Original article

Characterization of human primary chondrocytes of osteoarthritic cartilage at varying severity

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Keywords: *osteoarthritis; chondrocyte; differentiation*

Background There is a difficulty in evaluating the *in vivo* functionality of individual chondrocytes, and there is much heterogeneity among cartilage affected by osteoarthritis (OA). In this study, *in vitro* cultured chondrocytes harvested from varying stages of degeneration were studied as a projective model to further understand the pathogenesis of osteoarthritis.

Methods Cartilage of varying degeneration of end-stage OA was harvested, while cell yield and matrix glycosaminoglycan (GAG) content were measured. Cell morphology, proliferation, and gene expression of collagen type I, II, and X, aggrecan, matrix metalloproteinase 13 (MMP-13), and ADAMTS5 of the acquired chondrocytes were measured during subsequent *in vitro* culture.

Results Both the number of cells and the GAG content increased with increasing severity of OA. Cell spreading area increased and gradually showed spindle-like morphology during *in vitro* culture. Gene expression of collagen type II, collagen type X as well as GAG decreased with severity of cartilage degeneration, while expression of collagen type I increased. Expression of MMP-13 increased with severity of cartilage degeneration, while expression of ADAMTS-5 remained stable. Expression of collagen type II, X, GAG, and MMP-13 substantially decreased with *in vitro* culture. Expression of collagen type I increased with *in vitro* cultures, while expression of ADAMTS 5 remained stable.

Conclusions Expression of functional genes such as collagen type II and GAG decreased during severe degeneration of OA cartilage and *in vitro* dedifferentiation. Gene expression of collagen I and MMP-13 increased with severity of cartilage degeneration.

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Osteoarthritis (OA) is a leading cause of disability in older patients that creates a huge strain on medical treatment and care as well as a loss of the work force.[1] Much effort has been invested into understanding the mechanism behind OA and its effective medical intervention; however, there remains difficulty in identifying the *in vivo* functionality of chondrocytes in osteoarthritic cartilage. An alternative approach involving *in vitro* cultured chondrocytes from OA patients has been broadly investigated as a projective research model to study the OA mechanism.[2,3] This approach has also been used to assess the possibility of transplanting chondrocytes harvested from less-affected areas of OA joints in autologous chondrocyte implantation (ACI) and for use in *in vitro* chondrogenesis models.[4] However, as the roles of individual chondrocytes in pathogenesis of OA are heterogeneous and ill-defined, researches often lead to conflicting results due to varying methodologies especially in terms of the site of chondrocyte isolation.[5]

It is well documented that an overall up-regulation of cartilage matrix gene expression was observed in chondrocytes from OA-affected cartilages at varying degrees, in a futile effort to counter the abundant expression of degradation enzymes. Gene expression of Collagen I, II, and III increased sharply at end-stage OA compared with normal and early-stage OA, while a shift of phenotypes towards fibroblasts was indicated by the drop in collagen II/I ratio with OA development.[6-8] The increased expression of collagen II was not supported by results from intra-joint comparisons.[9] There was also a discrepancy in the gene expression of matrix metalloproteinase 13 (MMP-13) in advanced OA, with both up-regulation and suppression reported in OA cartilage.[2,9] Detailed exploration has been done by comparing gene expression of macroscopically intact areas and the degenerated areas in OA affected cartilages, as well as among cartilage of different zones. Though the

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expression of cartilage matrix genes (collagen IIA, aggrecan and link protein) was highly enhanced in macroscopically intact areas, only mild enhancement was found in the degenerated areas.[9] Further analysis with zonal categorization showed that suppression of cartilage matrix genes (collagen II and Aggrecan) was not detected at the superficial zone but was apparent at the middle and deep zones in OA cartilage.[10] Variation of chondrocyte genotypes among individuals and different anatomical locations makes the interpretation difficult and sometimes conflicting.[5,11]

Frequently, chondrocytes harvested from less-affected areas of osteoarthritic joints are used for *in vitro* study. During *in vitro* monolayer culture, chondrocytes proliferate largely at the expense of a lost of phenotype known as dedifferentiation, indicated by decreasing gene expression levels of matrix proteins and proteases. This differentiated phenotype of cells could be recovered with proper treatments, such as implantation on three-dimensional scaffolds.[12,13] It is unclear during this *in vitro* expansion process at which key point the dedifferentiated chondrocytes irreversibly lose their ability to re-differentiate and whether chondrocytes harvested from varying degrees of OA-affected cartilage will undergo similar dedifferentiation processes or not. Though some molecular markers have been proposed to monitor the phenotype of *in vitro* cultured chondrocytes with an aim to improve and stabilize efficiency of ACI,[14] there is no general consensus on optimal *in vitro* culture protocol to balance quantity and phenotype maintenance of cultured chondrocytes. Except for the aforementioned *in vivo* heterogeneity of chondrocytes in OA affected cartilages, the variation in the harvesting procedure, enzymatic digestion, plating density and passage frequency makes these findings from different research studies difficult to compare.

Previous studies on chondrocytes harvested from OA cartilages only classified sampling cartilages as macroscopically intact (preserved) or non-intact (degenerated), which somewhat over-simplified the heterogeneity of chondrocytes in OA-affected cartilage.[9,11] Gene expression analysis and some biochemical assays were not able to provide a comprehensive view of this situation. We hypothesized that a study on properly graded OA cartilage could provide a comprehensive understanding of the pathogenesis and functionality of individual groups of chondrocytes. In the current study, cell yield and matrix glycosaminoglycan (GAG) content were assessed during digestion of cartilage harvested from varying degrees of end-stage OA cartilage. The acquired chondrocytes were further expanded in a monolayer culture for 40 days, while cell morphology, proliferation, and gene expression were investigated with an aim to outline a comprehensive view of chondrocytes during the *in vitro* expansion process and to project the *in vivo* functionality of these individual groups of chondrocytes. Expression of key genes, which regulate functionality of chondrocytes, including collagen type I, II, III, and X, aggrecan, MMP-13 and ADAMTS 5, were evaluated.

METHODS

Tissue harvest and grading

Human articular cartilage tissue was obtained from late-stage OA donors undergoing total knee joint replacement surgery (n=17; 51–79 years; mean age 66 years). The acquisition of tissue and subsequent experiments were approved by the Ethics Committee of Peking University First Hospital. Articular cartilages were kept aseptic and graded according to Dougados classification:[15] Grade 0 (macroscopically normal), Grade I (softening and swelling), Grade II (fibrillation and fissuring on the surface), Grade III (fragmentation and fissuring to middle and deep zone of the cartilage), and Grade IV (completely exposed subchondral bone). Cartilages at different grades were removed separately for histological processing, biochemical assays, RNA extraction, or subsequent *in vitro* cell culture.

Histology

Cartilages with subchondral bones at different grades were fixed in 10% formalin for one day, decalcified in 10% formic acid, and subsequently embedded in paraffin and sectioned into 4 μm slices. The sections were stained with hematoxylin & eosin for histological observation.

Primary human chondrocytes culture

Harvested cartilages at different grades were minced and digested with 0.1% of collagenase II (17101-015, Gibco, USA) in Dulbecco's modified Eagle's medium (DMEM, 31600-034, Invitrogen, USA) with 1 g tissue /5 ml collagenase solution. After the system was incubated at 37°C for 24 hours, the cells were collected through a strainer mesh (200 mesh, 74 μm, Solarbio, Fr) and counted with a hemocytometer. The cells were planted at a density of 4000 cells/cm^2, cultured with DMEM, and supplemented with 10% fetal bovine serum (FBS, SV30087.02, Gibco) with 100 U/ml PS (Penicillin, 0741; Streptomycin, 0832, Amresco, USA). The systems were incubated at 37°C in a humidified environment with 5% CO_2. Culture media was changed every three days, while cells were passaged every 10 days. Cells were counted and implanted at a density of 4000 cells/cm^2 at each passage, and three passages were done in total.

Sulfated glycosaminoglycan (sGAG) quantification

Sulfated GAG concentration of cartilage digestive solution was determined with dimethylmethylene blue (DMMB, 341088, Sigma).[16] One milliliter of working DMMB solution (16 μg/ml DMMB, 2.5% ethanol, 1 mol/L GuHCl, 0.2 g/L sodium formate, and 2% formic acid) was briefly mixed with 100 μl of digestive solution, vortexed for 30 minutes, and then centrifuged at 9600 × g for 10 minutes. After the supernatant was discarded, 1 ml of decomplexation solution (50 mmol/L sodium acetate

solution buffer (pH 6.8), 10% propan-1-ol, 4 mol/L GuHCl) was added to the pellet and incubated for 30 minutes. Absorbance was measured at 656 nm. Quantities of sGAG content were extrapolated from the standard curve. Six replicates were done.

Cell proliferation

Cells were seeded into a 96-well plate at a density of 1000 cells per well at each passage. After culturing for 72 hours, cell numbers were determined with Hoechst 33258 (14530, Sigma) according to the user's manual. Briefly, each well was washed thoroughly with PBS after the culture media was removed, before 100 μl of sterilized water was added to each well in the 96-well plate. After incubated for half an hour, acquired solutions from each well were collected and kept at –20°C. Fluorescence assay was done by mixing one hundred microliters of assay solution (0.1 μg/ml Hoechst 33258) with an equal amount of acquired sample solution from individual wells and detecting fluorescence at 360 nm/460 nm as excitation/emission wave length with Multi-Mode Microplate Reader (Synergy, BioTek, USA). Cell numbers were extrapolated from the standard curve. Six replicates were done.

Cell doubling time (T) was calculated with the following formula:

$$T=t\times lg2/(lgN_t-lgN_{0)}$$

N_0 and N_t are the cell numbers at the beginning and the end of the passage respectively, while t represented proliferation time.[17]

Cell morphology

At the 5th day of every passage, the cultured cells were observed with a microscope (CKX41-RC, Olympus, Japan) and pictures were taken with a CCD (DP35). Morphology of the cells was analyzed with Image pro plus 6.0 (IPP 6.0, Media Cybernetics, USA). Cell spreading areas and ellipticity of individual cells were also measured. Cells at 6 microscopic fields or more were analyzed at each passage. R_{max} and R_{min} denoted maximal and minimal distance between a cell's centroid and contour respectively.

Quantitative real-time PCR

Total RNA was extracted with Trizol (15596-026, Invitrogen), following the manufacturer's instructions. Reverse transcription reaction was performed using a PCR thermal cycler (Mycycler, Bio-Rad Inc., CA, USA). cDNA synthesis was performed with M-MLV reverse transcriptase (C28025, Sigma) and oligo(dT) (A3477L, TOYOBO, Japan). 500 ng samples of total RNA in 20 μl reaction volumes were separately incubated at 25°C for 10 minutes, at 37°C for 50 minutes, and at 70°C for 15 minutes. The quantitative real-time PCR was performed on the DNA Engine Opticon 2 System (Bio-Rad) in PCR Tubes Strip (PCR-0208-C, Axygen, USA) using RealMasterMix SYBR Green PCR master mix (FP302-02, Tiangen, China) following the manufacturer's protocol.

Table. Real-time PCR primers

Genes		Primers
GAPDH	Forward	ATGGGGAAGGTGAAGGTCG
	Reverse	TAAAAGCAGCCCTGGTGACC
Collagen I	Forward	CAGCCGCTTCACCTACAGC
	Reverse	TTTTGTATTCAATCACTGTCTTGCC
Collagen II	Forward	GGCAATAGCAGGTTCACGTACA
	Reverse	CGATAACAGTCTTGCCCCACTT
Collagen X	Forward	CAAGGCACCATCTCCAGGAA
	Reverse	AAAGGGTATTTGTGGCAGCATATT
Aggrecan	Forward	TCGAGGACAGCGAGGCC
	Reverse	TCGAGGGTGTAGCGTGTAGAGA
MMP13	Forward	TTGAGCTGGACTCATTGTCG
	Reverse	TGCAAACTGGAGGTCTTCCT
ADAMTS5	Forward	TACTTGGCCTCTCCCATGAC
	Reverse	TTTGGACCAGGGCTTAGATG

The real-time PCR reaction was performed at 94°C for 3 minute (activation), 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 68°C for 20 seconds (amplification), and GAPDH was used as an internal control to normalize the amount of cDNA in each sample. Specificity of primers was checked by detecting the amplified and melting curve of 25, 2.5 and 0.25 ng cDNA with each gene compared to GAPDH. The primers used are listed in Table.

Data processing and statistics

During *in vitro* culture, collected data on proliferation, morphology, and gene expression were divided into 12 groups (each of the three experimental groups of cells from varying degeneration of cartilages (Grade 0, I, II/III) was further evaluated at four different time points (Passage 1, 2, 3, 4). In order to summarize the change of detected gene expression along with the progression of cartilage degeneration during *in vitro* culture, data from the aforementioned 12 groups were regrouped into three or four groups according to either degeneration grade or culture passage number.[3] SPSS V13.0 (SPSS Inc., IL, USA) was utilized to analyze the data, using the one-way analysis of variance (ANOVA) test procedure (Fisher LSD and turkey) with a significance level of 0.05.

RESULTS

Cartilage grading and grouping

Though the majority of the cartilages harvested had degenerated severely, individual anatomical locations of the cartilages were in varying stages of degeneration, ranked from Grade 0, I, II, III or IV, accordingly (Figure 1). As it was difficult to visually distinguish Grade II and III cartilage degeneration by judging if the fissures reached the middle layers of the cartilages or not, Grade II and III cartilage were combined as a single experimental group. Since there was almost no visible cartilage remaining for cases of Grade IV cartilage degeneration, no cartilage was harvested from this grade of cartilage degeneration in the current study.

Histology

The accuracy of sample grading was verified by histology.

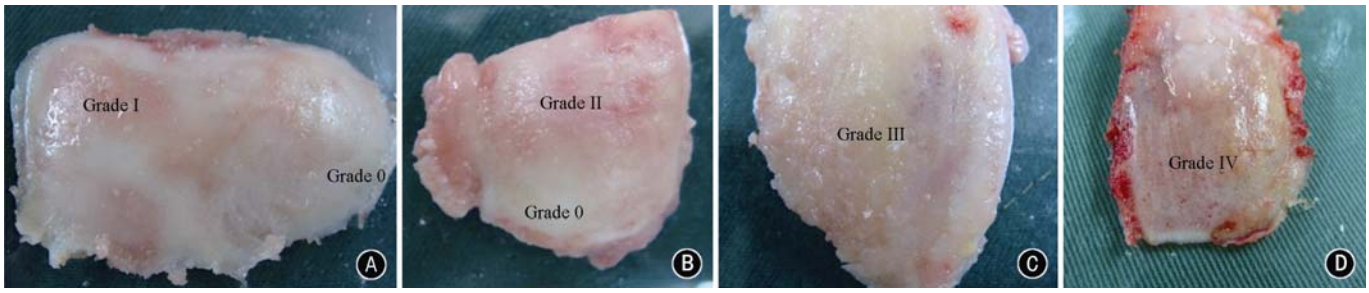

Figure 1. Classification of osteoarthritis cartilage. **A:** Grade 0 and Grade I; **B:** Grade 0 and Grade II; **C:** Grade III; **D:** Grade IV.

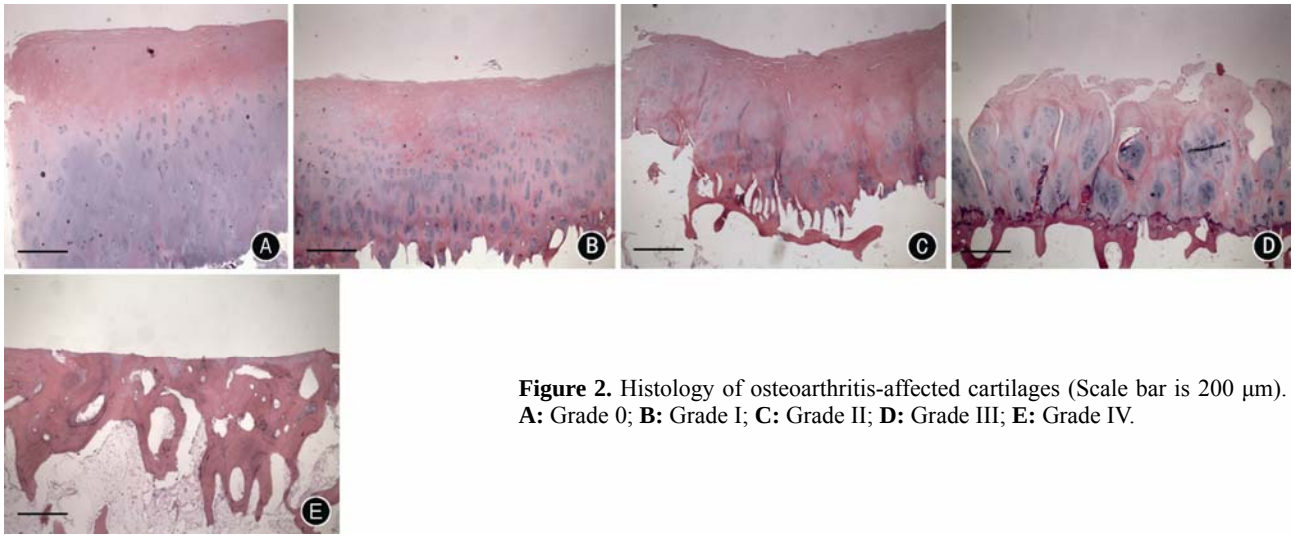

Figure 2. Histology of osteoarthritis-affected cartilages (Scale bar is 200 μm). **A:** Grade 0; **B:** Grade I; **C:** Grade II; **D:** Grade III; **E:** Grade IV.

At Grade 0, cartilage was composed of intact superficial, middle, and deep zones with distinct distribution of cells and matrix, as described previously.[1] Chondrocytes at superficial layers were small and lined parallel to the surface; chondrocytes dispersed in the matrix increased in size at the middle zone; chondrocytes at the deep zone were larger and lined perpendicularly to the surface (Figure 2A). For Grade I cartilage, the thickness of superficial zone decreased while surface integrity was broken occasionally as softening and swelling occurred (Figure 2B). The cartilage layer became even thinner for Grade II cartilage with some small fissures appearing (Figure 2C). For Grade III cartilage, the superficial zone of cartilage was totally lost with multiple fissures extending from the middle zone to the deep zone. Chondrocyte clusters were easily distinguishable and surrounded by a reduced extracellular matrix (Figure 2D). Full-thickness cartilage was completely absent for Grade IV tissue (Figure 2E).

sGAG

The amounts of sGAG per gram of cartilage increased substantially with the degeneration of cartilages (Figure 3). From Grade 0 cartilage ((6.4±4.4) μg/g tissue) and Grade I cartilage ((6.9±2.6) μg/g tissue) to Grade II/III cartilage ((8.4±3.0) μg/g tissue), the sGAG content increased about 1.3 times. However, there was no statistical difference (One-way ANOVA, LSD & Turkey, P >0.05).

Cell yield

Cell yields increased substantially from Grade 0 cartilage ((29.5±18.4)×10^4 cell/g tissue) and Grade I cartilage ((26.6±15.5)×10^4 cell/g tissue) to Grade II/III cartilage ((41.9±17.2)×10^4 cell/g tissue) (Figure 4). There was a statistically significant difference between Grade I and Grade II/III cartilage (One-way ANOVA, LSD, P=0.039).

Proliferation

Chondrocytes from mildly degenerated cartilage Grade I ((2.90±0.41) fold) proliferated slightly faster than those from either normal cartilages (Grade 0, (2.75±0.30) fold) or severely degenerated cartilage (Grade II/III, (2.84±0.31) fold) during the subsequent 40 day of *in vitro* expansion processes. However, no statistical significant difference was found (One-way ANOVA, LSD/Turkey, P >0.05). There was a general tendency for the chondrocytes from all groups to show an accelerated growth rate during *in vitro* cultures, which could have led to a statistical difference between P1 and P4 of chondrocytes from Grade 0 (One-way ANOVA, LSD, P=0.042), as well as between P1 and P3/P4 of chondrocytes from Grade I (One-way ANOVA, LSD, P=0.034/0.008). No significant difference was observed between passages of Grade II/III (Figure 5).

When cell proliferation was evaluated in a precisely controlled environment and time period, primary chondrocytes (P1) from severely degenerated cartilage

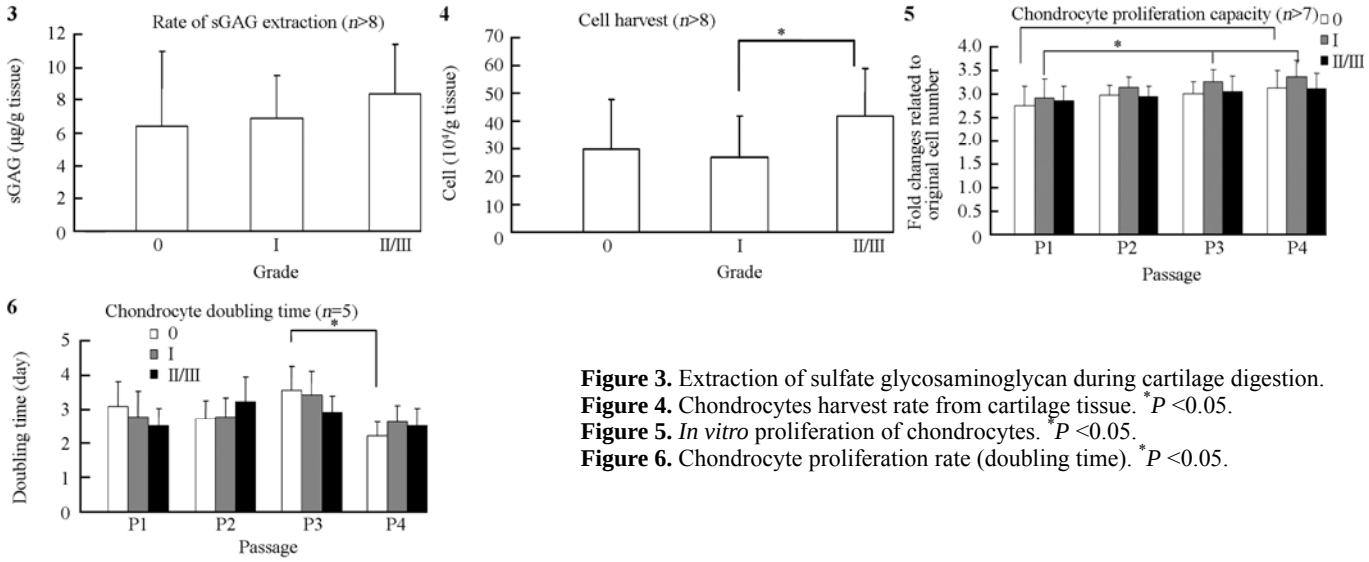

Figure 3. Extraction of sulfate glycosaminoglycan during cartilage digestion.
Figure 4. Chondrocytes harvest rate from cartilage tissue. $^{*}P$ <0.05.
Figure 5. *In vitro* proliferation of chondrocytes. $^{*}P$ <0.05.
Figure 6. Chondrocyte proliferation rate (doubling time). $^{*}P$ <0.05.

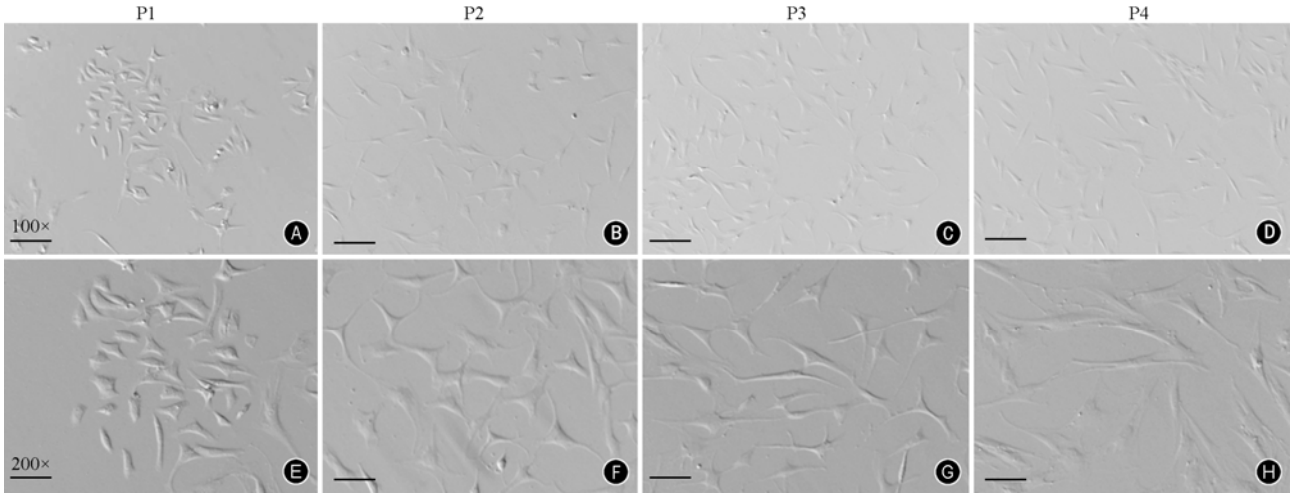

Figure 7. Change of cell morphology during *in vitro* culture (Scale bar = 200 μm). **A**, **E:** P1; **B**, **F:** P2; **C**, **G:** P3; **D**, **H:** P4.

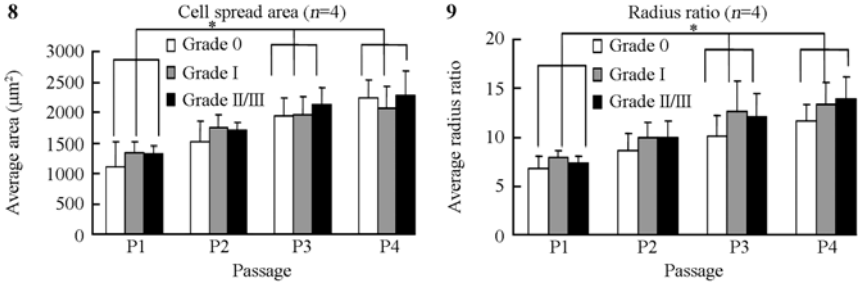

Figure 8. Spreading areas of cells during *in vitro* culture. $^{*}P$ <0.05.
Figure 9. Ellipticity of cells during *in vitro* culture. $^{*}P$ <0.05.

(Grade II/III) proliferated fastest, verified by the shortest doubling time of (2.50±0.53) days, when compared with P1 cells from relatively "healthy" cartilage ((3.08±0.66) days, Grade 0) and mildly degenerated cartilage ((2.78±0.60) days, Grade I). However the differences in cell proliferation rates showed no statistical significance. After *in vitro* culture for 30 days (P4), proliferation of chondrocytes from Grade 0 cartilage increased significantly with a doubling time of (2.21±0.41) days when compared with P3 cells with a doubling time of (3.54±0.72) days (One-way ANOVA, LSD, P=0.034) (Figure 6).

Morphology

The cell spreading area of primary chondrocytes (Figure 7) increased with severity of degeneration, from (1116±396) μm^2 (Grade 0) to (1334±168) μm^2 (Grade I) and (1328±109) μm^2 (Grade II/III), while spreading area of chondrocytes from all groups increased substantially with *in vitro* culture time. Cell area increased sharply from P1 to P3 and then remained stable at P4 significance between primary and P3 cells, One-way ANOVA, LSD, P <0.001 (Grade 0); P=0.008 (Grade I); P=0.001 (Grade II/III) (Figure 8). Cell spreading area increased to about 2200 μm^2 when primary chondrocytes were passaged to

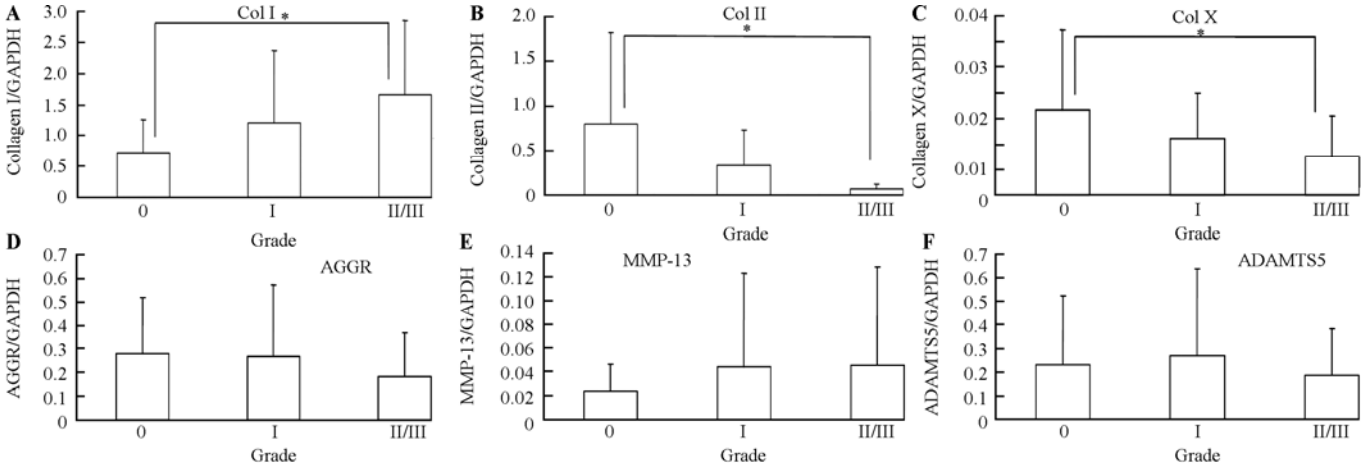

Figure 10. Summary of gene expression according to grade classification. **A:** Collagen I; **B:** Collagen II; **C:** Collagen X; **D:** AGGR; **E:** MMP-13; **F:** ADAMTS5. $^{*}P$ <0.05.

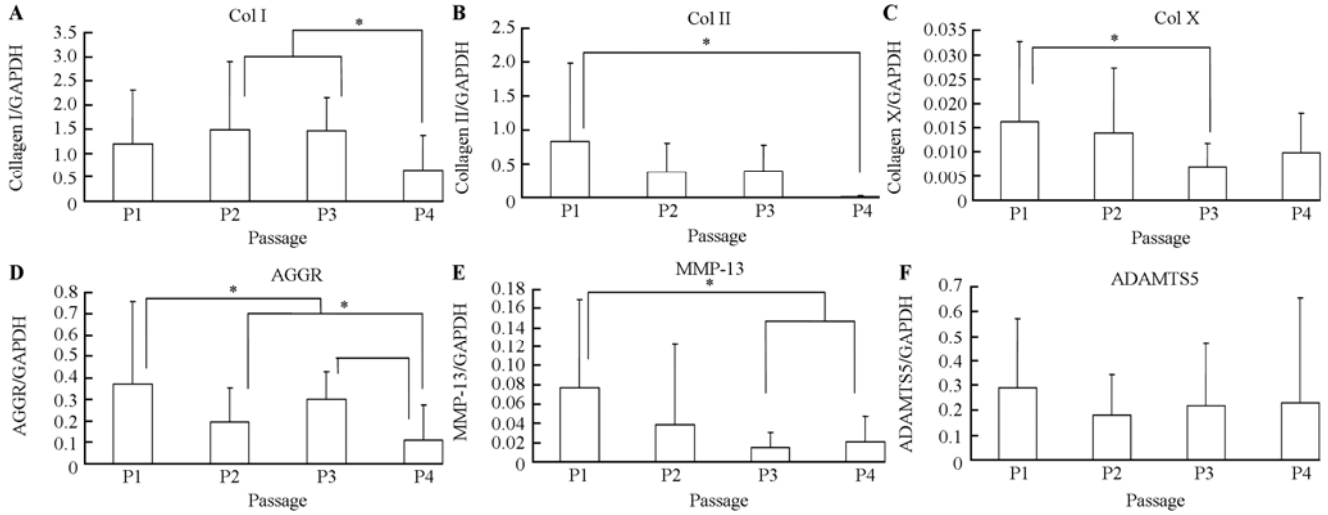

Figure 11. Summary of gene expression according to culture passages. **A:** Collagen I; **B:** Collagen II; **C:** Collagen X; **D:** AGGR; **E:** MMP-13; **F:** ADAMTS5. $^{*}P$ <0.05.

P4, while no grade-related difference in cell spreading area was observed among chondrocytes from all cartilage samples.

Apart for cell spreading areas, morphology of the *in vitro* cultured chondrocytes changed with time, from initial elliptical form (with ratio of radius of maximal to minimal about 7) to typical fibroblast-like morphology either with a spindle or stellate shape (with radius ratio about 12 at later stages, passage 3). Similarly, cell morphology of P3 and P4 cells was different from primary chondrocytes (significance between primary and P3 cells, One-way ANOVA, LSD, P=0.018 (Grade 0)/ P=0.007 (Grade I)/ P=0.004 (Grade II/III); significance between primary and P4 cells, One-way ANOVA, LSD, P=0.002 (Grade 0)/ P=0.003(Grade I)/ P <0.001 (Grade II/III)), while cell morphology varied little between different grades of chondrocytes at the same passage (Figure 9).

Real-time PCR

The relative mean expression levels of each gene were compared both among chondrocytes from cartilage with varying grade of degeneration (Figure 10) and between different passages (Figure 11). There were significant differences in gene expression profile of primary and passaged chondrocytes from varying degenerated cartilages.

Expression of collagen type I increased with either increasing severity of cartilage degeneration (Figure 10A) or *in vitro* culture time (Figure 11A). However, expression of collagen type I dropped at prolonged *in vitro* culture periods (P4), more obvious in chondrocytes from Grade 0 cartilage (One-way ANOVA, LSD, P=0.041) (Figure 10A).

In general, expression of collagen type II/X and aggrecan all decreased accordingly with either increasing severity of cartilage degeneration (Figure 10B, C, and D) or *in vitro* culture time (Figure 11B, 11C, and 11D). Significant grade-related difference was only found between expression of collagen type II in chondrocytes from Grade 0 and Grade I cartilage (One-way ANOVA, LSD,

P=0.008), and from Grade 0 and Grade II/III cartilage (One-way ANOVA, LSD, *P*=0.003), separately. Passage-related significant difference between primary and passaged chondrocytes from varying degenerated cartilage is detailed below. Among the detected gene expression of matrix proteins, relative expression levels of collagen type X were 10–100-fold lower than collagen type I/II and aggrecan.

Expression of MMP-13 increased in accordance with the severity of cartilage degeneration (Figure 10E) and decreased with *in vitro* culture time (Figure 11E), but no significant difference was found between different grade cartilages (One-way ANOVA, LSD, *P*=0.05). Expression of ADAMTS5 remained stable among Grade 0, Grade I, and Grade II/III (Figure 10F and 11F).

DISCUSSION

Cartilage is composed of collagen and aggrecan predominantly. Collagen type II, a marker for hyaline cartilage, together with aggrecan, are the predominant proteins in the extracellular matrix of cartilage.[18] Collagen type I, characteristic product of dedifferentiated chondrocytes[19,20] is normally not expressed in healthy cartilage. Expression of collagen type X typically indicates a phenotypic shift of chondrocytes towards hypertrophic.[21] The cartilage degeneration during OA progression is a complex process involving different enzymes. Loss of aggrecan is considered to take place earlier than degradation of collagen II.[22] Aggrecan is degraded by aggrecanases from the ADAMTS family of proteinases, including ADAMTS4 (aggrecanase 1)[23] and ADAMTS5 (aggrecanase 2).[24] ADAMTS4 is expressed only at a very low level in normal cartilage and is only slightly up-regulated in OA cartilage. ADAMTS5 is the most strongly expressed Aggrecanase during OA progression.[2,25] Although the loss of aggrecan is thought to be a reversible process, degradation of collagen II is considered irreversible and its degradation is central to the loss of structural and functional integrity of cartilage.[22] Matrix metalloproteinases (MMPs) are a family of enzymes that cleave different components of the extracellular matrix. Collagen II is specifically cleaved by MMP-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3).[26] Among these MMPs, MMP-13, specifically expressed in OA cartilage but not in normal cartilage, plays the most important role during OA pathogenesis of human articular cartilage.[26,27]

There have been different and sometimes contradictory reports on expression of collagen II and Aggrecan as well as their tendency to change in monolayer culture with chondrocytes from normal and OA cartilage.[3] In a previous study, expression of collagen type II decreased in half of the patients with advanced OA cartilage while it increased or remained unchanged in the other half of patients.[9] It was reported that anabolic activity of chondrocytes was enhanced in initial stage of OA before chondroocytes in OA undergo phenotypic changes.[6,28] Others studies reported significant increase in gene expression of collagen type II and Aggrecan in chondrocytes from late stage osteoarthritic cartilage both *in vivo*[8] and *in vitro*[29] investigation.

As OA-affected cartilage shows obvious heterogeneity in degeneration, it can be envisaged that chondrocytes isolated from these heterogeneous tissue would have undergone differing phenotypical changes and not have equivalent gene expression. Taking this into consideration, degeneration stages of the OA tissue need to be carefully graded and chondrocytes be isolated in segregation in order to correlate expression of genes to degeneration severity. Previous studies on chondrocytes from varying degenerated cartilages and differential cartilage layers revealed that up-regulation of anabolic genes were limited to macroscopically intact areas (equivalent to Grade 0 and Grade I cartilage in the current study) but not at severely degenerated cartilage (equivalent to Grade II/III cartilage).[11] In our study, expression of collagen type II decreased with severity of degeneration while level of aggrecan was comparable lower in Grade II/III cartilage than in Grade 0 and Grade I cartilage. Compared with other matrix protein genes (collagen type I/II and aggrecan), gene expression of collagen type X was maintained at a low level in all grades of cartilage, which was in contrast to previous studies involving rabbit and mice models.[30,31] This might be due to these studies utilizing animal articular cartilage that had higher expression levels of collagen type X than the human articular cartilage used in our study. Inversely to the expression of hyaline cartilage marker (collagen type II), expression of fibroblastic marker, collagen type I, increased with severity of degeneration, indicating a lost of cartilage phenotype that was associated with OA degeneration. Fibroblast-like phenotypic changes of chondrocytes was reported to occur only in severely degenerated cartilage.[9,10] In our study, chondrocytes from severely degenerated cartilage showed similar morphology with those from mildly degenerated cartilage despite significant changes in gene expression of collagen type I, II and X.

Gene expression of MMP-13 increased with severity of cartilage degeneration. By comparison, expression of ADAMTS5 remained constant in varying grades of OA cartilage which was in agreement with a previous study.[10] The distinction may be related to the different stages of MMP-13 and ADAMTS5 occurrence and their different enzymatic role.[22] ADAMTS5 managed degradation of Aggrecan and is considered to take place earlier in OA progression and thus might have been highly expressed in early stages of OA and maintain the expression to later OA stages.

The "primary" chondrocytes used in the current study were actually cultured *in vitro* for 10 days before first

passage. It was assumed that they still keep their original phenotype and gene expression profiles to some extent. As predicted, the difference in morphology, cell spreading areas, and gene expression between varying degenerated cartilages was not apparent after 20–30 days of *in vitro* culture.[29] *In vitro* cultured chondrocytes have been broadly adopted to investigate chondrogenesis and pathogenesis of OA, as it is difficult to directly evaluate the functionality of chondrocytes *in vivo*[3] and to obtain enough chondrocytes for primary culture study. It was found that gene expression profiles, though different between primary chondrocytes from normal and OA cartilage, were minimized after *in vitro* dedifferentiation as chondrocytes undergone de-differentiation when grown in monolayer regardless of cartilage source. In the current study, different expression levels of collagen II and Aggrecan between primary chondrocytes of different grade was reduced drastically by passage 2 and was indistinguishable by passage 4. Comparatively, level of collagen I in Grade 0 chondrocytes was kept at relatively low level throughout the passage when compared to grade II/III chondrocytes, which maintained its high level of collagen II expression. These expression trend for collagen type II and I was in agreement with previous reports.[20,32]

Once chondrocytes were cultured *in vitro*, expression of MMP-13 decreased sharply.[2,10,29] These outcomes may be attributed to the highly elevated MMP in chondrocytes isolated from OA tissue due to the pathological progression and/or the loss of 3D extracellular matrix support in monolayer culture that induced transformation to a dedifferentiated phenotype that does not generate MMP.[3]

Chondrocytes from all groups proliferated steadily *in vitro*, and there was no significant difference between their proliferation rates. Although chondrocytes from severely degenerated cartilage initially proliferated faster than healthy cartilage (verified by a relatively shorter doubling time), this preliminary advantage could not be sustained since chondrocytes from relatively healthy cartilages gradually dedifferentiated into fibroblast-like cells with increasing cell spreading area with higher passage number regardless of cartilage grade, thereby accelerating the speed of proliferation. Chondrocytes experienced dedifferentiation characterized by a change in morphology and gene expression during *in vitro* 2D culture.[20,32] However, there was no established correlation between morphological appearance and type of collagen the cell produced.[20] After prolonged four-passage culture, proliferation and differentiation of chondrocytes from different grades of OA cartilage tended to be similar, suggesting that *in vitro* culture had a stronger influence, compared with OA pathogenesis, which coincides with findings from a previous study.[3,29]

With cartilage degradation, chondrocytes became condensed, while GAG content did not change significantly during cartilage degeneration. Expression of collagen type I increased significantly while expression of collagen type II and type X decreased significantly. With *in vitro* culture, expression of collagen type I increased substantially up to 30 days and then declined. Both expression of anabolic genes (Col II, X and Aggrecan) and catabolic genes (MMP-13) dropped with time, and difference in gene expression among cells obtained from cartilage of different degeneration grade diminished with prolonged expansion.

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