



Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis

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Abstract Our work aimed at evaluating the role of adipose stem cells (ASC) on chondrocytes from osteoarthritic (OA) patients and identifying the mediators involved. We used primary chondrocytes, ASCs from different sources and bone marrow mesenchymal stromal cells (MSC) from OA donors. ASCs or MSCs were co-cultured with chondrocytes in a minimal medium and using cell culture inserts. Under these conditions, ASCs did not affect the proliferation of chondrocytes but significantly decreased camptothecin-induced apoptosis. Both MSCs and ASCs from different sources allowed chondrocytes in the cocultures maintaining a stable expression of markers specific for a mature phenotype, while expression of hypertrophic and fibrotic markers was decreased. A number of factors known to regulate the chondrocyte phenotype (IL-1 β , IL-1RA, TNF- α) and matrix remodeling (TIMP-1 and -2, MMP-1 and -9, TSP-1) were not affected. However, a significant decrease of TGF- β 1 secretion by chondrocytes and induction of HGF secretion by ASCs was observed. Addition of a neutralizing anti-HGF antibody reversed the anti-fibrotic effect of ASCs whereas hypertrophic markers were not modulated. In summary, ASCs are an interesting source of stem cells for efficiently reducing hypertrophy and dedifferentiation of chondrocytes, at least partly via the secretion of HGF. This supports the interest of using these cells in therapies for osteo-articular diseases.

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Introduction

Osteoarthritis (OA) is the most frequent rheumatic disease, characterized by a degeneration of articular cartilage, mainly due to changes in the activity of chondrocytes in favor of

catabolic activity as well as reduced cartilage cellularity (Bijlsma et al., 2011; Loeser et al., 2012). The capacity of adult articular chondrocytes to regenerate the normal cartilage matrix architecture declines with aging, due to cell death (apoptosis) and abnormal responsiveness to anabolic stimuli. OA chondrocytes lose their capacity to secrete the specific components of the extracellular matrix, such as collagen type IIB or aggrecan, and adopt a hypertrophic or fibroblastic phenotype. Currently, no treatment capable of markedly altering the progression of OA exists and therapeutic options are essentially pain management and surgical intervention (Hunter, 2011). Indeed, new innovative therapeutic strategies for cartilage protection/repair are currently being evaluated mainly based on stem cell-mediated approaches.

Mesenchymal stem cells or stromal cells which reside in bone marrow (MSCs) or in adipose tissue (adipose-derived stromal cells or ASC) are capable of self-renew and are characterized by their differentiation capabilities toward three lineages namely, chondrocytes, osteoblasts and adipocytes (Dominici et al., 2006). Beside this property, MSCs produce a number of secreted factors, such as cytokines, chemokines or growth factors, which mediate diverse functions (Doorn et al., 2012; Meirelles Lda et al., 2009; Salgado et al., 2010). Indeed, the use of MSCs for stem cell-based therapy of degenerative diseases such as OA may rely on two modes of action: MSC differentiation into chondrocytes, or prevention of cartilage degeneration through secretion of bioactive factors. Previous studies have demonstrated the potent role of MSCs combined with biomaterials and chondrogenesis-inducing factors for cartilage engineering (Vinatier et al., 2009). Moreover, the co-culture of human MSCs with OA chondrocytes allowed the differentiation of MSCs towards chondrocytes in the absence of growth factors and prevented their hypertrophic differentiation frequently occurring during in vitro growth factor-induced chondrogenesis (Aung et al., 2011). The possibility that MSCs, through their trophic role, may prevent cartilage degradation or stimulate cartilage formation would be of interest for the treatment of OA. Recent works have shown that trophic factors secreted by MSCs regulate the chondrocyte phenotype when the two cells were mixed in pellet- or alginate-based co-cultures (Mo et al., 2009; Wu et al., 2011, 2012). Altogether, these studies have highlighted the role of the cross-talk between MSCs and chondrocytes with an improved chondrogenic differentiation of MSCs by factors secreted by chondrocytes and the positive role of MSC trophic mediators on chondrocyte phenotype. Most of these studies were conducted using bone marrow-derived MSCs and less data are available on ASCs. These last cells have been intensively tested in various pre-clinical models since their initial characterization and are considered as a more accessible source of cells than MSCs with comparable properties (Pittenger et al., 1999; Zuk et al., 2001). However, neither the effect of ASC or MSC secretome when these cells are cultured with chondrocytes in the absence of direct contact, nor the identification of the proteins responsible for the paracrine effects, has been investigated.

In the present study, we therefore evaluated in vitro the effects of factors secreted by ASCs on the proliferation, apoptosis and phenotype of OA chondrocytes in a co-culture assay where chondrocytes were physically separated from ASCs by a porous membrane and cultured in a minimal medium. Our main findings show that ASCs reduce the

expression of both hypertrophic and fibroblastic markers on OA chondrocytes and that HGF is one key mediator involved in the anti-fibrotic effect of ASCs.

Materials and methods

Tissue samples

Samples for ASC isolation were obtained from patients undergoing plastic surgery or total joint replacement surgery (hip or knee hofa). Subcutaneous abdominal fat (SC-ASC) was obtained from 17 patients (15 women and 2 men; mean age: 44.9 ± 4.3 years). Infrapatellar Hoffa (Hoffa-ASC) and hip fat (Hip-ASC) were also obtained from OA patients. Chondrocytes and MSCs were isolated from patients with OA undergoing total knee replacement surgery. Articular cartilage was harvested from the femoral condyles of 12 patients (10 women and 2 men; mean age: 68.9 ± 2.3 years; body max index (BMI): 26.6 ± 1.4). MSCs were from 4 patients (3 women and 1 man; mean age: 68 ± 3.7 years). Consent of donors was approved by the French Ministry of Research and Innovation (approvals DC2009-1052 and DC-2010-1185).

Cell isolation

For ASC isolation, adipose tissue was digested with 250 U/mL collagenase type II for 1 h at 37°C and centrifuged (300 g for 10 min) using routine laboratory practices. The stroma vascular fraction was collected and cells filtered successively through a 100 μm , 70 μm and 40 μm porous membrane (Cell Strainer, BD-Biosciences, Le-Pont-de-Claix, France). Single cells were seeded at the initial density of 4000 cell/cm² in α MEM supplemented with 100 U/mL penicillin/streptomycin (PS), and 2 mmol/mL glutamine (Glu) and 10% fetal calf serum (FCS) was from PAA laboratories (Mureaux, France). After 24 h, cultures were washed twice with PBS. After 1 week, cells were trypsinized and expanded at 2000 cells/cm² till day 14 (end of passage 1), where ASC preparations were used. Clinical grade ASCs were isolated and expanded in platelet-enriched plasma (PLP) as described (Bourin et al., 2011). Typically, the doubling time of ASCs was evaluated to be 1.25 ± 0.05 days.

For MSC isolation, trabecular bone pieces were flushed out and cultured as previously described (Bouffi et al., 2010b). For chondrocyte isolation, knee cartilage slices were incubated in 2.5 mg/mL pronase (Sigma-Aldrich, Saint-Quentin-Fallavier, France) for 1 h at 37°C followed by 2 mg/mL collagenase type II (Sigma), overnight at 37°C . Digested pieces were filtrated through a 70 μm cell strainer and the cell suspension was cultured in DMEM/PS/Glu/10% FCS (proliferative medium) at the density of 25,000 cell/cm² till the end of passage 1.

Flow cytometry analysis

ASCs (1×10^5) in PBS containing 0.2% bovine serum albumin (BSA) were incubated with different antibodies (BD-Biosciences): fluorescein isothiocyanate (FITC)-conjugated CD31 or CD106 antibodies, peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated CD34 antibody, phycoerythrin (PE)-conjugated CD73 or CD105 antibodies, Allophycocyanin (APC)-conjugated

CD13 or CD90 antibodies, APC-H7-conjugated CD45 antibody, V450-conjugated CD11b or CD14 antibodies and V500-conjugated HLA-DR antibody or the respective isotype controls for 20 min at 4 °C in the dark. The labeled cells were then analyzed by multiparameter flow cytometry using a FACSCanto cytometer and the Diva software (BD-Bioscience).

Differentiation of ASCs

For adipogenesis, ASCs were seeded at the density of 10,000 cells/cm² in proliferative medium for 5 days. The medium was changed by DMEM-F12, PS, 16 μmol/L biotin, 18 μmol/L pantothenic acid, 100 μmol/L ascorbic acid, 5% FCS, 1 μmol/L dexamethasone, 60 μmol/L indomethacin, 50 μmol/L IBMX and 10 μmol/L rosiglitazone, up to 21 days. Adipogenesis was assessed by RT-qPCR and lipid droplets were visualized by microscopy. Osteogenesis was induced by culture at low density (3000 cells/cm²) in DMEM/PS/Glu/10% FCS, 10 mM β-glycerophosphate, 0.1 μmol/L dexamethasone and 50 μg/mL ascorbic acid for 21 days. Osteogenic differentiation was assessed by RT-qPCR and extracellular matrix mineralization detected by alizarin red S staining. Chondrogenic differentiation was induced by culture of ASCs (2.5 × 10⁵ cells) in micropellet as described (Bouffi et al., 2010b). Chondrogenesis was assessed by RT-qPCR and immunohistochemistry on paraffin sections of pellets using a 1/1000 dilution of anti-aggrecan antibody (Chemicon, Millipore, Molsheim) and the "Ultravision detection system anti-polyvalent HRP/DAB" kit (Lab Vision, Francheville, France). Sections were counterstained with Mayer's hematoxylin (Lab Vision) for 3 min and mounted with Eukitt (Sigma-Aldrich).

Co-culture assay

Chondrocytes (500,000 cells/well) were plated at high density on the bottom of 6-well plates and cultured with ASCs or MSCs (70,000 cells/insert) (ratio 7:1) in cell culture inserts (PET membranes, 0.4 μm pore porosity, BD Biosciences). These plating conditions correspond to confluent cultures in each compartment. Cultures were maintained for 7 days in 3 mL of minimal medium (DMEM supplemented with PS, proline (0.35 mmol/L), ascorbic acid (0.17 mmol/L) and sodium pyruvate (1 mmol/L)). Chondrocytes and ASCs were collected for RT-qPCR analysis and supernatants were stored at -20 °C. When indicated, isotypic control or anti-HGF antibody (100 ng/mL; R&D Systems, Lille) was added during the coculture.

Proliferation assay

Chondrocytes were plated at low density (40,000 cells/well) in a 24 well plate with or without ASCs (20,000 confluent cells/insert) (ratio 2:1) or in proliferative medium. After 2 days, chondrocyte number was evaluated using PrestoBlue™ assay (Life-Technologies, Courtaboeuf, France).

Apoptosis evaluation

High density co-cultures (ratio 7:1) were initiated for 2 days in the presence and in the absence of ASCs or 10% FCS. ASCs

were then removed, media changed and 40 μM of camptothecin was added on chondrocytes for 6 h (Bohm et al., 2010). Chondrocytes were trypsinized and 1 × 10⁵ cells in suspension were labeled with PE-conjugated annexin V antibodies (BD-Biosciences) in PBS containing 0.2% BSA for 20 min at 4 °C in the dark. Apoptotic chondrocytes were quantified as the percentage of PE-annexin V positive cells by flow cytometry analysis as described above. A second assay for the detection of apoptotic cells relied on the use of the Caspase-Glo® 3/7 Assay according to the manufacturer's recommendations (Promega, Charbonnières, France). Results were expressed as the average Caspases 3/7 activities detected in supernatants of lysed cells, either treated or not with camptothecin, and normalized to the cell number as measured using the CellTiter-Glo® Luminescent Cell Viability Assay as recommended by the supplier (Promega).

RT-qPCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Courtaboeuf, France). RNA (0.5 μg) was reverse-transcribed using the M-MLV enzyme (Fisher scientific, Illkirch, France). Primers were designed using Primer3 software (see Table 1) and purchased by MWG (Eurofins-Genomics, Courtaboeuf, France). PCR reaction was carried out on 25 ng of cDNA samples using 5 μmol/L of each primer and 5 μL 2 × SybrGreen PCR Master Mix (Roche, Meylan, France). The following conditions were used: 95 °C for 5 min; 40 cycles at 95 °C for 15 s; 64 °C for 10 s and 72 °C for 20 s in a LightCycler 480 system (Roche diagnostics, Meylan, France) and analyzed with the dedicated software. All values were normalized to RPS9 housekeeping gene and expressed as relative expression or fold change using the respective formulae $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$.

Quantification of secreted factors

Supernatants from co-cultures were used to quantify IL-1 receptor antagonist (IL1-RA), IL-1β, TNF-α, MMP-1, MMP-9, TIMP-1, TIMP-2, TSP-1, TGF-β1, and HGF by specific Enzyme-linked immunosorbent assays (ELISA; R&D Systems). Inducible NO synthase activity was quantified using colorimetric assays as reported (Bouffi et al., 2010a).

Statistical analysis

Data were expressed as the mean ± SEM. Statistical analysis was performed with GraphPad Prism software. The comparison between the different groups was analyzed by repeated one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test or with a Student's *t* test for two groups.

Results

ASC phenotype and differentiation abilities

In concordance with our previous report, SC-ASCs expanded in a medium containing fetal calf serum (FCS) exhibit a fibroblastic morphology at passage 1 (Fig. 1A). They expressed

Table 1 Primer sequences.

Gene	Sequence forward	Sequence reverse
Aggrecan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA
AP	CCACGTCTTCACATTTGGTG	GCAGTGAAGGGCTTCTTGTC
aP2	ATGGGATGGAAAATCAACCA	GTGGAAGTGACGCCTTTCAT
Collagene I	CCTGGATGCCATCAAAGTCT	CGCCATACTCGAAGTGAAT
Collagen IIA	ACGTGAAAGACTGCCTCAGC	AGGAGGTCCTTGGGTCTTA
Collagen IIB	CAGACGCTGGTGCTGCT	TCCTGGTTGCCGGACAT
Collagen III	CGCCCTCCTAATGGTCAAGG	AGGGCCTGAAGGACCAGCTT
Collagen VI	AGCCGAAAACACCCTGCAAT	CGTGAGGTCAGGCTTGGAAA
Collagen X	TGCTGCCACAATACCCTTT	GTGGACCAGGAGTACCTTGC
HGF	CCCCATCGCCATCCCCTATG	ACATCTATTAGCACATTGGTCTGCAGT
Link	TTCCACAAGCACAACTTTACACAT	GTGAAACTGAGTTTTGTATAACCTCTCAGT
LPL	GTCCGTGGCTACCTGTCAAT	TGGATCGAGGCCAGTAATTC
MMP13	GACTTCCCAGGAATTGGTGA	TACCCCAATGTCTTTCAGG
RSP9	ATGAAGGACGGGATGTTTAC	GATTACATCCTGGGCTGAA
PPAR γ	CCAGAAAGCGATTCTTTCAC	TGCAACCACTGGATCTGTTC
Osteocalcin	GGCGCTACCTGTATCAATGG	TCAGCCCACTCGTCACAGTC
RunX2	CGGAATGCCTCTGCTTTAT	TTCCCGAGGTCCATCTACTG
Sox9	AGGTGCTCAAAGGCTACGAC	GTAATCCGGGTGGTCTTCT
Vimentin	AAGGCTACCGAGGCGATGAG	AGCCCTCTGTGCCGTTTATC

the classical MSC markers: CD13, CD73, CD90 and CD105 and were negative for hematopoietic and endothelial markers: HLA-DR, CD11b, CD14, CD31, CD34, CD45 and CD106 (Fig. 1A). As expected, while the CD34 marker is detected on tissue-resident MSCs (Lin et al., 2012; Maumus et al., 2011b), its expression disappeared at the end of P1 after propagation in culture. After induction of differentiation towards the three specific lineages, ASCs gave rise to adipocytes, as shown by the increased expression of peroxysome proliferator-activated receptor (PPAR)- γ , lipoprotein lipase (LPL), fatty acid binding protein (FABP)-4 and the presence of lipid droplets in cultures, and compared to control ASCs cultured in proliferative medium. ASCs differentiated into osteoblasts, as shown by an increase in Runx2, alkaline phosphatase (AP), osteocalcin (OC) expression and Alizarin Red S staining. They also differentiated into chondrocytes, as indicated by an enhanced expression of Sox9, aggrecan, collagen (col) type II markers and anti-aggrecan immunostaining (Fig. 1B). Similar results were obtained with the different sources of mesenchymal stem cells or expansion protocol (data not shown).

Effect of ASC secretome on chondrocyte function

In order to evaluate the effect of ASCs on chondrocytes, a co-culture assay was designed using the transwell system to avoid cell–cell contact. We used chondrocytes and sub-cutaneous (SC)-ASCs that were isolated and cultured in proliferative medium containing 10% FCS which corresponds to routine laboratory culture conditions. Chondrocytes (lower part) and ASCs (upper part) were plated in a minimal medium to get rid of effects mediated by FCS. First, we investigated the effect of ASCs on the proliferation of chondrocytes. We therefore plated chondrocytes at low density allowing proliferation of the cells. Chondrocytes, alone or in co-

cultures with ASCs, did not proliferate in minimal medium, while they proliferated in the presence of FCS (Fig. 2A). Second, we analyzed the role of ASCs on confluent chondrocytes induced to apoptosis using camptothecin and high density conditions. In these conditions, the number of annexin V⁺ chondrocytes was significantly decreased when co-cultured with ASCs (Fig. 2B). Partial inhibition of apoptosis was confirmed using a second assay where reduced levels of apoptosis were observed both when chondrocytes were cultured with ASCs or in the presence of FCS (Fig. 2C). Finally, we evaluated the phenotype of chondrocytes in the co-culture assay. In the presence of ASCs, chondrocytes maintained the expression of aggrecan (Agg) and Sox9 while Col IIB expression significantly decreased and Hyaluronan and Proteoglycan Link Protein 1 (Link) expression was increased (Fig. 2D). However, the expression of markers of hypertrophic chondrocytes: metalloproteinase (MMP)-13, alkaline phosphatase (AP), and Runx2 was significantly decreased and Col X tended to diminish. Similarly, the expression of fibrotic markers: collagens type I, III, VI and vimentin was all highly significantly down-regulated in the presence of ASCs. Altogether, these data demonstrate a potent role of SC-ASCs in the protection of chondrocytes from apoptosis and loss of mature chondrocyte phenotype to hypertrophic and fibrotic phenotypes which are characteristics of OA.

Effect of culture conditions and tissue origin of ASC preparations on chondrocyte markers

Because clinical grade ASCs may be expanded using platelet-enriched plasma (PLP) instead of FCS, we investigated whether the culture conditions may influence the effect of SC-ASCs on chondrocyte phenotype. We therefore compared clinical grade SC-ASCs (Fig. 3A) and ASCs expanded with FCS

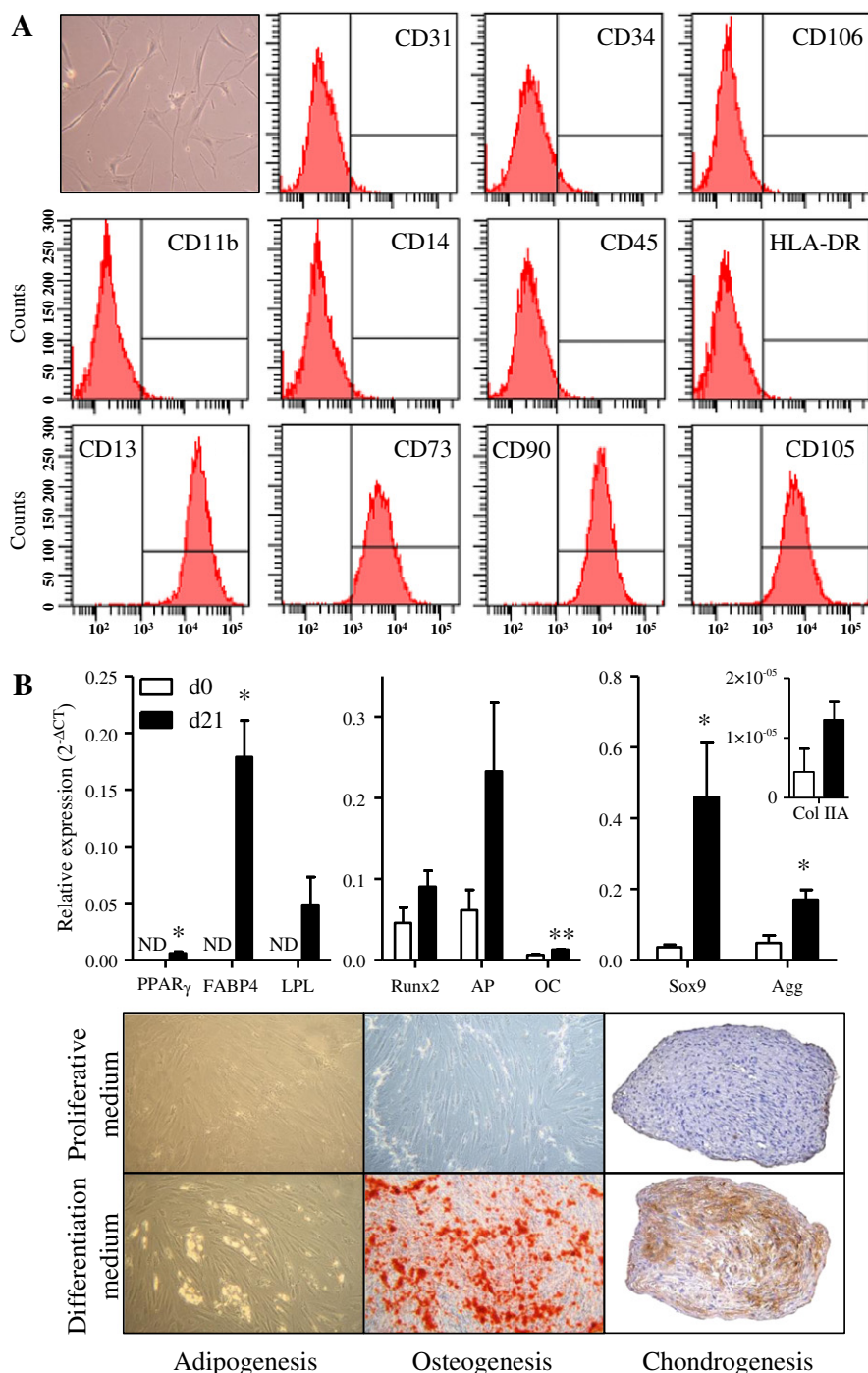


Figure 1 (A) Phenotype of human SC-ASCs: representative photomicrograph and histograms of flow-cytometry analysis of ASCs stained with different antibodies. (B) Differentiation of SC-ASCs: adipogenesis is characterized by expression of peroxysome proliferator-activated receptor (PPAR)- γ , FABP4 and lipoprotein lipase (LPL) and by the visualization of lipid droplets by phase contrast microscopy in differentiation versus proliferative medium at day 21 (d21) versus d0. Osteogenesis is characterized by the expression of Runx2, osteocalcin (OC), alkaline phosphatase (AP) and by Alizarin Red 5 positive staining in differentiation versus proliferative medium. Chondrogenesis is characterized by the expression of Sox9, collagen IIA (col IIA) and aggrecan (Agg) and by anti-aggrecan positive staining on pellet sections (lower panel) versus undifferentiated control section (upper panel). Results are expressed as relative expression ($2^{-\Delta CT}$) and represented as mean \pm sem for 3 independent biological replicates. ND: not detected; * $p < 0.05$, ** $p < 0.01$.

using routine laboratory conditions (Fig. 2C). Both cell samples were equally efficient to reduce the expression of a set of hypertrophic and fibrotic markers on chondrocytes

(data not shown for the other markers). Moreover, clinical grade SC-ASCs were able to maintain the expression of Col IIB in chondrocytes although mRNA levels of Sox9 were

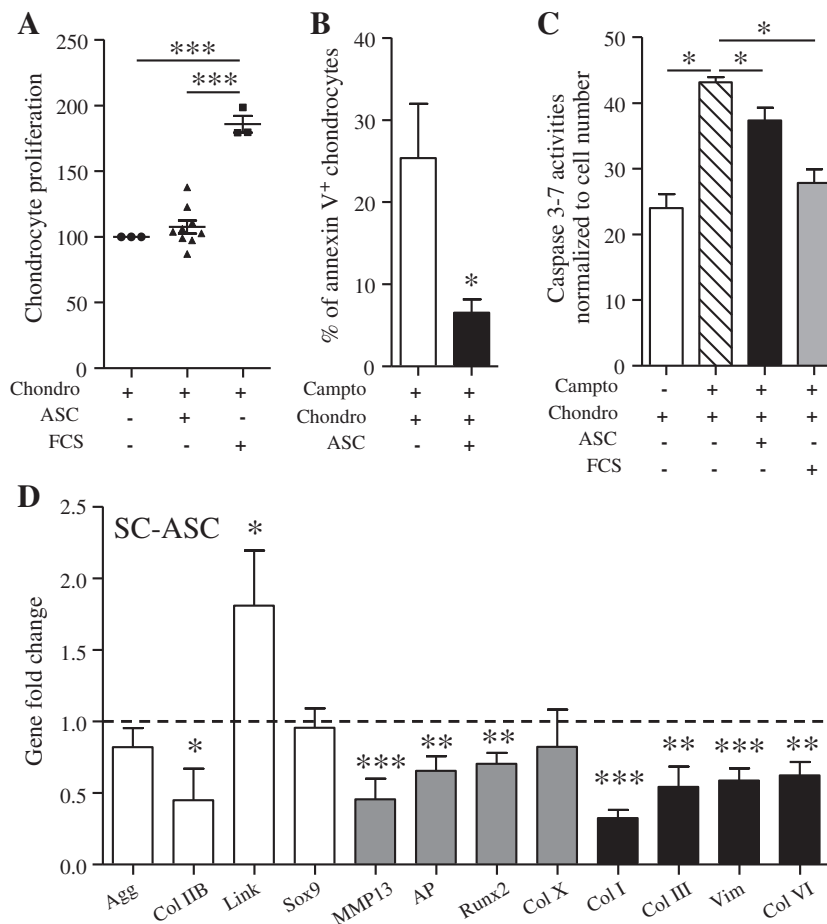


Figure 2 (A) The effect of ASCs on chondrocyte proliferation was evaluated after 2 days of co-culture in minimal medium using a PrestoBlue assay. Results are expressed as the percentage of control (chondrocytes alone) and represented as mean \pm sem (n = 9 biological replicates). (B) Percentage of annexin V⁺ apoptotic chondrocytes as evaluated by flow-cytometry analysis of camptothecin-treated chondrocytes after 2 days of co-culture in minimal medium (mean \pm sem, n = 9 biological replicates). (C) Evaluation of apoptosis in camptothecin-treated chondrocytes after 2 days of co-culture in minimal medium, with or without ASCs or 10% FCS, and determined by caspase 3/7 activities normalized to the number of cells (mean \pm sem, n = 4 biological replicates). (D) Expression of chondrocytic (aggrecan (Agg), collagen IIB (Col IIB), Link and Sox9), hypertrophic (MMP13, alkaline phosphatase (AP), Runx2 and collagen X (Col X)) and fibroblastic (Col I, III, VI and vimentin (Vim)) markers in chondrocytes co-cultured with ASCs from subcutaneous abdominal fat (SC-ASCs). Results are expressed as fold change of gene expression compared to chondrocytes alone (mean \pm sem (n = 10 biological replicates)). *p \leq 0.05, **p < 0.01, ***p < 0.001.

decreased (Fig. 3A). We also evaluated ASCs isolated from diverse locations. Clinical grade expanded Hoffa- and Hip-ASCs were able to reduce the expression level of at least two tested markers. Hoffa-ASCs did not reduce the expression of fibrotic markers (Fig. 3B), while Hip-ASCs significantly decreased expression of Agg, Col IIB and Sox 9 (Fig. 3C). Finally, we evaluated bone marrow-derived MSCs and obtained similar effects as SC-ASC on the expression of fibrotic and mature chondrocyte markers, except for Sox9 and AP whose expression was not down-regulated with BM-MSCs (Fig. 3D). Although direct comparison between ASCs from diverse locations and MSCs was prevented by the difficulty to get cells from the same subjects, our data suggest that SC-ASCs, when expanded under good laboratory practice conditions in the presence of PLP, appeared to be

an interesting source of stem cells for a protective effect on OA chondrocytes.

Secretory profile of chondrocyte and ASC co-cultures

We then quantified the secretion of several factors known to be modulated during the course of OA. Passage 1 chondrocytes and ASCs, alone or in co-culture, did not secrete mediators involved in inflammation such as interleukin (IL)-1 β , Tumor Necrosis Factor (TNF)- α , Matrix Metalloproteinase (MMP)-9 or nitric oxide (NO). IL-1Receptor Antagonist (IL1-RA) was highly produced by chondrocytes and poorly by ASCs while its secretion was not modulated in co-cultures (Fig. 4). Same results were obtained for factors involved in matrix

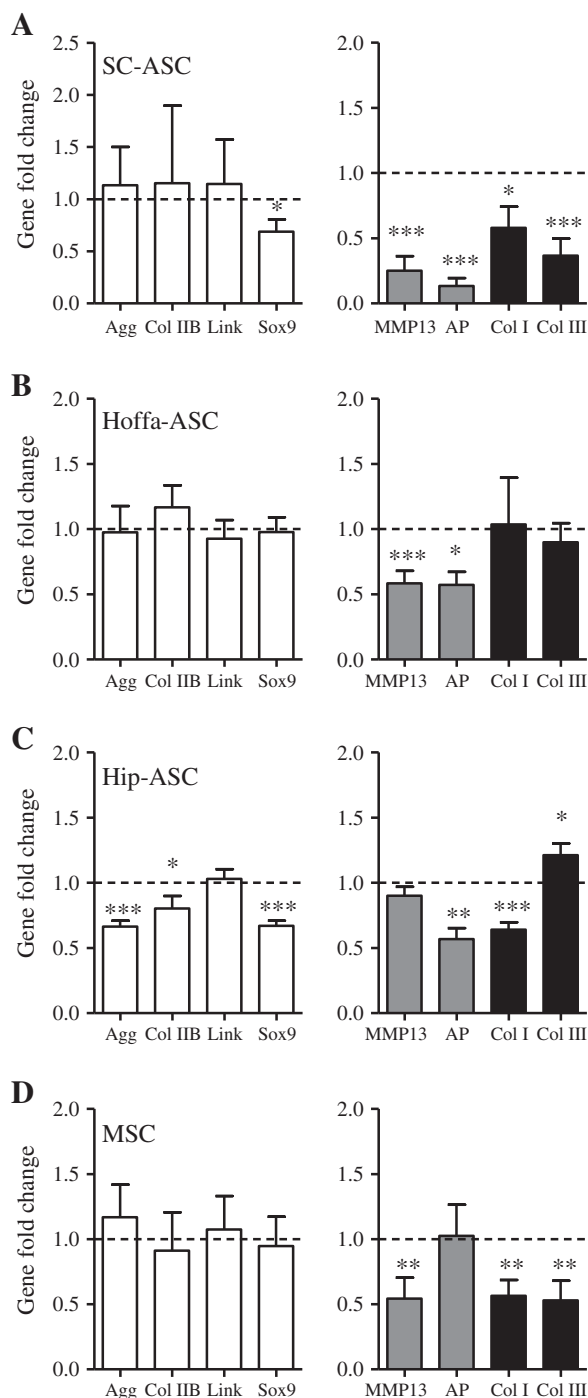


Figure 3 Expression of chondrocytic (aggrecan (Agg), collagen IIB (Col IIB), Link and Sox9), hypertrophic (MMP13 and alkaline phosphatase (AP)) and fibroblastic (Col I and III) markers on chondrocytes co-cultured with ASCs from subcutaneous abdominal fat (SC-ASCs) (A), infrapatellar fat (Hoffa-ASCs) (B), subcutaneous Hip-ASCs (C) or bone-marrow MSCs (D). Results are expressed as fold change of gene expression compared to chondrocytes alone (mean \pm sem (n = 7–18 biological replicates)). *p < 0.05, **p < 0.01, ***p < 0.001.

remodeling namely, thrombospondin (TSP)-1, Tissue Inhibitor of Metalloproteinase (TIMP)-1, TIMP-2 and MMP-1. On the contrary, Transforming Growth Factor (TGF)- β 1 which is a

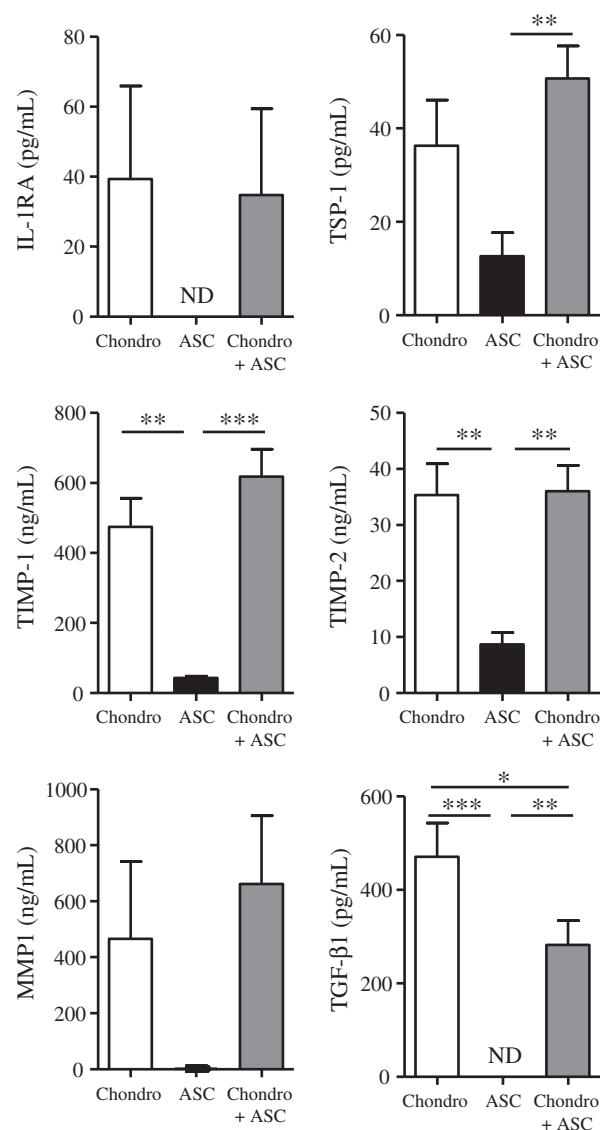


Figure 4 Quantification of different proteins in the supernatants of chondrocytes alone (white bar), SC-ASCs alone (black bar) or the co-culture (gray bar) by ELISA. Results are expressed as the concentration of cytokines (mean \pm sem, n = 18 biological replicates). *p < 0.05, **p < 0.01, ***p < 0.001.

known pro-fibrotic factor was secreted only by chondrocytes and not ASCs. Interestingly, in the co-cultures, the secretion of TGF- β 1 was significantly reduced by 40% further suggesting an anti-fibrotic effect of ASCs on OA chondrocytes.

The anti-fibrotic effect of ASCs is mediated by HGF

In the search for an anti-fibrotic factor secreted by ASCs, we focused on Hepatocyte Growth Factor (HGF). Indeed, high levels of HGF were detected in the supernatants of ASCs or co-cultures but not in chondrocyte supernatants (Fig. 5A). We then quantified the level of mRNA in chondrocytes and ASCs, when cultured alone or together, to identify the HGF-producing cells. Although low level of HGF mRNA was expressed in chondrocytes, high expression was detected in

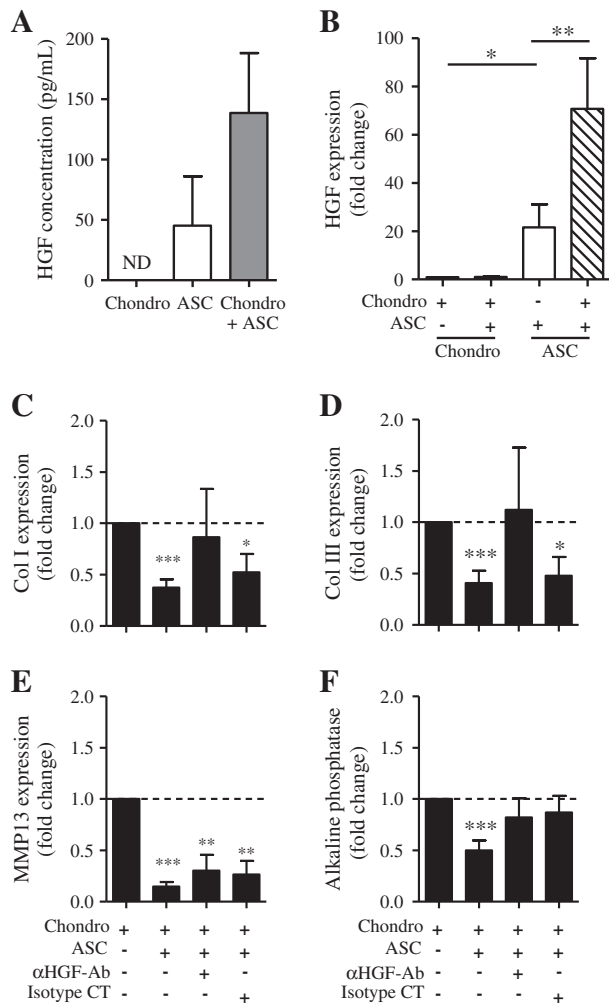


Figure 5 (A) HGF protein was quantified in the supernatants of chondrocytes alone (white bar), ASCs alone (black bar) or the co-culture (gray bar) by ELISA. Results are expressed as the concentration of cytokine (mean \pm sem, $n = 15$ biological replicates). (B) HGF mRNA level was measured by RT-qPCR in cells alone (Chondro or ASC) or co-cultured. Results are expressed as fold change of gene expression compared to chondrocytes alone ($2^{-\Delta\Delta CT}$) and represented as mean \pm sem ($n = 15$ biological replicates). (C–F) Expression of markers on chondrocytes co-cultured with SC-ASCs in the presence or absence of a neutralizing anti-HGF antibody or its isotype control (100 ng/mL) for 7 days. Results are expressed as fold change of gene expression compared to chondrocytes alone (mean \pm sem ($n = 13$ replicates)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ND = not detected.

ASCs and it was significantly increased in the co-cultures (Fig. 5B). To confirm the role of HGF, we used a neutralizing antibody directed against HGF in the cocultures. Addition of the neutralizing antibody reversed the effect of ASCs on expression levels of the fibrotic markers Col I and Col III while the isotypic control did not (Figs. 5C–D). Importantly, the anti-HGF or isotypic antibodies did not modify the expression of MMP13 while the reversion of AP expression was observed both with the anti-HGF and isotypic control antibodies (Figs. 5E–F). These results suggest that HGF secretion is not responsible for the anti-hypertrophic effect observed with

ASCs and demonstrate that chondrocytes activate ASCs to display an anti-fibrotic effect that is mediated to a large extent by HGF.

Discussion

The present study demonstrates for the first time that ASCs secrete molecules that exert a chondroprotective effect through the reduction of hypertrophic and fibrotic phenotypes, which characterize OA chondrocytes, as well as a protective role from apoptosis. We further establish that HGF is at least partly responsible for the anti-fibrotic effect mediated by ASCs.

While ASCs are considered a more accessible source of cells than MSCs, both cells exhibit similar properties and are considered as promising tools for tissue engineering and cell therapy applications (Maumus et al., 2011a). Previously, a number of studies investigated the cross-talk between MSCs and chondrocytes and most of the studies reported the role of chondrocytes on the differentiation of MSCs. Differentiation of MSCs, showed by up-regulation of Col II and aggrecan expression as well as GAG production, occurred via the secretion of factors such as Parathyroid Hormone related Peptide (PTHrP), TGF β , Insulin Growth Factor (IGF)-1 or Bone morphogenetic protein (BMP)-2 by chondrocytes (Acharya et al., 2012; Aung et al., 2011; Fischer et al., 2010; Liu et al., 2010). Conversely, other studies reported that the addition of MSCs increased cartilage matrix production by chondrocytes in co-culture (Giovannini et al., 2010; Mo et al., 2009; Wu et al., 2011, 2012). However, all these reports used MSCs in direct contact with chondrocytes cultured in pellet and under chondroinductive conditions. In these studies, the addition of fat- or BM-derived MSCs resulted in the absence or slight increase of cartilage matrix production by chondrocytes or enhanced proliferation. We therefore investigated the role of ASCs on chondrocytes using a co-culture assay in which OA chondrocytes are cultured in monolayer which is a condition close to the altered phenotype occurring during OA and in a minimal medium to see a direct effect of ASCs, independently of FCS or growth factors contained in chondroinductive medium. We also cultured chondrocytes in a transwell culture system to avoid direct contact with ASCs and evaluated the effect of diffusible molecules. Contrary to previous reports, in our conditions, we did not observe an increased proliferation rate or enhanced production of cartilaginous matrix by chondrocytes, likely due to the absence of chondro-inductive components. However, we were able to rather demonstrate a dual protective role of ASCs on chondrocytes, which was not described previously. ASCs decrease the expression of fibrotic markers, indicative of dedifferentiation towards fibroblast-like cells, and of hypertrophic markers, both hallmarks of OA chondrocytes. These properties were broadly shared by mesenchymal stem cells isolated from bone marrow or fat from different locations. ASCs from diverse fat tissue locations were tested because they display distinct secretomes and may differ in terms of functional properties (Jurgens et al., 2008, 2009; Schipper et al., 2008). Our findings therefore validate the interest of using mesenchymal stem cells, in particular from adipose tissue, not only for chondroinductive effect as shown by some other studies but also for a chondroprotective role decreasing the propensity of OA chondrocytes to become

fibrotic or hypertrophic. Moreover, the present study provides mechanistic insight with which MSCs or ASCs may exert their therapeutic effect in vivo when implanted in the joints of animals with osteoarthritis (Diekman et al., in press; Murphy et al., 2003; Ter Huurne et al., 2012).

We showed that the mechanism of chondroprotection is associated with the down-regulation of TGF- β 1 in chondrocytes. Although not investigated and not demonstrated here, the reduced TGF- β 1 secretion by chondrocytes when co-cultured with ASCs might down-regulate ALK1-Smad1/5/8 signaling that has been described to induce Runx2 activation and MMP13 secretion in OA chondrocytes and therefore explain the lower expression of hypertrophic markers (van der Kraan et al., 2012). Since TGF- β 1 is also known to be pro-fibrotic, its down-regulation might also explain the anti-fibrotic effect of ASCs (Biernacka et al., 2011). Together with the decrease of TGF- β 1 in chondrocytes, we observed the increase of HGF secretion by ASCs. HGF, which is mainly secreted by stromal cells, is known to participate in tissue protection and regeneration, promoting cell survival, proliferation and stimulating extracellular matrix degradation (Nakamura et al., 2011). The anti-fibrotic effect of MSCs through HGF secretion is documented in several diseases such as heart failure, bladder or kidney fibrosis but was never reported in OA (Liu et al., 2011; Shabbir et al., 2009; Song et al., 2012). Although it was reported that chondrocytes do not produce functional HGF (Bau et al., 2004; Guevremont et al., 2003; Pfander et al., 1999), the protein is detected in synovial fluid and its concentration is positively correlated with the severity of OA (Dankbar et al., 2007) while HGF increases proteoglycan synthesis in rabbit chondrocytes (Bau et al., 2004; Takebayashi et al., 1995). In our settings, we showed that HGF mediates the decrease of Col type I and III expression by OA chondrocytes. Moreover, up-regulation of HGF secretion by ASCs co-cultured with chondrocytes argues for a cross-talk between the two cell types. Even though the cross-talk clearly influences the range of HGF secretion, it is likely not required for a measurable effect on chondrocytes, as suggested by another study in a model of heart failure where the intramuscular injection of MSC conditioned medium allowed a regenerative process on the infarcted myocardium (Shabbir et al., 2009). The mechanism by which HGF mediates an anti-fibrotic role in chondrocytes has still to be investigated. However, possible explanation may be through the induction of connective tissue growth factor (CTGF), which suppresses collagen I synthesis (Inoue et al., 2003). It may also be explained by the induction of various matrix metalloproteinases, in particular MMP-1, -2, -9 which are crucial in matrix remodeling by denaturing collagens type I, III or IV (Lee et al., 2011; Sherriff-Tadano et al., 2006). While we identified HGF as a secreted factor playing a key role in the anti-fibrotic effect of ASCs on OA chondrocytes, we cannot however exclude that other released factors may contribute to this effect. Proteomic analysis of supernatants from co-culture and that of cells cultured alone might help identifying other molecules potentially involved in the chondroprotective role of ASCs on chondrocytes (Skalnikova et al., 2011).

Besides their direct effect on cartilage, ASCs might act on the other joint tissues as synovial membrane, ligaments, meniscus and subchondral bone that are also altered in OA. Although OA is not considered an inflammatory disease, pro-inflammatory cytokines, namely IL-1 β and TNF- α , are

secreted by OA chondrocytes and synoviocytes and, participate to joint tissue alterations. Results from a collaborative work report that several pro-inflammatory cytokines are significantly down-regulated in chondrocytes when cultured with ASCs suggesting that ASCs may also be protective through the down-regulation of inflammatory mediators (Manferdini et al., 2013). Moreover, using the pre-clinical murine model of collagenase-induced OA, we recently described that a single ASC injection in the knee joint of mice inhibited synovial activation and formation of chondrocyte/osteocyte in joint ligaments as well as cartilage destruction, probably by suppressing synovial macrophages (Ter Huurne et al., 2012). All these data argue in favor of a trophic action of ASCs for protecting endogenous cartilage from degradation. Here, we demonstrated a pleiotropic action of ASCs on fibrosis, hypertrophy and apoptosis and, proposed HGF as one soluble mediator that participates to this overall effect. These results are promising preclinical data which confirm the interest of using ASCs in cell-based therapies for osteo-articular diseases by mediating chondroprotection in the joints of patients. Ongoing clinical trials should help addressing the efficacy of ASCs in the treatment of OA.

Author disclosure statement

No competing financial interest exists.

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