Human Cartilage-Derived Progenitor Cells From Committed Chondrocytes for Efficient Cartilage Repair and Regeneration

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ABSTRACT

Articular cartilage is not a physiologically self-renewing tissue. Injury of cartilage often progresses from the articular surface to the subchondral bone, leading to pathogenesis of tissue degenerative diseases, such as osteoarthritis. Therapies to treat cartilage defects using autologous chondrocyte-based tissue engineering have been developed and used for more than 20 years; however, the challenge of chondrocyte expansion in vitro remains. A promising cell source, cartilage stem/progenitor cells (CSPCs), has attracted recent attention. Because their origin and identity are still unclear, the application potential of CSPCs is under active investigation. Here we have captured the emergence of a group of stem/progenitor cells derived from adult human chondrocytes, highlighted by dynamic changes in expression of the mature chondrocyte marker, COL2, and mesenchymal stromal/stem cell (MSC) marker, CD146. These cells are termed chondrocyte-derived progenitor cells (CDPCs). The stem cell-like potency and differentiation status of CDPCs were determined by physical and biochemical cues during culture. A low-density, low-glucose 2-dimensional culture condition (2DLL) was critical for the emergence and proliferation enhancement of CDPCs. CDPCs showed similar phenotype as bone marrow mesenchymal stromal/stem cells but exhibited greater chondrogenic potential. Moreover, the 2DLL-cultured CDPCs proved efficient in cartilage formation both in vitro and in vivo and in repairing large knee cartilage defects (6–13 cm²) in 15 patients. These findings suggest a phenotype conversion between chondrocytes and CDPCs and provide conditions that promote the conversion. These insights expand our understanding of cartilage biology and may enhance the success of chondrocyte-based therapies. STEM CELLS TRANSLATIONAL MEDICINE 2016;5:733–744

SIGNIFICANCE

Injury of cartilage, a non-self-repairing tissue, often progresses to pathogenesis of degenerative joint diseases, such as osteoarthritis. Although tissue-derived stem cells have been shown to contribute to tissue renewal and homeostasis, the derivation, biological function, and application potential of stem/progenitor cells found in adult human articular cartilage are incompletely understood. This study reports the derivation of a population of cartilage stem/progenitor cells from fully differentiated chondrocytes under specific culture conditions, which have the potential to reassume their chondrocytic phenotype for efficient cartilage regeneration. These findings support the possibility of using in vitro amplified chondrocyte-derived progenitor cells for joint cartilage repair.

INTRODUCTION

Articular cartilage is a non-self-repairing tissue. Injury of cartilage often marks the initiation of tissue degeneration, and the progressive cartilage loss and subchondral bone sclerosis lead to degenerative joint diseases [1], such as osteoarthritis (OA). Localized articular cartilage defects can be repaired by transplantation of autologous chondrocytes into the injury site, thus slowing disease progress [2, 3] (e.g., chondrocyte-based cell therapies, such as autologous chondrocyte implantation or transplantation and matrix-assisted autologous chondrocyte transplantation [MACT]), were developed and have been used safely in clinical practice [2, 4]. However, uncertainty regarding number and identity of chondrocytes after in vitro expansion remains a major challenge in chondrocyte-based therapies [5]. Search for better cells sources was then warranted. Tissue-derived stem cells contribute to tissue renewal and homeostasis (e.g., in skeletal muscle, satellite cells contribute to both myofiber repair...
and stem cell repopulation [6]). There was evidence of self-repair in human articular cartilage, albeit at a low capacity, and cartilage stem/progenitor cells (CSPCs) are thus expected and are currently under investigation (review by Jiang and Tuan [7]). In general, CSPCs were isolated on the basis of their colony-forming ability and identified by virtue of their multilineage differentiation and different surface marker combinations, such as CD105 [8–10], CD166 [8, 9, 11], and STRO-1 [12–14], but their derivation remains unclear.

In the context of developmental origin, multipotent cell sub-populations were found in human embryonic cartilaginous tissue. For example, a CD166(low−)CD73(−)/CD146(+) cell subpopulation was identified in human embryonic limb buds at weeks 5–6 [15]; Quintin et al. also reported that cells possessing chondrogenic, adipogenic, and osteogenic plasticity were found in fetal femurs at weeks 14–16 [16]. However, because of the spatiotemporal inconsistency in their derivations and phenotypes [7], the fate and lineage information of CSPCs are largely unknown.

Several recent studies have demonstrated that parenchymal cells exhibit reserved stemness; for example, in the stomach epithelium, fully differentiated chief cells act as reserve stem cells to generate all epithelial lineages [17], and epithelial cells in the stem cell niche could repopulate the lost stem cells in hair follicles [18]. In vitro culture conditions, including supplements, growth factors, and substrate properties, could also substantially alter cell fate, including cell phenotype and function [19], and pluripotency [20].

In this study, we hypothesized that terminally differentiated human articular chondrocytes possess “reserved stemness” (i.e., stem cell-like potency properties could emerge after culture) and can generate chondrogenic progenitor cells capable of cartilage repair. We report here our observation of the emergence of a group of stem/progenitor cells that were derived from adult human chondrocytes, highlighted by dynamic changes in expression of the mature chondrocyte marker COL2 [21] and the mesenchymal stromal/stem cell (MSC) marker CD146 [21]. These cells are termed chondrocyte-derived progenitor cells (CDPCs). The transition between the stemness and differentiation status of CDPCs was regulated and determined by physical, biochemical, and cell density cues during culture. Specifically, our goal was to probe the emergence and enhancement of these chondrocyte-derived progenitor cells, as well as to evaluate their efficacy on cartilage regeneration both in vitro and in vivo in a clinical study.

**Materials and Methods**

**Cell Isolation, Cultivation, Characterization, and Differentiation**

The object of our study was to trace the derivation of the adult human articular cartilage stem cells, the fate decision of their stem cell characteristics, and their effectiveness for cartilage repair. Cells from human adult articular cartilage were isolated and analyzed. Adult articular cartilage samples (47–71 years old; mean, 53.3 years; n = 51) were dissected from nonlesion surface areas of the knee joints of patients without signs of rheumatoid involvement undergoing total knee replacement surgery. Patient consent and protocol approval were obtained from the Medical Ethics Committee, Zhejiang University, and from the Institutional Review Board (IRB), University of Pittsburgh and University of Washington. Histological slides of adult healthy articular cartilage (n = 3) were donated by the Department of Anatomy, School of Medicine, Zhejiang University. Primary human bone marrow-derived mesenchymal stromal/stem cells (BMSCs) (age 27–46 years, n = 5) were isolated with IRB approval from bone marrow and used as a control (details are provided in the supplemental online data). Samples were randomly selected for all analyses; the specific number of biological replicates (i.e., donors) used for each experiment is indicated in the figure legends. Primary chondrocytes were isolated from distal femoral condyles by enzymatic digestion. Briefly, articular cartilage tissue was cut into ~1-mm³ pieces and digested for 6 hours at 37°C in 0.2% (wt/vol) collagenase (Collagenase Type I, Life Technologies, Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com). Cells were transferred to monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM)/F12 Nutrient Mixture 1:1 (Thermo Fisher Scientific Life Sciences) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Life Sciences) and penicillin/streptomycin (50,000 U/50 mg), then cultured under standard conditions. In the glucose concentration study, cells were cultured in DMEM of different glucose concentrations (Life Technologies, Thermo Fisher Scientific Life Sciences). To observe the dynamics of cell phenotype changes, single-cell suspensions were cultured at low density (100–300 cells per cm²) in low-glucose DMEM containing 10% FBS. Cell proliferation rates were tested in a 2% FBS culture condition and were determined by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan, http://www.dojindo.com).

**Light Microscopy and Immunostaining**

Cartilage tissue was fixed in 4% buffered paraformaldehyde and cryosectioned at 14-μm thickness. Cell cultures in 24-well plates were fixed in 4% buffered paraformaldehyde followed by 0.1% Triton X-100 for 30 minutes, washed, and blocked in 1% bovine serum albumin (BSA), then incubated with 200 μl primary antibody diluted 1:50 in phosphate-buffered saline (PBS) at 4°C overnight. After washing, for immunofluorescence, a fluorescently labeled secondary antibody diluted 1:500 was added for 20 minutes at room temperature. Two washing steps with PBS and 4’,6-diamidino-2-phenylindole (Life Technologies, Thermo Fisher Scientific Life Sciences) staining were performed thereafter. For double immunostaining, primary antibodies derived from different species and corresponding, noncross-reacting secondary antibodies were used (information on antibodies is given in the supplemental online data). Cells were examined with an epifluorescence microscope (Olympus X71, Nagano, Japan, http://www.olympus-global.com). For immunohistochemistry, a similar protocol was used except for the use of peroxidase-labeled secondary antibodies followed by diaminobenzidine-based detection.

**Flow Cytometry Analysis and Cell Sorting**

Cultured cells were detached and incubated with fluorescently labeled antibodies (1 × 10⁵ cells per milliliter, >200 μl/test) at room temperature in the dark, resuspended and washed in PBS, and then pelleted by centrifugation (300g for 10 minutes). Isotype-matched IgGs (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) were used as controls, and at least 10,000 live events were analyzed on an FC 500 MCL/MPL Flow Cytometer. Data were evaluated with the aid of COP Software v2.2 (Beckman Coulter, Sharon Hill, NJ, http://www.beckman.com) (information on antibodies is available in the supplemental online data). For cell cycle test, alcohol-fixed cells were stained with propidium iodide (Life Technologies, Thermo Fisher Scientific Life Sciences). For cell sorting, at least 5 × 10⁶ cells were collected and incubated with fluorescein isothiocyanate-conjugated mouse antihuman CD146 antibody (EMD Millipore, Billerica, MA, http://www.emdmillipore.com/) for 30 minutes at room temperature.

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Both CD146(+) and CD146(−) live cell subpopulations were sorted, counted on a Becton Dickinson fluorescence activated cell sorting (FACS) Aria II Flow Cytometer (McGowan Institute, Pittsburgh, PA, http://www.mirm.pitt.edu/), and collected for further study.

**Colony Formation Analysis**

One hundred cells were seeded in a 10-cm (diameter) dish and cultured with 20% FBS, low-glucose culture medium for the first week. FBS was then reduced to 10%, and the medium was changed every 3 days. After 9–12 days, the cultures were fixed in 1% paraformaldehyde and stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, https://www.sigmaaldrich.com/) in methanol for 10 minutes. All cell colonies formed with diameters >2 mm were counted and their size was estimated.

**Multilineage Differentiation and Quantification**

Osteogenesis, adipogenesis, and chondrogenesis of CDPCs and BMSCs were evaluated (experimental details are provided in the supplemental online data). To generate hyaline three-dimensional (3D) cartilage-like tissue in vitro, 2 × 10^6 CDPCs were seeded within the insert in a Transwell culture dish (to avoid medium change that disturbs tissue formation) (Corning, Corning, NY, https://www.corning.com) and treated with chondrogenic medium containing transforming growth factor (TGF)-β3 (10 ng/ml, PeproTech, Rocky Hill, NJ, https://www.peprotech.com) and/or bone morphogenetic protein 4 (BMP4, 10 ng/ml, PeproTech, Rocky Hill, NJ) [22, 23]. Samples were collected at week 9 for histological, gene expression, and immunoblot analyses.

**Ectopic Implantation of CDPCs in Nude Mice**

To test the potential of amplifiedCol2(−)/CD146(+) CDPCs to generate hyaline cartilage in vivo, 4 × 10^5 CDPCs at passage 6 were mixed with 0.2 ml fibrin gel (Tissuel, Baxter, Old Toongabbie, NSW, Australia, http://www.baxterhealthcare.com.au) per site and injected sc. 4 × 10^6 CDPCs at passage 6 were mixed with 0.2 ml fibrin gel (Tissuel, Baxter, Old Toongabbie, NSW, Australia, http://www.baxterhealthcare.com.au) per site and injected sc. Century, Boston, MA, http://www.century; and/or bone morphogenetic protein 4 (BMP4, 10 ng/ml, PeproTech, Rocky Hill, NJ) [22, 23]. Samples were collected at week 9 for histological, gene expression, and immunoblot analyses.

**Immunoblotting**

Proteins were extracted by using radioimmunoprecipitation assay buffer containing protease inhibitors cocktail (Sigma-Aldrich), boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (Bio-Rad, Hercules, CA, http://www.bio-rad.com/), and analyzed by SDS-PAGE (6% stacking gel and 12% separating gel). Proteins were detected on polyvinylidene fluoride membrane (0.45 μm; EMD Millipore), which was then incubated overnight with the respective primary antibodies (1:1,000; 1% BSA) at 4°C (information on antibodies is given in the supplemental online data). Immunoreactive protein bands were detected after incubation with appropriate secondary antibodies based on ECL signal (GE Healthcare, Chicago, IL, http://www3.gehealthcare.com; Pierce, Thermo Fisher Scientific), and visualized with a FOTO/Analyzer1 Fx CCD imaging system (Fotodyne Inc., Hartland, WI, http://www.fotodyne.com/). Results were analyzed with ImageJ 1.45s software (National Institutes of Health, Bethesda, MD, https://imagej.nih.gov/ij/). Gel loading was assessed on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunostaining.

**Acellular Cartilage-Derived Matrix**

Acellular cartilage matrix (AM) was prepared by using a combination of physical, chemical, and enzymatic treatments [24]. In brief, fresh bovine articular cartilage and meniscus cartilage were minced, frozen, and pulverized in liquid nitrogen with a freezer mill (6870, SPEX SamplePrep, Metuchen, NJ, http://www.spexsampleprep.com/) and the tissue powder was decellularized by Triton X-100 (Sigma-Aldrich) and DNase and RNase ( Worthington Biochemical, Lakewood, NJ, http://www.worthington-biochem.com) (additional experimental details are given in the supplemental online data). CDPCs were then mixed with the respective AM powder (5 × 10^5 cells per 20 mg AM powder in 1 ml of serum-free medium in a 1.5-ml tube) and cultured for 7 days. Relative mRNA expression levels were compared.

**mRNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction Analysis**

Monolayer cultures were extracted by using the RNeasy Mini Kit (Qiagen, Hilden, Germany, https://www.qiagen.com). 3D cultures were extracted by lysis in Trizol (Thermo Fisher Scientific Life Sciences) followed by treatment with RNase-Free DNase (Qiagen) and fractionated on RNeasy columns. After reverse transcription using random primers (Super Script III First-Strand System, Life Technologies, Thermo Fisher Scientific Life Sciences), quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed (SYBR Green PCR Master Mix, Life Technologies, Thermo Fisher Scientific Life Sciences; StepOne Plus Real-Time PCR system, Applied Biosystems, Thermo Fisher Scientific Life Sciences) and results were calculated using the 2^−ΔΔCts method. 18S rRNA, RPL13a, and GAPDH were used as housekeeping genes. The genes, NBI gene ID, primer sequences (5′–3′), and expected amplimer size are listed in the supplemental online data.

**Pilot Clinical Study**

This study was approved by the Ethics Review Committee of School of Medicine, Zhejiang University, China. The patients were consentsed for MACT as a two-stage process, with a follow-up arthroscopy procedure including biopsy approximately 1 year after the second stage of the procedure (details are available in the supplemental online data). Magnetic resonance (MR) imaging was performed with a 3.0-T magnet using a knee coil before surgery and at 1 year after surgery. Imaging was performed in the sagittal plane, and a series of T1-weighted and T2-weighted images were obtained as routine sequences. Fat-suppressed three-dimensional fast spoiled gradient-recalled sequences were also performed to obtain additional morphological details, with 1.5-mm slice thickness. MR images were evaluated by two skilled musculoskeletal radiologists. All 15 patients were evaluated using the International Knee Documentation Committee (IKDC) score and Lysholm score before and after surgery. Postsurgical evaluation was carried out 1 year after surgery. Additional details are provided in the supplemental online data.

**Statistical Analysis**

Data from at least three independent experiments are reported, and statistical analysis was carried out with data from separate specimens by using SPSS software (version 16.0; IBM, Armonk, NY). Results are presented as mean values and standard deviation (SD) or standard error (SEM). For cell proliferation and CD146 positivity test, after testing for normal distribution and variance homogeneity, a one-way analysis of variance (ANOVA) and post hoc pairwise comparison of mean values were performed, with
statistical significance defined as $p < .05$. For gene expression levels of CD146 in high- and low-density cultures, a two-way ANOVA was performed, with statistical significance defined as $p < .05$. Student’s t tests were applied to determine statistical significance of differences measured in cell cycle analysis, colony-forming unit (CFU) tests, differentiation quantifications, and clinical scores, with statistical significance defined as $p < .05$.

RESULTS

Articular Cartilage-Derived Chondrocytes Progressively Adopt a Stem/Progenitor Cell-Like Phenotype During Culture In Vitro

We observed that chondrocytes isolated from adult human articular cartilage gradually adopted a stem cell-like phenotype. To profile the dynamics of this phenotype transition, CD146, an early mesenchymal lineage associated surface marker (melanoma cell adhesion molecule), and collagen type II (COL2), a characteristic extracellular matrix component of mature chondrocytes, were used as indicators to trace the kinetics of the emergence of stem cell phenotype from cartilage tissue (Fig. 1). The avascular articular cartilage tissue exhibited a COL2-rich matrix, and it did not harbor any CD146$^+$ cells (Fig. 1A; supplemental online Fig. 1). When chondrocytes were freshly isolated from healthy cartilage and maintained in high-density, monolayer primary culture condition, the cells gradually became positive for the early-stage MSC marker, CD146 (Fig. 1B). The COL2$^+$CD146$^+$ transition stage, as indicated by the presence of double-immunopositive cells captured by immunofluorescence (Fig. 1A) and flow cytometry (Fig. 1B).

Physical, Biochemical, and Cell Density Culture Regulate Emergence and Proliferation of Stem/Progenitor Cell

A critical culture condition promoted the emergence and maintenance of CD146$^+$ cells from the articular cartilage-derived cell cultures: seeding at a low cell density (500 cells/cm$^2$) as monolayer cultures in a low-glucose medium (2DLL culture: monolayer, low density, and low glucose). Low-density seeding reduced cell-cell interactions and allowed cell cycle re-entry (Fig. 2A). Culturing in low-glucose (5.5 mM) medium, generally considered to favor the maintenance of stem cells and to prevent cell aging, resulted in higher proliferation of the cartilage-derived cells, compared with culturing under high glucose (25 mM) (Fig. 2B). Expression levels of genes related to MSC maintenance, self-renewal, and multipotency [25], such as RAB3B, Frizzled 7 (FZD7), and actin filament-associated protein (AFAP), also increased significantly during 2DLL culture (Fig. 2C).

To further characterize the CD146$^+$ cells, we sorted both CD146$^+$ and CD146$^-$ cells from early-passage chondrocyte cultures by FACS and tested their ability to generate CFUs immediately after sorting (Fig. 3B). The CFU activity of CD146$^+$ cells was significantly higher than that of the CD146$^-$ cells ($p < .05$), suggesting that CD146$^+$ cells possessed a stronger self-renewal ability. Interestingly, the CD146$^-$ cells became CD146$^+$ (∼50%) after 10 additional days of culturing, suggesting the intrinsic potency of the CD146$^-$ cells.

Figure 1. Fully differentiated human chondrocytes change phenotype after isolation. (A): COL2 and CD146 immunostaining in healthy articular cartilage from human femoral condyles and isolated chondrocytes. Tissue immunostaining was detected with diaminobenzidine (reddish brown), with hematoxylin nuclear counter stain (bar, 50 µm); passage 4 cell staining was based on immunofluorescence (COL2, green; CD146, red) and DAPI nuclear counterstaining (bar, 20 µm). (B): Time course of changes in COL2 and CD146 expression in chondrocytes during monolayer high-density culture and passage. Primary isolated normal human chondrocytes were collected at different passages (P1, P3, and P5), coimmunostained for COL2 and CD146, and analyzed by flow cytometry ($n = 3$); images were from representative sample. CD146$^+$ cells are gated in E1 and E2, COL2$^+$ cells are in E2 and E4, and CD146 and COL2 double-positive cells are in E2. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; H&E, hematoxylin and eosin.
Dynamic Transition Between Stem/Progenitor Cell-Like and Chondrocyte Phenotypes

We estimated ~44% CD146(+) cells emerged upon the second passage of 2DLL cultures, with enrichment of the CD146(+) cell population to ~90% after three passages of 2DLL cultures (Fig. 3A). We also found that CD146(1)/2 phenotype in chondrocyte-derived cells could be regulated by the in vitro culture environment (Fig. 3). Particularly significant was that the articular cartilage-derived cells were able to transition between a COL2(1)CD146(2) chondrocyte phenotype in 3D culture and a COL2(2)CD146(1) CDPC phenotype in two-dimensional (2D) culture. Thus, when the sorted CD146(1) CDPCs were allowed to condense in a high-density pellet and maintained in TGF-β-containing chondrogenic medium, the CD146 signal was lost and COL2 protein accumulated (Fig. 3B). However, when the cells migrated out from the chondrifying pellets and were subsequently seeded at low density in monolayer culture, the CD146(1) phenotype reappeared (Fig. 3B). Also, chondrocytes seeded at low, noninteractive cell density showed higher CD146 gene expression during culture (Fig. 3D). These dynamic, coordinated changes strongly suggest that subpopulations of stem/progenitor cells originated from chondrocytes, as a result of their gradual transition to a mesenchymal-like phenotype upon culturing in the absence of cell-cell interactions under monolayer and low-glucose condition (Figs. 2, 3). These cells are termed CDPCs.

Comparison of Multilineage Differentiation Potential of CDPCs and Human Bone Marrow-Derived Mesenchymal Stromal/Stem Cells

To further assess the stem cell characteristics of CDPCs, we compared them with BMSCs with respect to cell surface marker profiles and differentiation multipotency (Fig. 4). Flow cytometry showed that well-recognized MSC-associated surface markers (CD90, CD73, CD105, CD166, CD44, and CD29) were positive in both CDPCs and BMSCs, whereas the hematopoietic stem cell-associated markers (CD34 and CD45) were both negative, indicating similar cell surface epitope profile for CDPCs and BMSCs (Fig. 4A). Both cell types underwent induced differentiation into osteogenic, adipogenic, and chondrogenic lineages (Fig. 4B), although the extent of differentiation was not identical. BMSCs showed higher osteogenic and adipogenic ability than CDPCs, as indicated by stronger alizarin red staining and Oil Red O.
staining, respectively (Fig. 4B). In the pellet chondrogenesis assay, although no significant differences were found in Safranin O histological total scoring, the resulting cell morphologies were different. More fibroblast-like cells were found in BMSCs pellets, whereas more round cells were found in CDPC pellets (Fig. 4B).

Efficient Cartilage Formation With CDPCs In Vitro and In Vivo
To assess whether the high chondrogenic ability of CDPCs can result in the formation of hyaline-like cartilage, CDPCs were placed in 3D cultures in the presence of chondrogenic growth factors. Specifically, 2 million CDPCs were seeded in a Transwell insert and supplemented with the chondro-inductive factors, TGF-β3 and BMP4, which also inhibited hypertrophy in long-term chondrogenic cultures of MSCs and chondrocytes, respectively [22, 23]. After 9 weeks of culture as a condensed cell pellet, a compact, smooth, and translucent structure, which exhibited intense Safranin O staining of matrix sulfated glycosaminoglycans, was obtained (Fig. 5A). The cartilage matrix genes, COL2 and aggregan (AGN), were expressed at higher levels in the 3D cultures compared with 2D cultured cells, particularly with the addition of...
BMP4, with detectable activation of signaling via the downstream targets of BMPs, the SMA (small body size)- and MAD (mothers against decapentaplegic)-related proteins (SMADs) (increased pSMAD2, decreased pSMAD1, 5) (Fig. 5A).

To assess the potential application of their intrinsic chondrocytic ability for cartilage repair, CDPCs were seeded directly and cultured within AMs [26] prepared from bovine joint surface cartilage (hyaline cartilage) and meniscus (fibrocartilage) to mimic host tissue environments in vivo upon CDPC transplantation.

COL2 and AGN, and SOX9 gene expression by CDPCs increased significantly upon seeding and culture within hyaline cartilage-derived AM, whereas only the 

Figure 4. Stem cell characteristics of CDPCs versus BMSCs. (A): Cell surface epitope profiles analyzed by flow cytometry. CD90/73/105/166/29/6 SD; n = 5 each; *, p = .004. (B): Osteogenic, adipogenic, and chondrogenic differentiation potential of human CDPCs and human BMSCs. (B, left): Alizarin red staining (bar, 200 μm) represented the osteogenic potential and was quantified (values are mean ± SD; n = 3 each; *, p = 2.17 × 10⁻²⁴). (B, middle): Oil Red O staining (bar, 100 μm) represented adipogenic potential and was quantified (values are mean ± SD; n = 3 each; *, p = .009). (B, right): Safranin O staining (bar, 100 μm) represented chondrogenic potential and was quantified by pellet score (values are mean ± SD; n = 3 each; *, p = .004). Abbreviations: BMSC, bone marrow-derived stem cell; CDPC, chondrocyte-derived progenitor cell.

Clinical Repair of Large Cartilage Defects With CDPCs

A pilot clinical study was designed to evaluate the feasibility of the therapeutic application of CDPCs. Only knee joints without other concomitant joint problems, such as OA inflammation, are considered eligible as the host tissue for CDPC transplantation. Thus, confounding variables, such as proinflammatory cytokines [27] that could act to inhibit matrix production, or induce matrix degeneration [28], and cell apoptosis [29], are eliminated. Patients without inflammatory lesion and joint instability, with a cartilage defect more than 6 cm² in size, were enrolled in the prospective pilot study. Fifteen patients (2 female and 13 male patients) with a mean age of 25.0 years (range, 16–36 years) were treated with CDPCs in a matrix-assisted cell transplantation procedure. The sites of the defects were as follows: medial femoral condyle (n = 9), lateral femoral condyle (n = 5), and medial femoral condyle extending to trochlear groove (n = 1). The average size of the defects was 8.5 cm² (range, 6–13 cm²) and the defects were classified as grade III and IV according to International Cartilage Repair Society criteria (supplemental online Table 1).

After a standard diagnostic procedure, full-thickness cartilage specimens (200–300 mg) were harvested with a sharp gouge from a non-weight-bearing area of the trochlear ridge or intercondylar notch under arthroscopy. Chondrocytes were isolated from the cartilage specimen and culture-expanded to the desired cell number under Good Manufacturing Practice conditions. After 4 weeks, histologically hyaline cartilage-like tissues were formed in 4 of 6 injection sites, displaying round cells sited in lacunae surrounded by Safranin O-positive matrix (Fig. 4C). Taken together, these in vitro and in vivo observations strongly suggest cartilage regenerative potential of CDPCs.

Clinical Scores

The clinical scores indicated satisfactory maintenance of the loading capacity of the treated knee joint short term, as well as stable clinical improvement. Clinical evaluation showed significant improvement on the basis of IKDC scores (from 60.1 ± 15.4 before
surgery to 91.6 ± 4.3 at 12 months after surgery; \( p < .05 \) and Lysholm score (from 52.1 ± 15.1 to 89.3 ± 5.5; \( p < .05 \)) after the CDPC-based procedure.

**MRI Evaluation**

All patients were followed prospectively after CSPC implantation, and the repair tissue site was graded as completely attached, partially attached, or detached. In all 15 patients, completely attached grafts were found (Fig. 6A; supplemental online Table 2). The cartilage defect site was totally covered by the implanted repair tissue, which showed equivalent intensity compared with the surrounding cartilage in 13 patients and lower intensity in the remaining two patients. The contour of the subchondral bone was smooth and regular in 14 patients and irregular in 1 patient. Tissue hypertrophy and infection have been reported to be the most common adverse events of cell-based cartilage repair, and a second intervention was often required after graft failure [30]; in our study, we did not observe any postoperative complications (\( n = 0 \)).

**Histology**

Postoperative second-look arthroscopy (Fig. 6A) was performed on four patients. In all four patients, the grafted area appeared to be matrix-rich, with well-preserved hyaline-like cartilage architecture. Histological examination of biopsy samples from the 4 patients showed that compared with the adjacent arthritic cartilage, the repair tissue demonstrated the presence of chondrocyte-like cells and hyaline cartilage-like structure and matrix (i.e., COL2[+], COL1[-], and COL10[-]) (Fig. 6B). All four biopsy specimens were free of ectopic calcification and vascularization. No evidence of inflammation was observed.
Pain and Functional Evaluation

As indicated earlier, the IKDC and Lysholm scores from questionnaires regarding pain, swelling, blockage of knee, and general condition of the knee suggested generally favorable outcomes. All patients had scored poorly before the operations. One year after CDPC-based MACT, all patients had reduced knee pain and swelling, and locking sensation had disappeared (supplemental online Table 2). In all 15 patients, the mean IKDC score increased from 60.1 to 91.6 1 year after surgery (Fig. 6C), demonstrating a significant improvement ($p < .05$) compared with the preoperative findings. Concomitantly, the Lysholm score improved from 52.1 to 89.3 ($p < .05$) (Fig. 6D). Fourteen patients returned to normal daily living and sports activities within 1 year after treatment.

DISCUSSION

Although autologous chondrocyte-based therapies have been in use for more than 20 years [2, 4], the challenge of in vitro
chondrocyte expansion remains. A promising cell source, CSPCs, has recently attracted considerable attention, and their origin and identity are being investigated.

In this study, the emergence of the stem/progenitor cell phenotype from chondrocytes was for the first time captured kinetically by profiling the expression of the chondrocyte marker COL2 and a MSC marker, CD146. Our results strongly suggest that fully differentiated chondrocytes possess "reserved stemness," which is reactivated during in vitro expansion, gradually displaying multipotent stem/progenitor cell characteristics. We have termed these cells as chondrocyte-derived progenitor cells (CDPCs) and have shown that their stem and differentiation status is regulated as a function of their placement in low-density 2D versus high-density 3D cultures. We then demonstrated that the amplified CDPCs are capable of functional cartilage repair both in vitro and in a clinical study on patients with large articular cartilage defects (6–13 cm²). The reversible phenotype transition between chondrocytes and progenitor cells reported here provides information on the origin of human articular cartilage stem/progenitor cells and brings new insights into cartilage biology and the development of chondrocyte-based therapies.

To trace the origin of CSPCs, a mature chondrocyte-associated marker, COL2 [21], and an early mesenchymal lineage surface marker, CD146 [21, 31, 32], were used to profile the kinetics of the emergence of CSPCs from cartilage tissue. The avascular articular cartilage tissue exhibits COL2-rich matrix but does not harbor any CD146(+) cells, which are generally considered to be primarily located at perivascular sites [31]. Once placed in monolayer culture, primary chondrocytes are known to dedifferentiate [33] and to become fibroblast-like and lose their chondrocytic phenotype (i.e., COL2[−]) [34]. Here we observed that chondrocytes gradually express CD146, an early-stage MSC marker, during in vitro culture (Fig. 1). The conversion of COL2(+) CD146(−) chondrocytes to the COL2(−)CD146(+) mesenchymal progenitor-like phenotype is marked with a short transitional state of COL2(+)CD146(+) phenotype, detected as double-immunopositive cells by immunofluorescence and flow cytometry.

We observed that low cell density, monolayer culture in a low-glucose medium (2DLL culture) greatly promoted the emergence and maintenance of CD146(+) cells. Consistent with the reported promotion of stem cell maintenance [35] and prevention of cell aging [36] in low-glucose cultures, we also observed higher cell proliferation in low-glucose cultures (5.5 mM) than in high-glucose cultures (25 mM). Upon 2DLL culture, approximately 40% of cells were CD146(+) after first passage, and this population was enriched to ~90% after three passages (Fig. 3). In addition, higher cell cycle re-entry was seen in cultures seeded at low density (Fig. 2), concomitant with higher CD146 gene expression (Fig. 3D). Analysis of CFU activity of FACs-sorted CD146(+) and CD146(−) cells from early-passage chondrocytes (Fig. 3B) showed that the former consistently produced more clonal colonies (Fig. 3C). Interestingly, the CD146(−) cells appeared to transition to CD146(+) cells (~50%) after 10 additional days of 2DLL culturing, suggesting that the time-dependent emergence of CD146(+) cells in the primary culture was likely the result of both the proliferation of CD146(+) cells and change in phenotype of the originally CD146(−) cells (Fig. 3B).

We also found that these chondrocyte-derived CD146(+) cells exhibited the phenotype of mesenchymal stem/progenitor cells: (a) They displayed similar stem cell surface markers as bone marrow-derived/stromal cells (Figs. 3A, 4A); (b) they could be induced to undergo osteo-, adipo- and chondrogenesis (Fig. 4B); (c) they showed higher CFU activity than CD146(−) cells (Fig. 3C); and (d) they could form cartilage tissue both in vitro and in vivo (Figs. 4, 5). Besides the changes in COL2/CD146 expression, additional evidence supporting the chondrocyte-stem/progenitor phenotypic transition is also noted during the in vitro 2DLL expansion, including (a) the upregulation of expression of stem cell-related multipotency genes [25], such as RAB3B, FZD7, and AFAP (Fig. 2C); (b) cell-cycle re-entry (Fig. 2A); and (c) expression of MSC-related cell surface markers after 2 passages of 2DLL cultures, although not synchronized with CD146 expression (Fig. 3A). Taken together, these dynamic, coordinated changes support the notion that the CDPCs originate from chondrocytes, which gradually change to mesenchymal-like cells upon culturing under minimized cell-cell interaction conditions.

The concept of “dedifferentiation” has different meanings in the context of stem cell/developmental biology compared with chondrocyte biology [33, 37]. In stem cell biology, dedifferentiation describes the reverse developmental process in which differentiated cells with specialized functions become undifferentiated progenitor cells. Dedifferentiation and subsequent proliferation thus provide the basis for tissue regeneration and the formation of new stem cell lineages [37]. In chondrocyte biology, the phenotype shifting of chondrocytes to a fibroblast-like state when cultured in monolayers was referred to as “dedifferentiation” [5, 33]. In this process, cells underwent morphological changes and stopped producing a cartilage-specific matrix, but regression to an earlier biopotent or multipotent state was not implied [33]. Interestingly, Tallheden et al. [38] reported that some of the culture-expanded normal human articular chondrocytes demonstrated multipotency [38]. Therefore, the exact mechanisms underlying the reversibility of this process are unknown and deserve further investigation.

Our finding of the emergence of stem cell phenotype from chondrocytes presents a biological scenario distinct from the conventional unidirectional stem cell hierarchies (i.e., that a population of mature chondrocytes possesses intrinsic “stemness,” which could reverse into progenitor stage during in vitro culture expansion). This observation supports an alternative cellular feature that mature, differentiated cells are able to revert to a stem/progenitor cell stage and contribute to tissue regeneration. Of relevance is a recent study on fully differentiated chief cells of the stomach epithelium that act as reserve stem cells to generate cells of all lineages [17].

Studies on adult tissue-specific stem cells have suggested that they are likely present as a stem/progenitor cell population that is kept quiescent and is activated when needed, and may be identified and traced with a specific cell marker from early stages of development; examples include muscle satellite cells [6] and hematopoietic stem cells [39]. Wu et al. identified a CD166(low−)/CD73(−)/CD146(+) cell subpopulation in human embryonic limb buds at weeks 5–6 and showed that the CD146(+) phenotype disappeared in 8-week embryo [15]. We also were unable to detect any CD146(+) cells in adult human articular cartilage in vivo. Therefore, it is still unclear whether CSPCs are quiescent stem cell that are left from earlier development, remain reserved in tissue, and then become reactivated upon in vitro culture or instead whether most mature chondrocytes have such potential to become progenitors. A more in-depth analysis of the stem/progenitor cell populations in a future study is needed.
We next investigated the extrinsic factors that can regulate the transition between chondrocytes and stem/progenitor cells. We found that cells are able to change between COL2(+)CD146(-) chondrocyte phenotype in 3D culture and COL2(-)CD146(+) CDPC phenotype in 2D culture (Figs. 1, 3A). When the sorted CD146(+) cells were condensed to form a chondrogenic pellet, the CD146 signal was lost and accumulation of COL2 protein was seen (Fig. 3B); interestingly, CD146(+) cells reappeared when the cells migrated out from the chondrifying pellets and maintained their CD146(+) phenotype when subsequently seeded at low density in monolayer culture (Fig. 3B). These observations point toward the bidirectional nature of cell lineage commitment to adopt new fates in a manner regulated by different host microenvironments. The functional extent of this phenotype transition was further tested both in vitro and in vivo. We cultured CDPCs in native acellular matrices of different cartilage environment, derived from hyaline- and fibro-cartilage tissues [26] and observed that the former more favorably promoted CDPC chondrogenesis than did the latter (Fig. 5B). Upon ectopic implantation in immunodeficient mice in vivo, CDPCs produced robust cartilage tissue without the addition of any chondrogenic growth factors (Fig. 5C), indicating that the CDPCs are capable of forming cartilage.

Taken together, these results support the potential application of CDPCs for the repair of cartilage defects. Specifically, CDPCs are intrinsically highly chondrogenic, and the host hyaline cartilage tissue matrix may support chondrogenesis of the implanted cells. Thus, CDPCs may be used to form neo-cartilage in a cartilage defect without extrinsic inductive influences.

We therefore initiated a pilot clinical study to evaluate the application of CDPCs in cell therapy for cartilage repair using a standard MACT procedure. The 2DLL-cultured CDPCs showed efficacy in the repair of large knee cartilage defects (defect size, 6–13 cm²) in all 15 patients tested. The results reported here, including subjective and objective evaluations, are highly promising. By the first 6 months of follow-up, patients were able to do light work or sports; after 1 year, none of the patients showed moderate or severe limitation in daily activities, such as walking or climbing stairs. All patients were satisfied with their clinical outcomes and reported good quality of life. Both IKDC and Lysholm scores improved significantly at 1 year after surgery. The clinical and functional results of this study are similar or superior to those previously reported in patients who underwent other chondrocyte-based implantation procedures [30, 40], with no increased risk for complications during the first year of follow-up. We note that a previous report showing that CSPCs derived from OA cartilage populate diseased tissue in vitro [41]. In our study, we have a strictly controlled cell source and host environment (i.e., the CDPCs used were derived from healthy cartilage and placed in defects of joints without observable OA-associated inflammation). As such, confounding variables such as proinflammatory cytokines [27] that could act to inhibit matrix production, or induce matrix degeneration [28] and cell apoptosis [29] are eliminated. As far as the enrollment in the clinical study is concerned, only 15 young patients were included; further evaluations in older patients, as well as a larger patient population with longer-term follow-ups, are clearly needed.

CONCLUSION

We report here that a population of cartilage stem/progenitor cells can be derived from fully differentiated chondrocytes that have the potential to reassert their chondrocytic phenotype for efficient cartilage regeneration. This novel concept supports the possibility of using in vitro amplified chondrocyte-derived cells, the CDPCs, for joint repair. Importantly, it provides an alternative view to the widely accepted model that dedifferentiated chondrocytes are merely fibroblastic cells with minimal tissue regenerative ability. These findings strongly suggest that identification of the extrinsic influences that determine the fate of CDPCs is of great value in manipulating CDPCs for their therapeutic applications for cartilage repair.

AUTHOR CONTRIBUTIONS

Y.J.: conception and design, administrative support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Y.C.: provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; W.Z., C.H., and T.T.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; Z.Y.: conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; P.L. and S.Z.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript; D.N.: data analysis and interpretation, manuscript writing, final approval of manuscript; R.S.T.: financial support, administrative support, experimental conception and design, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript; H.W.O.: conception and design, financial support, administrative support, provision of study material or patients, experimental conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.
REFERENCES


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