Biological Potential Alterations of Migratory Chondrogenic Progenitor Cells during Knee Osteoarthritic Progression

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Abstract

Background: Although studies have demonstrated that chondrogenic progenitor cells (CPCs) remain present in human osteoarthritic cartilage, the heterogeneity of CPCs subpopulations, the biological alterations of CPCs, and their contributions to the progression of osteoarthritis remain to be investigated.

Methods: CPCs were isolated from paired grade 1-2 and grade 3-4 osteoarthritic cartilage by virtue of cell migratory capacities. The cell morphology, immunophenotype, self-renewal, multidifferentiation, and cell migration of these CPCs were evaluated. Additionally, the distributions of CPCs in articular cartilage were determined by immuno-histochemical staining. Furthermore, a high-through mRNA sequencing was performed to explore the underlying mechanisms.

Results: Migratory CPCs (mCPCs) robustly outgrew from collagenases-digested osteoarthritic cartilages for 2 weeks after the initial culture. The mCPCs from grade 3-4 cartilages (mCPCs, grade 3-4) harbored morphological characteristics, cell proliferation and colony formation capacity that were similar to those of the mCPCs from the grade 1-2 cartilages (mCPCs, grade 1-2). However, the mCPCs (grade 3-4) highly expressed CD271. In addition, the mCPCs (grade 3-4) showed enhanced osteo-adipogenic activities and decreased chondrogenic capacity. Furthermore, the mCPCs (grade 3-4) exhibited stronger cell migration in response to osteoarthritis synovial fluids. More CD105+ cells resided in grade 3-4 superficial articular cartilages. Moreover, the results of mRNA sequencing showed that mCPCs (grade 3-4) expressed higher migratory molecules.

Conclusions: Our data suggest that more mCPCs (grade 3-4) migrate to injured articular cartilages but with decreased cartilage-repairing capacity, which might accelerate cartilage degradation. Thus, these dysfunctional mCPCs might be a novel cell target to alleviate cartilage lesions.

Introduction

Knee osteoarthritis (KOA) is one of the most common degenerative disorders in joints and has been anticipated to be the fourth leading cause of disability worldwide by the year 2020[1, 2]. It is mainly characterized by slowly progressive degeneration and loss of the articular cartilage. Unfortunately, incomplete understanding of the pathogenesis of KOA confined the development of therapeutic strategies, and there are few curable treatments available so far for osteoarthritis (OA) until the end stage of the disease necessitates joint replacement [3, 4].

In the past decades, articular cartilages have been considered as hypocellular and hypovascular tissues and possessed poor capacities to self-repair. Promisingly, recent investigations have shown that the normal and OA articular cartilage containing tissue specific stem/progenitor cells, named chondrogenic progenitor cells (CPCs), with high proliferative, clonogenic, and multi-differentiaiton capacities[5, 6]. In addition, CPCs were capable of migrating to injured sites after cartilage trauma[7]. Furthermore, CPCs have recently attracted interest due to their immuno-regulatory properties [8, 9] and phagocytic capacity[10], which have been suggested as valuable potentials for cell-based therapies[6, 11–13].

However, heterogeneous and limited information from independent teams indicate inconclusive and somewhat contradictory information in understanding CPCs activity in different phase of knee OA progression. Seol et al. reported that CPCs represented a transient emergence and homing after cartilage mechanical injuries [7]. In addition, Tong et al. showed that CPCs harbored a transient proliferative response in early OA and became gradual quiet as OA process [14]. Many details of the biological mechanisms governing these cells remain to be elucidated, and CPCs in vivo distribution and any potential contribution to the development of OA remain poorly understood.

Fortunately, a portion of OA patients with total knee arthroplasty (TKA) present Outerbridge grade 3–4 cartilage lesions in the medial compartment accompanied by grade 1–2 cartilage lesions in lateral side[15, 16], which provide an opportunity to understand the CPCs difference present in different grades of osteoarthritic cartilage in a donor matched manner to avoid individual heterogeneity[17], Xia al. compared the relative percentage, proliferation activity, multi-lineage differentiation potential and miRNA expression profile of CPCs with CD105 and CD166 co-expression identification isolated from the degraded cartilage in the medial condyle and relatively normal cartilage on the lateral side from OA patients[18]. Also, CPCs derived from paired grade 1–2 cartilage on the lateral femoral condyle and grade 3–4 cartilage on the medial femoral condyle were assayed by a standardized colony-forming-unit assay using automated image-analysis software[17]. However, all of the CPCs were obtained from the released cells post collagenase digestion either by cell colony formation cell expansion or flow cytometry cell sorting, obtaining the subpopulation which may partially lose their biological function after in vitro expansion[19, 20]. All of these cells were obtained from femoral condyles, neither of them separated the CPCs from the osteoarthritic tibial plateau cartilage.

Our previous study[11] reported an effective strategy of isolating functional CPCs from human cartilage in which a short-time collagenase digestion efficiently loosened the cartilage microstructure and facilitated intact CPCs outgrowth from human articular cartilages. This subpopulation of CPCs exhibited high cell proliferation and cartilage regenerative capacity than that of released cells, which may benefit from mimicking the cellular niche in vitro [11, 21]. Therefore, we hypothesized that culturing the short-time collagenase-digested OA cartilage fragments may obtain a novel subpopulation of CPCs and exploring their functional characteristics may be helpful to understand progression of KOA. In the current study, we cultured CPCs from paired grade 1–2 OA on the lateral tibial plateau and grade 3–4 OA on the medial tibial plateau cartilage from the same donor by virtue of cell migrations. The CPC immunophenotype, self-renewal, multi-differentiation, in vivo distribution and their mRNA sequencing were also investigated and compared.

Methods

Patient characteristics

This study was approved by the institutional ethical review board of our Hospital (Rapid review of scientific research projects for use of discarded biological material), and informed consent was obtained

from all donors. Twenty-eight patients (9 male and 19 female, mean age, 63.6 years [range, 53-73 years]; mean body mass index, 26.0kg/m^2 [range, 22.7-30.8 kg/m^2]; mean disease duration 7.3 years [range, 3-15 years]) (Supplementary Table 1) who were diagnosed with late-stage idiopathic KOA according to the criteria of American College of Rheumatology [22] with varus malalignment of the lower extremity and scheduled for elective TKA were recruited. Radiographs exhibited a relatively spared lateral femoral compartment (joint space>3mm). Cartilage morphology was scored according to the Whole-organ magnetic resonance imaging score [23] (mean cartilage scores 19.4 [range, 12.0-25.0] for medial femorotibial joint; mean cartilage scores 6.2 [range, 3.0-10.0] for lateral femorotibial joint) (Supplementary Table 1). Patients were excluded if they had secondary arthritis related to systemic inflammatory arthritis or if their history included previous systemic or intra-articular injection glucocorticoids, prior ipsilateral knee surgery, knee injury, infection, or osteonecrosis.

Isolation, expansion and identification of mCPCs

During the arthroplasty procedure, an osteochondral specimen of the tibial plateau was harvested with the initial proximal tibial cut. Samples of Outerbridge grade 1-2 cartilage were obtained from the lateral tibial plateau, and samples of grade 3-4 cartilage were obtained from the medial tibial plateau (Fig. 1B). Grade 1-2 cartilage includes cartilages with an intact surface (grade 1) and minimal fibrillation (grade 2), and grade 3-4 cartilage includes cartilage with fissures to subchondral bone[24]. The methods used to harvest the CPCs have been described in previous studies [5, 11, 25, 26] with minor modifications. In brief, the cartilaginous tissues were separated from the osteoarthritic articular cartilages without contaminated subchondral bones and were minced into pieces (about 1.0mm×1.0mm×1.0mm, Fig. 1B), and then digested in 0.1% collagenase II (Sigma) for 2 hours. The released cells were abandoned and the digested cartilage chips were incubated in alpha-minimal essential medium (α-MEM) with 10% vol/vol fetal bovine serum (FBS) (Invitrogen Life Technologies) at 37℃ in an atmosphere of 5% CO_2. The mCPCs outgrew from cartilage chips within 10 days and the adhesive cells rapidly reach 60-80% confluence in another 5 days. Importantly, the cartilage chips were retained and maintained until passage 3 to mimic the cellular niche ex vivo and allow more CPCs outgrowth. The morphological characteristics of CPCs were observed with reverted light microscope (Olympus BX71).

Flow cytometry analysis

The cell surface antigen profile of mCPCs was analyzed by. mCPCs were harvested at passage 3 by trypsin digestion, and antibodies were stained individually (phycoerythrin (PE)-conjugated monoclonal antibodies against human CD29, CD44, CD73, CD166, fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human CD45, CD90, CD271, and allophycocyanin (APC)-conjugated antibodies against CD31, CD105, eBio-Science) for 30 min in the dark at 4℃. After 2 washes with phosphate-buffered saline (PBS), the cells were collected with a fluorescence-activated cell sorting (FACS) Aria II Flow Cytometer (Becton Dickinson Biosciences) and the data were analyzed using FlowJo 7.6 software.

Growth kinetics and CCK-8 assay

The growth kinetics were determined using the trypan blue exclusion cell count method for hemocytometer cell counting [27]. Briefly, mCPCs were cultured in 48-well plates at 2×10^4 cells/well and harvested every 3 days for hemocytometer cell counting during a period of 19 days. The Cell Counting Kit 8 (CCK-8, Dojindo, Japan) assay was conducted according to a previous study [28]. In brief, CPCs at passage 4 were seeded in 96-well plates (1×10^3/well, 5 wells in each group) and maintained in culture medium, and the CCK-8 solution was added at a ratio of 100μl/ml and incubated at 37°C for 1 hour. The absorbance was measured at a wavelength of 450 nm on days 1, 4, 7, 10, 13, 16 and 19.

Colony-forming unit fibroblast formation (CFU-F) assay

Passage 4 mCPCs in each group were adjusted to different cell numbers (1×10^3, and 5×10^3cells/well). Aliquots of cell suspensions were added to 6-well culture plates and were maintained in culture for 10 days. Crystal violet was used to stain the colonies, and their vertical gross appearances were imaged by digital photography.

Evaluation of the multipotency of mCPCs

Osteogenic, adipogenic, and chondrogenic differentiation was assayed. The previously reported protocols for CPCs differentiation were used with minor revision in the current study.[21] Briefly, for osteogenic differentiation, cells were harvested and incubated in osteogenic induction medium (10 mM of glycerol-2-phosphate, 0.1 μM of dexamethasone and 20 μM of ascorbic acid) for 14 or 28 days. The osteogenic activity was assessed at day 14 by alkaline phosphatase (ALP) staining and at day 28 by Von Kossa staining, respectively. For adipogenic differentiation, CPCs were cultivated at 1×10^4 cells/well in 48-well cell culture plates, adipogenic induction medium (1 μM of isobutylmethylxanthine and 10^{-3}μM of dexamethasone) was supplemented, and Oil red O staining (day 14) was performed to assess the adipogenic potency. For chondrogenic differentiation, 4×10^5 CPCs were centrifuged in polypropylene tubes to form a pelleted micromass and maintained in chondrogenic induction medium consisting of α-MEM supplemented with 10^7M of dexamethasone, 1% (vol/vol) insulin-transferrin-sodium selenite, 50 μM of ascorbate-2 phosphate, 1 mM of sodium pyruvate, 50μg/ml (wt/vol) of proline, and 20ng/ml (wt/vol) of transforming growth factor (TGF-β_3). On day 28, the pellets were fixed and sectioned. The development of chondrocytes and accumulation of the cartilage matrix were evaluated by hematoxylin-eosin, toluidine blue and Safran O staining. The expression of Sox-9 (SRYtype high-mobility group box-9) and Col-II (collagen type II) were detected by immunohistochemical assays. The images were captured using a microscope under brightfield mode.

Histologic and immunohistochemical analysis

The osteochondral specimens of initial proximal tibial cut during the arthroplasty procedure were also collected for histologic immunohistochemical analysis. Samples were placed in 10% formalin before

processing. For each patient, separate lateral and medial tibial plateau pieces were decalcified using 10% ethylenediaminetetraacetic acid (EDTA, Sigma) for 3-4 months and then mounted on paraffin blocks. Decalcified tissue specimens were stained with hematoxylin and eosin. Immunohistochemistry for CD105 and CD271 (NGF receptor) staining was performed. Mouse anti-human CD105 and CD271 monoclonal antibody (Abcam) was used at a dilution of 1:50. Digital image analysis was performed to evaluate relative cartilage damage (including the cartilage–bone interface) and CD105 and CD271 cells in vivo distribution. For each sample, the whole tissue area was scanned using an OlympusX71 microscope under brightfield mode depending on the size of the section, 2-5 images were captured for the cartilage area (including the cartilage–bone interface).

OA synovial fluid-mediated migration of mCPCs

Migration of mCPCs on stimulation with OA synovial fluid was analyzed in 24-well transwell plates (8-um pore size of polycarbonate membranes, Corning) in triplicate as described previously[29]. In brief, 5×10^4 mCPCs in serum-free α-MEM medium were seeded in the upper wells. The lower wells were filled with 0%, 20% and 40% OA synovial fluid (only 1 patient, 62 years old, idiopathic KOA). After 10 and 20 hours incubation at 37°C, cells that migrated through the polycarbonate membrane were fixed with acetone/methanol (1:1, vol/vol). Non-migrating cells on top of the membrane were removed. Migrated cells were stained by 4', 6-diamidino-2-phenylindole (DAPI) and crystal violet and counted microscopically. Three representative photographs (left, right, and central) of each well were taken, migrated cells per picture were counted using Image J (National Institutes of Health, Bethesda, MD), and the total number of migrated cells was extrapolated to the total well. Then the migration rates were calculated.

Real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to further evaluate their multilineage differentiation and RNA Sequencing validation. After maintaining in osteogenic, adipogenic, and chondrogenic differentiation media at a density of 5×10^4 cells/well in 6-well cell culture plates for 10 days, the total RNA was extracted using Trizol reagent (Fermentas), and reverse transcribed using an mRNA Selective PCR Kit (TaKaRa) according to the manufacturer's instructions. Human runt related transcription factor 2 (RUNX2), osteocalcin (OCN), CCAAT/enhancer binding protein alpha (CEBP/α), peroxisome proliferator-activated receptor gamma (PPARγ), sex determining region Y-box 9 (Sox-9), and collagen type II (Col-II) cDNA were amplified by real-time PCR using a SYBR PCR Master Mix Kit (Sigma-Aldrich). The primers were synthesized by Invitrogen (Shanghai, China), and the sequences are shown in Supplementary Table 2. The mRNA levels were normalized to the value of β-actin. Mean fold changes were calculated.

mRNA expression profile of mCPCs by RNA sequencing analysis

We used equal amounts of total RNA from each of 6 patients' paired mCPCs from grade 1-2 and grade 3-4 osteoarthritic cartilage. Gene expression profile analysis quality control and quantification of total RNA samples were conducted firstly. Gene expression experiments were performed according to the

manufacturer's protocols. We then selected relative expression of genes associated with OA pathogenesis (involved in mesenchymal stem cell [MSC] tripotentiality, collagen metabolism, chemotaxis, angiogenesis, and control of osteoclast activation and other genes). We clustered the significantly increased and decreased genes according to various biological processes, cellular component, molecular function and analyzed the differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [30]. Expression of arbitrarily selected dysregulated genes were validated by RT-qPCR.

Statistical analysis

Data were presented as mean values and standard deviation (SD). After testing for normal distribution, a paired *t*-test was employed to analyze the statistical significance between mCPCs from paired grade 1-2 and grade 3-4 cartilage for flow cytometric measurements, CFU-F assays, growth kinetic parameter, gene expression and migration rates. A *P*-value <0.05 was considered statistically significant. All tests were performed using IBM SPSS Statistics 20.0

Results

The morphological characteristics of mCPCs of Knee OA patients

Approximately 10 days after the primary culture, fibroblast like cells migrated out from the digested cartilage fragments and adhered to the dish (Fig. 1C) in both grade 1-2 and grade 3-4 groups. A culture confluence of 60-80% was achieved within 15 days at P0. Under the same enzymatic digestion conditions, more migrated cells can be seen around the grade 3-4 cartilage fragments. The cell morphology of mCPCs is macroscopically identical. An adherent layer of vortex-shaped cells developed and cartilage pieces can still be seen within 28 days at P3 (Fig. 1C).

mCPCs from grade 3-4 cartilage highly expressed CD271

The results of immuno-phenotyping showed that mCPCs from both grade 1-2 and grade 3-4 cartilage were homogenously negative for CD34, and CD45, and positive for CD29, CD44, CD73, CD90 CD105, and CD166 expression(Fig. 1D). However, the expression of CD271 was significantly higher in grade 3-4 cartilage (32.5±17.3%) in comparison with grade 1-2 cartilage (22.5±10.8%) (p=0.034) (Fig. 1E).

The mCPCs (grade 3-4) exhibited similar proliferation potential and self-renewal capacity to that of mCPCs (grade 1-2)

To investigate the proliferation ability, hemocytometer cell counting and a CCK-8 assay were performed. The results of the hemocytometer cell counting proliferation assay (Supplementary Fig. 1B) showed that mCPCs in both grade 1-2 and grade 3-4 cartilage exerted similar proliferation ability (P>0.05). Consistently, similar cell proliferation is also reflected by the CCK-8 assay (Supplementary Fig. 1C). Self-renewal potential was measured in a CFU-F assay. mCPCs in both grade 1-2 and grade 3-4 cartilage performed similarly with comparable clonogenic ability (Supplementary Fig. 1D and E).

mCPCs from the grade 3-4 cartilage showed stronger osteogenic, adipogenic and weaker chondrogenic potential

mCPCs from both grade 1-2 and grade 3-4 cartilage were able to differentiate toward the osteogenic, adipogenic and chondrogenic lineage. In particular, mCPCs in grade 3-4 cartilage display enhanced osteo- and adipogenic differentiation capacity compared to mCPCs in grade 1-2 cartilage. Analysis of osteogenic differentiation showed a higher amount of calcium deposition (Fig. 2A) and higher ALP activity (Fig. 2B) in mCPCs from grade 3-4 cartilage than in CPCs from grade 1-2 cartilage. Also, analysis of adipogenic differentiation showed a higher amount of intracellular Oil-Red O stained lipids accumulation in mCPCs from grade 3-4 cartilage than in mCPCs from grade 1-2 cartilage (Fig. 2C). Consistent with the results of cytochemical staining analysis, mCPCs from grade 3-4 cartilage after induction exhibited high levels of mRNA expression of osteogenic markers (RUNX-2 and OCN) (Fig. 2E) and adipogenic transcription factor (CEBP/α and PPARγ) (Fig. 2F) compared to mCPCs from grade 1-2 cartilage. However, the analysis of chondrogenic differentiation showed that more mCPCs in grade 1-2 cartilage developed into positive toluidine blue, Sox-9, and Collagen 2 immune staining cell pellets after 28 days of chondrogenic differentiation compared to mCPCs from grade 1-2 cartilage (Fig. 2D). The mRNA expression of chondrogenic markers (Sox-9 and Collagen 2) (Fig. 2G) confirmed that mCPCs in grade 1-2 cartilage exhibited significantly increased chondrogenic potential compared to CPCs in grade 3-4 cartilage.

mCPCs from the grade 3-4 cartilage showed stronger migration potential

mCPCs from both grade 1-2 and grade 3-4 cartilage were cultured for 10h and 20h under the conditions of 0%, 20%, 40% OA synovial fluid. Crystal violet and DAPI staining showed that relatively small number of CPCs pass out from the bottom of the transwell chamber under the condition of 0% OA synovial fluid. However, CPCs from both grade 1-2 and grade 3-4 cartilage showed strong migration potential under the condition of 20%, 40% OA synovial fluid(Fig. 3A). In addition, the migration rate of CPCs from the grade 3-4 cartilage was higher than that of CPCs from the grade 1-2 cartilage after 10h or 20h and under 20% or 40% OA synovial fluid condition (Fig. 3B).

Histologic features and in vivo distribution of CD271 and CD105 CPCs in grade 1-2 and grade 3-4 cartilage specimens

We performed histologic assessment of bone and cartilage in grade 1-2 and grade 3-4 cartilage specimens from 18 donors. Following decalcification, 12 of 18 paired tissue samples had sufficient tissue quality to enable histologic analysis, grade 1-2 cartilage shows relatively smooth of cartilage, or slight fibrillation (Fig. 4A). Grade 3-4 cartilage is characterized by fissured or denuded surface with chondrocyte clusters, multiple tidemarks and thicken trabecular area in the subchondral bone (Fig. 4B). We investigated and compared the distribution of immunohistochemistric positive CD105 and CD271 CPCs of bone and cartilage in paired grade 1-2 and grade 3-4 cartilage from 8 randomly selected patients. Although a highly heterogeneous distribution of CD105 and CD271 positivity (due to the large inter-subject heterogeneity of bone pathology and cartilage OA architectural changes) did not allow reliable

quantification of immunohistochemistric positive cells using digital imaging analysis for comparison. CD105 staining was distributed in the superficial cartilage surface and reticular pattern in bone marrow cavities in the subchondral bone area (Fig. 4C-F) in both grade 1-2 and grade 3-4 cartilage. Moreover, CD105 expression in the grade 3-4 superficial cartilage surface(Fig. 4E) and the bone lining locations near the osteochondral junction area (Fig. 4F) were more pronounced suggesting that CPCs had migrated toward and accumulated at regions of cartilage damage. CD271 staining was distributed as expected in a perivascular (Fig. 4J) and reticular pattern in bone marrow cavities[31] (Fig. 4H). Additionally, CD271 positivity was detectable in bone lining locations (Fig. 4H, J), while CD271 expression was lacking in the superficial cartilage surface (Fig. 4G).

Comparative gene expression of mCPCs from grade 1-2 and grade 3-4 cartilage

We compared messenger RNA expression profiles of mCPCs from grade 1-2 and grade 3-4 cartilage obtained from same donor. Six pairs were evaluated. After normalization mCPCs in grade 1-2 cartilage were set as $\log_2$fold change≧1.0 and $P<0.05$ to determine the differentially expressed mRNAs. mCPCs in grade 3-4 cartilage showed 134 genes were changed (with 105 gens up-regulated, 29 down-regulated), according to various biological processes, cellular component, molecular function, the expression of some genes related to the cell proliferation and intracellular signal transduction, plasma membrane and extracellular space, protein heterodimerization activity and growth factor activity (Fig. 5A). Nineteen dysregulated genes known to be involved in human OA were selected including CXCL6, CXCL1, FGF1, BMP4, FGF10, ALDH3A1, RERG, CACNA2D3, FGF9, GUCY1A3, SMOC2, LMX1B, FBN2, HPD, KIAA1244, LAMA5, FGF5, LRP2BP, and HGF (Fig. 5C). Four selected dysregulated genes validated by RT-qPCR showed that the expressions of CXCL6, and CXCL1 genes encoding chemokines proteins were increased, and HGF, and LAMA5 genes encoding chondroprotective proteins were decreased (Fig. 5D).

Discussion

In the present study, we isolated novel subpopulations of CPCs from paired cartilaginous tissues by virtue of their cell migration capacity instead of CPC immunophenotying. We found that mCPCs from Outerbridge grade 1–2 and grade 3–4 cartilage shared similar cell proliferation and the self-renewal ability, but the mCPCs (grade 3–4) showed enhanced osteo-adipogenic activities and decreased chondrogenic capacity. Importantly, the mCPCs (grade 3–4) exhibited stronger cell migration in response to OA synovial fluid. More CD105 + cells were found residing in grade 3–4 superficial articular cartilages and areas of osteochondral conjunction. Additionally, different gene expression profile encoding chemokines and chondroprotective proteins was observed.

The imbalance of extracellular matrix degradation and synthesis in the progress of OA caused by the combination of mechanical and biochemical factors were considered as fundamental factors contributing the destruction of tissue homeostasis. In recent years, increasing attention has been focused on the fact that the pathological changes of tissue-specific stem cells in articular tissues may be closely involved in the development of osteoarthritic diseases. However, controversial results were observed in

previous studies. As for chondrogenic capacity, an earlier study reported declined potential of CPCs from OA patients, and upregulation of the osteogenic transcription factor RUNX-2 decreased the expression of the chondrogenic transcription factor Sox-9 [5]. Another study described that adult CPCs, particularly those from moderately affected regions of the osteoarthritic joints, demonstrate superior chondrogenic potential [32]. In addition, the independent studies pursued by Xia et al. and Mantripragada et al. demonstrated that CPCs isolated from the degraded cartilage in the medial condyle and relatively normal cartilage on the lateral side from OA patients showed comparable chondrogenic potential [13, 17, 18].

The controversial data might result from the heterogenous CPCs isolating protocols and functional investigations in independent labs. First, almost all of the CPCs above were obtained from the released cells post long-time collagenase digestion with either cell colony formation cell expansion[6] or flow cytometry cell sorting [18, 33] except Koelling et al. obtained cells that migrated from human cartilage [5]. Our previous study [11] harvested CPCs migrated from human non-osteoarthritc cartilage, based on in vitro and in vivo studies, results demonstrated that mCPCs might represent more regenerative cell subpopulation in cartilages than that of released cells harvested by collagenase digestion. Therefore, in the current study, we initiated CPCs culture by cultivating short time digested OA cartilage chips to maintain their potential characteristics. Second, the released cells may partially lose their biological functions after in vitro expansion. Importantly, not only did studies show the risk of the cells changing properties and phenotype once dissociated from their native micro-environment [19, 20], but also works have demonstrated that maintaining the bone marrow niche in primary culture benefit for MSC properties [21, 34]. Thus, the cartilage chips were retained during cell passaging and kept in the cell culture dishes until passage 3 to mimic the cellular niche in vivo and allow more CPCs outgrowth. Third, no CPCs in previous studies were separated from osteoarthritic tibial plateau cartilage which is badly damaged in KOA due to its unique mechanical status. To the best of our knowledge, the differentiation fates of tissue specific stem/progenitor cells were greatly influenced by mechanical factors. Discher et al. described that a local biochemical and mechanical niche with complex and dynamic regulation control stem cells sense [35]. Yang et al. reported that stem cells remember past physical signals, and mechanical memory and dosing influence stem cell fate [36].

In addition to cell multi-potency, the migratory ability of seed cells is essential for cartilage regeneration. It has been reported that both trauma and degenerative lesions activate endogenous CPCs migration, and trauma and OA inflammatory factors can chemotactically exogenous CPCs migration [5, 7, 26, 37]. In the current study, we found that both mCPCs from grade 1–2 and grade 3–4 cartilage showed apparent migration capacity in response to OA synovial fluid. Interestingly, mCPCs (grade 3–4) exhibited stronger cell migration. The data of CPCs in vivo distribution in cartilage may be helpful to understand the phenomenon. In the current study, the in vivo CD105 + CPCs number was higher in the grade 3–4 cartilage group. In addition, these cells mainly accumulate in superficial cartilage lesions and areas of osteochondral junction. Moreover, CPCs have also been reported to be chemotactic migratory with nerve growth factor (NGF) treatment and result in extracellular matrix catabolism indicated by increased sulfated glycosaminoglycan release and matrix metalloprotease (MMP) expression [38]. Consistently, our

flow cytometry results showed that higher expression of CD271 in mCPCs derived from grade 3–4 cartilage, which may be a contributing factor in late stage OA articular cartilage degeneration.

To further explore the target genes of osteoarthritic CPCs, we performed an analysis of the gene expression profile of mCPCs from grade 1–2 and grade 3–4 cartilage. Notably, mCPCs (grade 3–4) overall exhibited higher inflammatory/catabolic and lower chondroprotective response as evidenced by higher levels of chemokines (CXCL-1, CXCL-6) and lower growth factors (HGF, LAMA5) than mCPCs (grade 1–2). Previous study have suggested innate associations between OA severity and synovial fluid CXCL1 concentration[39] while the upregulation of CXCL-1 and CXCL-6 are also responsible for stronger migration of mCPCs (grade 3–4). Downregulation of HGF may be responsible for decreased chondrogenic performance of mCPCs (grade 3–4) cartilage because study have demonstrated that HGF-rich exosome play a pivotal role in promoting cartilage repair[40]. Also, the downregulated expression of gene LAMA5 has been suggested to hamper the maintenance and function of the extracellular matrix (ECM) which is critical components in stem cell niche. Study has shown that the heterozygous LAMA5 mutation are closely associated with OA via regulating ECM proteins (COL1A1, MMP1 and MMP3) [41].

Thus, we speculated a pathological model of mCPCs in the progression of OA (Fig. 6). Although more mCPCs migrated rapidly to the degenerative cartilage of lesion site with the rapid progress of later staged OA, the chondro-generative capacity of these cells are impaired, which change the articular tissue structures and even worsen the pathological status. Notably, these findings suggested that mCPCs may be a novel cell target for OA treatment. Blocking impaired mCPCs migration may delay the articular degeneration. Additionally, rescuing the multi-potency of mCPCs may be helpful to alleviate tissue disruption in later-stage OA.

There were some limitations in our study. First, although the paired CPCs were isolated from grade 1–2 and grade 3–4 cartilage of the same OA patients, the CPCs in grade 1–2 degenerative cartilages are not equated with the fully healthy CPCs. Second, we cannot exclude the possibility that CPCs properties were influenced by anatomical (medial-to-lateral or superficial-to-deep)and/or mechanical differences in osteoarhritic cartilage[25]. Third, the cell surface markers of our ex vivo cultured mCPCs is different from that of in vitro culture-expanded[42] and further investigations are needed to clarify the reasons and their significance for cartilage regeneration.

Conclusions

We have isolated migratory progenitor cell populations from both grade 1–2 and 3–4 human OA cartilage, Although mCPCs in grade 3–4 OA cartilage present stronger migratory potential, the chondro-generative capacities of these cells are impaired (indicated by stronger osteogenesis, stronger adipogenesis, weaker chondrogenesis). Our findings may be helpful in understanding the role of mCPCs in the pathogenesis of OA progression. In addition, they are relevant for direct therapeutic exploitation of mCPCs in OA joint regeneration.

Declarations

Ethics approval and consent to participate

This study was approved by the institutional ethical review board of our Hospital (Rapid review of scientific research projects for use of discarded biological material).

Consent for publication

All donors' data (including demographic, clinical, and imaging details) was consent for publication. The informed consent was obtained from all donors.

Availability of data and materials

The datasets generated and analysed during the current study are available in the Figure 1-6, Supplementary Figure 1, and Supplementary Table 1-2. The more detail datasets are also available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

YW, ZZ, and QW conceived of the study, carried out the experimental design, data acquisition, and statistical analysis, drafted and revised the manuscript. HZ, and ZL participated in study conception and design, and revised the manuscript. YH, SZ, WH, JL, PL carried out the sample collections, experimental research, and data acquisition. HW, NM, CW participated in analysis and interpretation of data.

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Abbreviations

ALP: alkaline phosphatase; APC: allophycocyanin; CCK-8: Cell Counting Kit 8; CEBP/α: CCAAT/enhancer binding protein alpha; CFU-F: Colony-forming unit fibroblast formation; Col-II: collagen type II; CPCs; chondrogenic progenitor cells; DAPI: 4', 6-diamidino-2-phenylindole; EDTA: ethylenediaminetetraacetic acid; FACS: fluorescence-activated cell sorting; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOA: Knee osteoarthritis; mCPCs: migratory CPCs; MMP metalloprotease MSC: mesenchymal stem cell; NGF: nerve growth factor; Sox-9: SRYtype high-mobility group box-9; TGF-β3: transforming growth factor; OCN: osteocalcin; OA: osteoarthritis; PBS: phosphate-buffered saline; PE: phycoerythrin; PPARγ: peroxisome proliferator-activated receptor gamma; RT-qPCR: real-time quantitative polymerase chain reaction: RUNX2: runt related transcription factor 2; SD: standard deviation; α-MEM: alpha-minimal essential medium.

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Figures

Figure 1

. Isolation, expansion, and immuno-phenotypes of mCPCs from paired grade 1-2 and grade 3-4 cartilage. A, Representative MRI image late-stage idiopathic KOA before total knee arthroplasty shows Outerbridge grade 3-4 cartilage lesions in the medial tibial plateau and grade 1-2 cartilage lesions on the lateral side. B, Osteoarthritic cartilage specimen of the tibial plateau obtained from the same patient. Locations used for the harvesting of cartilage are indicated by black lines, the cartilage tissue was separated from the subchondral bone, minced into pieces, and incubated. C, mCPCs migrating from a paired OA cartilage pieces ex vivo after 15 days of cultivation, the cartilage pieces were still retained and maintained until passage 3 (arrowhead); scale bars=200 μm. D, Immuno-phenotype markers (CD29, CD31, CD44, CD45, CD73, CD90, CD105, CD166, and CD271) of mCPCs from paired grade 1-2 and grade 3-4 cartilage, red lines indicate isotype controls. E, the expression of CD271 in mCPCs from grade 1-2 cartilage was significantly lower compared with those from grade 3-4 cartilage (p=0.034). mCPCs: migratory chondrogenic progenitor cells; KOA: knee osteoarthritis.

Figure 2

Multi-differentiation of mCPCs derived from paired grade 1-2 and grade 3-4 cartilage. A-C, Representative photomicrographs of Von Kossa (A), ALP (B) and oil red O(C) staining of mCPCs. D, Representative H-E, Thionin blue, Safran O staining , Col-II and Sox-9 immunostaining of cartilage pellets formed with mCPCs

from paired grade 1-2 and grade 3-4 cartilage derived CPCs. (n = 5 donors with 5 pellets per donor) following 4 weeks of chondrogenic induction. Scale bar= 200μm (A and B), 100μm (C and D). E-G, The expression profile of osteogenesis (RUNX2 and OCN), adipogenesis(CEBP/α and PPARγ) and chondrogenesis markers (Col-II and Sox-9) by real-time polymerase chain reaction in mCPCs derived from paired grade 1-2 and grade 3-4 cartilage. Error bars denote the means ± SD, Data were normalized to β-actin. *p<0.05, ***p<0.001. ALP, alkaline phosphatase; H-E, hematoxylin and eosin; RUNX2, runt-related transcription factor 2; OCN, osteocalcin; CEBP/α, CCAAT/enhancer-binding protein alpha; PPARγ, peroxisome proliferator-activated receptor gamma; Col-II, collagen type II; Sox-9: sex determining region Y-box 9.

Figure 3

Migration potential of mCPCs derived from paired grade 1-2 and grade 3-4 cartilage A, Representative photomicrographs of migration in paired grade 1-2 and grade 3-4 cartilage derived mCPCs shown by DAPI and crystal violet staining. Scale bars = 100μm. B, Increased migration rate of transmigrated cells were observed in the grade 3-4 cartilage derived mCPCs group both at 10 h and 20 h under 10% and 20% OA synovial fluid condition. *P0.05.

Figure 4

Histologic and in situ CD105 and CD271 immunohistochemical distribution of paired grade 1-2 and grade 3-4 cartilage. A, B, Photomicrographs of H-E stained paired human osteoarthritic knee tibial plateau present Outerbridge grade 1-2 cartilage lesions(A), grade 3-4(B) cartilage lesions. C-J, CD105 cell distribution in grade 1-2 cartilage(C, D), grade 3-4(E, F) and CD271 cell distribution in grade 1-2 cartilage(H,I), grade 3-4(J, K) cartilage specimens as assessed by immunohistochemistry. More CD105 expression was observed in the grade 3-4 cartilage, both in the superficial cartilage surface (E) and the bone lining locations (arrowhead) near the osteochondral junction area (F). Grade 1-2 cartilage showing lacking CD271 expression in the superficial cartilage surface (G). Higher-magnification view of boxed area in G, CD271 expression was clearly detectable in bone lining (arrowhead) near the osteochondral junction regions and reticular pattern in subchondral bone marrow cavities(arrow) (H). Representative grade 3-4 cartilage specimen showing CD271 staining was distributed in a perivascular (asterisk) and subchondral bone lining area (arrow)(I and J). Higher-magnification view of boxed area in I (J). Scale bars = 500μm (A, B, G, I), 100 μm (C-F, H, J).

Figure 5

mRNA sequencing analysis of mCPCs derived from paired grade 1-2 and grade 3-4 cartilage. A, Cluster of significantly changed genes according to various biological processes, cellular component, molecular

function. B, The differentially expressed genes were analyzed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. C, Histogram showing levels of mean log2fold change in 19 selected relative genes associated with OA pathogenesis in mCPCs from paired grade 3-4 versus grade 1-2 cartilage; n=6 paired samples. D, Validation of CXCL6, CXCL1, HGF, and LAMA5 mRNA expression by real-time quantitative polymerase chain reaction, the 2-△Ct value was normalized to β-actin. *P < 0.05, by paired t-test.

Figure 6

Schematic representing a pathological model of mCPCs in the progression of OA. Although more mCPCs migrated rapidly to the grade 3-4 OA cartilage, the chondro-generative capacities of these cells are impaired (indicated by stronger osteogenesis, stronger adipogenesis, weaker chondrogenesis).

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