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ARE ANKLE CHONDROCYTES FROM DAMAGED FRAGMENTS A SUITABLE CELL SOURCE FOR CARTILAGE REPAIR?

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ABSTRACT

Objective: Characterize the post-expansion cartilage-forming capacity of chondrocytes harvested from detached fragments of osteochondral lesions of ankle joints (Damaged Ankle Cartilage Fragments, DACF), with normal ankle cartilage (NAC) as control.

Design: DACF were obtained from 6 patients (mean age: 35years) with symptomatic osteochondral lesions of the talus, while NAC were from 10 autopsies (mean age: 55years). Isolated chondrocytes were expanded for two passages and then cultured in pellets for 14 days or onto HYAFF®-11 meshes (FAB, Italy) for up to 28 days. Resulting tissues were assessed histologically, biochemically (glycosaminoglycan -GAG-, DNA and type II collagen -CII-) and biomechanically.

Results: As compared to NAC, DACF contained significantly lower amounts of DNA (3.0-fold), GAG (5.3-fold) and CII (1.5-fold) and higher amounts of type-I collagen (6.2-fold). Following 14 days of culture in pellets, DACF-chondrocytes generated tissues less intensely stained for Safranin-O and CII, with significantly lower GAG contents (2.8-fold). After 28 days of culture onto HYAFF®-11, tissues generated by DACF chondrocytes were less intensely stained for Safranin-O and CII, contained significantly lower amounts of GAG (1.9-fold) and CII (1.4-fold) and had lower equilibrium (1.7-fold) and dynamic pulsatile modulus (3.3-fold) than NAC chondrocytes.

Conclusion: We demonstrated that DACF-chondrocytes have inferior cartilage forming capacity as compared to NAC-chondrocytes, possibly resulting from environmental changes associated with trauma/disease. The study opens some reservations on the use of DACF-derived cells for the repair of ankle cartilage defects, especially in the context of tissue engineering-based approaches.

Keywords: chondrogenic differentiation, osteochondral fragments, talar chondrocytes, autologous chondrocyte implantation

INTRODUCTION

For chondral and osteochondral lesions (OCL) of the talus (ankle joint), different cell-based therapies such as the autologous chondrocyte transplantation (ACT) and matrix associated ACT are established operative procedures[1-3]. For these procedures, chondrocytes are commonly harvested from the healthy and not affected knee joint[3-5]. However, a cartilage biopsy in a joint, even if harvested from a non-load bearing site, represents an additional injury to the cartilage surface, and has been reported to be detrimental to the surrounding healthy articular cartilage[6].

Alternatively, cartilage biopsies could be harvested from healthy areas of the osteochondral defected ankle joint[7]. Indeed, promising clinical results were reported when autologous chondrocytes derived from cartilage cylinders taken from the anterior part of talus were used to restore of full-thickness cartilage defects of such joint[4]. Moreover, our recent finding that chondrocytes isolated from ankle cartilage exhibit similar proliferation ability and post-expansion tissue-forming capacity than chondrocytes isolated from knee cartilage[8] suggest that talar chondrocytes may represent a suitable cell source for the repair of cartilage lesions of the talus. The harvest of talar chondrocytes from healthy areas of defected ankle joints, however, could be detrimental to the function of this already injured joint[9].

To overcome the aforementioned possible problems caused by the harvesting of cartilage biopsies from healthy knee or healthy areas of the already affected ankle joint, a rational and convenient approach would be represented by the utilization of chondrocytes isolated from already detached OCL fragments which are debrided during surgery. In this regard, it was reported that viable cells can be isolated from detached osteochondral fragments from human articular joints[10-11]. Moreover, the implantation of such chondrocytes in patients was reported to yield good clinical results 12 months post-operatively[11]. However, the chondrogenic capacity of cells isolated from detached OCL fragments remains controversial. While Giannini et al (2005)[11] reported that these cells have the potential to re-express or synthesise

to a certain extent cartilage specific genes and proteins during culture in three-dimensional porous scaffolds, Kuroki et al (2002)[12], showed that constructs generated by chondrocytes from naturally occurring OCL of the humeral head of *dogs* and cultured in agarose gel formed tissues containing inferior amount of glycosaminoglycan, type II collagen and hydroxyproline and superior amounts of type I collagen as compared to those generated by chondrocytes from healthy cartilage. Similarly, Garvican et al (2008)[13] found that chondrogenic differentiation of *equine* chondrocytes from DACF was inferior to that of aged-mached normal chondrocytes.

The goal of this study was thus to assess the quality of cartilaginous tissues generated by human chondrocytes harvested from the detached fragments of OCL of ankle joints (Damaged Ankle Cartilage Fragments, DACF), using chondrocytes derived from normal ankle cartilage (NAC) as control cells. Cells were cultured in micromass pellets or in scaffolds in clinical use for the delivery of human chondrocytes (HYAFF®-11), and the resulting tissues assessed phenotypically biochemically and biomechanically.

MATERIALS AND METHODS

Cartilage biopsies

Damaged ankle cartilage fragments (DACF) were harvested from the talus of 6 patients (female:male = 3:3, mean age: 36, age range: 30-43 years) scheduled for surgery of symptomatic talar osteochondral lesions of this joint (Table 1), following informed consent. Lesions, localized in the middle third of the medial talus edge or at the lateral side, were from traumatic origin and not associated with diagnosed instability of the ankle joint. The mean time elapsed between the traumatic event and surgery was 6.5 months, which is typically sufficient to allow for the onset of early degenerative changes in the joints (e.g., cartilage fibrillation in the border of the lesion). The osteochondral lesions, graded according to the widely used Berndt & Harty classification (Grade I – V; grade I: subchondral compression; grade II: partially avulsed fragment; grade III: detached but not displaced fragment; grade IV: displaced fragments; grade V: subchondral cyst)[14] were of grade III (5/6) or grade II (1/6) (Table 1). During surgery the OCL fragments were found as talar in situ dissecates, carefully harvested, and immediately sent from the operation room to the laboratory for further analyses.

As control, normal ankle cartilage (NAC) biopsies were harvested from the healthy areas of the talus of 10 cadavers (female:male = 4:6, mean age 55, range 32-79 years) in accordance with the Local Ethical Committee[8]. The chondral layers of DACF and NAC biopsies were cleaned meticulously from fibrous tissue and bone, and rinsed several times in a sterile saline solution.

DACF and NAC biopsies were chopped in small pieces which were processed in parallel for histology and immunohistochemistry (about 50 mg), for biochemistry (about 50 mg), or for chondrocyte isolation (about 100 mg) as described below.

Chondrocytes isolation and expansion

Samples derived from DACF and NAC were digested with 5 ml of type II collagenase (0.15% in basic medium – see below – supplemented with 5% foetal bovine serum – FBS –) for 22 hours[8]. The isolated chondrocytes were counted using trypan blue, plated in tissue culture flasks at a density of 10,000 cells/cm^2 and cultured for two passages in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml D-Glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.29 mg/ml L-glutamine (basic medium) supplemented with 10% FBS, 1 ng/ml of Transforming Growth Factor-β1 (TGF-β1) and 5 ng/ml of Fibroblast Growth Factor-2 in a humidified 37°C/5% CO2 incubator with medium changes twice a week, as previously described[8]. Expanded DACF- and NAC-chondrocytes were harvested for mRNA analysis or subsequently cultivated in pellets or in 3D scaffolds, according to previously established methods and as briefly described below.

Chondrocyte cultivation in pellets

The chondrogenic capacity of expanded DACF- and NAC-chondrocytes was investigated using a simple and broadly used model, namely pellet cultures in a defined serum-free medium[14]. Briefly, cells were suspended in basic medium supplemented with ITS^{+1} (Sigma Chemical, St. Louis, MO; i.e., 10 µg/ml insulin, 5.5 mg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml bovine serum albumin, 4.7 mg/ml linoleic acid), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml human serum albumin, 10^{-7} M dexamethasone and 10 ng/ml TGF-β1 (chondrogenic medium, CHM). 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 and immunohistochemical or biochemical analysis as described below. Each analysis was performed independently in at least two entire pellets for each primary culture.

Chondrocyte cultivation in 3D scaffolds

The ability of expanded DACF- and NAC-chondrocytes to generate neo-cartilage was also investigated by cultures in esterified hyaluronic acid non-woven meshes (HYAFF®-11, Fidia Advanced Biopolymers, Abano Terme, IT), currently in clinical use for cartilage repair[15] since such model allows more extensive cartilaginous tissue maturation than in pellets over prolonged culture times[16]. Chondrocytes were loaded statically on the scaffolds (6 mm diameter, 2 mm thick disks) at a density of 4×10^6 cells/scaffold. Cell-scaffold constructs were cultured in basic medium supplemented with 0.1 mM ascorbic acid, 10 μg/ml Insulin and 10 ng/ml Transforming Growth Factor-β3, with medium changes twice a week, as previously described[8]. After 14 or 28 days of static culture, the resulting tissues were analysed histologically, immunohistochemically, biochemically and biomechanically, as described below.

Analytical Methods

Proliferation rate

Cell proliferation rate was calculated as the ratio of T to $\log_2(N/N_0)$, where N_0 and N are the numbers of cells respectively at the beginning and the end of the expansion phase, and T is the time required for the expansion[17].

Histological and immunohistochemical analyses

DACF and NAC tissues and engineered tissues (pellets and constructs) were rinsed with PBS, fixed in 4% formalin, embedded in paraffin, and cross-sectioned (5 µm thick for pellets and 7 µm thick for constructs and native tissues). Sections were stained with Safranin-O for sulfated glycosaminoglycans (GAG), with or without additional staining with 5% Silver nitrate solution (von Kossa staining for mineralized matrix detection), or processed for immuno-histochemistry using antibodies against type I collagen (Quartett, Berlin, Germany) and type

II collagens (II-II6B3, Hybridoma Bank, University of Iowa, USA) as previously described[18-19].

Biochemical Analysis

DACF, NAC and engineered constructs were cut in different pieces, part of them were lyophilized the remaining parts were blotted dry. Specimens were first weighed and subsequently digested for 15 hours at 56°C with protease K (1.0 ml of 1 mg/ml protease K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide and 10 µg/ml pepstatin-A). Pellets were digested with the same protocol but using 0.5 ml protease K. GAG amounts were measured spectrophotometrically after reaction with dimethylmethylene blue[20], with chondroitin sulfate as a standard[21]. The DNA amount was measured spectrofluorometrically using the CyQUANT® Kit (Molecular Probes, Eugene, USA) following the kit's instruction with calf thymus DNA as a standard. GAG contents were reported as % GAG/ dry weight tissue for DACF and NAC and engineered constructs or as GAG/DNA for pellets. DNA contents were reported as % DNA/ dry weight tissue for DACF and NAC.

For the determination of type I and II collagens, DACF NAC and cartilaginous constructs were first lyophilized. The dried samples were fully solubilised by digestion with 2 mg/ml Tosylamide-2-phenylethyl chloromethyl ketone-treated bovine pancreatic trypsin in 50 mM Tris-HCl, pH 7.6, containing 1mM iodoacetamide, 1mM EDTA and 10 µg/ml pepstatin A, using an initial incubation of 15 h at 37ºC followed by a further 2 h incubation at 65ºC after the addition of fresh trypsin. Samples were boiled for 15 min to inactivate the enzyme[22]. Amounts of type II collagen were assayed by inhibition ELISA using a mouse IgG monoclonal antibody to denatured type II collagen[23]. Amounts of type I collagen were assayed by inhibition ELISA using a rabbit anti-peptide antibody to type I collagen[22].

Real-time quantitative RT-PCR assays

RNA of expanded DACF- and NAC-chondrocytes was extracted using Trizol (Life Technologies, Basel, Switzerland), according to the Manufacturer's protocol. RNA was

treated with DNAseI using the DNA-freeTM Kit (Ambion, USA) and quantified spectrophotometrically. cDNA was generated from 3 μg of RNA by using 500 μg/ml random hexamers (Catalys AG, CH) and 1 μl of 50 U/ml StratascriptTM reverse transcriptase (Stratagene, NL), in the presence of dNTPs. PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). Cycle temperatures and times as well as primers and probes used for the reference gene (18-S rRNA) and the genes of interest (type I collagen and type II collagen), were as previously described[24]. For each cDNA sample, the threshold cycle (Ct) value of 18-S was subtracted from the Ct value of the target gene, to derive ΔCt. The levels of expression of type I and type II collagen were calculated as $2^{\Delta Ct}$ and represented in logarithmic scale. Each sample was assessed at least in duplicate for each gene of interest.

Biomechanical analysis

Mechanical tests on constructs were conducted in a standard miniature test instrument in unconfined compression (Synergie 100, MTS Systems Corp., Eden Prairie MN, USA) to measure the Equilibrium modulus (E_{EQ}) and the Pulsatile dynamic modulus (E_{PD}), as previously described in detail[25]. Briefly, E_{EQ} was determined from a linear regression of the data pairs of equilibrium stress/incremental strain, following application of five incremental strains of 5% and computation of the corresponding equilibrium stress. E_{PD} was calculated as the slope of the stress/strain curve, after exposing specimens to five cycles of compressive loading/unloading at 0.17 mm/s, reaching a strain of 20% and with each strain period followed by a no-load period of time equal to that for loading/unloading.

Statistical analysis

Unless otherwise stated, values are presented as mean ± standard error of measurements (SEM) using cells from 6 donors for the experimental group (i.e.: DACF) and cells from 10 donors for the control group (i.e.: NAC). Statistical analyses were performed using the Sigma

Stat software (SPSS Inc., Version13). Differences between groups were assessed by Mann-Whitney test and considered statistically significant when p values were lower than 0.05.

RESULTS

Cellular and extracellular matrix (ECM) contents in Damaged Ankle Cartilage Fragments (DACF) and Normal Ankle Cartilage (NAC) biopsies

Macroscopically, DACF appeared rough and dull. As compared to NAC biopsies, DACF were less cellular, as evidenced by a statistically significant lower amount of DNA (3.0-fold). Quantitative assessments of the ECM indicated that DACF contained statistically significant lower amounts of GAG (5.3-fold) and type II collagen (1.5-fold) but higher amounts of type I collagen (6.2-fold) (Table 2). Histological and immunohistochemical assessments indicated that: (i) staining for GAG and type II collagen were faint and/or scattered in restricted areas of the DACF but strongly and uniformly distributed in NAC, (ii) staining for type I collagen was diffuse in DACF whereas localized in the thin superficial layer of NAC, (iii) staining for Von Kossa was diffuse in DACF whereas restricted at the cartilage-bone interface in NAC, (iv) cells with fibroblastic morphology were present within the DACF but absent or localized in the thin superficial layer of NAC, (v) clusters of cells were present abundantly only within the DACF (Fig. 1).

Cell yield, proliferation rate and differentiation stage of DACF- and NAC-chondrocytes

In agreement with the DNA quantification results, DACF yielded statistically significant lower number of cells as compared to NAC (respectively $1.7 \pm 0.6 \times 10^5$ and $3.5 \pm 0.6 \times 10^5$ cells/100mg of tissues processed for cell expansion) following enzymatic digestion of specimens.

DACF- and NAC-chondrocytes cultured in monolayers exhibited similar elongated and spindle-shaped morphologies characteristic of de-differentiated chondrocytes (Fig 2A). As compared to NAC-chondrocytes, DACF-chondrocytes (i) proliferated at higher rate (p>0.05)

(cells underwent respectively 7.8 ± 1.2 and 10.4 ± 1.0 doublings in 15 ± 2 days; Fig. 2B) and (ii) expressed higher type I collagen (4.2-fold; p>0.05) and lower type II collagen (3.1-fold; p>0.05) mRNA levels, with consequently lower (21.6-fold) type II/type I collagen mRNA ratio (Fig. 2C). The above described trends were not, however, statistically significant. The extent of cell expansion within the 2 passages was sufficient to generate at least about 50×10^6 cells from 100 mg biopsy, which was sufficient for the subsequent differentiation assays and would be clinically relevant for implantation or for engineering of a cartilage graft.

Post-expansion chondrogenic capacity in cell pellets

Expanded DACF-chondrocytes exhibited a generally poor re-differentiation capacity in pellet culture. Tissues generated with this model in fact (i) resulted negatively stained for GAG (5/6) or positively stained only in scattered regions (1/6, *donor 3*) and (ii) contained cells with necrotic or fibroblastic appearances. Instead, pellets generated by NAC-chondrocytes were uniformly stained for GAG and contained cells with the typical chondrocytic round morphology (Fig. 3A). Biochemical assessment confirmed a statistically significant lower content of GAG (2.8-fold) in pellets generated by DACF-chondrocytes. DACF-chondrocytes from only one donor (*donor 3*) accumulated GAG at levels approaching the average ones measured for NAC-chondrocytes[8] (Fig 3B). DNA contents of pellets generated by the two cell types were similar (less than 10% of variation between the two groups).

Immunohistochemical analysis showed that type II collagen was absent (5/6) or accumulated only in limited region of pellets (1/6, *donor 3*) generated by DACF-chondrocytes. In contrast, pellets generated by NAC-chondrocytes were uniformly stained for type II collagen (Fig. 3C).

Tissue-forming capacity in 3D scaffolds

After 14 days of culture in HYAFF-11® meshes, tissues based on DACF-chondrocytes were negatively stained for GAG and type II collagen while the tissues based on NAC-chondrocytes were faintly stained for both proteins. At the same time, tissues generated by both DACF- and NAC-chondrocytes had similar amounts of DNA and contained cells with

fibroblastic appearance (data not shown). After 28 days of culture, tissues generated by DACF-chondrocytes remained negatively stained for GAG (5/6) or accumulated ECM faintly stained for GAG (1/6, *donor 3*) in restricted regions, and contained predominantly fibroblastic cells. At this time point, tissues generated by DACF chondrocytes had significantly lower DNA content than those generated by NAC-chondrocytes (1.5-fold), likely indicating the reduced capacity of the former chondrocytes to proliferate within the scaffolds. Staining for type II collagen was negative in all constructs. Instead, tissues generated by NAC accumulated large amounts of ECM positively stained for GAG and type II collagen and contained a large fraction of cells with a chondrocytic morphology (Fig. 4A).

Biochemical analysis generally confirmed the histological observations. GAG and type II collagen as a % of dry weight did not increase from 14 to 28 days of culture in constructs based on DACF-chondrocytes, and thus remained at statistically significant lower levels than those measured in constructs based on NAC-chondrocytes (1.9- and 1.7-fold respectively). Instead, type I collagen markedly increased with culture time (3.3-fold), resulting, at 28 days, in higher amounts (1.5-fold) in constructs based on DACF-chondrocytes than in those based on NAC-chondrocytes (Fig. 4B). At the latest culture time the ratio of type II to type I collagen was 2.9-fold higher in constructs based on NAC-chondrocytes.

The measured biomechanical properties of tissues captured the biochemical differences (Fig. 4C). In fact, equilibrium modulus and dynamic pulsatile modulus (i) did not significantly increase between 14 and 28 days of culture in constructs based on DACF-chondrocytes, and (ii) were significantly lower in constructs based on DACF-chondrocytes as compared to those based of NAC-chondrocytes (1.7- and 3.3-fold respectively) after 28 days of culture.

DISCUSSION

In this study, chondrocytes harvested from the detached fragments of osteochondral lesions (OCL) of ankle joints (Damaged Ankle Cartilage Fragments, DACF) and from normal articular cartilage (NAC) of the talus of cadavers were compared in terms of their growth and de-differentiation during two-dimensional culture, as well as of their post-expansion tissue forming capacity. DACF chondrocytes displayed more fibroblastic characteristics, as evidenced by the trends of higher proliferation rate and higher extent of de-differentiation. When induced to re-differentiate in pellet culture or in porous scaffolds, DACF-chondrocytes produced tissues with lower GAG and type II collagen contents and lower biomechanical properties.

Histological and biochemical assessments of the harvested DACF indicate that such tissues contained markedly lower amount of proteoglycans and type II collagen and higher amounts of type I collagen as compared to NAC. Interestingly, a large portion of the dry weight of DACF remains unaccounted for by the measured extracellular components and likely consisted of macromolecules produced to a larger extent by more fibroblastic cells (e.g., type III collagen and versican) or of mineral deposits, since in fact large areas of fibrocartilage and mineralized cartilage were observed in the DACF. Although our harvested DACF were from slightly older patients as compared to those of Giannini et al (2005)[11] (mean age: 36.3 years and 30.5 years, respectively), our data do not support the previously reported results[11]. Moreover our study is the first to more thoroughly compare the re-differentiation capacity of DACF-chondrocytes to that of NAC-chondrocytes, including a biochemical and biomechanical characterization of the *in vitro* generated tissues. The findings are generally in line with those reported by Aurich et al (2005)[10] and Garvican et al (2008)[13], describing that OCL chondrocytes display rather fibroblastic features.

Our results clearly show that DACF-chondrocytes exhibit a reduced re-differentiation capacity in pellets as compared to NAC-chondrocytes, as assessed by a reduced accumulation

of GAG and type II collagen. Due to the relatively limited amount of DACF analysed, we could not observe clear correlations between chondrogenesis and either patient age or the interval between trauma and the operation. Although the models here used to investigate the cartilage forming ability have been previously validated using a variety of different chondrogenic cell types[16,26], we cannot exclude that the presence of growth factors during the phases of cell expansion/differentiation could have critically determined the observed differences.

Similarly to our results, Garvican et al (2008)[13] have recently found that pellets generated by chondrocytes from equine OCL contained significantly lower GAG and expressed significantly less amount of Sox9 than pellets created from normal chondrocytes. Interestingly in the same study the authors observed that OCL chondrocytes expressed higher levels of MMP-13 mRNA as compared to normal chondrocytes. Therefore, the impaired capacity of OCL (vs normal) chondrocytes to re-differentiate could be a consequence of both decreased synthesis and increased degradation of ECM. The observations reported for OCL chondrocytes should not be generalized to other cartilage pathologies, since it was recently demonstrated that chondrocytes from osteoarthritic patients display a normal chondrogenic differentiation capacity[27].

We are aware that the models used in the present study to characterize the post-expansion chondrogenic capacity of DACF chondrocytes (i.e., static culture in the presence of chondrogenic factors), have not been validated to directly predict the reparative ability of the cell types when implanted in the talar lesions. In a recent work, Giannini et al (2008)[28] reported results of a clinical study in which 46 patients with focal talar dome osteochondral lesion were treated with ACI using HYAFF-11[®] and chondrocytes harvested from the detached OCL fragments or from tissue harvested from the margin of the lesion or from the anterior margin of the tibia. The authors however did not describe differences in the clinical outcome of patients treated with the different cell sources. Controlled randomized clinical

trials are therefore necessary to compare clinical outcome of cell based cartilage repair technique based on the DACF- and NAC-chondrocytes.

In conclusion, our results indicate that chondrocytes isolated from DACF have inferior cartilage forming capacity as compared to chondrocytes from normal ankle cartilage tissues, probably resulting from environmental changes occurring during and after OCL trauma/disease. The study opens some reservations on the use of DACF-derived cells for the repair of ankle cartilage defects, especially in the context of tissue engineering-based approaches, since DACF-chondrocytes would not be able to generate functional cartilaginous tissues *in vitro*. Further investigations are required to assess whether the use of culture conditions including different biochemical and biomechanical cues, or sorting of subpopulations expressing higher levels of markers associated with the cell chondrogenic capacity[29], could enhance the cartilage forming ability of DACF-chondrocytes.

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CONFLICT OF INTEREST STATEMENT

No Author has any interest that is potentially in conflict with this work

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LEGEND TO FIGURES

Figure 1. Histological appearance of detached fragments of osteochondral lesions of ankle joints (Damaged Ankle Cartilage Fragments, DACF) and normal articular cartilage (NAC).

Safranin O (**A**, **B**), Von Kossa/Safranin O staining (**B**), type II collagen and type I collagen immunohistochemical staining (A) of DACF harvested from two different, representative donors: *donor 3* (**I**) and *donor 5* (**II**) (see Table 1 for the donor information) and of normal articular cartilage (NAC) harvested from a cadaveric joint of a young individual (male, 32 years) (**III**). Bar = 100 μm. Arrows indicate cartilage/bone interface, asterisks show cell clusters, h = hyaline or hyaline-like cartilage, f = fibrocartilage, c = calcified cartilage.

Figure 2. Growth and differentiation stage of chondrocytes isolated from Damaged Ankle Cartilage Fragments (DACF) and normal articular cartilage (NAC)

(**A**) Representative phase-contrast pictures of expanded DACF-chondrocytes (I) and NAC-chondrocytes (II) (see Table 1 for the donor information). Bar = 100 μm. (**B**) Proliferation rate of DACF- and NAC-chondrocytes. Values are the mean ± SEM of measurements obtained from 6 different DACF or 10 different NAC. (**C**) Real time reverse transcriptase-polymerase chain reaction analysis of the expression of mRNA for type I (CI) and type II (CII) collagens (left y axis labelling) or the CII/CI ratio (right y labelling axis). Values are the mean ± SEM of measurements obtained from 4 different DACF or 4 different NAC.

Figure 3. Differentiation capacity of chondrocytes isolated from Damaged Ankle Cartilage Fragments (DACF) and normal articular cartilage (NAC) in pellets

(**A**) Safranin O stainings of representative pellets generated by DACF-chondrocytes harvested from two different donors: *donor 3* (**I**), *donor 5* (**II**) (see Table 1 for the donor information) and NAC-chondrocytes harvested from a cadaveric joint of a young individual (male, 32 years) (**III**) Bar = 100 μm. (**B**) Sulfate glycosaminoglycan (GAG) content normalized to the amount of DNA in pellets. Values are the mean ± SEM of triplicate pellets. The plotted lines

indicate mean values measured in pellets generated by cells from NAC (upper line, 10 donors, mean ± SEM = 13.1 ± 1.6) or DACF (bottom line, 6 donors, mean ± SEM = 4.6 ± 1.6). (**C**) Type II collagen immunohistochemical stainings of representative pellets generated by DACF-chondrocytes harvested from two different donors: *donor 2* (**I**), *donor 3* (**II**) and NAC-chondrocytes harvested from a cadaveric joint of a young individual (male, 32 years) (**III**).

Figure 4. Differentiation capacity of chondrocytes isolated from Damaged Ankle Cartilage Fragments (DACF) and normal articular cartilage (NAC) in HYAFF-11® scaffold.

Safranin O stainings (**A**) and type II collagen immunohistochemical stainings (**B**) of representative constructs generated by DACF-chondrocytes harvested from two different donors [*donor 3* (**I**), *donor 5* (**II**) (see Table 1 for the donor information)] and NAC-chondrocytes harvested from a cadaveric joint of a young individual (male, 32 years) (**III**) cultured in chondrogenic medium for 28 days. Bar = 100 μm. h = hyaline-like cartilage, f = fibrocartilage (**C**) Amounts of sulphate glycosaminoglycan (GAG), type I collagen and type II collagen accumulated in constructs generated by DACF-chondrocytes (bars, 6 donors) and NAC-chondrocytes (plotted line, 10 donors, values as mean ± SEM equal to: 9.9 ± 1.5, 4.2 ± 0.6 and 1.3 ± 0.4 respectively for GAG, type I collagen and type II collagen) expressed as a percentage of tissue dry weight. (**D**) Equilibrium modulus and dynamic pulsatile modulus of constructs generated by DACF-chondrocytes (bars, 6 donors) and NAC-chondrocytes (plotted line, 10 donors, values as mean ± SEM equal to: 29.9 ± 4.4 and 1.6 ± 0.1 respectively for equilibrium modulus and dynamic pulsatile modulus). * = significantly different from the NAC group at the corresponding time point, ° = significantly different from 2 weeks.

Table 1 Patient information for osteochondral lesions (OCL)

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Gender	female	male	male	female	male	female
Age	30 years	43 years	39 years	36 years	33 years	37 years
Joint side	medial	medial	lateral	medial	lateral	medial
OCL Grade (Berndt & Harty)	III	III	III	II	III	III
Interval trauma/operation	14 months	3 months	6 months	6 months	4 months	6 months