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# Trophic Effects of Mesenchymal Stem Cells in Chondrocyte Co-Cultures are Independent of Culture Conditions and Cell Sources

Ling Wu, M.Sc.,<sup>1</sup> Henk-Jan Prins, Ph.D.,<sup>2,3</sup> Marco N. Helder, Ph.D.,<sup>4</sup>  
Clemens A. van Blitterswijk, Ph.D.,<sup>1</sup> and Marcel Karperien, Ph.D.<sup>1,\*</sup>

Earlier, we have shown that the increased cartilage production in pellet co-cultures of chondrocytes and bone marrow-derived mesenchymal stem cells (BM-MSCs) is due to a trophic role of the MSC in stimulating chondrocyte proliferation and matrix production rather than MSCs actively undergoing chondrogenic differentiation. These studies were performed in a culture medium that was not compatible with the chondrogenic differentiation of MSCs. In this study, we tested whether the trophic role of the MSCs is dependent on culturing co-culture pellets in a medium that is compatible with the chondrogenic differentiation of MSCs. In addition, we investigated whether the trophic role of the MSCs is dependent on their origins or is a more general characteristic of MSCs. Human BM-MSCs and bovine primary chondrocytes were co-cultured in a medium that was compatible with the chondrogenic differentiation of MSCs. Enhanced matrix production was confirmed by glycosaminoglycans (GAG) quantification. A species-specific quantitative polymerase chain reaction demonstrated that the cartilage matrix was mainly of bovine origin, indicative of a lack of the chondrogenic differentiation of MSCs. In addition, pellet co-cultures were overgrown by bovine cells over time. To test the influence of origin on MSCs' trophic effects, the MSCs isolated from adipose tissue and the synovial membrane were co-cultured with human primary chondrocytes, and their activity was compared with BM-MSCs, which served as control. GAG quantification again confirmed increased cartilage matrix production, irrespective of the source of the MSCs. EdU staining combined with cell tracking revealed an increased proliferation of chondrocytes in each condition. Irrespective of the MSC source, a short tandem repeat analysis of genomic DNA showed a decrease in MSCs in the co-culture over time. Our results clearly demonstrate that in co-culture pellets, the MSCs stimulate cartilage formation due to a trophic effect on the chondrocytes rather than differentiating into chondrocytes, irrespective of culture condition or origin. This implies that the trophic effect of MSCs in co-cultures is a general phenomenon with potential implications for use in cartilage repair strategies.

## Introduction

**D**ESPITE THE SUCCESS of autologous chondrocyte implantation in treating large-size cartilage defects, there are some disadvantages of this treatment that limit its broader clinical application. One major issue is the requirement of relatively large quantities of chondrocytes from the patient,<sup>1</sup> which are obtained by *in vitro* expansion. The partial replacement of the chondrocytes with mesenchymal stem cells (MSC) has been proposed to tackle this problem. Many studies have evaluated the feasibility of this idea by the co-

culturing of MSCs and chondrocytes.<sup>2</sup> Indeed, cartilage matrix deposition was found to be improved in the co-cultures of MSCs and chondrocytes compared with the cultures of pure chondrocytes or MSCs.<sup>3,4</sup> In our previous report,<sup>5</sup> we have shown that pellet co-cultures of bovine primary chondrocytes (bPCs) and human MSCs benefit cartilage matrix formation and that cartilage matrix genes were mainly expressed by bovine chondrocytes. In addition, we also showed that the ratio of MSCs decreased dramatically due to the massive cell death of MSCs by apoptosis. Chondrocyte proliferation was increased by either co-culturing

<sup>1</sup>Department of Tissue Regeneration, MIRA-Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands.

<sup>2</sup>Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam, Amsterdam, The Netherlands.

Departments of <sup>3</sup>Oral and Maxillofacial Surgery and <sup>4</sup>Orthopaedics, VU Medical Center, Amsterdam, The Netherlands.

\*Current affiliation: Department of Developmental BioEngineering, MIRA-Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands.

with MSCs or culturing with MSCs conditioned medium. These findings were also confirmed by an independent study performed by Acharya *et al.* (2011).<sup>6</sup> This and our study demonstrated a new mechanism of cellular interaction in the co-culture of MSCs and chondrocytes, in which the trophic effects of MSCs stimulate chondrocyte proliferation and cartilage matrix deposition rather than actively undergoing chondrogenic differentiation.

The concept of MSCs as a trophic mediator of tissue repair is introduced by Arnold Caplan (2006),<sup>7</sup> who borrowed the term trophic from neurobiology, in which it refers to the bioactive molecules produced by nerve terminals, which are not neurotransmitters.<sup>8</sup> In relation to MSCs, the term trophic was first used to describe the process in which MSCs secrete factors that stimulate nearby cells to release functionally bioactive molecules.<sup>7</sup> Later, the term also related to the effect of the factors produced by MSC on the viability, proliferation, and matrix production of the neighboring cells. This concept has resulted in a paradigm shift in the way MSCs are involved in tissue repair. While traditionally it was believed that MSCs mainly repair damaged tissue by differentiating into specific cell types and replacing lost cells,<sup>9</sup> nowadays, the trophic role of the MSC in tissue repair is considered more important.<sup>10</sup> It was reported that MSCs, when introduced either directly or systemically into stroked brain of rats, promote the gain of coordinated functions without differentiating into neurons or any other supporting cell type.<sup>11</sup> Other examples are from the trophic role of MSCs in stimulating cardiomyocyte proliferation *in vitro*,<sup>12</sup> and vascular regeneration *in vivo*.<sup>13</sup>

So far, most of the reports dealing with the trophic effects of MSCs are based on studies using bone marrow-derived MSCs (BM-MSCs). There are many more sources from which MSCs can be isolated. For examples, adipose tissue can give rise to multipotent stromal cells,<sup>14,15</sup> and the human synovial membrane also contains an MSC population.<sup>16</sup> So far, MSCs have been isolated from many other tissues.<sup>17,18</sup> The MSCs isolated from other sources share many common features with BM-MSCs,<sup>19,20</sup> and have been applied in tissue-engineering strategies.<sup>21–25</sup> Nevertheless, only a few papers documented the paracrine or autocrine effects of adipose tissue-derived MSCs in tissue regeneration.<sup>26,27</sup> The trophic effects of MSCs isolated from a nonbone marrow origin have not yet been systematically and thoroughly studied.

In a previous study, we had co-cultured human BM-MSCs and bPCs in pellets in a chondrocyte proliferation medium,<sup>5</sup> and showed that increased cartilage matrix formation was not due to the chondrogenic differentiation of MSCs. These data indicated that in pellet co-cultures, signals from the chondrocytes alone are insufficient to induce the chondrogenic differentiation of MSCs, at least under these culture conditions. In this study, we performed experiments in which BM-MSCs and bPCs were co-cultured in a chondrogenic differentiation medium to investigate the influence of growth factors in the culture medium on MSCs' trophic effects. In addition, we examined whether MSCs from multiple sources (bone marrow, adipose tissue, and synovial membrane) have similar trophic effects by co-culturing them with human primary chondrocytes (hPCs). Our data presented here demonstrated that the trophic effects of MSCs on chondrocytes in pellet co-cultures is a general feature of MSCs, independent of culture conditions and their source.

## Materials and Methods

### Cell culture and expansion

bPCs were isolated from full-thickness cartilage of knee biopsies from cows that were approximately 6 months old. hPCs were obtained from macroscopically healthy cartilage obtained from the knee or hip biopsies of patients with end-stage osteoarthritis undergoing total knee or hip replacement. Both bovine and human cartilages were digested for 20–22 h in collagenase type II (0.15%) dissolved in chondrocytes proliferation medium. The components of the chondrocytes proliferation medium are Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1× nonessential amino acids, 0.2 mM ascorbic acid 2-phosphate (AsAP), 0.4 mM proline, 100 U/mL penicillin, and 100 µg/mL streptomycin. More details about chondrocyte isolation are described elsewhere.<sup>28</sup> Bone marrow mesenchymal stem cells (BM-MSCs) were separated from human bone marrow aspirates as previously described.<sup>29</sup> Human adipose tissue mesenchymal stem cells (AT-MSCs) and synovium MSCs were isolated according to the procedures in previous publications.<sup>30–31</sup> MSCs from all kinds of sources were cultured in MSC proliferation medium ( $\alpha$ -MEM supplemented with 10% fetal bovine serum, 1% L-glutamin, 0.2 mM ascorbic acid, 100 U/mL penicillin, 10 µg/mL streptomycin, and 1 ng/mL basic fibroblast growth factor).

The use of all human materials in this study has been approved by a local Medical Ethics Committee. All reagents used for cell culture were purchased from Gibco, Invitrogen, unless otherwise stated. Common chemicals were purchased from Sigma-Aldrich.

### Pellet culture and chondrogenic differentiation

For mono-cultures, 200,000 cells of hPCs or MSCs were seeded in one well of a round-bottom 96-well plate (non-tissue culture treated). For the co-cultures, 200,000 cells were seeded in an 80% MSC/20% hPC or bPC ratio. The cells were initially seeded in a chondrocyte proliferation medium and centrifuged for 5 min at 500 g. The medium was changed to a chondrogenic differentiation medium (DMEM supplemented with 40 µg/mL of proline, 50 µg/mL of ITS-premix, 50 µg/mL of AsAP, 100 µg/mL of Sodium Pyruvate, 10 ng/mL of TGF $\beta$ 3, 10<sup>-7</sup> M of dexamethasone, 500 ng/mL of BMP6, 100 U of penicillin/mL, and 100 µg/mL of streptomycin) 1 day after seeding when the stable pellets had been formed. The cell pellets were cultured for 4 weeks before the analysis.

### Histology

The cell pellets were fixed with 10% formalin for 15 min, dehydrated with ethanol, and embedded in paraffin using routine procedures. A microtome was used to cut 5 µm-thick sections. The slides were then deparaffinized and stained for sulfated glycosaminoglycans (GAG) with Alcian blue. Nuclei were counterstained with nuclear fast red.

### Quantitative GAG and DNA assay

Cell pellets ( $n=6$ ) were washed with phosphate-buffered saline (PBS) and stored at -80°C for 16–20 h. Subsequently, they were digested in digestion buffer (1 mg/mL proteinase

K in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.6) containing 18.5 µg/mL iodoacetamide and 1 µg/mL pepstatin A) for more than 16 h at 56°C. The GAG content was spectrophotometrically determined with 1,9-dimethylmethylene blue chloride (DMMB) staining in PBE buffer (14.2 g/L Na<sub>2</sub>HPO<sub>4</sub> and 3.72 g/L Na<sub>2</sub>EDTA, pH 6.5) using an ELISA reader (TECAN) at an absorbance of 520 nm with the chondroitin sulfate as a standard. Cell numbers were determined by the quantification of total DNA using a CyQuant DNA Kit (Molecular Probes).

#### *DNA isolation, RNA isolation, and quantitative polymerase chain reaction*

DNA samples of pellets were extracted with the DNA Mini Kit (Promega) according to the manufacturer's protocols. RNA samples of cell pellets were isolated with the NucleoSpin RNA II Kit (Macherey-Nagel). Total RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was performed on genomic DNA or cDNA samples by using the iQ SYBR Green Supermix (Bio-Rad). PCR reactions were carried out on the MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad) under the following conditions: cDNA was denatured for 5 min at 95°C, followed by 45 cycles, consisting of 15 s at 95°C, 15 s at 60°C, and 30 s at 72°C. For each reaction, a melting curve was generated to test primer dimer formation and nonspecific priming. The sequences of the primers for real-time PCR, either species specific or cross-species specific, were as previously described.<sup>5</sup> For the PCR on genomic DNA, standard curves were generated by using the serial dilutions of genomic DNA as a template. Bio-Rad iQ5 optical system software (version 2.0) was used to calculate copy numbers for each condition using the standard curves as reference. The relative signal was defined as the proportion of human or bovine GAPDH copy numbers as the percentage of the total copy numbers of both human and bovine genes. The calculation of relative expression was performed with the Bio-Rad iQ5 optical system software (version 2.0) using the double delta Ct method.<sup>32</sup> Cross-species-specific GAPDH primers were used for normalization.

#### *Cell tracking with organic fluorescent dyes*

The organic fluorescent dye CM-DiI (Molecular Probes) was used for cell tracking in co-cultures. The cells were labeled according to the manufacturer's protocol. Briefly, the cells were trypsinized and resuspended in PBS at a concentration of  $2 \times 10^6$  cells/mL. The cells were incubated with 4 µM of CM-DiI at 37°C for 5 min, followed by an incubation at 4°C for 15 min. The cells were washed with PBS and applied in co-culture experiments.

#### *5-ethynyl-2'-deoxyuridine labeling and staining*

Cell proliferation in pellets was examined with the Click-iT<sup>®</sup> EdU Imaging Kit (Molecular Probes). The cell pellets were cultured in a chondrogenic differentiation medium containing 10 µM EdU (5-ethynyl-2'-deoxyuridine) for 24 h before harvesting. At day 2, the cell pellets were washed with PBS and fixed with 10% formalin for 15 min. Sections of 10 µm were cut with a cryotome. The sections were per-

meabilized with 0.5% of Triton×100 for 20 min and stained for EdU with Alexa 488 cocktail. Nuclei were counterstained with Hoechst 33342 (provided in the same kit).

#### *Image acquisition and analysis*

Histological images were acquired with a Nikon E300 microscope. Fluorescent images were taken with a BD pathway 435 confocal microscope (BD Biosciences). Details of image quantification were published elsewhere.<sup>5</sup> Values represent the mean ± standard deviation of at least 3 biological replicates.

#### *Short tandem repeats analysis*

Genomic DNA samples were extracted from pellets with the QIAamp DNA Mini Kit (Qiagen). The sixteen loci of the kit PowerPlex 16 System (Promega) were amplified, typed, sequenced, and analyzed by ServiceXS B.V.. Specific alleles for the donor of hMSCs and the donor of hPCs were found in several loci. These alleles were used to define the origin of cells in the allogeneic co-culture of hMSCs and hPCs. The amount of DNA present for each donor was calculated from the areas of the electropherogram for each locus of the hMSCs' or hPCs' specific alleles, and the ratio of hMSCs and hPCs was determined.

#### *Statistical analysis*

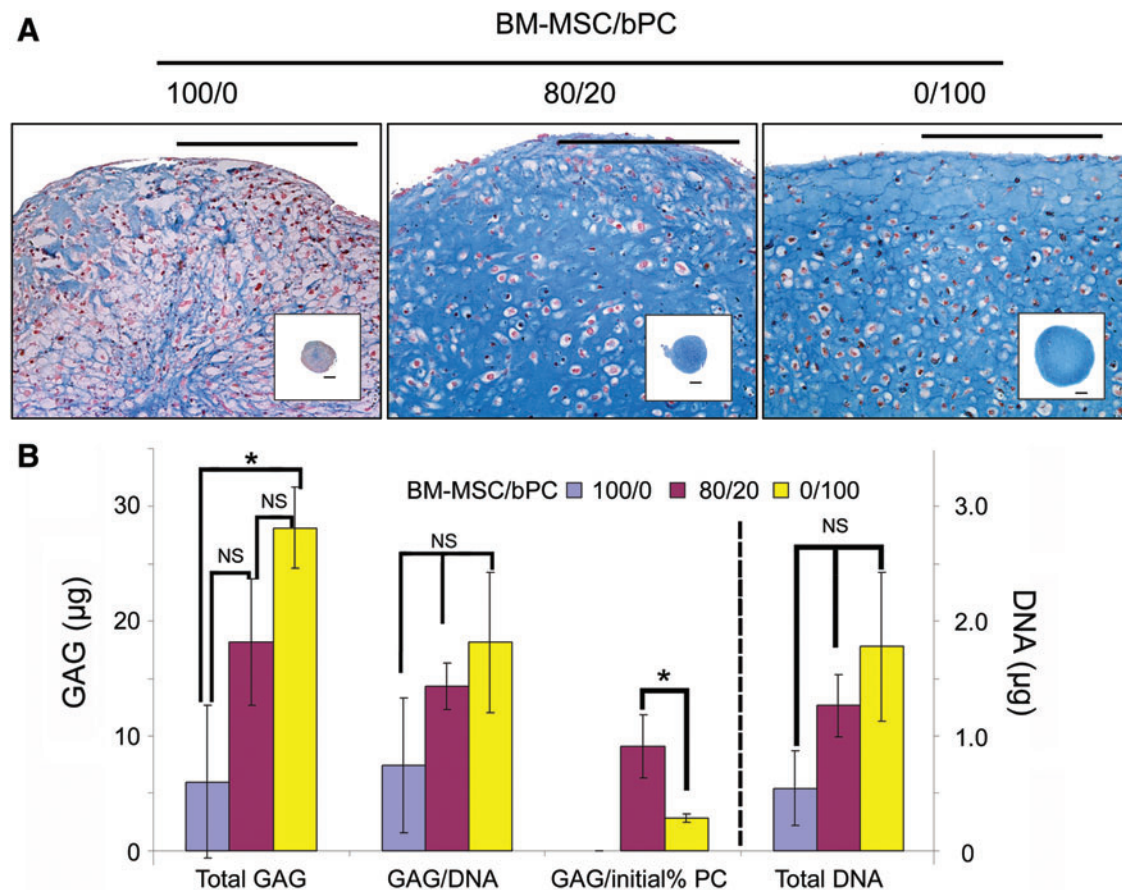
GAG and DNA quantifications were examined for statistical significance with the one-way analysis of variance followed by the Tukey's Honestly Significant Difference Test. Statistical analysis of EdU-positive cells was performed by using the Student's *t* test. *p*-Values of <0.05 were considered statistically significant.

## **Results**

### *Co-culture pellets in chondrogenic differentiation medium show increased cartilage formation*

To test whether growth conditions, favorable for the chondrogenic differentiation of MSCs, affect the trophic effects of MSCs in pellet co-cultures, we examined the cartilage matrix formation of BM-MSCs and bPCs in a chondrogenic differentiation medium. Each of the 3 BM-MSC donors were fully capable of differentiating into the chondrogenic, osteogenic, and adipogenic lineage and expressed typical cell surface markers of MSCs (data not shown), although some variability was observed in the chondrogenic differentiation potential. After 4 weeks of co-culture, histology and GAG assays were performed to evaluate cartilage formation. As shown in Figure 1A, the presence of GAG is indicated by Alcian blue staining in both mono-cultures and co-culture pellets. Cells in the positively stained areas were embedded in lacunae and showed typical characteristics of chondrocytes, round shape, and the presence of an Alcian blue-positive extracellular matrix. Similar data were obtained using Toluidine blue staining (data not shown). Quantitative analysis showed that both total GAG and total DNA increased as the ratio of MSCs decreased (Fig. 1B). In line with previous studies, the co-culture pellets contained more GAG than the pellets seeded with 100% chondrocytes, when normalized to the initial seeding percentage of bPCs, indicating





**FIG. 1.** Cartilage matrix formation is increased in co-culture pellets cultured in a chondrogenic differentiation medium. Pellets (200,000 cells) of BM-MSCs from 3 donors and bPC in 3 different ratios (100:0; 80:20 and 0:100 of BM-MSC:bPC) were cultured in a medium that was compatible with the chondrogenic differentiation of MSCs. After 4 weeks, the pellets were collected for a histological analysis. **(A)** Alcian blue staining was performed to examine the deposition of sulfated GAGs in midsagittal paraffin sections. Inserts indicate the overview of each pellet. Pictures show the results of a representative donor of MSCs, while 3 donors were tested. Scale bar = 100 µm. **(B)** GAG and DNA of cell pellets ( $n=6$  per donor) were quantitatively measured 4 weeks after the co-culture. Averages of 3 donors of BM-MSCs are shown. Ratios of hMSCs and bPCs are indicated by the bar colors. The left  $y$ -axis is for "Total GAG," "GAG/DNA," and "GAG/initial%PC," while the right  $y$ -scale is for "Total DNA." Asterisk represents  $p < 0.05$ . NS, Not Significant. Data are presented as a mean  $\pm$  standard deviation (SD). BM-MSCs, bone marrow-derived mesenchymal stem cells; bPC, bovine primary chondrocyte; GAG, glycosaminoglycans. Color images available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)

that the beneficial effect on cartilage matrix formation in co-cultures is preserved when the pellets are cultured in growth factors-containing medium.

#### *Proliferation of chondrocytes causes ratio changes of two cell types in pellets cultured in chondrogenic differentiation medium*

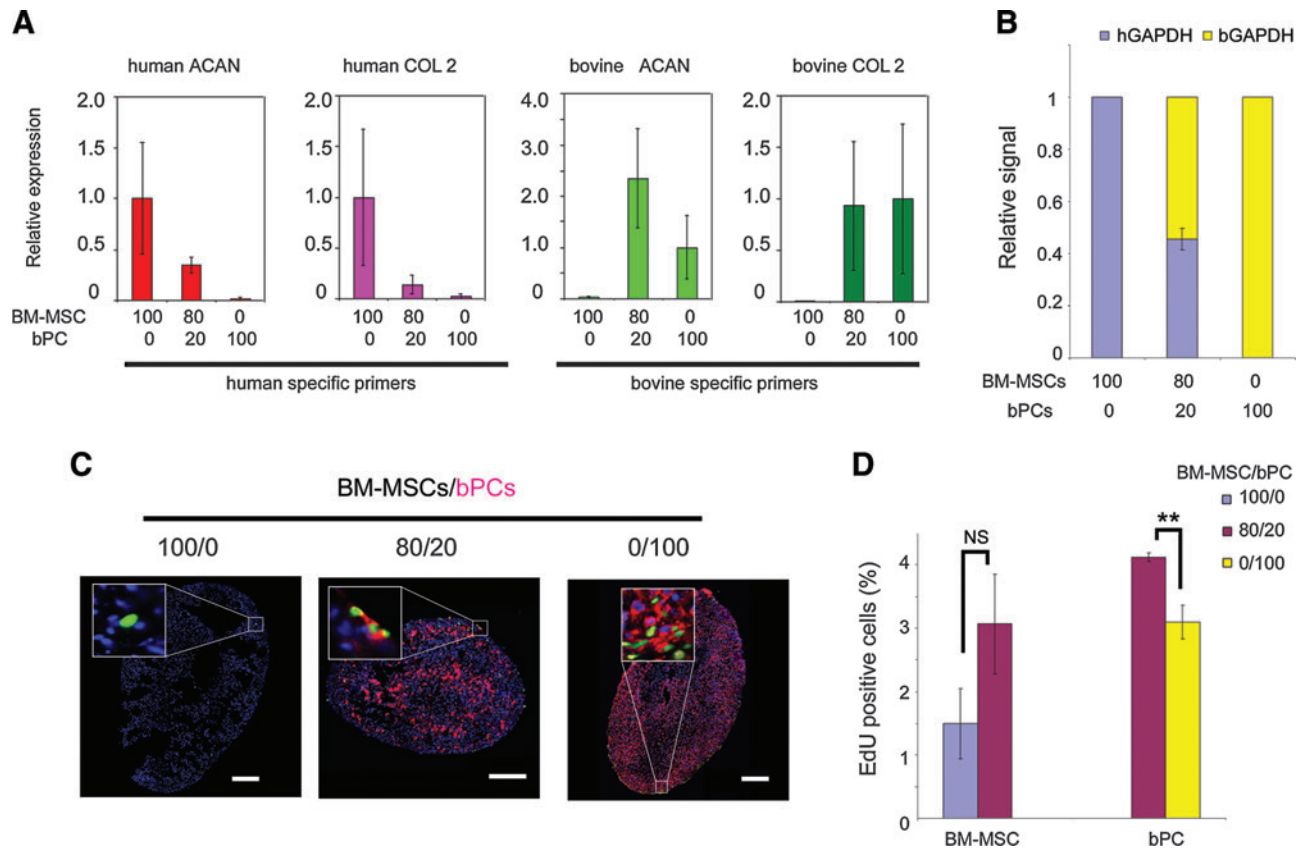
Species-specific qPCR was performed to study the origin of the chondrogenic marker genes in co-culture pellets (Fig. 2A). At week 4, the expression levels of both human Aggrecan (*ACAN*) and human Collagen 2 (*COL2*) were much lower in co-culture pellets than in pure MSCs pellets. On the other hand, the expression levels of bovine *ACAN* or *COL2* in co-culture pellets were either higher or similar to those of pure bPCs pellets. These data indicated that the cartilaginous matrix in co-culture pellets is mainly of bovine origin. At week 4 of culture, genomic DNA was isolated from the cell pellets, and used as a template in species-specific qPCR. The ratio of human/bovine cells dropped from 80% (initial

seeding percentage after 1 day) to approximately 40% after 4 weeks of culture (Fig. 2B).

Cell proliferation in co-culture pellets was studied using EdU incorporation combined with cell tracking. For this, bovine chondrocytes were labeled with CM-Dil (red). Proliferation was studied at day 2. As shown in Figure 2C, the EdU-positive cells were mainly found at the periphery of the pellets. Cell tracking showed that co-cultures predominantly increased the proliferation of the chondrocytes with a minor effect on the proliferation of the MSCs (Fig. 2D).

#### *Co-culture of hPCs and MSCs from multiple sources increases cartilage matrix formation*

To investigate whether the source of MSCs influences the degree of cartilage formation in co-culture pellets, MSCs derived from human adipose tissue and synovium were used in co-culture pellets with hPCs, while BM-MSCs served as a control. Alcian blue staining shows the deposition of GAGs, to some extent, in all groups (Fig. 3). GAG and DNA



**FIG. 2.** Enhanced cartilage matrix formation is caused by trophic effects of MSCs. Co-culture pellets of BM-MSCs and bPC were established and cultured in a chondrogenic medium as previously described. **(A)** After 4 weeks, the samples were collected for an analysis of the expression levels of ACAN and COL2 mRNA by species-specific qPCR. RNA samples were extracted from pellets ( $n=3$  per MSC donor). Relative expression levels were obtained by the normalization of human or bovine-specific signals to cross species-specific GAPDH. For human-specific genes, the values are relative amounts to the 100/0 hMSC/bPC group. For bovine-specific genes, the values are relative amounts to the 0/100 hMSC/bPC group. Data are presented as an average of 3 BM-MSCs donors  $\pm$  SD. **(B)** After 4 weeks of culture, a species-specific qPCR on genomic GAPDH was performed after DNA analysis ( $n=6$  per donor). Data represent the average of 3 BM-MSCs donors  $\pm$  SD. **(C)** After 2 days of culture, proliferation was assessed using an EdU assay. In this experiment, bPCs were labeled with CM-Dil (red). EdU incorporation into newly synthesized DNA was visualized by Alexa 488 (green), and nuclei were counterstained with Hoechst 33342 (blue) on 10  $\mu$ m freeze sections. Pictures show the results of a representative BM-MSCs donor out of the 3 donors tested. Scale bar = 100  $\mu$ m. **(D)** Quantification of EdU-positive cells. The initial ratios of hMSC and bPC are indicated by bar colors. Data from 3 donors of the BM-MSCs measured in triplicate were analyzed for statistic significance. Double asterisk represents  $p < 0.01$ . NS, Not Significant. Error bar reflects SD. ACAN, Aggrecan; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Color images available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)

contents of each pellet were then determined by chemospectrophotometric and fluorescent assays (Fig. 4). MSCs from different sources performed in a similar manner in GAG formation when co-cultured with hPCs. An average of 3 donor pairs of MSCs and hPCs showed that co-culture pellets contained more GAG than 100% chondrocyte pellets, when normalized to the initial seeding percentage of hPCs.

#### Co-culture of hPCs and MSCs promotes chondrocyte proliferation independent of the MSCs origin

EdU incorporation and cell tracking were used to investigate cell proliferation in the pellets. At day 2 after cell seeding, the EdU-positive cells were detected in all conditions tested (Fig. 5A). Quantitative data are shown in Figure 5B and C. Percentages of EdU-positive MSCs in co-culture pellets were close to those of 100% MSCs. No significant

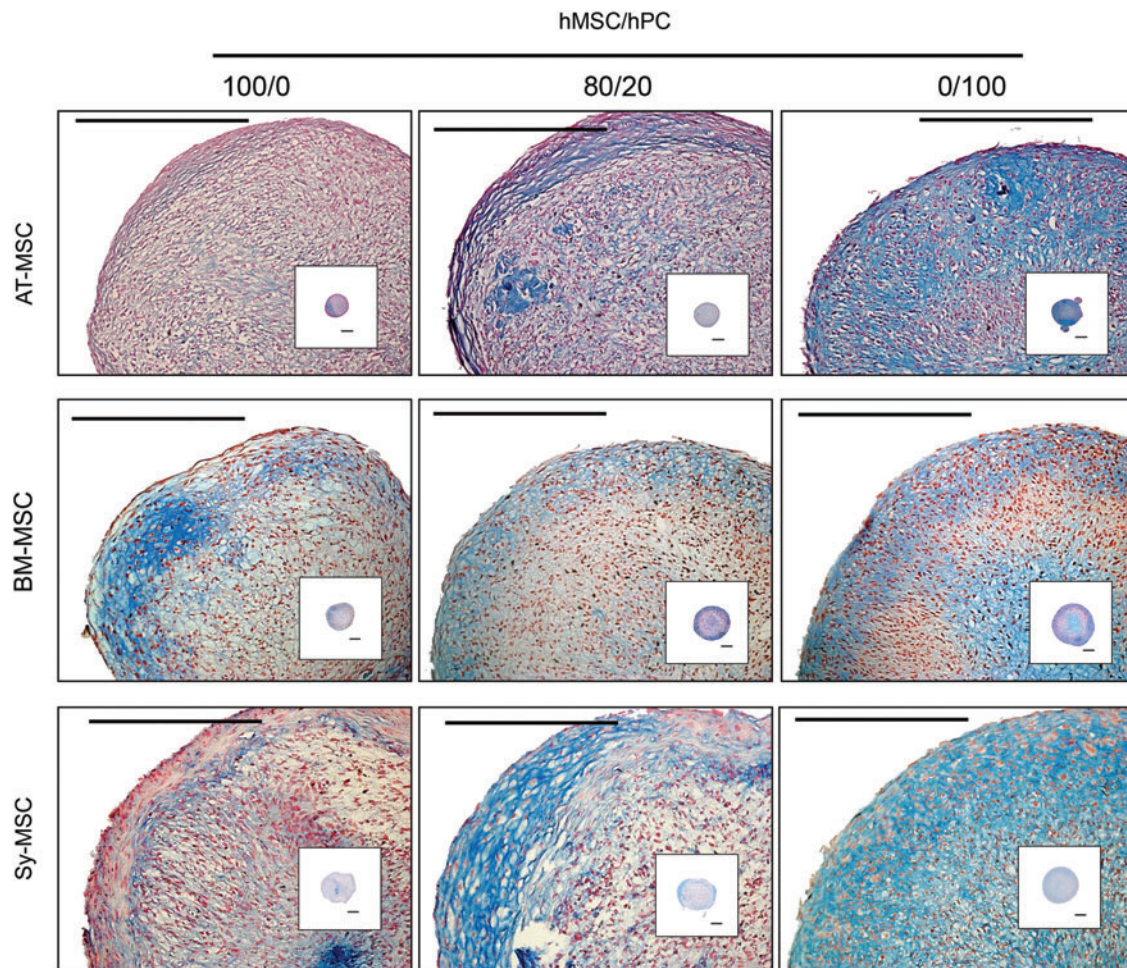
differences were observed. Percentages of proliferating hPCs in co-culture pellets were significantly higher as compared with 100% hPCs.

After 4 weeks of co-culture, the ratios of cells derived from hPC or MSC donors were determined by short tandem repeats analysis (Fig. 5D-F). The distinguishable loci between hPC and MSC donors are illustrated, which varied between the pairs of MSCs and hPCs. On average, these loci showed a clear change in the ratio of MSCs and hPCs compared with the initial seeding density, irrespective of the source of MSCs. Three MSC donors were tested for each co-culture combination with essentially comparable results.

#### Discussion

Multiple mechanisms have been postulated in order to explain increased cartilage matrix formation in the co-culture





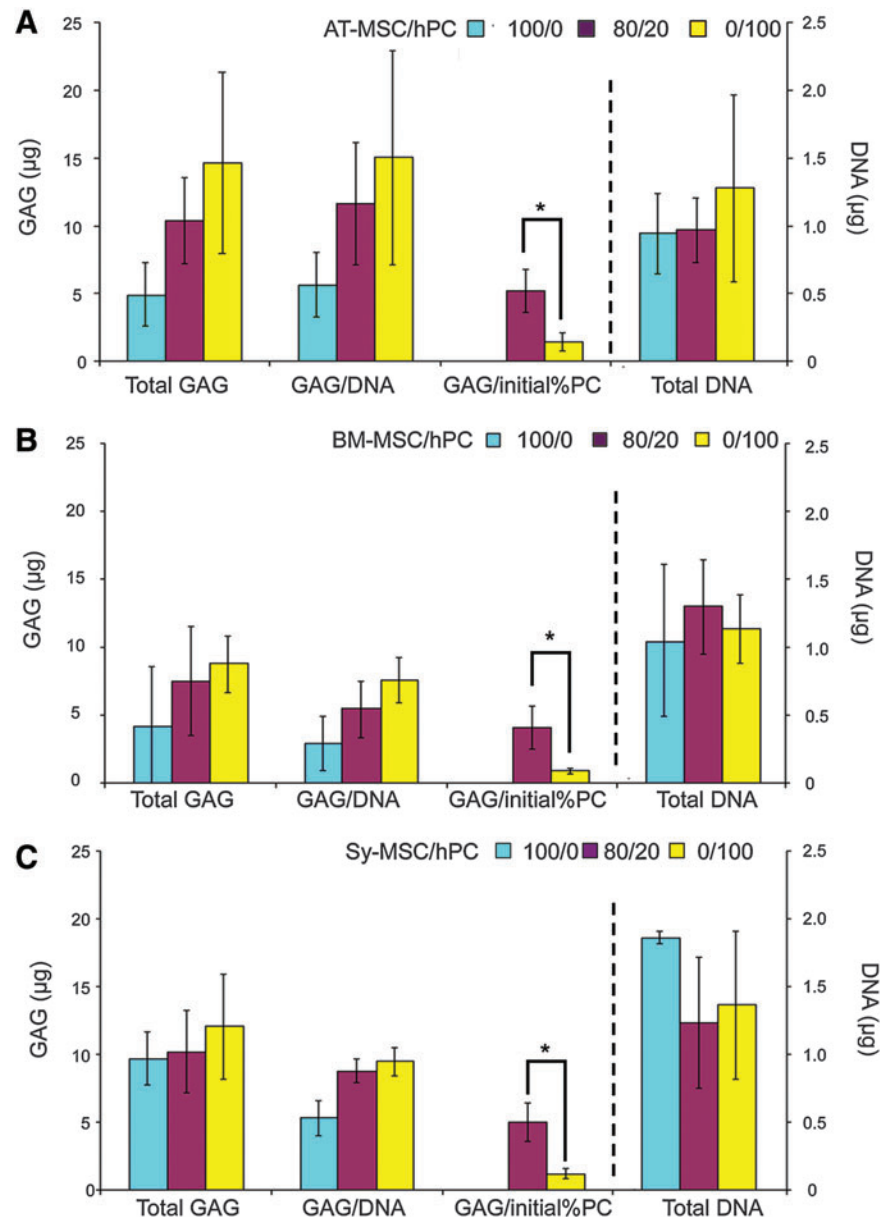
**FIG. 3.** GAG formation in co-culture pellets of hPCs and MSCs from different sources. Human chondrocytes were co-cultured with MSCs from different sources in 3 different seeding ratios (MSC versus PC; 100:0; 80:20; 0:100) for 4 weeks in a chondrogenic differentiation medium. Alcian blue staining was performed on midsagittal paraffin sections. Nuclei were counterstained with nuclear fast red. Pictures show a representative donor of MSCs, while 3 donors were tested. Scale bar = 100  $\mu$ m. Color images available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)

pellets of MSCs and chondrocytes.<sup>2</sup> When this beneficial effect of the co-culture was first discovered,<sup>3</sup> it was believed that chondrocytes stimulated the chondrogenic differentiation of MSCs, and that this contributed to the increased cartilage matrix formation in co-cultures. Several studies supported this idea.<sup>3,33-34</sup> It was shown that a conditioned medium of chondrocytes induced the osteo-chondrogenic differentiation of MSCs,<sup>33,35</sup> and co-culturing with chondrocytes in pellets induced chondrogenic gene expression in MSCs.<sup>34</sup> Results from these experiments, however, were not conclusive on the role of each cell type in the co-culture, as long-term cell tracking was not performed. Earlier, we had shown that increased cartilage formation in co-culture pellets is not due to the chondrogenic differentiation of MSCs, but is predominantly caused by MSCs exerting a trophic effect on the chondrocytes stimulating cell proliferation and matrix deposition. The effect of MSCs on chondrocytes in pellet co-cultures can be discerned in various effects: (1) MSCs stimulate the proliferation of chondrocytes at an early stage of the cell pellet cultures; (2) MSCs stimulate GAG formation and extracellular matrix production by the chondrocytes in the co-culture pellets; (3) As a consequence of the increased

chondrocyte proliferation, the initial seeding ratio between chondrocytes and MSCs changes over time in favor of the chondrocytes; (4) This effect is further exaggerated by the preferential cell death of MSCs in the co-culture pellets. These findings were essentially confirmed in a recent publication of Acharya *et al.*<sup>6</sup>

Our previous study had raised a number of additional unresolved questions: First, is the trophic effect of the MSCs in co-cultures dependent on the culture conditions? Our previous pellet co-cultures were performed in a serum-containing medium lacking chondrogenic factors that are essential for stimulating the chondrogenic differentiation of MSCs *in vitro*. This raises the question whether the absence of MSC differentiation and the disappearance of MSCs over time in these co-culture experiments were the result of culture conditions that are unfavorable for MSCs. To address this issue, we have repeated our co-culture experiments in a medium that is supportive of the chondrogenic differentiation of MSCs. We have performed the experiments with 3 donors who showed the capacity to differentiate into chondrocytes, albeit in a variable degree. Our results demonstrated that BM-MSCs essentially exerted a similar trophic

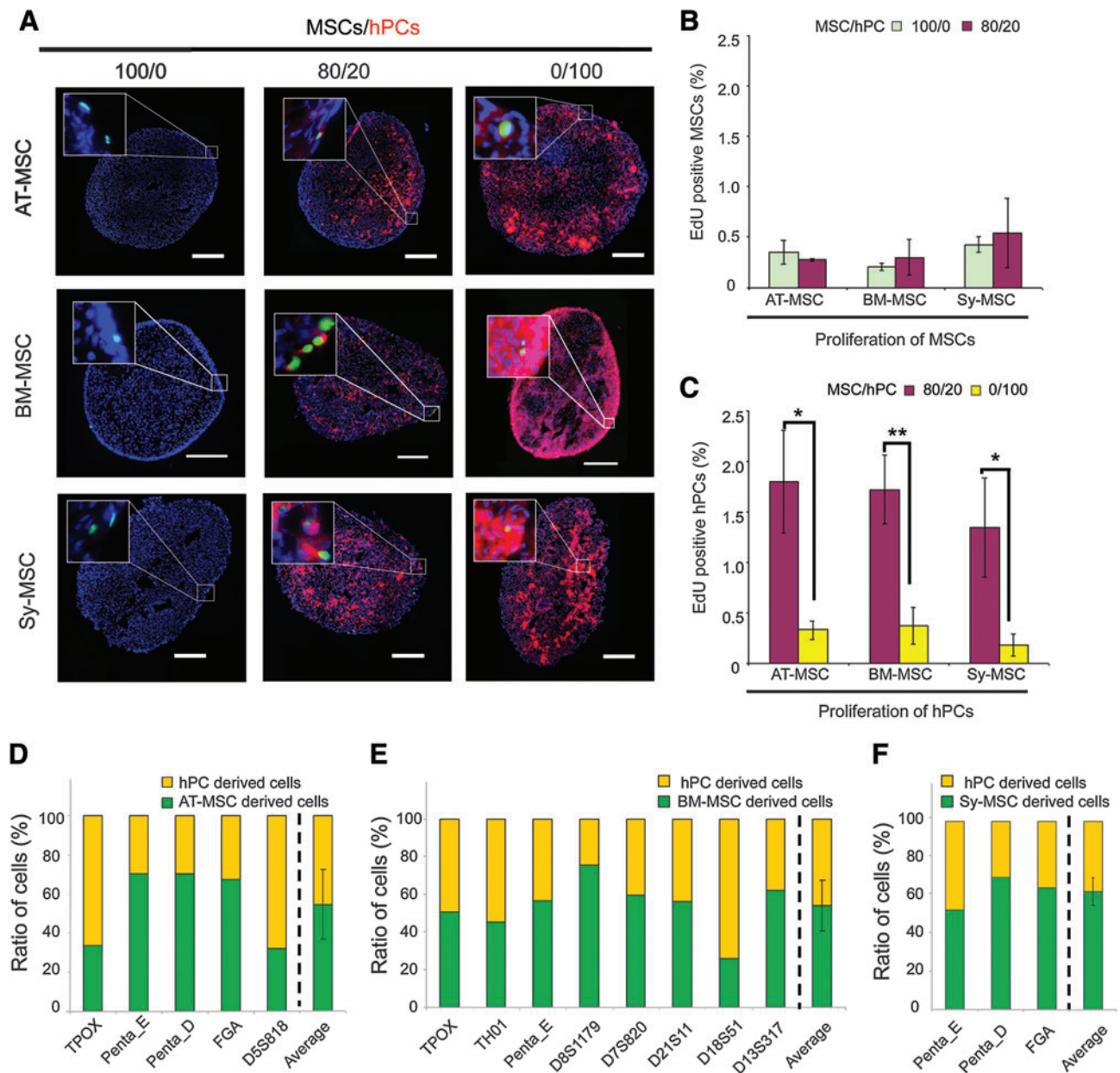
**FIG. 4.** Trophic effects in co-cultures are independent of the origin of the MSC. Human chondrocytes were co-cultured in pellets with AT-MSCs (**A**), BM-MSCs (**B**), or Sy-MSCs (**C**) in 3 different ratios in the chondrogenic differentiation medium. The GAG and DNA of cell pellets ( $n=6$  per donor) were quantitatively measured 4 weeks after the culture. Ratios of MSCs and bPCs are indicated by the bar colors. Scale on the left is for "Total GAG," "GAG/DNA," and "GAG/initial % PC," while the scale on the right is for "DNA." Asterisk represents  $p < 0.05$ . Data are the average of 3 donors per cell source  $\pm$  SD. AT-MSCs, adipose tissue mesenchymal stem cells; Sy-MSCs, synovium mesenchymal stem cells. Color images available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)



role in the co-cultures, irrespective of the culture conditions. In addition, in a chondrogenic medium, we did not observe the overt differentiation of the MSCs in chondrocytes. Based on the results of the present study, it appears that there is a common mechanism in both culture conditions, although we do find some differences in the fate of MSCs in co-cultures depending on the culture medium. The most notable difference is that the ratio change in the chondrogenic differentiation medium is not as dramatic as that in the proliferation medium. As reported, MSCs mixed with bPCs almost disappeared in the co-culture pellets in the absence of chondrogenic factors, while around 40% of MSCs remained in the co-culture pellets in the differentiation medium.<sup>5</sup> This suggests that the MSCs cultured in pellets can survive better in a chondrogenic differentiation medium than in a proliferation medium. We did not find experimental evidence stating that the better survival of the MSCs was due to the stimulation of chondrocyte differentiation. In fact, the expression of human-

specific *ACAN* and *COL2* after 4 weeks of culture was markedly reduced in the co-culture pellets compared with the pellets of pure MSCs. In addition, the production of GAGs corrected for the initial seeding percentage of chondrocytes did not differ between the co-culture pellets cultured in a proliferation medium<sup>5</sup> and those cultured in a chondrogenic medium (this study). Although we cannot conclude from these experiments that none of the MSCs had differentiated into chondrocytes, this effect appears to be marginal in both culture conditions. The most marked difference between both studies is that the ratio of MSCs dropped from 80% to below 5% after 4 weeks when the pellets were co-cultured in a proliferation medium, while only a reduction to 40% was seen in the chondrogenic medium. Despite this difference, similarities in both models were more common. For instance, in both models, a homogenous distribution of cartilage matrix compounds was found in the co-culture pellets even though the MSCs in the





**FIG. 5.** MSCs from multiple sources act as trophic mediators when co-cultured with hPCs. Human chondrocytes were co-cultured in pellets with AT-MSCs (**A**), BM-MSCs (**B**), or Sy-MSCs (**C**) in 3 different ratios in a chondrogenic differentiation medium, as described in the Materials and Methods section. (**A**) EdU staining was performed at day 2 to show the proliferating cells in the pellets. hPCs were labeled with CM-DiI (red). The EdU incorporation into newly synthesized DNA was visualized by Alexa 488 (green). The nuclei were counterstained with Hoechst 33342 (blue). The sources of MSCs are indicated on the left. Pictures are representative for each condition (3 pellets analyzed per donor) and are 10- $\mu$ m mid-sagittal freeze sections. The insets are magnifications of the boxed areas. Scale bar = 100  $\mu$ m. (**B**) and (**C**) Quantification of EdU-positive MSCs (**B**) or hPCs (**C**). The initial ratios of MSC and hPC are indicated by bar colors. The sources of MSCs are indicated at the bottom. Data represent the average from 3 donors of MSCs, each measured in triplicate  $\pm$  SD. Asterisk represents  $p < 0.05$ . Double asterisk represents  $p < 0.01$ . (**D**), (**E**), and (**F**) STR analysis was performed on genomic DNA isolated from the co-culture pellets of MSCs from adipose tissue (**D**), bone marrow (**E**), or synovium (**F**) and hPCs at an initial seeding ratio of 80:20 after 4 weeks of the culture. Only informative loci that could discriminate between the MSCs and hPCs are shown. The loci are listed at the bottom of the Figure. An average of these loci is shown by the rightmost bar  $\pm$  SD. For each combination, 3 donor pairs were tested. A representative example of each donor pair of MSCs and hPCs is shown. Color images available online at [www.liebertonline.com/tea](http://www.liebertonline.com/tea)

co-culture pellets with chondrocytes preferentially end up at the center of the pellet, irrespective of the culture conditions.<sup>5</sup>

The second question raised by our previous study is whether the observed role of the MSCs in the co-cultures is dependent on the source of MSCs. Our previous results were

obtained with BM-MSCs. However, the beneficial effects of co-cultures for cartilage matrix formation was also found in a combination of chondrocytes and a variety of other cell types, such as adipose-tissue-derived stem cells, human embryonic stem cells, fibroblasts, and meniscus cells.<sup>36-39</sup> It



is still unclear whether MSCs derived from adipose tissue or synovium have a comparable trophic role in the co-culture. To answer this question, we report that the MSCs isolated from adipose tissue and synovial membrane had similar trophic effects and similar behavior as BM-MSCs in the co-culture with chondrocytes. We show that MSCs, irrespective of their origin, stimulate chondrocyte proliferation and increase total GAG corrected for the initial seeding percentage of chondrocytes to a comparable extent. Furthermore, we show a similar decrease in the percentage of MSCs in pellet co-cultures over time. Given these remarkably similar observations, we concluded that the MSCs from various cell sources have likely exerted similar roles.

Adipose tissue has long been considered as an alternative to bone marrow as an MSCs source, as it can be obtained relatively easily in large quantities with a relatively low donor morbidity and contains a much higher frequency of MSCs, as compared with bone marrow.<sup>15,30</sup> Piles of documents had pointed out the potential use of AT-MSCs in a variety of tissue-engineering applications.<sup>40-43</sup> Although less studies have been performed with synovium-derived MSCs, the synovium has recently received attention as a promising cell source for cartilage tissue engineering.<sup>24,31,44</sup> Here, we report for the first time that MSCs isolated from bone marrow, adipose tissue, and synovium behave similarly in co-culture pellets with MSCs by acting as trophic mediators stimulating chondrocyte proliferation and matrix production. Our data suggest that the trophic effects of MSCs could be a general mechanism by which the MSCs from different origins orchestrate tissue function repair.

Taken together, our results demonstrate that in co-culture pellets, MSCs stimulate cartilage formation due to a trophic effect with chondrocytes rather than differentiating into chondrocytes, irrespective of the culture conditions or their origin. This implies that the trophic effect of MSCs in co-cultures is a general phenomenon that may have potential implications for the use of MSCs in cartilage repair strategies.

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### Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Marcel Karperien, Ph.D.

Department of Developmental BioEngineering

MIRA Institute for Biomedical Technology

and Technical Medicine

University of Twente

P.O. box 217, 7500AE

Enschede

The Netherlands

E-mail: h.b.j.karperien@tnw.utwente.nl

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