MICROMASS CO-CULTURE OF HUMAN ARTICULAR CHONDROCYTES AND HUMAN BONE MARROW MESENCHYMAL STEM CELLS TO INVESTIGATE STABLE NEOCARTILAGE TISSUE FORMATION *IN VITRO*

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Abstract

Cell therapies for articular cartilage defects rely on expanded chondrocytes. Mesenchymal stem cells (MSC) represent an alternative cell source should their hypertrophic differentiation pathway be prevented. Possible cellular instruction between human articular chondrocytes (HAC) and human bone marrow MSC was investigated in micromass pellets. HAC and MSC were mixed in different percentages or incubated individually in pellets for 3 or 6 weeks with and without TGF-β1 and dexamethasone (±T±D) as chondrogenic factors. Collagen II, collagen X and S100 protein expression were assessed using immunohistochemistry. Proteoglycan synthesis was evaluated applying the Bern score and quantified using dimethylmethylene blue dye binding assay. Alkaline phosphatase activity (ALP) was detected on cryosections and soluble ALP measured in pellet supernatants. HAC alone generated hyaline-like discs, while MSC formed spheroid pellets in ±T±D. Co-cultured pellets changed from disc to spheroid shape with decreasing number of HAC, and displayed random cell distribution. In -T-D, HAC expressed S100, produced GAG and collagen II, and formed lacunae, while MSC did not produce any cartilage-specific proteins. Based on GAG, collagen type II and S100 expression chondrogenic differentiation occurred in -T-D MSC co-cultures. However, quantitative experimental GAG and DNA values did not differ from predicted values, suggesting only HAC contribution to GAG production. MSC produced cartilage-specific matrix only in +T+D but underwent hypertrophy in all pellet cultures. In summary, influence of HAC on MSC was restricted to early signs of neochondrogenesis. However, MSC did not contribute to the proteoglycan deposition, and HAC could not prevent hypertrophy of MSC induced by chondrogenic stimuli.

Keywords: Human articular cartilage, human articular chondrocytes, co-culture, human bone marrow mesenchymal stem cells, pellet cultures.

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Introduction

Articular cartilage is an avascular, highly specialized connective tissue lining the extremity of the bones within the diarthrodial joints. Cartilage provides a low-friction interface and a wear-resistant surface for load support, load transfer and motion (Schulz and Baker, 2007). The innate avascularity, low cellularity and low cell turnover of the tissue represent major hurdles for natural regeneration process upon injuries. Lesions, associated with joint pain and disturbed function, are generally believed to progress to severe forms of osteoarthritis (Richter, 2007). Therefore, to avoid further degeneration of focal articular defects, development of successful treatments allowing regeneration of cartilage lesions represents a major challenge.

Autologous Chondrocytes Implantation (ACI), a cell-based cartilage repair treatment, is one of the most promising techniques to obtain a long-term regeneration of the tissue (Brittberg *et al.*, 1994; Peterson *et al.*, 2000; Saris *et al.*, 2008). With chondrocytes contributing to less than 5% of the total tissue volume, and the limited biopsy size that can be collected from a minor weight-bearing area, only a small number of cells can be retrieved from the patient. Thus, to obtain the required cell number necessary for re-implantation, human articular chondrocytes (HAC) need to be expanded *in vitro*, a process during which the cells de-differentiate and lose their chondrogenic capacities to re-build stable hyaline-like tissue upon re-implantation (Mandelbaum *et al.*, 2007). Chondrocyte de-differentiation has been characterized by changes in cell morphology, gene expression, extracellular matrix synthesis, surface marker expression patterns (Von der Mark *et al.*, 1977; Schnabel *et al.*, 2002; Marlovits *et al.*, 2004; Diaz-Romero *et al.*, 2005), as well as a progressive loss of intrinsic potential to form stable cartilage-like tissue *in vitro* (Giovannini *et al.*, 2010), and after intramuscular injection in nude mice (Dell'Accio *et al.*, 2001). These observations could explain why fibrocartilaginous tissue, rather than hyaline cartilage, is often found in patients following ACI treatment. Due to their chondrogenic differentiation potential *in vitro*, mesenchymal stem cells (MSC) have been considered as an attractive alternative cell source for the treatment of cartilage defects. However, important limiting factor for the resulting cartilage-like tissue is further chondrogenic differentiation towards hypertrophy, characterized by the upregulation of hypertrophic markers, namely collagen type X, MMP-13 and alkaline phosphatase (Barry *et al.*, 2001; Ichinose *et al.*, 2005; Pelttari *et al.*, 2006).

To overcome these problems, a possible approach would be mixing the cells with chondrogenic potential (HAC) with other cells with multilineage potential (MSC) in a co-culture system. Such system could allow for cellular crosstalk, instruction and stabilisation of the chondrogenic phenotype. Several *in vitro* cell culture models have shown that cells can be influenced by other cells and the surrounding environment. In a transwell system, co-culture of human immortalized chondrocytic line with immortalized MSC line on each side of the insert lead to an upregulation of chondrogenic genes but not of osteogenic markers in the early committed MSC (Chen *et al.*, 2009). Soluble factors released by rabbit articular chondrocytes were shown to inhibit terminal differentiation of growth plate chondrocytes with a suppression of alkaline phosphatase activity and calcification (Jikko *et al.*, 1999). Human embryonic stem cells (hES) were able to produce more glycosaminoglycans in the presence of primary chondrocytes in a transwell system (Vats *et al.*, 2006). The chondrogenic potential of hES could also be enhanced in high density micromass pellet co-culture with irradiated human chondrocytes (Bigdeli *et al.*, 2009). Furthermore, rat MSC cultured in alginate expressed SOX9, a marker of chondroprogenitor cells, and repressed collagen type X, a marker of hypertrophic cells, in the presence of rat hip cartilage tissue (Ahmed *et al.*, 2007). Finally, passaged bovine chondrocytes co-cultured with primary bovine chondrocytes were induced to re-differentiate and form cartilage like-tissue *in vitro* (Gan and Kendall, 2007).

To date, no studies have investigated the outcome of human chondrocytes co-culture with human adult bone-marrow derived MSC in a micromass pellet *in vitro* model. In our previous study, we have established a threshold of HAC cumulative population doublings identifying cells with retained intrinsic chondrogenic potential i.e. the ability to re-differentiate into cartilage-like tissue in a defined serum-free 3D system devoid of chondrogenic factors, and possibly instruct other cells (Giovannini *et al.*, 2010). In this study we investigated whether HAC with retained intrinsic chondrogenic potential could instruct human MSC towards chondrogenesis and could stabilize MSC phenotype by preventing hypertrophic differentiation.

Materials and Methods

Isolation and expansion of human articular chondrocytes and human mesenchymal stem cells

Hyaline articular cartilage was obtained at autopsy from femoral condyles of knee joints from 11 human donors (age range 45-64 years, mean = 66.3±5.2) without history of joint disease, according to local ethical committee guidelines (Bernische Ethik-Kommission). Isolation and culture of HAC was carried out as previously described (Diaz-Romero *et al.*, 2005; Giovannini *et al.*, 2010). Briefly, cells were released from the extracellular matrix by sequential enzymatic digestion using 2.5mg/ml pronase (Roche, Rotkreuz, Switzerland) for 1h followed by 250µg/ml collagenase-P (Roche) overnight. HAC were expanded for 8 days in DMEM-F12 (Life Technologies, Basel, Switzerland) containing 10% foetal bovine serum (FBS) (Biosera, Sissach, Switzerland), 100U/ml penicillin and 100µg/ml streptomycin (Life Technologies), and 10µg/ml L-ascorbate-2-phosphate (Sigma, Buchs, Switzerland).

Bone marrow was collected from 11 human donors (age range 34-75 years, mean = 59.2±4.7) undergoing a spine fusion surgical procedure, according to the local ethical committee permission (Bernische Ethik-Kommission). Bone marrow was aspirated from the iliac crest and processed as previously described (Diaz-Romero *et al.*, 2008). Mononuclear cells were obtained by Ficoll gradient centrifugation (GE Health Care, Otelfingen, Switzerland). Cells were seeded at 1.4 x 10^5 cells per cm^2 in Cambrex culture medium (Lonza, Basel, Switzerland). At subconfluence, MSC were trypsinised and further expanded for 3 passages.

3D pellet culture

Chondrogenesis was performed in micromass pellet cultures as previously described (Giovannini *et al.*, 2008). Briefly, HAC and MSC were harvested, washed once with phosphate buffered saline (PBS) and resuspended in serum free medium consisting of Dulbecco's Minimal Essential Medium (DMEM) high glucose (4.5g/l) supplemented with 1% ITS+ (1mg/ml insulin from bovine pancreas, 0.55mg/ml human transferrin, 0.5µg/ml sodium selenite, 50mg/ml bovine serum albumin, 470 µg/ml linoleic acid (Sigma)), 0.1mM L-ascorbate-2-phosphate, 0.4mM proline (Sigma) in the absence (-T-D) or presence (+T+D) of chondrogenic factors: 10^{-7}M dexamethasone (Sigma) and 10ng/ml human recombinant TFG-β1 (Acris GmbH, Herford, Germany). HAC and MSC were mixed at different percentages: 50-50, 25-75 and 10-90. HAC alone (100) and MSC alone (100) were used as references. Cell viability for both cell types exceeded 90%. Upon mixing, a total of 5 x 10^5 cells per pellet was centrifuged in 15ml polypropylene tubes at 170g and maintained at 37°C in 5% CO_2. The medium was replaced every 3 days. After 3 or 6 weeks of culture, pellets were collected and processed for histology and immunohistochemistry or analyzed for glycosaminoglycan (GAG) and DNA content. Supernatants were collected after 6 weeks to determine alkaline phosphatase (ALP) activity.

Cell labelling

To examine the cell distribution in pellets, HAC were labeled with the green fluorescent dye PKH67, and MSC with the red fluorescent dye PKH26 (both from Sigma) prior to pellet co-culture. The labelling procedure was performed according to the manufacture's protocol using a final concentration of 2x10^{-6}M of dye per 10^6 cells. Labelled cells were co-cultured for up to 3 weeks as described above. For cryopreservation, pellets were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen at -80°C. Fluorescence was observed on 5-7µm sections with an Axioplan 2 microscope (Zeiss, Feldbach, Switzerland), using fluorescein isothiocyanate (FITC) (green) and rhodamine (red) filters. The threshold for PKH67 and PKH26 was set by evaluating the highest exposure where unlabeled HAC or MSC pellets showed no autofluorescence.

Histology and immunohistochemistry

Pellets were fixed for 4 hours in 4% paraformaldehyde, embedded in paraffin and cut into 4µm thick sections. Alcian blue and Safranin-O staining was performed for detection of GAG deposition. The Bern Score visual grading system was used for the assessment of pellets (Grogan *et al.*, 2006). Three characteristics were considered for the evaluation: (1) the uniformity and intensity of Safranin-O staining; (2) the distance between cells and the amount of matrix produced; (3) the cell morphology (condensed/necrotic/pycnotic bodies, spindle/fibrous, rounded chondrogenic morphology).

Immunohistochemistry (IHC) for collagen type II, collagen type X and S100 was performed as previously described (Giovannini *et al.*, 2010). After pretreatment with 0.1% trypsin for 20min at 37°C for collagen type II and S100, or 2mg/ml hyaluronidase for 60min followed by 0.02mg/ml Protease XXIV for 60min for collagen type X, the sections were incubated overnight with a monoclonal primary antibody against collagen type II (II-II6B3, Hybridoma Bank, University of Iowa) at a dilution of 1:500 or with a monoclonal antibody against collagen type X (clones X-34 and X-53, a kind gift from Dr. K. von der Mark) at a dilution of 1:100, in a humid chamber at 4°C. Bound antibodies were detected using Dako REAL™ Detection System K5005 (Dako, Baar, Switzerland) according to the manufacturer's protocol. For detection of S100, sections were incubated with a polyclonal rabbit primary antibody against S100 at a dilution of 1:1000 (Dako Z0311) at room temperature for 2 hours. Staining was revealed with Polymer-HRP (Dako EnVision®+Dual Link System-HRP (DAB+) K4065) for 30 min. Incubation without the primary antibody was used as a negative control.

Alkaline phosphatase enzyme activity

Detection of ALP enzyme activity on 3 weeks pellet cryosections was performed with an alkaline phosphatase staining kit (Sigma). Sections were fixed with acetone-citrate-formaldehyde for 30 seconds, rinsed with distilled water and incubated with alkaline-dye for 15min.

ALP activity was measured in pellet supernatants according to previously established protocol (Dickhut *et al.*, 2009). In brief, after the last media change, replacing regular medium with the phenol red free equivalent, three days old culture supernatants were collected at the end of the pellet culture (6 weeks). Specific ALP activity was assayed as the release of *p*-nitrophenol from *p*-nitrophenolphosphate and measured spectro-photometrically at 405nm. Standard curves were established using bovine intestinal mucosal alkaline phosphatase (Sigma).

Biochemical analysis

Biochemical analysis of pellets was performed as previously described (Giovannini *et al.*, 2010). Pellets were digested with proteinase K (0.25ml of 1mg/ml proteinase K in 50mM Tris) (Sigma) overnight at 56°C. The sulphated GAG content was measured using dimethylmethylene blue dye binding assay with chondroitin sulphate (Sigma) as a standard. The cellularity was measured based on the DNA content using CyQuant® kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) with lambda DNA as a standard. Cellularity (DNA) and glycosaminoglycan (GAG) contents of pellets were quantified and expressed as the GAG/DNA ratio.

Statistical analysis

Values of GAG, DNA, GAG/DNA and ALP are reported as the mean ± standard error of the mean (SEM). A two-tailed, unpaired Student's *t*-test was used to determine the significance of changes between predicted and experimental values under -T-D and +T+D conditions. *P*-values < 0.05 were considered statistically significant.

Results

HAC and MSC produce pellets of different shape

HAC and MSC expanded in monolayer culture were mixed and incubated in pellets for 3 weeks at two different percentages (50-50, 25-75) and for 6 weeks at three different percentages (50-50, 25-75, 10-90). 10-90 HAC-MSC percentage was included to investigate whether a lower number of HAC during longer incubation time would suffice to induce chondrogenesis in MSC. Regardless of the presence or absence of TGF-β1 and dexamethasone (±T±D), HAC and co-cultured cells formed pellets within 24 hours, with their size continuously increasing during the incubation. Pellets formed from MSC alone (100) in -T-D condition remained small. Independently of the condition tested (±T±D), HAC alone (100) generated hyaline-like disc-shaped translucent pellets that resembled the thin cartilage surface lining the bone extremities (Fig. 1). In contrast, pellets formed from MSC alone (100) produced spheroid pellets of softer consistency. By decreasing the number of HAC relative to MSC, co-cultured pellets acquired more spheroid shape yet were larger compared to MSC alone. The sizes of co-cultured 50-50 and 25-75 pellets were similar in -T-D compared to +T+D condition. In contrast, 100 MSC pellets and co-cultured 10-90 pellets incubated in +T+D condition were larger compared to pellets made in -T-D condition. 100 HAC pellets had a slightly larger diameter in -T-D compared to +T+D condition.

To investigate cell distribution within co-cultured pellets, pellets formed by individual cell types alone (HAC labelled green, MSC labelled red), or in different percentages were examined after 3 weeks of incubation. In all co-cultured pellets, HAC and MSC were randomly distributed without any particular pattern, independently of the condition tested (Fig. 1B).

Chondrogenic differentiation occurs in co-cultured pellets

The extracellular matrix produced in pellet cultures was evaluated for the presence of sulphated proteoglycans and collagen type II. Moreover, to identify cells undergoing chondrogenesis, cells were stained for S100 protein, an intracellular calcium binding protein found in all chondroid

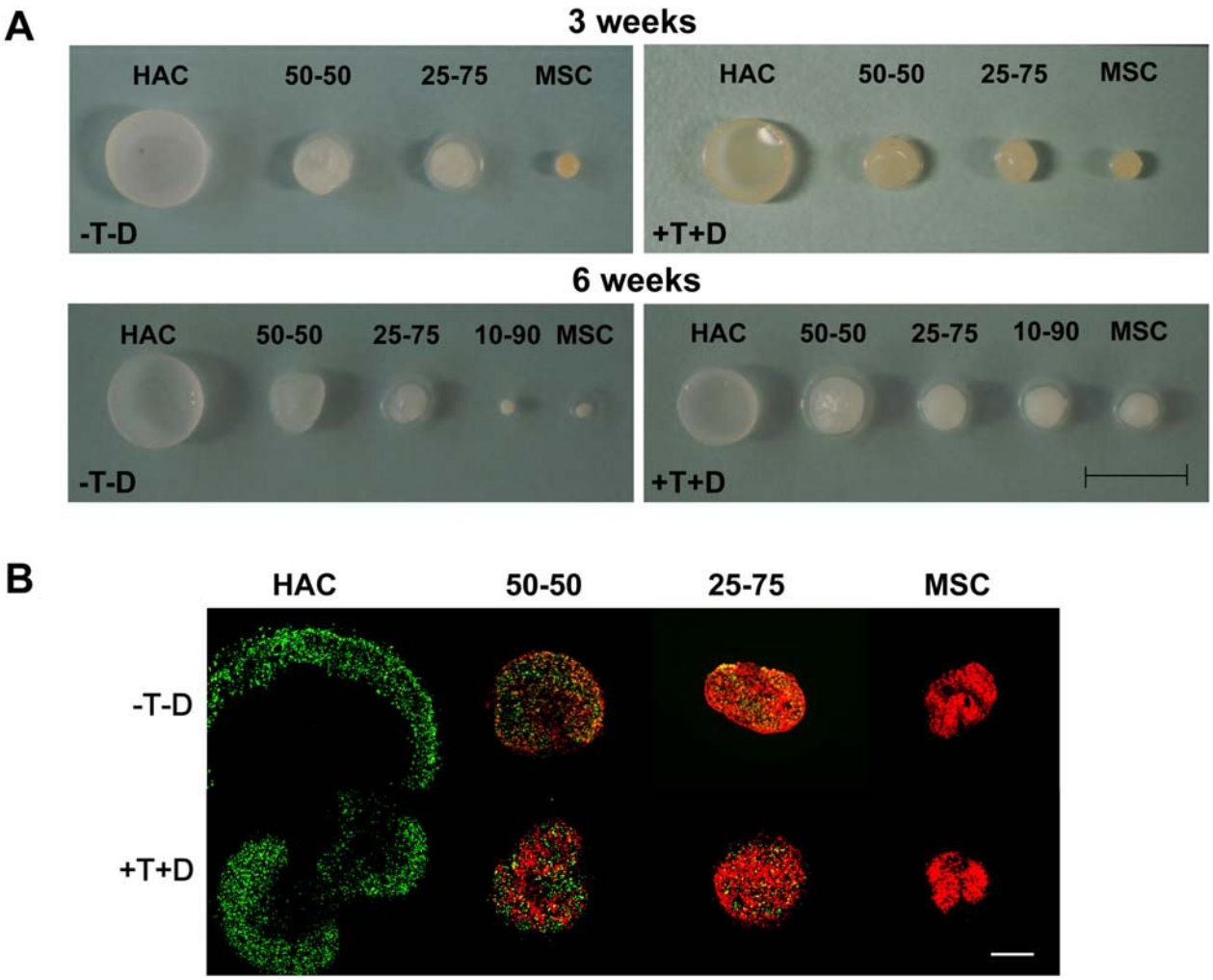

Fig. 1. HAC and MSC produce pellets of different shape and distribute randomly within the co-cultured pellets. (A) Macroscopic morphology of pellets cultured for 3 or 6 weeks in a serum free media in the absence (-T-D) or presence (+T+D) of TGF-β1 and dexamethasone. Pellets were prepared with HAC or MSC alone or mixed at 50-50, 25-75 and 10-90 HAC-MSC percentages. Bar=0.5cm. **(B)** HAC and MSC distribution in pellets using fluorescent microscopy. HAC were labelled with the green fluorescent dye PKH67 and MSC with the red fluorescent dye PKH26 prior to pellet preparation. Pellets were prepared with HAC or MSC alone or mixed at 50-50 and 25-75 HAC-MSC percentages and incubated in -T-D and +T+D for 3 weeks. Magnification 5x, bar=500μm.

tissues studied and expressed early during chondrogenic differentiation process (Mohr *et al.*, 1985; Wolff *et al.*, 1992).

After 3 weeks of culture, independently of the condition tested (±T±D), 100 HAC pellets expressed S100 and produced extracellular matrix rich in sulphated proteoglycans and collagen type II (Fig. 2). In agreement with our previous findings (Giovannini *et al.*, 2010), higher amount of matrix with more lacunae formation was observed in -T-D pellets, resulting in higher Bern score value (8.8) compared to +T+D 100 HAC pellets (7.2). In contrast, 100 MSC pellets underwent partial chondrogenesis only in +T+D, with S100 expression, production of proteoglycans (Bern score of 2.2) and collagen type II only in some areas of the pellets. While in 100 MSC pellets -T-D condition did not result in production of cartilage specific extracellular matrix (only fibrous tissue, Bern score of 0), the presence of 25 or 50 HAC lead to deposition of sulphated proteoglycans and the production of collagen type II within entire co-cultured pellets, resulting in high Bern score values (6.2 and 6.7, respectively). Furthermore, almost all cells within the co-cultured -T-D pellets expressed S100, suggesting chondrogenic differentiation of both cell types. Presence of 25 and 50 HAC within the co-cultured pellets in +T+D condition also resulted in prominent sulphated proteoglycan and collagen type II deposition, as well as S100 protein expression. In +T+D condition, 100 MSC and co-cultured pellets contained a layer of flattened cells at the periphery that did not undergo chondrogenic differentiation (no matrix deposition or detection of S100) but formed a perichondrium-like structure. This cellular organisation was not observed in -T-D pellets. In contrast, more matrix synthesis accompanied with more lacunae formation was seen in co-cultured pellets in -T-D condition, resulting in higher Bern score values compared to +T+D pellets.

Similar data were obtained after 6 weeks of culture, with proteoglycans and collagen type II deposition and S100 protein expression in 100 HAC and co-cultured

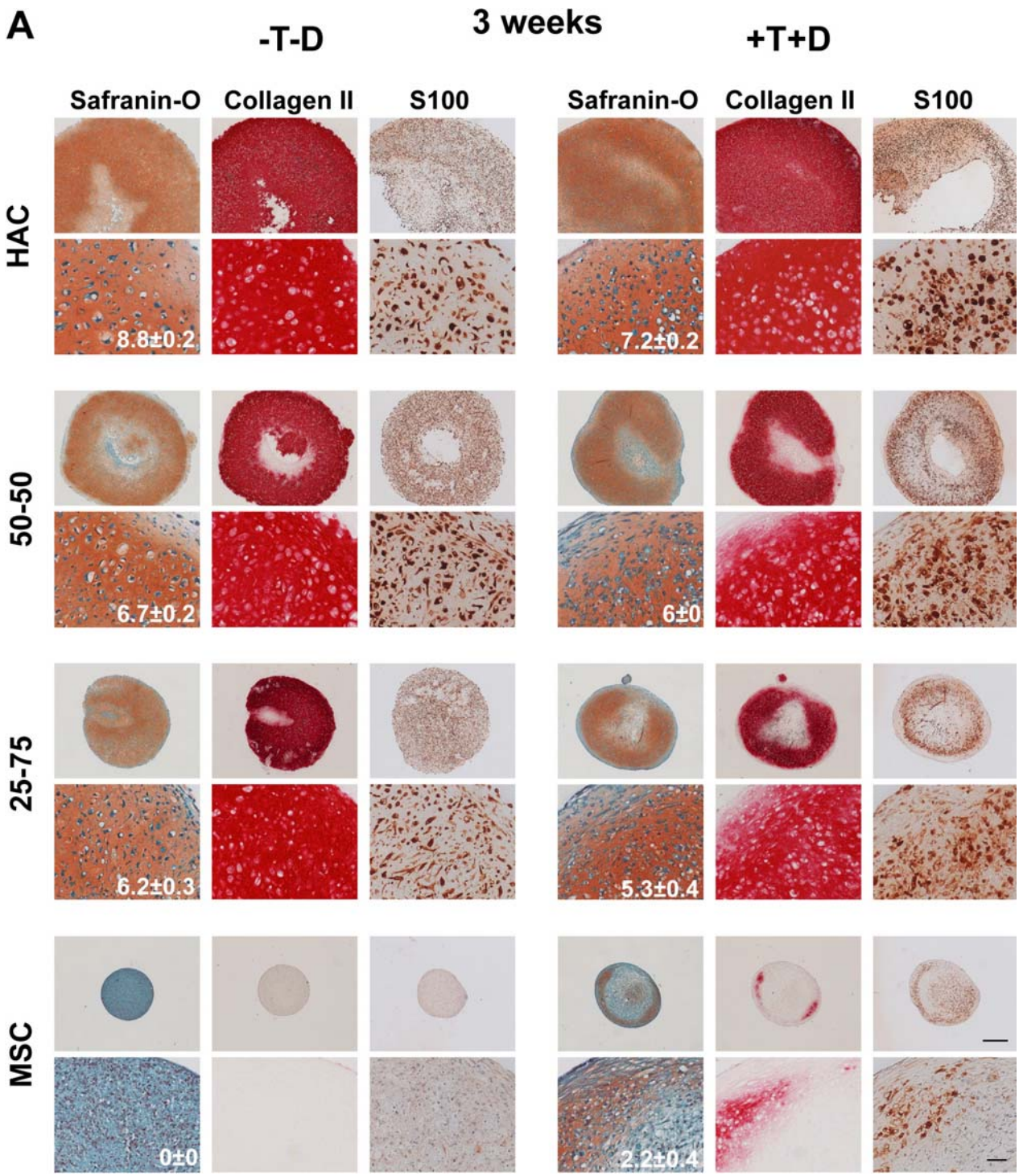

Fig. 2. Co-culture of HAC and MSC results in neocartilagenous tissue formation. Chondrogenic differentiation of 100 HAC, co-cultured 50-50, 25-75 HAC-MSC and 100 MSC was investigated in pellets incubated without or with TGF-β1 and dexamethasone (±T±D) after 3 weeks. Proteoglycan deposition was assessed with Safranin-O staining, and collagen type II deposition and S100 protein expression using immunohistochemistry. Representative data from one donor are shown at magnification 5x, bar=500µm and magnification 40x, bar=50µm. Bern scores are shown as the mean±SEM from 3 different donors.

pellets, independently of the incubation condition (Fig. 3). Substantially higher extracellular matrix deposition was observed in 100 MSC +T+D compared to -T-D pellets (Bern score 3.7 compared to 2.2). Proteoglycans, collagen type II and S100 were detected in parts of -T-D and throughout the +T+D 10-90 co-cultured pellets with corresponding Bern score values of 3.4 and 5.5. Importantly, in comparison to 100 MSC, where in -T-D condition neochondrogenesis was not detected, the presence of 10 HAC resulted in deposition of proteoglycans, collagen type II and S100 expression. Similarly, 10 HAC induced improvement in neochondrogenesis in +T+D when compared to MSC alone.

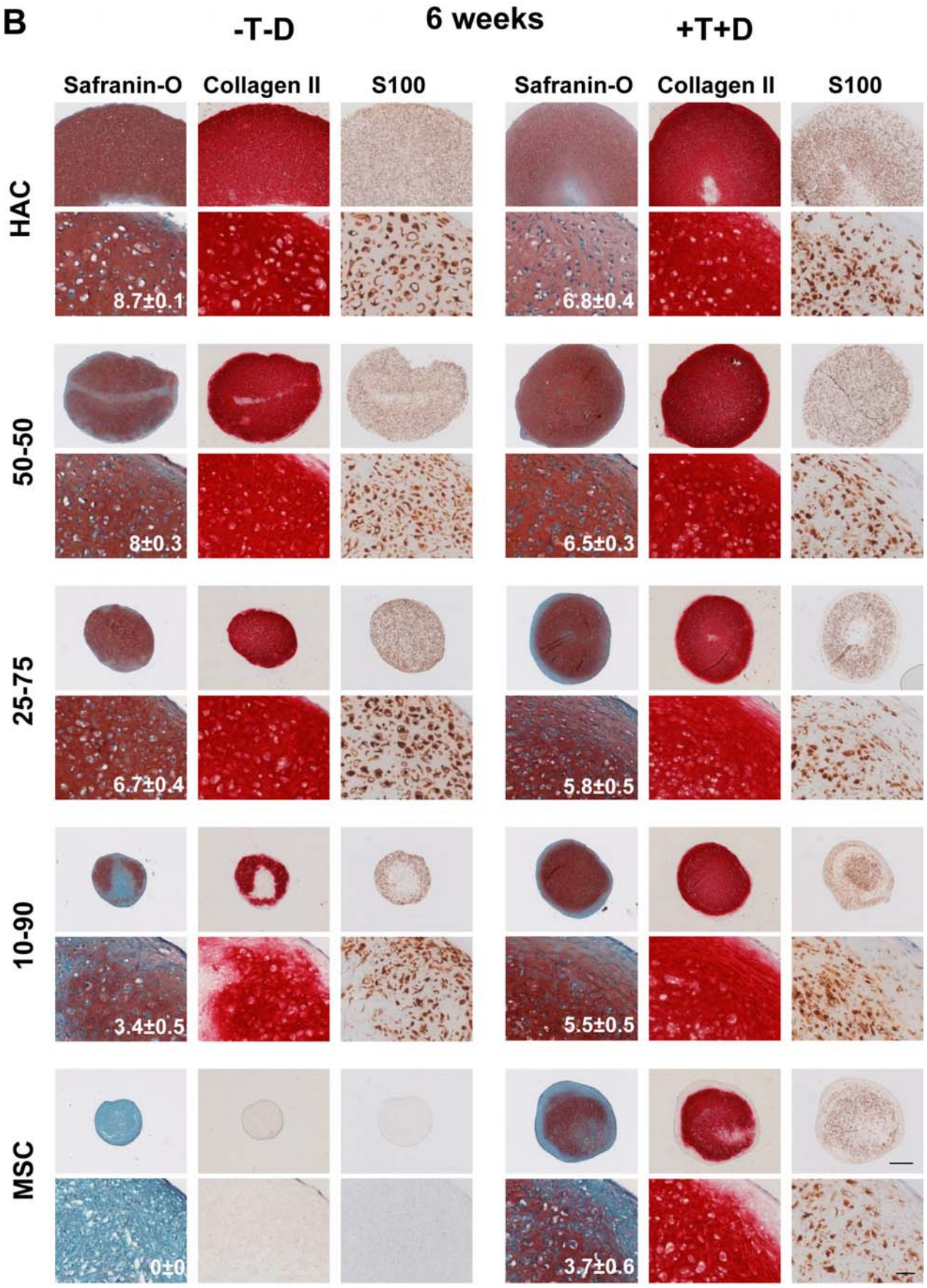

Fig. 3. Longer co-culture of HAC and MSC results in increased neocartilage tissue formation. Chondrogenic differentiation of 100 HAC, co-cultured 50-50, 25-75, 10-90 HAC-MSC and 100 MSC was investigated for 6 weeks. Proteoglycan deposition was assessed with Safranin-O staining, and collagen type II deposition and S100 protein expression via immunohistochemistry. Representative data from one donor are shown at magnification 5x, bar=500µm and magnification 40x, bar=50µm. Bern scores are shown as the mean±SEM from 5 different donors.

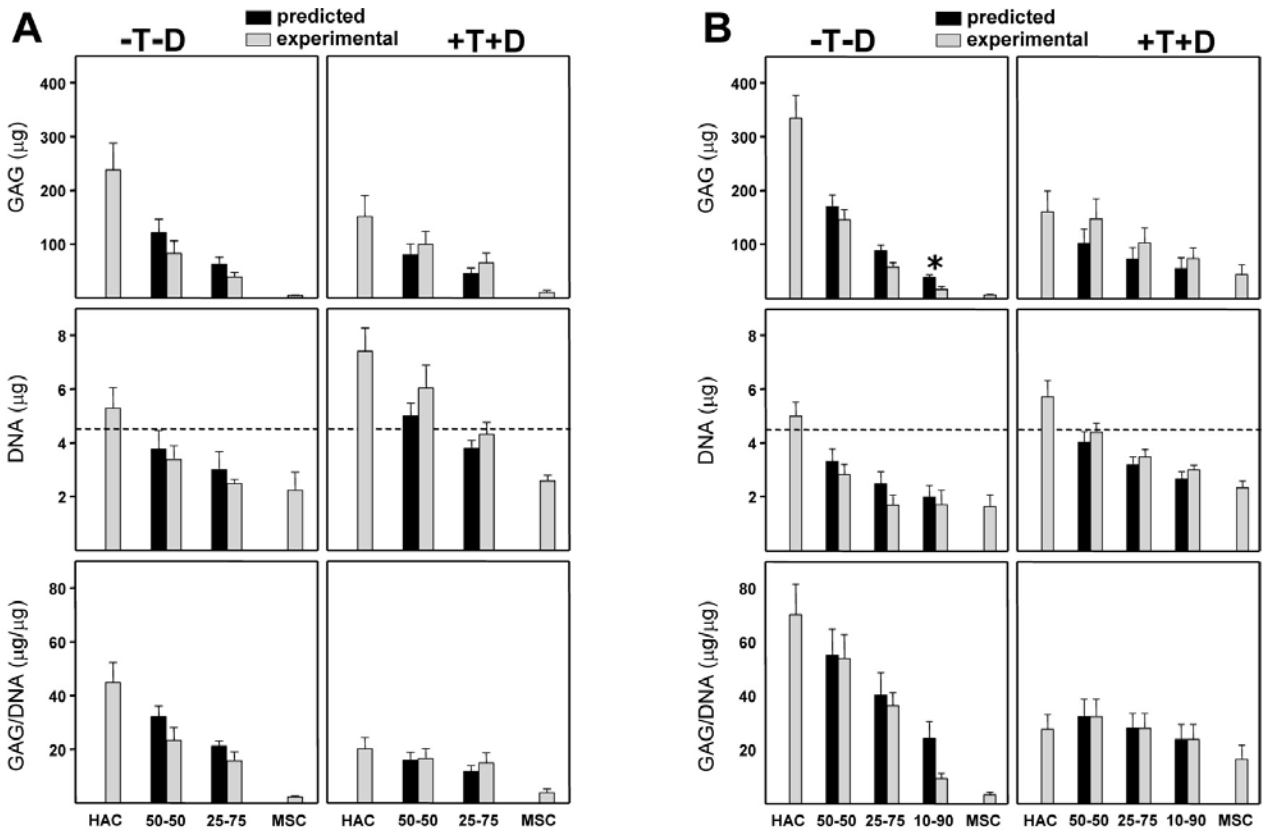

Fig. 4. Biochemical analysis of GAG deposition in pellets. Pellets cultured for 3 **(A)** or 6 weeks **(B)** were analyzed for GAG content (upper panels), DNA content (middle panels) and GAG content normalized by DNA content (lower panels). The dotted lines indicate the initial DNA content at the beginning of cultures. The experimental values of co-cultured pellets were compared with predicted values, calculated as the sum of the corresponding values obtained for HAC and MSC pellets alone corrected for the HAC-MSC percentages in the co-cultured pellets. The results are expressed as mean±SEM (error bars) from 3 (3 weeks cultures) or 5 (6 weeks cultures) different donors. Statistical significance difference (P<0.05) between experimental and predicted values of co-cultured pellets is indicated with an asterisk.

Co-culture of HAC and MSC does not improve proteoglycan production

To evaluate proteoglycan deposition quantitatively, biochemical analysis was performed on all pellet cultures. Analysis of pellets from each cell type demonstrated that the amount of deposited GAG as well as the DNA content was substantially lower in 100 MSC compared to 100 HAC pellets in 3 and 6 weeks in both conditions (±T±D) (P < 0.05 in all cases) (Fig. 4A,B). Based on the initial DNA amount calculated at the time of pellet preparation, cell death occurred in 100 MSC pellets, while proliferation occurred in 100 HAC pellet. The +T+D condition did not prevent cell death in MSC pellets, yet induced proliferation in 100 HAC pellets mainly during the first 3 weeks of culture. The differential cell type response to the presence/absence of chondrogenic factors was emphasized during time of culture albeit without statistical significance: GAG deposition increased in HAC but not MSC after 6 weeks in -T-D. Conversely, longer incubation resulted in improved GAG production in MSC but not HAC in +T+D condition, where the inhibition of GAG production was noted, confirming previous data (Giovannini *et al.*, 2010). Normalization of GAG to the DNA content revealed an inhibitory effect of chondrogenic factors in 100 HAC at 3 and 6 weeks, a stimulatory effect on MSC at 6 weeks (P < 0.05) yet no effect on 100 MSC at 3 weeks.

A decrease in GAG production and DNA amount occurred with decreasing amount of HAC in all co-cultured pellets, although less pronounced in +T+D at 6 weeks. Overall, more GAG was produced in pellets incubated for 6 relative to 3 weeks, indicating continuous matrix deposition during incubation.

After normalization, the GAG/DNA values decreased in -T-D with decreasing number of HAC in all co-cultured pellets. In contrast, in +T+D the GAG/DNA values remain similar to the values for HAC alone. Furthermore, an increase in GAG/DNA values in both conditions was observed at 6 weeks in comparison to 3 weeks.

To evaluate individual HAC and MSC contribution in co-cultured pellets, predictive values were calculated based on the sum of values obtained from individual HAC and MSC pellets corrected for the percentage of each cell type within each pellet. We anticipated that the comparison between the predictive and experimental values could

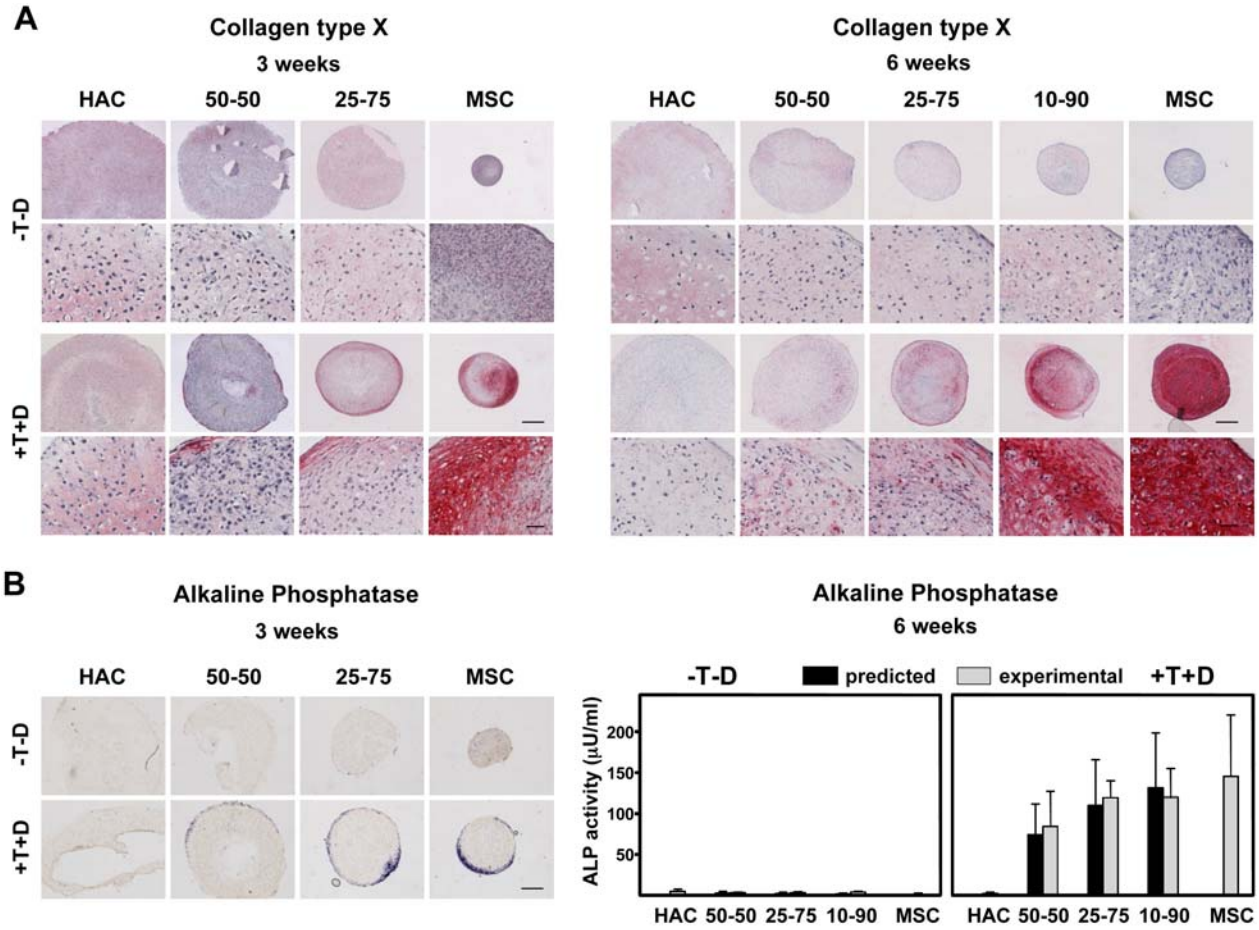

Fig. 5. Hypertrophic differentiation of MSC is induced only in the presence of chondrogenic factors and is not influenced by HAC. (**A**) Immunohistochemical staining for collagen type X was performed on pellet sections after 3 or 6 weeks of culture. Overview of the entire pellets section (magnification 5x, bar=500µm) and higher magnification of a representative pellet area (magnification 40x, bar=50µm) are shown. (**B**) Alkaline phosphatase activity was detected in cryofixed sections after 3 weeks (magnification 5x, bar=500µm). Soluble ALP activity was quantified in culture supernatants after 6 weeks. The results of soluble ALP activity are expressed as mean±SEM (error bars) from 3 different donors. Predicted values were calculated as in Figure 4 and compared with experimental values. No statistically significant differences ($P<0.05$) were observed between experimental and predicted values of co-cultured pellets.

clarify whether HAC and MSC interactions in the co-cultures result in an increase or decrease of GAG production.

In comparison to predicted values for GAG and DNA under -T-D condition, experimental values demonstrated a tendency of decrease without statistical significance in all co-cultured pellets except for 10-90 after 6 weeks ($P<0.05$). The opposite trend was observed in +T+D condition. While the experimental GAG/DNA values in -T-D condition demonstrated a tendency of a slight decrease compared to predicted values, in +T+D no difference was observed. Importantly, no statistical significance was noted between experimental and predicted values in all co-cultured pellets, indicating that the overall GAG production was not influenced by the co-culture.

HAC does not prevent MSC chondrogenic differentiation towards hypertrophic phenotype

Collagen type X deposition and alkaline phosphatase activity, markers of hypertrophy (Barry *et al.*, 2001; Ichinose *et al.*, 2005; Mueller *et al.*, 2008) were examined for the stability of the chondrogenic phenotype. Collagen type X was not detected in 100 HAC pellets after either 3 or 6 weeks of culture, independently of the condition tested (±T±D) (Fig. 5A). In contrast, strong collagen type X production, overlapping with proteoglycan and collagen type II synthesis (as shown in Figures 4 and 5), was detected in 100 MSC but only under +T+D condition, indicating further differentiation of MSC towards hypertrophic phenotype solely upon stimulation with chondrogenic factors. Importantly, collagen type X was not detected in any co-cultured -T-D pellets, indicating the production of a phenotypically stable cartilage-like tissue. In +T+D condition, increasing number of HAC within the pellets resulted in reduction of collagen type X deposition (Fig. 5A,B). ALP activity was also quantified in pellet supernatants after 6 weeks of culture. Similar to the observations with collagen type X expression, the amount of ALP remained barely detectable in -T-D condition, while in +T+D condition ALP was detected in all pellets containing MSC. A reduction of ALP activity

occurred concomitantly with an increasing number of HAC/decreasing number of MSC.

To evaluate potential contribution of each cell type in ALP secretion upon co-culturing, predictive values were calculated as described above. No difference was observed between predicted and experimental values, indicating that ALP production by MSC is not influenced by the presence of HAC.

Discussion

One major limitation for cell-based therapeutic approaches in articular cartilage regeneration is the lack of sufficient numbers of differentiated HAC at the time of implantation (Gikas *et al.*, 2009). Bone marrow-derived MSC represent an alternative providing that their default hypertrophic pathway could be prevented. We hypothesized that chondrocytes with retained intrinsic chondrogenic potential co-cultured with bone marrow derived MSC in a micromass pellet system in the absence of chondrogenic stimuli could result in stable chondrogenic phenotype of MSC.

Co-culturing articular chondrocytes and MSC has recently gained considerable attention. Monolayer co-culture of immortalized human articular chondrocytes with immortalized human MSC in the absence of exogenous TGF-β resulted in chondrocytic differentiation of MSC based on collagen type II protein expression (Chen *et al.*, 2009). Human adipose-derived stem cells contributed to chondrogenesis when co-cultured with passaged human articular chondrocytes in the presence of TGF-β, at the level of extracellular matrix genes yet collagen type II was not detected at the protein level (Hildner *et al.*, 2009). Chondrogenic effect of bovine chondrocytes on human MSC was not observed in pellet co-cultures induced with TGF-β, although co-culture with MSC increased the growth and upregulated chondral phenotype of chondrocytes (Tsuchiya *et al.*, 2004). Similar findings have been described in co-cultures of rabbit articular chondrocytes and human MSC in alginate constructs induced with TGF-β (Mo *et al.*, 2009). These co-culture studies were performed either in proliferation medium supplemented with FBS or in the presence of chondrogenic factors (TGF-β), thereby preventing proper attribution of the improved chondrogenesis to cell-cell interactions. In the light of these limitations, well designed *in vitro* studies examining the effects of human articular chondrocytes on human MSC are needed to distinguish inhibitory or stimulating influences occurring in co-culture (Richter, 2009).

In vitro models of MSC and HAC chondrogenesis rely on 3D culturing models and differentiation stimulating factors such as TGF-β, BMP, IGF, FGF and dexamethasone (Johnstone *et al.*, 1998; Mackay *et al.*, 1998; Pittenger *et al.*, 1999; Barbero *et al.*, 2003; Indrawattana *et al.*, 2004; Im *et al.*, 2006; Stenhamre *et al.*, 2008). The micromass pellet culture is commonly used in studies investigating cell chondrogenesis *in vitro* and has been recently demonstrated as superior in comparison to encapsulation of chondrocytes into gel-like biomaterials (alginate-based systems) (Bernstein *et al.*, 2009). Pellet culture allows for cell-cell interactions, cell-ECM interactions and exchange/response to soluble factors that could occur during cellular cross-talk in a co-culture system. Our recent study has demonstrated the inhibitory effect of TFG-β1 and dexamethasone on extracellular matrix synthesis by HAC that underwent less than one cumulative population doubling, and demonstrated that defined serum-free media lacking these factors supported intrinsic chondrogenesis using pellet culture (Giovannini *et al.*, 2010). Therefore, in this study human articular chondrocytes with retained intrinsic chondrogenic capacities were chosen as these are most likely to provide chondrogenic stimuli, and co-cultured with human bone marrow derived MSC. To distinguish between effects of chondrocytes from the effects of external chondrogenic factors, namely TFG-β1 and dexamethasone, we incubated pellets in the presence or absence of these factors.

Differential behaviour of HAC and MSC was observed in relation to morphological appearance of pellets, proteoglycan synthesis, cell survival/proliferation and response to chondrogenic stimuli. Morphological analysis of pellet cultures demonstrated differences in shape depending on the cell type used, with HAC producing disc-like and MSC spheroid pellets. These differences in shape could be attributed to the presence/absence of specific adhesion proteins on HAC and MSC. Condensation similar to the one occurring during embryogenesis (DeLise *et al.*, 2000) is supported by the adhesion of cells to one another through cell surface adhesion molecules. Cadherins (transmembrane adhesion proteins) mediate cell adhesion through homophilic calcium-dependent interaction of their extracellular domain (Leckband *et al.*, 2000), and different types of cadherins have been described to lead to different forms of cell aggregations. While N-cadherin-mediated cell-cell interactions resulted in spherical aggregates of N-cadherin transfected fibroblasts, transfection with cadherin-11 caused the formation of a sheet-like cell aggregates (Kii *et al.*, 2004). Our observation of differential pellet shape could suggest that different types of cadherins could be expressed on HAC and MSC.

In the individual cell pellet cultures HAC produced more cartilaginous matrix and slightly proliferated, while MSC did not produce matrix and demonstrated significant cell death during culture. The presence of TGF-β1 and dexamethasone in pellets from differentiated HAC decreased chondrogenesis, as previously observed (Giovannini *et al.*, 2010), yet remained necessary to induce chondrogenesis of MSC, in accordance with other studies (Johnstone *et al.*, 1998; Pittenger *et al.*, 1999; Caplan *et al.*, 2001). Additionally, TGF-β1 and dexamethasone could not rescue cell death in MSC pellets. Apoptosis of MSC induced towards chondrogenesis in pellet cultures has been previously reported (Sekiya *et al.*, 2002; Wang *et al.*, 2009) raising the question whether *in vitro* 3D models represent inappropriate culturing condition for MSC. Finally, a distinctive characteristic of MSC proved to be the production of ALP in all MSC pellets incubated with chondrogenic factors. Our data thus emphasize caution in

interpretation of data obtained with differentiation factors due to different cell-specific responses and masking of the potential cellular cross-talk.

Macroscopical analysis of co-cultured pellets showed changes in pellet shape from disc-like to spheroid with the decreasing number of HAC regardless of the presence or absence of chondrogenic factors. In the absence of chondrogenic factors, as little as 25 HAC in co-cultured pellets resulted in phenotypically stable hyaline-like cartilage tissue composed of sulphated proteoglycans, collagen type II and lacking collagen type X. However, the presence of 10 HAC proved insufficient for production of homogenous cartilaginous matrix deposition. Histology, including Bern score, and immunohistochemistry analysis of 25-75 and 50-50 HAC-MSC pellets demonstrated cartilage matrix deposition and S100 protein expression throughout the entire pellet, suggesting early differentiation of both cell types towards chondrogenic phenotype. However, biochemical analysis of proteoglycan production revealed that the deposition of GAG corresponded to the percentage of HAC within the pellets. Similar to a co-culture study performed using primary and dedifferentiated bovine articular chondrocytes, the amount of accumulated extracellular matrix did not reach the levels attained by the culture of HAC alone (Gan and Kandall, 2007). These data indicate that although Bern score provides valid qualitative assessment of neocartilage formation *in vitro* as previously published (Giovannini *et al.*, 2010), biochemical analysis remains necessary for a more accurate assessment. Although MSC appear to be induced towards early chondrogenesis based on S100 expression, they do not participate to the GAG synthesis within the co-cultured pellets even after 6 weeks of co-culture.

A combination of HAC and the presence of chondrogenic factors in co-cultures did not prevent MSC death. Proliferation of HAC and death of MSC most probably affected relative cell percentages in co-cultured pellets. Given that the predictive and experimental values for GAG, DNA and GAG/DNA did not show significant differences, it could be concluded that MSC did not contribute to GAG deposition.

While MSC hypertrophic differentiation beyond chondrogenesis is well established in the *in vitro* models (Weiss *et al.*, 2010), phenotypic stability of MSC was investigated only in one co-culture study where the presence of rat hip cartilage pieces strongly suppressed collagen type X gene expression in rat MSC (Ahmed *et al.*, 2007). Our data demonstrated that MSC co-cultured with HAC in the absence of dexamethasone and TGF-β1 did not show signs of hypertrophy even after 6 weeks of incubation. The presence of HAC decreased collagen type X deposition in +T+D co-cultured pellets indicating a decrease in hypertrophic differentiation. However, quantitative measurements suggest lack of decrease in ALP secretion of MSC with increasing amount of HAC. These data indicate that MSC chondrogenic differentiation can be induced only in the presence of TGF-β yet inevitably leads to further hypertrophic differentiation, in accordance with a recent study (Weiss *et al.*, 2010). During the time the current manuscript was under review, Fischer *et al.* published that HAC improved chondrogenesis and decreased hypertrophy of bone marrow derived MSC, by employing HAC conditioned media or direct co-culture (Fischer *et al.*, 2010). Several differences exist in comparison to our data, which may explain the contradictory results. In contrast to our study, the number of cells for pellet preparation in co-cultures differed between the individual cell pellets. It has been shown that the correlation in the increase of cell numbers and GAG production in pellets in not linear (Huang *et al.*, 2008), suggesting that direct comparison of pellets containing different cell numbers could be misleading. Furthermore, the physiological properties of cells in the two studies were dissimilar; while we employed HAC with retained intrinsic chondrogenic potential (Giovannini *et al.*, 2010), Fischer *et al.* (2010) used HAC which required the presence of TGFβ1 for chondrogenesis. Additionally, individual donor variation of MSC was observed by Fischer *et al.* (2010), which combined with the different MSC expansion media in the two studies could have resulted in differential chondrogenic as well as hypertrophic responses.

Age and species related cell differences could affect the ability of "instructor" cells to produce the expected signals as well the responsiveness of "instructed" cells to these signals. A recent study has shown that freshly isolated bovine chondrocytes produced soluble factor(s) that could induce re-differentiation of passaged bovine chondrocytes, while freshly isolated human chondrocytes failed to induce re-differentiation of human passaged chondrocytes (Taylor *et al.*, 2009), suggesting species-specific differences. Furthermore, primary bovine chondrocytes from old donors could not induce re-differentiation of passaged bovine chondrocytes, suggesting a role of donor's age for the cells ability to produce "instructing factor(s)". In another study immature cartilage demonstrated higher potential to form neocartilage and to repair tissue upon injury compared to mature cartilage further underlining the importance of age (Otsuki *et al.,* 2010). While most animal studies rely on skeletally immature animals, human cartilage and bone marrow are mostly obtained from adult patients. Given that the cartilage regeneration cell-based therapies in human patients are mainly intended for adults, future co-culture studies should aim at elucidating the observed species and age specific differences and/or age for cellular interactions.

Conclusion

Taken together our data indicate that chondrocytes with retained intrinsic chondrogenic potential failed to instruct MSC to undergo full chondrogenesis *in vitro* in a micromass co-culture model in the presence or absence of external chondrogenic stimuli. Importantly, our results demonstrate that MSC did not contribute to the proteoglycan deposition, and that HAC failed to prevent hypertrophy of MSC induced by chondrogenic stimuli. These data underline the importance of the choice of cell species as well as donor age, and culturing conditions in the interpretation of co-culturing data for future potential clinical application.

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Discussion with Reviewers

Reviewer I: As mentioned above, one of the most interesting aspects of the study relates to the differential changes in cell number (as reflected by DNA measurements) over the course of the experiment. How do the authors explain the markedly different survival profiles of the chondrocytes and MSCs and how does this differential survival impact the interpretation of co-culture results after 3 or 6 weeks?
Authors: The differential response of HAC and MSC under the given experimental conditions – pellet cultures incubated in serum media with or without the addition of chondrogenic factors, could only be explained by the cells intrinsic characteristics: while MSC die in pellet cultures, chondrocytes slightly proliferate, in particular under +T+D condition and mostly during the first 3 weeks. Thus, one can envision that indeed at the end of culture, based on the individual data for each cell type, half of MSC die and are partially substituted by chondrocytes. However, based on quantitative analysis of GAG, predicted values for individual contribution do not differ from experimental values, most probably indicating the lack of MSC contribution to GAG production. Similarly, ALP production by MSC in co-cultured pellets does not appear to be influenced by HAC. Thus, the only observed chondrogenic effect of HAC and MSC co-culture is the expression of S100, which does not suffice for full chondrogenesis of MSC.

Reviewer I: Given that there were few of any interactive effects of HACs on MSC chondrogenesis in this study, how do the authors reconcile their findings with those of published studies suggesting a positive phenotypic influence of HACs on differentiating stem cell populations?
Authors: Our study was based on co-culture of adult human chondrocytes with adult human bone marrow derived MSC in a micromass pellet culture in the absence of serum or external chondrogenic stimuli. The 3D culturing system represents a prerequisite for chondrogenesis and the presence of factors in serum or externally supplied prevents proper attribution of the improved chondrogenesis to cell-cell interactions.

Previous studies investigated potential cellular cross-talk leading towards improved chondrogenesis with chondrocytes in different developmental stages (de-differentiated, differentiated, immortalized), and MSC from different sources, and even included co-culturing cells from different species. Various culture systems and conditions were employed including monolayer, micromass pellets, alginate beads, and culture on filter inserts, with media supplemented with FBS or chondrogenic factors (TGF-β). The efficiency of chondrogenesis was assessed with different methods, including specific gene expression and/or histology and immunochemistry evaluation, yet without quantitative cartilage specific protein synthesis assessments in most cases.

Given the degree of variability, it is not surprising that data from these studies led to contradictory conclusions. Two studies indicated positive effect of chondrocytes on MSC (Chen *et al*., 2009; Hildner *et al*., 2009; text references). Positive effect was also noted in co-culture of human embryonic stem cells with chondrocytes (Vats *et al*., 2006; Bigdeli *et al*., 2009; text references). However, other studies did not demonstrate contribution of MSC towards chondrogenesis in co-culture with chondrocytes, but rather "assistance" of MSC for more efficient chondrogenesis of chondrocytes (Mo *et al*. 2009; Tsuchiya *et al*. 2004; text references).

In addition to differences in experimental systems, another important aspect involves the age of cell donors employed. Cartilage regeneration studies in animals rely on the ability of chondrocytes from mainly skeletally immature animals to produce neocartilage, whereas ACI represents a treatment for patients decades past skeletal maturity. A recent study by Taylor *et al*. (2009) (text reference) co-culturing primary and expanded bovine chondrocytes demonstrated that a) the "instruction" ability declined with the age of primary bovine chondrocytes; b) aged passaged (dedifferentiated) bovine and human chondrocytes remained irresponsive to instruction by young bovine primary chondrocytes, c) human primary chondrocytes failed to instruct human passaged chondrocytes. The last observation is in line with our data, and could be explained by the age of human cells and/or specie-specific differences. Previous evidence indicates decline of chondrogenic potential of human articular chondrocytes with age (Barbero *et al*., 2004), and a recent paper (Adkisson *et al*., 2010) demonstrated enormous differences in chondrogenic capacity of juvenile (younger than 10 years) *versus* adult, skeletally mature human articular chondrocytes. Therefore, the lack of "instruction" in our study could be attributed to the use of human chondrocytes with reduced capacity to produce the "instructing" factor(s).

Reviewer I: How do the current findings relate to *in vivo* MSC implantation strategies?
Authors: Although discovery of MSC held big promises for cartilage regeneration due to MSC capacity to differentiate towards chondrogenic phenotype *in vitro*, understanding the physiological behaviour of these cells remains insufficient. This is reflected in the lack of clinical studies employing MSC for cartilage regeneration. Our data confirm the necessity for chondrogenic induction by factors such as TGFβ that at the same time induce hypertrophic differentiation. The result that chondrocytes with intrinsic chondrogenic potential could not "instruct" *in vitro* expanded bone marrow-derived MSC could be a consequence of either inability of chondrocytes to instruct (most probably age-related) and/or MSC to respond. The later can be a consequence of age and/or physiological change due to *in vitro* expansion. Whether implanted human MSC would behave differently inside the joint, where "instruction" comes from the surrounding cartilage, synovial fluid and mechanical stimulation remains to be

investigated. Several studies have employed small (rabbit) and large (swine and sheep) animal models (at the border of skeletal maturity), where different scaffolds were seeded with isolated and expanded MSC and implanted in full thickness osteochondral defects (Løken *et al*., 2008; Li *et al*., 2009; Diao *et al*., 2009; Zscharnack *et al*., 2010; Centeno *et al*., 2010 supplementary references). Although hyaline cartilage formation has been observed in some of the studies, it should be noted that the full-thickness defect does not represent an ACI like approach for which substitution of chondrocytes by MSC is intended, as native bone marrow contributes to neocartilage formation. Application of autologous MSC in orthopaedics is only emerging, and is restricted to safety and possible complications (Centano *et al*., 2010). Given that the proof of concept studies should mimic the intended clinical indication as closely as possible, future studies should aim at learning more about MSC changes due to *in vitro* handling, and choosing appropriate animal models in terms of age, cartilage thickness, type and critical size of defect and allow long enough follow-up period.

Reviewer II: Why did you select TGFβ1, as TGFβ3 seems more relevant in the literature?
Authors: Members of the TGFβ-family, namely TGFβ1, 2 and 3 have all been used for chondrogenic differentiation of MSC, as reviewed in Lin *et al*. (2006). The fact that TGFβ3 has been recently more employed may not reflect that it is more relevant but more potent as demonstrated in Barry *et al*. (2001). Given that our previous study was performed with TGFβ1, we did not wish to change our experimental settings. Importantly, our major conclusions are based on the data obtained in the absence of chondrogenic factors making the choice of the better chondrogenic factors less important.

Reviewer II: What is the impact of chondrocytes on senescence of the stromal cells?
Authors: Chondrocyte senescence has been described in relation to age and osteoarthritic development (Nesic *et al*., 2006; Loeser, 2009). Additionally, senescence of MSC and chondrocytes has been described in terms of proliferation during the *in vitro* culture (e.g., Ksiazek, 2009; Moussavi-Harami *et al*., 2004).

We have isolated chondrocytes from healthy donors who had no signs of arthritis, and thus we do not believe these cells would induce senescence. Additionally, chondrocytes were cultured for only 8 days, and MSC for 3 passages during which during which no senescence has been reported.

Reviewer II: Could another way to analyse the results in the discussion be to argue that MSC induce chondrocyte dedifferentiation?
Authors: Chondrocyte dedifferentiation is related to proliferation, i.e. the number of population doublings. While we did observe an increase in chondrocyte cell numbers in our pellet cultures (in particular in +T+D condition), the increase is insufficient to account for the number of population doubling indicative of dedifferentiation. Additionally, 3D environment, allowing cell-cell contact simulating condensation during endochondral bone formation is likely to induce differentiation and not proliferation (leading to dedifferentiation). On the other hand, we cannot exclude an unfavourable effect of MSC on chondrocytes in co-cultured pellets. Should that be the case, based on our results, MSC would contribute to chondrogenesis to the same extent to which chondrocytes would have reduced chondrogenic efficiency, which although possible, is highly unlikely. If MSC would induce chondrocyte dedifferentiation, this would have been reflected in a decreased GAG content in co-cultures compared to the predicted values, what was not the case in the present study.

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