Comparative Potential of Juvenile and Adult Human Articular Chondrocytes for Cartilage Tissue Formation in 3D Biomimetic Hydrogels

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Abstract

Regeneration of human articular cartilage is inherently limited and extensive efforts have focused on engineering the cartilage tissue. Various cellular sources have been studied for cartilage tissue engineering including adult chondrocytes, as well as embryonic or adult stem cells. Juvenile chondrocytes (from donors below 13 years of age) have recently been reported to be a promising cell source for cartilage regeneration. Previous studies have compared the potential of adult and juvenile chondrocytes or adult and osteoarthritic (OA) chondrocytes. To comprehensively characterize the comparative potential of young, old and diseased chondrocytes, here we examined cartilage formation by juvenile, adult and OA chondrocytes in 3D biomimetic hydrogels composed of poly(ethylene glycol) and chondroitin sulfate. All three human articular chondrocytes were encapsulated in the 3D biomimetic hydrogels and cultured for 3 or 6 weeks to allow maturation and extracellular matrix formation. Outcomes were analyzed using quantitative gene expression, immunofluorescence staining, biochemical assays, and mechanical testing. After 3 and 6 weeks, juvenile chondrocytes showed a greater upregulation of chondrogenic gene expression than adult chondrocytes, while OA chondrocytes showed a downregulation. Aggrecan and type II collagen deposition and GAG accumulation were high for juvenile and adult chondrocytes but not for OA chondrocytes. Similar trend was observed in the compressive moduli of the cartilage constructs generated by the three different chondrocytes. In conclusion, the juvenile, adult and OA chondrocytes showed differential responses in the 3D biomimetic hydrogels. The 3D culture model described here may also provide a useful tool to further study the molecular differences among chondrocytes from different stages, which can
help elucidate the mechanisms for age-related decline in the intrinsic capacity for cartilage repair.
Introduction

Despite years of effort, engineering articular cartilage remains an unmet challenge. With a poor inherent capacity for repair, injuries to articular cartilage even in young healthy adults require medical intervention and can lead to an early onset of osteoarthritis. Current cell-based approaches towards cartilage regeneration have had limited success. Although autologous chondrocyte implantation (ACI) is approved for clinical treatment of focal cartilage defects, the challenges with utilizing the adult chondrocytes are associated donor site morbidity and a paucity of cells. In addition, monolayer expansion of autologous chondrocytes can lead to dedifferentiation, resulting in the formation of functionally inferior fibrocartilage rather than articular cartilage. Various sources of stem cells have been explored for cartilage repair including adult stem cells such as bone marrow or adipose-derived mesenchymal stem cells (MSCs), as well as pluripotent stem cells like embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs).

Cartilage repair has long been observed to be superior in children as compared to healthy adults in the clinic. Recent reports have suggested juvenile chondrocytes, isolated from young adults less than 13 years old, as a promising cell source for cartilage regeneration. In contrast to adult chondrocytes, juvenile chondrocytes resulted in neo-cartilage formation without chondrocyte hypertrophy in the absence of scaffolds. In addition, mixed co-culture of mesenchymal stem cells with juvenile chondrocytes in pellet or 3D biomimetic hydrogels resulted in significantly enhanced proteoglycan formation. Moreover, juvenile chondrocytes have been shown to be immune-privileged, making it possible to utilize them as an allogeneic source for cartilage repair.
Previous studies have compared adult and neonatal human chondrocytes, or adult and OA human chondrocytes using 2D or 3D culture platforms. However, how chondrocytes from different stages (juvenile, adult and diseased) compare in their potential for forming cartilage remains poorly understood. The aim of this study was to compare the potential of human adult and juvenile articular chondrocytes for cartilage formation and contrast it with osteoarthritic chondrocytes. To better mimic the physiological conditions, we chose a 3D biomimetic hydrogel culture given its ability to support chondrogenic phenotype for prolonged periods of culture since adult chondrocytes have a tendency to dedifferentiate in 2D culture. In addition, the biomimetic hydrogels can be used as a matrix to guide cartilage formation in situ and may provide useful insights for its potential use in vivo.

All three human articular chondrocytes were encapsulated in 3D biomimetic hydrogels and cultured over a period of 3 and 6 weeks to allow maturation and extracellular matrix formation. Outcomes were analyzed using quantitative gene expression, immunofluorescence staining, biochemical assays, and mechanical testing.

Methods

Chondrocyte isolation and culture

Normal adult (34 years, Male) and juvenile/neonatal human articular chondrocytes (6 months, Male) were purchased commercially from Lonza (Clonetics™, Lonza Walkersville Inc., Walkersville, USA) and cultured in chondrocyte growth medium (Clonetics™ CGM™, Lonza Walkersville Inc., Walkersville, USA). Articular chondrocytes were harvested from OA cartilage tissues obtained during total knee arthroplasty (74 years, Male), under protocols approved by Human subjects Institutional...
Review Board of Stanford University. Macroscopically intact cartilage was dissected and the chondrocytes were dissociated from the matrix as described previously. Chondrocytes were cultured in monolayer using Dulbecco's modified Eagle's medium (Hyclone, Thermo Scientific Inc.) supplemented with 25 μg/mL ascorbate, 2 mM L-glutamine, antimicrobials (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone), and 10% FBS (Gibco, Invitrogen, Carlsbad, CA) for 24 hours at 37°C.

**Polymer Synthesis**

Chondroitin sulfate methacrylate (CS-MA) was synthesized by modifying a method reported previously. Briefly, chondroitin sulfate sodium salt from bovine trachea (Sigma, C9819) was reacted with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in an MES buffer for 5 minutes followed by the addition of 2-aminoethyl methacrylate (AEMA). Chondroitin sulfate, NHS, EDC, and AEMA was mixed at a molar ratio of 2.2:1:2:1 for 24 hours at room temperature. The polymer solution was then dialyzed against water for 4 days using dialysis tubing (12-14kDa MWCO), and the purified solution was lyophilized and stored at -20 °C until use.

Unless otherwise stated, all reagents were purchased from Sigma (St Louis, MO). Poly(ethylene glycol diacrylate) (MW 5000 g/mole) was purchased from Laysan Bio (Laysan Bio Inc., Al).

**CS-MA Synthesis & Characterization**

Chondroitin sulfate-methacrylate (CS-MA) was synthesized as previously described. CS-MA was dissolved in deuterated water (D₂O) and nuclear magnetic resonance (NMR)
spectra were obtained on Inova 300 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA). The degree of substitution of methacrylate side groups was determined as previously described.\(^\text{19}\)

*Cell encapsulation in 3D hydrogels*

On the day of cell encapsulation, chondrocytes were thawed, recounted and used without further expansion. Based on previous studies for chondrocytes and mesenchymal stem cells encapsulation using UV-crosslinkable 3D hydrogels\(^\text{10, 20, 21}\), \(1.5 \times 10^6\) cells/mL cells were suspended in a hydrogel solution consisted of 5% weight/volume (w/v) poly(ethylene glycol diacrylate) (PEGDA, MW=5000 g/mole), 3% w/v chondroitin sulfate-methacrylate (CS-MA), and 0.05% w/v photoinitiator (Irgacure D 2959, Ciba Specialty Chemicals, Tarrytown, NY) in DPBS. The cell-hydrogel suspension was pipetted into a custom-made cylindrical gel mold with 70µl volume and exposed to UV light (365nm wavelength) at 3mW/m\(^2\) for 5 minutes to induce gelation. Cell-laden hydrogels were cultured in Dulbecco’s modified Eagle’s medium (Hyclone, Thermo Scientific Inc.) with 10% FBS (growth media). To confirm cell viability, live dead staining was performed 24 h post-encapsulation using live dead viability/cytotoxicity kit (Life Technologies, Carlsbad, CA). All samples were cultured for 3 weeks or 6 weeks before harvest for analyses.

*Gene expression analyses*

2D monolayer culture- Total RNA was extracted from high-density monolayer chondrocyte cultures using the RNeasy mini kit (Qiagen, Valencia, CA). One mg of RNA
from each sample was reversed transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan Gene Expression Arrays for examination of gene expression for metalloproteinase 1, 3, 9, 13 (MMP1, 3, 9, 13), Type II collagen – chain alpha 1 (Col2a1), Sox9 and Aggrecan (AGC) with a universal mastermix (Applied Biosystems). Gene expression levels were normalized internally to GAPDH.

3D hydrogel culture- Total RNA was extracted from cell-hydrogel constructs (n=3) using TRIzol (Invitrogen, Carlsbad, CA). One mg of RNA from each sample was reversed transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan Gene Expression Arrays for examination of gene expression for Type II collagen – chain alpha 1 (Col2a1), Type VI collagen – chain alpha 1 (Col6a1) and Aggrecan (AGC) with a universal mastermix (Applied Biosystems). Gene expression levels were normalized internally to GAPDH.

The method used for the real-time PCR analysis is the comparative Ct method described by Schmittgen and Livak. ΔCt value is calculated as follows: (CT gene of interest - CT internal control -GAPDH) and $2^{-\Delta\text{Ct}}$ equation is calculated and plotted.

PCR conditions included a 2-minute incubation at 50°C to inactivate previous amplicons with uracil-DNA glycosylase, followed by a 10-minute incubation at 95°C to activate the Taq polymerase. Forty cycles of PCR, consisting of 15 seconds at 95°C, and 1 minute at 60°C, were performed.

**Biochemical analyses**
Cell-hydrogel constructs (n=4) were weighed wet, lyophilized, weighed dry, and digested in papainase solution (Worthington Biochemical, Lakewood, NJ) at 60°C for 16 hours. DNA content was measured using the PicoGreen assay (Molecular Probes, Eugene, OR) with Lambda phage DNA as standard. Sulfated glycosaminoglycan (sGAG) content was quantified using the 1,9-dimethylmethylene blue (DMMB) dye-binding assay with shark chondroitin sulfate (Sigma, St. Louis, MO) as standard. GAG content of the acellular hydrogels was determined as a negative control, and subtracted from the amount of GAG released by the encapsulated cells during the 3-6 weeks of culture.

**Immunohistochemistry**

2D monolayer culture- For immunostaining of cells in monolayer, human articular chondrocytes grown in 6-well plates to 50-70% confluence were fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 10 minutes and then permeabilized with cold 0.4% Triton X-100 (Sigma, St. Louis, MO) in PBS for 15 minutes. The cells were then incubated for 1 hour in PBS containing 1% BSA - 10% FBS - 0.4% Triton X-100 (for blocking). Antibodies against Aggrecan (1:500), Col2a1 (Abcam, 1:100), Col10 (Abcam, 1:100), were added overnight in blocking buffer. The following day cells were washed three times with PBS and then incubated for 1 hour at room temperature with Alexa 594-conjugated goat anti-rabbit secondary antibody (Gibco, Invitrogen; 1:250). Cellular DNA was counterstained with DAPI (Life Technologies, Carlsbad, CA). The antibody anti-AGC used for this study was a kind gift from Prof. R.L. Smith.

3D hydrogel culture- For 3D culture staining, hydrogels were fixed in 4% paraformaldehyde overnight, transferred to 70% Ethanol and included in paraffin
(Histoserv Inc.). Sections were then deparaffinized through xylene and graded alcohols. A step of enzymatic antigen retrieval with 0.1% Trypsin in PBS was performed prior to blocking (2% Goat Serum - 3% BSA - 0.1% Triton X-100) for 1 hour. Antibodies against Aggrecan (1:500), Col2a1 (Abcam, 1:100), Col10 (Abcam, 1:100), Sox9 (Santa Cruz, 1:100), Col6a1 (Santa Cruz, 1:100) were added overnight in blocking buffer. Secondary antibody incubation was performed as described above.

**Mechanical testing**

Unconfined compression tests were conducted using an Instron 5944 materials testing system (Instron Corporation, Norwood, MA) fitted with a 10 N load cell (Interface Inc., Scottsdale, AZ). Cell-hydrogel constructs were tested on day 1, day 21 and day 42 of culture (n=4). During testing, cell-hydrogel constructs were submerged in a PBS bath at room temperature. Compressive strain of 5-20% (at a strain rate 1%/s) has been typically utilized for investigating the mechanical properties of cartilage tissue\textsuperscript{26,27} since the physiological strain experienced by cartilage tissue under loading condition has been reported to be 10-20%\textsuperscript{28,29}. Constructs were therefore compressed at a rate of 1% strain/sec to a maximum strain of 15%. Stress vs. strain curves were created and curve fit using a third order polynomial equation. The compressive tangent modulus was determined from the curve fit equation at strain values of 15%.

**Measurement of MMP activities**

Total MMPs activities were measured using a fluorogenic peptide substrate (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH\textsubscript{2}, ES010, R&D, USA). High-density chondrocyte
culture media was collected, concentrated with centrifugal filter units (Amicon Ultra, 3K, Millipore) and 10ul of concentrated media were applied to the peptide substrate in a black 96-well plate. After 1 hour incubation at 37°C, the fluorescent intensity was measured with a microplate reader (Molecular Devices), excitation at 320 nm and emission at 405 nm.

Results

Characterization of juvenile, adult and OA chondrocytes in monolayer culture

In order to characterize the juvenile, adult and OA chondrocyte populations, we first studied their growth patterns, gene and protein expression in monolayer culture with limited cell passages (1-2) to prevent dedifferentiation. Cell growth was measured using a quantitative and sensitive fluorescence based assay reflecting the metabolic activity of live cells (Prestoblue, Life technologies). We observed similar growth for all the three chondrocyte cell types (n=3 for each) with no statistically significant difference over a time period of 6 days (without cell passage) (Fig. 1a). Gene expression analyses of metalloproteinases (MMPs), a family of enzymes associated with OA, showed a 2 and 20-fold higher expression of MMP 1 and 3 and 400 and 300-fold higher expression of MMP 9 and 13 by OA chondrocytes compared to the normal juvenile and adult chondrocytes (Fig. 1b). Gene expression was analyzed for cartilage markers to characterize the three chondrocyte cell types in the 2D monolayer culture. A higher expression of Col2a1 and Sox9 was observed in the juvenile and OA chondrocytes compared to adult chondrocytes (Fig. 1b, Fig. S1). Among the three cell types, OA cells showed the highest level of AGC expression, whereas comparable level of AGC was observed in normal adult and juvenile chondrocytes (Fig. S1). Immunostaining with
specific antibodies however showed comparable AGC expression at protein level among the three chondrocyte samples while a higher expression of Col2a1 protein was detected in juvenile chondrocytes in comparison to adult or OA chondrocytes (Fig. 1c). In contrast, little or no expression of the chondrocyte hypertrophy marker, Collagen X (Col10) was observed in the juvenile, adult or OA chondrocyte samples (Fig. 1c). The higher Col2a1 or AGC gene expression in OA compared to normal chondrocytes did not result in increased protein levels (Fig. S1 and 1c). Such a lack of correlation between Col2a1 mRNA and protein expression in OA chondrocytes has been reported previously as well 30, 31.

**Chondrocyte culture in 3D using biomimetic hydrogels**

To study the behavior of chondrocytes from various sources in 3D culture, we chose a bioactive hydrogel (synthetic biological composite) containing PEG and CS moieties that had been previously described (Fig. 2a) 10, 18, 20. Photo-polymerization was used to homogeneously encapsulate cells in the hydrogel as previously described 10 with minimal cytotoxicity. The degree of CS methacrylation was ~18% as determined after NMR analysis (Fig. S2). Live/dead staining was used to estimate cell viability the day after photo-polymerization, and a cell viability of 90-95% was observed (data not shown).

**Differential response of juvenile, adult and OA chondrocytes in 3D biomimetic hydrogels**

Juvenile, adult and OA chondrocytes were encapsulated at a density of 15 million/ml in the CS-PEG hydrogels and cultured in growth media (see methods). After 3 weeks of culture, juvenile chondrocytes showed a marked increase in Col2a1 and Col6a1 gene
expression compared to day 1. Adult chondrocytes showed a similar increase in gene expression of Col2a1 and Col6a1, although the fold of increase in Col2a1 expression was less than juvenile chondrocytes (Fig. 2b). In contrast, OA chondrocytes showed over 90% decrease in the expression of Col2a1, while Col6a1 expression was comparable to day 1. No significant change was observed in AGC expression in juvenile and adult chondrocytes, while the AGC expression in OA chondrocytes was over 50% lower after 3 weeks of culture. These results showed that juvenile and adult chondrocytes continue to mature in the 3D biomimetic hydrogels, whereas OA chondrocytes gradually lose their chondrocyte phenotype during the 3D culture. Juvenile chondrocytes showed a greater response in the up-regulation of Col2a1 (250-fold compared to day 1) than adult chondrocytes (80-fold compared to day 1). Both juvenile and adult chondrocytes showed a similar 5-fold upregulation of Col6a1. Immunostaining analyses showed a higher accumulation of AGC and Collagen II at protein level in juvenile and adult chondrocytes compared to the OA chondrocytes at 3 weeks (Fig. 3) and compared to day 1 after cell encapsulation (Fig. S3). A similar pattern was observed when the juvenile, adult and OA chondrocytes were cultured for 6 weeks (Fig. S4). Collagen II protein levels were similar in juvenile and adult chondrocytes as opposed to the mRNA expression. In contrast, the hypertrophy marker, Col X was low in juvenile and adult but not in OA chondrocytes after 3 weeks of culture. Therefore, the CS-PEG hydrogels could be a useful tool for normal (juvenile and adult) chondrocyte expansion without hypertrophy (Fig. 3). Expression of the chondrogenic regulator, Sox9 was comparable across the juvenile, adult and OA chondrocytes while Collagen VI expression was higher in the juvenile chondrocytes as compared to the adult and OA chondrocytes (Fig. 3).
GAG accumulation and biomechanical characterization of cartilage constructs

Cell density was analyzed by measuring DNA content after encapsulation and after three weeks of culture. As reported previously, chondrocyte growth is minimal in the CS-PEG hydrogels. While cell density remained constant over 3 weeks of culture for the juvenile and adult populations, OA chondrocytes exhibited cell death over the culture period with the final density being 50% lower than the initial cell density (Fig. 4a). We used the DMMB method to quantify the total GAG content deposited by the cells after 3 weeks of culture in the CS-PEG hydrogels. To accurately quantify the GAG produced by cells from the CS component in the hydrogels, we have included acellular hydrogels as a negative control for our experiments and cultured them the same way as the cell-containing hydrogels. By subtracting the GAG content of the acellular gels from cell-containing hydrogels, we obtained an accurate quantification of the amount of GAG released by the encapsulated cells during the in vitro culture. Similar to the accumulation of AGC and Collagen II, there was equivalent accumulation of total GAG in juvenile (170μg) and adult (150 μg) chondrocyte populations after 3 weeks of culture in the CS-PEG hydrogels (Fig. 4b). The GAG accumulation in OA chondrocytes was however minimal. Upon normalization to the DNA content, the GAG production by both juvenile and adult chondrocytes was ~ 4 μg of GAG per μg of DNA (Fig. 1c).

To define the biomechanical properties of the hydrogel constructs relative to native human cartilage, we did unconfined compression tests on the different constructs as defined previously (details in methods). The compressive modulus of native human cartilage is well documented to range from 0.4-2 MPa. Despite exhibiting a similar
biochemical composition as the hydrogel construct containing adult chondrocytes, the compressive modulus of the hydrogel construct containing juvenile chondrocytes was slightly higher than adult chondrocytes after 3 weeks of culture (Fig. 4d). At 6 weeks, the compressive modulus of the hydrogel construct containing adult chondrocytes was slightly higher than juvenile chondrocytes, however neither of these differences were statistically significant (Fig. 4e). Nonetheless, the compressive modulus of the hydrogel construct containing OA chondrocytes was significantly lower than the constructs containing juvenile or adult chondrocytes at week 3 as well as 6 (Fig. 4d,e). Interestingly, the compressive modulus of the OA chondrocytes containing hydrogel construct was lower than those with juvenile and adult chondrocytes after initial encapsulation itself. These results highlight the biomechanical differences in the cartilage tissue generated by juvenile, adult and OA chondrocytes.

**Discussion**

The intrinsic limitations of cartilage repair and regeneration, on one hand emphasize the need for successful cartilage engineering and on the other hand highlight the challenges for the same. Despite extensive efforts towards developing cell-based therapy for cartilage repair, the optimal cell type for cartilage engineering remains a matter of debate. The only clinically approved cell source for cartilage repair are adult autologous chondrocytes in autologous chondrocyte implantation (ACI). It has been claimed that allogeneic juvenile chondrocytes derived from individuals 13 or younger have a better capacity to repair cartilaginous defects. Only a handful of studies are available on the potential of juvenile chondrocytes and their comparison with adult chondrocytes. We
therefore sought to study human juvenile, adult as well as osteoarthritic (OA) chondrocytes in order to directly compare and contrast their chondrogenic potential in a 3D biomimetic hydrogel containing chondroitin sulfate.

Previous studies have shown that age can influence the metabolic activity of chondrocytes as well as their response and sensitivity to external factors \(^8, 12, 13, 33-37\). It is generally agreed that chondrocytes’ proliferative potential, cartilage synthesis rate, as well as their response to anabolic growth factors decline with age \(^33-37\). Specifically, juvenile chondrocytes were reported to proliferate faster as well as deposit proteoglycans at a much higher rate than adult chondrocytes after culture in cell pellets in serum-free media \(^8\). On the contrary, a recent study by Saha et al. \(^13\) compared the chondrogenic potential of adult and juvenile chondrocytes in 3D cultures in PLGA scaffolds \textit{in vitro} and \textit{in vivo}, and concluded that adult chondrocytes maintained their chondrogenic phenotype long-term in a TGF-\(\beta\) supplemented chondrogenic medium while the juvenile/neonatal chondrocytes did not. In the present study, we cultured the juvenile, adult and OA chondrocytes in serum-containing media to facilitate the understanding of the different biological responses of these three cell sources in the presence of growth factors. In our study, cell proliferation was not significantly different among the different chondrocyte populations. As such, the observed differences in ECM deposition and mechanical properties of the resulting tissues reflect a direct comparison of the ability of different chondrocyte cell types to form cartilage tissues.

Using a 3D biomimetic hydrogel, we compared the tissue regeneration potential of human chondrocytes from three different aged populations \textit{in vitro} for up to 6 weeks, evaluating cell phenotype as well as the biochemical and biomechanical properties of the
cell-hydrogel constructs. We have chosen to utilize a 3D hydrogel model in the present study, which provides a physiologically relevant condition for chondrocytes than conventional 2D culture, allowing cells to maintain their round morphology and retain their phenotype \(^{15,38}\). Moreover, it contains a bioactive component chondroitin sulfate (CS), a major constituent of proteoglycans found in cartilage. CS is known to exhibit a number of useful biological properties and a beneficial anti-inflammatory role in wound healing and in the arthritic joint. The incorporation of CS in the hydrogel composition enables enzymatic degradation by cell-secreted chondroitinase and has been shown to enhance MSC chondrogenesis \(^{20}\). It can also be chemically modified to enhance tissue-biomaterial integration during cartilage repair \(^{39,40}\), and may serve as a potential scaffolding material for cell delivery.

Our results indicated that chondrocytes from different ages or disease state exhibited differential response in 3D biomimetic hydrogel culture. Both juvenile and adult chondrocytes responded favorably when cultured in 3D biomimetic hydrogels with enhanced chondrogenic phenotype until 6 weeks. Col2a1 as well as Col6a1 expression was up-regulated while aggrecan expression was maintained. Specifically, juvenile chondrocytes exhibited the highest Col2a1 up-regulation (~250-fold) while adult chondrocytes had a moderate increase in Col2a1 expression (~80 fold). This suggests that the CS-containing biomimetic hydrogels support the maturation of juvenile and adult chondrocytes. Similar to our findings, previous studies showed that incorporation of CS into PEG hydrogels enhanced chondrogenic differentiation of MSCs \(^{20}\). Unlike juvenile and adult chondrocytes, OA chondrocytes in 3D biomimetic hydrogels showed a gradual loss of cartilage markers, with decrease in both aggrecan and Col2a1 expression. These
results suggest that the ability of chondrocytes to respond to bioactive niche cues like CS may decline with disease progression. Previous studies have shown that sensitivity to anabolic growth factors such as IGF-1 and TGF-beta declines with the age of the chondrocytes \(^{33, 35}\).

In addition to the marked enhance chondrogenic gene expressions in 3D culture as well as total GAG and collagen content, juvenile and adult chondrocyte-seeded hydrogels also resulted in higher compressive moduli than the OA chondrocyte-seeded hydrogels. Compressive modulus for the OA chondrocytes was lower than the juvenile and adult chondrocytes even upon initial seeding when the number of cells was comparable, and further decreased as the cell density declined over time. Given there is minimal difference in ECM compositions at day 1, we speculate such change in mechanical modulus is caused by the cellular components. Consistent with the trend observed in our study, Hsieh et. al. has reported that the stiffness of human OA chondrocytes \(^{41}\) is 64\% lower compared to the stiffness of normal chondrocytes using atomic force microscopy measurement. Together, these results suggest that decreased mechanical property of OA chondrocyte-containing hydrogels at day 1 is likely caused by the lower mechanical property from the cell components. The further decrease in mechanical property of OAC-containing hydrogels over time may be contributed by the decreased GAG-production and increased rate of apoptosis of OA chondrocytes \(^{37, 42}\). In addition, our results show that OA chondrocytes generally exhibited higher level of MMP activities (Fig. 1), which may lead to faster hydrogel degradation and decrease in the mechanical property of cell-containing hydrogels over time. Similarly, a previous study showed that bovine chondrocytes from young animals (1-2 years) formed cartilage
constructs with superior mechanical properties compared to those formed by aged animals (5-7 years) 42.

In summary, here we report a comparative study of human chondrocytes from juvenile, adult, and OA population side-by-side using 3D biomimetic hydrogels. Our findings suggest that both juvenile and adult chondrocytes continue to mature in 3D biomimetic hydrogels, resulting in elevated chondrogenic gene expression and the formation of cartilage constructs with comparable mechanical properties. The 3D biomimetic gels therefore appear to be advantageous for adult chondrocyte culture as the adult chondrocytes function as well as the juvenile chondrocytes. In contrast, OA chondrocytes showed minimal GAG production and gradually lost hyaline cartilage marker expression and protein deposition, and the use of 3D biomimetic hydrogels was not sufficient to salvage the chondrocyte phenotype of OA cells. Our results highlight the importance of considering the age and disease state of chondrocytes used for cartilage tissue engineering. In addition, the model of culturing juvenile or adult chondrocytes in 3D biomimetic hydrogels may provide a useful tool for understanding the molecular basis for the age-associated decline in the intrinsic capacity of cartilage to regenerate.

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Figure Legends
Comparative Potential of Juvenile and Adult Human Articular Chondrocytes for Cartilage Tissue Formation in 3D Biomimetic Hydrogels (doi: 10.1089/ten.TEA.2014.0070)

This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.
Figure 1. Characterization of juvenile, adult and OA chondrocytes in monolayer culture. 

a, Growth curves for juvenile, adult and OA chondrocytes in monolayer culture. Data represent three biological replicates and are relative to Day 0. 
b, Quantitative gene expressions for MMP1, MMP3, MMP9, MMP13 and Col2a1. All data represent three biological replicates, are relative to adult chondrocytes and expressed as mean ± SD. The single asterisk (*) represents a statistical significance at p <0.05 as determined by a two-tailed Student t test. 
c, Depositions of cartilage markers by juvenile, adult and OA chondrocytes in monolayer culture. Red: immunofluorescence from staining for aggregan (AGC), Collagen 2A1 (Col2A1) or Collagen 10 (Col10); Blue: cell nuclei stained with DAPI. Scale bar= 30 μm.
Figure 2. a, Schematic of hydrogels used for culturing chondrocytes in 3D, containing polyethylene glycol (PEG) and chondroitin sulfate (CS). b, Quantitative gene expression of cartilage markers by juvenile, adult and OA chondrocytes after being cultured in 3D biomimetic hydrogels for 3 weeks. Values are normalized to gene expression level of Day 1. Error bars represent mean± SD. The single asterisk (*) represents a statistical significance at p <0.05 as determined by a two-tailed Student t test.
**Figure 3.** Deposition of cartilage markers by juvenile, adult and OA chondrocytes after 3 weeks of culture in 3D biomimetic hydrogels. Red: immunofluorescence staining for aggregan (AGC), Collagen 2A1 (Col2A1), Collagen 10 (Col10), Sox9 and Collagen 6A1 (Col6A1); Blue: cell nuclei stained with DAPI. *Scale bar*= 30 μm. Insets show a higher magnification image of cells in each field.
**Figure 4.** Characterization of cartilage tissue formation by juvenile, adult and OA chondrocytes after 3 weeks of culture in 3D biomimetic hydrogels. 

- *a*, DNA content.
- *b*, total GAG content.
- *c*, GAG content normalized to DNA content.
- *d*, compressive moduli at week 3.
- *e*, compressive moduli at week 6. Error bars represent mean ±SD. The single asterisk (*) represents a statistical significance at p <0.05 as determined by a two-tailed Student t test.


Supplementary Figure 1. a, Quantitative gene expressions for the chondrocyte markers Sox9 and AGC in monolayer culture of juvenile, adult and OA chondrocytes. All data represent three biological replicates, are relative to adult chondrocytes and expressed as mean ± SD. b, Total MMP activities in normal and OA chondrocytes culture media measured using a fluorogenic probe. All data represent three biological replicates and are expressed as mean ± SD. The single asterisk (*) represents a statistical significance at p < 0.05 as determined by a two-tailed Student t-test.
Supplementary Figure 2. 1H NMR spectra of chondroitin sulfate methacrylate (CS-MA). Methacrylate substitution is illustrated by peaks at 5.6 and 6 ppm. The degree of methacrylation was ~18%.
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