Arthritis & Rheumatism
WILEY

Identification of markers to characterize and sort human articular chondrocytes with enhanced in vitro chondrogenic capacity

scholarONE™
Manuscript Central

Identification of markers to characterize and sort human articular chondrocytes with enhanced in vitro chondrogenic capacity

Shawn Patrick Grogan (Ph.D)[1][§][†], Andrea Barbero (Ph.D)[2][§], Jose Diaz-Romero (Ph.D)[1], Anne-Marie Cleton-Jansen (Ph.D)[3], Stephan Soeder (M.D)[4], Robert Whiteside (Ph.D)[1], Pancras C.W. Hogendoorn (Ph.D)[3], Jian Farhadi (M.D.)[2], Thomas Aigner (M.D.)[4], Ivan Martin[2] (Ph.D), Pierre Mainil-Varlet (M.D.)[1].

1. Institute of Pathology, University of Bern, Murtenstrasse 31, 3010, Bern, CH.

2. Depts of Surgery and of Research, University Hospital Basel, Hebelstrasse 20, Basel, CH.

3. Dept of Pathology, Leiden University Medical Center, Albinusdreef 2, Leiden, NL.

4. Institute of Pathology, Liebigstrasse 26, 04103, Leipzig, FRG.

[§] Both authors contributed equally

[†]Present address of S.P.G is The Division of Arthritis Research, The Scripps Research Institute, 10555 N. Torrey Pines Road, MEM-161, La Jolla, California, 92037, USA.

Corresponding author: Ivan Martin, Institute for Surgical Research and Hospital Management, University Hospital Basel, Hebelstrasse 20, ZLF, Room 405, 4031 Basel, Switzerland. tel: + 41 61 265 2384; fax: + 41 61 265 3990; e-mail: imartin@uhbs.ch

Running headline: Markers of chondrogenic capacity

ABSTRACT

Objective. To identify markers associated with the chondrogenic capacity (CC) of expanded human articular chondrocytes and to use these markers for sorting of more chondrogenic subpopulations.

Methods. The CC of chondrocyte populations derived from different donors (n=21) or different clonal strains from the same cartilage biopsy (n=21) was defined based on the glycosaminoglycan (GAG) content of tissues generated using a pellet culture model. Selected cell populations were analysed by microarray and cytofluorimetry. In some experiments, cells were sorted using antibodies against molecules found to be associated with differential CC and again assessed in pellet cultures.

Results. Significance analysis of microarrays indicated that chondrocytes with low CC expressed greater levels of insulin-like growth factor-1 and of catabolic genes (e.g., metalloproteinase-2, aggrecanase-2), while chondrocytes with high CC expressed greater levels of genes involved in cell-cell or cell-matrix interactions (e.g., CD49c, CD49f). Cytofluorimetry analysis showed that CD44, CD151 and CD49c were expressed at significantly greater levels in chondrocytes with higher CC. Cytofluorimetrical analysis of clonal chondrocyte strains indicated that CD44 and CD151 can also identify more chondrogenic clones. Chondrocytes sorted for brighter CD49c or CD44 signal expression produced tissues with higher GAG/DNA (up to 1.4-fold) and collagen type II mRNA (up to 3.4-fold) than unsorted cells.

Conclusion. We identified markers enabling to characterize the capacity of monolayer expanded chondrocytes to form *in vitro* cartilaginous tissue and to enrich for subpopulations with higher CC. These markers might be used as a mean to predict and possibly improve the outcome of cell-based cartilage repair techniques.

Cellular therapy and tissue engineering are promising strategies for the repair of missing, degenerated or diseased tissues in the human body. In the past few decades, the search for innovative cell-based repair strategies has been particularly intense for the treatment of cartilage lesions, due to the large number of clinical cases, the limited inherent capacity of articular cartilage to heal, and the limitations of current treatment methods (1,2). Current cell-based strategies to induce cartilage repair include autologous chondrocyte implantation (3) and a more recent alternative movement towards prefabrication of cartilaginous implants (4-11). Both methods rely on the expansion of a limited population of chondrocytes derived from a small cartilage biopsy, intrinsically associated with cellular de-differentiation (12), and on the ability of the expanded cells to re-differentiate and generate cartilaginous tissue. However, the chondrogenic capacity (CC) of chondrocytes has been shown to be highly dependent on a wide variety of factors, including the biopsy site (13-15), the culture medium supplements (16,17), and the duration of expansion (18). Even using identical harvest and culture conditions, we previously demonstrated that human articular chondrocytes from different individuals display extreme variability in the in vitro CC; such variability is clearly age-related, but can be observed even among individuals within the same age range (19). Before cell-based cartilage regeneration can be included as a standard method in the routine tool kit of medical application and widely accepted by healthcare systems, it will be necessary to identify means of predicting and possibly overcoming biological variations in different chondrocyte preparations.

We recently established that clonal populations of human chondrocytes derived from the same biopsy also exhibit a large variability in the post-expansion re-differentiation capacity (20). This finding suggests that the identification of markers predictive of the cell CC could not only help identify the quality of expanded chondrocytes, but also allow enrichment of more chondrogenic cell subpopulations, to possibly improve the quality of

the generated tissues. However, markers to predict human chondrocyte CC are not yet available. Previous studies reported that a reduction of cartilage forming capacity upon extensive cell expansion is related to changes in the expression of a number of molecules, including a reduction in fibroblast growth factor receptor 3, bone morphogenic protein 2 and integrin alpha 3, accompanied by increased production of activin receptor-like kinase 1 (18,21). However, these studies were limited to comparison of the same primary cultures at different phases of expansion: thus, the expression of the candidate molecules was likely related to specific stages of chondrocyte differentiation, and might not capture inter- or intra-individual differences in cell CC.

In this study, we first aimed at identifying markers differentially expressed by human articular chondrocyte populations displaying high or low CC, using a combination of microarray- and cytofluorimetry-based strategies. We then tested whether cell sorting using some of the identified markers could enhance the CC of expanded chondrocyte populations. The CC of cell preparations, derived from different donors or different clonal strains, was assessed in vitro using a well established pellet culture model.

MATERIALS AND METHODS

Cell isolation and culture.

Human articular cartilage tissues were collected from 21 donors (age range: 40 to 60 years; Mean±SE: 50.0±2.7; male=15, female=6) *post mortem* under local ethical guidelines (Bernische Ethik-Kommission). Only tissue from joints showing no sign of degenerative changes was used. Isolation and expansion of cells from the cartilage biopsies were carried out as previously described (19). In brief, each biopsy was digested in 0.15% type II collagenase and the remaining cells were expanded for two passages (average of 8-9 doublings) in DMEM with 10% fetal bovine serum, further

supplemented with 1ng/ml Transforming Growth Factor-β1 (TGFβ1), 5ng/ml Fibroblast

Growth Factor-2 and 10ng/ml Platelet Derived Growth Factor-BB (expansion medium).

The expanded cells were then used for pellet cultures or cryopreserved for later

microarray and cytofluorimetry analyses, as detailed below.

Pellet culture, histology and biochemical analyses.

The chondrogenic capacity (CC) of post-expanded chondrocytes was investigated in

pellet cultures using a defined serum-free medium, as previously described (19). Briefly,

chondrocytes were suspended in DMEM supplemented with ITS^{+1} (Sigma Chemical, St.

Louis, MO), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml human serum albumin, 10^{-7}

M dexamethasone and 10 ng/mL TGFβ1. Aliquots of 5×10^5 cells/0.5 ml were

centrifuged at 250 g for 5 minutes in 1.5 ml polypropylene conical tubes (Sarstedt,

Nümbrecht, Germany) to form spherical pellets, which were placed onto a 3D orbital

shaker (Bioblock Scientific, Frenkendorf, Switzerland) at 30 rpm. Pellets were cultured

for 2 weeks, with medium changes twice per week, and subsequently processed for

histological, immunohistochemical, biochemical or mRNA analysis as described below.

Each analysis was performed independently in at least two entire pellets for each primary

culture and expansion condition.

For histology and immunohistochemistry, the pellets were fixed in 4% formalin,

embedded in paraffin, cross-sectioned (5 μm thick) and stained with Safranin O for

sulfated glycosaminoglycans (GAG) or processed for immunohistochemistry to visualize

collagen type II (II-II6B3, Hybridoma Bank, University of Iowa, USA), as previously

described (23).

For biochemistry, the pellets were digested in Proteinase K (0.5 ml of 1 mg/ml protease

K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin-A for

15 hours at 56°C) and then assessed for GAG content as described previously (24). The

DNA content was also measured in these samples by means of the CyQUANT Kit (Molecular Probes, Eugene, OR) using calf thymus DNA as a standard. GAG contents are reported as µg GAG per µg DNA. Each analysis was performed independently in at least two entire pellets for each primary culture and expansion condition.

Clonal study.

Cell cloning was performed by "limiting dilution" of cells from an additional cartilage biopsy from a 30 year old donor, as previously described (20). In brief, chondrocytes, freshly released enzymatically from the collected cartilage, were suspended at 5 cells/ml in expansion medium and 100 µl aliquots of the cell suspension were plated in 96 well plates. Cell populations arising from single cells were cultured till confluency and then passaged in 12 well plates. Upon reaching confluence, each clonal population was either cultured as pellet and processed histologically and biochemically, or cryopreserved for later surface marker analysis studies as detailed below.

Definition of chondrocyte populations with low and high chondrogenic capacity (CC).

The chondrogenic capacity (CC) of chondrocyte populations from different donors or from different clones was defined based on the distribution of the GAG/DNA contents of the resulting pellets. In particular, cells were considered as *low CC* or *high CC* if respectively in the lower 33% or the higher 33% of the distribution (see Table 1). GAG/DNA contents of pellets were previously shown to correlate with the mRNA expression and deposition of collagen type II (19) and with histological grading evaluation (22).

Microarray analysis.

Cell populations from 9 donors were used for microarray analysis (5 cells populations with high CC and 4 cells populations with low CC). Since cells from 2 donors were assessed twice as technical replicate, in total 11 unique array data sets were generated (n=6 for high CC cells and n=5 for low CC cells).

Total RNA from each cell population, expanded in monolayer for 2 passages, was isolated using the Qiagen RNeasy kit (Qiagen) in combination with DNase on column treatment (Qiagen). Total RNA (10µg) was prepared for hybridization on the Human Genome U133A 2.0 Array with 14,500 genes with >22,000 probe sets (Affymetrix, Inc. Santa Clara, CA, USA) according to standard protocols (technical manual, version 701021 Rev. 4; Affymetrix).

Microarray Data analysis.

Data were normalized using the GC-Robust Multi-Array Average (GC-RMA) (25), an algorithm provided by the Bioconductor project (www.bioconductor.org). Unsupervised hierarchical clustering was performed using the Spotfire[TM] decision site for functional genomics (www.spotfire.com). Significance analysis of microarrays (SAM) was preformed on two different subsets of the data, selected either with an average expression of at least 5-fold difference or with a variance of at least 0.025 (26). Significant differentially expressed genes are reported for high CC cells as fold change from low CC cells (positive = higher in high CC; negative = higher in low CC). The q-value (%) was reported as a measure of the proportion of false positives incurred (called the false discovery rate) when the statistical test was called significant.

Flow cytometric analysis and cell sorting.

Surface marker expression on chondrocytes was quantified using cryopreserved cells according to a previously described protocol (27). In summary, cells were thawed, washed in 10ml of PBS and centrifuged at 400g for 5 minutes. After centrifugation the

cell concentration was adjusted to 5×10^6 cells per ml in PBS/BSA 1%. Three-color immunofluorescence analysis for different surface markers was performed by simultaneous labeling with mAb-FITC, mAb-PE, and 7-AAD. Each tube contained 10^5 cells. All incubation steps were performed at room temperature for 15 minutes in the dark and all washing steps were completed by centrifugation (5 minutes, 400g). Cells were kept at 4°C until they were analysed using a FACScan flow cytometer (Becton-Dickinson). For each sample, a region for live cells (cells excluding 7-AAD) was defined, and at least 10,000 live chondrocytes were analyzed. Data were analyzed using FlowJo software (version 3.4, Tree Star Inc., San Carlos, CA). Non-specific staining was assessed using fluorochrome-, isotype- and species- matched immunoglobulins (isotype controls). The level of marker expression was calculated as the ratio between geometric mean fluorescence intensity (MFI) of sample cells and that of the isotype control.

Cell sorting of chondrocytes was performed on a FACS Vantage flow cytometer (Becton-Dickinson). After culturing for two passages, cells were enzymatically detached, washed in PBS and centrifuged at 400g for 5 minutes. Cells were resuspended at 8×10^6 cells in PBS-BSA 1% and transferred to 12 x 75 mm Falcon tubes (Becton-Dickinson). Chondrocytes were then labelled with mAb against CD49c (4 individuals) or CD44 (1 individual) and incubated for 15 minutes at room temperature in the dark. Cells were then washed in 5ml of PBS-BSA 1% and centrifuged at 400g for 5 minutes. Pellets were finally resuspended in 2 ml of PBS-BSA 1% and the resulting cell suspension was passed through the cell sorter. Chondrocytes were collected without sorting (unsorted cells), or following sorting on the basis of the intensity of the labelling, using the mean fluorescence intensity as arbitrary threshold. Chondrocytes from the different populations ($2-3 \times 10^6$) were then cultured in pellets as described above. Antibodies used for cytofluorimetry and cell sorting are listed in Table 2.

Statistical analysis.

All values are reported as mean ± standard error of the mean. ANOVA was used to test whether differences existed in the levels of marker expression between the different CC groups (high and low). Post-hoc t-tests were used to identify significant relationships using the Holm's step-down procedure to control the family-wise error rate.

RESULTS

Gene expression profiling of chondrocyte populations with high and low CC.

Human articular chondrocyte populations with low CC or high CC (Table 1) were assessed via microarray to identify differentially expressed gene sets. Unsupervised hierarchical cluster analysis showed distinct separation of low CC and high CC cells (Figure 1a). Significance analysis of microarrays (SAM) revealed a number of differentially expressed genes (Figure 1b). Low CC was associated with an upregulation of extracellular matrix (ECM) catabolic enzymes, including metalloproteinase 2 (MMP2), and ADAMTS-5 (aggrecanase 2) (Figure 1b), and with a higher expression of insulin like growth factor 1 (IGF-1) and of bone morphogenetic protein type IA receptor (BMPRIA). High CC cells expressed higher levels of the chondrogenic factor TGFβ-2, of several surface molecules, including chondroitin sulfate proteoglycan 4, syndecan 2 and the integrin subunits alpha 3 (CD49c) and alpha 6 (CD49f), and of a number of intracellular proteins involved in the integrin signal transduction and cytoskeleton organization, such as actinin alpha 1, nebulette and embryonal Fyn-associated substrate (Figure 1b).

Relation between chondrogenic capacity and surface marker expression: cells from different donors

Microarray data suggested that the expression of surface molecules could discriminate between chondrocyte preparations with low CC or high CC. Expanded chondrocytes were thus assessed by cytofluorimetry using antibodies against several clusters of differentiation (CD), typically used to characterize the phenotype of mesenchymal progenitor cells (28-30), and recently introduced to determine the stage of differentiation of human articular chondrocytes (27). For these experiments, we used cells with low CC from 6 donors (GAG/DNA of resulting pellets: 3.5±0.1 μg/μg) and cells with high CC from 5 donors (GAG/DNA of resulting pellets: 8.9±0.3 μg/μg). Representative Safranin O stained sections of pellets are shown in Figure 2a.

Multiple comparison analysis indicated that cells with greater CC expressed higher levels of a number of surface molecules (Figure 2a; Table 3), all known to play a key role in mesenchymal condensations (31). These included: (i) integrin alpha 3 (CD49c), consistent with the microarray data; (ii) the hyaluronan receptor CD44; and (iii) the tetraspanin CD151.

Relation between chondrogenic capacity and surface marker expression: cells from different clonal populations of the same biopsy

In order to assess whether the same set of markers can identify more chondrogenic subpopulations within a heterogeneous primary culture, we performed cytofluorimetric analysis and chondrogenesis assays of different clonal populations derived from the same cartilage biopsy.

Starting from one cartilage biopsy, we expanded 21 clonal chondrocyte populations, which were found to generate pellets of highly variable GAG contents (ranging 0.8 - 7.2 μg/μg; Table 1). For these experiments we used 6 clones having low CC (GAG content of resulting pellets: 2.0±0.2 μg/μg) and 3 clones having high CC (GAG content of

resulting pellets: 6.6±0.3 µg/µg). Representative Safranin O stained sections of pellets are displayed in Figure 2b.

The molecules CD44, CD90, CD151, CD49c, CD49e, CD49f and CD166 were all expressed at greater levels by high CC clones, although due to large variability in the intensity levels among different clones, statistical significance was established only for the first 3 (Figure 2b; Table 3).

Sorting chondrocyte populations based on the expression level of surface molecules: enrichment of chondrogenic capacity

The clonal analysis strongly suggested that the expression levels of specific surface molecules may identify the CC of chondrocyte subpopulations expanded from the same biopsy. Thus, we tested whether cell sorting using antibodies against integrin alpha 3 (CD49c) and hyaluronan receptor (CD44) could enhance the CC of expanded chondrocyte populations. These molecules were selected based on their broad fluorescence signal profiles (see Figure 3a). Chondrocytes expressing these molecules at intensities *greater than the mean fluorescence intensity* (MFI) produced cartilage tissues containing higher amounts of type II collagen and GAG as compared to the unsorted chondrocyte populations (Figure 3c,d). Conversely, chondrocytes expressing CD49c or CD44 at levels *lower than the MFI* formed tissues with no detectable type II collagen and lower GAG content than the unsorted cells. Expression levels of type II collagen mRNA were consistent with the protein accumulation (Figure 3d). Interestingly, as compared to the unsorted chondrocyte populations, those with brighter CD49c signal showed a reduced inter-individual variability in CC, such that the coefficient of variation in the GAG content of pellets from the different donors (i.e., the standard deviation expressed as a percentage of the mean) decreased from 31.5% to 18.0% following cell sorting.

DISCUSSION

In this study, we compared chondrocyte preparations from different donors or from different clonal strains, displaying low or high chondrogenic capacities (CC) in vitro. Using microarray and cytofluorimetry tools, we identified that cells with higher CC express lower levels of catabolic mediators and higher levels of surface molecules involved in early stages of cartilage development, including those involved in establishing cell-cell and cell-matrix interactions (e.g., the hyaluronan receptor CD44; the alpha 3 integrin subunit CD49c and the tetraspanin CD151). Finally, we verified that the CC of chondrocyte populations can be enhanced by sorting cells expressing higher levels of CD49c and CD44.

Microarray analyses indicated that low CC chondrocyte populations expressed greater levels of catabolic mediators (i.e., matrix metalloproteinase 2 and aggrecanase 2), that would reduce the accumulation and retention of cartilage specific ECM in the reparative tissue. Low CC cells also upregulated the anabolic factor IGF1, possibly as an attempt to counteract the catabolic pathway and consistent with the characteristic concomitant activation of anabolic and catabolic pathways by chondrocytes in diseased joints (32). Another gene that was more expressed by low CC chondrocytes was BMPRIA, also known as activin receptor-like kinase (ALK)-3, a member of the TGFβ/BMP receptor type I. Interestingly, increased mRNA expression levels of ALK-1, another member of the same family, were previously shown to correlate with a reduced capacity of expanded chondrocyte to generate stable cartilage *in vivo* (18).

High CC cells expressed higher levels of genes involved in the establishment of cell-matrix interactions and in the transduction of integrin-mediated signals (i.e., actinin alpha 1, CD49c and CD49f). Since these processes are known to be important mediators of mesenchymal condensation, which is in turn required to initiate chondrogenesis (33-

35), it is possible that high expression levels of these genes would increase the propensity of the cells to differentiate and produce cartilage ECM.

Cytofluorimetrical analysis of monolayer expanded chondrocyte populations revealed that CD49c, CD151, CD44 were expressed at greater intensities in chondrocyte populations with high CC, in general agreement with the microarray results. Tetraspanins, such as CD151, are membrane proteins that bind to integrins and other proteins to generate functional complexes involved in cell-cell and cell-ECM interactions (36,37). CD151 has recently been shown to be part of a multimolecular complex consisting of integrin $\alpha 3\beta 1$ and E-cadherin in epithelial cells, and to regulate cell-cell adhesion (38). The major receptor for hyaluronan, CD44, is highly expressed during mesenchymal cell condensation and plays an important role in chondrogenesis (39). The increased expression of CD44 in high CC is consistent with a recent study reporting upregulation of CD44 on human chondrocytes expanded on collagen type II coated dishes, which exhibit a superior capacity to generate cartilaginous tissues (40).

Cytofluorimetrical analysis of clonal chondrocyte strains derived from a single cartilage biopsy indicated that CD44 and CD151 can also identify more chondrogenic clones within a heterogeneous primary culture. Markers characteristic of mesenchymal progenitor cells, i.e. CD90 and CD166 (29,41,42), were as well more expressed in high CC clones (although statistical significance was reached only for the former). This suggests that within a chondrocyte preparation, subpopulations with higher capacity to form cartilage might correspond to those with progenitor characteristics. Indeed, we previously demonstrated that some single colony-derived strains of expanded human chondrocytes display a multilineage differentiation potential (20), while other researchers have recently shown that different combinations of surface markers including CD90 and CD166 can be used to define sub-populations of chondrocytes with progenitor

features (43,44). In this context, it would be tempting to speculate that high CC clones reside in the surface layer of articular cartilage, which was postulated to contain a progenitor cell population (45).

Chondrocytes expanded from different donors were then sorted for the expression level of CD49c and CD44. The superior quality of the cartilaginous tissues generated by chondrocytes with brighter CD49c or CD44 signal as compared to unsorted cells suggests that the low CC of certain chondrocyte preparations is due to contaminating populations (i.e., cells with low CD49c and/or CD44 signal). In this regard, it is possible that the threshold used for cell sorting, so far set at the mean fluorescence intensity, may need to be optimized to reach an efficient compromise between purity and quantity of the separated cells. Although the number of sorting experiments was relatively low, the observed reduced variability in CC by chondrocytes from different donors following sorting indicates the potential of the technique to achieve higher levels of standardization in cartilage tissue formation. It remains to be verified whether cell populations expressing high levels of CD49c coincide or not with those expressing high levels of CD44 and whether the same markers could be used to identify high CC chondrocytes immediately after isolation from the native cartilage.

We are aware that the model used in the present study to characterize the CC of the cells (i.e., *in vitro* pellet culture), cannot directly predict the tissue-forming capacity of the cells when implanted in the joints of patients. On the other hand, studies using large animal models may not be helpful to validate our results, considering that human chondrocytes are known to be biologically different from those derived from other species. Ectopic implantation in nude mice might represent an alternative model to assess the cartilage forming capacity of human chondrocyte populations within an in vivo site (17,18). Although it does not mimic the complex biological and physical environment of

a load-bearing joint, the model would allow to investigate the intrinsic capacity of the human cells to form stable cartilage tissues, in a way that is not dependent upon TGF beta stimulation. Ultimately, however, our study prompts for the monitoring of the levels of expression of specific surface molecules in autologous chondrocyte populations, in parallel to their clinical use for cartilage repair. Establishing a correlation between the expression of such markers and clinical outcome could lead to the use of these molecules as a quality control to predict the result of autologous cell-based cartilage repair techniques and to possibly improve it by sorting specific cell subpopulations. This approach would exemplify how the combination of a diagnostic tool with the definition of a tailored therapy (i.e., the field recently called "theranostic") could be extended from drug treatment to cell-based regenerative medicine.

ACKNOWLEDGMENTS

REFERENCES

(1) Kuo CK, Li WJ, Mauck RL, Tuan RS. Cartilage tissue engineering: its potential and uses. Curr Opin Rheumatol 2006;18:64-73.

(2) Tuli R, Li WJ, Tuan RS. Current state of cartilage tissue engineering. Arthritis Res Ther 2003;5:235-8.

(3) Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889-95.

(4) Diduch DR, Jordan LC, Mierisch CM, Balian G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy 2000;16:571-7.

(5) Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. Chondrogenesis in a cell-polymer-bioreactor system. Exp Cell Res 1998;240:58-65.

(6) Freed LE, Martin I, Vunjak-Novakovic G. Frontiers in tissue engineering. In vitro modulation of chondrogenesis. Clin Orthop Relat Res 1999;(367 Suppl):S46-S58.

(7) Mainil-Varlet P, Rieser F, Grogan S, Mueller W, Saager C, Jakob RP. Articular cartilage repair using a tissue-engineered cartilage-like implant: an animal study. Osteoarthritis Cartilage 2001;9(Suppl A):S6-15.

(8) Marijnissen WJ, van Osch GJ, Aigner J, Verwoerd-Verhoef HL, Verhaar JA. Tissue-engineered cartilage using serially passaged articular chondrocytes. Chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM). Biomaterials 2000;21:571-80.

(9) Nehrer S, Breinan HA, Ramappa A, Shortkroff S, Young G, Minas T et al. Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro. J Biomed Mater Res 1997;38:95-104.

(10) Niederauer GG, Slivka MA, Leatherbury NC, Korvick DL, Harroff HH, Ehler WC et al. Evaluation of multiphase implants for repair of focal osteochondral defects in goats. Biomaterials 2000;21:2561-74.

(11) Ochi M, Adachi N, Nobuto H, Yanada S, Ito Y, Agung M. Articular cartilage repair using tissue engineering technique--novel approach with minimally invasive procedure. Artif Organs 2004;28:28-32.

(12) Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215-24.

(13) Archer CW, McDowell J, Bayliss MT, Stephens MD, Bentley G. Phenotypic modulation in sub-populations of human articular chondrocytes in vitro. J Cell Sci 1990;97:361-71.

(14) Klein TJ, Schumacher BL, Schmidt TA, Li KW, Voegtline MS, Masuda K et al. Tissue engineering of stratified articular cartilage from chondrocyte subpopulations. Osteoarthritis Cartilage 2003;11:595-602.

(15) Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. J Orthop Res 2003;21:132-8.

(16) Jakob M, Demarteau O, Schafer D, Hintermann B, Dick W, Heberer M et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. J Cell Biochem 2001;81:368-77.

(17) Moretti M, Wendt D, Dickinson SC, Sims TJ, Hollander AP, Kelly DJ et al. Effects of in vitro preculture on in vivo development of human engineered cartilage in an ectopic model. Tissue Eng 2005;11:1421-8.

(18) Dell'Accio F, De Bari C, Luyten FP. Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. Arthritis Rheum 2001;44:1608-19.

(19) Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. Osteoarthritis Cartilage 2004;12:476-84.

(20) Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 2003;48:1315-25.

(21) Schulze-Tanzil G, Mobasheri A, de SP, John T, Shakibaei M. Loss of chondrogenic potential in dedifferentiated chondrocytes correlates with deficient Shc-Erk interaction and apoptosis. Osteoarthritis Cartilage 2004;12:448-58.

(22) Grogan SP, Barbero A, Winkelmann V, Rieser F, Fitzsimmons JS, O'Driscoll S, Martin I, Mainil-Varlet P. Visual histological grading system for the evaluation of in vitro-generated neocartilage. Tisssue Eng 2006;12:2141-9.

(23) Grogan SP, Rieser F, Winkelmann V, Berardi S, Mainil-Varlet P. A static, closed and scaffold-free bioreactor system that permits chondrogenesis in vitro. Osteoarthritis Cartilage 2003;11:403-11.

(24) Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173-7.

(25) Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249-64.

(26) McAlinden A, Haag J, Bau B, Gebhard PM, Aigner T. Quantification of mRNA expression levels in articular chondrocytes with PCR technologies. Methods Mol Med 2004;100:79-100.

(27) Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol 2005;202:731-42.

(28) Boiret N, Rapatel C, Veyrat-Masson R, Guillouard L, Guerin JJ, Pigeon P et al. Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. Exp Hematol 2005;33:219-25.

(29) Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM et al. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 2002;46:3349-60.

(30) Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.

(31) Delise AM, Tuan RS. Analysis of N-cadherin function in limb mesenchymal chondrogenesis in vitro. Dev Dyn 2002;225:195-204.

(32) Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin Orthop Relat Res 2004;(427 Suppl):S27-S36.

(33) Delise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage 2000;8:309-34.

(34) Goldring MB, Tsuchimochi K, Ijiri K. The control of chondrogenesis. J Cell Biochem 2006;97:33-44.

(35) Tavella S, Bellese G, Castagnola P, Martin I, Piccini D, Doliana R et al. Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. J Cell Sci 1997;110:2261-70.

(36) Sterk LM, Geuijen CA, van den Berg JG, Claessen N, Weening JJ, Sonnenberg A. Association of the tetraspanin CD151 with the laminin-binding integrins alpha3beta1, alpha6beta1, alpha6beta4 and alpha7beta1 in cells in culture and in vivo. J Cell Sci 2002;115:1161-73.

(37) Yanez-Mo M, Tejedor R, Rousselle P, Madrid F. Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins. J Cell Sci 2001;114:577-87.

(38) Chattopadhyay N, Wang Z, Ashman LK, Brady-Kalnay SM, Kreidberg JA. alpha3beta1 integrin-CD151, a component of the cadherin-catenin complex, regulates PTPmu expression and cell-cell adhesion. J Cell Biol 2003;163:1351-62.

(39) Rousche KT, Knudson CB. Temporal expression of CD44 during embryonic chick limb development and modulation of its expression with retinoic acid. Matrix Biol 2002;21:53-62.

(40) Barbero A, Grogan SP, Mainil-Varlet P, Martin I. Expansion on specific substrates regulates the phenotype and differentiation capacity of human articular chondrocytes. J Cell Biochem 2006;98:1140-1149.

(41) Mareschi K, Ferrero I, Rustichelli D, Aschero S, Gammaitoni L, Aglietta M et al. Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 2006;97:744-54.

(42) Wang JF, Wang LJ, Wu YF, Xiang Y, Xie CG, Jia BB et al. Mesenchymal stem/progenitor cells in human umbilical cord blood as support for ex vivo expansion of CD34(+) hematopoietic stem cells and for chondrogenic differentiation. Haematologica 2004;89:837-44.

(43) Alsalameh S, Amin R, Gemba T, Lotz M. Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 2004;50:1522-32.

(44) Fickert S, Fiedler J, Brenner RE. Identification of subpopulations with characteristics of mesenchymal progenitor cells from human osteoarthritic cartilage using triple staining for cell surface markers. Arthritis Res Ther 2004;6:R422-R432.

(45) Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ et al. The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004;117:889-97.

Table 1. Glycosaminoglycans to DNA (GAG/DNA) content of donor and clonal cell populations screened in this study.

	Donor #	GAG/DNA	Clone #	GAG/DNA
	1	2.2	1	0.8
low CC 33%	2	3.1	2	1.3
	3	3.2	3	1.4
	4	3.3	4	1.5
	5	3.7	5	2.0
	6	3.8	6	2.2
	7	3.8	7	2.4
	8	4.5	8	2.5
	9	4.7	9	2.5
middle CC 33%	10	5.5	10	3.6
	11	6.0	11	3.9
	12	6.2	12	4.0
	13	6.5	13	4.1
	14	7.2	14	4.5
	15	8.1	15	4.6
	16	8.2	16	4.9
high CC 33%	17	8.9	17	5.7
	18	9.2	18	6.3
	19	9.3	19	6.3
	20	9.5	20	6.4
	21	11.2	21	7.2

Cell populations highlighted in grey were used in the array and/or FACS analysis as representative of low or high Chondrogenic Capacity (CC).

Table 2. Specification of antibodies presented in this study.

Antigen recognized	Isotype	Cat. # / Flurochrome	source
CD44 (hyaluronan receptor)	mIgG1	852.601.010 / FITC	Diaclone
CD49c (α3 integrin chain)	mIgG1	36615X / PE	Pharmigen
CD49e (α5 integrin chain)	mIgG2b	MCA698 / PE	Serotec
CD49f (α6 integrin chain)	mIgG2b	MCA956 / PE	Serotec
CD90 (Thy-1)	mIgG1	MCA90 / FITC	Serotec
CD151 (tetraspanin)	mIgG1	556057 / PE	Pharmigen
CD166 (ALCAM)	mIgG1	37615X / PE	Pharmigen

Table 3. Expression of surface markers in chondrocyte populations.

Surface marker	Donor-related populations (high CC/low CC)	Clonal-related populations (high CC/low CC)
CD44 (hyaluronan receptor)	1.5*	2.7*
CD49c (integrin alpha 3)	2.7*	2.4
CD49e (integrin alpha 5)	1.4	3.5
CD49f (integrin alpha 6)	2.1	2.2
CD90 (Thy-1)	1.2	1.6*
CD151 (tetraspanin)	1.4*	4.2*
CD166 (ALCAM)	1.5	3.1

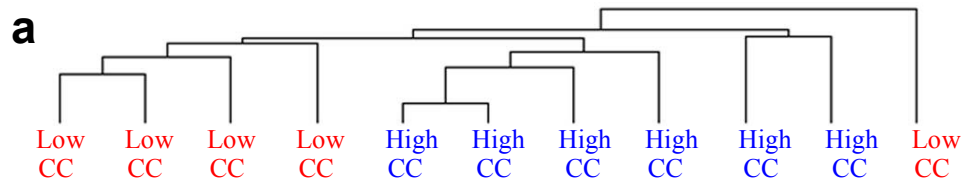* P <0.05 from low chondrogenic capacity (CC)

FIGURE LEGENDS

Figure 1. Gene expression analysis of low and high chondrogenic capacity (CC) cells. Unsupervised clustering dendrogram (**a**) has two major branches, indicating that high and low CC groups possess distinct and unique gene expression profiles. Significance Analysis of Microarrays (SAM) uncovered 229 genes differentially expressed, with a false discovery rate of 5.63% (Delta value of 1.14). The genes differentially expressed with statistical significance (**b**) are reported for high CC cells as fold change from low CC cells (positive = higher in high CC; negative = higher in low CC). Low CC cells display higher expression levels for insulin-like growth factor-I and various catabolic genes, while high CC cells have higher levels of expression of extracellular matrix, cytoskeleton and surface molecule genes that are known to be involved in early chondrogenesis. q-values (%) are a measure of false discovery rate.

Figure 2. Chondrogenic capacity (CC) and surface marker expression of cells from different donors or clonal populations.

Cells populations from different donors (**a**) and clones (**b**) were classified into low CC or high CC according to the glycosaminoglycan content of the generated tissues (see Methods and Table 1; representative Safranin-O stained histologies are presented) and characterized for the levels of expression of specific surface molecules. Overall, almost all of the surface molecules examined were expressed at higher levels in high CC cells derived from different donors or different clones, as compared to the low CC counterparts. A similar pattern of differences was observed using cells from different donors or from different clones. Values are reported as mean ± standard error of the mean. * = statistically significant difference between low and high CC cells.

Figure 3. Enhanced cartilage quality following cell sorting. (**a**) Typical expression profile of CD49c (integrin alpha 3 subunit) in cells with high and low chondrogenic capacity (CC) from different donors. CD44 (hyaluronan receptor) expression profile is similar to that of CD49c. To demonstrate the utility of employing such surface molecules to select high CC chondrocytes from mixed populations, cells were incubated with antibodies against CD49c or CD44 and sorted on the basis of the fluorescence intensity (low and high CD if respectively lower or higher than the mean value). (**b**) The cell population with brighter CD49c or CD44 signal produced tissues with greater GAG content and higher collagen type II at both the protein and gene expression level as compared to unsorted cells (**c-d**). Values are reported as mean ± standard error of the mean. * = statistically significant difference from unsorted chondrocytes.

a

b

DIFFERENTIALLY EXPRESSED GENES IN HIGH vs LOW CHONDROGENIC CAPACITY CELLS				
Accession number	**Gene Symbol**	**Gene Title**	**Fold Change**	**q-value (%)**
NM_000210	ITGA6	integrin, alpha 6 (CD49f)	4.76	5.5
NM_013230	CD24	CD24 antigen	4.76	5.1
NM_030820	COL21A1	collagen, type XXI, alpha 1	4.35	5.5
NM_005501	ITGA3	integrin, alpha 3 (CD49c)	3.23	5.5
NM_003238	TGFB2	transforming growth factor, beta 2	3.13	4.6
NM_005978	S100A2	S100 calcium binding protein A2	3.13	5.5
NM_001897	CSPG4	chondroitin sulfate proteoglycan 4	2.33	3.2
NM_213569	NEBL	nebulette	2.13	4.6
NM_002998	SDC2	syndecan 2 (heparan sulfate proteoglycan 1)	1.96	2.7
NM_002081	GPC1	glypican 1	1.85	5.5
NM_005022	PFN1	Profiling 1	1.82	4.6
NM_006888	CALM1	calmodulin 1 (phosphorylase kinase, delta)	1.67	5.1
NM_001024212	S100A13	S100 calcium binding protein A13	1.59	0.0
AA909121	ACTN1	actinin, alpha 1	1.45	0.0
NM_014782	ARMCX2	armadillo repeat containing, X-linked 2	1.43	4.6
NM_005864	EFS	embryonal Fyn-associated substrate	1.35	5.6
NM_014923	FNDC3A	fibronectin type III domain containing 3	-1.40	5.4
NM_004329	BMPR1A	bone morphogenetic protein receptor, type IA	-1.42	1.9
NM_002414	CD99	CD99 antigen	-1.53	5.1
NM_014350	TNFAIP8	tumor necrosis factor, alpha-induced protein 8	-1.56	3.8
NM_004530	MMP2	matrix metalloproteinase 2 (MMP2)	-1.64	0.0
NM_007038	ADAMTS5	aggrecanase-2	-1.67	5.4
NM_000618	IGF1	insulin-like growth factor 1 (somatomedin C)	-10.15	2.8

Figure 1

a **Donor comparison**

b **Clonal comparison**

Figure 2

Figure 3