Knee Surg Sports Traumatol Arthrosc (2010) 18:1407–1416
DOI 10.1007/s00167-009-1017-4

EXPERIMENTAL STUDY

Chondrogenesis in a hyaluronic acid scaffold: comparison between chondrocytes and MSC from bone marrow and adipose tissue

Rune B. Jakobsen · Aboulghassem Shahdadfar · Finn P. Reinholt · Jan E. Brinchmann

Received: 7 July 2009 / Accepted: 1 December 2009 / Published online: 18 December 2009

Abstract Treatment of focal lesions of the articular cartilage of the knee using chondrocytes in a hyaluronic acid (HA) scaffold is already being investigated in clinical trials. An alternative may be to use mesenchymal stem cells (MSC). We have compared articular chondrocytes with MSC from human bone marrow (BM) and adipose tissue (AT), all cultured in HA scaffolds, for their ability to express genes and synthesize proteins associated with chondrogenesis. The cells were expanded in monolayer cultures. After seeding into the scaffold, the chondrocytes were maintained in medium, while the two MSC populations were given a chondrogenic differentiation medium. Chondrogenesis was assessed by real-time RT-PCR for chondrocyte-associated genes, by immunohistochemistry and by ELISA for collagens in the supernatant. Redifferentiation of the dedifferentiated chondrocytes in the HA scaffold was shown by a modest increase in type II collagen mRNA *(COL2A1)* and reduction in *COL1A1*. BM-MSC expressed 600-fold higher levels of *COL2A1* than chondrocytes after 3 weeks in the scaffold. The levels of aggrecan *(AGC1)* and *COL1A1* were similar for chondrocyte and BM-MSC scaffold cultures, while *COL10A1* was higher in the BM-MSC. AT-MSC expressed levels of *COL2A1* and *COL1A1* similar to chondrocytes, but less *AGC1* and *COL10A1*. Surprisingly, little collagen II protein was observed in the scaffold. Instead, collagen II was found in the culture medium. Chondrogenesis in HA scaffolds was more efficient using BM-MSC than AT-MSC or chondrocytes. Some of the secreted collagen II escaped entrapment in the extracellular space and was detected in the culture medium.

Keywords Chondrocytes · Mesenchymal stem cells · MSC · Chondrogenesis · Hyaluronic acid scaffold · Type II collagen

R. B. Jakobsen · A. Shahdadfar · F. P. Reinholt · J. E. Brinchmann (✉)
Institutes of Immunology and Pathology, Rikshospitalet University Hospital, 0027 Oslo, Norway
e-mail: jan.brinchmann@rr-research.no

R. B. Jakobsen
e-mail: r.b.jakobsen@gmail.com

A. Shahdadfar
e-mail: aboulghassem.shahdadfar@rr-research.no

F. P. Reinholt
e-mail: finn.p.reinholt@rr-reserach.no

Introduction

The development of cell-based treatment strategies for the repair of articular cartilage injury has received considerable interest during the last decade. Autologous chondrocyte implantation (ACI) [8] and various forms of matrix-assisted ACI have shown promising results [31, 33, 41] but have not yet been proven in well-performed randomized controlled trials to be superior to more traditional surgical approaches [20, 46]. There are inherent problems associated with ACI therapy, including donor-site morbidity [47] and the switch from type II to type I collagen synthesis by chondrocytes cultured adherent to plastic [4, 11, 21, 38] in a process termed dedifferentiation [18]. ACI using dedifferentiated chondrocytes frequently results in the production of a mixed hyaline-fibrocartilaginous repair tissue following implantation [11, 24, 47]. Re-expression of type II collagen and ECM proteoglycans may occur in vitro if the chondrocytes are introduced into a three-dimensional culture system, either as pellet cultures [42] or supported

by scaffolds, without chondrogenic differentiation medium (CDM), in a process called redifferentiation [15].

Mesenchymal stem cells (MSC) can be isolated from several tissues, including bone marrow (BM-MSC) and adipose tissue (AT-MSC) [2]. They can be induced by CDM to differentiate to chondrocyte-like cells in vitro [7, 34, 36, 37, 49] and are thought to be the adult equivalent of the embryogenic cartilage and bone precursor cell. They have received attention as cell source candidates to improve the ACI procedure and have already been used clinically in a limited number of studies [26, 44, 45]. However, it is not yet clear what cell source is the most appropriate for cartilage engineering, or whether MSC may be able to equal or surpass chondrocytes in terms of articular cartilage-like extracellular matrix (ECM) production.

The glycosaminoglycan hyaluronic acid (HA) is abundant in ECM and binds to chondrocytes via the transmembrane receptor CD44 [22]. Blocking this interaction results in lysis of native chondrocytes [23]. The HA-based scaffold HYAFF-11 is used with chondrocytes in the clinical setting. Here, the chondrocytes are cultured in the scaffold without CDM [15, 31, 43]. Some studies have investigated BM-MSC cultured in this scaffold [9, 12, 28]. However, until now, a detailed comparison of chondrogenic gene expression between chondrocytes, AT-MSC and BM-MSC cultured in HYAFF-11 has not been performed.

Materials and methods

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless stated otherwise.

Cell sources and monolayer cultures

Approval for the collection of all tissue samples was obtained from the Regional Committee for Ethics in Medical Research. All patients and donors included in the study signed an informed consent. Surplus pieces of articular cartilage (200–300 mg) were harvested from a low weight-bearing area of the knee in three otherwise healthy individuals undergoing anterior cruciate ligament reconstruction (age 23–48, mean 30). Biopsies were transferred into a 50-ml tube (NUNC, Roskilde, Denmark) filled with saline (0.9%) and transported to the laboratory facility. Biopsies were minced manually in DMEM/F12 (Imperial Laboratories, Andover, UK) with 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Life Technologies-BRL) and subjected to 3–5 h of digestion in the same medium supplemented with collagenase type XI (1.2 μg/ml) and deoxyribonuclease I (0.1 μg/ml) in 37°C humidified room air with 5% CO_2. Digested biopsies were then filtered through 70-μm cell strainer (Becton–Dickinson, NJ), washed in medium and seeded in culture flasks (NUNC) with medium supplemented with 50 μg/ml ascorbic acid and 20% fetal bovine serum (FBS) (Bio Whittaker, Verviers, Belgium) until the first medium change, subsequently 10% FBS (culture medium). Amphotericin B was used only until the first medium change for all cell types. Culture medium was changed every 3–4 days, and the cells were passaged at 70% confluence.

AT-MSC were obtained from five female donors (age 29–50, mean 38) undergoing liposuction. AT-MSC were isolated from the liposuction material as previously described [6, 7]. Briefly, lipoaspirate (300–1,000 ml) was washed with Hanks' balanced salt solution (HBSS; Life Technologies-BRL, Paisley, UK) containing 100 IU/ml penicillin, 100 IU/ml streptomycin and 2.5 μg/ml amphotericin B. Washed AT was digested for 45 min on a shaker at 37°C in HBSS containing 0.1% collagenase I. The stromal vascular fraction was pelleted (400×*g*, 10 min), the pellets were then resuspended in HBSS containing 2% FBS and tissue clumps were allowed to settle for 1 min. Suspended cells were passed through 100-μm and then 40-μm cell sieves (Becton–Dickinson). After density-gradient centrifugation at 400×*g* for 20 min, the mononuclear cells were collected, washed with HBSS and counted. Immediately after separation, AT-MSC were separated from remaining cells using magnetic cell sorting. Cells with endothelial ($CD31^+$) and leukocyte ($CD45^+$) cell surface markers were removed using mouse anti-human monoclonal antibodies utilizing a superMACS magnet and LS columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. Cells were washed, resuspended in culture medium containing 20% FBS and seeded in 175-cm^2 culture flasks (NUNC). From the first medium change, 10% FBS was used instead of 20%.

BM-MSC were obtained from four volunteers (age 22–38, mean 30) as previously described [37]. Briefly, syringes coated with monoheparin were used to aspirate approximately 50 ml of bone marrow from the iliac crest. The aspirate was quickly diluted 1:3 in culture medium, then transferred to 50-ml tubes containing 15 ml Lymphoprep and centrifuged at 800×*g* for 20 min. The mononuclear cell layer was carefully transferred to new tubes and washed twice (300×*g*, 10 min). To reduce contamination by other adherent cells, CD14+ monocytes were removed using magnetic microbeads as described earlier. The CD14- cells were seeded in culture flasks with culture medium supplemented with 20% FBS. At day 1, non-adherent cells were discarded, and adherent cells were washed with culture medium containing 20% FBS three times. Medium was changed every 3–4 days, using 10%

instead of 20% FBS from the first medium change. MSC were passaged 1:3 at 45% confluence.

Cell cultures in HA scaffolds

The cells were placed in HA scaffolds after 3–5 passages in monolayer culture. At this stage, all the cell populations were in a stable log phase of growth. The cells were trypsinized, pelleted (260×*g*, 8 min), resuspended, viability tested and counted. For each three-dimensional culture, 2.5×10^6 cells were resuspended in 70 µl culture medium and carefully dripped onto a precut 0.5 cm^2 HYAFF-11 scaffold (generously provided by Fidia Advanced Biopolymers, Italy) placed in a 24-well plate, where the wells had been precoated with a 1% poly(HEMA) solution to avoid adherent cell growth [13]. Scaffolds were then placed in the incubator for 3 h after which cell attachment to the scaffold was verified by light microscopy. Subsequently, 2 ml culture medium was added. No cells were observed outside the scaffold, either immediately after medium was added or the next day. For chondrocytes, culture medium supplemented with ascorbic acid was used. To induce chondrogenic differentiation in the MSC, serum-free culture medium containing the following CDM was used [6, 35, 39]: high-glucose DMEM (4.5 g/l; Life Technologies-BRL) supplemented with 500 ng/ml bone morphogenic protein-6 (R&D Systems, Minneapolis, MN), 10 ng/ml recombinant human transforming growth factor-β1 (R&D Systems), 1 mm sodium pyruvate, 0.1 mm ascorbic acid-2-phosphate, 100 nm dexamethasone, 1% ITS (25 mg insulin, 25 mg transferrin and 25 µg sodium selenite) and 1.25 mg/ml bovine serum albumin. For each MSC donor, the following three scaffold cultures were established: 1) culture medium only for 21 days, 2) CDM until the first medium change (4 days), then culture medium and 3) CDM for the full duration of the scaffold culture (21 days). In all cultures, medium was changed every 4 days. At each change of medium, 2 ml of supernatant was frozen at −20°C for enzyme-linked immunosorbent assays (ELISA).

Real-time RT-PCR

At day 0, 7 and 21 of scaffold cultures for chondrocytes, and day 7 and 21 for MSC cultures, samples were processed for real-time RT-PCR. Total RNA was extracted using Trizol (Invitrogen, Gaithersburg, MD) according to the manufacturer's recommendation.

RNA to cDNA conversion was performed using the High-Capacity cDNA Achive Kit (Applied Biosystems, Rockville, CA). RNA samples were diluted to 50 µl total volume with RNase-free water and treated with DNase I to remove genomic DNA contamination. Subsequently, 0.5 µg RNA was reverse transcribed to cDNA according to the manufacturer's protocol. Real-time PCR was performed in triplicates using a cDNA input equivalent of 12.5 ng RNA per replicate on a 7300 Real-Time PCR System (Applied Biosystems) using standard Taqman Assays and protocols. All assays spanned an exon junction to prevent detection of genomic DNA. Results were analyzed using the $2^{-\Delta Ct}$-method [30] and presented as values normalized to the expression of GAPDH, which was used as endogenous control.

Immunohistochemistry

Scaffolds with MSC were fixed in 0.1 M phosphate-buffered 4% formalin for a week. The specimens were dehydrated in graded ethanol and embedded in paraffin blocks. Immunohistochemistry was performed on rehydrated 4-µm thick sections using antibodies to human type I collagen (mouse monoclonal antibody, MP Biomedicals, OH, USA, Cat. # 63170, dilution 1: 25) and type II collagen (rabbit polyclonal antibody, Cedarlane Laboratories Ltd, Ontario, Canada, Cat. # CL50111AP, dilution 1: 200). Demasking of epitopes was by trypsin in case of collagen I and by hyaluronidase in case of collagen II according to the manufacturers' recommendations. Envision kits (DAKO, Copenhagen, Denmark) were used to locate immunoreactivity with DAB as chromogen. Contrast staining with hematoxylin was performed followed by dehydration, mounting and qualitative light microscopic evaluation.

ELISA

ELISA specific for type I and type II collagen was performed on supernatants collected at day 0 and day 20 for all three cell types. For MSC, only cultures that were exposed to CDM throughout all 21 days were analyzed. Using the commercially available kits Collagen Type II ELISA (MD Biosciences, St. Paul, MN, catalogue no. CII96) and Human Type I Collagen Detection Kit (Chondrex Inc., Redmond, WA, catalogue no. 6008), the samples were processed according to the manufacturer's protocol. Plates were read at 492 and 450 nm for type I and II collagen, respectively, on a Multiskan Ascent (Thermo Electron Corp., Vantaa, Finland).

Statistics

One-way analysis of variance (ANOVA) with a Tukeys post hoc test was used to compare means of gene expression (ΔCt-values). Significance was assumed for $P < 0.05$. Data are presented as mean ± SE of biological replicates. Samples from each of the donors were run in triplicate. All graphing and statistical analyses were

performed in Graphpad Prism 5 (Graphpad Software, La Jolla, CA).

Results

Redifferentiation of chondrocytes

We compared three different cell populations for their ability to induce chondrogenesis in HYAFF-11: chondrocytes, BM-MSC and AT-MSC. Similar to the HYAFF 11/chondrocyte cultures used in the clinical setting, the chondrocyte cultures in the present study were not given CDM [15, 31, 43]. In addition to type II collagen mRNA *(COL2A1)*, we also tested for mRNA levels of Sry-related high mobility group box family member 9 (*SOX9),* the factor proposed to control *COL2A1* transcription [5, 16]. In addition, we measured the mRNA levels for the hyaline cartilage proteoglycan aggrecan (*AGC1)* for type I collagen *(COL1A1)* that is produced by dedifferentiated chondrocytes and contributes to a fibrocartilaginous phenotype [3] and for two molecules known to participate in the transition from proliferating to hypertrophic chondrocytes in embryological chondrogenesis, i.e. type X collagen *(COL10A1)* and runt-related transcription factor II (*RUNX2)* [14, 48]. The mRNA levels at the end of monolayer cultures (day 0) and on day 7 and 21 of scaffold cultures are shown in Fig. 1. The level of *COL2A1* was low on day 0, increased by day 7 of scaffold culture and then stabilized. This pattern was mirrored by the mRNA expression of *SOX9.* Consistent with a redifferentiation process, the level of *COL1A1* dropped following establishment of the chondrocytes in the HA scaffold but was found to be moderately increased again at day 21. *AGC1* and *RUNX2* followed a similar pattern. *COL10A1* expression was not changed over time.

Differentiation of BM-MSC and AT-MSC

For the MSC in scaffold cultures, we investigated whether transient exposure to CDM programs the cells for chondrogenesis, or whether continuous exposure to CDM was

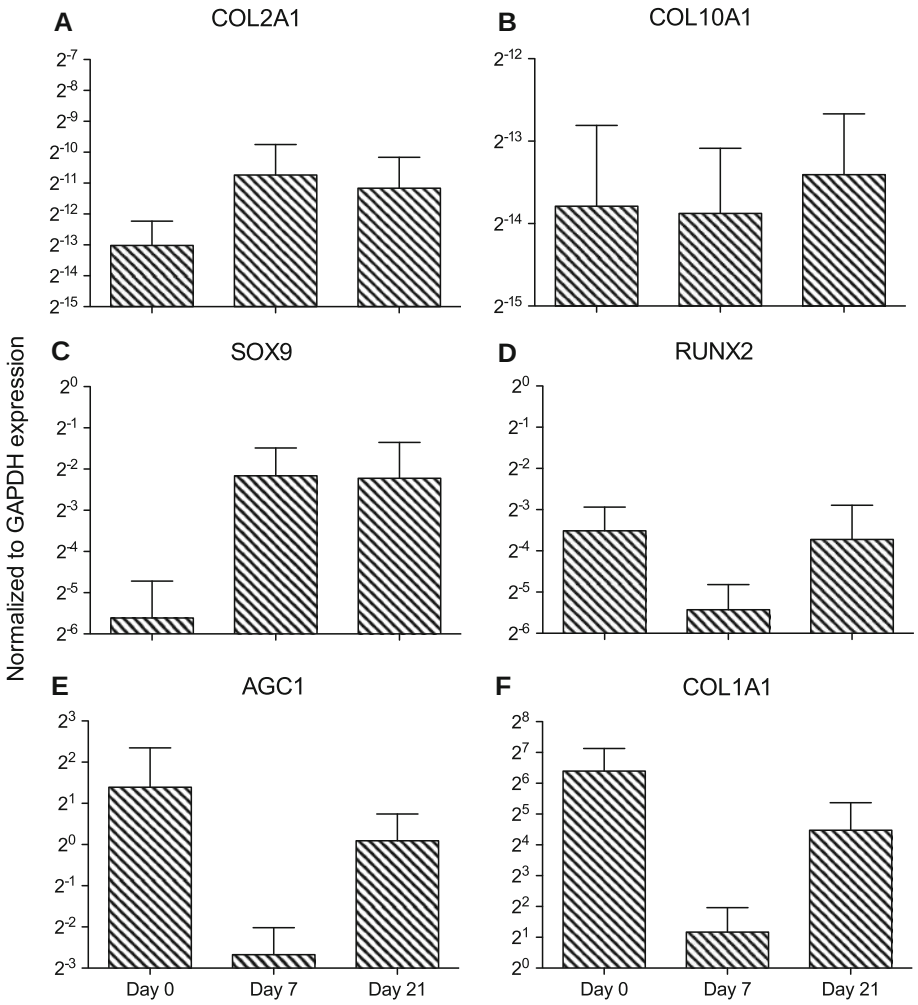

Fig. 1 Results of real-time RT-PCR for chondrocytes in monolayer and HA scaffolds. All *bars* are average of three donors (biological replicates) ± SE normalized to the expression of GAPDH

required. *COL2A1* mRNA was not detectable in any MSC monolayer cultures (data not shown). Figure 2 shows that establishment of the BM-MSC in HA scaffolds alone was sufficient to induce low levels of *COL2A1* in these cells. A brief exposure to CDM did not significantly improve on this. Under the continuous exposure of CDM, however, the *COL2A1* mRNA levels were similar to those observed in chondrocytes already at day 7 and were upregulated an average of 540-fold by day 21. The expression levels of *COL2A1* were partly mirrored by changes in expression of *SOX9*, but on a much smaller scale. The level of *AGC1* was low in undifferentiated BM-MSC at day 7. Exposure to CDM increased this by day 21, to levels similar to those observed for chondrocytes. The level of *COL1A1*, too, increased with time in HA scaffold and continuous exposure to CDM, to levels similar to those observed for chondrocytes. The expression of *COL10A1* was very high in BM-MSC compared with chondrocytes, even without CDM, and was greatly upregulated by day 21. This expression pattern was mirrored by *RUNX2,* albeit at a lower level of expression relative to chondrocytes.

In AT-MSC, the effect of 3D culture on the expression of *COL2A1* in the absence of CDM was similar to that observed for BM-MSC (Fig. 3). However, for AT-MSC, continuous exposure to CDM only increased the *COL2A1* expression marginally. Thus, at the end of 21 days of continuous exposure to CDM in HA scaffolds, the *COL2A1* expression was 1,000-fold higher in BM-MSC than in AT-MSC. Again, the levels of SOX9 mirrored the *COL2A1* expression levels. The expression of *AGC1* was lower in AT-MSC than both of the other cell populations, while the expression of *COL1A1* and *RUNX2* at the end of three weeks in HA scaffolds was similar for all cells and culture conditions. *COL10A1,* however, was much lower in AT-MSC after three weeks than in BM-MSC, but still higher than in chondrocytes.

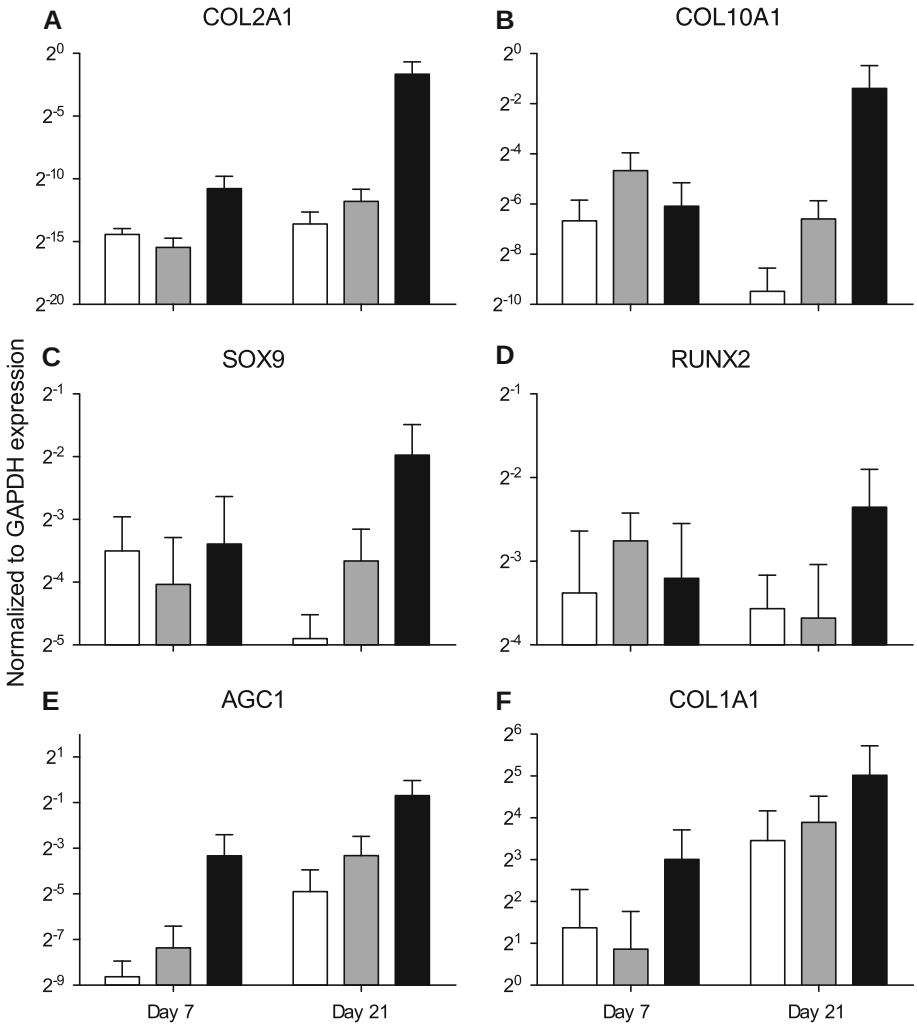

Fig. 2 Results of real-time RT-PCR for BM-MSC in HA scaffolds. All *bars* are average of four donors (biological replicates) ± SE normalized to the expression GAPDH. *White bars* represent culture medium only, *gray bars* represent CDM days 0–4 of scaffold culture, *black bars* represent continuous CDM

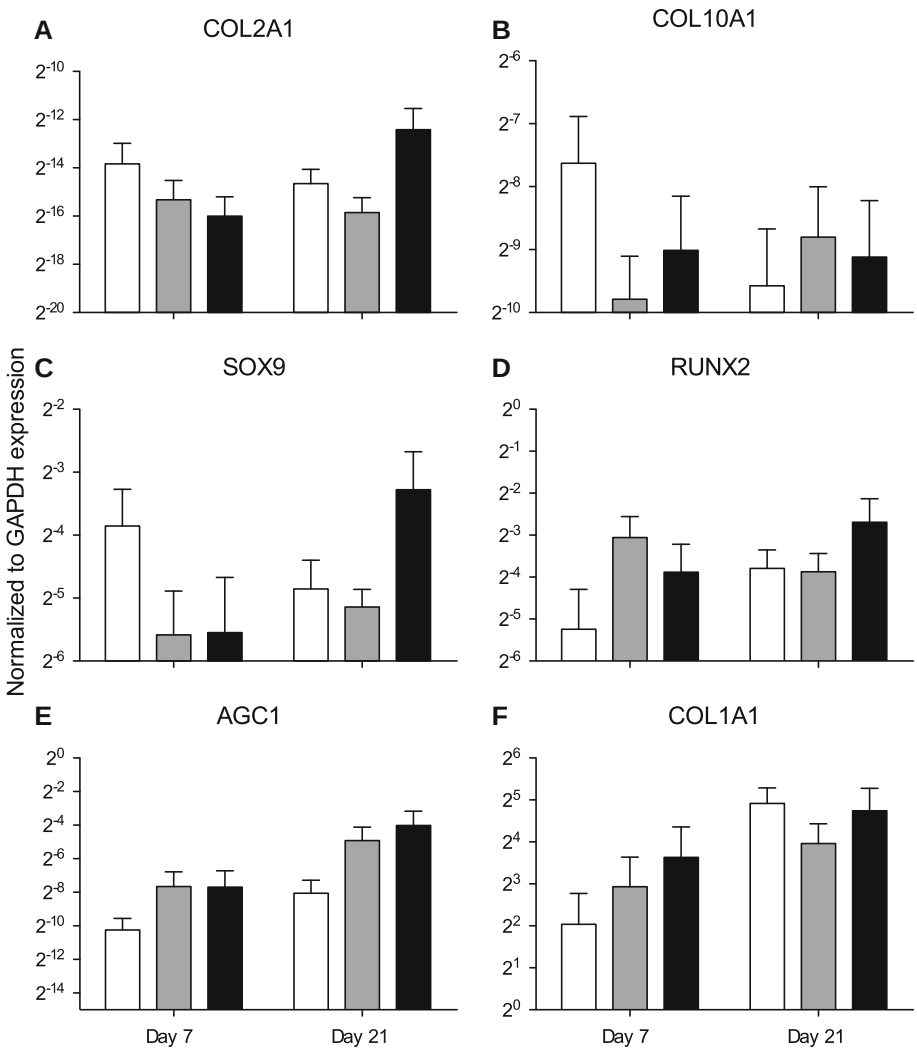

Fig. 3 Results of real-time RT-PCR for AT-MSC in HA scaffolds. All *bars* are average of five donors (biological replicates) ± SE normalized to the expression in chondrocytes at day 7. *White bars* represent culture medium only, *gray bars* represent CDM days 0–4 of scaffold culture, *black bars* represent continuous CDM

Synthesis and secretion of collagens

Light microscopy of sections of scaffolds revealed that the different cell populations were embedded in the scaffold at approximately the same density (data not shown). In the scaffold, most of the cells had a long, thin, fibroblast-like appearance (Fig. 4a, b). The ECM structure was mostly loose, with only a few fibrillar structures. By immunohistochemistry, type II collagen was observed in the cytoplasm, but only in small focal areas in the extracellular environment (Fig. 4a). A similar observation was made for type I collagen. As collagen was observed within the cells, but not in the loose ECM, we considered the possibility that the collagens were washed away from the immediate surroundings of the cells by the culture medium. Thus, we performed ELISA on the supernatants from day 20 for chondrocytes and MSC continuously exposed to CDM. The scaffolds had been in this medium for 4 days. The results for type II collagen are shown in Fig. 4c, and the corresponding mRNA results for each donor are shown for comparison in Fig. 4d. High levels of type II collagen were detected in all three supernatants from the scaffolds with BM-MSC cells and from two of the AT-MSC donors and one of the chondrocyte donors. Although obviously not linear, a correlation between mRNA expression and detection of type II collagen was indicated in the supernatants (Fig. 4c, d). Interestingly, we found no type I collagen in any of the supernatants (data not shown).

Discussion

The aim of this study was to compare chondrocytes, BM-MSC and AT-MSC cultured in a HA scaffold (HYAFF-11) known to support chondrogenic differentiation of MSC [28] and redifferentiation of dedifferentiated articular chondrocytes [12, 15]. We confirmed that chondrocytes in monolayer cultures redifferentiate when cultured in this scaffold as seen by the upregulation of *COL2A1* expression, the hallmark of hyaline cartilage, and

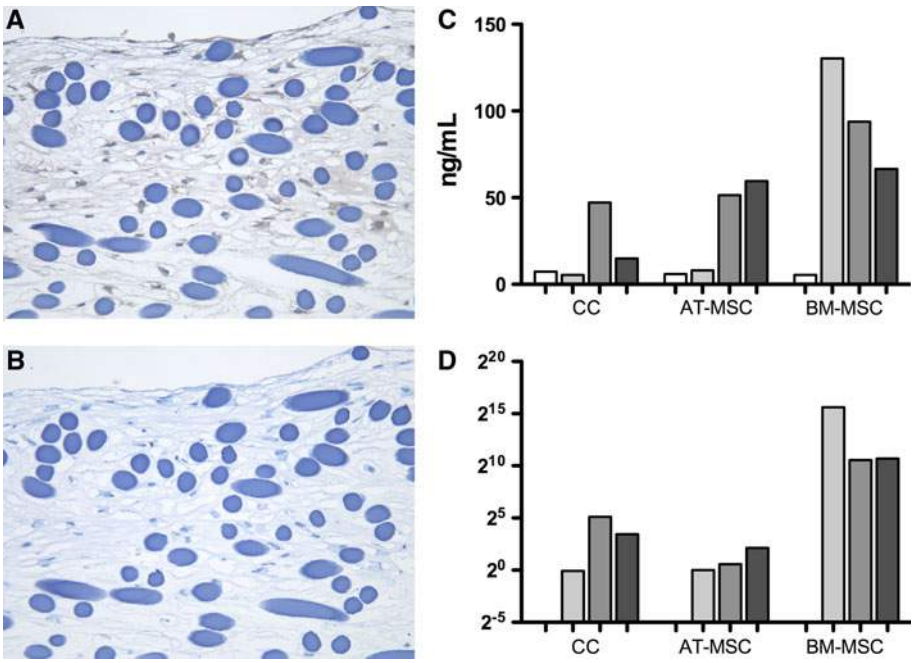

Fig. 4 Immunhistochemistry and ELISA analysis. In **a,** type II collagen is seen in the cell cytoplasm, but not in the ECM. HA fibers are *dark blue*. **b** shows section stained with isotype control antibody. **c** shows the results of ELISA analysis of supernatants from day 20 from chondrocytes (CC), and from AT-MSC and BM-MSC continuously exposed to CDM. *White bars*: negative controls. In **d**, the corresponding *COL2A1* mRNA results are shown. Note that these results represent 3 of the 5 AT-MSC donors and 3 of the 4 BM-MSC donors normalized to the results in chondrocytes day 20, which explains minor differences from the mean values presented in Figs. 2 and 3. Same donor, same *shade* of *gray*

downregulation of *COL1A1* expression, the collagen typically produced by dedifferentiated chondrocytes and the main collagen component in fibrocartilage. Not fully in line with redifferentiation was the downregulation of *AGC1* at day 7 of scaffold culture and the upregulation of *COL1A1* expression at day 21. Following the establishment of the chondrocytes in HA scaffolds, the changes in *SOX9* exactly mirrored those observed for *COL2A1*, consistent with its known role as the main transcription factor responsible for type II collagen synthesis [5]. *COL10A1* was detected in these cells, but did not change upon culture in HA scaffold. Our results indicate that the use of chondrocytes in a HA scaffold may be beneficial when compared to single cell chondrocyte suspensions in clinical cartilage repair [31].

When BM-MSC were established in HA scaffolds, without CDM, the *COL2A1* levels rose to a level of expression similar to that observed in chondrocytes. The *COL2A1* levels in BM-MSC exposed to CDM for 4 days also did not exceed those observed for chondrocytes. In cultures continuously exposed to CDM, however, the *COL2A1* levels far exceeded the chondrocyte levels at day 21. This observation could impact on treatment strategies involving BM-MSC in scaffolds. Although it is possible that certain factors in a cartilage lesion may induce differentiation in the BM-MSC following implantation of cells in scaffolds, it seems more likely that a period of in vitro culture exposed to CDM will enhance the ability of BM-MSC in scaffolds to produce *COL2A1* mRNA. However, dense cartilaginous ECM cannot be expected to be produced by differentiated MSC in HA scaffolds in vitro, at least not under the culture conditions provided in our experiments. True, type II collagen was found within the cells, but only small focal areas of ECM could be demonstrated at three weeks, with very little collagen deposition. In stead, substantial amounts of type II collagen were detected in the culture supernatants. As can be seen from the immunohistochemistry images, there is a lot of space between the cells and scaffold fibers. Presumably, type II collagen molecules have been synthesized and secreted by the cells, but due to lack of solid material in the pericellular environment for collagen to attach to, a large proportion was washed away by the medium to be recovered in the supernatant.

Changes in the levels of *SOX9* mRNA in BM-MSC followed those observed for *COL2A1* mRNA, but never exceeded the level of expression seen in chondrocytes despite the very much higher levels of *COL2A1* mRNA observed at day 21 in the BM-MSC. As SOX9 is a DNA-binding transcription factor, a one-to-one relationship between *COL2A1* and *SOX9* is not expected [10], but it is interesting that the same level of *SOX9* expression in the two cell types leads to great difference in the *COL2A1* expression. *L-SOX5* and *SOX6* are known to work in concert with *SOX9* during embryological chondrogenesis [27]. One may speculate that these factors may be differentially expressed in the two cell populations and that this in turn may explain the differences observed in type II collagen mRNA expression.

During embryological articular chondrogenesis, the transition from proliferating to hypertrophic chondrocytes is mediated in part by RUNX2 [14]. Hypertrophic chondrocytes synthesize type X collagen and stimulate perichondrial cells to become osteoblasts, which produce bone matrix. Mineralization and vascularization of the bony

collar is stimulated by factors secreted by hypertrophic chondrocytes. The hypertrophic chondrocytes then undergo apoptotic cell death. Osteoblasts invade the cartilage matrix left behind and change the matrix to bony primary spongiosa which eventually becomes bone [25]. Thus, while *RUNX2* and *COL10A1* are markers of hypertrophic chondrocytes in embryological chondrogenesis, these cells eventually die and are not directly involved in bone formation. The exact role of these molecules in adult chondrogenesis in vitro is not known. An upregulation of *COL10A1* has been observed in pellet cultures of MSC and was found to correlate with subsequent calcification and vascular invasion following subcutaneous implantation into SCID mice [32]. However, when the same group implanted MSC in cartilage lesions, no evidence of bone formation was found [40]. We observed high levels of *COL10A1* and *RUNX2* in BM-MSC in HA scaffolds compared with chondrocytes. While this implies a resemblance to hypertrophic chondrocytes, the MSC in the present study also expressed high levels of *SOX9* and *COL2A1*, which are not found in hypertrophic chondrocytes [25]. Thus, the importance of the expression of *RUNX2* and *COL10A1* during in vitro chondrogenesis remains to be determined.

Like *COL2A1,* the level of *AGC1* was very low in undifferentiated BM-MSC at day 7, but increased with time and continuous exposure to CDM. *COL1A1* levels increased as a result of prolonged culture in three weeks in HA scaffolds, even in the absence of CDM. However, only small amounts of type I collagen were observed in the ECM, and nothing was found in the supernatants. Our present observations are in line with a recent study of articular chondrocytes expanded in vitro using a new culture strategy; here, we were able to show that these cells expressed high levels of *COL1A1* mRNA and intracellular protein, but that the fibrils in the ECM were all type II collagen [38].

The most important difference between AT-MSC and BM-MSC cultured in HA scaffolds was that the levels of *COL2A1* in AT-MSC at day 21 were >1,000-fold lower than the levels observed in BM-MSC. This shows that BM-MSC are more likely to outperform AT-MSC when it comes to produce hyaline cartilage in HA scaffolds. Other studies have also published results indicating that BM-MSC may be more easily differentiated into the chondrogenic lineages than AT-MSC [1, 19, 29] and that this reduced chondrogenic potential of AT-MSC may be due to reduced expression of the TGF-beta receptor [17].

In vivo, MSCs have been used without CDM in cartilage lesions in minipigs [40]. This resulted in the formation of some hyaline cartilage, with lower *COL10A1/COL2A1* and *MMP13/COL2A1* ratios than those observed in vitro. In vitro expanded, dedifferentiated chondrocytes have been used to treat human cartilage lesions. This resulted in the formation of a mixed hyaline-fibrocartilaginous repair tissue [11, 24, 47]. The two cell types have not been directly compared in any in vivo models of cartilage repair. Until this is done, preferably using a scaffold system both with and without in vitro CDM supplementation for both cell types, the best cell population for repair of cartilage lesions cannot be finally named.

Conclusion

We find that MSC, when cultured in CDM in a HA scaffold, respond with upregulation of chondrogenic markers measured at the mRNA level. For maximal effect, the MSC needed to be continuously exposed to the CDM. Under these conditions, BM-MSC induced much higher levels of type II collagen mRNA expression than AT-MSC and chondrocytes. However, the chondrocyte cultures used in this study were not supplemented with CDM, as this is the way chondrocytes are currently cultured for clinical applications. Further studies are needed to determine whether chondrocytes exposed to CDM may perform as well as BM-MSC. Type II collagen protein was noted within the cells in the scaffold. Surprisingly, only small amounts of extracellular type II collagen were observed, in stead considerable amounts of type II collagen were found in the culture supernatants. The clinical relevance of our studies is that BM-MSC should be chosen over AT-MSC for tissue engineering of hyaline cartilage. Also, it may be futile to expect hyaline ECM to be formed in vitro in this particular HA scaffold in the conditions studied here, because matrix molecules are washed out of the scaffold during cell culture.

Acknowledgments We are indebted to Fidia Advanced Biopolymers, Italy for generously providing us with HYAFF-11 scaffolds, to Lars Engebretsen, Sverre Løken and Asbjørn Årøen for providing samples of articular cartilage, to Yngvar Fløystrand for doing the bone marrow aspirates, to Lars Haasted, Christiania Park Clinic for providing us with liposuction material and to Linda T. Dorg for technical assistance in the morphological studies.

Conflict of interest statements The authors state that they do not have conflicts of interest to declare.

References

1. Afizah H, Yang Z, Hui JH, Ouyang HW, Lee EH (2007) A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. Tissue Eng 13:659–666
2. Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584
3. Benjamin M, Ralphs JR (2004) Biology of fibrocartilage cells. Int Rev Cytol 233:1–45

4. Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224
5. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) Sox9 is required for cartilage formation. Nat Genet 22:85–89
6. Boquest AC, Shahdadfar A, Brinchmann JE, Collas P (2006) Isolation of stromal stem cells from human adipose tissue. Methods Mol Biol 325:35–46
7. Boquest AC, Shahdadfar A, Fronsdal K, Sigurjonsson O, Tunheim SH, Collas P, Brinchmann JE (2005) Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture. Mol Biol Cell 16:1131–1141
8. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 331:889–895
9. Cristino S, Grassi F, Toneguzzi S, Piacentini A, Grigolo B, Santi S, Riccio M, Tognana E, Facchini A, Lisignoli G (2005) Analysis of mesenchymal stem cells grown on a three-dimensional HYAFF 11-based prototype ligament scaffold. J Biomed Mater Res A 73:275–283
10. de Crombrugghe B, Lefebvre V, Nakashima K (2001) Regulatory mechanisms in the pathways of cartilage and bone formation. Curr Opin Cell Biol 13:721–727
11. Diaz-Romero J, Nesic D, Grogan SP, Heini P, Mainil-Varlet P (2007) Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. J Cell Physiol 214:75–83
12. Facchini A, Lisignoli G, Cristino S, Roseti L, De Franceschi L, Marconi E, Grigolo B (2006) Human chondrocytes and mesenchymal stem cells grown onto engineered scaffold. Biorheology 43:471–480
13. Folkman J, Moscona A (1978) Role of cell shape in growth control. Nature 273:345–349
14. Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem 97:33–44
15. Grigolo B, Lisignoli G, Piacentini A, Fiorini M, Gobbi P, Mazzotti G, Duca M, Pavesio A, Facchini A (2002) Evidence for redifferentiation of human chondrocytes grown on a hyaluronan-based biomaterial (HYAff 11): molecular, immunohistochemical and ultrastructural analysis. Biomaterials 23:1187–1195
16. Hardingham TE, Oldershaw RA, Tew SR (2006) Cartilage, SOX9 and Notch signals in chondrogenesis. J Anat 209:469–480
17. Hennig T, Lorenz H, Thiel A, Goetzke K, Dickhut A, Geiger F, Richter W (2007) Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. J Cell Physiol 211:682–691
18. Holtzer H, Abbott J, Lash J, Holtzer S (1960) The loss of phenotypic traits by differentiated cells in vitro, I. Dedifferentiation of cartilage cells. Proc Natl Acad Sci USA 46:1533–1542
19. Im GI, Shin YW, Lee KB (2005) Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? Osteoarthritis Cartilage 13:845–853
20. Jakobsen RB, Engebretsen L, Slauterbeck JR (2005) An analysis of the quality of cartilage repair studies. J Bone Joint Surg Am 87:2232–2239
21. Kaps C, Frauenschuh S, Endres M, Ringe J, Haisch A, Lauber J, Buer J, Krenn V, Haupl T, Burmester GR, Sittinger M (2006) Gene expression profiling of human articular cartilage grafts generated by tissue engineering. Biomaterials 27:3617–3630
22. Knudson CB, Knudson W (2004) Hyaluronan and CD44: modulators of chondrocyte metabolism. Clin Orthop Relat Res 427(Suppl):S152–162
23. Knudson W, Loeser RF (2002) CD44 and integrin matrix receptors participate in cartilage homeostasis. Cell Mol Life Sci 59:36–44
24. Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grontvedt T, Solheim E, Strand T, Roberts S, Isaksen V, Johansen O (2004) Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. J Bone Joint Surg Am 86:455–464
25. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423:332–336
26. Kuroda R, Ishida K, Matsumoto T, Akisue T, Fujioka H, Mizuno K, Ohgushi H, Wakitani S, Kurosaka M (2007) Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. Osteoarthritis Cartilage 15:226–231
27. Lefebvre V, Behringer RR, de Crombrugghe B (2001) L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 9(Suppl A):S69–75
28. Lisignoli G, Cristino S, Piacentini A, Toneguzzi S, Grassi F, Cavallo C, Zini N, Solimando L, Mario Maraldi N, Facchini A (2005) Cellular and molecular events during chondrogenesis of human mesenchymal stromal cells grown in a three-dimensional hyaluronan based scaffold. Biomaterials 26:5677–5686
29. Liu TM, Martina M, Hutmacher DW, Hui JH, Lee EH, Lim B (2007) Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. Stem Cells 25:750–760
30. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408
31. Marcacci M, Berruto M, Brocchetta D, Delcogliano A, Ghinelli D, Gobbi A, Kon E, Pederzini L, Rosa D, Sacchetti GL, Stefani G, Zanasi S (2005) Articular cartilage engineering with Hyalograft C: 3-year clinical results. Clin Orthop Relat Res 435:96–105
32. Pelttari K, Winter A, Steck E, Goetzke K, Hennig T, Ochs BG, Aigner T, Richter W (2006) Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum 54:3254–3266
33. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A (2000) Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop Relat Res 374:212–234
34. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
35. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ (2005) Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 320:269–276
36. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ (2002) In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci USA 99:4397–4402
37. Shahdadfar A, Fronsdal K, Haug T, Reinholt FP, Brinchmann JE (2005) In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. Stem Cells 23:1357–1366
38. Shahdadfar A, Loken S, Dahl JA, Tunheim SH, Collas P, Reinholt FP, Engebretsen L, Brinchmann JE (2008) Persistence of collagen

type II synthesis and secretion in rapidly proliferating human articular chondrocytes in vitro. Tissue Eng Part A 14:1999–2007
39. Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ (2004) Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells 22:823–831
40. Steck E, Fischer J, Lorenz H, Gotterbarm T, Jung M, Richter W (2009) Mesenchymal stem cell differentiation in an experimental cartilage defect: restriction of hypertrophy to bone-close neocartilage. Stem Cells Dev 18:969–978
41. Steinwachs M, Kreuz PC (2007) Autologous chondrocyte implantation in chondral defects of the knee with a type I/III collagen membrane: a prospective study with a 3-year follow-up. Arthroscopy 23:381–387
42. Tallheden T, Dennis JE, Lennon DP, Sjogren-Jansson E, Caplan AI, Lindahl A (2003) Phenotypic plasticity of human articular chondrocytes. J Bone Joint Surg Am 85(Suppl 2):93–100
43. Tognana E, Borrione A, De Luca C, Pavesio A (2007) Hyalograft C: hyaluronan-based scaffolds in tissue-engineered cartilage. Cells Tissues Organs 186:97–103
44. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 10:199–206
45. Wakitani S, Mitsuoka T, Nakamura N, Toritsuka Y, Nakamura Y, Horibe S (2004) Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant 13:595–600
46. Wasiak J, Clar C, Villanueva E (2006) Autologous cartilage implantation for full thickness articular cartilage defects of the knee. Cochrane Database Syst Rev 3:CD003323
47. Whittaker JP, Smith G, Makwana N, Roberts S, Harrison PE, Laing P, Richardson JB (2005) Early results of autologous chondrocyte implantation in the talus. J Bone Joint Surg Br 87:179–183
48. Yoshida CA, Komori T (2005) Role of Runx proteins in chondrogenesis. Crit Rev Eukaryot Gene Expr 15:243–254
49. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228