media formulation, which have the potential to alter cellular responses to biomaterials. For example, the presence of a phosphate source, which was absent in our culture medium, may impact the extent of osteogenesis. Alternatively, altered presentation of the FnIII7-10 between two-dimensional and three-dimensional may influence the activity and integrin specificity of the ligand. Much work is clearly still required to understand the complex and context-dependent roles of cell-ECM interactions in differentiation, and new insights in this area could lead to improved biomaterials and regenerative therapies.

Although functionalizing hydrogels with ECM mimetic ligands may be detrimental for *in vitro* chondrogenesis, this strategy may be advantageous for other tissue engineering applications. Adhesion to RGD peptides can enhance initial cell viability within gels, and the incorporation of enzymatically cleavable tethers allows these ligands to be removed during later stages of differentiation (Salinas and Anseth, 2008; Kloxin *et al.*, 2009). Patterning of adhesive ligands may also allow for undifferentiated progenitors to be retained within specified regions of an engineered tissue. Finally, graded levels of bioactive molecules could be a useful strategy for engineered heterogeneous tissues or interfaces such as fibrocartilage or osteochondral replacements, where controlling the extent of the chondrogenic response to initial biochemical cues may allow the development of tissues with desired spatial patterns of phenotypic heterogeneity.

Acknowledgments

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Discussion with Reviewers

Reviewer I: There appears to be an increase in collagen production in basal conditions in FN-modified gels. Please comment. Is this type I or type II collagen? Was any immunostaining performed to verify that gene expression changes were reflected in matrix accumulation and type?

Authors: As additional patterns among ligands in basal medium may have been obscured by the large difference in matrix production between chondrogenic and basal medium, we re-examined the matrix synthesis rates and matrix accumulation for constructs in basal conditions only by means of a one factor general linear model with Tukey's test for pairwise comparisons. Indeed, the FnIII7-10-conjugated group was the only group to demonstrate collagen accumulation significantly greater than that in unmodified gels in basal medium ($p = 0.0053$). Furthermore, the sGAG accumulation, sGAG synthesis rate, and protein synthesis rate were all significantly greater in FnIII7-10-conjugated gels than in any other group in basal medium ($p < 0.0001$, $p < 0.0001$ and $p = 0.0011$, respectively). The patterns of response to ligands in basal medium thus appear to be roughly opposite to those in chondrogenic medium (Fig. 3). It is tempting to ascribe these effects to differences among ligands, perhaps related to the specific integrins involved in adhesion to the scaffold. However, while the ligand concentrations were chosen based on comparable effects on cell spreading at 24 h, it is also possible that similar effects on matrix production would be seen for the other ligands at higher densities. This issue certainly merits exploration in future studies.

We previously observed that interactions with RGD-modified alginate inhibited both collagen II gene expression and collagen II deposition by BMSCs (Connelly *et al.*, 2007, text reference), and that cyclic tensile loading of BMSCs in fibrin enhanced both collagen I gene expression and collagen I deposition (Connelly *et al.*, 2010, additional reference). We would thus expect a general correspondence between gene expression and protein deposition patterns in

the current study, but did not examine at the protein level the specific collagen types produced in the different gels.

Reviewer I: What would the effects be over a longer time course as the cells accrete their own pericellular matrix? Also, does cell spreading continue or does the 24 h time point represent the steady state condition? This would be important in interpreting the longer-term responses, given the authors finding that shape is such a strong determinant of phenotypic conversion.

Authors: Cell spreading persists for at least one week, but beyond this point production of a pericellular matrix likely would interfere with access to the engineered ligands. We previously observed that cells in RGD-modified alginate gels accumulated a fibronectin-rich extracellular matrix within 6 days (Connelly *et al.*, 2008a, text reference) and also observed that sGAG synthesis rates in the RGD-modified and unmodified gels were similar after two weeks in culture, although total sGAG accumulation still lagged in the RGD-modified gels (unpublished). These results suggest that the chondrogenic supplements are sufficient stimuli to eventually promote chondrogenesis in ligand-functionalized gels, although it is not yet clear whether the cells and matrix will eventually catch up or if the initial cell-scaffold interactions are sufficient to permanently alter the cell phenotype or behavior.

Reviewer I: Were any measures of viability taken in these different contexts (i.e., with FN and GFOGER ligands)? Does having any attachment improve overall cell health/viability as it appears to do with RGD?

Authors: We did not examine cell viability in this study, although we did examine the total DNA content of the gels. As expected, DNA content was significantly greater in chondrogenic medium than in basal medium for all ligand groups (all $p < 0.0001$). Among basal medium groups, RGD- and RGE-conjugated gels had higher DNA contents than unmodified, GFOGER- or FnIII7-10-conjugated gels (all $p < 0.0001$). Among chondrogenic groups, all conjugated gels had lower DNA contents than unmodified gels ($p \leq 0.018$), and the DNA contents of GFOGER- and FnIII7-10-conjugated gels were significantly lower than those of all other groups (all $p < 0.0001$). Consistent with the effects on matrix production and gene expression, interactions with the integrin ligands generally inhibit the cellular response to the chondrogenic supplements.

Reviewer I: It is not clear how many times this work was repeated. Is this the result of a single donor? If so, have other replicates been performed to validate the findings?

Authors: The results presented involve cells from a single bovine donor, and describe one experiment examining matrix synthesis and accumulation and a second, independent experiment examining gene expression, with good agreement between the two studies. Additionally, patterns of sGAG synthesis and accumulation were similar in two other related experiments, which consistently showed inhibition by all ligands. The effects of RGD-interaction are similar to our previous findings involving RGD-modified alginate and agarose (Connelly *et al.*, 2007;

Connelly *et al.*, 2008a, text references). Furthermore, we have recently observed reduced chondrogenic gene expression and matrix production by human BMSCs in (adhesive) fibrin gels relative to (non-adhesive) alginate gels (unpublished). We are thus fairly confident in our general findings. The issue of donor variability is an important one of substantial relevance to clinical implementation of stem cell-based therapies, but will be more appropriately explored for human donors.

Reviewer II: The main results the authors present demonstrate that these ligands are not suitable for 3D chondrogenesis in hydrogels. Their discussion of the likely reasons is sound and reasonable, but is it likely that anybody would wish to attempt this approach? There are some potentially interesting findings in relation to the osteogenesis specific genes. However, much further work would be warranted to elucidate the mechanisms of action. Please comment!

Authors: We believe that the results of this study are interesting and relevant for several reasons. First, assuming that the only relevant goal is the generation of an engineered articular cartilage, information on strategies that do not produce a favorable outcome are just as valuable as information on strategies producing a desired effect. Results indicating that a strategy does not “work,” particularly if they arise from well controlled, carefully executed studies, are quite important to the field as a whole. A focus purely on what “worked,” rather than on understanding how or why one approach works while another does not, was an endemic problem in the early development of tissue engineering as a discipline.

Second, we believe that these results are quite relevant. Adhesive scaffolds (both engineered and natural protein-based) are widely used in tissue engineering studies, including many studies on chondrogenesis. Our results are highly relevant to understanding cell behaviors in a wide range of scaffolds currently under investigation and may aid in the improvement of those scaffolds or understanding the tradeoffs involved in using such

scaffolds. The fact that ligands targeting different integrins all inhibit chondrogenesis suggests that this should be a general concern for chondrogenesis in any scaffold material providing integrin binding domains. Furthermore, identification of aspects of the cellular environment that inhibit chondrogenesis has bearing on understanding potential reasons for inadequate chondrogenesis in clinical treatments such as microfracture.

Third, we disagree with the notion that approaches that do not directly enhance the extent of hyaline-like chondrogenesis are inherently not useful or interesting, as there are situations in which inhibition of chondrogenesis may in fact be a desirable or necessary outcome. For example, the cells from fibrocartilaginous tissues such as the knee meniscus, temporomandibular joint disc, acetabular labrum, and annulus fibrosus are similar to, but not identical to, articular chondrocytes, and furthermore display notable regional heterogeneity. Most current tissue engineering approaches to such tissues are similar to those employed for articular cartilage, and do not involve approaches designed to specifically promote fibrocartilaginous differentiation. Understanding how to tune the chondrogenic differentiation of progenitors with instructive scaffolds may in fact be quite useful for engineering non-hyaline cartilages.

Regarding the effects on osteogenic genes, we agree that this is interesting (as noted in the discussion). However, exploration of the differential effects of different ligands on osteogenesis is a separate issue well beyond the scope of this study.

Additional Reference

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