Case Series

International Journal of Clinical Rheumatology

Chondrocyte implantation for treatment of articular cartilage

Anvar Khodiev*

Department of Chemical Engineering, Alzahra University, Tehran, Iran

***Author for Correspondence:**

khodiev@yahoo.com

Background: Because articular chondrocyte-based Autologous Chondrocyte Implantations (ACIs) have restrictively restored articular cartilage defects, alternative cell sources as a new therapeutic option for cartilage repair have been introduced.

Purpose: To assess whether implantation of a Costal Chondrocyte–Derived Pellet-Type (CCP) ACI allows safe, functional, and structural restoration of full-thickness cartilage defects in the knee.

Study Design

Case series: Level of evidence, Methods: In this first-in-human study, 7 patients with symptomatic, full-thickness cartilage lesions were enrolled. The chondrocytes isolated from the patients' costal cartilage were expanded, followed by 3-dimensional pellet culture to prepare the CCP-ACI. Implantation of the pellets was performed via minimal arthrotomy and secured with a fibrin sealant. Clinical scores, including the International Knee Documentation Committee (IKDC) subjective, Lysholm, and Tegner activity scores, were estimated preoperatively and at 1, 2, and 5 years postoperatively. High-resolution magnetic resonance imaging was also performed to evaluate cartilage repair as well as to calculate the MOCART (magnetic resonance observation of cartilage repair tissue) score.

Results: The costal chondrocytes of all patients formed homogeneous-sized pellets, which showed the characteristics of the hyaline cartilaginous tissue with lacunae-occupied chondrocytes surrounded by glycosaminoglycan and type II collagen-rich extracellular matrix. There were no treatment-related serious adverse events during the 5-year follow-up period. Significant improvements were seen in all clinical scores from preoperative baseline to the 5-year follow-up (IKDC subjective score, 34.67 to 75.86; Lysholm score, 34.00 to 85.33; Tegner activity score, 1.17 to 4.67; and MOCART score, 28.33 to 83.33). Two patients had complete defect filling on magnetic resonance imaging evaluation at 1 year. Moreover, at 5 years postoperatively, complete defect filling was observed in 4 patients, and hypertrophy or incomplete defect filling (50%-100%) was observed in 2 patients.

Conclusion: The overall results of this clinical study suggest that CCP-ACI can emerge as a promising therapeutic option for articular cartilage repair with good clinical outcomes and structural regeneration and with stable results at midterm follow-up.

Keywords: knee ● articular cartilage ● articular cartilage resurfacing ● clinical assessment/grading scales ● tissue engineering ● costal chondrocyte

Introduction

The intrinsic regenerative capacity of articular cartilage is limited given the absence of vascular supply and low cellularity. Furthermore, scattered chondrocytes in this tissue are surrounded by compact Extracellular Matrices (ECMs) that prevent migration of nearby cells to the site of injury, unlike most tissues. Therefore, articular cartilage injuries that are associated with pain and impairment of joint function leading to severe osteoarthritis remain a clinical challenge [1-5]. Since the first report of Brittberg et al., [4] several studies have confirmed positive clinical results for articular chondrocyte–based Autologous Chondrocyte Implantation (ACI) [5-8]. However, the reproducibility and durability of restored cartilage structure and function, as well as the cost-effectiveness, remain to be proved [9, 10]. Although articular cartilage can be easily

collected from healthy and non-weight bearing regions by arthroscopy, it has been extensively reported that this procedure may increase donor-site morbidity which increases the likelihood of developing posttraumatic osteoarthritis [11-14]. Furthermore, the size of donor tissues available from biopsy is significantly limited. The number of chondrocytes that can be isolated from articular cartilage and the expansion ability and cartilage-forming capacity of the cells vary by donor, which is one explanation for the age limitation regarding the application of conventional ACI [15, 16].

Nonarticular cartilage, such as nasoseptal, auricular, and costal cartilage, has been introduced as an alternative cell source for articular cartilage regeneration [17-21]. Among these options, costal cartilage is the largest permanent hyaline cartilage in the mammalian body. Although retrogressive change of calcification is often reported, actively proliferating chondrocytes were detected in patients aged ≥80 years, and a significant amount of costal cartilage is available in patients <60 years old [22, 23]. Because costal and articular cartilage originates from so-called somites, which are bilaterally paired blocks of paraxial mesoderm of the embryo, costal cartilage might have significant similarity to the articular cartilage [24]. Given its easy surgical accessibility and expandability in vitro, numerous reports support the idea of using chondrocytes from costal cartilage as an ideal cell source for articular cartilage regeneration [25]. Lee et al. [26] and Isogai et al. [27] reported costal cartilage as a promising source of chondrocytes for hyaline cartilage regeneration and confirmed that the chondrocytes derived from this source were comparable with those obtained from articular cartilage, with superior expandability and better capacity to generate hyaline-like cartilage tissues. Furthermore, costal chondrocytes can produce superficial zone proteins in vitro, indicating that they possess a regeneration capacity for full ranges of articular cartilage, even though costal cartilage is a non-articular cartilage in its native form [28]. Experimental animal studies that used costal chondrocytes to cure articular cartilage defects in a rabbit model showed an adequate healing outcome, with no age dependency for their healing capacity [29-32]. However, no human studies that used costal chondrocytes for articular cartilage regeneration have been reported. CartiLife (Biosolution Co, Ltd) is a small pellet-type ACI that is manufactured from the chondrocytes of a patient's own costal cartilage, followed by expansion culture and 3-dimensional (3D) pellet culture. During the pellet culture, chondrocytes are initially aggregated and induced to differentiate into cartilage to produce their own ECM that is similar to the natural ECM of the hyaline cartilage [33, 34]. This first-in-human study aims to evaluate the feasibility, safety, and efficacy of Costal Chondrocyte–Derived Pellet-Type (CCP) ACI for the treatment of full-thickness cartilage defects of the knee. Magnetic resonance imaging (MRI) analysis and clinical outcomes were used to evaluate efficacy through 5 years of clinical follow-up.

Methods

Study Design and patients

This first-in-human study was conducted at Kyung-Hee University Medical Center. The inclusion criteria were as follows: age ≥19 years and International Cartilage Repair Society grade III or IV isolated chondral lesions of the knee (2 cm^2 to10 cm^2 in area, up to 4 cm^3 in volume). The chondral lesions were diagnosed and sized according to MRI findings. The exclusion criteria were as follows: inflammatory arthritis, arthritis associated with autoimmune diseases, a history of radiotherapy and chemotherapy within the past 2 years, and pregnancy. The protocol was written according to the good clinical practice principles and the Declaration of Helsinki [35, 36].

Preparation of the CCP-ACI

Patients underwent arthroscopic examination 4 weeks to 5 weeks before implantation to determine the definitive size of the cartilage defect of the knee. At this time, cartilage biopsy (approximately 500 mg) was performed from the 8th, 9th, or 10th costal cartilage region according to standard surgery instructions. After the skin incision (≤2 cm) was made, blunt dissection of the subcutaneous tissue and the external abdominal oblique muscle was performed to expose the perichondrium. Subsequently, the perichondrium was incised and separated, and costal cartilage tissue was harvested with a gouge (**Figures 1A and 1B**).

The cartilage biopsy specimen was stored in sterile Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies), which was sent to the good manufacturing practices facility at Biosolution Co, Ltd, for isolation and culture of autologous cells according to the following protocol. The costal cartilage biopsy specimen was minced into 1- to 2-mm^3 pieces, and the cartilage tissue fragments were then digested with 0.5% pronase and 0.2% type II collagenase overnight in an incubator (37°C, 5% CO_2). The isolated cells were suspended in MSCGM (Lonza) with FGF-2 (1 ng/mL; R&D Systems) and then plated onto culture dishes at a cell density of 1×10^4 cells/cm^2. The medium was changed

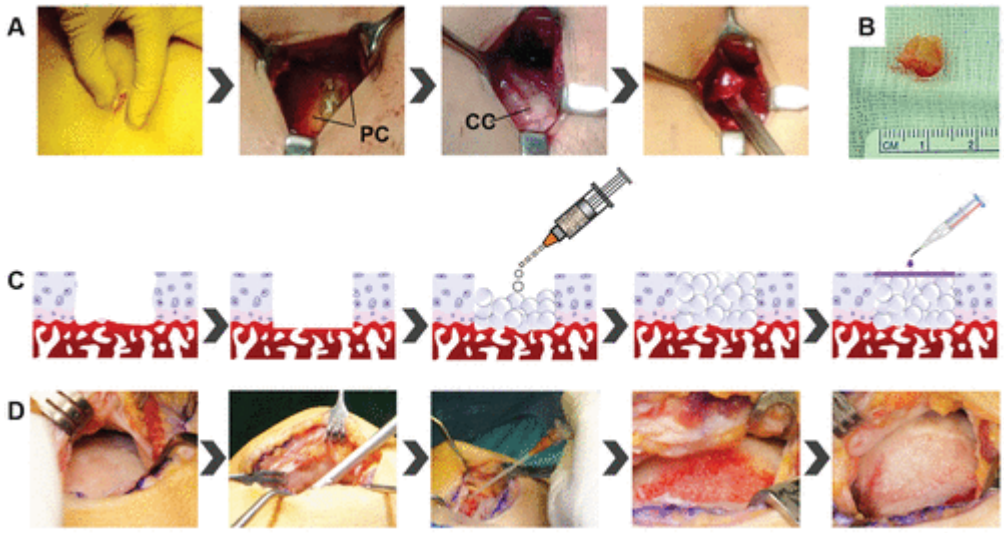

Figure 1. (A, B) Harvesting costal cartilage tissue from the patient. (C) Schematic illustration and (D) photos of the implantation procedure of costal chondrocyte–derived pellet-type autologous chondrocyte implantation. After debridement of the damaged cartilage, an intravenous catheter (14-gauge) was connected to the prefilled syringe, and the pellets were implanted into the defect by slowly pushing the plunger. During this step, the pellets were transplanted to the level of the adjacent cartilage in a manner to ensure that they did not protrude or sink. Fibrin glue was applied to the surface to fix the pellets in the defect. CC: Costal Cartilage; PC: Perichondrium.

twice a week, and the primary cells were subcultured at full confluence up to passage 6. To prepare the pellet-type cartilaginous tissues without an exogenous scaffold, the cells were suspended in chondrogenic medium, and the suspension was distributed into the wells of a 96-well plate with a V-shaped bottom (Axygen Scientific, Inc) at a density of 1.0×10^5 cells/well. The chondrogenic medium comprised DMEM, 1% ITS+3, 100nM dexamethasone, 50-µg/mL ascorbic acid, 40-µg/mL proline (Sigma-Aldrich), and 10-ng/mL TGF-β_3 (ProSpec TechnoGene). After centrifugation at 1200 rpm for 5 minutes, the cells were 3D cultured in an incubator (37°C; 5% CO_2). After 10 days of 3D culture, 480 pellets from five 96-well plates were collected. The collected pellets were then suspended in 3 mL of phenol red-free DMEM (Lonza) and packaged into a 5-mL prefilled syringe. The volume of 480 pellets of a prefilled syringe is approximately 1 mL, which is capable of filling a lesion of 1 cm^3.

Cell viability and total cell number were assessed by a trypan blue exclusion method. After the pellet was digested in the final product with 0.5% type II collagenase at 37°C for 30 minutes, the cell suspension was stained with 0.4% trypan blue in 1:1 concentration. The unstained viable cells and blue-stained dead cells were manually counted on the hemocytometer. For morphometric analysis, digital images of 12 randomly selected pellets were taken with a digital camera–attached inverted microscope (Olympus CKX41; Olympus Optical Co), and the pellet size (maximum diameter) was then measured with Image J software (National Institutes of Health). For reverse transcription (RT) polymerase chain reaction (PCR) analysis, RNA was isolated from the pellets with Trizol Reagent (Gibco Laboratories, Life Technologies). A total of 1 mg of RNA was reverse transcribed with an RT polymerase kit (Takara Bio Inc), followed by PCR with human gene–specific type II collagen primers (GenBank accession NM_001844; product size, 257 bp). After PCR amplification, PCR products were analyzed by electrophoresis in 2% agarose gel. Early-passage (passage 1-3) costal chondrocytes and dermal fibroblasts were used as positive and negative controls for type II collagen expression, respectively. The Glycosaminoglycan (GAG) content of the pellet was quantitatively determined with the 1,9-dimethyl methylene blue assay (Blyscan Glycosaminoglycan Assay; Biocolor Ltd). The pellet was digested with papain solution (125 µg/mL), and the subsequent process carried out according to the manufacturer's specification. For histological and biochemical assessments, the pellet samples were fixed with 10% neutral buffered formalin and cryosectioned at a thickness of 5 µm. The thin sections were stained with safranin O or fast green or were subjected to immunofluorescence staining with antibodies against type II collagen (Millipore), aggrecan (R&D Systems), type I collagen (SouthernBiotech Associates Inc), and type X collagen (Sigma).

Surgical procedure, rehabilitation, and assessment

Implantation of CCP-ACI was performed through

miniarthrotomy of the knee. The cartilage defect was prepared by removing all damaged cartilage down to the subchondral bone. The defect size was reassessed after debridement of the lesion area. An intravenous catheter (14 gauge; BD Medical) was connected to the prefilled syringe, and the pellets were implanted into the defect by slowly pushing the plunger of the syringe to the height of the adjacent cartilage. Fibrin glue (Greenplast; Green Cross Corp) was applied on the top of the defect to fix the pellets (**Figure 1**). A manual range of motion test of the knee was performed before wound closure to ensure pellet fixation. All patients were encouraged to complete a postoperative rehabilitation protocol. Continuous passive motion within postoperative 24 hours was recommended and continued for 4 weeks to 6 weeks, 6 hours to 8 hours daily. Partial weightbearing walking was recommended at 2 weeks postoperatively with a gradual increase to 6 weeks after transplantation, followed by full weightbearing for 6 weeks to 12 weeks to maximize tissue repairing. Patients who undertook surgery in the trochlear groove were strongly advised to actively refrain from high-loading activities on this area, such as squatting. Eight weeks after transplantation, all patients were monitored for signs of immune reactions through blood analyses, such as leukocyte counts and immunoglobulin M levels. Other safety parameters monitored included Adverse Events (AEs) and anomalies in laboratory tests, vital signs, and physical examinations during the 48 weeks of follow-up. The severity of each AE was graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (v 4.0) [37-39]. Safety assessments based on physical examinations and efficacy assessments based on International Knee Documentation Committee (IKDC) subjective, Lysholm, and Tegner activity scores were conducted during the 5-year extended follow-up period. Clinical scores for preoperative baseline were obtained before surgery on the day of transplantation. High-resolution MRI with a 3.0-T MRI system (3-T Achieva; Philips Medical Systems) was also performed to evaluate the level of structural improvement of the regenerated cartilage. After imaging, the MOCART (magnetic resonance observation of cartilage repair tissue) score was used to define the extent of tissue repair, including thickness, surface, and signal intensities. Evaluations were performed by independent orthopaedic specialists not involved in the treatment of patients in this study [40].

Statistical Analysis

All statistical analyses were performed with SAS (v 9.3; SAS Institute Inc.). The data were statistically analyzed with a repeated measures analysis of variance. The statistical data are presented as mean ± standard deviation. *P* values <.05 were considered statistically significant.

Results

Baseline participant characteristics

From July 2012 to January 2013, 7 patients with full-thickness articular cartilage defects of the knee joint were treated with CCP-ACI. Safety was evaluated for 48 weeks on all 7 patients, whereas efficacy evaluation and the extended 5-year follow-up study were performed in 6 patients because 1 patient was excluded owing to violation of the inclusion/exclusion criteria after implantation of CCP-ACI. The mean age and body mass index of the 7 participants who received the treatment were 40.00 ± 8.72 years and 27.53 ± 2.94 kg/m^2. The mean defect area and volume were 4.32 ± 2.04 cm^2 and 2.17 ± 1.36 cm^3, respectively.

Characteristics of the pellet-type ACI derived from costal cartilage

Cells were successfully isolated from the costal cartilage of all the patients at a mean 655 ± 168 cells/mg of costal cartilage tissue. The mean duration of expansion culture was 24.29 ± 5.88 days, and the entire manufacturing process from biopsy to the final product took about 4 weeks to 6 weeks. The mean population doubling time during the expansion culture was 1.72 ± 0.59 days (**Table 1**). During the expansion culture, the costal chondrocytes progressively exhibited a dedifferentiated phenotype characterized by fibroblast-like morphology and type I collagen expression (**Figures 2A and 2B**). After a 10-day chondrogenic pellet culture, the cells formed solid, smooth, and glossy white pellets with diameters of 1.1 mm to 1.5 mm (**Figures 2C-E**). The costal chondrocytes from all patients formed pellets with a relatively constant size and GAG content. The characteristics of the pellets in the final product for each patient are shown in **Table 1**. To determine the chondrogenic phenotype of the pellets, type II collagen, aggrecan, type I collagen, and type X collagen expressions were evaluated by RT-PCR or immunofluorescence staining. Furthermore, cell morphology and GAG distribution in the pellets were evaluated after safranin O staining. Based on RT-PCR evaluation, all pellets from each patient expressed type II collagen gene (**Figure 2F**). Histologically, the cells in the entirety of the pellet, excluding the outermost surface, were chondrocyte-typical round, homogeneously distributed, and enveloped in a GAG-positive matrix (**Figure 2G**).

Table 1. In-process and final product characteristics.					
Patient	**Isolated Cells**	**Mean PDT**	**Viability**	**Cell Number**	**GAG**
R01	762	1.14	99.90	7.11	13.27
RO2	655	0.97	100	6.61	13.26
RO3	610	1.26	99.90	6.91	13.30
RO4	684	2.36	100	8.09	14.34
RO5	404	2.37	99.92	8.25	15.18
RO6	933	2.05	99.88	5.61	11.70
RO7	536	1.91	100	5.73	13.03

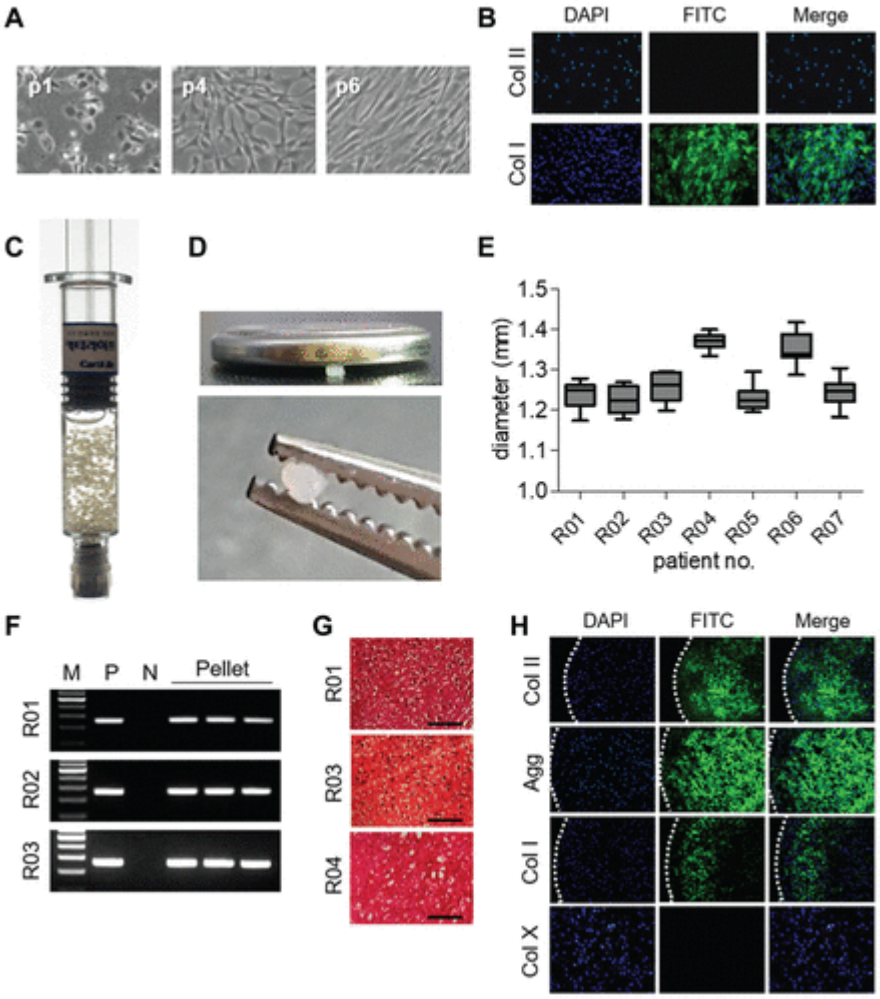

Figure 2. Characteristics of costal chondrocytes before 3-dimensional culture and costal chondrocyte–derived pellet-type autologous chondrocyte implantation. (A) Cell morphology during the cell expansion culture (passages 1, 4, and 6). (B) Immunofluorescent staining for type I and II collagen on costal chondrocyte at passage 6. (C) Macroscopic appearance of CartiLife. (D) A solid pellet enduring the weight of a lithium battery (CR2032). (E) Diameter ranges of the pellets for each patient (n = 20). Values are presented as median (line), interquartile range (box), and 95% CI (error bars). (F) Reverse transcription polymerase chain reaction analysis for type II collagen expression. M, DNA marker; N, negative control (dermal fibroblast); P, positive control (early passage costal chondrocyte). (G) Microscopic appearance of the pellets assessed with safranin O staining. Scale bar, 100 µm. (H) Immunofluorescence staining for type II collagen, aggrecan, type I collagen, and type X collagen. White dot line, pellet surface. Agg: Aggrecan; Col I: type I collagen; Col II: type II collagen; Col X: type X collagen; DAPI: 4′,6-diamidino-2-phenylindole; FITC: Fluorescein Isothiocyanate.

Immunohistochemical staining was performed against type II collagen and aggrecan as specific markers of dedifferentiated chondrocytes to confirm the phenotype of the cells. Although the expression of type II collagen was weak, it was observed throughout the pellets except for the outer layer. In addition, the strong expression

of aggrecan was detected in the entire pellets. However, type I collagen was detected in the outer layer of the pellet, and expression of type X collagen, a hypertrophic chondrocyte–specific marker, was barely observed (**Figure 2H**).

AEs and safety assessment

No signs of immune reactions were detected in any participants. In total, 7 AEs occurred, the most common being procedural pain (n = 4) attributed to surgery for costal cartilage biopsy and CCP-ACI transplantation. The other AEs were arthralgia, headache, and nausea (n = 1, respectively) during the 48-week clinical trial. All AEs were mild and resolved without sequelae or complications. There were no clinically important trends in the data for physical examination, vital signs, and laboratory tests during the 48-week clinical trial. During the extended 5-year follow-up, no specific adverse reactions, including immune reactions, osteogenesis, or tumorigenesis, were observed in the 6 participants. No participants underwent additional knee surgery because of pain, aggravation, or functional impairment during the follow-up period. One patient experienced an ipsilateral patella fracture 41 months postoperatively. However, this patient recovered from the fracture and exhibited improved clinical scores at the 5-year follow-up visit.

Clinical outcomes

In all 6 participants, CCP-ACI was associated with improved clinical scores over the 5-year follow-up period. The mean improvements of the IKDC subjective score were 23.95 ± 13.80, 30.08 ± 19.63, and 41.19 ± 17.26 at the 48-week (1 year), 2-year, and 5-year follow-up visits, respectively. Lysholm score improvements at the same visits were 24.50 ± 11.88, 35.67 ± 21.55, and 51.33 ± 21.65, and those of the Tegner activity score were 2.33 ± 1.86, 3.17 ± 1.33, and 3.50 ± 1.05. Statistically significant improvements from baseline were observed for all clinical scores at the 48-week, 2-year, and 5-year follow-up visits ($P < .05$ or $P < .005$). Also, the Lysholm score improved significantly between 48 weeks and 5 years as well as between 2 years and 5 years ($P = .0127$ and .0206, respectively). The clinical outcome scores of each patient are presented in **Figures 3A-C**.

Magnetic resonance imaging

High-resolution MRI was performed before and 48 weeks (1 year), 2 years, and 5 years after implantation to evaluate structural improvements induced by CCP-ACI. The mean improvements of the MOCART score were 44.17 ± 14.97, 51.67 ± 14.02, and 55.00 ± 17.32 at 48 weeks, 2 years, and 5 years after implantation, respectively. Significant improvements from the baseline were seen at all 3 follow-up visits ($P = .0008$,

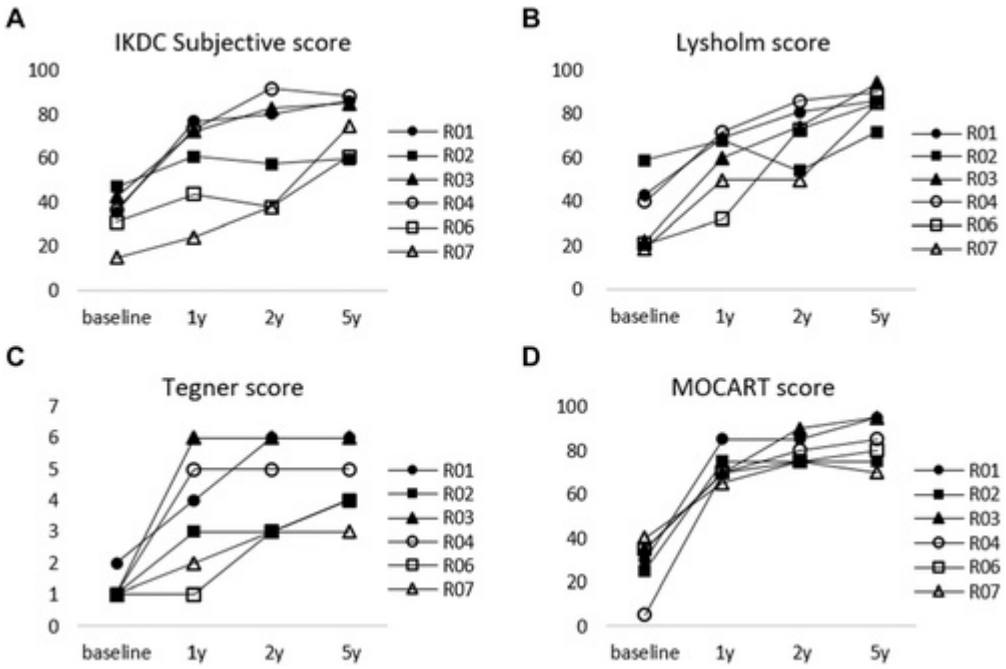

Figure 3. Changes of the (A) IKDC subjective knee score, (B) Lysholm score, (C) Tegner activity score, and (D) MOCART score during the 5-year follow-up period. All clinical scores and MRI score at 48 weeks (1 year), 2 years, and 5 years postoperatively were significantly improved when compared with the baseline. Each line represents 1 patient. IKDC, International Knee Documentation Committee; MOCART, magnetic resonance observation of cartilage repair tissue; MRI: Magnetic Resonance Imaging.

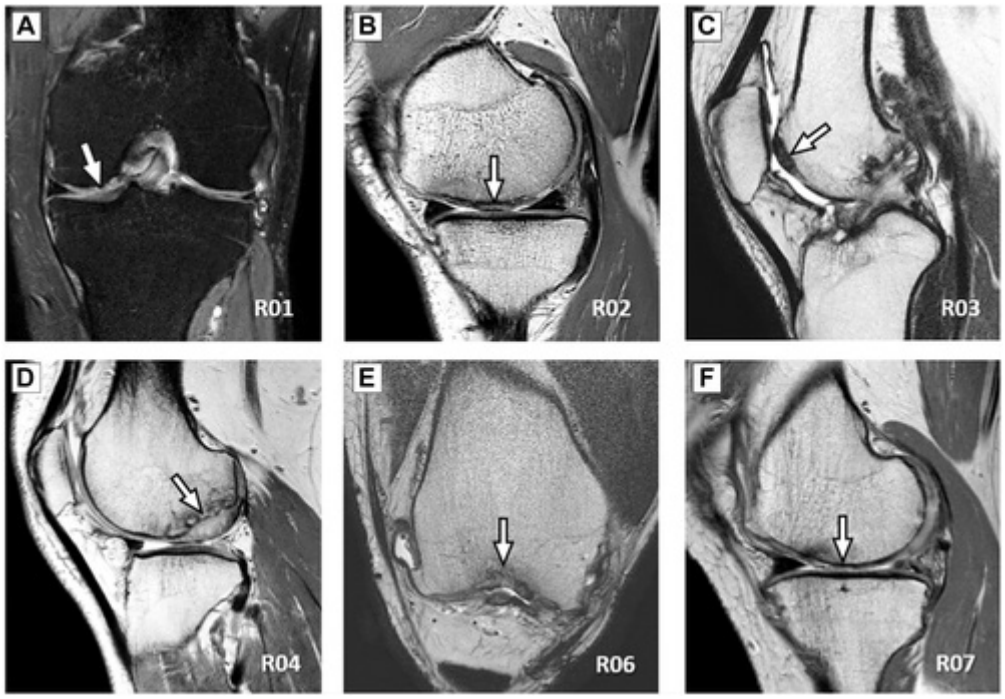

Figure 4. MRI evaluation of cartilage regeneration after costal chondrocyte–derived pellet-type autologous chondrocyte implantation transplantation. At 5 years after implantation, MRI revealed that the repair tissues were (C) thicker, (F) thinner, or (A, B, D, E) nearly identical to the surrounding normal cartilage. White arrow, regenerative cartilage. MRI: Magnetic Resonance Imaging.

.0003, and .0006). The comparison between 48 weeks and 5 years showed the same trend (P = .0274). The MOCART score of each patient is presented in Figure 3D. Complete filling of the defect was detected in 2, 3, and 4 patients at 48 weeks, 2 years, and 5 years after implantation, respectively, regarding the thickness of the repair tissue as compared with that of the adjacent native cartilage (Figure 4). Hypertrophic repair tissue was detected at 48 weeks and 2 years in 2 patients, 1 of whom exhibited hypertrophic cartilage after 5 years. No patient displayed worsening of the defect filling over time. In all patients, the signal intensity of the repair tissue in fast spin echo (dual T2-FSE) and fat-suppressed gradient echo (3D-GE-FS) sequences was isointense as compared with the adjacent native cartilage at 5 years after implantation. In addition, adhesion was not detected preoperatively or during the 5-year follow-up period. The preoperative synovitis detected in 2 patients completely disappeared postoperatively (Figure 4).

Although we evaluated the relationships between the clinical and MRI scores, no significant correlations were obtained given the small number of patients.

Discussion

In our phase 1 cohort study, we showed the feasibility of using costal chondrocytes as an alternative cell source for ACI. The study confirmed that CCP-ACI is a safe and effective treatment for articular cartilage defects in terms of improvement of symptoms and knee function as well as structural regeneration. Treating cartilage lesions is an important challenge because of the difficulty in restoring the damaged areas with hyaline cartilage, which is a prerequisite for long-lasting regeneration and improved functionality [32]. Several long-term clinical studies for suspension-type and tissue-engineered ACIs have reported that the clinical and MRI scores were highest at 1- or 2-year follow-up and deteriorated at 6- or 7-year follow-up [11,38]. Conventional ACIs produce predominantly fibrocartilage and a mixture of hyaline and fibrocartilage. These cartilages lack biomechanical properties as compared with the normal hyaline cartilages, perhaps because the dedifferentiated chondrocytes during in vitro expansion cultures are implanted. It is also possible that the implanted cells undergo apoptosis because of physical stress and inflammation. However, in our study, all clinical scores as well as MOCART scores at 1 year were significantly improved as compared with the presurgical values, with greater increases observed after 5 years. Based on a previous animal study, transplantation of costal chondrocyte–derived tissue-engineered ACI regenerated cartilage defects into hyaline cartilage, which was confirmed by histological

and immunohistochemical analysis [21]. In addition to the animal study data, the hyaline cartilaginous ECM of CCP-ACI produced by the costal chondrocytes during in vitro 3D cultivation allows us to expect that hyaline-like repair tissue produced by CCP-ACI will result in superior and more durable clinical results. The biochemically and mechanically supportive microenvironment provided by the cartilaginous matrix surrounding the implanted cells in CCP-ACI can exert a cytoprotective effect, which might have potentiated the functionality of the chondrocytes and resulted in the improved clinical outcome. To confirm the hyaline cartilaginous nature of newly restored cartilage tissues, we should perform multiparametric MRI or histological analyses of the repaired tissues in our future clinical trials. The size of the CCP-ACI pellet is about 1.1 to 1.5 mm in diameter, which allows its implantation via less invasive surgery, such as miniarthrotomy or arthroscopy, and is easily applicable regardless of contour, curvatures, and thicknesses of the cartilage defects. CCP-ACI is implanted in a filling-up manner depending on the depth of the defect; therefore, it is possible to obtain a result where the cartilage defect is filled with hyaline cartilage–like tissue immediately after implantation. To produce a pellet with appropriate size and properties, we optimized the number of cells per pellet and the duration of its 3D chondrogenic culture. The culture period for in vitro cartilage formation is important for determining its optimal implantation status. Biochemical and biomechanical variables are the criteria for assessing tissue maturity and the timing of implantation [13, 20]. Mature in vitro cartilage tissues with good biomechanical characteristics might not be a desirable option, mostly because of their poor ability to integrate with the surrounding host tissue. Despite insufficient mechanical properties exhibited by the immature cartilage tissues, these tissues with a higher biosynthetic activity would be more desirable knee cartilage. According to a previous study, pellets obtained from chondrogenic culture of costal chondrocytes for 7 days to 14 days had the most significant active biosynthetic activity based on the results of biochemical and protein expression assays [23]. Therefore, we decided to set the chondrogenic culture period to 10 days. After 10 days of 3D chondrogenic culture, the cells in the pellet were enveloped in a matrix that strongly expressed GAG, existed in high density, and displayed the morphology of the immature hyaline cartilage tissues (**Figures 2F-H**). Graft hypertrophy is known as a characteristic complication after ACI surgery, occurring in up to 30% of all patients [15]. Although some epidemiological data suggest that its incidence is closely associated with the use of periosteal grafts for first-generation ACIs, graft hypertrophy has also been reported in about 25% patients after tissue-engineered ACI surgery [9] indicating that this is not exclusively related to the use of the periosteal flap. Graft hypertrophy can be considered transient excessive growth of the regenerative cartilage tissue rather than chondrocyte hypertrophy related to terminal differentiation of the chondrocyte.[28] In our study, graft hypertrophy was observed in 2 patients with cartilage lesions located in the trochlear groove. Henderson et al. [15] suggested that graft hypertrophy may be of cellular origin. Therefore, the high cell density in pellets and active ECM synthetic activity of the cells may have showed a combined effect resulting in excessive growth of regenerative cartilage tissue. Another possibility is that during the implant process, the pellets were not filled to the level of the surrounding tissue but rather planted with an excess number of pellets, thus resulting in cartilage hypertrophy. It remains unclear whether this occurs only in the trochlear groove; however, the difference in the biomechanical environment among different locations of the cartilage could affect the growth of newly developing cartilage tissue and the regression of the repair tissue. In this study, graft hypertrophy was not a persistent or systematic complication or cause for reoperation. The clinical results of this study suggest that CCP-ACI could emerge as a promising therapeutic option for articular cartilage repair in terms of safety, positive clinical results, and durability. Limitations of our study include the following: first, the nonhomologous use of costal cartilage for the regeneration of articular cartilage defects; second, the difficulty in confirming its reproducibility and feasibility in patients aged >50 years, who have increased risk of osteoarthritis, given the inclusion of a small number of relatively young patients with no active control. To define whether CCP-ACI is reproducible and feasible in a heterogeneous patient population and is significantly superior to standard treatment for cartilage defects, we are conducting a multicenter randomized active-controlled (microfracture) phase 2 clinical study (NCT03545269) and are planning to report the results.

Conclusion

This study demonstrated that the costal chondrocytes of all patients formed homogeneous-sized pellets, which showed the characteristics of the hyaline cartilaginous tissue with lacunae-occupied chondrocytes surrounded by glycosaminoglycan and type II collagen-rich extracellular matrix. There were no treatment-related serious adverse events during the 5-year follow-up period. Significant improvements were seen in

all clinical scores from preoperative baseline to the 5-year follow-up. Moreover, at 5 years postoperatively, complete defect filling was observed. In summary, we demonstrate that CCP-ACI is feasible and safe. It also provides improvement in terms of clinical outcomes and cartilage repair during 5 years of follow-up.

References

1. Andriolo L, Merli G, Filardo G *et al*. Failure of autologous chondrocyte implantation. *Sports. Med. Arthrosc. Rev.* 25(1), 10-18 (2017).
2. Barbero A, Grogan S, Schäfer D *et al.* Age related changes in human articular chondrocyte yield, proliferation and post–expansion chondrogenic capacity. *Osteoarthritis. Cartilage*. 12(6), 476-484 (2004).
3. Bartlett W, Skinner JA, Gooding CR *et al.* Autologous chondrocyte implantation versus matrix–induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study. *J. Bone. Joint. Surg. Br.* 87(5), 640-645 (2005).
4. Brittberg M, Lindahl A, Nilsson A *et al.* Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* 331(14), 889-895 (1994).
5. Buckwalter JA, Mankin HJ. Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation. *Instr. Course. Lect.* 47, 487-504 (1998).
6. Caplan AI, Elyaderani M, Mochizuki Y *et al*. Principles of cartilage repair and regeneration. *Clin. Orthop. Relat. Res.* 342, 254-269 (1997).
7. Dewan AK, Gibson MA, Elisseeff JH *et al.* Evolution of autologous chondrocyte repair and comparison to other cartilage repair techniques. *Biomed. Res. Int*. 2014, 272481 (2014).
8. DiBartola AC, Everhart JS, Magnussen RA *et al.* Correlation between histological outcome and surgical cartilage repair technique in the knee: a meta–analysis. *Knee.* 23(3), 344-349 (2016).
9. Ebert JR, Smith A, Fallon M *et al.* Incidence, degree, and development of graft hypertrophy 24 months after matrix–induced autologous chondrocyte implantation: association with clinical outcomes. *Am. J. Sports. Med.* 43(9), 2208-2215 (2015).
10. El Sayed K, Haisch A, John T *et al.* Heterotopic autologous chondrocyte transplantation—a realistic approach to support articular cartilage repair?. *Tissue. Eng. Part. B. Rev*. 16(6), 603-616 (2010).
11. Filardo G, Kon E, Di Martino A *et al*. Arthroscopic second–generation autologous chondrocyte implantation: a prospective 7–year follow–up study. *Am. J. Sports. Med.* 39(10), 2153-2160 (2011).
12. Gooding CR, Bartlett W, Bentley G *et al.* A prospective, randomised study comparing two techniques of autologous chondrocyte implantation for osteochondral defects in the knee: periosteum covered versus type I/III collagen covered. *Knee.* 13(3), 203-210 (2006).
13. Grande DA, Breitbart AS, Mason J *et al*. Cartilage tissue engineering: current limitations and solutions. *Clin. Orthop. Relat. Res*. 367, S176-S185 (1999).
14. Harris JD, Siston RA, Brophy RH *et al*. Failures, re–operations, and complications after autologous chondrocyte implantation—a systematic review. *Osteoarthritis. Cartilage*. 19(7), 779-791 (2011).
15. Henderson I, Gui J, Lavigne P. Autologous chondrocyte implantation: natural history of postimplantation periosteal hypertrophy and effects of repair–site debridement on outcome. *Arthroscopy.* 22(12), 1318-1324 (2006).
16. Huey DJ, Hu JC, Athanasiou KA. Unlike bone, cartilage regeneration remains elusive. *Science.* 338(6109), 917-921 (2012).
17. Isogai N, Kusuhara H, Ikada Y *et al.* Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. *Tissue. Eng*. 12(4), 691–703 (2006).
18. Knutsen G, Drogset JO, Engebretsen L *et al.* A randomized trial comparing autologous chondrocyte implantation with microfracture: findings at five years. *J. Bone. Joint. Surg. Am*. 89(10), 2105–2112 (2007).
19. Kreuz PC, Steinwachs M, Erggelet C *et al.* Classification of graft hypertrophy after autologous chondrocyte implantation of full–thickness chondral defects in the knee. *Osteoarthritis. Cartilage*. 15(12), 1339–1347 (2007).
20. Lee CR, Grodzinsky AJ, Hsu HP *et al.* Effects of a cultured autologous chondrocyte–seeded type II collagen scaffold on the healing of a chondral defect in a canine model. *J. Orthop. Res*. 21(2), 272–281 (2003).
21. Lee J, Lee E, Kim HY *et al.* Comparison of articular cartilage with costal cartilage in initial cell yield, degree of dedifferentiation during expansion and redifferentiation capacity. *Biotechnol. Appl. Biochem*. 48, 149–158 (2007).
22. Lee J, Lee JY, Chae BC *et al.* Fully dedifferentiated chondrocytes expanded in specific mesenchymal stem cell growth medium with FGF2 obtains mesenchymal stem cell phenotype in vitro but retains chondrocyte phenotype in vivo. *Cell. Transplant.* 26(10), 1673–1687 (2017).
23. Lee J, Lee JY, Lee E *et al.* Serum deprivation induces efficient chondrogenic differentiation of fully dedifferentiated human costal chondrocytes. *Tissue. Eng. Regen. Med.* 6(4–11), 957–962 (2009).
24. Mori R, Kataoka H, Kuriwaka M *et al.* Articular cartilage restoration with costal cartilage previously fused with bone. *Clin. Orthop. Relat. Res.* 406, 262–274 (2003).
25. Mumme M, Barbero A, Miot S *et al.* Nasal chondrocyte–based engineered autologous cartilage tissue for repair of

articular cartilage defects: an observational first–in–human trial. *Lancet.* 388(10055), 1985–1994 (2016).

26. Mumme M, Steinitz A, Nuss KM *et al.* Regenerative potential of tissue–engineered nasal chondrocytes in goat articular cartilage defects. *Tissue. Eng. Part. A*. 22(21–22), 1286–1295 (2016).
27. Murphy MK, DuRaine GD, Reddi A *et al.* Inducing articular cartilage phenotype in costochondral cells. *Arthritis. Res Ther.* 15(6), R214 (2013;).
28. Niemeyer P, Uh M, Salzmann GM *et al*. Evaluation and analysis of graft hypertrophy by means of arthroscopy, biochemical MRI and osteochondral biopsies in a patient following autologous chondrocyte implantation for treatment of a full–thickness–cartilage defect of the knee. *Arch. Orthop. Trauma. Surg.* 135(6), 819–830 (2015).
29. Niethammer TR, Pietschmann MF, Horng A *et al.* Graft hypertrophy of matrix–based autologous chondrocyte implantation: a two–year follow–up study of NOVOCART 3D implantation in the *knee. Knee. Surg. Sports. Traumatol. Arthrosc.* 22(6), 1329–1336 (2014).
30. Ochi M, Uchio Y, Tobita M *et al.* Current concepts in tissue engineering technique for repair of cartilage defect. *Artif. Organs*. 25(3), 172–179 (2001).
31. Ogura T, Mosier BA, Bryant T *et al*. A 20–year follow–up after first–generation autologous chondrocyte implantation. *Am. J. Sports. Med.* 45(12), 2751–2761 (2017).
32. Peterson L, Minas T, Brittberg M *et al*. Two– to 9–year outcome after autologous chondrocyte transplantation of the knee. *Clin. Orthop. Relat. Res*. 374, 212–234 (2000).
33. Popko J, Szeparowicz P, Sawicki B *et al*. Rabbit articular cartilage defects treated with cultured costal chondrocytes (preliminary report). *Folia. Morphol.* 62(2), 107–112 (2003).
34. Quintarelli G, Dellovo MC. Age changes in the localization and distribution of glycosaminoglycans in human hyaline cartilage. *Histochemie.* 7(2), 141–167 (196).
35. Roberts S, Hollander AP, Caterson B *et al*. Matrix turnover in human cartilage repair tissue in autologous chondrocyte implantation. *Arthritis. Rheum*. 44(11), 2586–2598 (2001).
36. Saadeh PB, Brent B, Mehrara BJ *et al.* Human cartilage engineering: chondrocyte extraction, proliferation, and characterization for construct development. *Ann. Plast. Surg.* 42(5), 509–513 (1999).
37. Szeparowicz P, Popko J, Sawicki B *et al.* Is the repair of articular cartilage lesion by costal chondrocyte transplantation donor age–dependent? An experimental study in rabbits. *Folia. Histochem. Cytobiol.* 44(3), 201–206 (2006).
38. Takazawa K, Adachi N, Deie M *et al.* Evaluation of magnetic resonance imaging and clinical outcome after tissue–engineered cartilage implantation: prospective 6–year follow–up study. *J. Orthop. Sci.* 17(4), 413–424 (2012).
39. Trotti A, Colevas AD, Setser A *et al.* CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin. Radiat. Oncol.* 13(3), 176–181 (2003).
40. Wong CC, Chen CH, Chiu LH *et al.* Facilitating in vivo articular cartilage repair by tissue–engineered cartilage grafts produced from auricular chondrocytes. *Am. J. Sports. Med*. 46(3), 713–727 (2018).
41. Zhang Z, McCaffery JM, Spencer RG *et al.* Hyaline cartilage engineered by chondrocytes in pellet culture: histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants. *J. Anat.* 205(3), 229–237 (2004).