

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

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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 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 *AGC1* and *COL1A1* were similar for chondrocyte and BM-MSC scaffold cultures, while *COL10A1* was

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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 [1, 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 [11, 21, 38] in a process termed dedifferentiation [18]. ACI using dedifferentiated chondrocytes frequently results in the production of a mixed hyaline- and 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 culture [42] or supported

by scaffolds, without chondrogenic differentiation medium. Digested biopsies were then filtered through a 70- μ m cell strainer (Becton Dickinson, NJ), washed in medium and seeded in culture flasks (NUNC) with medium supplemented with 50 μ g/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 μ g/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 a 100- μ m 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-cm² 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 [6]. 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 filtered with saline (0.9%) and transported to the laboratory facility. Biopsies were minced manually in DMEM/F12 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%

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 the 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) and 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 μ g/ml streptomycin and 2.5 μ g/ml amphotericin B (Life Technologies-BRL) and subjected to 3–5 h digestion in the same medium supplemented with collagenase type XI (1.2 μ g/ml) and deoxyribonuclease I (0.1 μ g/ml) in 37 C humidified room air with 5% CO₂.

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

Cell cultures in HA scaffolds

The cells were placed in HA scaffolds after 3D5 passages to prevent detection of genomic DNA. Results were analyzed using the $2^{-\Delta Ct}$ -method [30] and presented as values were in a stable log phase of growth. The cells were normalized to the expression of GAPDH, which was used as endogenous control.

For each three-dimensional culture,

2.5×10^6 cells were resuspended in 70 culture medium

and carefully dripped onto a pre-cut 0.5 cm² HYAFF-11

scaffold (generously provided by Fidia Advanced Bio-Scaffolds with MSC were fixed in 0.1 M phosphate-buffered saline (pH 7.4) containing 4% formalin for a week. The specimens were dehydrated in graded ethanol and embedded in paraffin blocks. Immunohistochemistry was performed on rehydrated 4- μ m thick sections using antibodies to human type I collagen (mouse monoclonal antibody, MP Biomedicals, Irvine, CA, 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 chondrogenic differentiation in the MSC, serum-free culture medium containing the following CDM was used [35, 39]: high-glucose DMEM (4.5 g/l; Life Technologies-BRL) supplemented with 500 ng/ml bone morphogenetic protein-6 (R&D Systems, Minneapolis, MN), 10 ng/ml recombinant human transforming growth factor- β 1 (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 μ g sodium selenite) and ELISA

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 throughout all 21 days. In all cultures, medium was changed every 4 days. At each medium change, 2 ml of supernatant was frozen at -20 C for enzyme-linked immunosorbent assays (ELISA). 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).

processed for real-time RT-PCR. Total RNA was extracted using Trizol (Invitrogen, Gaithersburg, MD) according to Statistics

the manufacturer's recommendation.

RNA to cDNA conversion was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Rockville, CA). RNA samples were diluted to 50 total volume with RNase-free water and treated with DNase I to remove genomic DNA contamination. Subsequently, 0.5 μ g RNA was reverse transcribed to cDNA according to

triple. All graphing and statistical analyses were performed using One-way analysis of variance (ANOVA) with a Tukey's 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 \pm SE of biological replicates. Samples from each of the donors were run in triplicate.

Real-time RT-PCR

Real-time RT-PCR

At day 0, 7 and 21 of scaffold cultures for chondrocytes, respectively, on a Multiskan Ascent (Thermo Electron Corp., Vantaa, Finland).

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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 a transient exposure to CDM programs the cells for chondrogenesis, or whether continuous exposure to CDM was

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

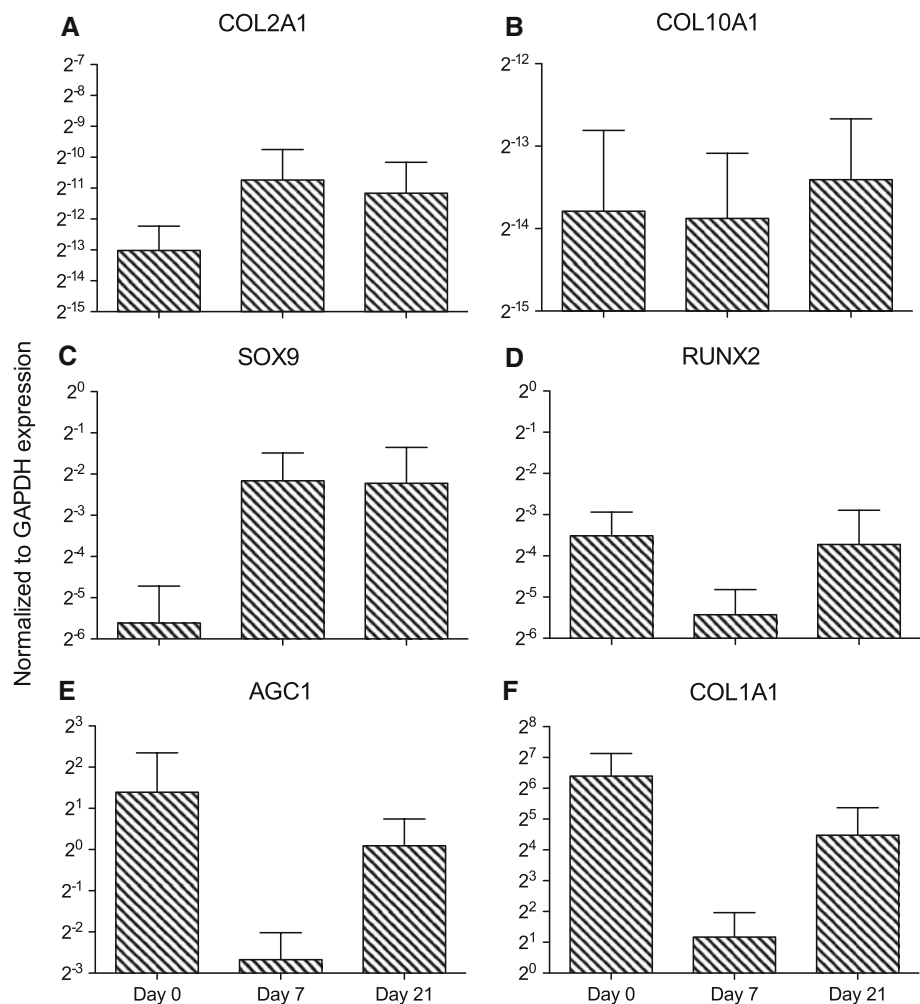
This pattern was mirrored by the mRNA expression of *SOX9*. Consistent with a redifferentiation process, the level of *COL1A1* dropped following establishment of the chondrocytes in the HA scaffold but was moderately increased again at day 21. *COL1A1* expression was not changed over time.

SOX9, the high mobility group box family member 9 factor proposed to control *COL2A1* transcription [5, 16]. In

Differentiation of BM-MSC and AT-MSC

addition, we measured the mRNA levels for the hyaline cartilage proteoglycan aggregate (AGC1) for type I collagen. For the MSC in scaffold cultures, we investigated whether

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



required. *COL2A1* mRNA was not detectable in any MSC CDM, and was greatly upregulated by day 21. This monolayer cultures (data not shown). Figure 2 shows that expression pattern was mirrored by *RUNX2*, albeit at a lower level of expression relative to chondrocytes. A brief exposure to CDM did not significantly improve on of *COL2A1* in the absence of CDM was similar to that observed for BM-MSC (Fig 3). However, for AT-MSC, *COL2A1* mRNA levels were similar to those observed in continuous exposure to CDM only increased the *COL2A1* chondrocytes already at day 7 and were upregulated an expression marginally. Thus, at the end of 21 days of average of 540-fold by day 21. The expression levels of continuous exposure to CDM in HA scaffolds, the *COL2A1* *COL2A1* were partly mirrored by changes in expression of expression was 1,000-fold higher in BM-MSC than in AT-MSC. Again, the levels of *SOX9* mirrored the *COL2A1* low in undifferentiated BM-MSC at day 7. Exposure to expression levels. The expression of *COL1A1* was lower in CDM increased this by day 21, to levels similar to those AT-MSC than both of the other cell populations, while the observed for chondrocytes. The level of *COL1A1*, too, expression of *COL1A1* and *RUNX2* at the end of three increased with time in HA scaffold and continuous expo-weeks in HA scaffolds was similar for all cells and culture to CDM, to levels similar to those observed for chondrocytes. *COL10A1*, however, was much lower in chondrocytes. The expression of *COL10A1* was very high AT-MSC after three weeks than in BM-MSC, but still in BM-MSC compared with chondrocytes, even without 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

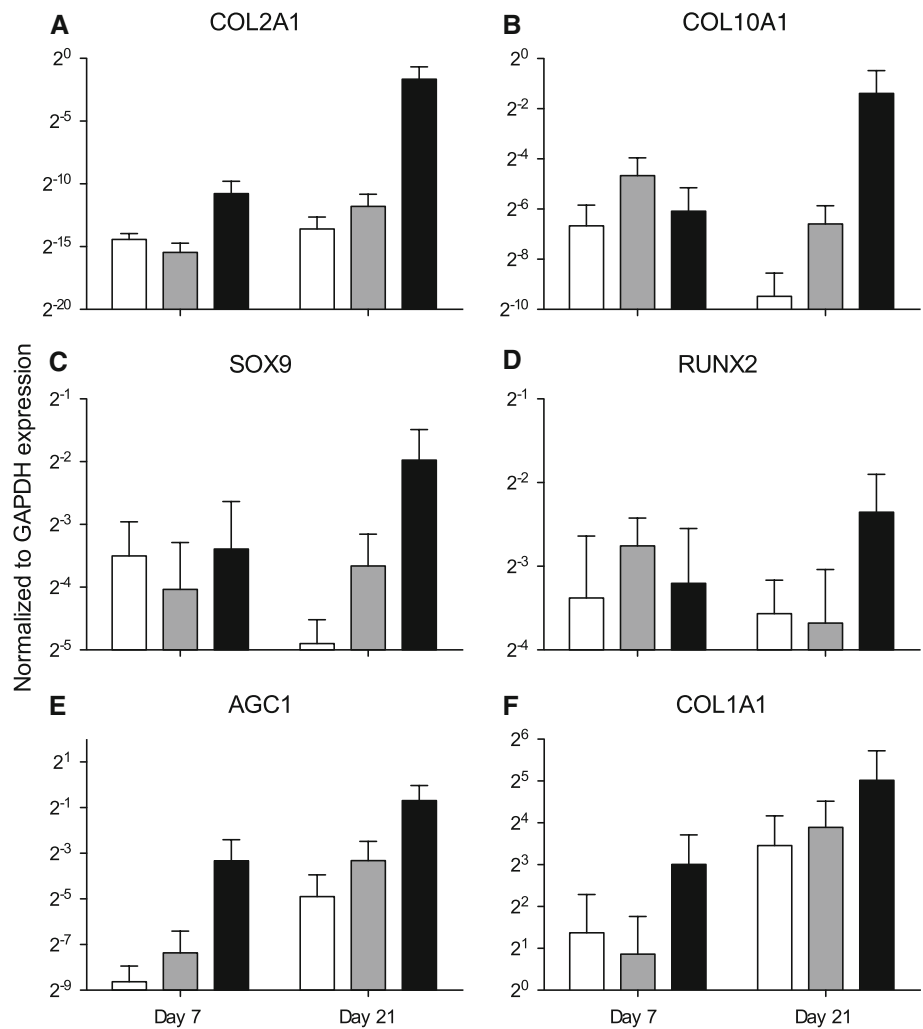
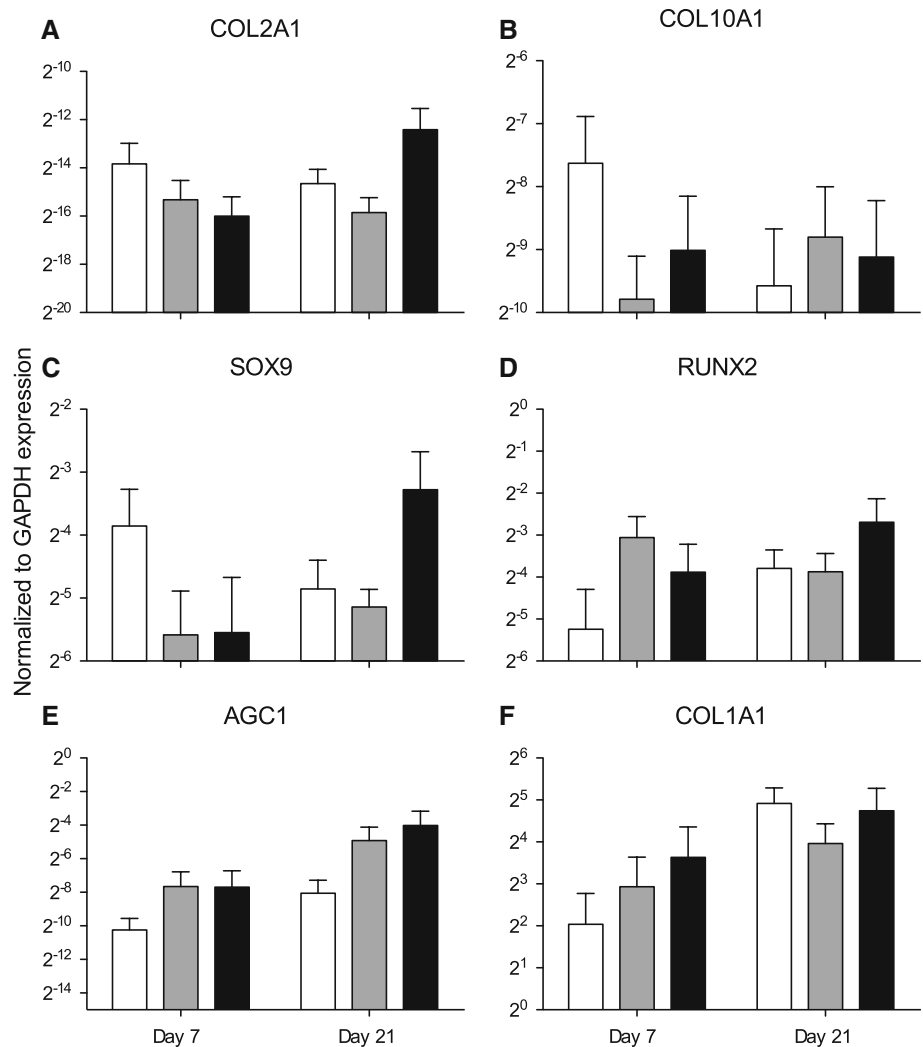


Fig. 3 Results of real-time RT-PCR for AT-MSC in HA scaffolds. All bars are average of five donors (biological replicates) \pm SE normalized to the expression in chondrocytes at day 7. *White bars* represent culture medium only, *gray bars* represent CDM days 0–4 of scaffold culture, *black bars* represent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



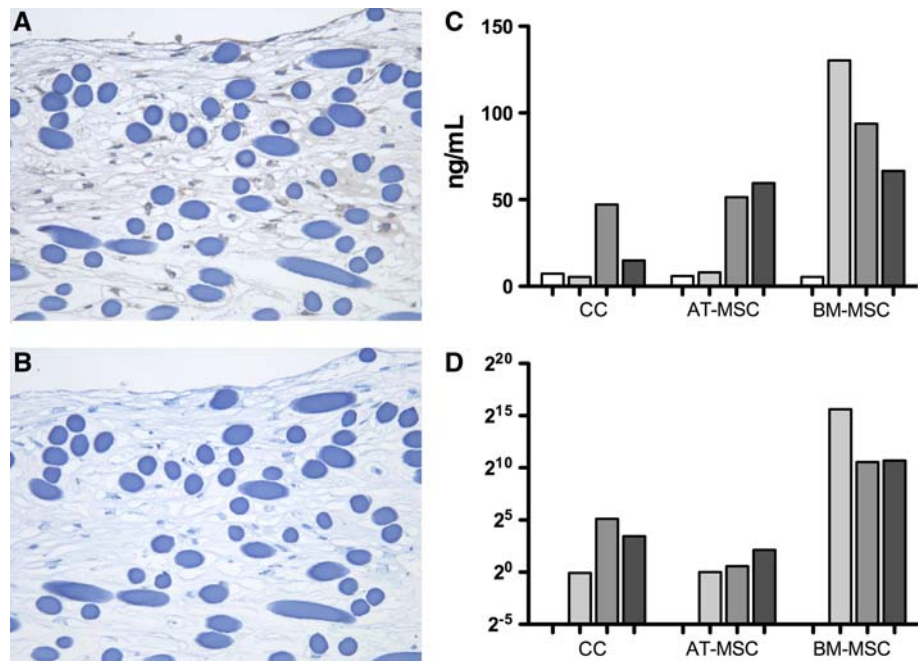
Synthesis and secretion of collagens

Light microscopy of sections of scaffolds revealed that the BM-MSC cells and different cell populations were embedded in the scaffold above the chondrocyte donors. Although obviously not approximately the same density (data not shown). In the linear, a correlation between mRNA expression and scaffold, most of the cells had a long, thin, fibroblast-like detection of type II collagen was indicated in the super-appearance (Fig. 4a, b). The ECM structure was mostly natural (Fig. 4c, d). Interestingly, we found no type I 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 Discussion type I collagen. As collagen was observed within the cells,

but not in the loose ECM, we considered the possibility. The aim of this study was to compare chondrocytes, that the collagens were washed away from the immediate BM-MSC and AT-MSC cultured in a HA scaffold surroundings of the cells by the culture medium. Thus, we performed ELISA on the supernatants from day 20 of MSC (HYAFF-11) known to support chondrogenic differentiation of chondrocytes and MSC continuously exposed to CDM. The 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

comparison in Fig. 4d. High levels of type II collagen were detected in all three supernatants from the scaffolds with the BM-MSC cells and from two of the AT-MSC donors and different cell populations were embedded in the scaffold above the chondrocyte donors. Although obviously not approximately the same density (data not shown). In the linear, a correlation between mRNA expression and scaffold, most of the cells had a long, thin, fibroblast-like detection of type II collagen was indicated in the super-appearance (Fig. 4a, b). The ECM structure was mostly natural (Fig. 4c, d). Interestingly, we found no type I 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 Discussion type I collagen. As collagen was observed within the cells,

Fig. 4 Immunohistochemistry and ELISA analysis. **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. **Ind**, 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 type II is demonstrated at three weeks, with very little collagen deposited in the scaffold. In contrast, 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.

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 [1].

When BM-MSC were established in HA scaffolds, despite the very much higher levels of *COL2A1* mRNA 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. It is interesting that the same level of *SOX9* expression in the cultures continuously exposed to CDM, however, exceeded the level of expression seen in chondrocytes. *COL2A1* mRNA expression in BM-MSC followed those observed for *COL2A1* mRNA, but never exceeded the level of expression seen in chondrocytes. 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 and are 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 perlecan synthesis. In our chondrial 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 spongiosa which eventually becomes bone. 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-MSCs 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-MSCs at day 7, but increased with time and continuous exposure to CDM. *COL1A1* levels increased as a result of prolonged culture in three weeks HA scaffolds, even in the absence of CDM. However, only small amounts of type I collagen were observed in the 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-MSCs and BM-MSCs cultured in HA scaffolds was that the levels of *COL2A1* in AT-MSCs at day 21 were >1,000-fold lower than the levels observed in BM-MSCs. This shows that BM-MSCs are more likely to outperform AT-MSCs when it comes to produce hyaline cartilage in HA scaffolds. Other studies have also published results indicating that BM-MSCs may be more easily differentiated into the chondrogenic lineages than AT-MSCs [19, 29] and that this reduced chondrogenic potential of AT-MSCs may be due to reduced expression of the TGF- β 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

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

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