

Extracellular Matrix Domain Formation as an Indicator of Chondrocyte Dedifferentiation and Hypertrophy

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Cartilage injury represents one of the most significant clinical conditions. Implantation of expanded autologous chondrocytes from noninjured compartments of the joint is a typical strategy for repairing cartilage. However, two-dimensional culture causes dedifferentiation of chondrocytes, making them functionally inferior for cartilage repair. We hypothesized that functional exclusion of dedifferentiated chondrocytes can be achieved by the selective mapping of collagen molecules deposited by chondrogenic cells in a three-dimensional environment. Freshly isolated and *in vitro* expanded human fetal or adult articular chondrocytes were cultured in a thermoreversible hydrogel at density of 1×10^7 cells/mL for 24 h. Chondrocytes were released from the gel, stained with antibodies against collagen type 2 (COL II) or COL I or COL X and sorted by fluorescence activated cell sorting. Imaging flow cytometry, immunohistochemistry, quantitative polymerase chain reaction, and glycosaminoglycan (GAG) assays were performed to evaluate the differences between COL II domain forming and COL II domain-negative cells. Freshly dissected periarticular chondrocytes robustly formed domains that consisted of the extracellular matrix surrounding cells in the hydrogel as a capsule clearly detectable by imaging flow cytometry (ImageStream) and confocal microscopy. These domains were almost exclusively formed by COL II. In contrast to that, a significant percentage of freshly isolated growth plate pre-hypertrophic and hyperdrophic chondrocytes deposited matrix domains positive for COL II, COL I, and COL X. The proportion of the cells producing COL II domains decreased with the increased passage of *in vitro* expanded periarticular fetal or adult articular chondrocytes. Sorted COL II domain forming cells deposited much higher levels of COL II and GAGs in pellet assays than COL II domain-negative cells. COL II domain forming cells expressed chondrogenic genes at higher levels than negative cells. We report a novel method that allows separation of functionally active chondrogenic cells, which deposit high levels of COL II from functionally inferior dedifferentiated cells or hypertrophic chondrocytes producing COL X. This approach may significantly improve current strategies used for cartilage repair.

Introduction

RESTORATION OF ARTICULAR cartilage represents a major challenge for orthopedic surgeons due to the lack of self-regeneration of cartilage tissue after injury. Autologous chondrocyte implantation (ACI) is one of the treatments used for restoration of moderate-to-large cartilage defects in young patients.¹ ACI is a two-stage procedure that requires expansion of autologous chondrocytes *ex vivo*, and subsequent reimplantation into the site of injury. This approach has been limited by the fact the cultured cells elaborate

hyaline-like fibrocartilage with inferior histologic and biomechanical properties as compared to native hyaline cartilage. It has been demonstrated that during *in vitro* expansion, the phenotype of chondrocytes is unstable and rapidly lost during passaging in monolayer cultures.^{2,3} This process of losing the chondrogenic phenotype is termed dedifferentiation and is characterized by the loss of cellular ability to synthesize cartilaginous extracellular matrix (ECM) molecules, such as type II collagen (COL II).⁴ Morphologically, chondrocytes cultured in monolayers rapidly lose their typical round shape and transform into flat fibroblast-like cells.

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It has been proposed that three-dimensional (3D) culture in pellets better preserves the chondrogenic phenotype and that dedifferentiated chondrocytes can re-express COL II when cultured in 3D.^{5,6} Nevertheless, not all dedifferentiated chondrocytes regain their chondrogenic phenotype in 3D culture. In long-term cultures, a portion of articular chondrocytes can also undergo hypertrophic transformation and deposit COL X.⁷ Separation or enrichment of COL II producing cells from dedifferentiated or hypertrophic chondrocytes could enhance the efficacy of ACI.

Cells are usually sorted or separated based on their differences in cell surface antigens or cytoplasmic density. Cellular differences in surface antigens (CD markers) enable cell sorting by fluorescent-activated cell sorting (FACS) or magnetic-activated cell sorting. Cellular differences in cytoplasmic density help to separate different cells by density gradient centrifugation. CD marker expression profile of cultured, dedifferentiated chondrocytes has been compared to freshly isolated chondrocytes by several groups.^{8,9} A group of CD markers such as CD49c, CD49f, and CD44 have been proposed to predict the chondrogenic capacity of monocultured chondrocytes.¹⁰ However, all previously published studies are based on a complex combination of CD markers, which has an indirect, partial correlation with the chondrogenic phenotype. Currently, cell sorting technologies have not been based on the detection of ECM molecules produced by the cells.

In this study, we proposed a new method of cell sorting that we have termed extracellular matrix domain (EMD) detection (EMDD), which allows for the enrichment of functionally active, COL II-producing chondrocytes and the exclusion of dedifferentiated and hypertrophic cells expressing COL X. EMDD is based on a thermoreversible gel system that creates a 3D culture environment for chondrocytes and permits the isolation of cells without disruption of the ECM capsule produced around secretory active chondrocytes. The goal of this methodology is to select cells that possess a functional phenotype predisposed to COL II synthesis, the predominant constituent of hyaline cartilage, rather than COL I synthesis more typical of the fibrocartilage ECM.

Methods

Cell culture and expansion

Human fetal tissues were obtained from Novogenix Laboratories, LLC following informed consent and elective termination. All donated material was anonymous and carried no personal identifiers. The developmental age was determined by ultrasound. Periarticular chondrocytes or growth plate fetal chondrocytes were isolated under the dissection microscope from 17-week-old human fetuses. Adult articular chondrocytes were isolated from full thickness cartilage dissected from knee biopsies of a patient undergoing total knee replacement. Isolation of chondrocytes was carried out as previously described.^{11,12} In short, cartilage was digested for 20–22 h in collagenase type II (0.15%) in the Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). Chondrocytes were then cultured in the chondrocyte proliferation medium (DMEM/F12 supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin). The medium was

refreshed every 3–4 days. Upon confluence, chondrocytes were trypsinized. Half of the cells were cryopreserved; the other half was replated. All chondrocytes (fetal or adult) used in this article have experienced at least one cycle of freeze-and-thaw. All reagents used for cell culture were purchased from Invitrogen, unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

3D culture in thermoreversible hydrogel or agarose

To assess the collagen expression signature of freshly isolated chondrogenic cells, either fetal periarticular, growth plate chondrocytes or adult articular chondrocytes were first resuspended in a thermoreversible hydrogel (Cosmo Bio [Tokyo, Japan]) on ice at a 1×10^7 cell/mL density (Fig. 1A). Next, hydrogels with cells were solidified by incubating at 37°C and cultured in the chondrogenic medium (the StemPro base medium with 50 µg/mL of ascorbic acid, 10 ng/mL of transforming growth factor-β3 (TGFβ3), and 100 U penicillin/mL and 100 µg/mL streptomycin) stimulating ECM production. Twenty-four hours later, hydrogels were liquefied by cooling plates on ice. Chondrocytes were released from hydrogels, washed with phosphate-buffered saline (PBS), and stained with antibodies against COL II, COL I, or COL X, respectively, or in combination (as indicated in figure legends), and analyzed by FACS. The entire procedure is summarized in Figure 1. To visualize ECM domains on histology sections, we cultured fetal periarticular chondrocytes (Passage 1) in an agarose gel for 7 days in the chondrogenic medium. The agarose hydrogel was used because of scientifically better quality of sections after paraffin embedding as compared to the thermoreversible hydrogel. Toluidine Blue staining was performed to show glycosaminoglycan (GAG) formation, and immunohistochemistry was carried out to indicate the expression of COL II.

FACS and Imagestream analysis

Noncultured fetal periarticular and growth plate chondrocytes, adult articular chondrocytes or *in vitro* expanded periarticular (passage 1–6) chondrocytes were suspended in liquefied thermoreversible hydrogel at a density of 10 million cells per mL on ice; 100 µL of gel was used for each sample. After the addition of cells and mixing, hydrogels were solidified by incubating at 37°C for 5 min. Hydrogel droplets with trapped cells were cultured in the chondrogenic medium for 24 h before sorting. After 24 h, culture plates were placed on ice, the hydrogel was liquefied, and recovered live cells incubated with rabbit antibodies against COL II, COL I, or COL X (Abcam) or the mouse antibody against CD29 followed by washing with PBS containing 1% bovine serum albumin (BSA). For dedifferentiation studies, cells were next incubated with secondary antibodies conjugated with Alexa-488 or –594 fluorochrome, and then washed with PBS containing 1% BSA. Control samples were processed without incubation with a primary antibody. For simultaneous detection of two collagens in matrix domains, primary antibodies were labeled with Zenon® Alexa 594 or Alexa488 IgG2a Labeling Kits (Invitrogen). Stained samples were sorted using a BD FACSAria or analyzed using the LSRII cytometer (BD Biosciences). Data files were exported and analyzed with FACSDiva software (BD Biosciences). The same cells were also analyzed using the ImageStream IS100

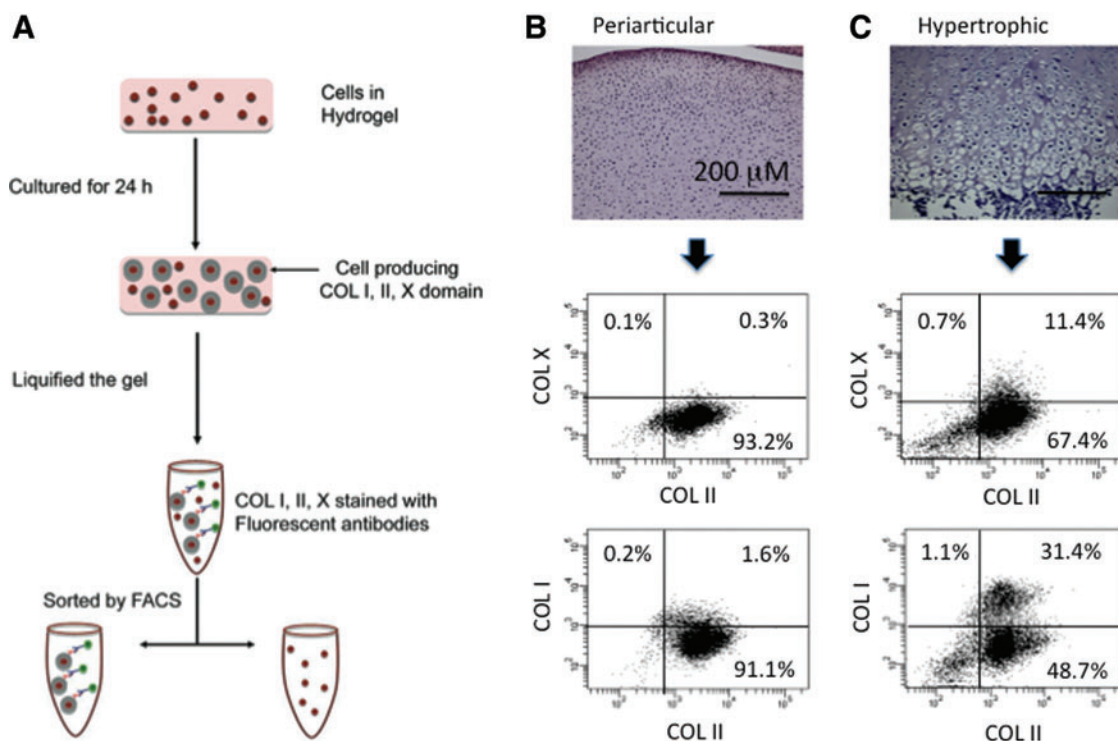


FIG. 1. Chondrocytes produce collagen-rich extracellular matrix (ECM) domains in hydrogels. **(A)** Schematic diagram of experimental design. Chondrocytes were first resuspended in the thermoreversible hydrogel (liquid phase). Then, the hydrogel with cells was solidified and cultured for 24 h. To release cells with intact matrix domains, the hydrogel was liquefied by placing on ice and fluorescence activated cell sorting (FACS) performed to separate collagen type 2 (COL II) producing cells from nonproducing cells. **(B)** Representative image of the periarticular region, 17 weeks of development. Below the image, FACS plots showing matrix domain formation by fetal freshly isolated periarticular chondrocytes. **(C)** Representative image of the growth plate hypertrophic chondrocytes, 17 weeks of development. Below the image are FACS plots showing matrix domain formation by fetal freshly isolated growth plate chondrocytes isolated at 17 weeks of development. Quantitative analysis of matrix domain forming cells is shown as percentage of total analyzed live cells (average of three independent experiments). Color images available online at www.liebertpub.com/tec

imaging flow cytometer (Amnis) and analyzed using IDEAS software (Amnis). Data were obtained from a minimum of 5000 cells. ImageStream is a combination of flow cytometry and fluorescence microscopy. On one hand, it can analyze a large number of cells based on their fluorescent labels, on the other hand, it also enables detailed morphometric analysis based on fluorescent images for individual cells.¹³

RNA isolation and quantitative polymerase chain reaction

Gene expression analysis of cells in monolayer culture was performed as described.^{11,12} RNA samples were isolated with the RNeasy Micro Kit (Qiagen). For quantitative polymerase chain reaction (qPCR), 1 μ g of total RNA was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen). qPCR was performed on cDNA samples by using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). PCR reactions were carried out on ViiATM 7 Real-Time PCR System (Life Technologies). For each reaction, a melting curve was generated to test primer dimer formation and nonspecific priming. The primers for real-time PCR are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tec). Relative expression was calculated using the double delta Ct method.¹⁴ A geo-

metric average of B2M (β 2 microglobulin), TBP (TATA-box binding protein), and RPL7 (ribosomal protein L7) was used for gene normalization.

Pellet culture and chondrogenic differentiation

Passage 2 of fetal periarticular chondrocytes was sorted based on the expression of Col II domains. Fifty thousand sorted cells (either positive or negative) were seeded in one well of a round-bottom 96-well plate (nontissue culture treated). Cells were aggregated by centrifugation at 500 g for 5 min. Cell aggregates were cultured in the chondrogenic differentiation medium (the StemPro base medium supplemented with 50 μ g/mL of ascorbic acid, 10 ng/mL of TGF β 3, and 100 U penicillin/mL and 100 μ g/mL streptomycin) for 1 week. The medium was refreshed every other day.

Histology and immunohistochemistry

Three cell aggregates formed by COL II domain-positive or -negative chondrocytes were fixed in 10% formalin for 15 min, dehydrated with ethanol, and embedded in paraffin using routine procedures. A microtome (Leica) was used to cut 5- μ m sections. Slides were then deparaffinized and stained for sulfated GAG with Toluidine Blue. For immunohistochemistry, paraffin sections were processed using

routine techniques. Rabbit antibodies against COL I and COL II (both Abcam) were used, followed by incubation with horseradish peroxidase-conjugated secondary antibodies against rabbit immunoglobulin G (Vector Laboratories). Antibodies were then visualized with Peroxidase substrate Kit DAB (Vector Laboratories). Images were acquired using either the confocal TCS SP2 AOBS laser-scanning microscope system (Leica Microsystems, Inc.) with 63×(1.4 numerical aperture [NA]) oil-immersion objectives or using the Zeiss AxioVision software version 4.8 Carl Zeiss Microscope (Carl Zeiss) equipped with ApoTome.2: modules for Axio Imager.2 and Axio Observer with 10×, 20×, 40×(1.3 NA), and 63×(1.4 NA) oil-immersion objectives.

Quantitative GAG and DNA assay

Cell aggregates ($n=3$) were washed with PBS and stored at -80°C for 16–20 h. Subsequently, they were digested in a buffer (1 mg/mL proteinase K in the Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.6) containing 18.5 $\mu\text{g}/\text{mL}$ iodoacetamide and 1 $\mu\text{g}/\text{mL}$ pepstatin A) for more than 16 h at 56°C . The GAG content was spectrophotometrically determined with 1,9-dimethylmethylene blue chloride staining in the phosphate buffer with ethylenediamine-

tetraacetic acid buffer (14.2 g/L Na_2HPO_4 and 3.72 g/L Na_2EDTA , pH 6.5) using an FLUO star Omega microplate reader (BMG LABTECH) at an absorbance of 520 nm with chondroitin sulfate as a standard. Total DNA amounts in aggregates were determined by a CyQuant DNA Kit (Molecular Probes).

Statistical analysis

All experiments were repeated at least three times. GAG and DNA quantifications were examined for statistical significance with the paired Student's t test. For statistical analysis of qPCR data, the ΔCt for positive and negative cells were subject to a simple t -test. p Values of <0.05 were considered as statistically significant.

Results

Freshly isolated chondrocytes form ECM domains in thermoreversible hydrogel system

Freshly dissected fetal periarticular chondrocytes robustly formed domains that consisted of the ECM surrounding cells in the hydrogel as a capsule-like structure. These domains were almost exclusively formed by COL II (Fig. 2). In

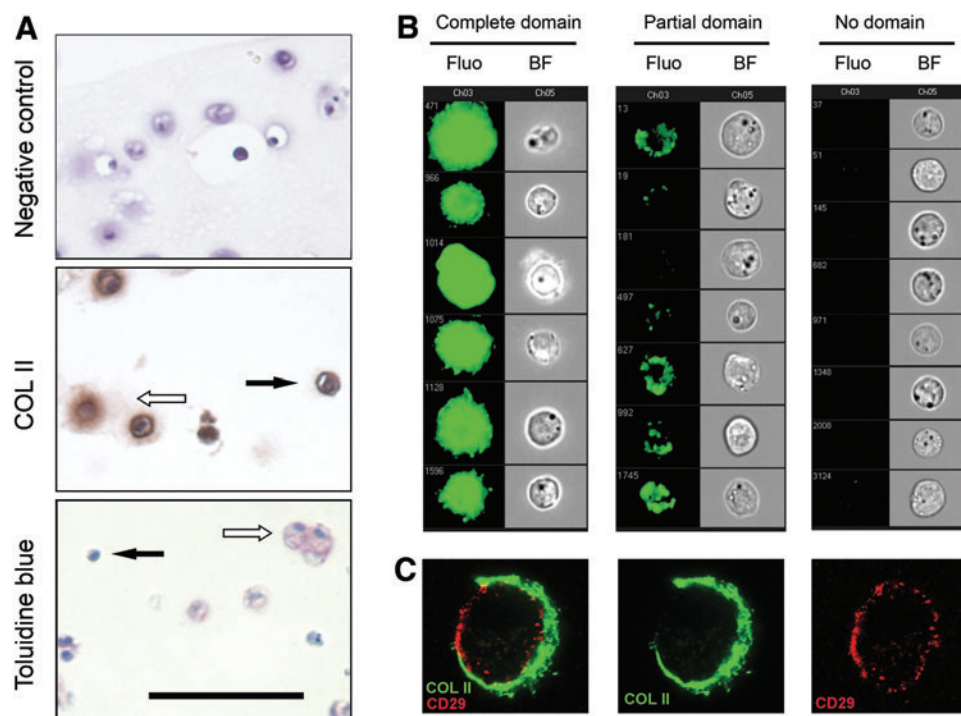
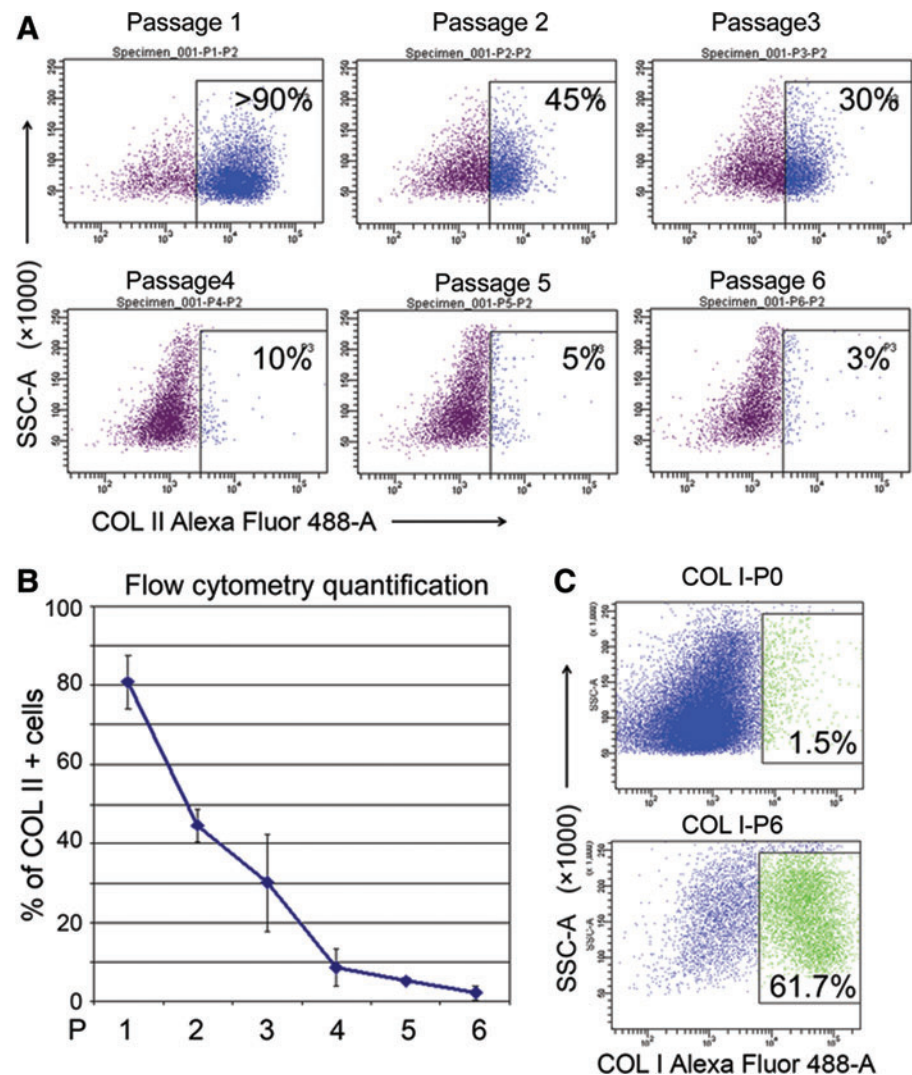


FIG. 2. Visualization of the ECM domains. **(A)** Fetal periarticular chondrocytes (passage 2) were cultured in the agarose gel for 7 days before immunohistochemical analysis. Next, cultures were fixed, embedded in paraffin, sectioned, and analyzed by immunohistochemistry. The primary antibody against COL II, followed by the secondary antibody labeled with horseradish peroxidase and DAB substrate were used to reveal ECM domains (brown color). The negative control was treated with the same procedure without primary antibody incubation. Toluidine Blue staining was also performed to demonstrate deposition glycosaminoglycans (purple color). Bar = 50 μm . Black arrow indicates cells without ECM domains. Empty arrow indicates cells producing ECM domains. **(B)** Imaging flowcytometry (ImageStream analysis; Amnis) was performed on fetal periarticular chondrocytes (P2) cultured in thermoreversible gels for 24 h. COL II domains were visualized by COL II antibody staining followed by a secondary antibody conjugated with Alexa 488 fluorochrome (green). Fluo = fluorescent images; BF = bright field. Analysis showed the presence of viable chondrocytes forming complete or partial COL II domains. Dedifferentiated chondrocytes did not form COL II matrix domains. **(C)** The high-resolution confocal image of a live chondrocyte with ECM domain. Chondrocytes were released from the gel and stained with primary antibodies against COL II (green) to mark extracellular collagen deposition, or CD29 (red) to visualize the plasma membrane. Color images available online at www.liebertpub.com/tec

FIG. 3. The ratio of COL II producing cells decreases as chondrocytes dedifferentiate. **(A)** Fetal periarticular chondrocytes from passages 1 to 6 were cultured in the thermoreversible gel; 24 h later, cells were released from the gel and analyzed by FACS. A representative donor ($n=3$) is shown. **(B)** Quantification of chondrocyte dedifferentiation by FACS analysis. Percentages of chondrocytes producing COL II ECM domains are shown. Results are shown as mean \pm standard error (SE) of three independent experiments. Each experiment represents one donor. P=passage number. **(C)** Robust detection of COL I domains by FACS; percentage of cells forming COL I matrix domains markedly increased with the passage number. Chondrocytes at P0 and P6 were cultured in the thermoreversible gel for 24 h. FACS plots for a representative donor are shown; percentage of COL I domain forming cells represent an average of three independent donors analyzed separately. $N=3$. Color images available online at www.liebertpub.com/tec



contrast to that, a significant percentage of freshly isolated growth plate chondrocytes deposited matrix domains positive for COL II, COL I, and COL X. This is consistent with the expression signature of hypertrophic cartilage cells (Fig. 1C).

Decrease of COL II producing cells reflects the dedifferentiation of chondrocytes

Morphology of chondrocytes expanded in two-dimensional (2D) for up to six passages is shown in Supplementary Figure S1. From passage 1 to 6, chondrocytes turned into fibroblast-like cells with a flat and spindle-like appearance different from their original small, polygonal shape. The most dramatic change in morphology happened between passage 3 and passage 4. qPCR confirmed that the expression of chondrogenic genes dropped rapidly during the 2D expansion (Supplementary Fig. S2). In agreement with morphological findings, the level of tested chondrogenic genes also dropped quickly from passage 1 to passage 2. Less than 1% of expanded periarticular chondrocytes expressed COL X after six passages (not shown), and therefore, the next studies focussed on COL I and II. We next tested if dedifferentiation of chondrocytes correlates with the formation of COL II domains in hydrogel. Up to 90% of cells showed robust COL

II-rich matrix domain formation at passage 0–1, but this potential progressively decreased with the passage number (Fig. 3A, B). As shown in Figure 3C, the ratio of fetal chondrocytes producing COL I-rich domains markedly increased (from 1–5% to 60–70%) after *in vitro* expansion for 6 passages (Fig. 3B). It is important to mention that fetal synovial or bone marrow-derived stromal cells obtained from the same fetuses produced no COL II or COL I matrix domains with a 24–48-h interval.

Cultured adult articular chondrocytes also showed the ability to form EMD in the thermoreversible gel. However, only a small subset (~ 10) of chondrocytes from adult articular cartilage deposit ECM domains can be detected by COL II antibody staining (Supplementary Fig. S3) after *in vitro* expansion (passage 3).

Chondrocytes that maintain their phenotype can be separated from dedifferentiated cells based on matrix domain

We next tested if heterogeneous *in vitro* expanded periarticular chondrocytes could be separated on the basis of their collagen deposition signature. First, to visualize ECM domains on histological sections, chondrocytes were

cultured in an agarose gel for 7 days before histological assessment. COL II antibody-based labeling was used to detect ECM domains surrounding chondrocytes (Fig. 3A). GAGs were also deposited into ECM domains by some of the gel-trapped chondrocytes (Fig. 3A), indicating functional heterogeneity of the cultured cells. ImageStream analysis and high-resolution confocal microscopy were carried out next to confirm the specific morphological extracellular appearance of matrix domains produced by chondrocytes cultured in the thermoreversible hydrogel (Fig. 3B, C, respectively).

Next, FACS analysis was performed to sort COL II matrix domain producing chondrocytes after 2 passages in 2D culture (Fig. 4A). No difference in viability was observed between these two subsets as detected by the quantitative viability test (Fig. 4B). Isolated COL II domain-positive or COL II-negative cells were seeded to form aggregates, respectively (Fig. 5A). Immunohistochemical assessment of cell aggregates showed that COL II domain forming cells almost exclusively deposited COL II proteins, while COL II-negative cells produced COL I (Fig. 5A). qPCR data from 3D culture of sorted cells also confirmed higher expression of the chondrogenic genes in COL II domain-positive cells (Fig. 5B), while COL I expression was much higher in COL II domain-negative cells, indicating that they represent dedifferentiated chondrocytes (Fig. 5C). Although immunostaining of cell aggregates with Toluidine Blue showed a minimal difference in GAG deposition (Supplementary Fig. S4), quantitative GAG production analysis normalized to DNA levels indicated that COL II-positive cells produced more GAG than negative cells, further indicating that COL II domain forming cells are functionally superior (Fig. 5D). To further confirm the differences of aggregate sizes formed by sorted EMD-positive and -negative cells, we quantified the diameters of aggregates. As shown in Figure 5E, aggregates formed by positive cells were significantly bigger. Finally, the expression levels of COL II isoforms A and B were determined by qPCR in the EMD-positive and -negative populations sorted by FACS. Positive cells expressed significantly higher levels of both isoforms than negative cells (Fig. 5F). The same data with different calculations indicated that both positive and

negative cells express more COL II isoform A than isoform B (Fig. 5G).

Discussion

In this study, we used a thermoreversible hydrogel to permit ECM domain formation by cultured chondrocytes and then separated COL II producing cells by FACS. COL II domain-positive or -negative cells demonstrated very distinct characteristics in regard to morphology, histological appearance, and gene expression, suggesting that cells retaining their chondrogenic properties can be enriched based on their production of COL II. In particular, the data clearly demonstrate the superiority of sorting using COL II domain generation rather than GAG matrix domains since COL I-positive cells still produced substantial GAG after culture in aggregates under conditions suitable for re-expression, while failing to recover substantial COL II production. The phenotypically normal COL II-positive cells can be defined/enriched by sorting COL II producing cells that may be more phenotypically stable upon implantation.

This study demonstrates for the first time functional cell sorting based on ECM production of two different markers by multiplex sorting. Current techniques for cell sorting are generally based on principles that employ either separation based on the cytoplasmic density or surface antigen detection. Density gradient centrifugation has long been used for rough separation of cells, although it is only applicable for separation of very distinct cell populations with obvious differences in cellular density.¹⁵ On the other hand, isolation of cells by CD markers is more broadly applicable. This principle is based on differences in cell surface antigen expression, which is used for immunophenotyping of the cells. CD markers have numerous physiological functions, often acting as receptors or ligands in specific cell signaling pathways. Some CD markers also function as adhesion molecules. Although cell sorting by CD markers has greatly advanced cell biology, the link between surface antigens and cell function is often indirect and empirical. Our study provides an important enhancement of current technologies used for cell sorting.

Previously, studies indicated that some articular chondrocytes undergo hypertrophy and deposit COL X in long-term cultures.⁷ Although no significant deposition of COLX by cultured periarticular chondrocytes was observed in this study, detection of COLX depositing cell subset was clearly observed after plating freshly isolated hypertrophic chondrocytes in hydrogels. Detection and labeling of the COLX depositing cells offer a unique tool for the isolation of highly purified hypertrophic chondrocytes.

Matrix-induced autologous chondrocyte implantation (MACI) is a very popular treatment for cartilage defects.¹⁶ Like the classical procedure of ACI, it includes arthroscopic excision of biopsies from low-weight-bearing areas of healthy cartilage, isolation and expansion of chondrocytes in the laboratory, and implantation of chondrocytes into the cartilage defect. However, instead of injection into defects as a cell suspension, chondrocytes were seeded on a bilayer of porcine-derived type I/type III collagen after *in vitro* expansion. One major reason that this procedure may fail is that dedifferentiated chondrocytes are introduced into the defects together with cartilage matrix-producing cells. Our

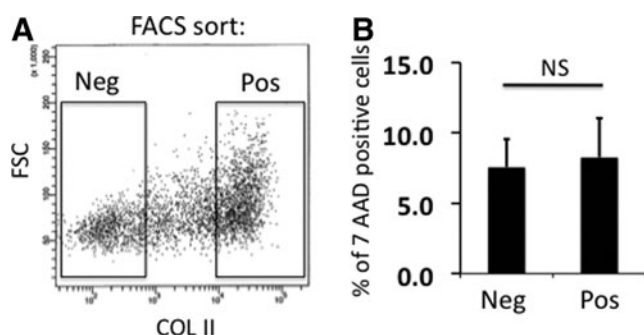


FIG. 4. Functional sorting of COL II matrix domain producing chondrocytes. **(A)** Fetal periarticular chondrocytes at passage 2 were sorted based on their production on COL II domains visualized by an antibody staining as described earlier. POS=positive, NEG=negative for COL II matrix domains. Representative sorting gates. **(B)** Viability of chondrocytes was tested by FACS using 7-aminoactinomycin D (7AAD) staining and showed no difference between pos and neg cells. NS=not significant.

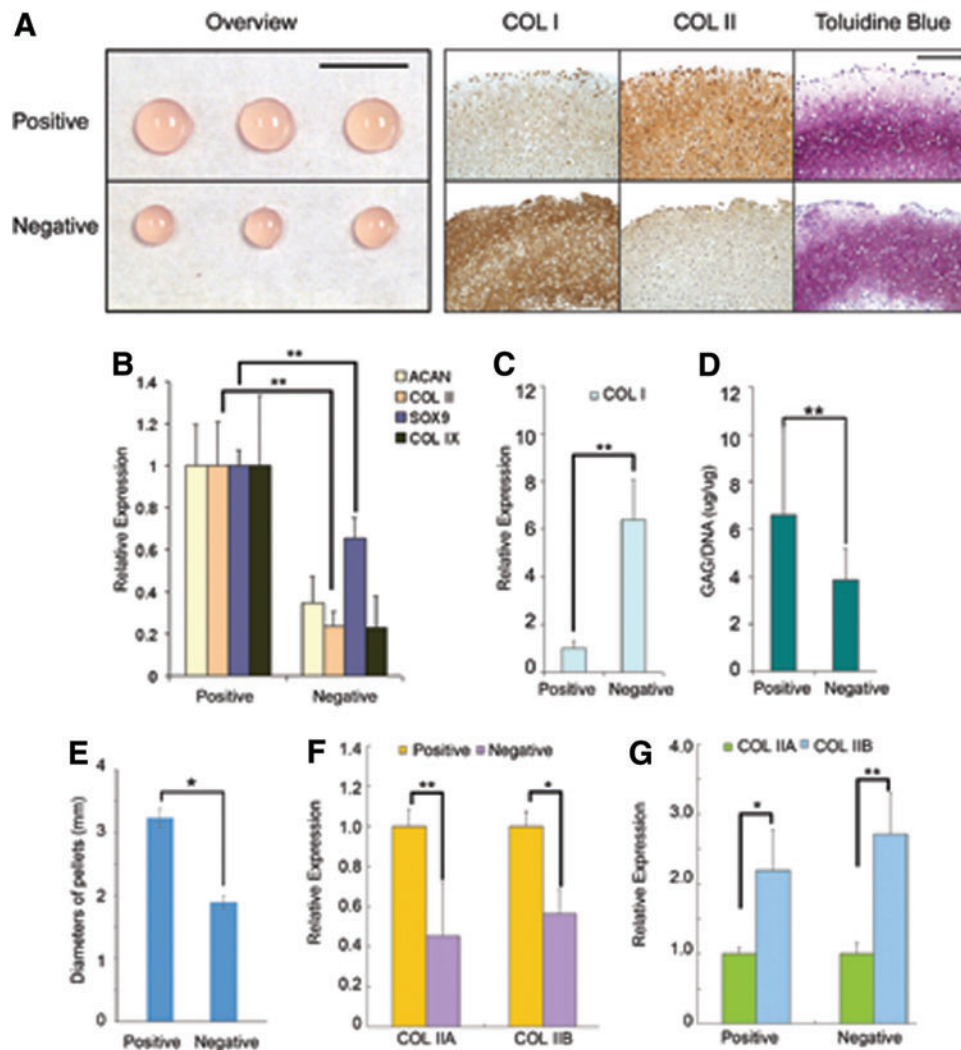


FIG. 5. Morphological and functional analysis of COL II domain- positive and COL II domain-negative chondrocytes. Fetal periarticular chondrocytes (passage 2) were sorted as shown in Figure 4(A) and seeded in 96-well plates at 50,000 cells per well density and cultured for 7 days. **(A)** An overview of the resulting pellets after 1 week of culture. Bar=0.5 cm; Histological examinations of chondrogenic pellets generated from COL II domain-positive or -negative cells. Bar=100 μm. Five microgram-thick sections were stained with primary antibodies against COL II or COL I; proteoglycan deposition was marked by Toluidine Blue staining. The positive immunohistochemical signal is shown in brown color; positive proteoglycan staining is shown in purple color. Nuclei were counterstained with hematoxylin. Scale bar shown for images is 20 mm. Bright field images were acquired using Zeiss Axiovision software version 4.8 Carl Zeiss Microscope (Carl Zeiss) equipped with ApoTome.2: Modules for Axio Imager.2 and Axio Observer with 10× objectives. **(B)** Expression of chondrogenic genes was significantly higher in COL II domain producing cells than in COL II-negative cells after 7 days of culture in aggregates. Results are shown as mean ± SE of three independent experiments. Double asterisk represents $p < 0.01$. **(C)** COL I mRNA expression was significantly lower in COL II-positive cells than in negative cells as determined by real-time polymerase chain reaction analysis. Results are shown as mean ± SE of three independent experiments. Double asterisk represents $p < 0.01$. **(D)** COL II domain-positive cells produced more glycosaminoglycans (GAG) than negative cells. Results are shown as mean ± SE of three independent experiments. **(E)** Diameters of aggregates (as shown in Fig. 5A) formed by extracellular matrix domain (EMD)-positive chondrocytes were significantly bigger than negative cells. Asterisk represents $p < 0.05$. **(F)** Expression of COL II isoforms A and B were significantly higher in EMD-positive cells compared to EMD-negative cells. Expression levels in EMD-negative cells were calculated against positive cells (set as 1). Results are shown as mean ± SE of three independent experiments. Asterisk represents $p < 0.05$; double asterisk represents $p < 0.01$. **(G)** Both EMD-positive cells and -negative cells express more COL II isoform A than isoform B. Expression levels of isoform B were first calculated against isoform A (set as 1). Results are shown as mean ± SE of three independent experiments. Asterisk represents $p < 0.05$; double asterisk represents $p < 0.01$. Color images available online at www.liebertpub.com/tec

method can be used to exclude dedifferentiated cells by sorting out chondrocytes producing COL II domains. With enrichment of COL II producing cells that are implanted into cartilage defects, the efficacy of ACI and MACI could potentially be improved. EMDD can be used as a sensitive

readout for factors that regulate matrix protein production in chondrocytes and other cell types. Similar to other antibodies previously used in clinical practice, antibodies against COL II can be manufactured in good manufacturing practice (GMP) facilities. Monoclonal antibodies have been approved

by FDA for many therapeutic applications, especially for oncology.¹⁷ To improve the performance of ACI or MACI, *in vitro* expanded chondrocytes can be sorted using GMP grade antibodies against COL II, or potentially, other ECM (COL II, I, VI, aggrecan, etc.) and implanted to patients.

Several significant improvements in cartilage tissue engineering have been reported recently.¹⁶ Coculture of chondrocytes with mesenchymal stromal cells, mechanical stimulation of chondrogenic cultures, and control of metabolic parameters allowed producing cartilage tissue with better mechanical and biological characteristics with relatively small chondrocyte numbers.^{18–21} In this study, we report a novel method that allows enrichment for functionally active chondrogenic cells, which may further advance existing cartilage tissue-engineering strategies.

It is important to mention that EMDD can be not only used to sort chondrocytes, but in principle, any cell type that produces ECM in hydrogels. Based on the direct link between ECM production and biological processes such as inflammation, tissue repair, and metastatic cancer invasion, EMDD provides a useful tool to study cell behaviors in such biological events.

In conclusion, our data show that live chondrogenic cells can be separated on the basis of their ECM production using functional cell sorting based on the methodology we called EMDD. This approach can be carried out in a xenogen-free environment and allows for functional enrichment of chondrogenic cells producing high levels of COL II and may lead to significant improvement of current therapeutic approaches in the area of cartilage restoration.

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Disclosure Statement

The authors declare no financial support or any other benefits from commercial sources received for the work reported on in the manuscript. D.E. and L.W. have submitted a patent disclosure to the UCLA office of intellectual property.

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