# Reconstruction of Hyaline Cartilage Deep Layer Properties in 3-Dimensional Cultures of Human Articular Chondrocytes

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**Background:** Articular cartilage (AC) injuries and malformations are commonly noticed because of trauma or age-related degeneration. Many methods have been adopted for replacing or repairing the damaged tissue. Currently available AC repair methods, in several cases, fail to yield good-quality long-lasting results, perhaps because the reconstructed tissue lacks the cellular and matrix properties seen in hyaline cartilage (HC).

**Purpose:** To reconstruct HC tissue from 2-dimensional (2D) and 3-dimensional (3D) cultures of AC-derived human chondrocytes that would specifically exhibit the cellular and biochemical properties of the deep layer of HC.

**Study Design:** Descriptive laboratory study.

**Methods:** Two-dimensional cultures of human AC–derived chondrocytes were established in classical medium (CM) and newly defined medium (NDM) and maintained for a period of 6 weeks. These cells were suspended in 2 mm–thick collagen I gels, placed in 24-well culture inserts, and further cultured up to 30 days. Properties of chondrocytes, grown in 2D cultures and the reconstructed 3D cartilage tissue, were studied by optical and scanning electron microscopic techniques, immunohistochemistry, and cartilage-specific gene expression profiling by reverse transcription polymerase chain reaction and were compared with those of the deep layer of native human AC.

**Results:** Two-dimensional chondrocyte cultures grown in NDM, in comparison with those grown in CM, showed more chondrocyte-specific gene activity and matrix properties. The NDM-grown chondrocytes in 3D cultures also showed better reproduction of deep layer properties of HC, as confirmed by microscopic and gene expression analysis. The method used in this study can yield cartilage tissue up to approximately 1.6 cm in diameter and 2 mm in thickness that satisfies the very low cell density and matrix composition properties present in the deep layer of normal HC.

**Conclusion:** This study presents a novel and reproducible method for long-term culture of AC-derived chondrocytes and reconstruction of cartilage tissue with properties similar to the deep layer of HC in vitro.

**Clinical Relevance:** The HC tissue obtained by the method described can be used to develop an implantable product for the replacement of damaged or malformed AC, especially in younger patients where the lesions are caused by trauma or mechanical stress.

**Keywords:** articular cartilage resurfacing; biology of cartilage; stem cell therapy; tissue engineering

Articular cartilage (AC) is a form of hyaline cartilage (HC) primarily composed of extracellular matrix (ECM), rich in collagen II and proteolglycans (PG)—especially aggrecan. It has a very limited regenerative capacity due to lack of blood vessels and low mitotic activity in the small number of cells that it contains (only 5% of the total AC tissue). AC, which is located in the diarthrodial joint, is a high mechanical stress–bearing tissue and therefore, structurally it represents the uniqueness of HC organization.[8,9,24,26] It is composed of 4 different layers (Figure 1) that differ in their protein content, proteoglycan composition, thickness of the collagen network, and cellular content.[3,9,16,26,30] Among all the layers, the deep layer (layer III [LIII]) of the native AC is the most stress resistant because it comprises thick collagen fibrils, high PG content, and decreased water concentration.[16,26,30] This layered structural organization of AC

gets disturbed to variable degrees because of excessive mechanical stress caused by sports-related trauma or other day-to-day activities, resulting in osteoarthritis.[5,6,11,15,34,38] The International Cartilage Repair Society has prescribed specific "grades of degradation" of AC, according to which when more than 50% thickness of LIII is degraded, AC tissue cannot be repaired without surgical intervention.[7,23] For management of these conditions, various cell-based techniques such as autologous chondrocyte implantation, matrix-assisted chondrocyte implantation, autologous mesenchymal stem cell transplantation, and microfracture are used to ameliorate the pain and arrest the degradation of the tissue.[2,3,15,17,37] New strategies are being developed for making scaffold-based tissue-engineered materials using synthetic and natural materials,[1] biomolecules, growth factors, ECM,[10] and genetically modified cells[21] that would help in repairing the degraded AC.[13,23,25] Based on the evidence,[10] we selected the collagen gel–based Culture Matrix system (Invitrogen) for growing the 3-dimensional (3D) cultures of AC. The main consideration for choosing this system was that the collagen is a natural biomaterial highly biocompatible and absorbable in the tissue.

The success of these procedures and strategies has been limited by several biological factors that restrict the establishment of HC-like properties in the regenerated tissue, which becomes fibrous and eventually nonfunctional.[7,29] For improving the existing strategies of AC repair, a better assessment of the depth and grade of the damage is necessary so that the regenerated tissue is without defects[28] and matches the cellular and matrix properties of native AC.[12]

We describe a method for growing normal human chondrocytes in a newly defined medium (NDM) that contains human umbilical cord serum (UCS), described elsewhere as a better alternative to fetal bovine serum (FBS) for culturing mesenchymal cells.[32,33] We have extensively compared the histological and molecular properties of cultured chondrocytes and the reconstructed 3D cartilage with the native AC properties. Our results clearly show that 3D cartilage reconstructed in NDM yields cellular and matrix properties that are very similar to that of the deep layer of HC. To maintain consistency and avoid batch-to-batch variation in NDM constitution, pooled UCS obtained from 5 independent sources was used.

## MATERIALS AND METHODS

### Materials

*Cell Culture Media.* The classical medium (CM) we used contained Dulbecco Modified Eagle Medium (DMEM high
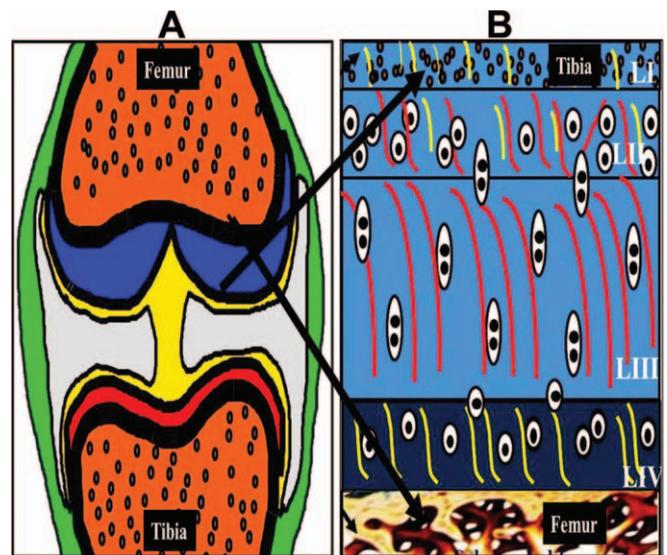


**Figure 1.** (A) Anterior view of the knee exposing the diarthordial joint. (B) The 4 layers of the hyaline cartilage. Layer I (LI), or the superficial layer, consists of round-shaped cells, thin collagen fibrils, and low PG content; layer II (LII), or the intermediate layer, has moderate cell number and thick and thin collagen fibrils; layer III (LIII), or the deep layer, shows sparse cell population with elongated chondrocytes, thick and long collagen fibers, and a high proteoglycan content; layer IV (LIV), or the calcified layer, contains rich concentration or calcium salts, low proteoglycan composition, and less protein synthesis.

glucose; Sigma-Aldrich), supplemented with $1\times$ Glutamax, $1\times$ penicillin streptomycin (both from Gibco), and 10% FBS (Sigma-Aldrich). The NDM contained the same ingredients as the CM, except 10% FBS was replaced with 10% human UCS. Serum was prepared from umbilical cord blood, obtained from voluntary donors at Sridevi Nursing Home, Hyderabad, India.

*Other Reagents.* Total RNA Isolation Reagent (cat No. 1015; Bioserve); collagen type I gel (cat No. 3447-020-01; Invitrogen); sterile Dulbecco phosphate-buffered saline (PBS), which was free of $Ca^{2+}$ and $Mg^{2+}$; 0.05% (wt/vol) hyaluronidase (cat No. H4272; Sigma-Aldrich); 0.25% (wt/vol) trypsin (cat No. T4049; Sigma-Aldrich); 0.2% (wt/vol) collagenase type IV (cat No. C5138; Sigma-Aldrich) were also used. All enzymes were prepared in $1\times$ PBS and filter sterilized using 0.22-$\mu$m syringe filters.

*Antibodies.* Antibodies included rabbit polyclonal to collagen I, collagen II, and aggrecan (ab292, ab34712, and ab36861, respectively) and rabbit monoclonal to CD44

(ab51037). All primary antibodies were Abcam products. Alexa Fluor 594 goat antirabbit IgG (A11012; Invitrogen) and Fluoroshield with DAPI (F6057; Sigma-Aldrich) were also used.

*Primers and Other Reagents.* High-performance liquid chromatography (HPLC)–purified DNA nucleotide primers of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen II, collagen I, aggrecan, and CD44 were obtained from Bioserve. The Super-Script III First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) (cat No. 18080-051; Invitrogen) and SafeView Classic (cat No. G108; Applied Biological Materials) were also included.

*Surgical Equipment and Plasticware.* Equipment consisted of scalpels, 100-μm nylon cell strainer, T25 culture flasks, 35-mm Petri dishes, 6- and 24-well culture plates (all from BD Falcon), and 6- and 24-well culture inserts (cat Nos. PIHT 30R48 and PIHT 12R 48, respectively; Millicell).

## Methods

*Collection of the Human Articular Tissue.* The unaffected region of the AC tissue was collected under aseptic conditions from osteoarthritis-affected patients undergoing total knee replacement after fulfilling the requirements of the Institutional Ethics Committee of Sathya Kidney Centre, Hyderabad, India (hospital registration No. 07F-APMCE-0029). The study was performed with 17 samples over a period of 2.5 years, with the average wet weight of the tissue being 2.78 g.

*Tissue Processing.* Primary chondrocytes from normal parts of the affected AC were prepared according to a modified protocol described elsewhere.[36] Details of the methodology and their 2D and 3D cultures are given below.

*Two-Dimensional Cultures.* The unaffected parts of the resected AC tissue was separated from the rest of the tissue and minced in small volumes of DMEM; the suspension was filtered through 100-μm nylon mesh. The tissue pieces remaining on top of the mesh were processed further. The AC pieces were sequentially treated in enzyme solutions in the following order: 0.05% hyaluronidase, 0.25% trypsin, and 0.2% collagenase at 37°C for 1 hour. Chondrocytes obtained after enzymatic treatment were collected by centrifugation and counted.

Freshly isolated chondrocytes were plated onto T25 culture flasks at an average seeding density of $1.6 \times 10^4$ cells/$cm^2$ and maintained either in CM or NDM for a period of 21 days, with alternate day media changes. On reaching confluence, the cells were subcultured once and further maintained for 14 days. After this, the cells were either cryopreserved or processed for 3D cultures and cellular/molecular analysis.

*Three-Dimensional Cultures.* Chondrocytes grown in 2D cultures for 21 (passage 0 [P0]) or 14 (P1) days were suspended in collagen type I gel (prepared at 2 mg/mL in alkaline PBS) at the concentration of $5 \times 10^4$ cells/mL; 100 μL of this cell suspension was added onto Millicell 24-well culture inserts placed in a 24-well culture plate, under aseptic conditions. Same volume of plain collagen gel without cells was used as a control. Both of the gels were left undisturbed for 30 to 40 minutes at 37°C, after which the gel solidified; at this point, 200 μL of CM or NDM was added in each well, and the cultures were maintained for 30 days with media changes twice a week. The same experiment was also replicated in Millicell 6-well culture inserts, but 1 mL of cell suspension and 2 mL CM or NDM were used for each insert. After this period, the control and experimental gels were processed for histology, immunofluorescence, and scanning electron microscopy (SEM).

*Histocytochemical Methods.* A 2.4-$cm^2$ piece of freshly isolated native AC tissue was fixed in 10% buffered formalin for 10 days; processed sequentially in grades of 70%, 80%, 95%, and 100% isopropyl alcohol and chloroform, 1 hour each; and embedded into wax blocks as per the protocols described elsewhere.[19] Sections 6 μm thick were cut using a Leica RM 2135 microtome and stained using hematoxylin and eosin (H&E), toluidine blue (TB) for total proteoglycans,[19] and Masson trichrome (MT) for total collagen (procedure No. HT15; Sigma-Aldrich).

*Immunofluorescence.* Collagen I (Col I) and collagen II (Col II), aggrecan (Agg), and CD44 expression in native AC, 3D-reconstructed cartilage, and isolated chondrocytes were studied using the respective antibodies as described above. For native AC and the 3D-reconstructed tissue, the immunofluorescence protocol for paraffin sections was used. Sections 6 μm thick were deparaffinized with different ethanol concentrations in sequence and treated with sodium citrate buffer (pH, 6.0) for heat-mediated antigen retrieval. For 2D cultures of chondrocytes, the cells were first grown on 22-$mm^2$ coverslips and then fixed with 4% paraformaldehyde and dried. For sections as well as cells, blocking of nonspecific antibodies was done by treatment with 3% bovine serum albumin (BSA) in PBS. The sections/cells were treated with 1:200 dilutions of the respective primary antibodies for 1 hour at room temperature, washed with PBS, then 1:1000 dilution of Alexa Fluor 594 goat antirabbit IgG was added and mounted with DAPI (4′,6-diamidino-2-phenylindole) Fluoroshield. Specific fluorescence for antibody and DAPI was visualized in a Zeiss Apotome fluorescence microscope equipped with a 20× Plan-Apochromatic objective and filter settings of 594 nm/620 nm and 350 nm/470 nm as the excitation/emission wavelengths. Image analysis was done using AxioVision Rel 4.7 software (Zeiss).

*Gene Expression Profiling.* Total RNA was extracted from native AC (1.4 mg/mL), chondrocyte monolayer (0.8 mg/mL), and 3D-reconstructed AC tissue (1 mg/mL) using TRIzol reagent (BioServe India). The extracted RNA was purified by DNAse (Invitrogen Cat. No. 18068-015) treatment, and 0.8 ng RNA of each sample was reverse transcribed using Super-Script III First-Strand Synthesis System for RT-PCR. The PCR was performed using the DNA Engine Peltier Thermal Cycler (Bio-Rad) for analyzing the expression of GAPDH (the house-keeping gene), Col II, Agg,[35] Col I,[22] and CD44[14] genes. Primer sequences for these genes are taken from the references mentioned above. To analyze GAPDH expression, the following PCR conditions were considered: hot start, 95°C, 2 minutes; 30 cycles of denaturation, 95°C, 30 seconds; annealing, 57.5°C, 30 seconds; and elongation, 72°C, 30 seconds. This was followed by final extension at 72°C, 5 minutes. The PCR
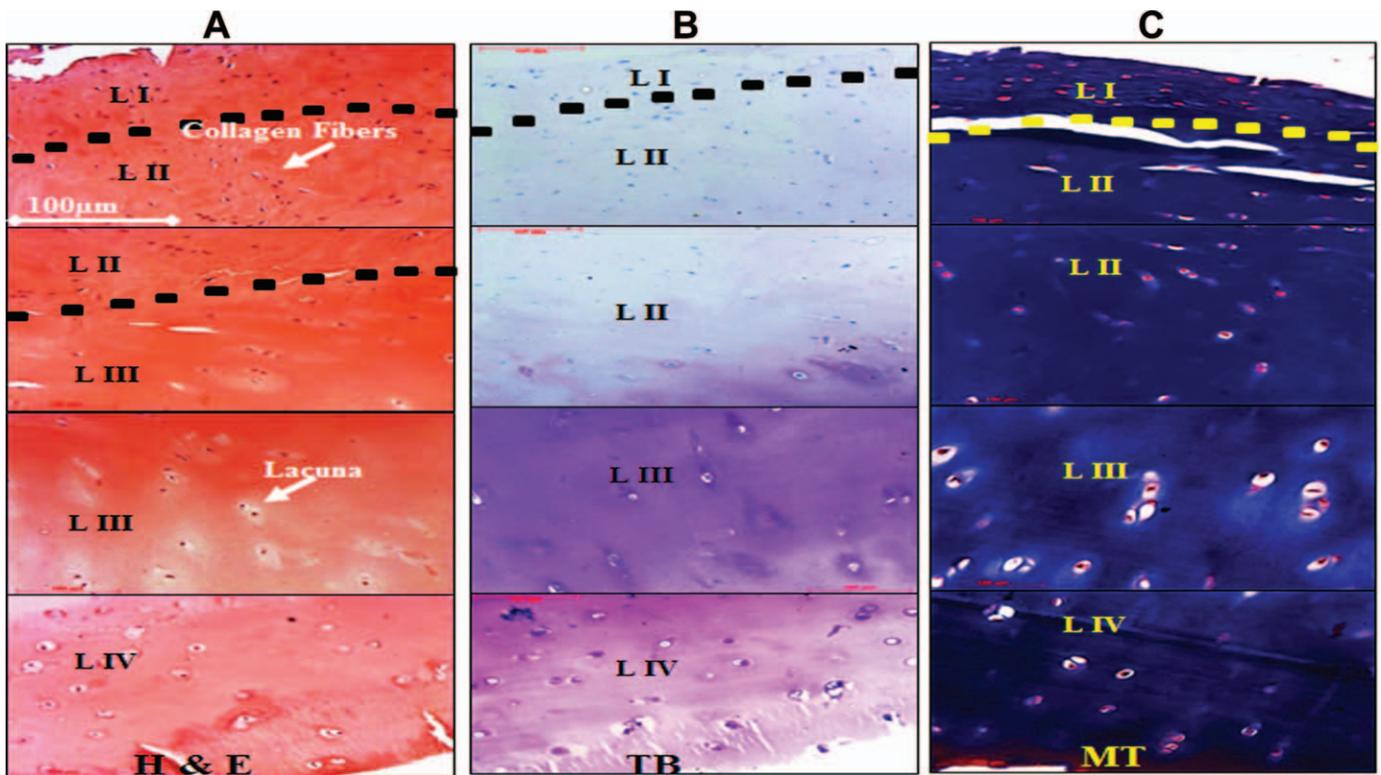
**Figure 2.** Histologic staining of the native hyaline cartilage to highlight the cellular and matrix properties. All panels are displayed at 20× magnification. However, portions from each layer (LI-LIV) have been exhibited to generate the continuity of the tissue. (A) Hematoxylin and eosin (H&E; cellular organization), (B) toluidine blue (TB; total proteoglycan), and (C) Masson trichrome (MT; total collagen) staining.

conditions to study the expression of the remaining genes were similar to those used for GAPDH, but with changes in the annealing temperatures of 60°C for Col II and CD44 and 57°C for Col I and Agg. SafeView Classic, a substitute for ethidium bromide in 1% agarose gel, was used to visualize the amplified products.

*Scanning Electron Microscopy of Normal Human AC and 3D Cultures.* Surface architecture of collagen gels (with and without cells) and native AC was studied according to a modified protocol[31] by SEM (S-3400N, Hitachi), exposing at 15.0 kV of voltage. The samples were fixed in 2.5% glutaraldehyde (Sigma-Aldrich) and 4% paraformaldehyde in PBS (pH, 7.2) for 4 hours at room temperature; sequentially dehydrated with 30%, 50%, 70%, 80%, 95%, and 100% ethyl alcohol and 100% acetone for 15 minutes each at room temperature; and followed by immediate critical point drying (CPD), sputter coating with gold, and observation.

## RESULTS

### Native AC Histology

Native AC sections stained with H&E, TB, and MT are shown in Figure 2A, 2B, and 2C, respectively. In panel A, we can visualize the organization of each layer of AC; thin collagen fibers are seen in LII, and elongated chondrocytes within their surrounding lacunae are noted in LIII. Panel B shows that LIII has the highest expression of total PG in

comparison with other layers, whereas panel C shows that total collagen expression in all the layers is similar. For the complete visualization of all 4 layers by each stain, please see Appendix Figure A1.

### Collagen and Proteoglycan Subtypes in LIII of AC

To confirm the type of collagen and PG expressed in the LIII of native AC, immunofluorescence using antibodies for Col II, Agg, Col I, and CD44 was done as described. Figure 3 shows that Col II and Agg are highly expressed, whereas Col I and CD44 are expressed at very low levels in LIII. Comparison of these markers in LIII versus LI, LII, and LIV can be seen in Appendix Figure A2. The results in Figures 2 and 3 confirm that Col II and Agg molecules are mainly expressed in LIII of AC, and it contains a very small number of cells.

### Tissue Disorganization in Grades of AC Injury

Comparison of the histocytochemical properties of native AC and 2 grades of AC injury can be seen in Appendix Figure A3. In grade IV damage, ECM distribution is nonhomogeneous, and it shows very low levels of Col II and Agg but high levels of Col I and CD44. In grade I AC damage, intermediate levels of the same changes are seen. Based on these observations, we propose that the regenerated AC should show the same levels and organization of these markers as are seen in the normal AC.
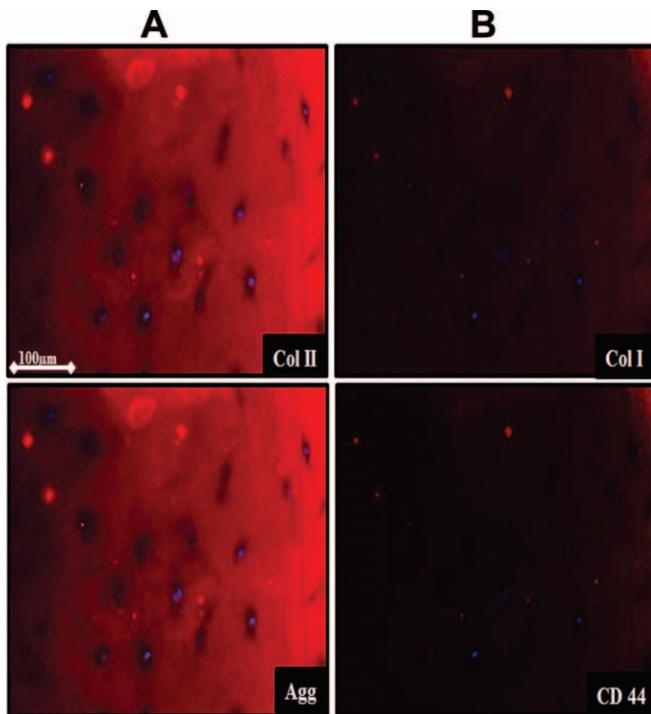
**Figure 3.** Immunofluorescence of LIII of hyaline cartilage at 20× magnification. (A) Highly expressed Col II and Agg; (B) Col I and CD44 expressed at very low levels.



**Figure 4.** Two-dimensional cultures of chondrocytes grown in either classical medium (CM) or newly defined medium (NDM), at 10× magnification. (A) Passage 0 (P0) cells on day 19; (B) passage 1 (P1) on day 10. Increased ECM formation is seen on P1 cultures grown in NDM compared with CM.

## Two-Dimensional Culture Studies

Primary chondrocytes from normal LIII of AC were maintained either in CM or NDM for a total period of 21 (P0) or 14 (P1) days. The phase contrast microscopy images of these cells are shown in Figure 4. We clearly noticed increased ECM formation in P1 chondrocytes grown in NDM as compared with CM. To confirm this observation, immunofluorescence of Col I, Col II, Agg, and CD44 was performed, and the results from a representative experiment are shown in Figure 5 (similar observations were seen in 8 different experiments). Col II and Agg were highly expressed, whereas Col I and CD44 were maintained at low levels in P1 chondrocytes grown in NDM as compared with the same grown in CM. Thus, our results reproducibly show that P1 chondrocytes grown in NDM exhibit matrix properties similar to that of LIII in native AC.

We analyzed the cell division potential of these chondrocytes based on 5 independent experiments by flow cytometry (Appendix Figure A4), where the statistical observations proved that the percentage of proliferative cells in CM was $11.56 \pm 0.5$ and in NDM was $8.43 \pm 0.4$. Hence, it can be inferred that the proliferative fraction of chondrocytes grown in NDM was approximately 3% to 5% less than those grown in CM, indicating that NDM maintained them at a relatively lower proliferation potential, another property that is similar to LIII in native AC.
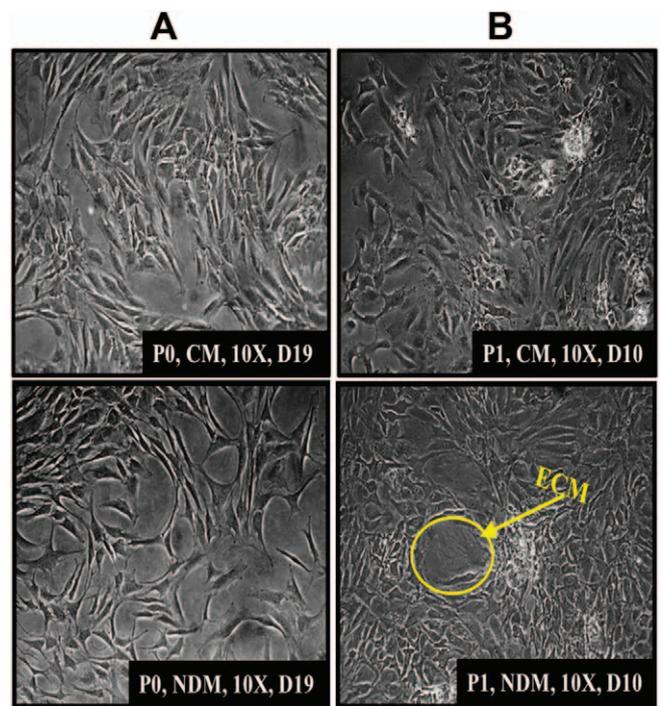
## Three-Dimensional Culture Studies

Primary chondrocytes grown in 2D cultures were placed in type I collagen gels in Millicell culture inserts as described; gels incubated without cells were considered controls. The 3D cultures were maintained for 30 days either in CM or NDM, after which they were processed for SEM, immunofluorescence, and molecular analyses. SEM results of the gel surfaces with cells grown in CM (A) or NDM (B) along with that of native AC (C) are shown in Figure 6. The surface of gels with chondrocytes grown in NDM showed thick and dense collagen fibers, similar to that of the native AC, whereas gels with chondrocytes grown in CM showed a flat and rough surface with few collagen fibers. Interestingly, gels placed in NDM without cells also showed a remodeled ECM surface having either smooth regions (labeled "1" in Appendix Figure A5) or regions covered with thick collagen fibers (labeled "2" in Appendix Figure A5). This indicated that components present in NDM alone could modify the gel surface organization. In contrast, control gels placed in CM showed no changes on the surface of the gels (Appendix Figure A5, part A). These observations were confirmed in 3 independent experiments.

The gels with chondrocytes were stained with H&E, TB, and MT, as seen in Figure 7. NDM-grown HC tissue clearly showed a 4-layered organization, with sparse cell number and high levels of total PG and collagen in LIII (Figure 7A, 7B, and 7C, respectively). Gels without the cells, maintained in similar experimental conditions, are shown in Appendix Figure A6, where higher levels of total PG and
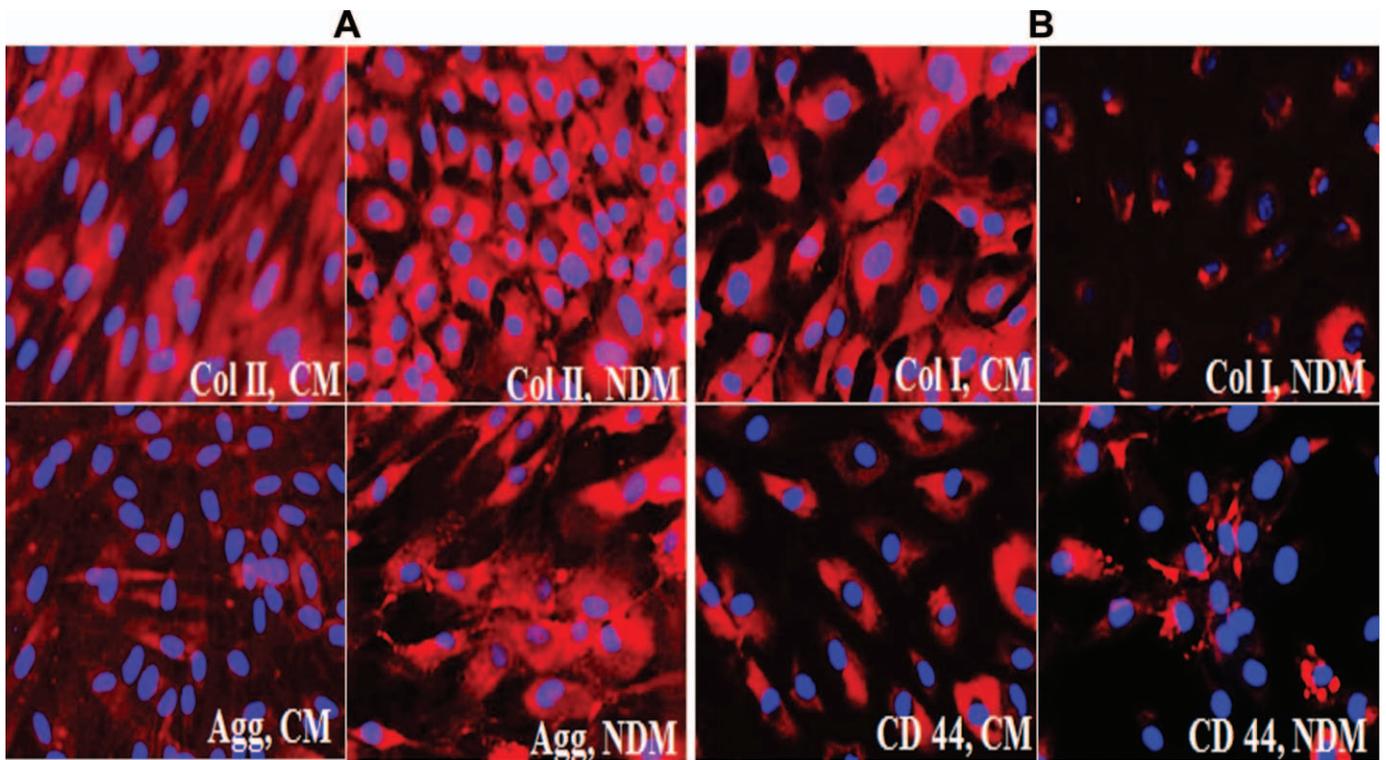
**Figure 5.** Immunofluorescence of 2D cultures of chondrocytes at passage 1, maintained in either classical medium (CM) or newly defined medium (NDM), at 20× magnification. (A) Col II and Agg are highly expressed in chondrocytes grown in NDM, whereas (B) Col I and CD44 are expressed at low levels.
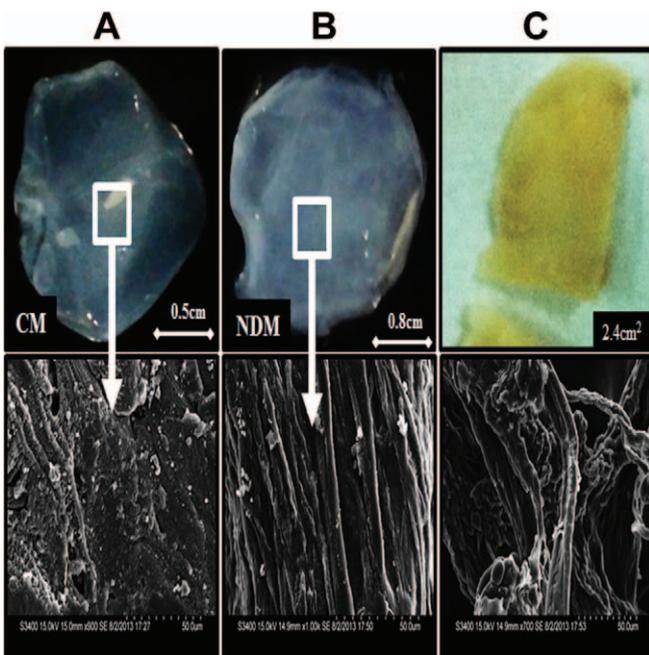


**Figure 6.** Scanning electron microscopy analysis of the 3D-reconstructed and native hyaline cartilage (HC). (A) Three-dimensional culture (0.5 cm) of passage 1 (P1) chondrocytes at day 30, grown in classical medium. (B) Three-dimensional culture (0.8 cm) of P1 chondrocytes at day 30, grown in newly defined medium (NDM). (C) Native HC (2.4 cm$^2$).
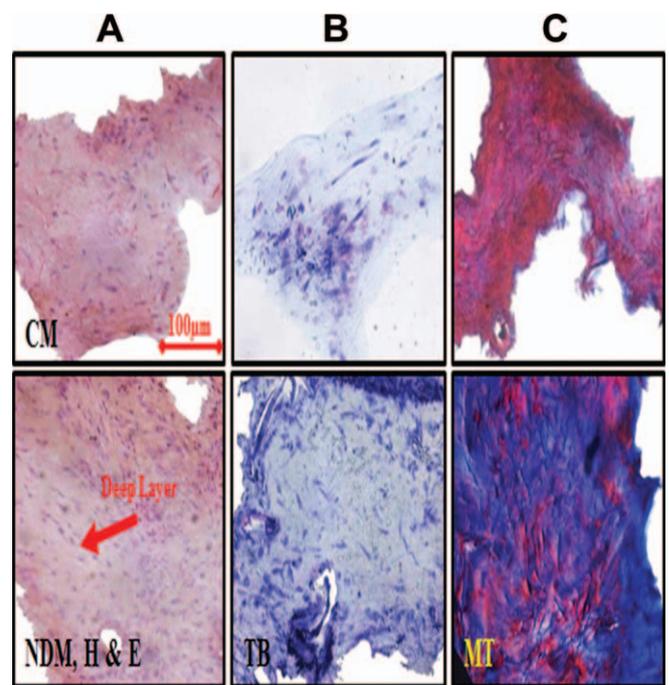


**Figure 7.** Histological studies of the 3D-reconstructed hyaline cartilage grown in either classical medium (CM) or newly defined medium (NDM), using collagen gel as a scaffold, at 20× magnification. (A) Hematoxylin and eosin (H&E) staining, (B) toluidine blue (TB) staining, and (C) Masson trichrome (MT) staining.
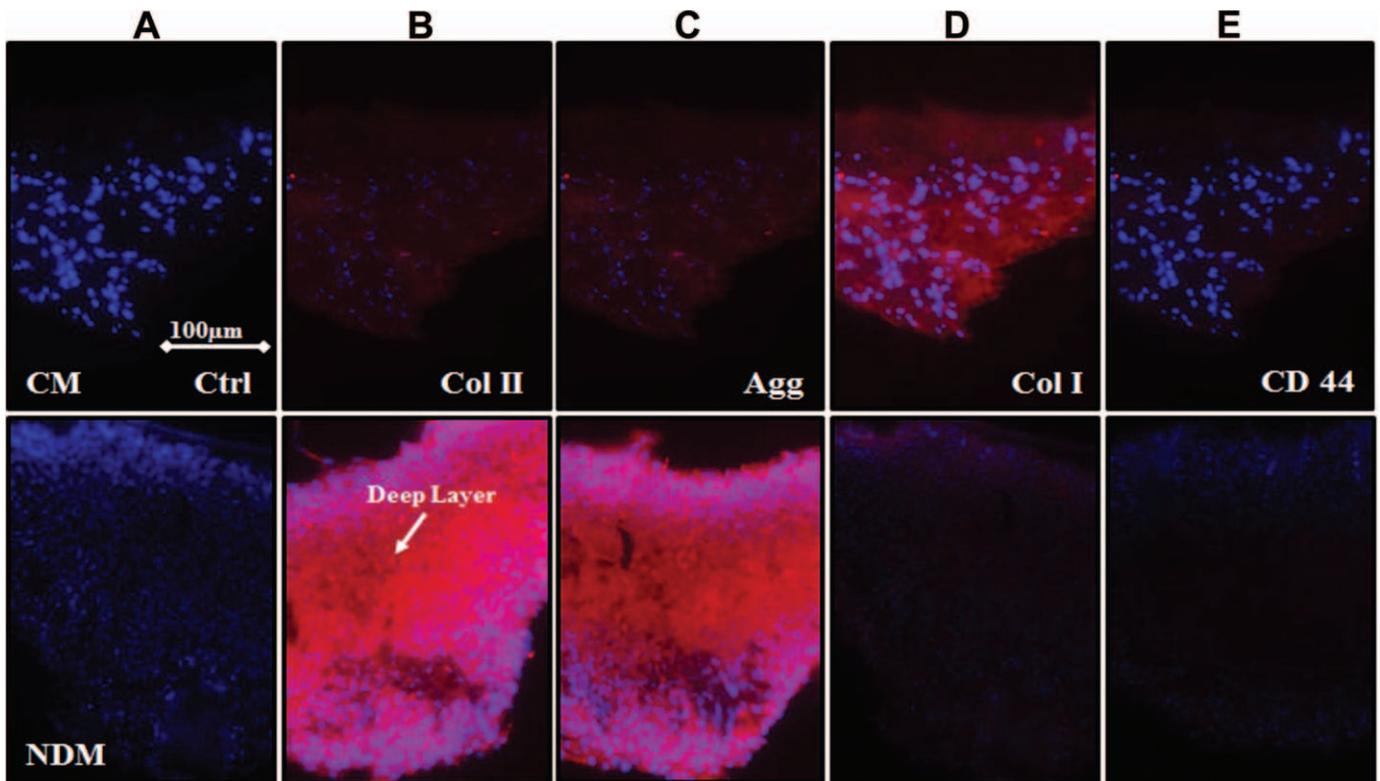
**Figure 8.** (A) Immunofluorescence of HC grown for 30 days in 3D cultures of classical medium (CM; upper row) or newly defined medium (NDM; lower row). Blue stain indicates the cellular nuclei and red indicates the Alexa Fluor 594 nm fluorescence for different antibodies. (B) Col II, (C) Agg, (D) Col I, (E) CD44. All pictures taken at 20× magnification.

collagen were observed in NDM than those in CM (Appendix Figure A6).

Immunofluorescence for Col II, Agg, Col I, and CD44 in 3D-regenerated HC tissue is shown in Figure 8. NDM-regenerated HC clearly showed the 4 distinguishing layers of AC, with enhanced LIII properties for Col II and Agg (Figure 8B and 8C, respectively). Features associated with fibrous and elastic cartilage such as Col I and CD44 were either very low or absent (Figure 8, D and E). Interestingly, even control gels that were maintained in NDM showed higher Col II and low levels of Col I than gels grown in CM (Appendix Figure A7, compare part A vs part B). This showed that Col I present in the original gel could be modified by the components of NDM alone.

Gene Expression Analysis

A comparative study of native AC and cells obtained from 2D/3D AC cultures was done by RT-PCR. Equal amounts of total RNA (0.8 ng) was used for estimating GAPDH (loading control), Col II, Agg, Col I, and CD44 expression levels. Eight independent experiments were performed, for which a representative experiment is shown in Figure 9. High levels of expression of Col II and Agg genetic markers were observed in NDM-grown 2D and 3D cultures, similar to that of the native AC, compared with that of CM-grown 2D and 3D cultures. Expression of Col I was seen at higher levels in

samples grown in CM, whereas samples grown in NDM showed minimal expression, thus maintaining the LIII property. Expression of CD44 is almost negligible throughout, indicating that the native HC-collected 2D and 3D cultures are unaffected and are completely healthy.

DISCUSSION

In this article, we have described a new method to regenerate multilayered AC tissue in 3D cultures of primary chondrocytes prepared from native AC. Our method provides cell biological evidence that properties of native AC could be sustained in our culture system in a better manner than by methods reported earlier.[8,17] The significant features of our methodology are discussed.

Primary 2D cultures of AC-derived chondrocytes from nonhuman sources have been reported[1,36]; however, reports on the culture of normal human AC are fewer.[4,8,36] Most of the reports on human chondrocytes have not described the quality and quantity of ECM secreted by the cultured cells[8,17] as they have mainly focused on the gene/protein expression profiles of these cells.[18,20,27] In our method, human chondrocytes grown in UCS-containing media showed extensive matrix formation that was rich in collagen II and aggrecan and showed the absence or a very low expression of CD44 and collagen I. This is the
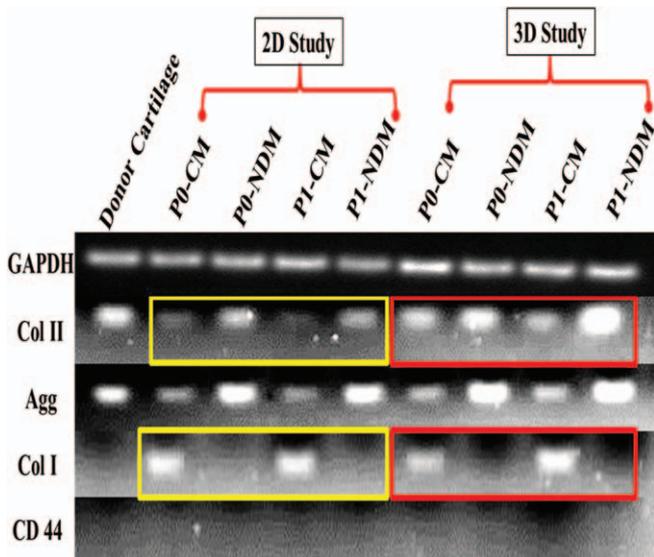
**Figure 9.** RT-PCR products for the respective markers as indicated in each row obtained from 2D and 3D cultures grown in classical medium (CM) or newly defined medium (NDM). GAPDH indicates the loading control.

first demonstration of sustenance of AC-like ECM properties in the 2D cultures of human chondrocytes. As the native AC comprises >70% ECM components, it is important that ECM properties of cultured chondrocytes be similar to that of native AC. Interestingly, in NDM-grown chondrocytes, we observed that ECM formation and gene profiles of cells were sustained, in fact better, in P1 cells than in P0 cells. Earlier reports on primary chondrocyte cultures had shown reduction in AC-specific markers after the first passage.[4,8]

P1 chondrocytes grown in 2D cultures were placed in collagen 1 gels and further cultured in NDM as a 3D module. We noticed that cells growing in 3 dimensions modified the collagen gel properties significantly after 30 days and yielded tissue of approximately 2 mm thickness that shared many features of native HC, such as multilayered organization with different levels of ECM-specific protein expression, variable cell density in the different layers, a middle layer with very similar properties as seen in LIII of native HC. Further analysis of this LIII-like region in the reconstructed tissue showed that it contained high levels of Col II and Agg and low levels of Col I and no CD44 and low cellularity. Thus, the reconstructed tissue portrayed most features of native HC except that LIII cells are not enclosed in lacunae.

Currently available methods fail to reproduce HC-like tissue properties, which results in the degradation of the implanted tissue/cells shortly after implantation. The HC tissue reconstructed in our 3D cultures exhibits a multilayered cellular and ECM organization similar to that of the native AC tissue. Although we have not done any specific compressive strength measurements of this tissue, we propose that, because of its similarity to native AC, the tissue grown in our cultures will be more durable when

implanted in vivo. Though not clinically tested, our tissue could also be used as a single-step procedure for allogenic cartilage implants because NDM-grown primary chondrocytes from 2D cultures are cryopreserved and can be used for 3D cultures whenever required.

## CONCLUSION

We have reported a novel scheme to regenerate AC tissue from primary chondrocyte cultures without using xenogenic media. As our method reproducibly yields AC with all the significant features of HC, we propose that it can be used in clinics for replacing grade III or IV degraded AC using a cost-effective procedure.

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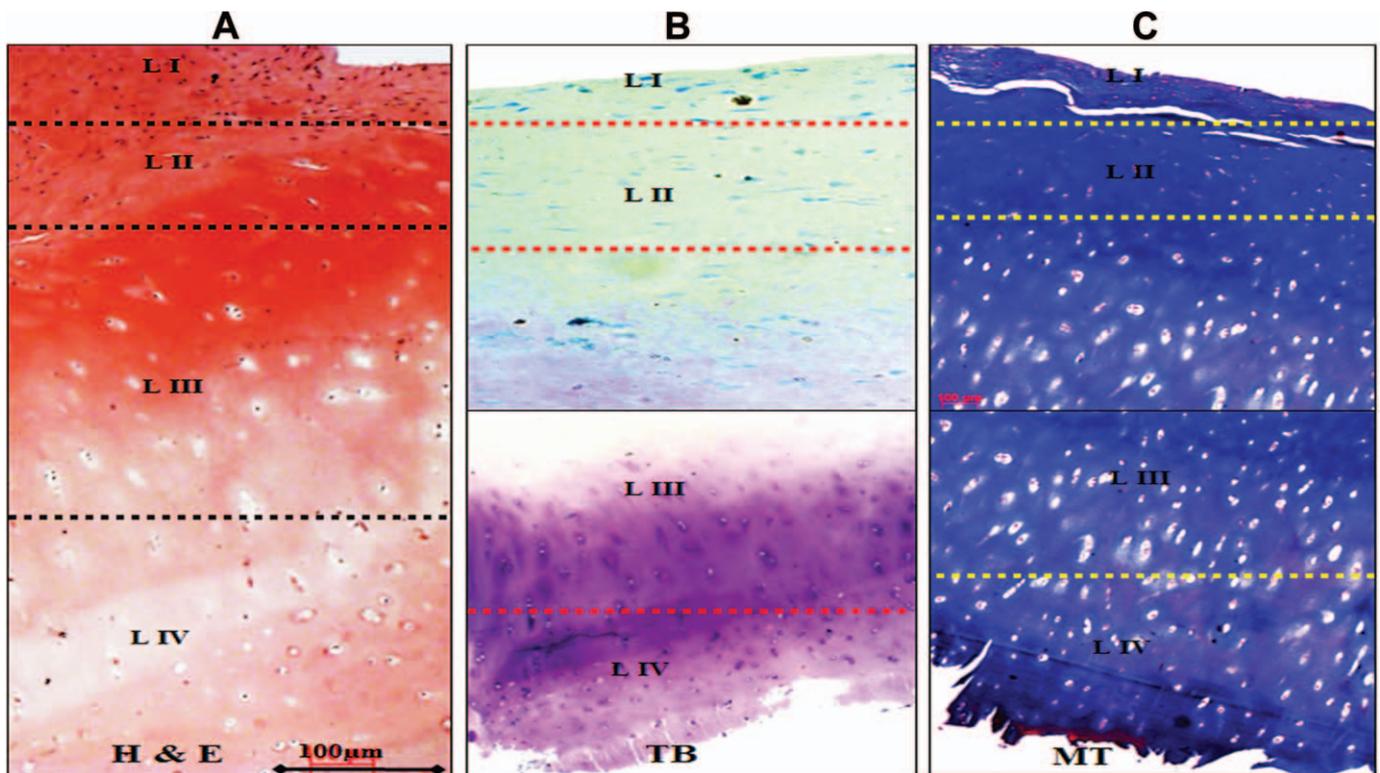
# Appendix



**Figure A1.** Histologic studies showing the organization of the layers of hyaline cartilage, at 5× magnification. (A) Hematoxylin and eosin (H&E) staining; (B) toluidine blue (TB) staining; (C) Masson trichrome (MT) staining.
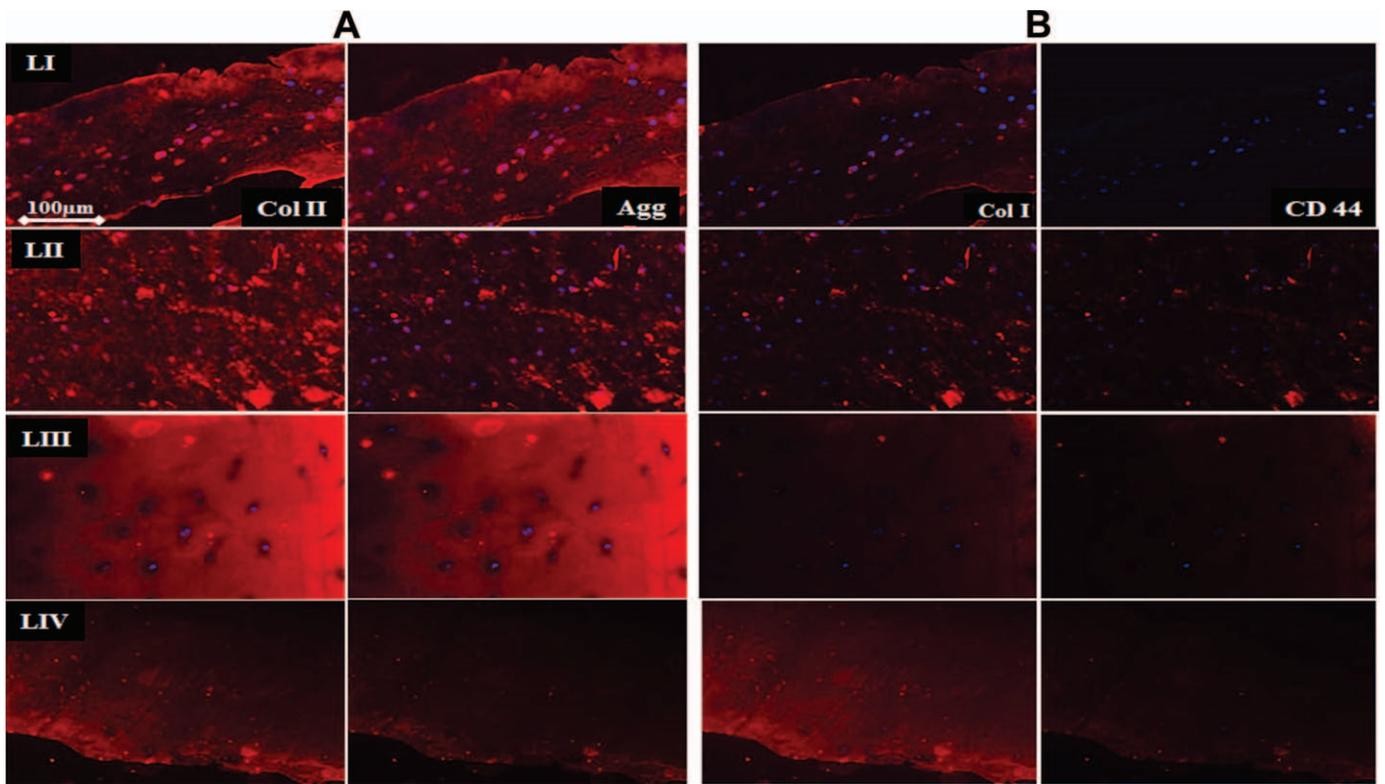
**Figure A2.** Immunofluorescence of the 4 layers of hyaline cartilage, at 20× magnification. (A) Col II and Agg; (B) Col 1 and CD44.
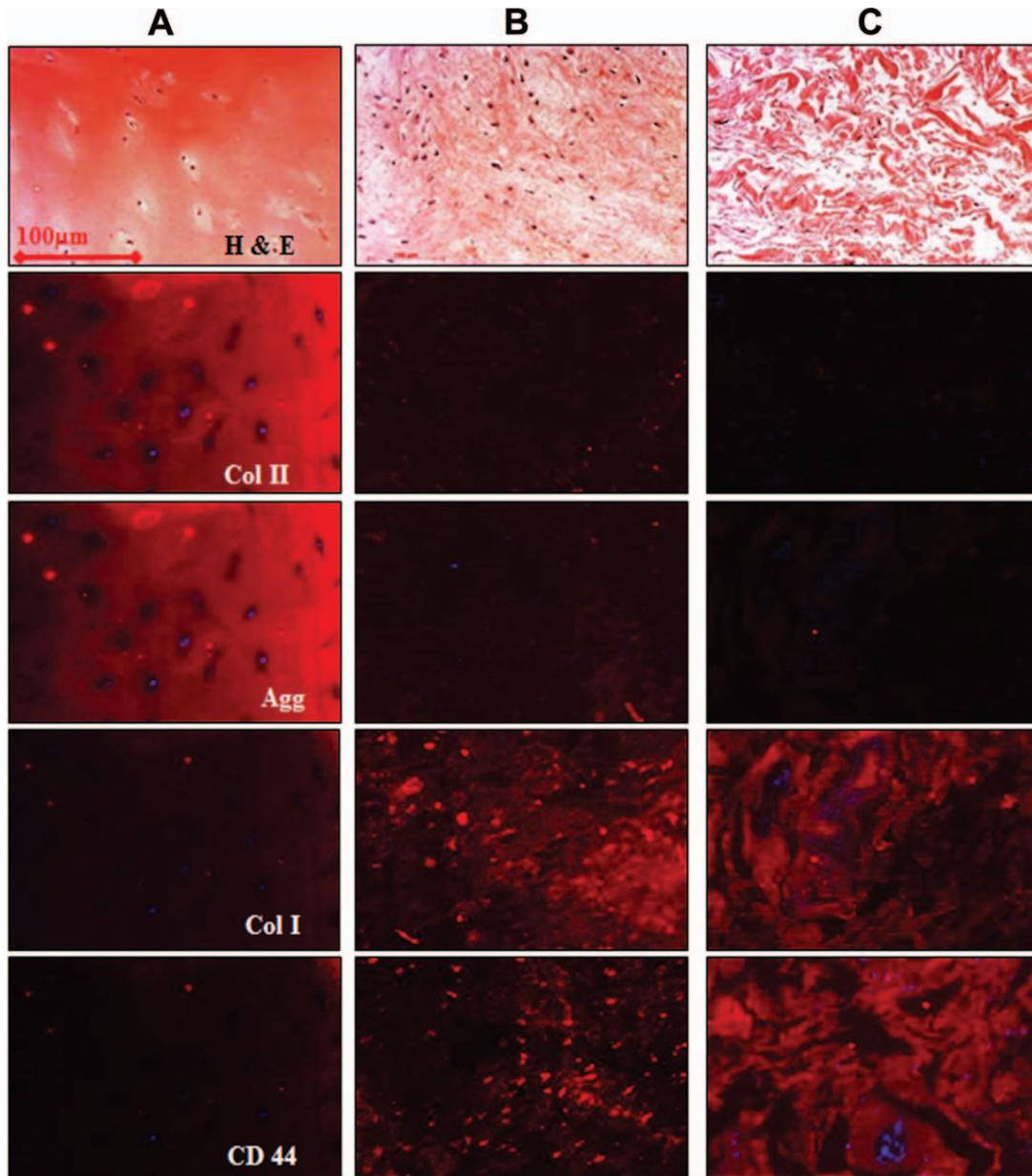
**Figure A3.** Grades of degradation of hyaline cartilage, from normal to completely degraded (grade IV) damage. (A) Hematoxylin and eosin (H&E) stain of the deep layer of normal hyaline cartilage followed by positive markers (Col II and Agg expression) and negative markers (Col I and CD44 expression). (B) Grade I damage. (C) Grade IV damage.
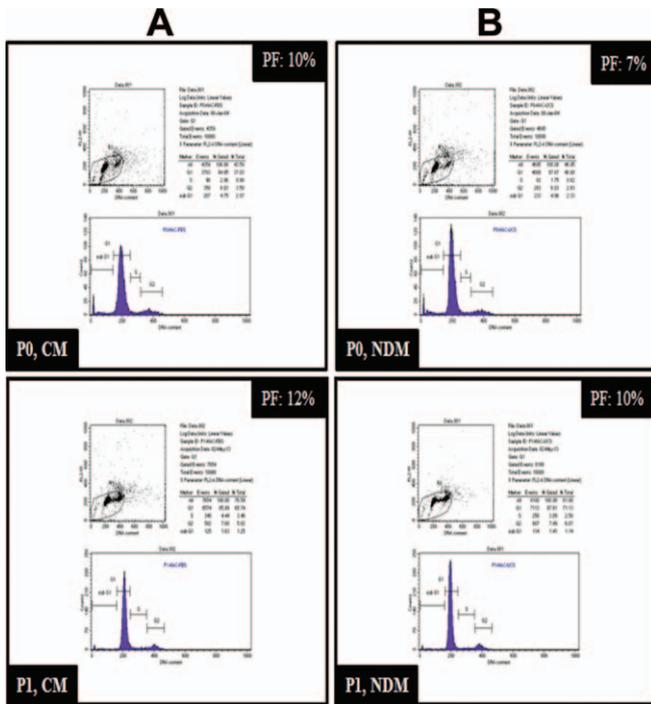
**Figure A4.** Cell proliferation analysis by FACS. (A) Proliferative fraction (PF) of passage 0 (P0) and passage 1 (P1) chondrocytes grown in classical medium. (B) PF of P0 and P1 chondrocytes grown in newly defined medium (NDM).
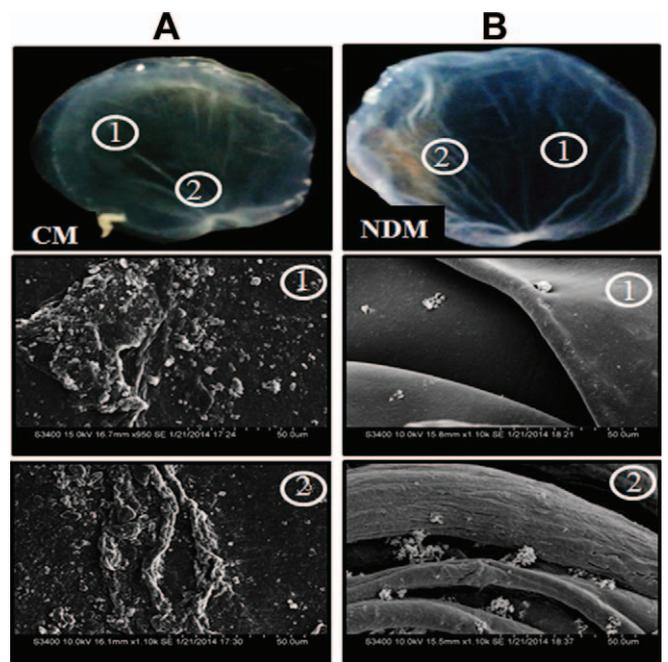


**Figure A5.** Scanning electron microscopic analysis of the 3D-reconstruction control tissue. (A) 3D culture (0.8 cm) at day 30, grown in classical medium (CM); A1 and A2 show no clear difference. (B) 3D culture (1.1 cm) at day 30, grown in newly defined medium (NDM); B1 and B2 show smooth surface and thick collagen fibers, respectively.
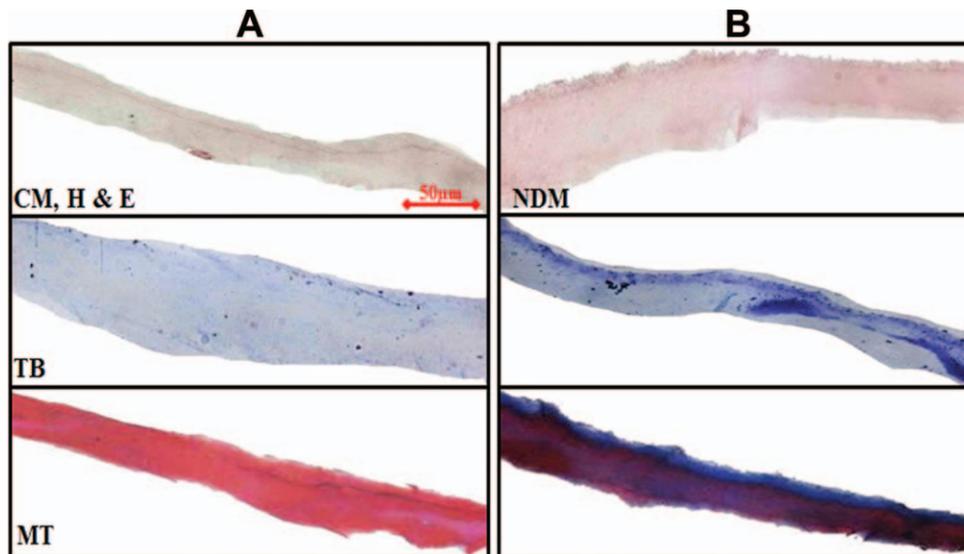


**Figure A6.** Histological studies of the 3D-reconstructed control tissue, using collagen gel as a scaffold, at 20× magnification. Three-dimensional control tissue grown in (A) classical medium (CM) and (B) newly defined medium (NDM) for 30 days and stained with hematoxylin and eosin (H&E), toluidine blue (TB), and Masson trichrome (MT).
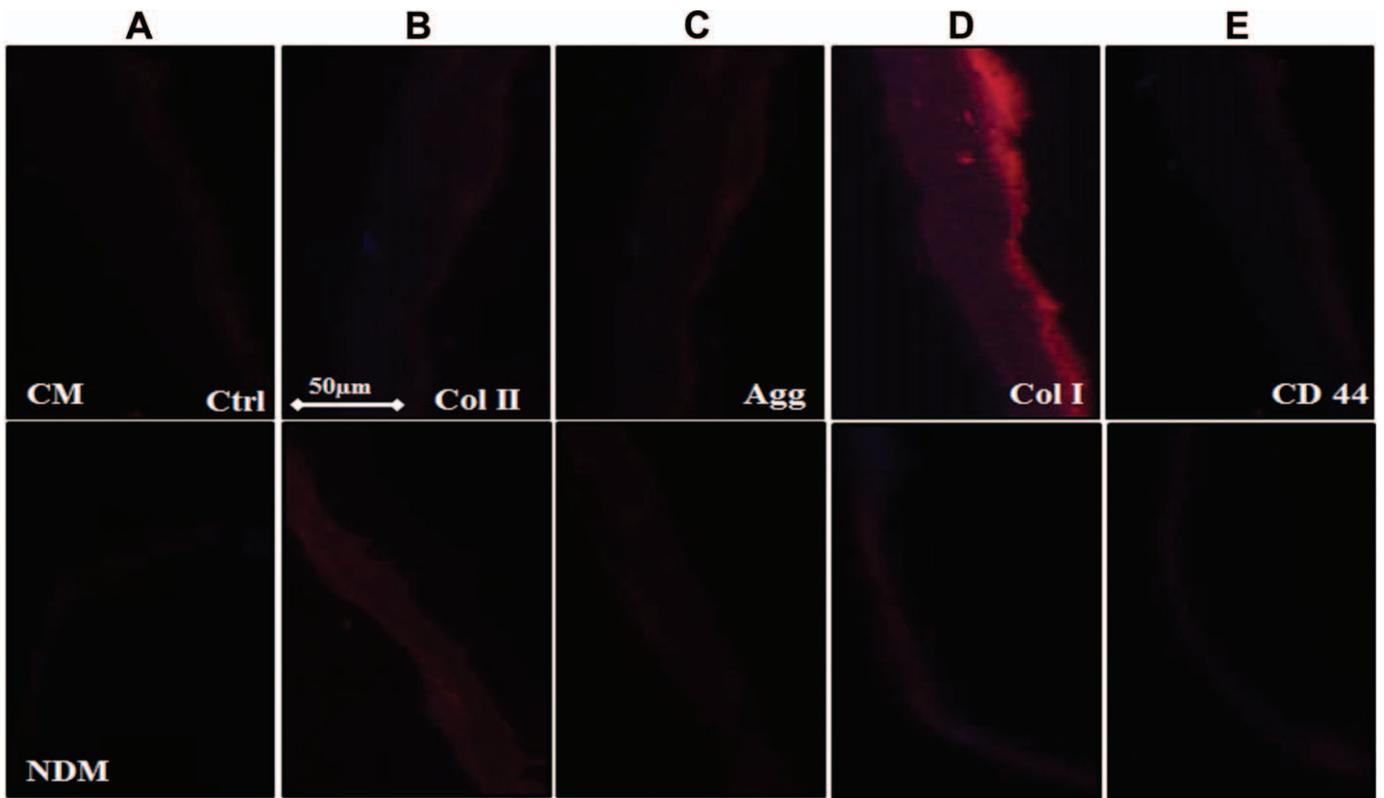
**Figure A7.** Immunofluorescence of the 3D-reconstructed control tissue grown in either CM or NDM, using collagen gel as a scaffold, at 20× magnification. (A) Control; (B) Col II; (C) Agg; (D) Col I; (E) CD44.