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Collagen VI Enhances Cartilage Tissue Generation by Stimulating Chondrocyte Proliferation

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Regeneration of human cartilage is inherently inefficient. Current cell-based approaches for cartilage repair, including autologous chondrocytes, are limited by the paucity of cells, associated donor site morbidity, and generation of functionally inferior fibrocartilage rather than articular cartilage. Upon investigating the role of collagen VI (Col VI), a major component of the chondrocyte pericellular matrix (PCM), we observe that soluble Col VI stimulates chondrocyte proliferation. Interestingly, both adult and osteoarthritis chondrocytes respond to soluble Col VI in a similar manner. The proliferative effect is, however, strictly due to the soluble Col VI as no proliferation is observed upon exposure of chondrocytes to immobilized Col VI. Upon short Col VI treatment in 2D monolayer culture, chondrocytes maintain high expression of characteristic chondrocyte markers like Col2a1, agc, and Sox9 whereas the expression of the fibrocartilage marker Collagen I (Col I) and of the hypertrophy marker Collagen X (Col X) is minimal. Additionally, Col VI-expanded chondrocytes show a similar potential to untreated chondrocytes in engineering cartilage in 3D biomimetic hydrogel constructs. Our study has, therefore, identified soluble Col VI as a biologic that can be useful for the expansion and utilization of scarce sources of chondrocytes, potentially for autologous chondrocyte implantation. Additionally, our results underscore the importance of further investigating the changes in chondrocyte PCM with age and disease and the subsequent effects on chondrocyte growth and function.

Introduction

Articular cartilage has a poor inherent capacity for regeneration.[1] Although tissue regeneration generally declines with age, cartilage injuries are problematic even in young adults and can often lead to post-traumatic osteoarthritis. Cartilage tissue engineering is, therefore, a relevant and attractive strategy for cartilage injuries. Current cell-based approaches for engineering cartilage have, however, had limited success. Cell sources commonly utilized for engineering cartilage include autologous chondrocytes, mesenchymal stem cells (MSCs), or adipose-derived stem cells (ADSCs).[1] The major challenges common to all these cell sources are the paucity of available cells and the generation of functionally inferior fibrocartilage rather than articular cartilage.[2,3] In addition, *in vitro* expansion of autologous chondrocytes can lead to dedifferentiation and loss of chondrogenic phenotype.[4] Expansion of MSCs and ADSCs *in vitro* is also challenging as it leads to a rapid loss of their stem cell properties.

Cartilage is a unique tissue as it is composed of a single cell type, that is, chondrocytes embedded in a self-secreted extracellular matrix (ECM). It is widely accepted that the ECM plays a critical role in cartilage function. Recent research has also highlighted the importance of the pericellular matrix (PCM) in articular cartilage function especially for maintaining its biochemical and biomechanical properties.[5] Chondrocytes have a unique and well-defined PCM consisting of the collagen types VI, II, and IX along with various proteoglycans and fibronectin.[6] The precise biochemical effects of these individual and combined components on chondrocyte fate and function are, however, just beginning to emerge. Understanding how the ECM and PCM components influence the cell–matrix and cell–cell communication in chondrocytes is, therefore, of significance fundamentally as well as for potentially enhancing the quality of engineered cartilage.

A major component of the chondrocyte PCM is the heterotrimeric protein, Collagen VI (Col VI) that consists of three different α-chains, α1, α2, and α3. Three alternate subunits α4, α5, or α6 have recently been discovered that can substitute for α3.[7,8] Alterations in Col VI expression have been associated with various muscular dystrophies and

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ligamentous disorders, including Ulrich congenital muscular dystrophy and Bethlem myopathy.[9] A recent report has also implicated Col VI in maintaining adult muscle stem cell function. Failure in skeletal muscle regeneration was observed in Col6a1$^{-/-}$ mice, attributed to impairment of muscle stem cell function due to the decreased ECM stiffness.[10] Col VI is of particular interest in cartilage as the double von Willebrand factor A domains (DVWA) susceptibility locus for knee osteoarthritis has recently been identified as a part of the col6a4 locus.[11] In addition, Col6a1$^{-/-}$ mice show accelerated osteoarthritic changes in the hip as well as decreased bone mass implicating perturbations in both secondary ossification and cartilage repair due to Col VI deficiency.[12,13] In the present study, we aimed to study the direct effect of Col VI on chondrocyte fate and function to test whether Col VI would have a potentially beneficial role in cartilage tissue engineering.

Methods

Materials and antibodies

Col VI (BD 354261) and Col I (BD 354265) were obtained from BD Biosciences and are endotoxin-free preparations from human placenta purified up to an electrophoretic homogeneity of >90%. An alternate bovine source of Col VI from Millipore (Col VI; Millipore CC086) was utilized, also purified from placenta up to >90% purity on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The respective solvent solutions were used as control treatment for each collagen type namely, a 1 M sodium chloride, 1.25 mM Tris solution for human Col VI (BD), a 0.05 M acetic acid solution for bovine Col VI (Millipore), and a 0.02 M acetic acid solution for human Col I (BD). Primary antibodies used were anti-human: Col 2a1 (Abcam; 1:100), Col 1 (Abcam; 1:100), Col 10 (Abcam; 1:100), Ki67 (ebiosciences; 1:500), Sox9 (Santa Cruz; 1:100), Aggrecan (1:500), Col6a1 (Santa Cruz; 1:100) at the specified dilutions. The antibody anti-Aggrecan used for this study was a kind gift from Prof. R.L. Smith.[14]

Chondrocyte isolation and culture

Normal adult (34 years, male) and juvenile human articular chondrocytes (normal Juvenile: 6 years, male; normal Juvenile: 2–6 months, male) were purchased commercially from Lonza Walkersville, Inc. (Clonetics™; Lonza) and expanded for limited passages (1–3) in high-density monolayer culture (to prevent dedifferentiation) in chondrocyte growth medium (Clonetics CGM™; Lonza Walkersville, Inc.). Additional normal chondrocytes were obtained from grossly normal cartilage pieces during arthroplasty or debridement from patients undergoing anterior cruciate ligament reconstruction who had no history of osteoarthritis (OA) symptoms (normal adult 2–18 years, male; normal adult 3–25 years, male). Osteoarthritic chondrocytes were harvested from articular cartilage samples obtained during total knee arthroplasty (OA1: 64 years, female; OA2: 74 years, male; OA3: 71 years, male) under the approved Human subjects Institutional Review Board protocols. Cartilage dissection and chondrocytes isolation were performed as described previously.[15] Isolated chondrocytes were then expanded in high-density monolayers for one to two passages in 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% fetal bovine serum (FBS) at 37°C.

Cell growth assays

Chondrocytes were plated at 1000 cells per well in triplicates in 96-well plates. Cell viability was assayed daily for each cell type with the PrestoBlue Cell Viability Reagent kit (Life Technologies) following the manufacturer's instructions. PrestoBlue reagent was added in the cell culture medium, and the cells were incubated at 37°C for 30 min. Fluorescence intensity of the PrestoBlue reagent reduced by living cells was measured at 690 nm (650 nm excitation wavelength) with a microplate reader (Molecular Devices). Fluorescence level was expressed in relative fluorescence unit.

Polymer synthesis

Chondroitin sulfate methacrylate (CS-MA) was synthesized by modifying a method reported previously.[16] Briefly, CS sodium salt from bovine trachea (Sigma; C9819) was reacted with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in an 2-(N-morpholino)ethanesulfonic acid buffer for 5 min followed by the addition of 2-aminoethyl methacrylate (AEMA). CS, NHS, EDC, and AEMA was mixed at a molar ratio of 2.2:1:2:1 for 24 h at room temperature. The polymer solution was then dialyzed against water for 4 days using a dialysis tubing (12–14 kDa MWCO), and the purified solution was lyophilized and stored at −20°C until use. Unless otherwise stated, all reagents were purchased from Sigma. Poly(ethylene glycol diacrylate) (PEGDA; MW 5000 g/mol) was purchased from Laysan Bio, Inc.

Cell encapsulation in 3D hydrogels

Before encapsulation, chondrocytes were treated with soluble Col VI for 2 days and on the day of cell encapsulation they were counted and used without further expansion. As previously described,[17–19] 15×10^6 cells/mL cells were suspended in a hydrogel solution consisting of 5% weight/volume (w/v) PEGDA (MW = 5000 g/mol), 3% w/v CS-MA, and 0.05% w/v photoinitiator (Irgacure D 2959; Ciba Specialty Chemicals) in Dulbecco's phosphate buffered saline. Seventy microliters of the cell–hydrogel suspension was pipetted into a custom-made cylindrical gel mold and gelation was induced through exposure to UV light (365 nm wavelength) at 3 mW/m^2 for 5 min. Cell-laden hydrogels were cultured in Dulbecco's modified Eagle's medium (Hyclone, Thermo Scientific, Inc.) with 10% FBS (growth media). The viability of the cells was confirmed by Live/Dead staining 24 h postencapsulation using a viability/cytotoxity kit (Life Technologies). All samples were cultured for 3 weeks before harvest for analyses.

Gene expression analyses

For the 2D cultures, total RNA was extracted from high-density monolayer chondrocyte cultures using the RNeasy mini kit (Qiagen). For 3D hydrogel cultures, total RNA was extracted from cell–hydrogel constructs ($n = 3$) using TRIzol (Invitrogen). One milligram of RNA from each sample was reverse transcribed into cDNA using the High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using TaqMan gene-specific expression arrays for metalloproteinase 1, 3, 9, 13 (*MMP1, 3, 13*), Type II collagen–chain alpha 1 (*Col2a1*), Type VI collagen–chain alpha 1 (*Col6a1*), *Sox9*, and Aggrecan (*Agc*) with a universal mastermix (Applied Biosystems). Gene expression levels were normalized internally to *GAPDH*. qPCR reactions included a 2-min incubation at 50°C to inactivate previous amplicons with uracil-DNA glycosylase, followed by a 10-min incubation at 95°C to activate the Taq polymerase. The amplification cycle, consisting of 15 s at 95°C, and 1 min at 60°C, was repeated 40 times. The relative expression levels were determined using the ΔCt method (CT gene of interest – CT internal control -GAPDH) and $2^{-\Delta Ct}$ equation is calculated as described by Schmittgen and Livak[20] and plotted.

Biochemical analyses

For the cell–hydrogel constructs ($n=3$), DNA and sulfated glycosaminoglycan (sGAG) production were quantified as follows: The hydrogels were lyophilized and digested in papainase solution (Worthington Biochemical) at 60°C for 16 h.[18,21] DNA content was measured using the Pico-Green assay (Molecular Probes) with Lambda phage DNA as standard. sGAG content was quantified using the 1,9-dimethylmethylene blue dye-binding assay with shark CS (Sigma) as standard.[22] 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 weeks of culture.

Immunofluorescence

For immunostaining of human chondrocytes, cells grown in six-well plates were fixed in 4% paraformaldehyde (Sigma) for 10 min and then permeabilized with cold 0.4% Triton X-100 (Sigma) in phosphate buffered saline (PBS) for 15 min. For ki-67, cells were permeabilized with cold methanol (Sigma) for 6 min at −20°C. Cells were then blocked for 1 h in PBS containing 2% goat serum, 3% bovine serum albumin (BSA), 0.1% Triton X-100, and incubated with primary antibody overnight. The following day cells were washed with PBS and incubated for 1 h with secondary antibody (1:250, goat anti-rabbit Alexa 594-conjugated; Gibco, Invitrogen) at room temperature. Cellular DNA was counterstained with DAPI (Life Technologies). For 3D culture staining, cell–hydrogel constructs were rinsed in PBS upon harvest, fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol and paraffin embedded (Histoserv, Inc.). Ten micrometer sections were then deparaffinized through xylene and graded alcohols. A step of enzymatic antigen retrieval with 0.1% Trypsin in PBS was performed for 15 min before blocking (2% goat serum, 3% BSA, 0.1% Triton X-100) for 1 h. Primary and secondary antibody incubation was performed as described above.

Mechanical testing

Unconfined compression modulus was determined using an Instron 5944 materials testing system (Instron Corporation) fitted with a 10 N load cell (Interface, Inc.). Cell–hydrogel constructs were tested on day 1 and 21 of culture ($n=3$). Specimens were submerged in a PBS bath at room temperature and compressed at a rate of 1% strain/s to a maximum strain of 15%[23,24] since the physiological strain experienced by cartilage tissue under loading condition has been reported to be 10–20%.[25,26] Stress versus 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%.

Results

Col VI stimulates chondrocyte proliferation

To test the effect of Col VI, we utilized both normal chondrocytes and OA chondrocytes isolated from OA cartilage tissues obtained from patients undergoing total knee arthroplasty (see Methods section). Four different chondrocyte preparations were utilized to test the effect of Col VI and study the underlying mechanisms—two representative samples of normal chondrocytes with no history of OA (34 years old male normal adult and 6 years old female normal juvenile) and two OA chondrocyte samples (64 years old female OA1 and 74 years old male OA2). All chondrocyte samples showed the characteristic gene expression for the chondrogenic markers—*Col2a1*, *Sox9*, and *Agc* whereas the OA1 and OA2 showed an increased expression of *MMP1*, *3*, and *13* compared with the normal samples, as expected (Supplementary Fig. S1a; Supplementary Data are available online at www.liebertpub.com/tea). Immunostaining with specific antibodies showed Col II protein expression in all the chondrocyte samples whereas little or no expression of the fibrocartilage marker Col I was observed (Supplementary Fig. S1b).

In the initial experiments, we observed an increase in total cell count in both normal and OA chondrocytes upon treatment with 2.5 μg/mL of soluble Col VI for 2 days (Supplementary Fig. S2a). No proliferative effect was, however, observed on the growth of primary osteoblasts upon a similar treatment with Col VI (Supplementary Fig. S2b). For a detailed analysis of growth pattern in the absence and presence of Col VI, a quantitative fluorescence-based assay reflecting the metabolic activity of live cells (PrestoBlue; Life technologies) was used to measure cell growth. In this assay, the reagent Resazurin is nonfluorescent, but is converted to fluorescent Resorufin in the reducing environment of living cells, allowing a quantitative measurement of relative cell numbers. The advantage of this reagent is its high sensitivity making it possible to detect relatively low numbers of cells such as 500 or 1000 cells reproducibly. This assay provided growth kinetics comparable to an actual counting of the cell numbers with time, either by a cell counter or by counting DAPI-stained nuclei of cells fixed with or without Col VI-treatment.

During a 2-day treatment with 2.5 μg/mL of Col VI or vehicle control added everyday, we observed a significant increase in the cell numbers of all Col VI-treated chondrocyte samples compared with control-treated chondrocytes (Fig. 1). Both normal (adult and juvenile) and OA samples responded to Col VI in a similar manner, with no significant differences observed among samples (Fig. 1). Higher dosage of Col VI (5 μg/mL) did not induce any further increase in cell proliferation (data not shown). In addition, a similar proliferative effect was observed upon using either human or bovine Col VI isolated from the placenta (to ≥90% purity,

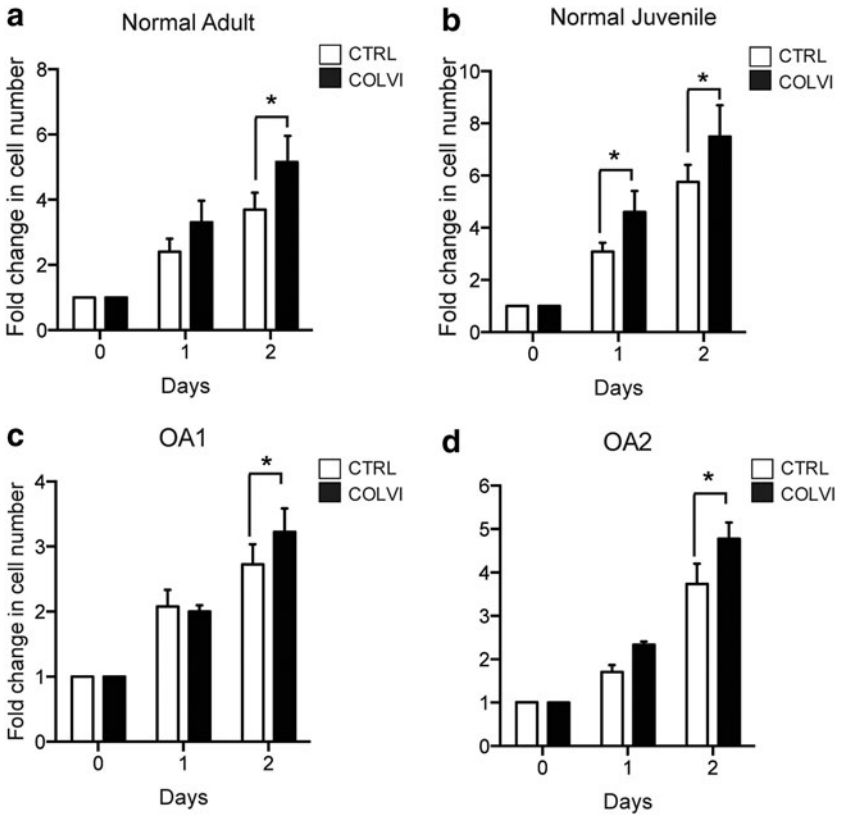


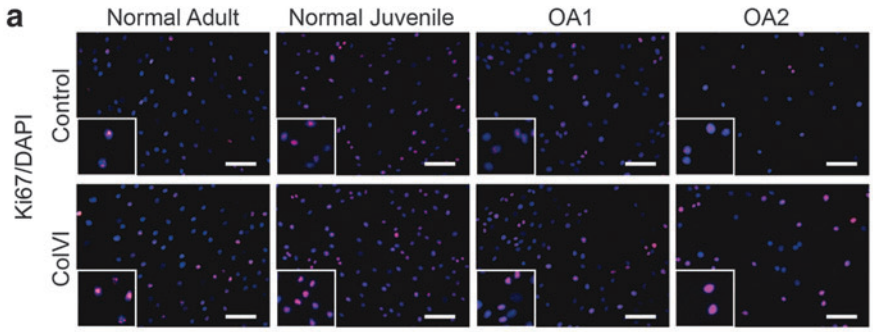
FIG. 1. Soluble collagen VI (Col VI) stimulates chondrocyte proliferation. **(a, b)** Normal and **(c, d)** osteoarthritis (OA) chondrocyte growth upon treatment with control (*white bars*) or Col VI (2.5 μg/mL) (*black bars*) in monolayer cultures for 2 days. Col VI is replenished daily. Fold increase in cell number is indicated relative to day 0. Data represent three biological replicates and are expressed as mean ± SD. $*p < 0.05$.

see Methods section). A treatment with soluble Col I, isolated from human placenta similar to Col VI (see Methods section), was utilized as an additional control and failed to increase proliferation in either normal or OA chondrocytes (Supplementary Fig. S2c), further demonstrating that the proliferative effect is specific to Col VI. Besides the four chondrocyte populations followed in detail, we observed a similar increase in cell count upon Col VI treatment in four additional human chondrocyte samples (three normal and one OA) confirming the effect on a larger cohort (Supplementary Fig. S2d).

To ascertain that the increase in cell numbers in the presence of Col VI was due to an increase in cell proliferation and not decrease in cell death, we utilized immunostaining for Ki67 that is an antigen associated with cellular proliferation. Cells were fixed before and after control or Col VI treatment for 2 days, and were immunostained for Ki67 (red) with nuclei counterstained for DAPI (blue) (Fig. 2a). The signal corresponding to Ki67 staining was enhanced in the chondrocytes treated with Col VI compared with control-treated chondrocytes as shown in representative images (Fig. 2a). DAPI-stained nuclei were counted manually for five representative microscopic fields along with cells immunostained for Ki67 (red) to determine the total number of cells and the Ki67 expressing cells. The table in Figure 2b reports the total and Ki67-positive cells averaged over five representative fields of view. Percentages of Ki67-expressing cells were then calculated for normal adult and OA samples upon control or Col VI treatment (Fig. 2b, c). An increase in the total number of Ki67-expressing cells was observed in all samples upon Col VI treatment confirming an increase in chondrocyte proliferation (Fig. 2c).

Col VI treatment maintains the chondrogenic cell fate

A common challenge for culturing chondrocytes (unless maintained as a high-density monolayer) is their tendency to dedifferentiate over multiple cell passages in 2D culture. Although the Col VI treatment was only for 2 days, we tested whether the increased proliferation due to Col VI treatment leads to dedifferentiation of chondrocytes. A hallmark of dedifferentiated chondrocytes is a decrease in the expression of the chondrogenic marker Col II and increased expression of Col I. It has been reported previously that changes in the mRNA expression of Col2a1 tend not to correlate with the protein expression.[27,28] In our study, we have similarly observed that a higher expression of Col2a1 in the OA chondrocytes as compared with the normal adult chondrocytes does not lead to an increase in protein levels (Supplementary Fig. S1). Therefore, we tested the expression of Col II at the protein level by immunostaining chondrocytes before and after 2 days of Col VI treatment. We chose immunostaining over western blot to obtain data at a single cell level rather than the bulk population. We observed that Col II is maintained in the Col VI-expanded chondrocytes in both normal and OA samples demonstrating that the cells maintained the chondrogenic phenotype (Fig. 3a). Upon similar staining for Col I, we observed that there was no increase in Col I staining after control or Col VI treatment showing that the cells did not dedifferentiate despite increased cell growth. Furthermore, expression of the hypertrophy marker, Col X remained low and largely unchanged in three out of four samples, although the OA2 sample showed a slight increase in Col X expression (Fig. 3c). In addition, Col VI treatment maintained chondrogenic



b

		Total cells	Ki67+	% Ki67+/tot	Fold Δ
Normal adult	Control	272	85	31.25	1.00
	ColVI	406	202	49.75	1.59
Normal Juvenile	Control	643	498	77.45	1.00
	ColVI	725	636	87.72	1.13
OA1	Control	304	154	50.66	1.00
	ColVI	421	238	56.53	1.12
OA2	Control	323	137	42.41	1.00
	ColVI	759	451	59.42	1.40

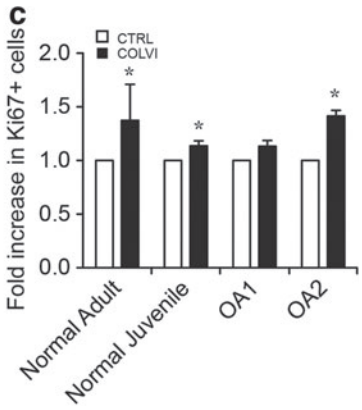
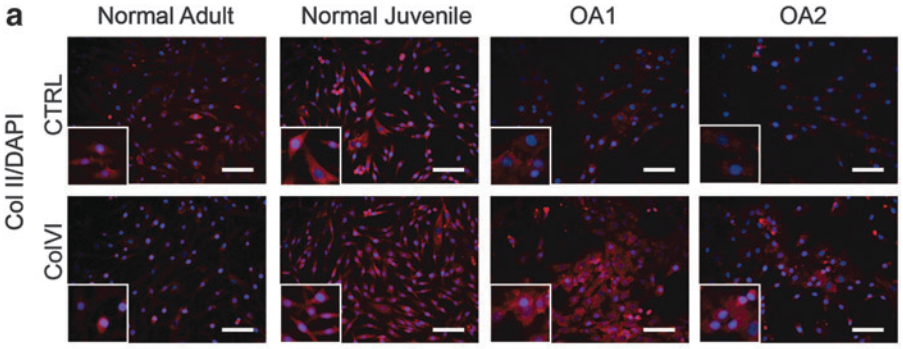
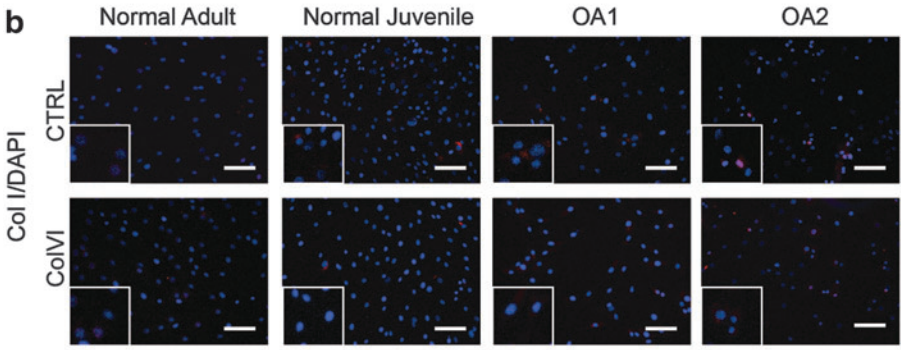


FIG. 2. Col VI induces Ki67 expression in proliferating chondrocytes. **(a)** Representative immunofluorescence images for Ki67 (*red*) expression in normal and OA chondrocytes, after control (*upper panel*) or Col VI (*lower panel*) treatment for 2 days. Nuclei (*blue*) are stained with DAPI. Scale bar = 30 μm. **(b)** Quantification of total number of cells and Ki67$^+$ cells (average of five fields), Ki67$^+$/total cells ratio and fold change upon control or Col VI treatment for 2 days. **(c)** Fold increase in Ki67$^+$ cells in normal and OA chondrocytes upon control (*white bars*) or Col VI treatment (*black bars*) for 2 days. Data are expressed as mean ± SD, $*p < 0.05$. *Insets* show a higher magnification image of cells in each field.





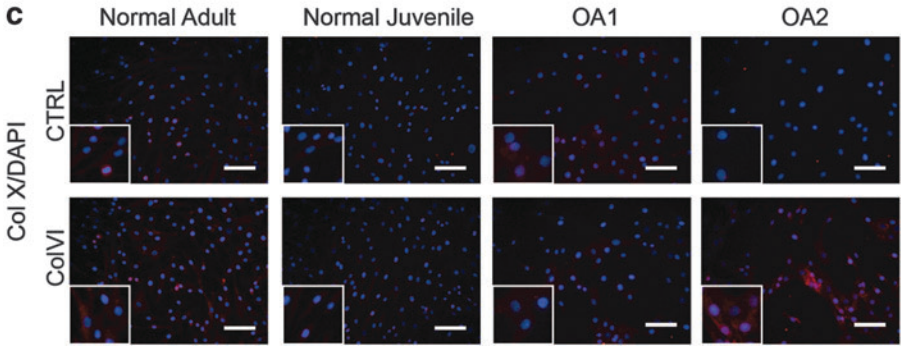


FIG. 3. Chondrocytes treated with Col VI maintain their chondrogenic phenotype. Representative immunofluorescence images for Col II **(a)**, Col I **(b),** and Col X **(c)** expression in normal and OA chondrocytes, upon treatment with control (*upper panel*) or Col VI (*lower panel*) for 2 days. Nuclei (*blue*) are stained with DAPI. Scale bar = 30 μm. *Insets* show a higher magnification image of cells in each field.

gene expression as shown by an unchanged expression of *AGC*, *SOX9*, *COL6A1*, and *MMP1* and *MMP3* in both normal and OA chondrocytes upon control or Col VI treatment (Supplementary Fig. S3). Collectively, these studies show that the chondrogenic fate is maintained during Col VI-mediated proliferation. Col VI may, therefore, be a useful biologic for culturing scarce primary chondrocytes for experimentation as well as for clinical applications.

Immobilized Col VI has no effect on chondrocyte proliferation

Next, we explored the mode of Col VI action mainly to test if Col VI stimulated chondrocyte proliferation through a biophysical effect of the ECM or through a biochemical effect acting as a ligand to a receptor. For this, we exposed the cells to immobilized Col VI as opposed to soluble Col VI and observed the effect on chondrocyte proliferation. Ninety-six-well plates were coated with 5 μg/μL Col VI and 1000 normal or OA chondrocytes were initially plated in each well and their growth was monitored as before using the PrestoBlue Viability assay. Fibronectin (5 μg/μL) or gelatin (5 μg/μL) coating was used as a control along with an uncoated surface. No increase in proliferation was detected when the normal and OA cells were grown on immobilized Col VI (Fig. 4). These results suggest that the proliferative effect induced by soluble Col VI is an effect of a cell–ligand rather than a cell–matrix interaction.

Potential of Col VI-treated normal chondrocytes to form cartilage tissue in 3D biomimetic hydrogels

To evaluate whether the expansion of chondrocytes in the presence of Col VI maintains their ability to form cartilage, we chose a 3D culture system that we have previously utilized to study the behavior of chondrocytes of various ages. The 3D culture system consists of a bioactive hydrogel (synthetic biological composite) containing PEG and CS moieties.[18] Photopolymerization was used to homogeneously encapsulate cells in the hydrogel as previously described[18] with minimal cytotoxicity. Upon using Live/Dead staining to estimate cell viability the day after photopolymerization, a cell viability of 90–95% is typically observed (data not shown).

Normal adult and juvenile chondrocytes were treated with control or Col VI for 2 days. An equal number of cells from both the control and Col VI-treated chondrocyte populations were then encapsulated at a density of 15 million/mL in the CS-PEG hydrogels and cultured in growth media (see Methods section). After 3 weeks of culture, the expression of *AGC*, *SOX9*, and *COL6A1* was similar for control or Col VI-treated chondrocytes for both juvenile and adult chondrocytes (Fig. 5a). However, Col VI-treated juvenile chondrocytes showed an increased expression of *COL2A1* as compared with the control-treated chondrocytes even after 3 weeks in 3D culture (Fig. 5a). In addition, even control-treated juvenile chondrocytes showed a greater response in the upregulation of *COL2A1* (250-fold compared with day 1) than adult chondrocytes (80-fold compared with day 1) after 3 weeks of culture. An increased accumulation of GAG was observed in the juvenile chondrocytes compared with the adult chondrocytes after 3 weeks of culture (Fig. 5b). While the GAG accumulation in the control and Col VI-treated adult chondrocytes was very similar, a slightly larger GAG accumulation was observed in the Col VI-treated juvenile chondrocytes as compared with the control-treated juvenile chondrocytes. To define the biomechanical properties of the hydrogel constructs, unconfined compression tests were carried out on the different constructs as previously reported (details in Methods section). In keeping with the similar biochemical composition, the compressive moduli of the hydrogel construct containing control and Col VI-treated chondrocytes were comparable for both juvenile and adult chondrocytes

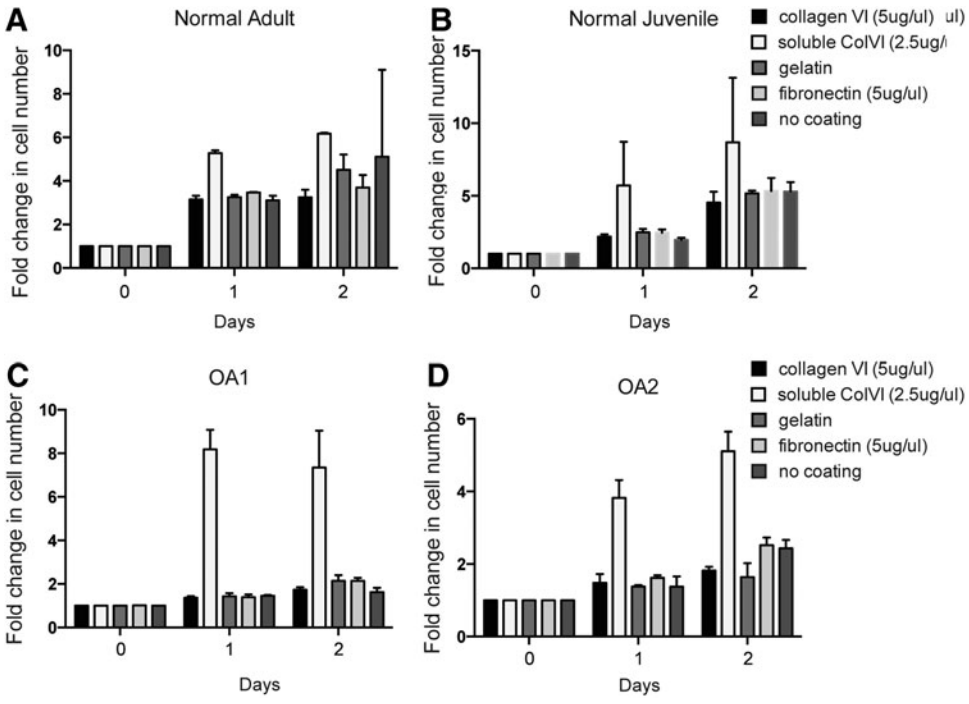


FIG. 4. Immobilized Col VI does not induce proliferation in chondrocytes. Cell growth for **(A, B)** normal and **(C, D)** OA chondrocytes cultured on uncoated (*dark gray bars*) wells or wells coated with Col VI (5 μg/μL) (*black bars*), Gelatin (5 μg/μL) (*medium gray bars*), or fibronectin (5 μg/μL) (*light gray bars*). Soluble Col VI (5 μg/μL) (*white bars*) is used as a positive control. Fold increase in cell number is indicated relative to day 0. Wells are coated with the indicated proteins 1 day before day 0 (day −1). Data represent three biological replicates and are expressed as mean ± SD.

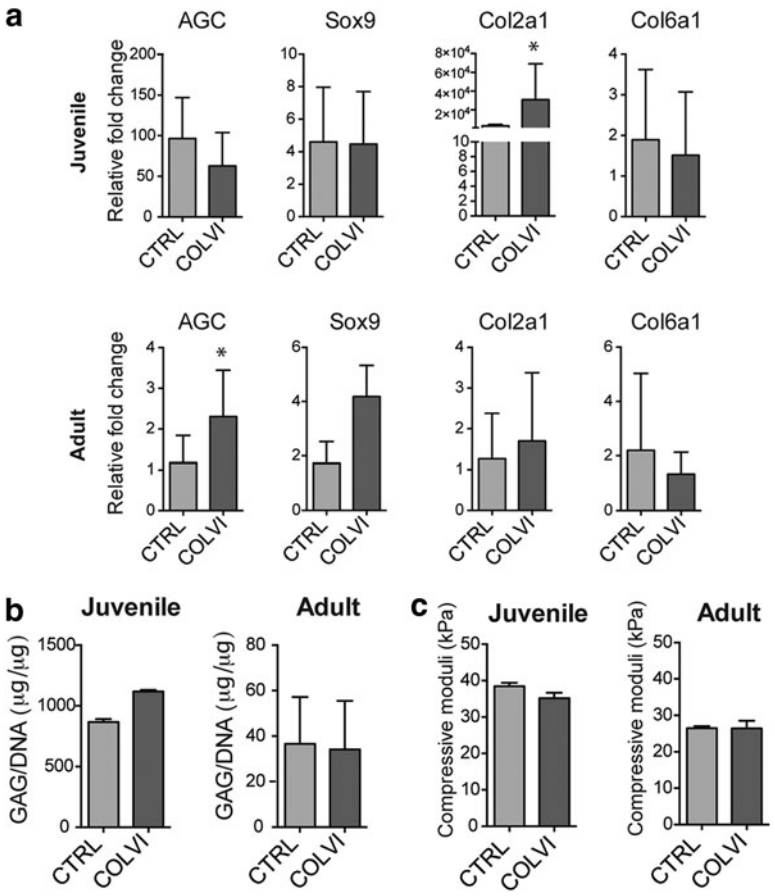


FIG. 5. Col VI-treated chondrocytes maintain chondrogenic phenotype after 3 weeks of culture in 3D biomimetic hydrogels. **(a)** Quantitative gene expression of chondrogenic markers in control (*light gray bars*) and Col VI-treated (*dark gray bars*) juvenile and adult chondrocytes following encapsulation and culture in 3D biomimetic hydrogels for 3 weeks. **(b)** Glycosaminoglycan content normalized to DNA content in hydrogels seeded with control (*light gray bars*) and Col VI-treated (*dark gray bars*) juvenile and adult chondrocytes after 3 weeks of culture. **(c)** Compressive moduli of hydrogels seeded with control (*light gray bars*) and Col VI-treated (*dark gray bars*) juvenile and adult chondrocytes after 3 weeks of culture. Error bars represent mean ± SD. The *single asterisk* (*) represents a statistical significance at $p < 0.05$ as determined by a two-tailed Student's *t*-test.

after 3 weeks of culture (Fig. 5c). Immunostaining analyses showed similar accumulation of Col II, AGC, and Col VI at protein level in juvenile and adult chondrocytes irrespective of control or Col VI treatment (Fig. 6). Similarly, the Sox9 protein expression was unchanged for control or Col VI-treated chondrocytes. The dedifferentiation marker Col I, as well as the hypertrophy marker Col X, also remained low upon Col VI treatment and subsequent culture for 3 weeks in the 3D hydrogels (Fig. 6). These results highlight that the biochemical and mechanical properties of cartilage constructs formed by control and Col VI-treated chondrocytes are comparable demonstrating the usefulness of Col VI for expansion of chondrocyte populations.

Discussion

Col VI is a major component of the ECM in multiple tissues, including skeletal muscle, skin, lung, blood vessels, brain, myocardium, and adipose tissue, where it forms a network of microfilaments interacting with other ECM components.[9,29] The unique character of the Col VI network in articular cartilage is its localization in the PCM immediately surrounding chondrocytes.[30] Our study provides the interesting finding that soluble Col VI stimulates both normal and OA chondrocytes to proliferate faster. Utilizing staining for Ki67, an antigen associated with cell proliferation, we observed an increase in the number of Ki67-expressing chondrocytes upon Col VI treatment, showing that the increase in cell number was indeed due to increased cell proliferation and not due to reduced cell death. The proliferative effect appears specific to soluble Col VI, as a related soluble Col I has no appreciable effect on proliferation. Col VI interacts with Col II and IX as well as other PCM components such as fibronectin and hyaluronan, and is supposed to anchor the chondrocyte PCM to the ECM.[5] The specific effect of soluble Col VI is, however, not mimicked by immobilized Col VI suggesting that the proliferative action is due to a ligand–receptor rather than a cell–ECM interaction.

Identification of COL6A4 as a susceptibility locus for OA as well as the accelerated OA observed in Col6a1-null mice provided an association between Col VI function and OA.[11,12] However, a precise molecular basis for Col VI function in articular cartilage is still unclear. Our results demonstrate that a deficiency in Col VI could lead to a reduction in the proliferation of chondrocytes or chondrogenic progenitor cells, leading to a possible regeneration defect and accelerated OA in Col6a1-null mice. Studies on Col6a1-null mice demonstrated a clear, early, and spontaneous onset of hip osteoarthritis as compared with wild-type mice.[12,13] In addition, the Col6a1-null mice showed perturbations in the trabecular bone structure and mineralization implicating a dysregulation of the endochondral ossification process.[13] The link between Col VI and chondrocyte proliferation provides a potential mechanistic basis for the altered ossification observed in Col6a1-null mice. Interestingly, Col VI has also been observed to be transiently upregulated during chondrocyte differentiation and maturation in isolated chicken

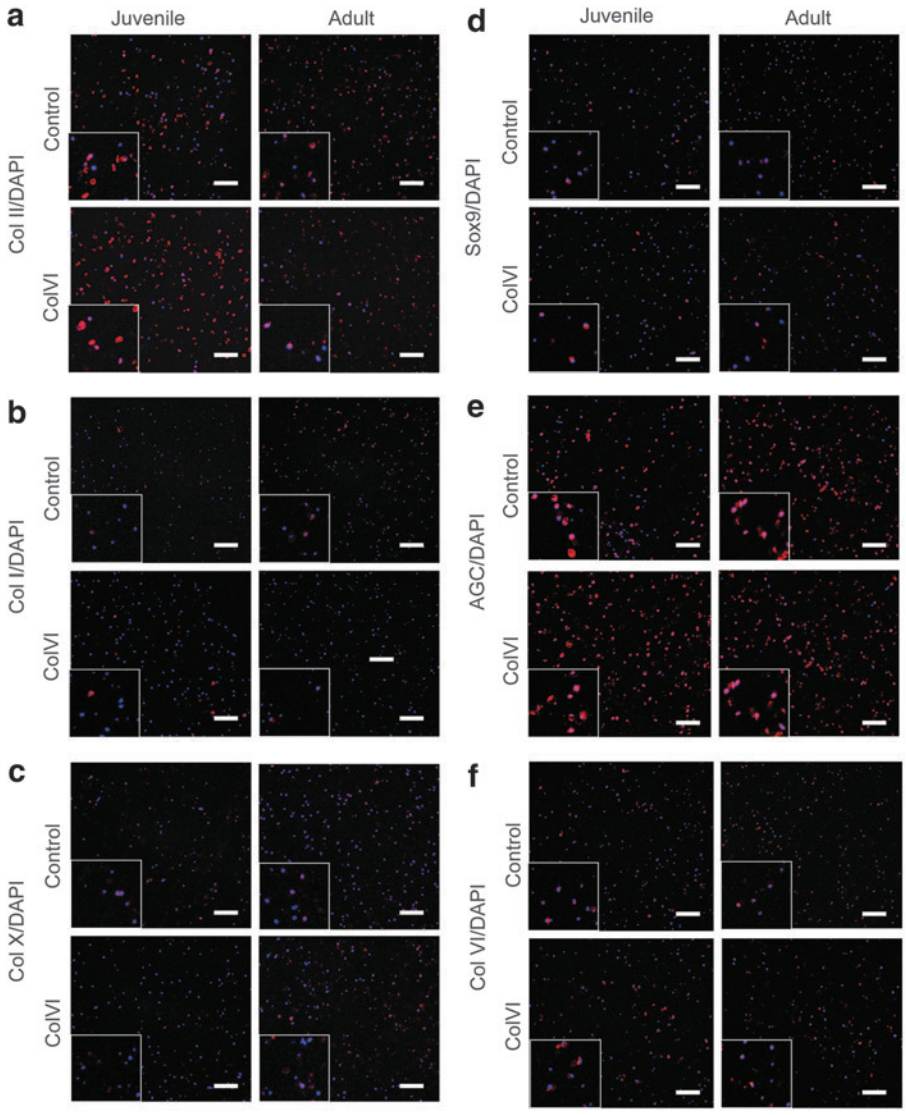


FIG. 6. Col VI-treated chondrocytes maintain a similar potential for engineering cartilage as untreated chondrocytes. Chondrogenic marker expression in three-dimensional (3D) biomimetic hydrogels seeded with control or Col VI-treated chondrocytes after 3 weeks of culture. *Red*: immunofluorescence staining for **(a)** collagen 2A1 (Col II), **(b)** collagen 1A1 (Col I), **(c)** collagen 10 (Col X), **(d)** Sox9, **(e)** aggrecan (AGC), and **(f)** collagen 6A1 (Col VI); *Blue*: cell nuclei stained with DAPI. Scale bar = 30 μm. *Insets* show a higher magnification image of cells in each field.

chondrocytes as well as in mouse and bovine embryos.[31–33] However, the precise stages and effect of Col VI expression and underlying molecular details are not understood. In the context of our findings, it will be interesting to investigate the effect of endogenous as well as soluble Col VI on chondrogenic proliferation and differentiation in the embryonic mesoderm and in adult stem cells.

Although cartilage tissue engineering is an attractive approach for cartilage injuries, the potential of this approach is limited by the scarcity of effective cell sources. For autologous chondrocyte implantation (ACI) procedures, both the low numbers of isolated chondrocytes obtained from patients as well as their effective expansion are challenging. Therefore, identification of Col VI as a soluble biologic for the efficient expansion of chondrocytes can be applied to cartilage tissue engineering and clinical applications, irrespective of the precise biological context of its function. The present study demonstrates the ability of soluble Col VI to expand chondrocytes in a 2D monolayer culture whereas maintaining a high expression of chondrogenic markers like Col II and Sox9 as well as a low expression of Col I and Col X markers for fibrocartilage and chondrocyte hypertrophy, respectively. These observations suggest that Col VI treatment would potentially be beneficial in overcoming another challenge that is the dedifferentiation of chondrocytes with increasing passages of cell culture. Soluble Col VI treatment can shorten the time required for chondrocyte expansion for procedures like ACI. In addition, presence of soluble Col VI does not lead to dedifferentiation of chondrocytes in the short culture time. Strategies applying various growth factors (like TGFβ, BMPs, and Wnt factors) or more simply 3D culture have been previously suggested for redifferentiation of chondrocytes.[28,34]

To assess the potential of normal chondrocytes expanded in the presence of soluble Col VI to engineer cartilage, we utilized a PEG-CS 3D biomimetic hydrogel that we have previously characterized for cartilage tissue generation.[18] Both control and Col VI-treated normal chondrocytes (adult and juvenile) showed robust cartilage tissue generation when seeded in equal numbers and cultured in the 3D biomimetic hydrogel for 3 weeks. Expression of chondrogenic markers like *AGC*, *SOX9*, *COL2A1,* and *COL6A1* was maintained or enhanced after 3 weeks of culture in both control and Col VI-treated chondrocytes. Similarly, all the chondrocyte-seeded

hydrogel constructs showed robust Col II and AGC protein expression as well as high expression for Sox9 and Col VI, whereas Col I and Col X expression remained minimal. In addition to the enhanced expression of chondrogenic factors in 3D culture, there was an equivalent accumulation of total GAG as well as high compressive moduli in the control and Col VI-treated chondrocyte-seeded hydrogels. Cumulatively, these data showed that the biochemical and biomechanical characteristics of cartilage engineered by Col VI-expanded chondrocytes are similar to untreated chondrocytes. Therefore, the Col VI-expanded chondrocytes effectively engineer cartilage tissue in 3D biomimetic hydrogels, comparable to the chondrocytes that have not been treated with Col VI.

Understanding the molecular factors that regulate cartilage function and regeneration is of great fundamental and translational interest for devising more effective and successful cartilage engineering strategies. This study highlights the potential of intrinsic cartilage PCM factors in regulating its growth and function. In particular, identification of additional factors like Col VI may provide novel means of modulating chondrocyte expansion to facilitate clinical transplantation of chondrocytes as well as cartilage tissue engineering.

Authors' Contributions

N.B. and P.S. designed the study; P.S., L.D., and J.H.L. performed experiments; S.B.G., J.L.D., and W.J.M. helped procure normal and patient samples; J.H.L. and F.Y. provided PEG hydrogels; N.B. and P.S. analyzed and interpreted data; R.L.S. and W.J.M. contributed to data interpretation and all authors contributed to writing the article.

Disclosure Statement

The authors declare no competing financial interests.

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