Facilitating In Vivo Articular Cartilage Repair by Tissue-Engineered Cartilage Grafts Produced From Auricular Chondrocytes

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Background: Insufficient cell numbers still present a challenge for articular cartilage repair. Converting heterotopic auricular chondrocytes by extracellular matrix may be the solution.

Hypothesis: Specific extracellular matrix may convert the phenotype of auricular chondrocytes toward articular cartilage for repair.

Study Design: Controlled laboratory study.

Methods: For in vitro study, rabbit auricular chondrocytes were cultured in monolayer for several passages until reaching status of dedifferentiation. Later, they were transferred to chondrogenic type II collagen (Col II)–coated plates for further cell conversion. Articular chondrogenic profiles, such as glycosaminoglycan deposition, articular chondrogenic gene, and protein expression, were evaluated after 14-day cultivation. Furthermore, 3-dimensional constructs were fabricated using Col II hydrogel-associated auricular chondrocytes, and their histological and biomechanical properties were analyzed. For in vivo study, focal osteochondral defects were created in the rabbit knee joints, and auricular Col II constructs were implanted for repair.

Results: The auricular chondrocytes converted by a 2-step protocol expressed specific profiles of chondrogenic molecules associated with articular chondrocytes. The histological and biomechanical features of converted auricular chondrocytes became similar to those of articular chondrocytes when cultivated with Col II 3-dimensional scaffolds. In an in vivo animal model of osteochondral defects, the treated group (auricular Col II) showed better cartilage repair than did the control groups (sham, auricular cells, and Col II). Histological analyses revealed that cartilage repair was achieved in the treated groups with abundant type II collagen and glycosaminoglycans syntheses rather than elastin expression.

Conclusion: The study confirmed the feasibility of applying heterotopic chondrocytes for cartilage repair via extracellular matrix–induced cell conversion.

Clinical Relevance: This study proposes a feasible methodology to convert heterotopic auricular chondrocytes for articular cartilage repair, which may serve as potential alternative sources for cartilage repair.

Keywords: chondrocytes; extracellular matrix; dedifferentiation; cell conversion; auricular cartilage; articular cartilage

Articular cartilage is an avascular tissue with limited capacity for self-repair. Because most chondrocytes are in the arrest status of the cell cycle, articular cartilage lacks intrinsic healing capacity in response to traumatic injury. Eventually, these cartilage defects may lead to osteoarthritis, a prevailing clinical problem that affects billions of patients worldwide. Currently, the therapeutics capable of promoting cartilage healing during osteoarthritis progression have not been routinely available for clinical practice. Nonetheless, for selected patients, limited cartilage defects can be treated by autologous cartilage grafting. Autologous chondrocyte transplantation is a technique that has been proposed for cartilage repair, but it requires articular biopsy specimens from nonweightbearing zones, which leads to further joint damage. Moreover, the amount of healthy cartilage available for harvest is very limited. A sufficient number of chondrocytes may be obtained through monolayer expansion of autologous cells, but this method is endowed with the risk of cell dedifferentiation. Hence, autologous chondrocyte transplantation is hindered by donor-site morbidity and the possibility of chondrocyte dedifferentiation.

It is believed that using nonarticular heterotopic chondrocytes for cartilage repair circumvents these restrictions...
because of easy accessibility and minimal morbidity of the donor sites. Many investigators have directly harvested heterotopic nonarticular chondrocytes for articular cartilage repair. However, the results were controversial. Although nasoseptal chondrocytes were easily ossified when cultured in vitro, when they were successfully transplanted into knee osteochondral defects they became hyaline-like repair tissue. When auricular chondrocytes were used to repair nucleus pulposus defects, hyaline-like tissue, rather than elastic cartilage, formed. Moreover, successful reconstruction of temporomandibular joint cartilage was achieved by implanting auricular chondrocytes. Similar results were also reported when articular cartilage was directly repaired by costal chondrocytes, and successful generation of cartilaginous tissue was observed. Although these studies show the possibility of applying heterotopic cartilage for articular cartilage repair, insufficient cell yield and implanted cells inborn with distinct original cellular features render direct transplantation of heterotopic chondrocytes difficult to implement. Therefore, heterotopic chondrocytes should be converted before transplantation for articular cartilage repair. The differentiation plasticity of heterotopic chondrocytes proven in these studies may be the key to potential solutions. There are several ways to convert the phenotypes of differentiated cells. Reprogramming using molecular and genetic tools reverts differentiated cells to undifferentiated states such that the cells regain their pluripotency capacity. However, laborious work and the risk of teratoma formation remain potential threats. Therefore, methods that bypass the use of pluripotent stem cells are prevailing. Many proof-of-concept studies have recently shown that somatic cells can be directly converted to another type of cells via transdifferentiation. Using transcription molecules and growth factors, cellular plasticity can be harnessed for specific processes. Due to the risk of vector-born complications and the presence of residual exogenous agents, the approaches of tissue engineering, particularly those approved for clinical use, such as extracellular matrices derived from natural origins, may be a more attractive method to induce cell conversion than genetic tools.

The plasticity and differentiation potential of heterotopic auricular chondrocytes in cartilage tissue engineering have been previously demonstrated. When suitable engineering strategies are applied, auricular chondrocytes can convert cellular phenotypes and functions. In addition, auricular cartilage provides high cell yields from a single tissue harvest and thus offers the cell densities required for cartilage repair. However, controversies and inconsistencies exist among different culture methods, scaffold preparations, materials fabrication, and cell origins of heterotopic chondrocytes. Among these variables, the specific characteristics of heterotopic cells inherited from original native tissue are regarded as the most critical part that needs to be solved. However, it has seldom been investigated. To increase the applicability of the use of heterotopic chondrocytes in articular cartilage repair, this study aimed to explore the methods of converting auricular chondrocytes for articular cartilage repair that comply with the regulation for clinical translation to facilitate therapeutic applications.

METHODS

Isolation and Culture of Chondrocytes

All procedures were carried out according to the Guide for the Care and Use of Laboratory Animals and approved by the institute. Autologous rabbit cartilage was harvested from the auricle or knee joints of male New Zealand White rabbits. Then, the harvested cartilage was diced into small, rectangular (20–25 mm²) fragments (Figure 1A). The diced cartilage fragments were digested with 0.05% (w/v) collagenase II (Gibco) for 18 hours at 37°C. The digestion suspension was centrifuged (5 minutes at 1200 rpm) in DMEM/F-12 media containing 10% FBS (Gibco) and then cultured at a density of 105 cells/cm² for 4 (P4). Morphological changes, such as cell shapes, sizes, and numbers, were recorded. The proliferation rate, as well as synthesies of glycosaminoglycans (GAGs), type I

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collagen, and elastin protein, was evaluated immunohistochemically from P1 to P4.

Preparation of Type II Collagen

Type II collagen (Col II) used in both monolayer and 3-dimensional culture was isolated and purified as previously described.11 Briefly, the minced cartilage pieces were pretreated with 4 M guanidine HCl overnight, followed by 4.5 M NaCl–50 mMTris, pH 7.5, for another 24 hours. Then, the tissue was extracted with 3 mg/mL pepsin in 0.5 M acetic acid followed by salting-out in 0.9 M NaCl–0.5 M acetic acid. The Col II pellet was washed 3 times with 70% ethanol in phosphate-buffered saline to remove excessive salts and acid and for sterilization. Finally, the Col II pellet was dissolved in 5 mM acetic acid, quantified, and stored at 4°C until use.

Conversion of Chondrocyte by Col II In Vitro

For the cell conversion experiment (Figure 1B), a Col II-coated plate was first prepared by loading 250 μg/mL exogenous Col II onto each well of a 6-well culture plate. The Col II solution was evenly distributed on the surface of the well and then lyophilized until completely dried. Later, the P2 dedifferentiated auricular chondrocytes were suspended with 4 M guanidine HCl overnight, followed by 4.5 M NaCl–50 mMTris, pH 7.5, for another 24 hours. Then, the tissue was extracted with 3 mg/mL pepsin in 0.5 M acetic acid followed by salting-out in 0.9 M NaCl–0.5 M acetic acid. The Col II pellet was washed 3 times with 70% ethanol in phosphate-buffered saline to remove excessive salts and acid and for sterilization. Finally, the Col II pellet was dissolved in 5 mM acetic acid, quantified, and stored at 4°C until use.

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Cell Proliferation Assay

Cell numbers of each group of triplicate wells were counted using a hemocytometer under a light microscope at a magnification of ×400. To accurately assess the total number of living cells, the trypan-blue exclusion method was performed before cell counting. Triplicate aliquots from each well of the group were counted for averaging.

GAG Staining

Accumulated GAG levels were measured using Alcian blue staining. Briefly, the cells were fixed followed by incubation with 0.0018 M H2SO4 for 30 minutes. Then, the acid
solution was completely removed before adding the Alcian blue solution (1% Alcian blue 8GX in 0.0018 M H2SO4). The staining was maintained for 3 hours and was followed by immediate washing with 0.018 M H2SO4 for another 3 hours to remove excess dye. Finally, the bound dye was eluted with a dissociation buffer. The absorbance was measured at 600 nm using a spectrophotometer.

Reverse Transcription Polymerase Chain Reaction (PCR) and Quantitative PCR

Total RNA of the cells after various treatments was extracted with TRIzol reagent and stored at –80°C for later use. Reverse transcription (RT) was performed according to the protocol described by the manufacturer (Superscript III Kit; Invitrogen). The RT-PCR reagents, including TRIzol and SuperScript III RT systems, were obtained from Invitrogen. The SYBR Green I qPCR system was obtained from Roche Applied Science.22 Aliquots of cDNA specimens in each group were further amplified by conventional PCR (Platinum Taq system; Invitrogen) or real-time PCR (Roche Light Cycler System) using the specific primer sets listed in Table 1. The expression intensity of each gene was normalized using GAPDH as an internal control.

Immunohistochemical Staining

The cells of each group were fixed in 4% paraformaldehyde and dehydrated in a gradient ethanol series. Then, immunohistochemical staining was permeabilized in 0.1% (v/v) Triton X-100 (Sigma), blocked with 5% (v/v) donkey serum (Biological), and rinsed in phosphate-buffered saline containing 2% (v/v) BSA. Goat polyclonal antibodies for collagen type Col II (Calbiochem), SOX 9 (Novus Biological), lubricin (BIOSS), and elastin (Novus Biological) were used as primary antibodies at a 1:100-1:1000 dilution, followed by the DAB secondary antibody (EnVisionTM, System-HRP) to detect protein expression. Briefly, after deparaffinization, the sections were immersed with the proteinase K working solution at 37°C for 15 minutes. After wash, the sections were incubated with peroxidase blocking at room temperature for 5 minutes. Later, primary antibodies were applied with indicated concentrations. The sections were then incubated at room temperature for 60 minutes. Secondary antibodies were then applied with labeled polymer HRP for 30 minutes at room temperature. Finally, the sections were counterstained with Mayer’s hematoxylin solution. Quantitative expression of protein deposition in each group was measured using Image J analytic software.

Preparation of Auricular Col II Constructs by Fabricating 3-Dimensional Scaffolds

For preparation of the auricular Col II constructs, 1 × 10^6 P2 auricular chondrocytes were suspended in 2 mL of 2× DMEM/F-12 medium containing 10% FBS, 40 mM L-proline, and 1× P/S/F. It was then mixed with an equal volume of the 2 mg/mL Col II solution prepared as mentioned above.

### Table 1

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Size</th>
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<tbody>
<tr>
<td>COL1A1</td>
<td>5'-GATGGGCTGAGGCTCAGA-3', 5'-GGTTTGTGAGGAGGCTG-3'</td>
<td>312</td>
</tr>
<tr>
<td>COL2A1</td>
<td>5'-GCACCATGACATGGGAGG-3', 5'-CACAGAGTACACATCC-3'</td>
<td>366</td>
</tr>
<tr>
<td>AGN</td>
<td>5'-GAGGGAGATGGAGGTTAGCTTTT-3', 5'-CTTCGCCCTTGAGAACGCTG-3'</td>
<td>313</td>
</tr>
<tr>
<td>DECORIN</td>
<td>5'-ACAAACTCTGCTAGACCTG-3', 5'-ATTGTTGTATCAGCAATAACGA-3'</td>
<td>330</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TCCACTCTCCAGGAGCGA-3', 5'-CACAATGCCGAAGTGTCGT-3</td>
<td>293</td>
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After polymerization of the collagen matrix at 37°C for 24 hours, 3-dimensional chondrocyte-collagen constructs were formed.10 The chondrocyte-collagen constructs were subsequently cultured with fresh DMEM/F-12 media containing 10% FBS, 40 mM L-proline, and 1× P/S/F for 3 days and were transferred to T-25 flasks. The chondrocyte-collagen constructs were then cultured for 14, 21, and 28 days with a medium change every 2 days. Then, all samples were fixed in 4% formalin for at least 24 hours and then embedded in paraffin, sectioned at 5 mm, and stained with hematoxylin, safranin O, Alcian blue, or Masson’s trichrome staining, respectively. For hematoxylin and eosin staining, sections were stained for 5 minutes in Mayer’s hematoxylin solution (Sigma-Aldrich), rinsed in water, and counterstained for 1 minute in eosin (Sigma-Aldrich). For Masson’s trichrome staining, the sections were incubated in Weigert’s iron hematoxylin working solution for 10 minutes and rinsed. They were incubated in the Biebrich scarlet-acid fuchsin solution for 10 minutes, followed by the phosphomolybdic acid solution for another 10 minutes. The sections were then transferred directly to the aniline blue solution and were stained for 10 minutes. Finally, the sections were rinsed briefly in distilled water and in the 1% acetic acid solution for 2 to 5 minutes. For Alcian blue staining, the sections were stained for 30 minutes in the Alcian blue solution. After wash, the sections were stained with the nuclear fast red solution for 5 minutes. For safranin O staining, the sections were incubated in the Weigert’s iron hematoxylin working solution for 10 minutes. After wash, the sections were stained with the fast green (Sigma-Aldrich) solution for 5 minutes. After quick rinse with the 1% acetic acid solution, the sections were finally stained with 0.1% safranin O solution for 5 minutes. For detection of specific chondrogenic protein expression, immunohistochemical staining was performed for histological and extra-cellular matrix evaluation.

Scanning Electron Microscopy

Auricular Col II constructs were harvested after 4 weeks’ incubation for scanning electron microscopy (SEM). SEM specimens were washed twice with sterile phosphate-buffered saline and then fixed in 4% paraformaldehyde (wt/vol) for 2
days. After fixation, the samples were dehydrated in a graded series of ethanol (10%-95%). The dehydrated samples were transferred to a vacuum desiccator until completely dry. The specimens were then gold sputter coated with the DESK II gold sputter coater (Denton) and examined using a Hitachi 3500 scanning electron microscope (Hitachi) in the secondary electron mode at 15.0 kV.

Dynamic Mechanical Testing

Determination of the modulus of the cartilage monotonic compression test was performed using a Bose Electroforce 3200 (Bose Corp) Dynamic Mechanical Analysis instrument equipped with 2 parallel compression plates. The thickness and diameter of the cartilage samples were measured to calculate the area. Cartilage specimens were preloaded in the Dynamic Mechanical Analysis instrument. After preconditioning, the samples were compressed at a rate of 0.01 mm/s until either the maximal displacement limit of the instrument was reached or the cartilage failed. The compressive modulus was calculated using the stress-strain data with the software provided by the manufacturers.

Transplantation of Converted Chondrocyte In Vivo

An animal model of the osteochondral defect was created in the rabbit knee joints to evaluate the repair effects of the auricular Col II constructs. A total of 16 male skeletally mature New Zealand White rabbits with an average 3 kg bodyweight were evenly divided into 4 groups assigned as the sham (nontreated), auricular cells, Col II (nonseeded with cells), and auricular Col II treated groups. Skeletally mature adult rabbits with ages more than 34 weeks were used in this study to minimize the bias of cartilage self-healing. Under sterile condition, a cartilage defect was created that was 3 mm in diameter and about 2 to 2.5 mm in depth. Then, the osteochondral defects were treated with indicated conditions (Figure 1C). Three months postoperatively, the rabbits were euthanized, and their femurs were harvested for histological analyses.

Statistical Analysis

For the quantitative assay, each data point derived from 3 independent experiments or an experiment of a triplicate assay was presented as a mean with the standard deviation. All analyses were performed using R 2.0.1. The statistical significance was set at \( P < .05 \). The qPCR and histological data were analyzed using 1-way analysis of variance (ANOVA) followed by the post hoc Scheffe’s test and the Dunnett’s test for multiple comparisons.

RESULTS

Loss of the Original Phenotypes of Auricular Chondrocytes by In Vitro Monolayer Culture

The chondrocytes of auricular cartilage were cultured and passaged in monolayers. In in vitro expansion and passage, auricular chondrocytes progressively lost their original auricular cartilage phenotypes, as evidenced by morphologic characteristics, cell proliferation rates, and extracellular matrix (ECM) expression profiles (Figure 2, A-C). With serial passages, the auricular chondrocytes converted to fibroblast-like phenotypes, the typical morphological features of chondrocyte dedifferentiation (Figure 2A). Expression of extracellular matrixes, including GAG, type I collagen, and elastin, progressively decreased in the serially expanded auricular chondrocytes (Figure 2B), with significant differences (Figure 2C). Dedifferentiation had been reported to proceed more rapidly in auricular chondrocytes than in articular chondrocytes, which was closely related to their proliferative capacity. In our results, the cell number decreased after passage 2 (Figure 2D). Based on these results, the auricular cells started the process of phenotypic conversion during in vitro monolayer expansion.

Cellular Phenotypic Conversion of Auricular Chondrocytes by Coll II Cultivation

These auricular chondrocytes were cultivated in a culture environment composed of coated Col II lasting for 2 consecutive 7-day periods. Various concentrations of Col II had been tested, and 250 \( \mu \)g/mL Col II was concluded to be appropriate for inducing re-expression of chondrogenic markers in quiescent chondrocytes. Figure 3A shows the morphology of chondrocytes of the control and Col II groups. After cultivation with Col II, cells of the control group became flattened, fully extended, and adherent to the substratum. On the contrary, cells appeared predominantly polygonal in the Col II group. The cell number increased in both groups on day 7 and day 14, but the proliferative rate of chondrocytes of the control group was higher than that of the Col II group at both time points. When the 2 groups were compared, auricular chondrocytes of the Col II group exhibited decreased cell numbers (Figure 3B). With increased culture time, the GAG levels did not increase in the control group, indicating that the cultured auricular chondrocytes lost their ability to synthesize GAG. Interestingly, in the Col II group, the expression of GAG increased, implying that Col II might stimulate GAG production in the cultured cells (Figure 3, C and D). The elevated level of total accumulated GAG in the Col II group was found in the first 7 days and increased to a significantly high level after the second 7-day culture period. GAG synthesis represents the anabolic activity of the cultivated chondrocytes. Compared with the control group, a 5-fold anabolic capacity was exhibited for the auricular chondrocytes cultivated with Col II. Together, the decreased proliferative rate and the increased GAG levels suggested that cell conversion of auricular chondrocytes was ongoing. The results indicated that the culture system composed of Col II biomaterials was capable of assisting cells to undergo cell conversion in a time-dependent manner.

Characterization of Converted Auricular Chondrocytes

To further characterize the effects of Col II on inducing cell conversion of auricular chondrocytes toward articular
chondrogenesis, expression of chondrocyte-specific protein was tested in both groups. The auricular chondrocytes cultured in the control group (noncoated plates) maintained their original elastin expression similar to the P0 auricular chondrocytes (Figure 4, A and B). On the contrary, the elastin expression level of the cells cultured in the Col II–coated plates decreased significantly from day 7 to 14 (Figure 4, A and B). The auricular chondrocytes cultured in the noncoated plates exhibited low Col II expression comparable with that of P0 articular chondrocytes. Nonetheless, it increased from day 7 to 14 in the auricular cells cultured in Col II–coated plates. Quantitatively, no significant differences of Col II expression were found between P0 articular and auricular chondrocytes cultured in Col II–coated for 14 days (Figure 4, C and D). For further confirmation of cell conversion, specific articular chondrogenic markers, including lubricin and SOX9, were tested. Auricular chondrocytes cultured on the noncoated plates had low levels of lubricin and SOX9 expression, whereas those cultured on the Col II–coated plates demonstrated a progressive increase of lubricin and SOX9 from day 7 to 14 (Figure 4, E-H). To further confirm the genotypes of converted cells, the mRNA expression patterns of Col I (COL1A1), Col II (COL2A1), aggrecan, and decorin were assessed in both groups on days 7 and 14 (Figure 4I). In the Col II group, Col II mRNA was upregulated from day 7 to 14. However, the expression of Col I mRNA exhibited a reverse pattern. The mRNA level of aggrecan increased from day 7 to 14, and decorin was found only in the cells of the Col II group. On the contrary, in the control group, the mRNA expression level of Col II and aggrecan gradually decreased, but Col I did not decrease. For quantitative PCR, the expression level of Col II was normalized to that of P0 articular chondrocytes, while the Col I expression level was normalized to that of P0 auricular chondrocytes for comparison. From day 7 to 14, the relative expression of the articular chondrogenic marker, Col II, increased from 0.7 to 0.91, while that of Col I reduced from 0.37 to 0.15 (Figure 4, J and K). The data demonstrated that Col II promoted articular markers but suppressed auricular markers during the cell conversion process.

Preparation and Characterization of the Auricular Col II Constructs

For tissue repair of cartilage defects, the converted auricular chondrocytes were prepared with 3-dimensional fabricated Col II scaffolds. Figure 5A shows the evolving process of the Col II constructs loaded with auricular chondrocytes. The gross view of the construct showed the white, elastic, and glossy texture after 28-day cultivation (Figure 5A). The Col II constructs loaded with articular chondrocytes were prepared as positive controls. Figure 5 (B and C) shows the histological features of the auricular Col II constructs and articular Col II constructs, respectively. A cluster of round chondrocytes resembling the native
articular cartilage tissue was observed. The high magnification views showed clustered cells with lacunae-like structures (arrowheads, Figure 5B), suggesting secreted cartilaginous matrices produced by the embedded chondrocytes. The constructs were further stained with safranin O for synthesized proteoglycan. The results revealed abundant proteoglycan fibers in the auricular Col II constructs (lower panels, Figure 5B). The magnified views also showed that secreted proteoglycans accumulated around the cell clusters, a typical histological feature similar to native cartilage. These data confirmed that auricular Col II constructs facilitated auricular chondrocytes to gain cellular features of articular chondrocytes and synthesized specific chondrogenic ECM. By SEM, the auricular Col II constructs were demonstrated to have plentiful porous architecture with auricular chondrocytes growing inside. Moreover, the auricular chondrocytes in the Col II scaffolds demonstrated abundant accumulation of ECM on the cellular surfaces, showing close cell-matrix and cell-cell contact among resident cells (Figure 5B). Increased expression levels of specific chondrogenic markers could also be detected in the 3-dimensional auricular Col II constructs after cultivation (Appendix Figure A2, available in the online version of this article). Upon testing their mechanical properties with monotonic compression tests, the stress-strain responses corresponding to these 2 constructs of converted auricular chondrocytes and articular chondrocytes were similar (Figure 5, E and F). An average Young’s modulus of 9.84 KPa was found in the articular Col II constructs, while an average Young’s modulus of 7.26 KPa was found in the auricular Col II constructs, without significant differences. The results suggested that the 3-dimensional constructs composed of auricular chondrocytes engineered by cell conversion procedures could generate tissue structures similar to those produced by articular chondrocytes.

In Vivo Cartilage Repair Using the Auricular Col II Constructs

The next step was to test the repairing ability of the auricular Col II constructs in vivo. A nontreated group (sham
Figure 4. Characterization of converted auricular (AU) chondrocytes. (A) The immunostaining results of elastin in each group during 2 different culture periods (day 7 and day 14) are shown. Passage 0 (P0) AU chondrocytes’ elastin expression was used as the positive control (scale bar, 10 mm). (B) The quantitative results of elastin expression in each group. (C) The immunostaining results of type II collagen (Col II) in each group during 2 different culture periods (day 7 and day 14). The Col II expression of P0 articular (AR) chondrocytes Col was used as the positive control (scale bar, 10 mm). (D) The quantitative result of Col II expression in each group. (E) The immunostaining results of lubricin in each group during 2 different culture periods (day 7 and day 14). The lubricin expression of P0 AR chondrocytes was used as the positive control (scale bar, 10 mm). (F) The quantitative result of lubricin expression in each group. (G) The immunostaining results of SOX 9 in each group during 2 different culture periods (day 7 and day 14). The SOX9 expression of P0 AR chondrocytes was used as the positive control (scale bar, 10 mm). (H) The quantitative result of SOX 9 expression in each group. (I) Reverse transcription polymerase chain reaction (PCR) of the expression levels of COL1A1, COL2A1, AGN, and DCN for the first (7D) and second 7-day (14D) culture periods of both groups. (J) The quantitative PCR showed the mean expression levels of COL1A1 in the converted AU chondrocytes after being normalized with P0 AU chondrocytes. (K) The quantitative PCR showed the average expression levels of COL2A1 in the AU chondrocytes after being normalized with P0 AR chondrocytes. *P < .05. **P < .01. ***P < .001.
group) with nothing implanted into the defect site and the groups implanted with auricular cells only or the Col II scaffolds only (no cell seeding) were prepared as controls (Figure 6A). Three months postoperatively, the treated condyles were demonstrated in sagittal and coronal views of all groups (Figure 6B). The gross defects in the groups of sham operation, the auricular cells, and the Col II scaffolds still presented nonuniform and irregular surfaces covered with a layer of fibrous-like tissue, suggesting that articular tissue had undergone fibrous degeneration for cartilage repair. However, in the auricular Col II group, the condylar articular tissue showed intact, smooth, and hyaline-like surfaces resembling normal articular tissue. The auricular Col II implant integrated with adjacent normal tissue, and the boundary between the implant and the native tissue was barely discernible. There were no signs of inflammation around the implanted auricular Col II constructs. Quantitatively, the macroscopic score of cartilage repair was significantly greater in the auricular Col II group compared with the groups of sham operation, the auricular cells, and the Col II scaffold only.

Histological Characterization of Repaired Cartilage by Auricular Col II Constructs

The harvested samples were then analyzed histologically. In the sham, the auricular cells, and the Col II groups, the articular condyles were coronally concave with nonuniform surfaces over the defect sites (Figure 7A). Cartilage fragmentation was also noted. Cancellous bone tissue beneath a thin layer of subchondral bone was observed. On the contrary, in the auricular Col II group, the condyle showed a contacting and smooth surface with a thin layer of subchondral bone, a typical histological feature present in healthy native condyles. The auricular Col II constructs also demonstrated adequate lateral integration with original cartilage when compared with other groups (Figure 7B). The results of the articular Col II groups, which served as controls, are also shown for comparison (Appendix Figure A1). For ECM syntheses, Alcian blue staining
showed that only auricular Col II group had prevailing positive staining in the defect area (Figure 7C), suggesting that GAG was produced and reformed into the defect with the auricular Col II construct.

The repaired tissues generated by the auricular Col II constructs were then compared with native articular cartilage. Native articular cartilage showed positive Col II (Figure 8A), whereas the original auricular cartilage exhibited positive elastin staining (Figure 8B). Furthermore, the auricular Col II constructs exhibited strongly positive staining for Col II, both in vitro and in vivo (Figure 8, C and E). Nonetheless, the elastic fibers usually produced by auricular chondrocytes were scarcely detected in the auricular Col II constructs both in vitro and in vivo (Figure 8, D and F). Similar results were found in the in vivo study when the repaired sites were compared with the uninjured regions. Based on Masson’s trichrome staining, the transplanted chondrocytes expressed abundant Col II in the defects repaired by the auricular Col II constructs (Figure 8, I and J), similar to those demonstrated in the unoffended sites (Figure 8, G and H). These results indicated that the auricular Col II constructs composed of converted auricular chondrocytes had typical tissue features of articular cartilage when transplanted in vivo. Accordingly, the data confirmed the efficacy of applying heterotopic chondrocytes for cartilage repair and regeneration.

DISCUSSION

This study confirmed the feasibility of converting heterotopic chondrocytes through naturally derived ECM. The cultivation microenvironment composed of Col II successfully synchronized auricular chondrocytes with articular chondrocytes in vitro as they exhibited comparable cell traits, which also contributed to cartilage repair in vivo. Based on the current methodology, Col II not only serves as the scaffold but also induces cell conversion of auricular chondrocytes. The potential plasticity of auricular chondrocytes can be modulated to fulfill the need of articular cartilage repair.

The plasticity of cell fate is a complicated concept that has evolved and been studied for decades. The traditional concept of Waddington’s epigenetic landscape indicates that the fates of somatic cells compulsorily follow downhill differentiation routes once determined. However, recent studies have indicated that some somatic cells possess high plasticity through artificial manipulation. Mature differentiated cells can be reprogrammed to a pluripotent state. As such, any alteration of cell differentiation fate requires external intervention by introducing a set of transcription factors, transferring nuclei, or environmental factors. In addition, the techniques of transdifferentiation or directed reprogramming make it possible to induce a differentiated cell to become another cell type without returning...
to the primitive status of pluripotency. This process efficiently bypasses the need to revert cells back to the pluripotent status before cell redifferentiation, which significantly reduces the time and risks associated with cell reprogramming. These techniques blur the boundaries between distinct cell fates and lower the ridge and flatten the hierarchy of differentiation barriers.

Auricular cartilage is derived from mesenchymal precursors of branchial arches during embryonic development. Despite the difference of tissue origins, auricular chondrocytes maintain their ability to express articular chondrogenic genes in vitro. It has been found that auricular chondrocytes cultured in tissue-engineered constructs expressed lubricin, a typical glycoprotein usually found

Figure 7. Histological characterization of in vivo cartilage repair of auricular type II collagen (AU Col II) constructs. (A) Histological sections of the cartilage harvested 3 months postoperatively from the sham, the AU cells, the Col II, and the AU Col II groups were stained with hematoxylin and eosin (HE). (The lower panels show the high-magnified views of the designated rectangular frames in the upper panels of each group; scale bars, 5 mm). (B) HE and (C) Alcian blue staining of the histological sections. The junctions between the unoffended cartilage and the repaired area are shown in 2 different magnifications. (Arrowheads: the defect sites; the asterisk marks the integration of the AU Col II constructs with the surrounding cartilage. Upper panels, ×40; lower panels, ×100; scale bar, 100 μm). (D) Semiquantitative histological analyses using the modified International Cartilage Repair Society (ICRS) score for harvested samples. ***P < .001.
only in articular chondrocytes. Auricular chondrocytes had similar ECM expression profiles as those of articular chondrocytes when cultured with alginate. In addition to showing a flexible capacity of synthesizing ECM, heterotopic chondrocytes also show plasticity in changing cellular phenotypes. Auricular chondrocytes can be converted into distinct phenotypes when incubated in specific microenvironments. When auricular chondrocytes were impregnated in the 3-dimensional polyglycolic acid, they demonstrated high chondrogenic potential and induced superior histological healing responses in vivo. In our study, we showed that auricular chondrocytes could be induced by ECMs to gain phenotypes similar to articular chondrocytes in 2-dimensional culture, 3-dimensional culture, and in vivo transplantation. Our results confirmed the feasibility of switching the cellular phenotypes of chondrocytes exclusively via the microenvironment created by ECM biomaterials.

There were 2 steps proposed in our method to induce the process of cell conversion of auricular chondrocytes for cartilage repair (Figure 1). The harvested auricular chondrocytes first lose their original phenotypes via monolayer expansion. Different types of chondrocytes underwent dedifferentiation during in vitro monolayer expansion. The status of chondrocyte dedifferentiation was characterized by alteration of cellular morphology, genetic profiles, and ECM synthesis. The dedifferentiation status facilitates cells to change their fates.

The next step of our method is to convert the dedifferentiated cells toward articular chondrocyte-like cells by specific ECM environment. Alteration of the chondrocyte ECM synthetic profile provides evidence to support the transition of cellular phenotypes. Auricular and articular chondrocytes have distinct gene expression profiles when cultured via monolayer expansion. Articular chondrocytes express much more Col II mRNA than do auricular chondrocytes, which makes Col II a typical marker to distinguish the types of chondrocytes. The initial 7-day Col II treatment tended to suppress Col I syntheses in auricular chondrocytes and enhanced Col II and aggrecan expression in a reciprocal manner. However, the 7-day treatment with Col II triggered auricular chondrocytes to partially differentiate into cells with an intermediate phenotype that exhibited both Col I and Col II expression. The results showed that a single-stage cultivation in a 7-day period might result in a mixed population of chondrocytes with different degrees of cell conversion. Therefore, an additional 7-day Col II cultivation was included in our methodology to strengthen the tendency of converting auricular chondrocytes toward articular-like cells.

![Figure 8](https://example.com/figure8.png)

Figure 8. Histological comparison of the native tissue, auricular type II collagen (AU Col II) constructs in vitro and AU Col II constructs in vivo. (A) Native rabbit articular cartilage was positively stained for Col II. (B) Native rabbit AU cartilage was positive for elastin staining. (C) The AU Col II constructs after 28 days of in vitro culture were positively stained for Col II, but (D) limited elastin positivity. (E) The AU Col II construct implanted for 3 months in vivo showed that the neomatrix was positive for Col II in the central portions, whereas (F) staining for elastin was scarcely detectable. (G, H) Collagen fibers in the uninjured sites of normal cartilage appeared blue upon Masson’s trichrome (MT) staining. (I, J) The AU Col II repair site showed similar homogeneous blue color staining. Scale bars in A-F, H, J: 50 μm; G, I: 200 μm.

Lubricin is a reliable cartilaginous marker reflecting the
differentiation status of chondrocytes since it is specifically synthesized by articular chondrocytes on the surface of articular cartilage for joint lubrication and synovial homeostasis. Comparable results were also found in GAG expression of converted auricular and articular chondrocytes. Accordingly, the results provide evidence that the 2-stage cell conversion protocol successfully induces auricular chondrocytes toward articular-like cells.

Col II has been widely used as a scaffold for cartilage engineering. In addition to its mechanical role, Col II facilitates chondrocyte proliferation and maintains phenotypes by modulating molecules specific to chondrocytes. Col II also affects several biological processes critical for cartilage homeostasis and repair. We previously compared the influence of distinct growth factors, including transforming growth factor β1, insulin-like growth factor, and fibroblast growth factor, with Col II on the chondrogenic potential of quiescent chondrocytes. Our results revealed that Col II alone could reactivates chondrogenic potential. Similar results have been reported when only exogenous Col II was employed to restore the genotypes in serially expanded chondrocytes. It is possible that the effect of Col II is due to signaling mediated by cell-matrix interaction. Col II has been reported to induce chondrocyte differentiation through activation of the FAK-ERK 1/2 and FAK-JNK signaling pathways. Further studies will help reveal the underlying molecular mechanism accounting for the competence of Col II in regulating cell differentiation. In addition to Col II, many reports also suggested that other ECM components might be beneficial for constructing the culture systems of regulating chondrocyte phenotypes.

Cartilage ECM is principally composed of type I and II collagen associated with extensive networks of proteoglycans. We have examined the roles of type I collagen, hyaluronic acid, and chondroitin sulfate in regulating chondrocyte differentiation. However, none of these factors have a better promoting effect than Col II. Hyaluronic acid and chondroitin sulfate have even been reported to inhibit chondrogenic gene expression of cultured chondrocytes. Therefore, the controversial effects of different ECM proteins on alteration of chondrogenic phenotypes needs further verification.

For osteochondral repair, the implanted constructs must be able to integrate with surrounding native tissue. The auricular Col II constructs showed successful in vivo integration with articular cartilage after transplantation. Auricular chondrocytes cultured in Col II scaffolds exhibited high viability, abundant proteoglycan syntheses, and increased expressions of articular cartilage markers. The cultivated cells within the auricular Col II constructs were induced to synthesize articular-specific ECM in the scaffolds, which helped to integrate the auricular Col II construct into the articular cartilage defects. Histologically, the defects repaired by the auricular Col II constructs were similar to those repaired by articular Col II constructs, as reported by our group and others. The articular Col II constructs were used as the controls in this study (Appendix Figure A1). On the other hand, to further delineate the important role of Col II in directing cell conversion, transplantsations of only auricular chondrocytes were employed as another control. However, auricular chondrocytes alone did not result in compatible healing outcomes (Figures 6 and 7). The results provided further evidence that using cells alone was not an effective approach.

Sufficient numbers of autologous chondrocytes are critical for the success of cell-based cartilage repair or tissue engineering. However, the number of cells harvested from articular cartilage for autologous transplantation is limited. Auricular cartilage produces nearly 2-fold cell yields compared with articular cartilage. Furthermore, auricular chondrocytes cultured in vitro have a 4-times greater proliferative rate than articular chondrocytes. Therefore, auricular chondrocytes are regarded as a cell source with efficient and sufficient cell expansion. Compared with the results of the articular Col II constructs (Appendix Figure A1), the auricular Col II constructs engineered by Col II also facilitated cartilage repair after transplantation in animals. Our method efficiently switches the cell phenotypes without harnessing the cells back to the very primitive status, which reduces time and cost and lowers the biological risks of complete cell reprogramming. These advantages extend the potential of clinical translation.

In this study, we tested the mechanical properties of constructs of converted auricular chondrocytes and articular chondrocytes by monotonic compression tests. We demonstrated that the stress-strain responses between the 2 constructs were similar. However, we have not yet confirmed functional mechanical properties of repaired cartilage. It is well known that the mechanical properties of tissue-engineered cartilage are not constant. When implanted in vivo, the mechanical features of engineered cartilage change dynamically with time, especially when the tissue further regenerates or integrates into the recipient cartilage. Therefore, the mechanical properties of these constructs may be different from the native cartilage. For potential future clinical translation, our next step will be to apply mechanical stimulation, such as hydrostatic pressure, dynamic shear, or compression, to enhance mechanical stiffness and ECM production in auricular Col II constructs. The effect will be tested by in situ biomechanical tests and nano-indentation examinations to ensure successful translation to future preclinical and clinical trials.

**CONCLUSION**

Chondrocytes harvested from auricular cartilage can be converted to articular cartilage–like cells exclusively by ECMs. The results confirmed the feasibility of engineering auricular chondrocytes to mitigate the drawbacks of applying heterotopic chondrocytes for cartilage repair. In this study, we propose a feasible and efficient methodology of applying autologous cartilage transplantation using heterotopic chondrocytes, which is expected to expedite the potential clinical application of cartilage repair.
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