

## CHONDROGENIC POTENTIAL OF MESENCHYMAL STEM CELLS FROM PATIENTS WITH OSTEOARTHRITIS

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### Summary

This study was initially designed to differentiate human bone marrow-derived mesenchymal stem cells (MSC) into chondrocyte-like cells. We cultured MSCs in a medium containing transforming growth factor  $\beta$ 3, insulin, dexamethasone and/or demineralized bone matrix. *In vitro* chondrogenic activity was measured as metabolic sulfate incorporation and type II collagen expression in pellet cultures. Our results indicate that culture-expanded MSCs from OA patients did not differ significantly from the normal population with respect to their chondrogenic potential *in vitro*. Capability of total protein and proteoglycan synthesis as well as collagen II mRNA expression by cell aggregates was similar for all cell preparations in the presence of the appropriate growth and differentiation factors.

These findings show that MSCs from OA patients possess similar chondrogenic potential as MSCs isolated from healthy donors, therefore these cells may serve as a potential new prospect in cartilage replacement therapy.

**Key words:** Chondrogenesis; mesenchymal stem cells; osteoarthritis.

### Introduction

Several pathological conditions associated with articular cartilage damage such as osteoarthritis (OA) show limited capacity for tissue regeneration due to the lack of vascularity and paucity of cellularity. Thus, clinical needs for cartilage-generating therapies are diverse, prevalent and increasing in concert with our aging population. Transplantation cell therapy combined with appropriate growth factors and scaffolds is able to regenerate the cartilage matrix specifically in damaged areas, hence opening new perspectives in curing these diseases (Risbud and Sittinger, 2002; Raghunath *et al.*, 2005; Kuo *et al.*, 2006). The capacity of multipotent mesenchymal stem cells (MSCs) to differentiate into tissues of mesenchymal lineages, including bone, cartilage, fat, tendon and muscle, according to culture conditions and specific growth factors, make these cells

ideal candidates for tissue engineering. MSCs are usually isolated from bone marrow (BM), but also obtained from adipose tissue or cord blood, and can be multiplied *ex vivo* without loss of phenotype or multipotentiality (Johnstone *et al.*, 1998; Pittenger *et al.*, 1999; Barry and Murphy, 2004; Krampera *et al.*, 2006). However, MSCs per se, in terms of their chondrogenic potential, have not been well characterized in OA (Oreffo *et al.*, 1998; Murphy *et al.*, 2002; Alsalameh *et al.*, 2004; Jones *et al.*, 2004). Thus, the question is still open as to whether MSCs isolated from OA patients are useful for tissue engineering purposes.

Therefore, the aim of the present study was to compare the protein and proteoglycan synthesis ability of normal, and OA MSCs *in vitro*. A microculture system in 96-well culture plates facilitating the chondrogenic differentiation of human MSCs was developed for this purpose.

## Materials and methods

### *Patients*

We studied 7 patients with OA (4 female, 3 male; age range 41–69 years) and 9 control adult volunteers (5 female, 4 male; age range 42–78 years) without any symptoms of OA. Patients had discontinued any medication for at least 2 weeks prior to BM aspiration.

### *Isolation and Cultures of Human BM-Derived MSCs*

To isolate human MSCs, BM samples of 2–10 ml were taken from the femoral shaft of normal donors and patients underwent bone surgery after informed consent and under a protocol approved by an institutional review board according to the Declaration of Helsinki. Nucleated cells were isolated with a density gradient and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% of a selected lot of fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin (GIBCO) (complete medium). All low-density cells were plated in 25-cm<sup>2</sup> flasks at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. After 2–4 days, nonadherent cells were discarded and adherent cells were expanded in complete medium. Fresh complete culture medium was added and replaced every 3 or 4 days. When cells grew to 80% confluence, they were harvested with 0.25% trypsin and 1mM EDTA for 5 min at 37 ° C and designated as passage 1. These cells were further expanded with 1: 3 to 1: 5 splitting in 175-cm<sup>2</sup> flasks. Adherent cells between passages 3 and 7 were used as MSCs in all experiments.

*Demineralized Bone Matrix.* DBM was a gift from Pasteur SA Bucharest.

### *In vitro Cartilage Formation*

For in vitro chondrogenesis studies, a novel micromass culture system, similar but not identical to that described by Penick *et al.* (2005), was developed. Briefly, a total of 2 x 10<sup>5</sup> MSCs per well were placed in 200 µl final volume in U-bottom tissue culture plates in DMED/F12 medium

supplemented with 10% of a selected lot of FCS, 10<sup>-7</sup>M dexamethasone, 10ng/ml TGF-β3 and 6.25 µg/ml insulin, with DBM alone (10 µl 10% v/w suspension in PBS) or with DBM plus growth factors. The 96-well tissue culture plates were then centrifuged at 1,200 rpm and incubated at 37°C in 5% CO<sub>2</sub>. Within 24h after incubation, the cells formed aggregates which did not adhere to the bottom of the plate. The medium was changed every 3–4 days, and cell aggregates were obtained at intervals of 14 days. Sulfated glycosaminoglycan was visualized with 1% 1,9-dimethyl-methylene blue.

### *Metabolic Sulfate Incorporation*

For quantification of proteoglycan synthesis, cultures received 1.0 µCi/well sodium <sup>35</sup>S-sulfate and 1.0 µCi/well sodium <sup>3</sup>H-leucine 24 h prior to the time point chosen for measurement of newly synthesized proteoglycans and protein, respectively. Incorporation of radioactivity was measured by liquid scintillation counting.

### *Quantitative RT-PCR for Type II Collagen*

Total RNA was prepared from undifferentiated MSCs and from the chondrogenic pellets cultured for 7 or 14 days using Trizol following the manufacturer's instructions. Two-microgram aliquots of RNA were subjected to cDNA synthesis with 200 U of MMLV reverse transcriptase and 0.5 µg of oligo(dT) 15 primer. Quantitative PCR was performed and analyzed on a capillary real-time thermocycler. Amplification was done in the presence of SYBR Green I as follows: in 20 µl of final volume, cDNA was mixed with LightCycler-Fastart DNA Master SYBR Green, 10 pmol each of forward primer and reverse primer, and 4 mM MgCl<sub>2</sub>. The following sense and antisense primers were used: type II collagen, 5'-CCAGGTCAAGATGGTC-3', 5'-CTTCAGCACCTGTCTCACCA-3'; GPDH, 5'-CGTCTTCACCATGGAGA-3', 5'-CGGCCATCACGCCACAGTTT-3'.

Glass capillaries were placed into the Light-Cycler rotor and the following run protocol was used: a predenaturing step at 95°C for 10min, amplification and quantification program repeated for 50 cycles at 95°C for 10s, annealing at 60°C for 10s and extension at 72°C for 30s. At the end of elongation at each cycle, SYBR Green I fluorescence was measured. Data were analyzed using the fit point method in the LightCycler software 3.3. Relative quantification was made against serial dilution of GAPDH cDNA used as a housekeeping gene.

#### *Histology and Transmission Electron Microscopy*

Cell aggregates (pellets) were harvested and fixed in 10% buffered formalin for 2 h at room temperature and then stained with 0.1% 1,9-dimethyl-methylene blue for 10 min. Alternatively, pellets were fixed in 2.5% glutaraldehyde in cacodylate buffer and then processed for embedding into resin. Semithin sections were stained with 1% toluidine blue, ultrathin sections were contrasted and examined with an electron microscope (Tokyo, Japan).

#### *Statistical Analysis*

All experiments were performed 3–5 times. Data are presented as mean values  $\pm$  SD. Statistical significance was assessed by a two-tailed unpaired Student t test.

## **Results**

#### *Chondrogenic Differentiation of BM-Derived Human MSCs in Micromass Culture System*

MSCs from normal donors were pelleted into micromasses and then differentiated in FCS containing DMEM/F12 medium further supplemented with TGF- $\beta$ 3 and insulin, with DBM or without DBM plus growth factors. Immediately after centrifugation, the cells appeared as flattened pellets at the bottom of the wells. One day later, the pellets became spherical without any increase in size.

As the pellets grew in size between days 4 and 14, a cartilage matrix was synthesized, as demonstrated by the significant increase in the levels of  $^{35}$ S-sulfate incorporation at days 7 (fig. 1,a–c) and 14 (fig. 1,d–f) compared with untreated control cultures. Maximal level of sulfate incorporation occurred in cultures containing TGF- $\beta$ 3, insulin and DBM.

Addition of TGF- $\beta$ 3 and insulin, or DBM alone also resulted in a significant increase in the levels of sulfate incorporation that is proteoglycan synthesis, compared to untreated samples.

Furthermore, similar changes of type II collagen mRNA levels were demonstrated by quantitative RT-PCR analysis (fig. 1g, h).

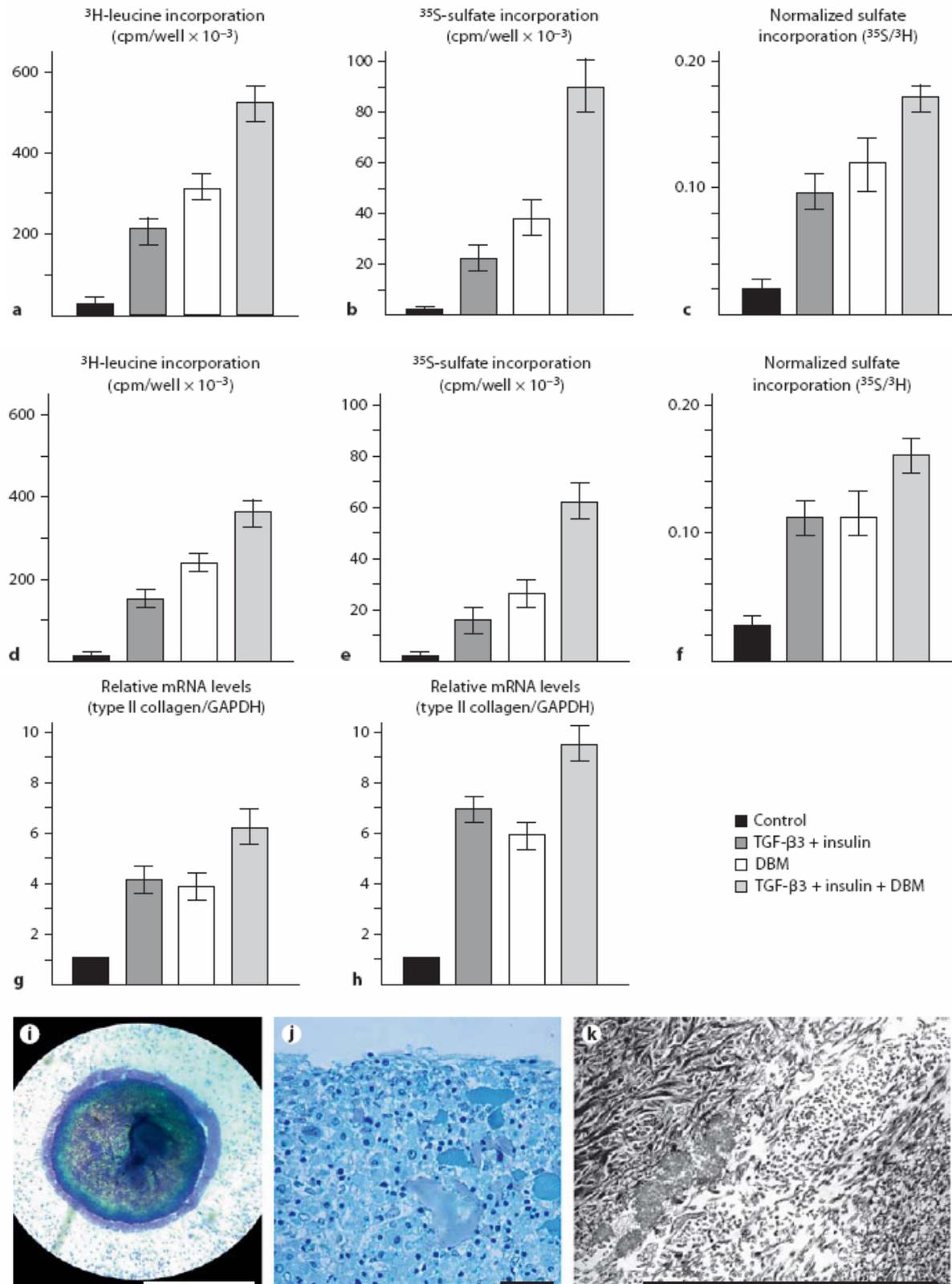
After 14 days of chondrogenic culture, the extent of chondrogenesis was also assessed histologically using methylene blue (fig. 1i) and toluidine blue (fig. 1j) staining, as well as by transmission electron microscopy (fig. 1k).

Histological examination of pellets confirmed sulfate incorporation, showing an abundant cartilage-like, metachromatic-stained matrix and a dramatic increase in pellet size in the TGF- $\beta$ 3, insulin and DBM treated cultures compared to untreated control cultures.

#### *Chondrogenic Differentiation of MSCs from OA Donors*

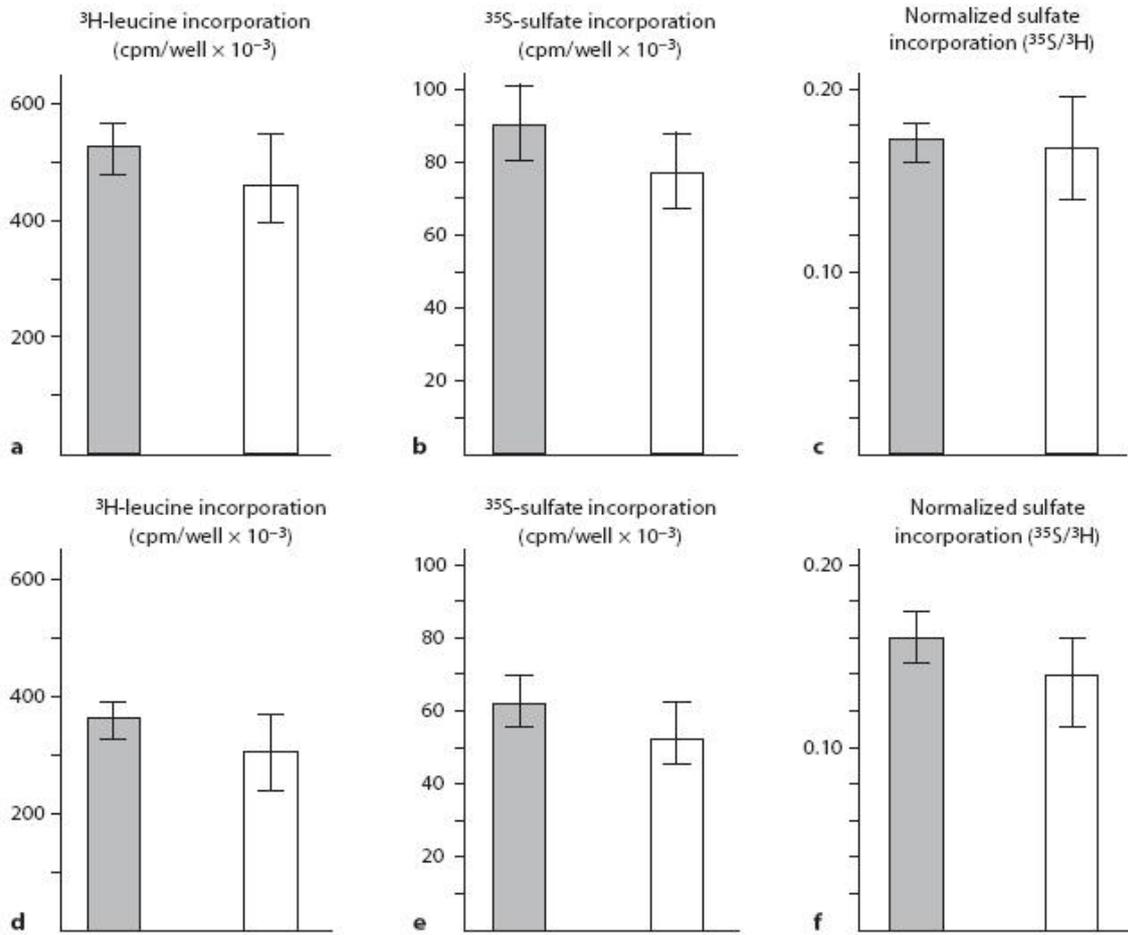
The capacity of MSCs from OA patients versus normal donors undergoing chondrogenic differentiation in response to combined treatment with TGF- $\beta$ 3, insulin and DBM was determined.

As shown in figure 2, all MSC preparations from either normal donors or OA patients were able to synthesize the same amount of proteins and proteoglycans after 7 (fig. 2 a–c) and 14 (fig. 2 d–f) days of culture in the presence of TGF- $\beta$ 3, insulin and DBM as determined by leucine and sulfate incorporation. Similar results were obtained when type II collagen mRNA levels were measured by quantitative RT-PCR (fig. 2 g, h).

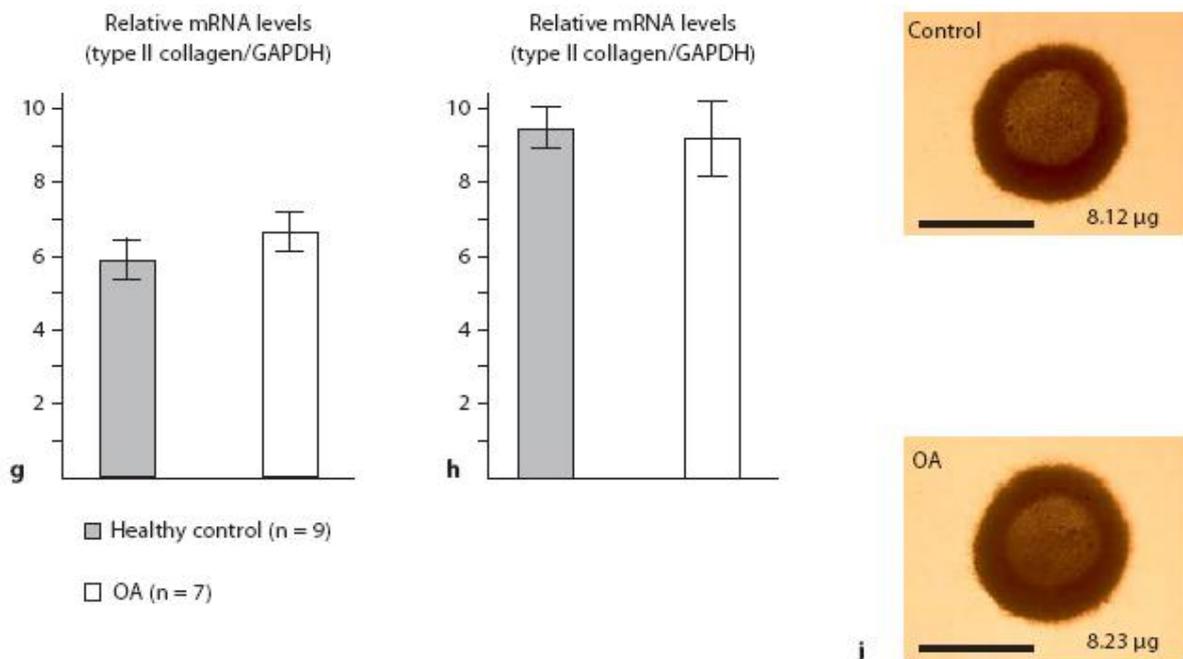


**Fig. 1 - Chondrogenic differentiation of normal human MSCs.** MSC aggregates ( $2 \times 10^5$  cells/well) were cultured either with medium alone, TGF-β3 plus insulin, DBM or TGF β3 plus insulin in combination with DBM. <sup>3</sup>H-leucine (a, d) and <sup>35</sup>S-sulfate (b, e) incorporation was measured on days 7 (a-c) and 14 (d-f), then normalized sulfate incorporation was calculated (c, f). The expression levels of type II collagen and GAPDH were quantified by real-time RT-PCR on days 7 (g) and 14 (h).

Results are expressed as means ± SD. i Methylene blue staining of a day 14 pellet maintained in the presence of TGF-β3, insulin and DBM. Scale bar = 1 mm. j Toluidine blue staining of a section from resin-embedded pellet cultured for 14 days in the presence of TGF-β3, insulin and DBM. Scale bar = 50 μm. k Transmission electron micrograph depicting the ultrastructure of extracellular matrix inside the chondrogenic pellet on day 14. Scale bar = 50 μm. Morphologically, 2 types of matrix could be observed.



**Fig. 2 - Chondrogenic potential of normal, and OA MSCs.** MSC aggregates ( $2 \times 10^5$  cells/well) were cultured with TGF- $\beta$ 3 plus insulin in combination with DBM.  $^3\text{H}$ -leucine (a, d) and  $^{35}\text{S}$ -sulfate (b, e) incorporation was measured on days 7 (a–c) and 14 (d–f), then normalized sulfate incorporation was calculated (c, f). The expression levels of type II collagen and GAPDH were quantified by real-time RT-PCR on days 7 (g) and 14 (h). Results are expressed as means  $\pm$  SD. i Size and dry weight of representative pellets after 14 days of culture (h). Scale bars = 1 mm.



**Fig. 2 - Chondrogenic potential of normal, and OA MSCs (cont.).**

Thus, the chondrogenic capacities of culture-expanded MSCs from pathological and healthy donors were not different in our experimental system. Similar results were obtained comparing the size and dry weight of pellets after 14 days of culture in chondrogenic medium (fig. 2 i).

## Discussion

For regeneration therapy of cartilage and bone, which are damaged either by degeneration, MSCs with chondrogenic potential are obviously superior to the mature cells of these tissues. The main reason is that MSCs have much greater capability to proliferate in vitro than mature cells. Moreover, the ex vivo expanded stem cells could readily turn into the desired cell types under the influence of respective in vitro and or in vivo signals (Fibbe, 2002; Jorgensen *et al.*, 2004; Kuo *et al.*, 2006). For example, normal MSCs are able to differentiate into chondrocytes in the presence of TGF- $\beta$ 3 plus insulin or insulin-like growth factor and/or DBM (Johnstone *et al.*, 1998; Gurevitch *et al.*, 2003; Zhou *et al.*, 2005). The proper choice of the source of MSCs for long-term tissue repair is not trivial.

Although allogeneic MSCs are usually not rejected by the immune system (Rasmusson, 2006), using autologous MSCs for cell and tissue replacement therapy is still more harmless than allogeneic stem cell transplantation. Moreover, it can bypass the necessity for volunteer marrow donors. Contrasting data have been reported, however, on the chondrogenic potential of MSC populations isolated from OA patients (Murphy *et al.*, 2002; Lee *et al.*, 2003; Alsalameh *et al.*, 2004; Luyten 2004, Scharstuhl *et al.*, 2007).

Unfortunately, the heterogeneity of the starting MSC populations (iliac crest vs. femoral head or shaft), subtle differences in cell preparation techniques, initial plating density and culture conditions for most of the chondrogenesis experiments render the

comparison of results between different groups difficult.

In the present study, we have used a new micromass culture system, carried out in 96-well tissue culture plates, that facilitates the chondrogenic differentiation of human MSCs (fig. 1). We have found that culture-expanded MSCs isolated from the femoral shaft of OA patients do not differ significantly from the normal population in respect to their chondrogenic differentiation in vitro as far as the total protein and proteoglycan synthesis as well as the expression of type II collagen mRNA are concerned (fig. 2).

## Conclusion

In conclusion, our results indicate that autologous MSCs from BM of RA and OA patients might be used for cartilage and/or bone replacement therapy in the future.

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