TISSUE ENGINEERING: Part A
Volume 17, Numbers 7 and 8, 2011

DOI: 10.1089/ten.tea.2010.0531

Coculture of Human Mesenchymal Stem Cells and Articular Chondrocytes Reduces Hypertrophy and Enhances Functional Properties of Engineered Cartilage

Liming Bian, Ph.D.,[1] David Y. Zhai,[1] Robert L. Mauck, Ph.D.,[1,2] and Jason A. Burdick, Ph.D.[1]

Mesenchymal stem cells (MSCs) are being recognized as a viable cell source for cartilage repair; however, it still remains a challenge to recapitulate the functional properties of native articular cartilage using only MSCs. Additionally, MSCs may exhibit a hypertrophic phenotype under chondrogenic induction, resulting in calcification after ectopic transplantation. With this in mind, the objective of this study was to assess whether the addition of chondrocytes to MSC cultures influences the properties of tissue-engineered cartilage and MSC hypertrophy when cultured in hyaluronic acid hydrogels. Mixed cell populations (human MSCs and human chondrocytes at a ratio of 4:1) were encapsulated in the hydrogels and exhibited significantly higher Young's moduli, dynamic moduli, glycosaminoglycan levels, and collagen content than did constructs seeded with only MSCs or chondrocytes. Furthermore, the deposition of collagen X, a marker of MSC hypertrophy, was significantly lower in the coculture constructs than in the constructs seeded with MSCs alone. When MSCs and chondrocytes were cultured in distinct gels, but in the same wells, there was no improvement in biomechanical and biochemical properties of the engineered tissue, implying that a close proximity is essential. This approach can be used to improve the properties and prevent calcification of engineered cartilage formed from MSC-seeded hydrogels with the addition of lower fractions of chondrocytes, leading to improved clinical outcomes.

Introduction

Mesenchymal stem cells (MSCs) have emerged as a clinically relevant cell source for regenerative medicine, especially for cartilage repair. MSCs undergo chondrogenesis and deposit a cartilage specific matrix in a variety of natural and synthetic scaffold materials in the presence of the appropriate growth factors.[1,2] In contrast to chondrocytes, which undergo de-differentiation with expansion *in vitro*, MSCs are readily isolated from patients and expanded for use.[3,4] These features make MSCs an attractive cell source for delivery for cartilage repair.

As a natural polymer, hyaluronic acid (HA) hydrogels provide a stable 3D environment that is conducive to the chondrogenesis of MSCs in the presence of growth factors, leading to the formation of neocartilage *in vitro*.[5] We have previously shown that neocartilage production by MSCs can be optimized by using HA hydrogels with tunable degradation[5] and that specific interactions of MSCs with HA can lead to enhanced chondrogenesis, particularly when compared to alternative and inert hydrogels.[6] However, there is still room for improvement in the overall properties of engineered cartilage obtained by MSCs in HA hydrogels.

Specifically, it still remains a challenge to recapitulate the functional properties of native articular cartilage using only MSCs, particularly when compared to donor-matched articular chondrocytes, as shown in a bovine model.[7] In addition, MSCs exhibit a hypertrophic phenotype under chondrogenic induction, resulting in extensive calcification of the extracellular matrix (ECM) after ectopic transplantation.[8] These limitations will prevent the utility and widespread adoption of MSCs as a clinically viable cell source for cartilage repair. Thus, new approaches must be taken to improve cartilage properties and reduce MSC hypertrophy. Factors such as mechanical loading, controlling cell and matrix interactions, and engineered biomaterials have been investigated toward this end, yet only modest improvements have been seen.[9]

Recent studies indicate that paracrine factors released by articular chondrocytes are able to induce the chondrogenesis of MSCs[10–12] and inhibit the terminal differentiation of growth plate chondrocytes[13,14] as well as hypertrophy of MSCs in coculture.[11,15] However, there have been few studies examining the effects of human MSC/chondrocyte coculture on the development of the functional properties of tissue engineered cartilage as well as MSC hypertrophy using a 3D scaffold system such as these HA hydrogels. Thus, the hypothesis of

[1]Department of Bioengineering and [2]McKay Orthopedic Research Laboratory, Department of Orthopedic Surgery, University of Pennsylvania, Philadelphia, Pennsylvania.

this study was that MSC/chondrocyte cocultures in engineered HA hydrogels could enhance the functional properties of tissue-engineered cartilage and inhibit the hypertrophic propensity of MSCs during chondrogenesis compared to MSC cultures alone. Importantly, this work is being performed using human MSCs and a potentially translatable and engineered hydrogel system, in an attempt to provide specific insight into clinical applicability. Also, this is a potentially clinically feasible technique, since low numbers of chondrocytes could be harvested and used without significant damage to healthy tissue and avoid the *in vitro* expansion and the corresponding issues related to de-differentiation.[16,17]

Materials and Methods

Macromer synthesis

Methacrylated HA was synthesized as previously reported.[18] Briefly, methacrylic anhydride (94%, molecular weight [MW]: 154.17, Sigma) was added to a solution of 1 wt% HA (sodium hyaluronate powder, research grade, MW ~74 kDa, Lifecore) in deionized water, adjusted to a pH of 8 with 5 N NaOH, and reacted on ice for 24 h. The macromer solution was purified via dialysis (MW cutoff 6–8 k) against deionized water for a minimum of 48 h with repeated changes of water. The final product was obtained by lyophilization and stored at −20°C in powder form before use. The final macromer products were confirmed by ^{1}H NMR to have a methacrylation rate of 27%. Lyophilized macromers were dissolved in phosphate buffered saline containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (I2959, Ciba) for polymerization.

Sample preparation and tissue culture

Human articular chondrocytes were obtained via an 11 h collagenase digestion (0.05 w/v%, Sigma) of discarded human cartilage from total knee replacements (three male patients aged from 55 to 77). Human MSCs (Lonza) were expanded to passage 3 in growth media consisting of α-minimum essential medium with 16.7% fetal bovine serum and 1% pen/strep. MSCs and/or chondrocytes were photoencapsulated in 1.5% methacrylated HA hydrogel disks (Ø5 mm, 2.5 mm thickness) and cultured in chondrogenic media (Dulbecco's modified Eagle's medium, 1% ITS+Premix, 50 μg/mL L-proline, 0.1 μM dexamethasone, 0.9 mM sodium pyruvate, and antibiotics) supplemented with ascorbate (50 μg/mL)[19] and transforming growth factor-β3 (TGF-β3; 10 ng/mL; R&D Systems), which was changed three times a week.[19] Cell viability was assessed using the LIVE/DEAD Assay Kit (Molecular Probes) where live cells are stained green with calcein-AM and dead cells stained red with ethidium homodimer.

Mechanical testing

At various time points (days 20, 42, and 80), samples were removed from the culture and the spatially averaged mechanical properties of construct disks were evaluated at selected time points using a custom table-top testing device as described previously.[20] Briefly, samples were first equilibrated in creep to a tare load of 2 g by a impermeable loading platen in a loading chamber filled with phosphate buffered saline, and from this offset, stress relaxation tests were performed with a single compression ramp at a speed of 10%/min until reaching 10% strain. The peak stress was obtained at the moment of reaching 10% strain. The equilibrium Young's modulus (E_Y) was determined by the equilibrium load obtained after 1000 s of relaxation under unconfined compression at 10% strain, followed by tests for dynamic moduli at 0.1, 0.5, and 1 Hz and 1% strain amplitude. The Young's moduli of the tested samples became undetectable if the equilibrium load at the end of stress relaxation was lower than the initial tare load and was plotted as zero.

Gene expression analysis

For short-term gene expression analysis (3 and 14 days of culture), samples were homogenized in Trizol Reagent (Invitrogen) with a tissue grinder, RNA was extracted according to the manufacturer's instructions, and the RNA concentration was determined using an ND-1000 spectrophotometer (Nanodrop Technologies). One microgram of RNA from each sample was reverse transcribed into cDNA using reverse transcriptase (Superscript II, Invitrogen) and oligoDT (Invitrogen). Polymerase chain reaction (PCR) was performed on an Applied Biosystems 7300 Real-Time PCR system using TaqMan primers and probes specific for *GAPDH* (housekeeping gene) and other genes of interest. Sequences of the primers and probes used are listed in Table 1. The relative gene expression was calculated using the $\Delta\Delta C_T$ method, where fold difference was calculated using the expression $2^{\Delta\Delta Ct}$. Each sample was internally normalized to GAPDH, and each group was normalized to the expression levels of MSCs at the time of encapsulation (i.e., after expansion and before differentiation). Relative expression levels >1 represent upregulation with culture, while relative expression levels <1 represent downregulation of that gene compared to that of initially encapsulated MSCs.

Biochemical analysis

One-half of each construct was weighed wet, lyophilized, reweighed dry, and digested in 0.5 mg/mL Proteinase-K (Fisher Scientific) at 56°C for 16 h. The PicoGreen assay (Invitrogen, Molecular Probes) was used to quantify the DNA content of the constructs with Lambda phage DNA (0–1 mg/mL) as a

Table 1. Sequences of Primers and Probes Used for Gene Expression Analysis

Gene	*Forward primer*	*Reverse primer*	*Probe*
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
COL I	AGGACAAGAGGCATGTCTGGTT	GGACATCAGGCGCAGGAA	TTCCAGTTCGAGTATGGC
COL II	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT	CTGCACGAAACATAC
ALP	CGGAACTCCTGACCCTTGAC	TGTTCAGCTCGTACTGCATGTC	TCGAAGAGACCCAATAGGT
Aggrecan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA

Sequences related to gene *MMP13* and type X collagen are proprietary to Applied Biosystems Inc. and not disclosed.

Group	Chondrocytes (mil/ml)	MSCs (mil/ml)
Mixed +	4	16
MSC	0	20
2 gels *	4	20
Chon4	4	0
Chon20	20	0

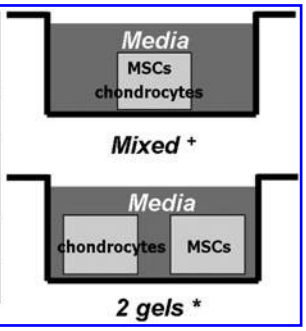

FIG. 1. Cell types (human MSCs and chondrocytes) and seeding densities of experimental groups. MSCs, mesenchymal stem cells.

standard.[21] For each sample, both the weight of the entire gel and the half gel used for the DNA assay was measured. The total amount of DNA per sample was calculated by scaling up the amount of DNA detected in the half gel by a weight ratio (total weight/half weight). The glycosaminoglycan (GAG) content was measured using the dimethylmethylene blue (DMMB, Sigma Chemicals) dye-binding assay with shark chondroitin sulfate (0–50 mg/mL) as a standard.[22] The overall collagen content was assessed by measuring the orthohydroxyproline content via dimethylaminobenzaldehyde and chloramine T assay. Collagen content was calculated by assuming a 1:7.5 orthohydroxyproline-to-collagen mass ratio.[23] The collagen and GAG contents were normalized to the disk wet weight.

Histological analysis

The other halves of the constructs were fixed in 4% formalin for 24 h, embedded in paraffin, and processed using standard histological procedures. The histological sections (8 μm thick) were stained for targets of interest using the Vectastain ABC kit and the DAB Substrate kit for peroxidase (Vector Labs). Briefly, sections were predigested in 0.5 mg/mL hyaluronidase for 30 min at 37°C and incubated in 0.5 N acetic acid for 4 h at 4°C to swell the samples prior to overnight incubation with primary antibodies at dilutions of 1:100, 1:200, and 1:3 for chondroitin sulfate (mouse monoclonal anti-chondroitin sulfate, Sigma), and type I (mouse monoclonal anti-collagen type 1, Sigma) and type II collagen antibodies (mouse monoclonal anti-collagen type II, Developmental Studies Hybridoma Bank), respectively. Nonimmune controls underwent the same procedure without primary antibody incubation.

Statistical analysis

Statistica (Statsoft) was used to perform statistical analyses using two-way analysis of variance and the Tukey HSD *post hoc* test of the means ($n = 4$ samples per group) with culture duration and experimental groups as independent factors.

Results

Human cells were photoencapsulated in HA hydrogels at a concentration of 20 million cells/mL, including MSCs alone, chondrocytes alone (at both 4 and 20 million cells/mL), or mixtures of both MSCs and chondrocytes in the same gel (Fig. 1). An additional control included culturing gels of either MSCs or chondrocytes alone in the same well (2gels) (Fig. 1). Live/dead staining of cells indicated that MSCs remained viable in all conditions (Fig. 2). In contrast, the viability of articular chondrocytes seeded separately in HA hydrogels without MSCs (Chon4 and Chon20, chondrocyte constructs of the 2gels group [not shown]) decreased over time, and the majority (>90%) of the chondrocytes were dead by day 35 (Fig. 2).

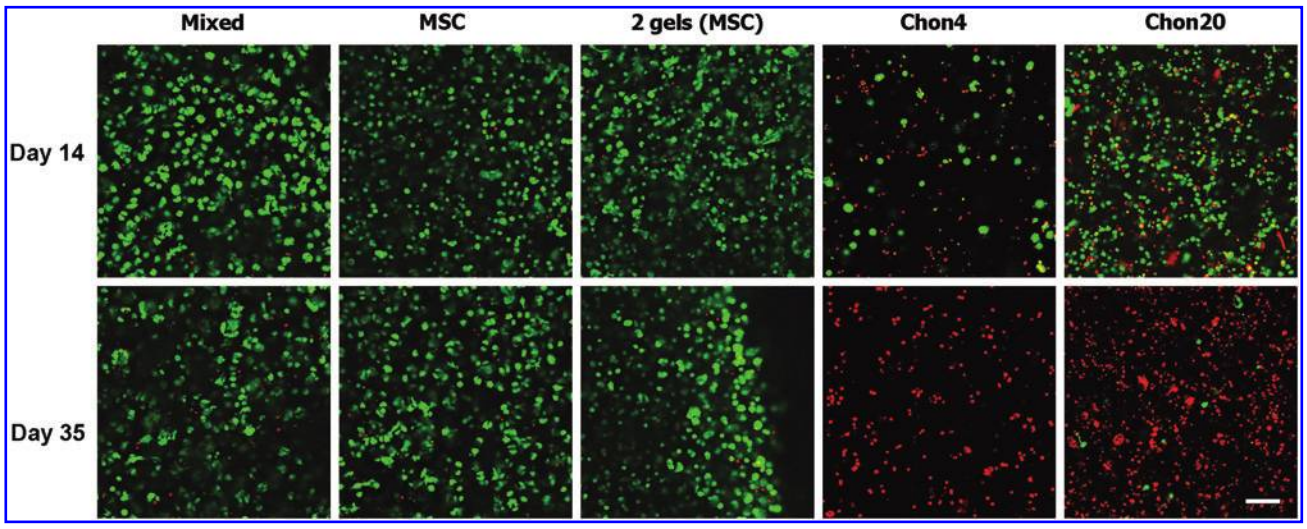

FIG. 2. Viability staining of cells in HA hydrogels on days 14 and 35. Green: live cells; red: dead cells; scale bar = 100 μm. HA, hyaluronic acid. Color images available online at www.liebertonline.com/tea.

Unconfined mechanical testing indicated that Mixed constructs seeded with a combination of MSCs and chondrocytes exhibited significant increases in peak stress, dynamic moduli, and Young's moduli over time, whereas these properties in all other groups remained relatively unchanged for up to 80 days of culture (Fig. 3A–C). Peak stress, dynamic moduli (1 Hz), and Young's moduli of the Mixed group were significantly higher than those of all other groups by day 42 and reached values of 28.5 ± 1.3 kPa, 339.8 ± 29.3 kPa, and 23.4 ± 1.11 kPa on day 80, respectively (Fig. 3A–C). On day 80, the Young's moduli of the MSC and 2gels groups became undetectable due to the equilibrium load at the end of stress relaxation being lower than the initial tare load.

The Mixed group also developed significantly higher GAG content by day 42 and total collagen content by day 80 than all other groups (Fig. 3D, E). GAG and total collagen content of the Mixed group reached $1.0\% \pm 0.1\%$ and $1.9\% \pm 0.1\%$ (mean $\pm$ standard deviation) of the sample wet weight, respectively (Fig. 3D, E). The GAG content of the Chon4 and Chon20 was very low and the total collagen content of these two groups was not detectable (Fig. 3D, E). The DNA content of the Mixed group increased slightly and was higher than the MSC group on day 80, whereas the DNA content of the MSC and 2gels groups remained constant over time (Fig. 3F).

Compared to undifferentiated monolayer cells, both the Mixed and MSC groups exhibited significant upregulation of type II collagen and aggrecan gene expression, which are major markers of chondrogenesis, whereas the expression of type I collagen remained unchanged from that of the pre-encapsulation MSCs (Fig. 4). Compared to the constructs seeded with MSCs alone (MSC), the Mixed group had elevated aggrecan expression on both days 14 and 42, but collagen X expression was reduced on day 42 (Fig. 4). The MMP13 expression of the Mixed group was also higher on day 14 but decreased to a level similar to the MSC group on day 42 (Fig. 4). No significant difference in the expression of alkaline phosphatase was found between these two groups (Fig. 4). Please note that the cells were normalized to MSCs alone at the time of encapsulation for both groups.

Immunohistochemical staining for type II collagen and chondroitin sulfate showed intense staining in the Mixed, MSC, and 2gels constructs on day 42, whereas there was

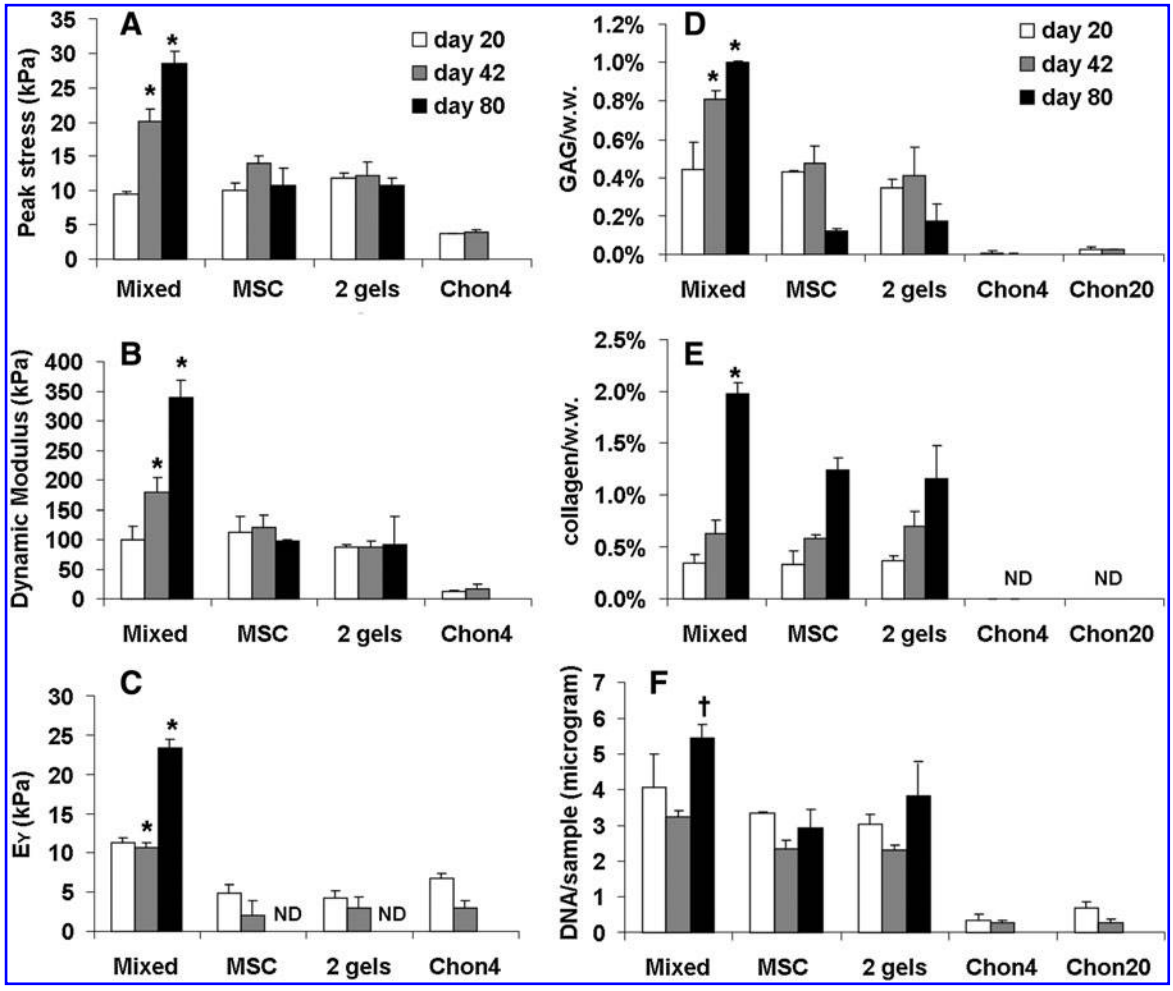

FIG. 3. Peak stress (**A**), dynamic modulus (1 Hz) (**B**), Young's modulus (**C**), GAG (**D**), total collagen (**E**), and DNA (**F**) content of all experimental groups (data presented as mean $\pm$ standard deviation). ND: not detectable, $^{*}p < 0.05$ versus all other groups at the same culture time; $^{\dagger}p < 0.05$ versus MSC at the same culture time ($n = 4$). The Chon20 group was not tested for mechanical properties due to poor early cell viability. GAG, glycosaminoglycan.

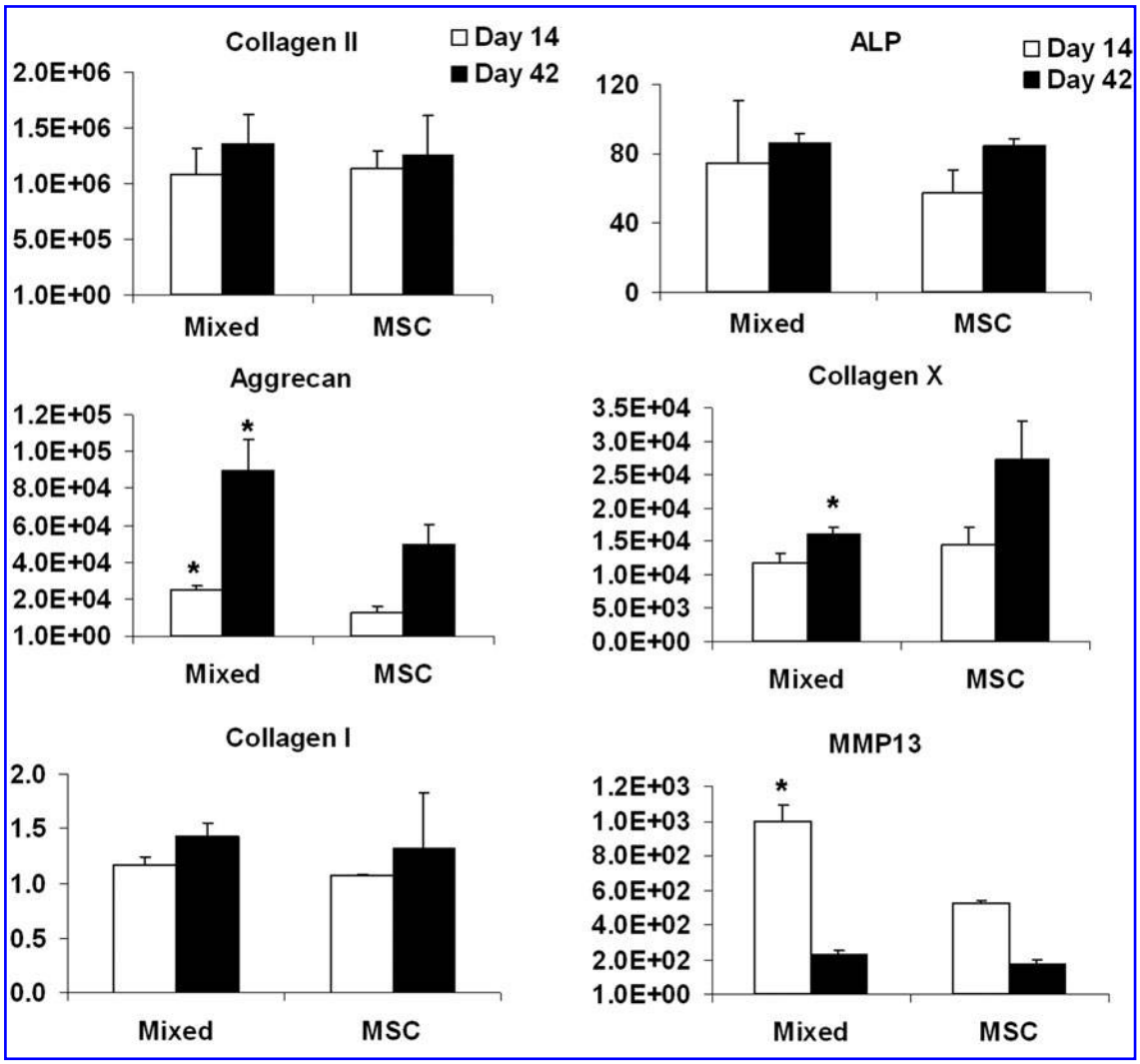

FIG. 4. Gene expression (in fold change) of selected chondrogenic and hypertrophic markers with time in culture (data presented as mean ± standard deviation). Only the Mixed and MSC groups were compared due to limited viability in other experimental groups. *$p < 0.05$ versus MSC group ($n = 3$).

little staining for type I collagen (Fig. 5). There was little matrix staining present in the groups (Chon4 and Chon20) seeded with chondrocytes alone (Chon20 not shown) (Fig. 5). Immunohistochemical staining for collagen 10A1 showed significantly lower collagen type X content in Mixed constructs than in MSCs alone (Fig. 6).

In a follow-up study, human MSCs and human articular chondrocytes were encapsulated in mixtures with varying ratios (19:1, 16:4, 10:10, and 20:0 million MSCs:chondrocytes per mL). The 10:10 and the MSC alone group (20:0) developed significantly lower Young's modulus and GAG content than the 19:1 and 16:4 group after 42 days of culture (Fig. 7). Although the DNA content was similar among all the groups, the 10:10 also had less total collagen content compared to all other groups (Fig. 7).

Discussion

HA scaffolds are finding application as cell carriers in the repair of cartilage defects. HA is a linear polysaccharide involved in cellular processes like cell proliferation, morphogenesis, inflammation, and wound repair and interacts with cells, including MSCs via cell surface receptors for HA (CD44, CD54, and CD168).[24–27] Recent studies demonstrated successful chondrogenesis of MSCs photoencapsulated in HA hydrogels formed through the radical polymerization of HA modified with methacrylate groups.[5,6,28] Despite the success, improvements in the functional properties of these constructs are still needed toward their clinical utility. In this study we showed that coculturing human MSCs with human articular chondrocytes in HA hydrogels enhances the mechanical properties and cartilage-specific ECM content of tissue-engineered cartilage. Furthermore, cocultures decreased the expression of collagen type X by MSCs, which is an important marker of MSC hypertrophy.

Several previous studies have shown that paracrine factors released by articular chondrocytes enhance the chondrogenesis of MSCs.[11,12] Grassel *et al.* showed that coculture of murine MSCs with murine cartilage explants resulted in the elevated biosynthesis of type II collagen compared to

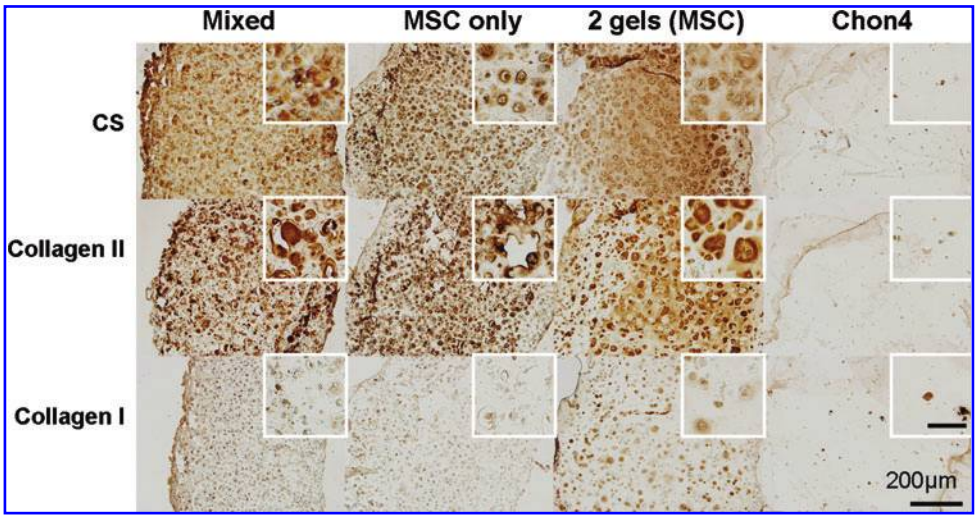

FIG. 5. Immunohistochemical staining for chondroitin sulfate (CS), type II collagen, and type I collagen on day 42; bar in inset = 50 μm. Color images available online at www.liebertonline.com/tea.

monoculture of MSCs.[11] Another study showed that differentiation of equine MSCs was enhanced by coculturing in association with mature articular chondrocytes, improving the expression of cartilage-specific genes such as *col2a1*, aggrecan, and *sox9*, and producing a more homogeneous ECM within the newly formed cartilage.[12] We observed significantly higher levels of aggrecan expression on day 42 and GAG content starting from day 42 in the engineered cartilage constructs seeded with a mixed population of human MSCs and articular chondrocytes (Mixed group) compared to constructs seeded with MSCs alone (MSC group). Although the expression of type II collagen was similar between these two groups, the total collagen content of the Mixed group was significantly higher than that of the MSC group on day 80. Based on our PCR results and immunohistochemical staining, which showed minimal expression and staining of type I collagen and significant upregulation of type II collagen expression, we postulate that the majority of the collagen content detected by the hydroxyproline assay performed was type II collagen. In addition, it has been shown that the mechanical properties (Young's modulus, dynamic modulus, etc.) of cartilage are generally positively correlated to the GAG and type II collagen content.[29,30] Therefore, the enhanced Young's modulus and dynamic modulus of the Mixed constructs can be attributed to the elevated GAG and type II collagen content, respectively.

MSCs have also been shown to exhibit a hypertrophic phenotype under chondrogenic induction by TGFs, resulting in extensive calcification of the ECM after ectopic transplantation.[8] Meanwhile, studies demonstrated that articular chondrocytes secrete parathyroid hormone-related protein (PTHrP),[15] which can inhibit hypertrophy of chondrocytes or MSCs during chondrogenesis.[31–34] In this study, the expression and biosynthesis of type X collagen, a major marker of hypertrophy, was significantly suppressed under the coculture condition (Mixed group) compared to the monoculture of MSCs (MSC group) on day 42. The expression of MMP13, another marker of MSC hypertrophy, in the Mixed group was higher on day 14 but decreased to a similar level as the MSC group on day 42. This suggested that the reduced

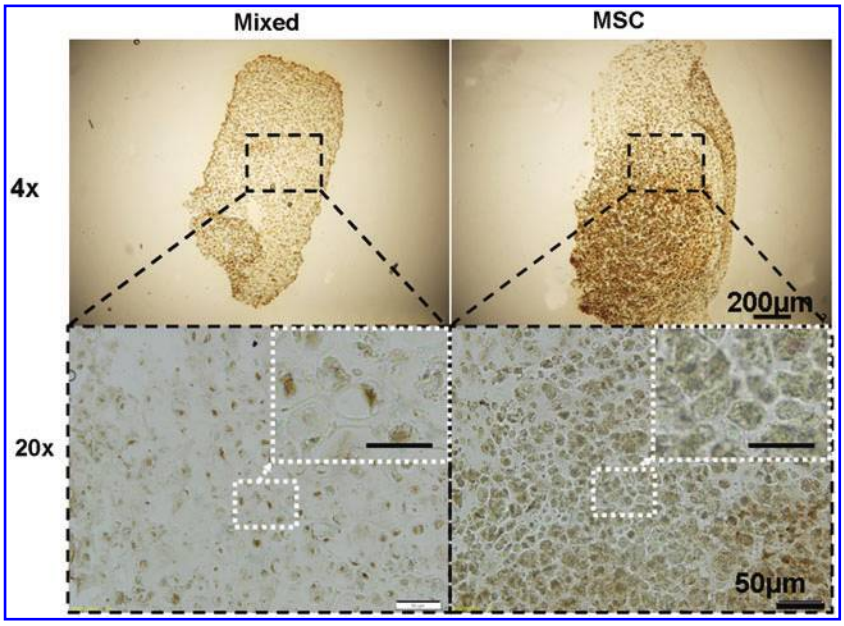

FIG. 6. Immunohistochemical staining for collagen 10A1 on day 42; bar in inset = 25 μm. Color images available online at www.liebertonline.com/tea.

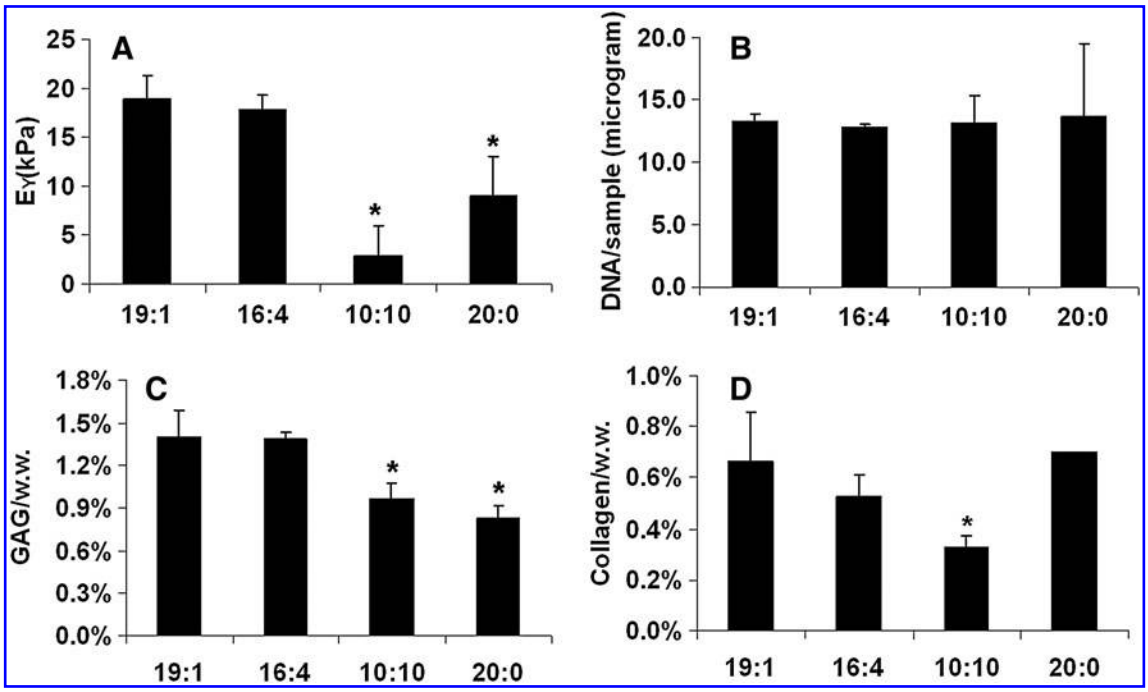

FIG. 7. Young's modulus (**A**), DNA (**B**), GAG (**C**), and total collagen (**D**) content of all groups on day 42. The entities 19:1, 16:4, 10:10, and 20:0 represent ratios of MSCs and chondrocytes seeded in mixture in terms of million cells/mL (i.e., 19:1 indicates a seeding density of 19 million MSCs and 1 million chondrocytes per mL of hydrogel and cell mixture, data presented as mean ± standard deviation). *$p < 0.05$ versus 19:1 and 16:4 group ($n = 4$).

deposition of type X collagen, a substrate of MMP13, in the Mixed group was unlikely due to enzymatic degradation by MMP13 but more likely due to a regulatory effect on its biosynthesis.

In this study, human articular chondrocytes in monoculture (Chon4 and Chon20 group) exhibited decreasing viability in HA hydrogels over time. Similarly, the chondrocytes in the 2gels group also showed a similar trend of decreasing viability, resulting in no significant improvements in the mechanical and biochemical attributes that were observed in the Mixed group. While long-term viability of the chondrocytes seeded in mixture with MSCs is difficult to analyze, short-term viability studies using cell trackers showed that chondrocytes seeded in the mixture with MSCs (Mixed group) remained mostly viable up to day 7 (data not shown). Based on the significantly higher GAG and collagen content of the Mixed group than that of the other groups, we speculate that chondrocytes seeded in mixture with MSCs remained viable after day 7 and enhanced chondrogenesis of the surrounding MSCs. To support this hypothesis, chondrocytes and MSCs were seeded in mixture with various ratios (MSCs:chondrocytes at 1:19, 4:16, and 10:10 million/mL). The constructs seeded with more chondrocytes (10:10 group) developed lower mechanical stiffness, GAG, and collagen content than those seeded with fewer chondrocytes (1:19 and 4:16 groups). This indicates that the increased biosynthesis of GAG and collagen in the Mixed group was likely due to enhanced chondrogenesis of MSCs rather than enhanced biosynthesis by the chondrocytes, which is in contrast to the findings of previous studies.[35,36] These results may be specific to the engineered hydrogel system that is used, as matrix interactions may control this response, particularly when changes in cell viability are observed.

However, future work is still needed to elucidate the specific mechanism of the interactions between MSCs and articular chondrocytes and its impact on MSC hypertrophy *in vivo*. For instance, the reason that seeding MSCs and chondrocytes in close proximity (as in the Mixed group) might have improved chondrocyte viability in the HA hydrogel still needs to be investigated. Furthermore, it was shown that HA hydrogel with a low macromer density as used in this study is fairly permeable to molecule diffusion, on the size scale of a range of growth factors.[28] Therefore, approaches to increase chondrocyte viability in the 2gels group (e.g., alternative material) may help determine whether the paracrine factors released by the chondrocytes located a greater distance will also promote the chondrogenesis of MSCs to a similar extent to that observed in the Mixed group where MSCs and chondrocytes were seeded in close proximity. In addition, candidate molecules such as tissue inhibitor of metalloproteinase1, tissue inhibitor of metalloproteinase2, MMP13, TGF-β, fibroblast growth factor-2, and PTHrP, were identified as prominent molecules secreted by the chondrocytes during coculture.[15,32] The exact molecules released by the chondrocytes that enhanced MSC chondrogenesis in this 3D system need to be identified. Finally, the impact of coculture on MSC hypertrophy *in vivo* needs to be investigated in animal studies. A recent study showed that pretreatment of MSCs using hypertrophy suppressive molecules such as PTHrP or fibroblast growth factor2 did not prevent substantial mineralization of MSC pellets ectopically implanted in nude mice.[32] This indicates that a continuous suppression of MSC hypertrophy after *in vivo* implantation is necessary. Future animal studies are needed to evaluate the efficacy of MSC/chondrocyte coculture in containing MSC hypertrophy at ectopic sites *in vivo*.

The findings from this study are potentially of clinical significance. When treating local cartilage defects, such as focal lesions, which are usually of small dimension but of irregular geometry, the advantage of this photocrosslinkable HA scaffold system is that the defects can be repaired with the HA hydrogel seeded with autologous MSCs in a precise manner. This allows close contact between the implanted MSCs and the articular chondrocytes in the surrounding host tissue, effectively forming a coculture system *in vivo*. As suggested by findings from this study, the interactions between these two populations of cells could result in enhanced chondrogenesis and reduced hypertrophy of the implanted MSCs and therefore lead to better clinical outcomes. Furthermore, when treating relatively large cartilage defects, it may be beneficial to deliver both MSCs and chondrocytes encapsulated in a mixture since host cartilage tissue might be too distant away for significant paracrine interactions to occur.

In conclusion, our findings demonstrate that coculturing human MSCs with human articular chondrocytes in HA hydrogels enhances the mechanical properties and cartilage-specific ECM content of tissue-engineered cartilage. Furthermore, coculture decreased the expression of collagen type X by MSCs, which is an important marker of MSC hypertrophy. These results indicate that the close proximity of chondrocytes in a defect filled with an MSC-containing hydrogel or the delivery of both MSCs and chondrocytes in one gel may improve neocartilage production and reduce hypertrophy compared to MSC alone hydrogels (e.g., *in vitro* culture) and lead to improved clinical outcomes. However, future work is needed to elucidate the specific mechanism of the interactions between MSCs and articular chondrocytes.

Acknowledgments

This work was supported by National Institutes of Health grants R01EB008722 and R01AR056624. Dr. Roshan Shah and Dr. Mara Schenker are gratefully acknowledged for helping obtain discarded human cartilage from total knee replacements.

Disclosure Statement

No competing financial interests exist.

References

1. Williams, C.G., Kim, T.K., Taboas, A., Malik, A., Manson, P., and Elisseeff, J. *In vitro* chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue Eng **9,** 679, 2003.
2. 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.
3. Friedenstein, A.J., Piatetzky, S., II, and Petrakova K.V. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol **16,** 381, 1966.
4. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., *et al.* Multilineage potential of adult human mesenchymal stem cells. Science **284,** 143, 1999.
5. Chung, C., Beecham, M., Mauck, R.L., and Burdick, J.A. The influence of degradation characteristics of hyaluronic acid hydrogels on *in vitro* neocartilage formation by mesenchymal stem cells. Biomaterials **30,** 4287, 2009.
6. Chung, C., and Burdick, J.A. Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis. Tissue Eng Part A **15,** 243, 2009.
7. Mauck, R.L., Yuan, X., and Tuan, R.S. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage **14,** 179, 2006.
8. Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., *et al.* Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum **54,** 3254, 2006.
9. Chung, C., and Burdick, J.A. Engineering cartilage tissue. Adv Drug Deliv Rev **60,** 243, 2008.
10. Vats, A., Bielby, R.C., Tolley, N., Dickinson, S.C., Boccaccini, A.R., Hollander, A.P., *et al.* Chondrogenic differentiation of human embryonic stem cells: the effect of the micro-environment. Tissue Eng **12,** 1687, 2006.
11. Ahmed, N., Dreier, R., Gopferich, A., Grifka, J., and Grassel, S. Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells. Cell Physiol Biochem **20,** 665, 2007.
12. Lettry, V., Hosoya, K., Takagi, S., and Okumura, M. Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells. Jpn J Vet Res **58,** 5, 2010.
13. Jikko, A., Kato, Y., Hiranuma, H., and Fuchihata, H. Inhibition of chondrocyte terminal differentiation and matrix calcification by soluble factors released by articular chondrocytes. Calcif Tissue Int **65,** 276, 1999.
14. D'Angelo, M., and Pacifici, M. Articular chondrocytes produce factors that inhibit maturation of sternal chondrocytes in serum-free agarose cultures: a TGF-beta independent process. J Bone Miner Res **12,** 1368, 1997.
15. Fischer, J., Dickhut, A., Richter, W., and Rickert, M. Articular chondrocytes secrete PTHrP and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. Arthritis Rheum **62,** 2696, 2010.
16. Oegema, T.R., Jr., and Thompson, R.C., Jr. Characterization of a hyaluronic acid-dermatan sulfate proteoglycan complex from dedifferentiated human chondrocyte cultures. J Biol Chem **256,** 1015, 1981.
17. West, C.M., Lanza, R., Rosenbloom, J., Lowe, M., Holtzer, H., and Avdalovic, N. Fibronectin alters the phenotypic properties of cultured chick embryo chondroblasts. Cell **17,** 491, 1979.
18. Smeds, K.A., Pfister-Serres, A., Miki, D., Dastgheib, K., Inoue, M., Hatchell, D.L., *et al.* Photocrosslinkable polysaccharides for *in situ* hydrogel formation. J Biomed Mater Res **54,** 115, 2001.
19. Byers, B.A., Mauck, R.L., Chiang, I.E., and Tuan, R.S. Transient exposure to transforming growth factor beta 3 under serum-free conditions enhances the biomechanical and biochemical maturation of tissue-engineered cartilage. Tissue Eng Part A **14,** 1821, 2008.
20. Mauck, R.L., Soltz, M.A., Wang, C.C.-B., Wong, D.D., Chao, P.-H.G., Valhmu, W.B., *et al.* Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng **122,** 252, 2000.
21. McGowan, K.B., Kurtis, M.S., Lottman, L.M., Watson, D., and Sah, R.L. Biochemical quantification of DNA in human

articular and septal cartilage using PicoGreen and Hoechst 33258. Osteoarthritis Cartilage **10,** 580, 2002.
22. Farndale, R.W., Buttle, D.J., and Barrett, A.J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta **883,** 173, 1986.
23. Hollander, A.P., Heathfield, T.F., Webber, C., Iwata, Y., Bourne, R., Rorabeck, C., *et al.* Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest **93,** 1722, 1994.
24. Chen, W.Y., and Abatangelo, G. Functions of hyaluronan in wound repair. Wound Repair Regen **7,** 79, 1999.
25. Zhu, H., Mitsuhashi, N., Klein, A., Barsky, L.W., Weinberg, K., Barr, M.L., *et al.* The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. Stem Cells **24,** 928, 2006.
26. Lisignoli, G., Cristino, S., Piacentini, A., Cavallo, C., Caplan, A.I., and Facchini, A. Hyaluronan-based polymer scaffold modulates the expression of inflammatory and degradative factors in mesenchymal stem cells: Involvement of Cd44 and Cd54. J Cell Physiol **207,** 364, 2006.
27. Lisignoli, G., Grassi, F., Zini, N., Toneguzzi, S., Piacentini, A., Guidolin, D., *et al.* Anti-Fas-induced apoptosis in chondrocytes reduced by hyaluronan: evidence for CD44 and CD54 (intercellular adhesion molecule 1) invovement. Arthritis Rheum **44,** 1800, 2001.
28. Erickson, I.E., Huang, A.H., Sengupta, S., Kestle, S., Burdick, J.A., and Mauck, R.L. Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels. Osteoarthritis Cartilage **17,** 1639, 2009.
29. Mauck, R.L., Seyhan, S.L., Ateshian, G.A., and Hung, C.T. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann Biomed Eng **30,** 1046, 2002.
30. Yan, D., Zhou, G., Zhou, X., Liu, W., Zhang, W.J., Luo, X., *et al.* The impact of low levels of collagen IX and pyridinoline on the mechanical properties of *in vitro* engineered cartilage. Biomaterials **30,** 814, 2009.
31. Kim, Y.J., Kim, H.J., and Im, G.I. PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs. Biochem Biophys Res Commun **373,** 104, 2008.
32. Weiss, S., Hennig, T., Bock, R., Steck, E., and Richter, W. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. J Cell Physiol **223,** 84, 2010.
33. Zhang, M., Xie, R., Hou, W., Wang, B., Shen, R., Wang, X., *et al.* PTHrP prevents chondrocyte premature hypertrophy by inducing cyclin-D1-dependent Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation. J Cell Sci **122,** 1382, 2009.
34. Guo, J., Chung, U.I., Yang, D., Karsenty, G., Bringhurst, F.R., and Kronenberg, H.M. PTH/PTHrP receptor delays chondrocyte hypertrophy via both Runx2-dependent and -independent pathways. Dev Biol **292,** 116, 2006.
35. Sharma, B., Hui, A., Mountziaris, P.M., Khan, M., and Elisseeff, J.H. Mesenchymal Stem Cells Stimulate Chondrocyte Function in a Bilayered Osteochondral Tissue Engineering Model. Presented at the Trans ORS, Chicago, IL, 2006.
36. Giovannini, S., Diaz-Romero, J., Aigner, T., Heini, P., Mainil-Varlet, P., and Nesic, D. Micromass co-culture of human articular chondrocytes and human bone marrow mesenchymal stem cells to investigate stable neocartilage tissue formation *in vitro*. Eur Cell Mater **20,** 245, 2010.

Address correspondence to:
Jason A. Burdick, Ph.D.
Department of Bioengineering
University of Pennsylvania
240 Skirkanich Hall, 210 S. 33rd St.
Philadelphia, PA 19104

E-mail: burdick2@seas.upenn.edu

Received: September 06, 2010
Accepted: December 08, 2010
Online Publication Date: January 7, 2011