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LARGE-SCALE SECRETOME ANALYSES UNVEIL THE SUPERIOR IMMUNOSUPPRESSIVE PHENOTYPE OF UMBILICAL CORD STROMAL CELLS AS COMPARED TO OTHER ADULT MESENCHYMAL STROMAL CELLS

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Abstract

Mesenchymal stromal cells (MSCs), given their regenerative potential, are being investigated as a potential therapeutic tool for cartilage lesions. MSCs express several bioactive molecules which act in a paracrine fashion to modulate the tissue microenvironment. Yet, little is known about the divergence of these signalling molecules in different MSC populations. The present study investigated secretomes of stromal cells harvested from Hoffa's fat pad (HFPSCs), synovial membrane (SMSCs), umbilical cord (UCSCs) and cartilage (ACs) by quantitative liquid chromatography-mass spectrometry (LC-MS/MS) proteomics. Also, multiplex protein arrays and functional assays were performed to compare the constitutive immunomodulatory capabilities of different MSCs. Proteins involved in extracellular matrix degradation and inflammation, such as matrix metalloproteinases (MMPs), interleukin (IL)-17 and complement factors, were downregulated in UCSCs as compared to adult cell sources. Additionally, secretion of transforming growth factor (TGF)-β1 and prostaglandin E2 (PGE2) was enhanced in UCSC supernatants. UCSCs were superior in inhibiting peripheral blood mononuclear cell (PBMC) proliferation, migration and cytokine secretion as compared to adult stromal cells. SMSCs significantly suppressed the proliferation of PBMCs only if they were primed with pro-inflammatory cytokines. Although all cell types repressed human leukocyte antigen-DR isotype (HLA-DR) surface expression and cytokine release by activated macrophages, only UCSCs significantly blocked IL-6 and IL-12 production. Furthermore, UCSCs supernatants increased aggrecan gene expression in two-dimensional chondrocyte cultures. The data demonstrated that UCSCs displayed superior anti-inflammatory and immunosuppressive properties than stromal cells from adult tissues. This allogeneic cell source could potentially be considered as an adjuvant therapy for articular cartilage repair.

Keywords: Secretome, umbilical cord stromal cells, chondrocytes, Hoffa's fat pad-derived stromal cells, synovial membrane stromal cells, mesenchymal stromal cells, immunomodulation, cartilage repair.

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Introduction

Articular cartilage lesions associate with pain, discomfort and inflammation of the synovial joint, which subsequently restrict the function of the joint. Mechanical trauma or degenerative disorders are the major causes of articular cartilage injuries (Chubinskaya *et al.*, 2015). Traumatic cartilage lesions may increase the risk of developing osteoarthritis (OA) by more than four times (Muthuri *et al.*, 2011). This fast-growing chronic disease is expected to be the fourth leading cause of disability by 2020 (Cross *et al.*, 2014). Commonly used surgical and nonsurgical OA treatment modalities include intra-articular injections of soluble materials, such as corticosteroids or hyaluronate, autologous blood products, nonsteroidal anti-inflammatory drugs (NSAIDs) and arthroscopic lavage. These procedures improve OA symptoms to a certain degree but do not heal completely the progressive loss of joint

functions (Lee and Wang, 2017; Wolfstadt *et al.*, 2015). Additionally, the treatment of localised cartilage injuries with cell-based therapies benefits patients with debilitative knee functions and delays the onset of secondary OA (Ogura *et al.*, 2017). Although autologous chondrocytes are used as an intuitive source for cell-based therapy (Knutsen *et al.*, 2016; Saris *et al.*, 2014), in recent years, mesenchymal stromal cells (MSCs) are gaining attention as an alternative and potentially effective therapeutic tool for cartilage lesions.

MSCs are successfully isolated and expanded *in vitro* from numerous tissues sources. MSCs represent a defined population of multipotent progenitor cells residing in the perivascular niche of nearly all human tissues, although different views exist (Crisan *et al.*, 2008; Guimaraes-Camboa *et al.*, 2017). MSC differentiation capacity and immunomodulatory properties are demonstrated *in vitro*, irrespective of tissue sources (Ghannam *et al.*, 2010). However, *in vitro* studies show that MSCs from different origins differ in their lineage-specific differentiation capacity and their functional potential (Garcia *et al.*, 2016; Islam *et al.*, 2016; Subramanian *et al.*, 2015). In addition, a systematic review of intra-articular injection of bone marrow MSCs in humans has concluded that articular stem cell therapies are safe (Peeters *et al.*, 2013). Some preclinical studies in animals demonstrate the *in vivo* efficacy of MSCs from different sources, including autogenic, allogeneic and xenogeneic, in OA management. However, none of these studies has compared the optimal source of MSCs (Ozeki *et al.*, 2016; Saulnier *et al.*, 2015; Singh *et al.*, 2014; Yang *et al.*, 2015). Therefore, the choice of optimal MSC source for a given clinical implication has yet to be elucidated.

In mechanistic terms, it is not well established how MSCs exert their effects *in vivo*. Liechty *et al.* (2000) propose that MSCs promote tissue regeneration by engraftment of cells in damaged areas and trans-differentiation into tissue-forming cells to promote repair. Recently, paracrine signalling and release of potent bioactive factors have emerged as modulators of the microenvironment during tissue healing (Gnecchi *et al.*, 2016; Iso *et al.*, 2007; Prockop, 2009). In the field of cartilage repair and OA, the fate of implanted cells during biological repair procedures and their contribution to rebuilding the damaged tissue is mostly unknown. Previous studies in animals suggest that most of the repaired tissue is composed of cells of unknown origin migrating to the lesion (Dell'Accio *et al.*, 2003; Grande *et al.*, 1989). A recent human clinical trial has concluded that allogeneic bone marrow MSCs function as a source of stimulatory and trophic factors, which orchestrate tissue repair rather than differentiating into the host tissue (de Windt *et al.*, 2017).

Clinical procedures based on autologous MSC transplantation, including bone marrow or adipose tissue MSCs, may provide beneficial effects, but are associated with invasive harvesting procedures, two-stage operations and long-time cell expansion *ex vivo*. Allogeneic MSCs harvested from umbilical cords, amniotic membrane and placenta might represent alternative sources for one-stage cell-based therapies. In addition to their pro-angiogenic properties, anti-inflammatory phenotype and multi-lineage differentiation potential, these allogeneic MSCs are well tolerated and elicit low immunogenic responses as their adult counterparts (Balasubramanian *et al.*, 2012; Donders *et al.*, 2015). Differently from the investigation into the neo-tissue-forming ability, the role of secreted bioactive molecules in the context of paracrine signalling and immunomodulation have not been comprehensively explored between cells from adult joints and young cells. The aim of the present study was to find the most suitable cell source to promote immunomodulation and chondrogenesis from the paracrine signalling perspective. The number of published studies presenting comparative analyses of secretory profiles between different cell types is very limited (Amable *et al.*, 2014; Dabrowski *et al.*., 2017). Among those, the present study was the only one applying both large-scale secretomics and multiplex protein analyses and integrating functional immune assays to validate the results.

The present study compared the secretome of culture-expanded cells harvested from four different tissues sources comprising articular cartilage (ACs), Hoffa's fat pad stromal cells (HFPSCs), synovial membrane stromal cells (SMSCs) and umbilical cords stromal cells (UCSCs). For analyses, mechanisms and pathways relevant to cartilage and joint physiology, including inflammation and

Table 1. Demographics of patients from which cells were isolated. M: male; F: female; ICRS: International Celestial Reference System (van den Borne *et al.*, 2007).

Donor	Gender	Age (years)	ICRS grade
1	M	40	I/II
2	F	46	II/III
3	M	47	II/III
4	F	54	II
5	F	60	III
6	M	60	II
7	M	61	III
8	F	67	III
9	F	70	II
10	F	70	III
11	F	71	II/III
12	M	73	III
13	M	74	III
14	M	76	II/III
15	M	77	III

immune regulation, extracellular matrix (ECM) remodelling, mitotic factors and chondro-inductive molecules, were considered. Proteins involved in ECM remodelling, such as matrix metalloproteinases (MMPs), complement factors and serpins, were significantly downregulated in UCSCs as compared to other cell types, whereas cell signalling molecules, such as transforming growth factor (TGF)-β1, monocyte chemoattractant protein-1 (MCP-1) and platelet derived growth factor D (PDGFD), were upregulated in UCSCs. Additionally, the effects of cell supernatants on cartilage signature genes expression in two-dimensional (2D) chondrocyte culture were investigated. UCSCs significantly enhanced the expression of aggrecan gene by dedifferentiated chondrocytes. To evaluate the constitutive abilities of the different MSCs as immunomodulators, the immunoregulatory properties of supernatants from the different cell types were compared by functional immune assays. Data revealed that UCSCs exhibited superior anti-inflammatory properties and low catabolic phenotypes as compared to ACs, HFPSCs and SMSCs.

Materials and Methods

Human materials and ethical statements

Human samples were collected from the University Hospital of Northern Norway (UNN). The Regional Ethical Committee (REK Nord 2014/920 and 2010/586) at the University of Tromsø approved the study. Adult stromal cells were isolated from knee joint tissues of 15 patients (40-77 years old) undergoing total knee replacement (TKR). Patients' demographics are summarised in Table 1. A macroscopic observation was made by the operating surgeon to assess the inflammatory state of the sample. Patients with inflammatory joint diseases and very advanced OA were excluded; however, both secondary post-traumatic and primary OA patients were included in the study. Fat pad and synovial membrane tissues were harvested from TKR patients. UCSCs were isolated from seven umbilical cords immediately after birth. Buffy-coats for isolation of peripheral blood mononuclear cells (PBMCs) were collected from healthy donors from the local blood bank (REK Nord 2014/401). All patients provided written informed consent.

Isolation and culture of human stromal cells

Macroscopically-normal-looking cartilage, corresponding to cartilage areas looking intact to the eye and presenting no signs of abnormalities, such as tears, scratches, rugose or eroded tissue, was collected from femoral condyles during total knee replacements and was used to isolate human chondrocytes. All cell types were isolated using a mixed enzymatic-explant method, as previously described (Islam *et al.*, 2016; Islam *et al.*, 2017). Briefly, all tissue specimens were washed three times with sterile Dulbecco's phosphate buffered saline (PBS; D8537, Sigma-Aldrich) and minced into small pieces for subsequent enzymatic digestion in collagenase XI solution (≥ 800 units/mg solid; C9407, Sigma-Aldrich) at a final concentration of 1.25 mg/mL at 37 °C on a shaker at 400 rpm. Cartilage tissue specimens were digested for 3-4 h, other adult tissue specimens were digested only for 1-1.5 h. UCSCs were isolated from 40 mm-long cord fragments (also known as mixed cords) previously sterilised by immersion in 90 % ethanol for 30 s and enzymatically digested for 1 h collagenase XI. Partially digested tissues were centrifuged for 10 min at 800 ×*g* and resuspended in high-glucose Dulbecco's modified Eagle medium (DMEM; D5796; Sigma-Aldrich) before plating in a T-75 culture flask (156499; ThermoFisher Scientific). The culture medium was supplemented with 62 mg/L L-ascorbic acid (103033E, BDH Laboratory, Pole, UK), 1 % penicillin and streptomycin (P/S; P4333, Sigma-Aldrich) and 20 % foetal bovine serum (FBS; S0115, Biochrom) to promote cell attachment. All cells were incubated in a humidified atmosphere containing 5 % CO_2 at 37 °C. After the initial 24 h, primary cultures were expanded in 10 % FBS-supplemented medium and the medium was changed every 3-4 d until the cultures became confluent. Cells from all tissue sources were used for experiments at passage (P) 3 and 4.

Preparation of conditioned medium (CM)

Serum-containing CM was used in the functional assays with PBMCs and macrophages, whereas serum-free CM was used for protein-arrays and secretomics. Upon reaching 70-80 % confluence, culture flasks were thoroughly washed with warm PBS and the medium was replaced with fresh high-glucose DMEM containing 1 % P/S, with or without 10 % FBS. Serum-free medium was supplemented with 1 μg/mL dexamethasone (PZN-3103491; Galenpharma, Kiel, Germany) and 1 : 1000 insulin-transferrin-selenium supplement (ITS; 354351; Corning). For priming of cells with pro-inflammatory cytokines, growth media were supplemented for 48 h with 50 ng/mL each of tumour necrosis factor alpha (TNF-α; 300-01A, PeproTech) and interferon gamma (IFNγ; 300-02, PeproTech). CM was collected after 48 h, centrifuged at 4500 ×*g* for 10 min, filtered through a 0.22 μm porous membrane (GSWP04700; Millipore) and used immediately for experiments or stored at – 70 °C for further analysis. Cells were counted for each culture condition and the value used to normalise the measured expression of cytokines and growth factors in the CM. In functional assays with PBMCs and macrophages, the fresh culture medium was diluted with serum-containing CM (1 : 1) from different stromal cells.

Quantitative and qualitative liquid chromatography-mass spectrometry (LC-MS/MS) analysis

6 mL of serum-free CM from all cultures were collected from T-75 culture flasks and concentrated

for 20 min at 4500 ×*g* in PBS to a final volume of 500-800 µL using a 5 kDa molecular weight cut-off (MWCO) Vivaspin 6 column (Z614440-25EA, Sigma-Aldrich). Protein concentration was measured using DC Protein Assay Kit (5000116, Bio-Rad). Protein samples were prepared as described before (Islam *et al.*, 2019). Briefly, protein samples (100 µg/tube) were reduced in 5 mM dithiothreitol (D9779, Sigma-Aldrich) for 30 min at 70 °C. Samples were alkylated by incubation with 375 mM iodoacetamide (90034, ThermoFisher Scientific) at room temperature for 30 min in the dark. Protein samples were collected as dry pellets after overnight precipitation in pre-chilled acetone (270725, Sigma-Aldrich) at − 20 °C. Dry pellets containing 100 µg of protein were resuspended in 100 µL of 2 M urea (U1250, Sigma-Aldrich) with 50 mM triethylammonium bicarbonate (TEAB; 90114, ThermoFisher Scientific). Only 25 µg of protein per sample were taken for further analysis. Samples were pre-digested for 6 h with 1 : 100 (w/w) LysC endopeptidase (125-05061, Wako Chemicals, Neuss, Germany) with 1 mM final concentration of $CaCl_2$, followed by further dilution with 50 mM TEAB in 1 M urea and digestion overnight in 1 : 20 (w/w) trypsin (V511A, Promega). 5 µL trifluoroacetic acid (10 %) (28904, ThermoFisher Scientific) were added to each tube and centrifuged at 16,000 ×*g* for 10 min. OMIX C18 tips were used for sample clean-up and concentration. Samples containing 0.2 % formic acid (FA; 28905, ThermoFisher Scientific) were loaded into a ThermoFisher Scientific EASY-nLC1000 system and EASY-Spray column (C18, 2 µm, 10 nm, 50 µm, 500 mm). Peptides were fractionated using a 2-100 % acetonitrile (51101, ThermoFisher Scientific) gradient in 0.1 % FA at a flow rate of 250 nL/min over 180 min. The separated peptides were analysed using a ThermoFisher Scientific Q-Exactive mass spectrometer. Data were collected by Top10 method in data-dependent mode. The raw data were processed using MaxQuant (v 1.5.6.0) for label-free protein quantification (LFQ). MS/MS data were searched against the UniProt human database (Web ref. 1) from November 2016 to yield protein identification [false discovery rate (FDR) = 0.01]. Parameters used for the search: fixed modification, carbamidomethylation of cysteines; variable modifications, oxidation of methionine and acetylation of protein N-terminal; ion mass tolerance, 4.5 ppm; fragment mass tolerance, 20 ppm; charge states, 2+, 3+ and 4+; maximum missed cleavages, 2; enzyme specificity, trypsin; minimum number of unique peptides, 2. Perseus 1.5.6.0 software (Max Planck Institute of Biochemistry, Germany) was used for statistical analysis of the identified proteins. All contaminants were filtered out before log_{10}-transformation of data for further analysis. The log_{10}-transformed intensities were normalised by subtracting the median. Data were grouped as ACs, HFPSCs, SMSCs and UCSCs and analysed using a *t*-test, with a minimum of three valid values in each group. Volcano plots for each comparison were generated to identify differentially expressed proteins using FDR < 0.01.

Multiplex protein arrays

A panel of 36 specific proteins including cytokines, chemokines, MMPs and growth factors was measured in the serum-free CM of all four stromal cell types by immune-based protein array. A human cytokine magnetic 25-plex kit (LHC0009M, ThermoFisher Scientific) was used to measure the concentration of 18 cytokines (1 : 4 dilution) involved in inflammation – including granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-α, IFN-γ, interleukin (IL)-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17 and TNF-α – and 7 chemokines (1 : 4 dilution) – including eotaxin, interferon-gamma-induced protein 10 (IP-10), MCP-1, MIG, macrophage inflammatory proteins (MIP)-1α, MIP-1β and RANTES. Fluorokine MAP human MMP base kit (LMP000, R&D Systems) was used to measure the concentration of MMP-1, MMP-3, MMP-7, MMP-9 and MMP-13 (1 : 5 dilution). Quantitative measurements (two replicates) were performed according to manufacturers' guidelines using Luminex Bio-Plex 200 system (Bio-Rad). In addition, quantification of PGE2 (KGE004B), IL-4 (DY204-05), IL-10 (DY217B-05) and the five growth factors TGF-β1 (DY240-05), BMP-2 (DY355-05), IGF-1 (DY291-05), PDGF-AB (DY222) and basic fibroblast growth factor (bFGF) (DY233-05) was performed by enzyme-linked immunosorbent assay (ELISA). All ELISA kits were purchased from R&D Systems and performed according to the manufacturer' instructions. Measured protein concentrations were normalised to cell number at specific culture conditions and expressed as pg/mL/10^6 cells.

Isolation and culture of human PBMCs

PBMCs were isolated from buffy-coats (n = 5) of whole blood using Lymphoprep™ (1114545, Alere Technologies, Oslo, Norway) following the manufacturer' instructions. Briefly, peripheral blood was diluted in sterile PBS (1 : 1) and slowly layered over a Lymphoprep™ gradient in a 50 mL tube. The tube was centrifuged at 800 ×*g* for 30 min at room temperature. PBMCs were collected from the gradient-interface with a Pasteur pipette, followed by washing three times for 10 min at 400 ×*g* with phosphate NaCl (PBSA) containing PBS and 0.2 % bovine serum albumin (BSA; 130-091-376, Miltenyi Biotec). PBMCs were cultured in growth medium containing Rosewell Park Memorial Institute medium (RPMI-1640; R8758, Sigma-Aldrich), 1 % P/S and 10 % FBS in a humidified atmosphere (5 % CO_2) at 37 °C.

PBMC proliferation assay

PBMC proliferation was assessed using the carboxyfluorescein succinimidyl ester (CFSE) dilution assay (10009853, Cayman). Cultured PBMCs were washed in pre-warmed sterile PBS and centrifuged at 400 ×*g* for 5 min before incubation with

CFSE for 15 min at 1 : 400 dilution. CFSE-stained PBMCs were cultured in RPMI, 1 % P/S and 10 % FBS in a 24-well plate at a density of 10^6 cells/well. PBMCs were stimulated with 10 μg/mL mitogen phytohemagglutinin (PHA; 1249738, Roche) for 5 d to induce proliferation. Half of the medium was replaced with fresh medium after the second day. The proliferation assay was performed on a BD FACSAria III flow cytometer (BD Biosciences) and the data were analysed by FlowJo software (Tree Star Inc., Ashland, OR, USA). CM from stromal cells was added to the culture of PHA-activated PBMCs from day 1 at 1 : 1 ratio with fresh culture medium. CM from the last 3 d of PBMC proliferation was collected to measure cytokine profiles. CM derived from PBMCs was centrifuged at 4500 ×*g* for 5 min and filtered through a 0.22 μm porous membrane (GSWP04700; Millipore) before analysing TNF-α (DY210-05; R&D Systems) and IFN-γ (DY285-05; R&D Systems) content by ELISA.

PBMC migration assay

PBMC migration assay was performed by Boyden chamber assay (Gorchs *et al.*, 2015). PBMCs were activated with 10 μg/mL PHA for 48 h before performing the assay. 100 ng/mL recombinant stromal cell-derived factor-1 (SDF-1; 300-28A, PeproTech) was used as a chemoattractant. 10^6 cells/100 μL PBMCs were added to the top chamber of Corning® Costar® Transwell® cell culture inserts (6.5 mm diameter, 8 μm pores; CLS3464-48EA, Sigma-Aldrich). Bottom chambers contained either growth medium or serum-rich CM from different stromal cells. After 2 h, PBMCs that migrated to the lower chamber were harvested and washed in PBSA by centrifugation at 400 ×*g* for 4 min followed by resuspension in 0.5 mL of PBSA. Lymphocytes population was gated using forward and side scatter and counted on a BD FACSAria III flow cytometer.

Isolation and culture of monocyte-derived macrophages

Monocytes were isolated from PBMCs using $CD14^+$ magnetic-activated cell sorting (MACS; 130-050-201, Miltenyi Biotec) with minor modification from manufacturer' guidelines. Briefly, PBMCs were washed in MACS buffer containing autoMACS rinsing solution (130-091-222, Miltenyi Biotec) and BSA (20 : 1) for 10 min at 4 °C. PBMCs were incubated with $CD14^+$ microbeads at a concentration of 10 μL beads/10^7 cells in 40 μL of MACS buffer for 15 min at 4 °C. PBMCs were rinsed in MACS buffer and resuspended in an appropriate volume before passing through the MS column. $CD14^+$ monocytes were eluted from the column and washed with ice-cold MACS buffer. The purity of eluted monocytes was checked by flow cytometry using anti-CD14-fluorescein isothiocyanate (FITC)-conjugated antibody (130-098-063, Miltenyi Biotec) and its isotype control mouse IgG2a-FITC (130-098-877). Fully transformed macrophages (M0-M) were achieved after 6 d incubation of $CD14^+$ monocytes in a macrophage growing medium containing RPMI-1640, 1 % P/S, 10 % FBS and 100 ng/mL macrophage colony-stimulating factor (M-CSF; 300-25, PeproTech) in a humidified atmosphere (5 % CO_2) at 37 °C. The medium was replaced with fresh medium after 3 d.

Macrophage polarisation assay

For induction of the M1 phenotype (M1-M), M0-M were plated in a 6-well plate at a density of 2.5 × 10^6 cells/well and stimulated for 48 h with 100 ng/mL lipopolysaccharides (LPS; L6529-1MG, Sigma-Aldrich) and 20 ng/mL IFN-γ. To investigate the effects of cell secretomes on M1-M polarisation, macrophage culture medium was diluted with serum-rich CM (1 : 1) from different stromal cells. Macrophages were harvested after 48 h and washed in MACS buffer before analysing on BD FACSAria III flow cytometer. FlowJo software was used for analysing surface markers expression.

M1-M polarisation was characterised by surface marker expression of CD40 (130-099-385, Miltenyi Biotec), CD64 (130-100-415, Miltenyi Biotec), CD80 (130-110-371, Miltenyi Biotec), CD86 (560957; BD Biosciences) and HLA-DR (560943, BD Biosciences). All antibodies were phycoerythrin (PE)-conjugated and analysed with respective isotype controls, including mouse IgG1 (130-098-845, Miltenyi Biotec), REA control (130-104-612, Miltenyi Biotec) and mouse IgG2a (555574, BD Biosciences). For induction of M2 phenotype (M2-M), M0-M were stimulated with 4 μg/mL dexamethasone for 48 h and characterised by surface marker expression of

Table 2. qPCR probes.

Gene name	Gene symbol	Assay ID
Collagen type I α1	COL1A1	Hs00164004_m1
Collagen type II α1	COL2A1	Hs00264051_m1
Aggrecan	ACAN	Hs00153936_m1
sex determining region Y (SRY)-box 9	SOX9	Hs00165814_m1
Ribosomal protein L13A	RPL13A	Hs04194366_g1

CD163-FITC; 130-099-969, Miltenyi Biotec) and its isotype control mouse IgG1-FITC (130-098-847). To further investigate the effects of CM from all cell types on M1-M polarisation, macrophage-CM was collected after 48 h, centrifuged at 4500 ×*g* for 5 min, filtered by 0.22 µm porous membrane (GSWP04700; Millipore) and used immediately for experiments or stored at − 70 °C for further analysis. The concentration of TNF-α, IL-6 (DY206-05) and IL-12 (DY1240-05) were measured by ELISA. All ELISA kits were purchased from R&D Systems.

qPCR

Dedifferentiated chondrocytes at P3 were cultured for 48 h in the presence of stimulated and non-stimulated CM. After 48 h, chondrocytes were harvested for qPCR to analyse the expression of cartilage signature genes. Dedifferentiated chondrocytes at P2 and P3 grown in the absence of CM were used as controls. The qPCR reaction was performed as described before (Hansen *et al.*, 2017). Briefly, RNA was extracted according to the manufacturer's guidelines using the RNeasy Plus Mini Kit (74134, QIAGEN). Each RNA template was reverse transcribed to cDNA using the qScript cDNA Synthesis Kit (95047, Quanta Biosciences). The qPCR reaction was run on a StepOnePlus™ Real-Time PCR system (Applied Biosystems). Probes used are summarised in Table 2. Gene relative expression was presented using the ΔCq value (Hansen *et al.*, 2017) and calculated by subtracting the gene of interest from the reference gene (RPL13A), making higher ΔCq value represents increased gene expression and *vice versa*.

Three-dimensional (3D) cultures

In vitro 3D chondrogenic assays of dedifferentiated chondrocytes was performed using a pellet culture method as described previously (Islam *et al.*, 2016). Briefly, chondrocytes were harvested at P3, centrifuged in a conical-bottom 96-well plate for 10 min at 1,100 ×*g* and incubated in culture medium for 48 h in low oxygen (3 % O_2) atmosphere. After 48 h, the newly formed neo-tissues were transferred to ultra-low attachment plate and cultured for 21 d in serum-free basal chondrogenic medium containing high-glucose DMEM, 62 mg/L L-ascorbic acid, 1 % P/S, 1 µg/mL dexamethasone, 1 : 1000 ITS in low oxygen (3 % O_2) atmosphere. Chondrogenesis was induced in the control samples by adding 10 ng/mL TGF-β1 (100-21C; PeproTech) and 100 ng/mL BMP-2 (120-02C; PeproTech) to the basal chondrogenic medium. To investigate the effect of stimulated and non-stimulated CM on chondrogenesis, the spheroids were incubated with CM in the absence of growth factors. Half of the CM was replaced with fresh CM twice a week. Spheroids were harvested after 21 d and fixed in 10 % formalin overnight for histological evaluation. Alcian blue solution (A5268, Sigma-Aldrich) was used for metachromatic staining of 4 µm-thick paraffin-embedded sections.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics version 24 (Chicago, IL, USA). Data were analysed using non-parametric Kruskal-Wallis test, unless an exception was mentioned, and significance values were adjusted by Bonferroni correction for multiple comparisons. The level of significance was set at $p < 0.05$. Results are presented as density graphs, where each donor plotted as a dot in the dataset.

Results

Comparative protein profiles in supernatants of different stromal cells by LC-MS/MS proteomics

The MS secretomics data (identifier PXD011762) were deposited to the ProteomeXchange Consortium through the PRIDE partner repository (Vizcaíno *et al.*, 2016). All cell types were characterised by MSC surface markers and retained similar characteristics, as shown by Islam *et al.* (2016). The cell secretome established in serum-free CM from each cell type (four unrelated donors per cell type) was analysed by LC-MS/MS proteomics. Only proteins identified in at least three donors in each group were considered for further analyses. Results showed more proteins identified in the supernatants of ACs (709) as compared to HFPSCs (641), SMSCs (567) and UCSCs (653) (Fig. 1**a**). Comparative analysis of identified proteins revealed 472 proteins present in the supernatants of all four cell types. Only a minor fraction of proteins was exclusively found in supernatants of specific cell types, including 50 differentially expressed by UCSCs, 44 by ACs, 22 by HFPSCs and 2 by SMSCs. Hierarchical clustering of identified proteins revealed two major clusters, where one cluster comprised the four donors of UCSCs and the second cluster comprised all stromal cells from adult tissues (Fig. 1**b**). Furthermore, among the stromal cells from adult tissues, the four AC donors were clearly separated from HFPSC and SMSC donors. Identified proteins were divided into six groups according to their functions using Gene Ontology Biological Process (GOBP) terms (Pazos and Chagoyen, 2009) (Fig. 2**a**). Qualitative comparison of proteins in different pathways revealed no major differences between cell sources. Proteins involved in ECM remodelling were abundant in the supernatants of all cell types. In addition, all stromal cells released similar percentage of proteins involved in immunoregulation (~ 20 %) and secretion (~ 13 %) (Fig. 2**a**).

Quantitative analyses of protein expression were performed using the LFQ approach (Patel *et al.*, 2009) (Fig. 2**b**). Six volcano plots representing all possible comparisons showed differentially expressed proteins by plotting $\log_{10}$ of the fold change on the X-axis and $-\log_{10}$ of the *p*-value on the Y-axis for each comparison (*e.g.* HFPSCs *vs.* ACs). Results revealed the largest differences in protein expression between

UCSCs and all other adult stromal cells (FDR < 0.01). Proteins involved in cell signalling, such as TGF-β1, PDGFD and MCP-1, were significantly upregulated in UCSCs (FDR < 0.01), while catabolic proteins, such as MMPs, serpins and complement factors, were downregulated as compared to stromal cells from adult origin (Fig. 2**b**). Notably, minor differences particularly in ECM remodelling proteins, such as MMPs and serpins, were observed while comparing stromal cells from cartilage and synovium (Fig. 2**b**). Protein profiles belonging to specific pathways (ECM remodelling, cell communication and inflammation) were compared among the four cell types (Fig. 3). Several MMPs, serpins, some complement factors and heat shock proteins were significantly downregulated in UCSCs (FDR < 0.01). On the other hand, cell signalling molecules including MCP-1, integrin (ITG)-β1, PDGFD, CSF-1, HLA-C and TGF-β1 were more abundant in the supernatants of UCSCs.

Determination of cytokines and growth factors in supernatants by multiplex protein arrays

A panel of 18 selected cytokines involved in inflammation and immunoregulation was measured in the supernatants of all stromal cells. Only IL-4, IL-6, IL-8, IL-12 and IL-17 were detected in supernatants of all cell types, whereas GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-5, IL-7, IL-13, IL-15 and TNF-α were not detected in any of the supernatants. From the panel of chemokines, MCP-1, MIP-1α and RANTES were detected in all supernatants, but eotaxin, IP-10, MIG and MIP-1β were not detected. Overall, the levels of IL-6, MCP-1 and PGE2 were increased in UCSCs supernatants as compared to other cell types, whereas the levels of IL-17, MIP-1α and RANTES were decreased (Fig. 4). The concentration of IL-17 and MIP-1α was significantly lower in the UCSC supernatant as compared to AC supernatant and the levels of PGE2 was significantly higher in UCSC supernatants as compared to HFPSC supernatant (Fig. 4). With regards to the expression of proteases, only MMP-13 was not detectable, whereas MMP-1, MMP-3, MMP-7 and MMP-9 were detected to some degree in all serum-free CM (Fig. 5). The secretion of MMP-1, MMP-3, MMP-7 and MMP-9 was in general lower in UCSCs as compared to all other cell types. Significant differences were found for MMP-3 and MMP-7 when comparing UCSCs and ACs. The anabolic growth factors TGF-β1, BMP-2 and bFGF were detected at low levels in supernatants of the four cell sources, whereas IGF-1 and PDGF-AB could not be detected. Additionally, TGF-β1 was significantly elevated in UCSCs as compared to HFPSCs (Fig. 5). To check for possible effects of serum supplementation on the production of cytokines by MSCs, the expression of TNF-α, IFN-γ,

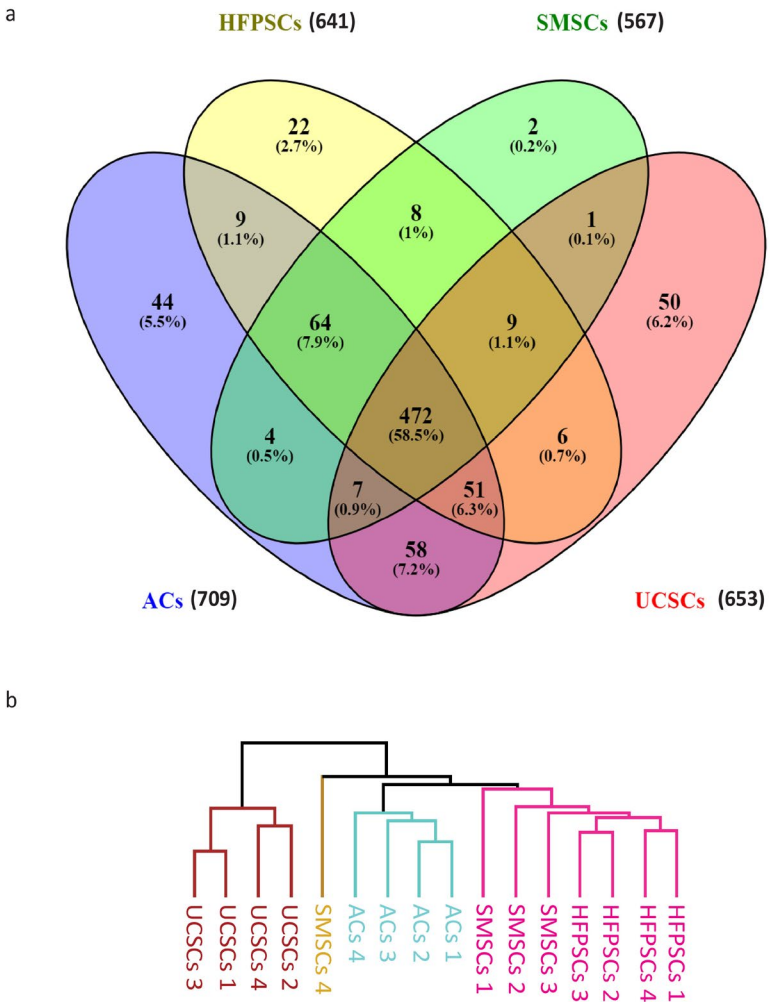

Fig. 1. Hierarchical clustering of identified proteins from secretomes of ACs, HFPSCs, SMSCs and UCSCs. (**a**) Venn diagram depicts the percentage of identified proteins shared among the four different stromal cell types (n = 4 donors/cell sources). (**b**) Dendrogram shows two major clusters of four different stromal cell types. All cell sources from adult mesenchymal origin clustered together, whereas the four donors of UCSCs from extra-embryonic origin clustered separately.

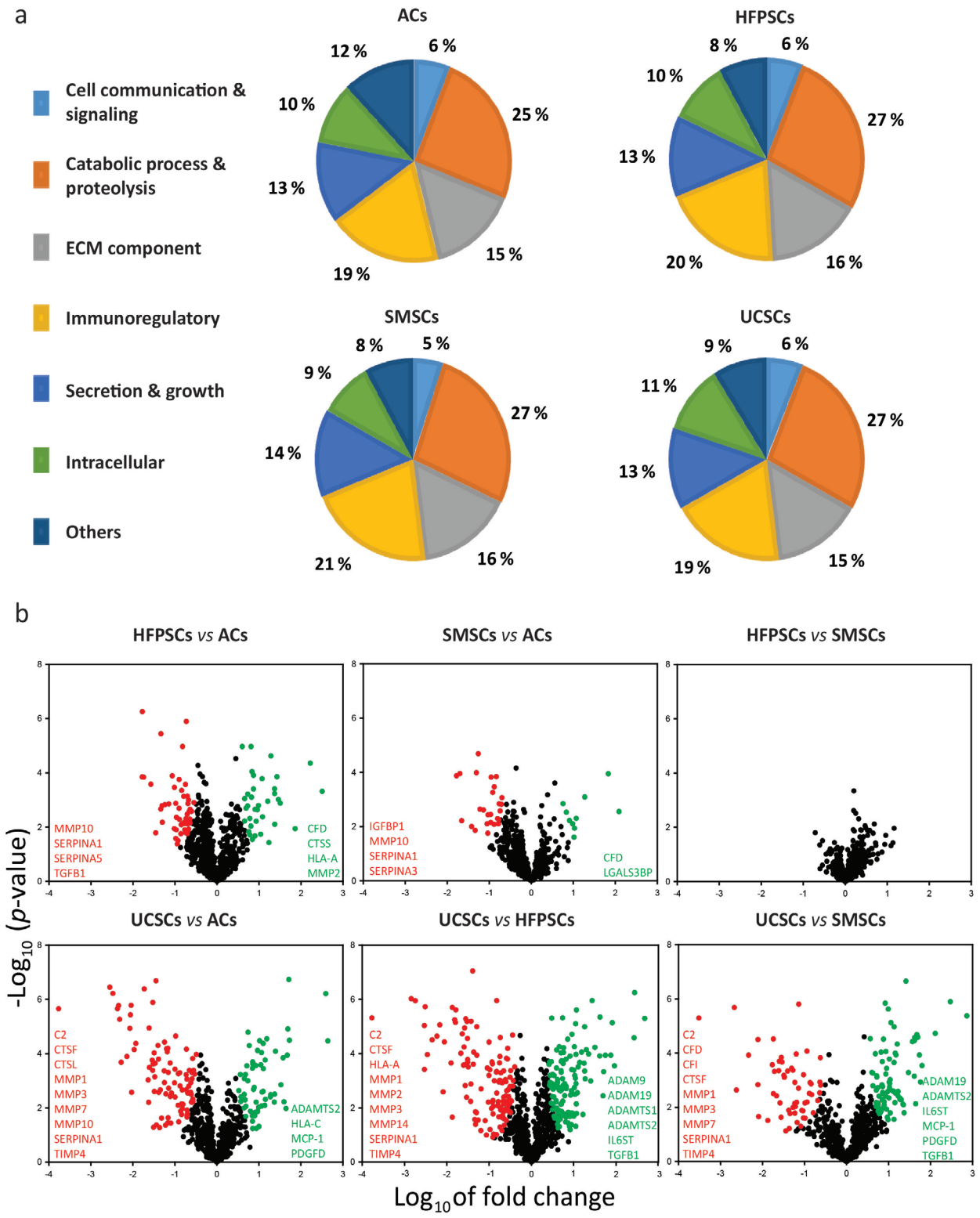

Fig. 2. Protein expression analysis by LC-MS/MS from CM of ACs, HFPSCs, SMSCs and UCSCs. (**a**) Distribution of identified proteins into six main categories according to their function annotated using GOBP terms. (**b**) Volcano plots illustrate the results of the six sets of statistical comparisons made between HFPSCs *vs.* ACs, SMSCs *vs.* ACs, HFPSCs *vs.* SMSCs, UCSCs *vs.* ACs, UCSCs *vs.* HFPSCs and UCSCs *vs.* SMSCs. These plots show each protein with $-\log_{10}$ (*p*-value) and $\log_{10}$ of fold change of the comparison on the Y-axis and X-axis, respectively. Proteins with larger fold change and lower *p*-value are plotted further away from zero on each axis. Proteins that are significantly up- and down-regulated ($p < 0.01$) are represented in green and red, respectively.

IL-6 and IL-12 in both serum-containing and serum-free CM from all four cell types was analysed. From the analysed cytokines, only the expression of IL-6 was considerably changed in the presence of serum (Fig. 6).

UCSC supernatants exerted stronger immunosuppressive effects on activated PBMCs

To investigate the immunomodulatory effects of cell supernatants on activated PBMCs, *in vitro* lymphocytes proliferation and migration assays were performed (Fig. 7**a**). PHA-activated PBMCs were incubated for 5 d in the presence or absence of serum-containing CM from the different cell sources. The proliferation assay revealed that UCSC supernatants blocked PBMC proliferation (p = 0.06) when compared with PHA-treated controls (Fig. 7**a**,**b**). CM from ACs did not block PBMC proliferation, while HFPSCs (71 ± 5 %) and SMSCs (68 ± 3 %) had a minor effect. In the migration assay, both UCSCs and SMSCs blocked the migration of activated PBMCs as compared to positive controls (38 ± 2.5 % and 38 ± 1.2 % *vs.* 44.2 ± 0.5 %, respectively) (Fig. 7**b**). To further investigate the immunomodulatory effects of MSC supernatants, the expression of TNF-α and IFN-γ in PBMCs-CM was measured. Values were normalised against residual expression levels present in supernatants of stromal cells. Supernatants from HFPSCs and SMSCs stimulated the production of TNF-α and IFN-γ above the levels achieved by PHA treatments. Importantly, supernatants from UCSCs suppressed the production of both TNF-α and IFN-γ by activated PBMCs, reaching significant differences when compared to HFPSCs (Fig. 7**c**).

Immunomodulatory properties of supernatants after stimulation of MSCs with pro-inflammatory cytokines

TGF-β1, PGE2, IL-4 and IL-10 were measured in the supernatants of stimulated and non-stimulated stromal cells by ELISA. Only TGF-β1 and PGE2 were detected in the supernatants of both stimulated and non-stimulated cells (Fig. 8**a**), whereas IL-4 and IL-10 were not detected. In supernatants of cells stimulated with pro-inflammatory cytokines, the level of TGF-β1 was increased in AC cultures (p = 0.09) but unchanged in SMSC and UCSC cultures. The amount of secreted PGE2 significantly increased in the supernatants of stimulated ACs and SMSCs (Fig. 8**a**). In addition, PBMC proliferation assay demonstrated superior blocking capacity of supernatants from primed adult stromal cells but not from UCSCs (Fig. 8**b**).

UCSC supernatants exerted superior anti-inflammatory effects on M1 activated macrophages

Macrophage polarisation assays were performed as previously reported (Ambarus *et al.*, 2012; Vogel *et al.*, 2014). A panel of co-stimulatory molecules and

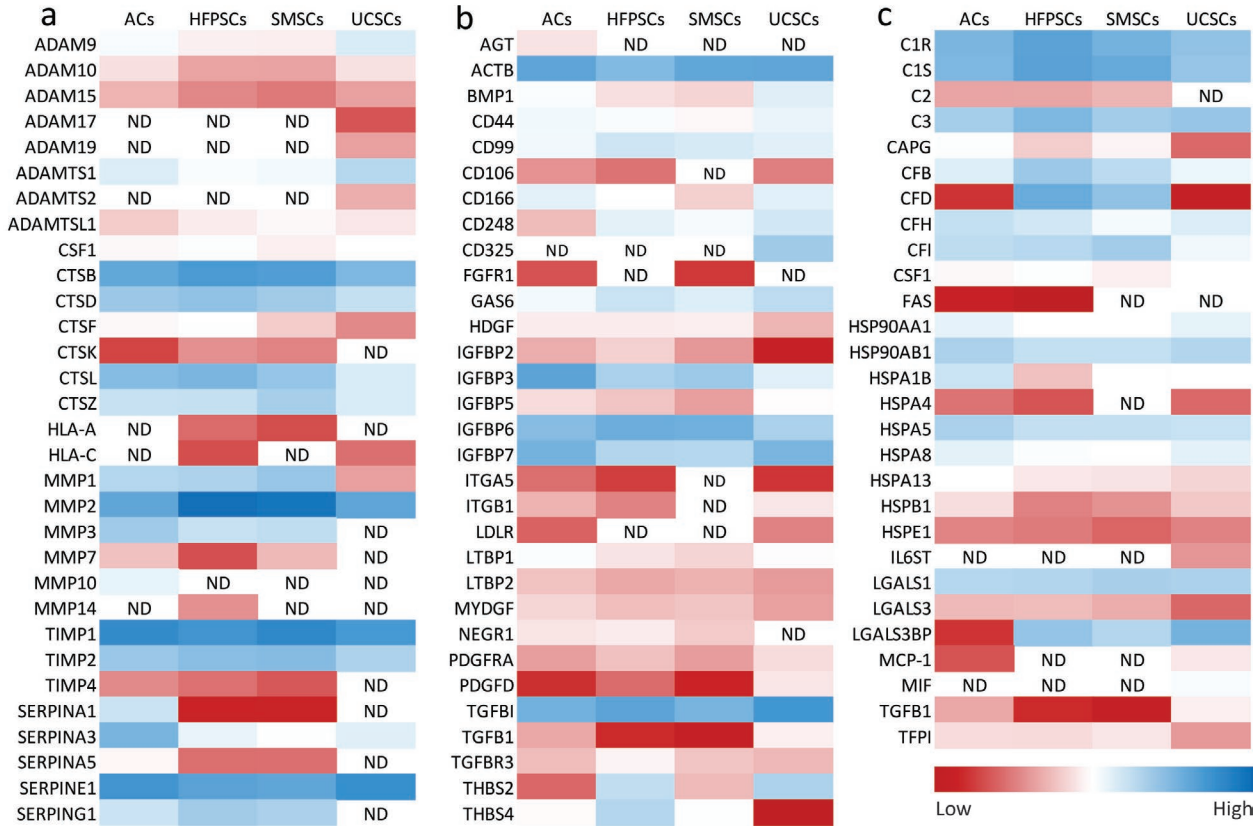

Fig. 3. Comparative expression of selected proteins identified by LC/MS-MS belonging to specific pathways. Heat map showing proteins involved in (**a**) ECM remodelling (proteases and inhibitors), (**b**) cell signalling (growth factors) and (**c**) inflammation/immune responses. Down-regulated proteins are indicated in red, whereas up-regulated proteins in blue.

cytokines to characterise macrophage polarisation was chosen based on validation tests using different stimulants. Divergent expression of these markers was observed in the presence of different stimulants. In addition, IL-10 expression resulted to be an irrelevant marker for M2 polarised macrophages. In the validation test, increased IL-10 production was observed in the presence of LPS and IFN-γ (for M1 induction) as compared to dexamethasone or TGF-β1 and IL-4 stimulation (for M2 induction) (data not shown). These discrepancies are also reported by Chanteux *et al.* (2007) and Vogel *et al.* (2014). Moreover, CD163 was a suitable marker for dexamethasone-stimulated M2 polarised macrophages but not for TGF-β1 and IL-4 stimulation. The discrepancy concerning the expression of M2 markers is also demonstrated by Jaguin *et al.* (2013).

The immunomodulatory effects on M1 polarised macrophages were investigated by characterisation of surface marker expression (CD40, HLA-DR, CD64, CD80, CD86) and inflammatory cytokines release (Fig. 9). Supernatants from all stromal cell types suppressed the surface expression of HLA-DR on activated macrophages, whereas only UCSC supernatants were able to significantly suppress the expression of CD40. On the other hand, supernatants from ACs were able to significantly increase the expression of the co-regulatory receptors CD80 and CD86 above the levels of M1 activation (Fig. 9**b**). In contrast to ACs and UCSCs, supernatants from HFPSCs and SMSCs increased the surface expression of CD64 above M1 activation levels. None of the supernatants was able to alter the expression of the M2 phenotype marker CD163. With regards to cytokine profiles, CM from all cell types was able to reduce the production of TNF-α, IL-6 and IL-12 by M1-M. Of note, a significant reduction of IL-6 and IL-12 concentration was only achieved by UCSCs (Fig. 9**c**).

Effects of UCSC CM on AC chondrogenesis

To check the chondrogenic potential of UCSC secretomes, the effects of UCSCs-CM on the expression of cartilage signature genes and the tissue-forming capacity of dedifferentiated chondrocytes were investigated. Incubation with UCSCs-CM positively influenced gene expression of COL2A1, SOX9 and ACAN as compared to non-stimulated

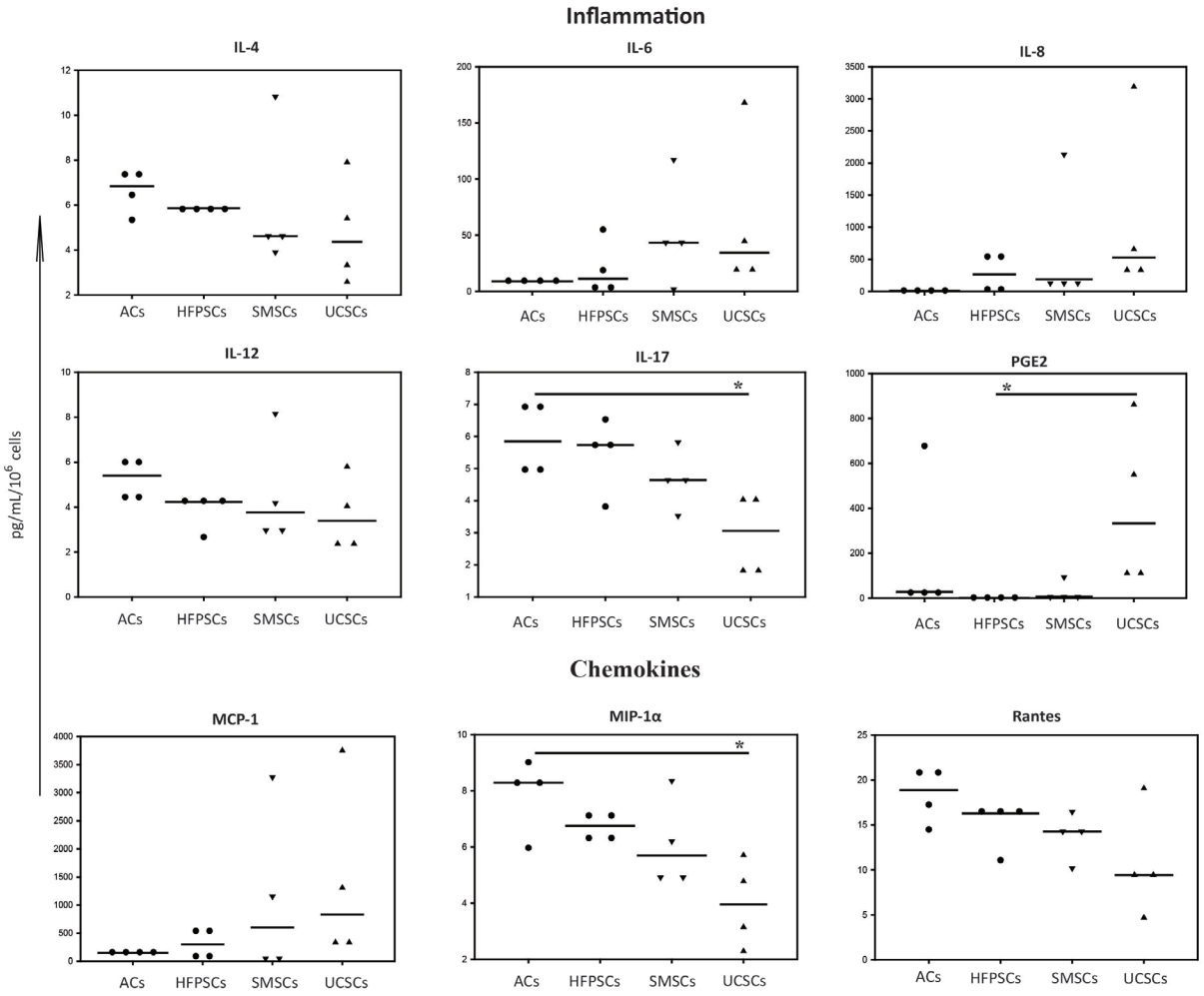

Fig. 4. Comparison of cytokines and chemokines from CM of ACs, HFPSCs, SMSCs and UCSCs. Dot density shows concentration of cytokines involved in inflammation (IL-4, IL-6, IL-8, IL-12, IL-17 and PGE2) and chemokines (MCP-1, MIP-1α and RANTES) detected in supernatants of four different stromal cell types. * $p < 0.05$; $n = 4$ donors/cell sources.

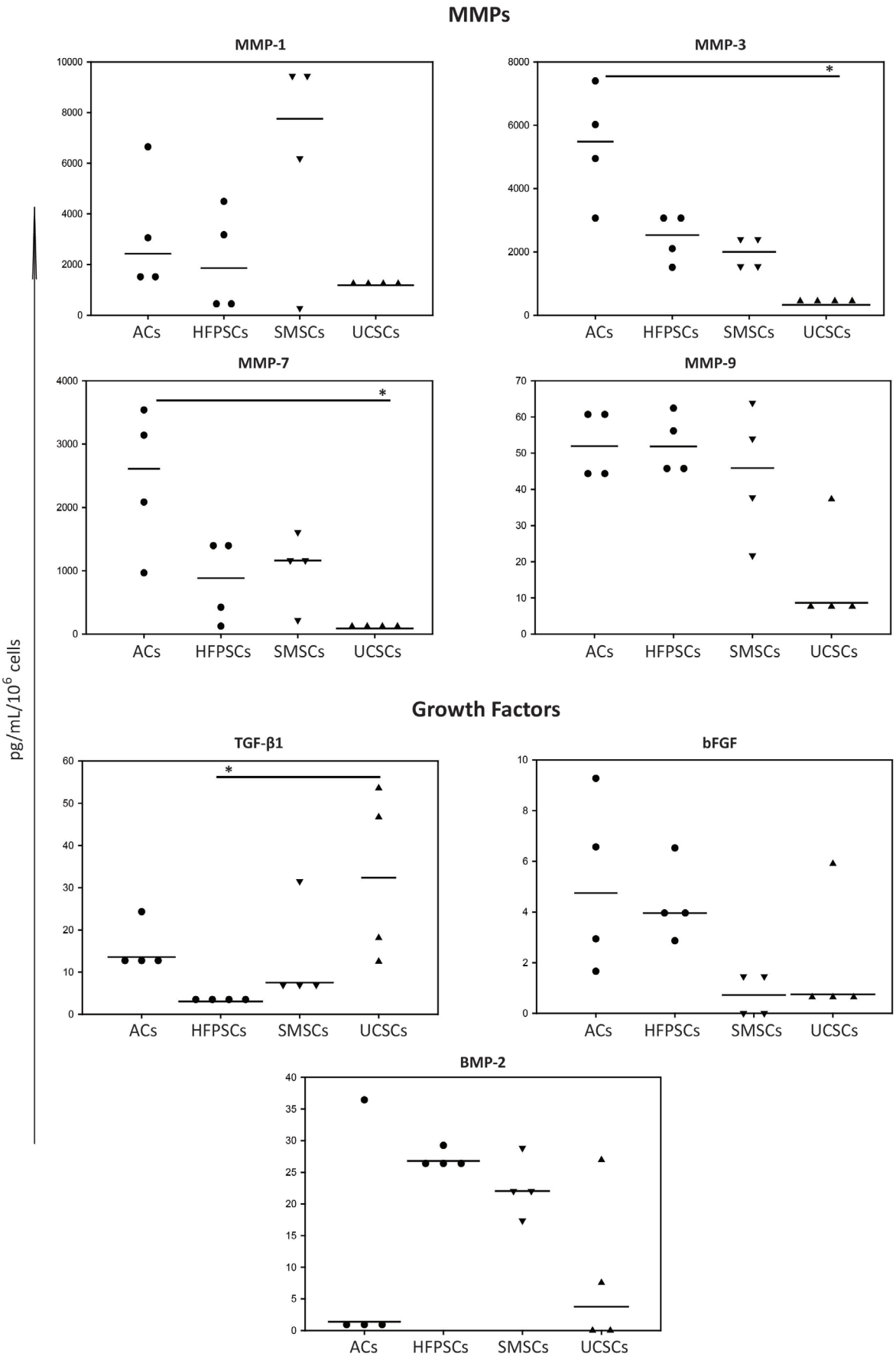

Fig. 5. Comparison of MMPs and anabolic factors from CM of ACs, HFPSCs, SMSCs and UCSCs. Dot density shows the concentration of MMPs (MMP-1, MMP-3, MMP-7 and MMP-9) and growth factors (TGF-β1, bFGF and BMP-2) detected in supernatants of four different stromal cell types. * $p < 0.05$; $n = 4$ donors/cell sources.

control P3 chondrocytes (Fig. 10); however, the differences were not significant except for ACAN. The CM of both stimulated and non-stimulated UCSCs significantly upregulated the expression of ACAN as compared to the control P3 chondrocytes (Fig. 10). On the other hand, in the 3D culture assays, no differences were observed in cartilage neo-tissue formation by histology when comparing pellets incubated in the presence or absence of UCSCs-CM (Fig. 11).

Discussion

The main objective of the study was to ascertain which source of stromal cells possesses the most favourable secretome to promote tissue repair. Given the importance of the MSC paracrine signalling, large-scale comparative analyses of cell secretomes were performed and functional studies with cell supernatants were conducted on immune cells to compare the constitutive immunomodulatory capabilities of different MSCs. Overall, the results demonstrated that stromal cells from umbilical cord matrix exhibited superior anti-inflammatory and trophic effects when compared with adult ACs, HFPSCs and SMSCs.

All cell sources were expanded in monolayer cultures in the serum-supplemented media for some weeks, as done in standard cell transplantation procedures. To facilitate the analyses of secretory profiles by LC-MS/MS proteomics, the media were conditioned under serum-free conditions. Multiplex protein arrays were performed with the same serum-free CM that was used for proteomics, allowing direct comparisons of results. However, functional assays with immune cells were performed with serum-supplemented CM, as serum deprivation affects proliferation and induces apoptosis in lymphocytes and macrophages, respectively (Sato *et al.*, 2009; Wei *et al.*, 2006). Short periods of serum deprivation do not affect the cell viability of mesenchymal cells (Boraldi *et al.*, 2008). However, some changes in the secretome could occur upon changes in serum supplementation. Significantly higher expression of IL-6 was only observed in the presence of serum in all four cell types (Fig. 6). Although only minor phenotypic changes were expected in cells associated with serum presence, alterations in the expression of some bioactive molecules could occur and should be taken into consideration.

Currently, MSCs are viewed as "drugstores" with the potential to modulate the phenotype, migration and activation of resident tissue and inflammatory cells (Caplan and Correa, 2011). This has led researchers to study MSC-mediated paracrine effects and profiles of secreted proteins from different mesenchymal stromal cell types. Previous studies comparing secretory profiles from different MSC sources highlight the existence of differentially expressed factors, with impact on angiogenesis, matrix remodelling, inflammation and immunosuppression (Amable *et al.*, 2014; Dabrowski *et al.*, 2017; Hsiao *et al.*, 2012; Li *et al.*, 2015). The qualitative analyses using a large-scale proteomic approach revealed similar protein profiles, where most of the identified proteins were present in all cell supernatants. However, after hierarchical clustering of protein profiles from all donors, UCSC secretomes single out from the other adult cell sources (Fig. 1**b**). Quantitative analyses of the secretome data revealed that proteins involved in cell signalling, such as TGF-β1 and PDGFD, were upregulated in UCSC supernatants, while catabolic proteins, such as MMPs, serpins and complement factors, were downregulated when compared to stromal cells from adult origin. TGF-β1 is a master driver of chondrogenesis and has pleiotropic effects in OA pathogenesis (Tang *et al.*, 2015; Zhang *et al.*, 2015; Zhen *et al.*, 2013). In addition, TGF-β1 has anti-apoptotic effects (Rehman *et al.*, 2004). Observations from other studies are disparate and include cell sources that were not used in the present study;

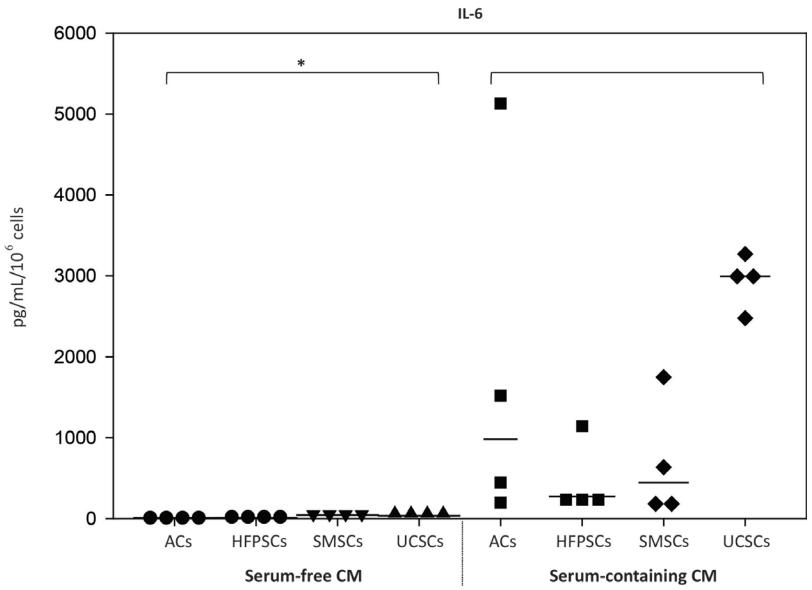

Fig. 6. Cytokine expression by all cell sources in serum-free and serum-supplemented conditions. The secretion of selected cytokines and factors was assessed by ELISA. TNF-α, IFN-γ and IL-12 were not detected. Data were analysed using Mann-Whitney U comparison between serum-free and serum-containing CM of each cell type. * $p < 0.05$; $n = 4$ donors/cell sources.

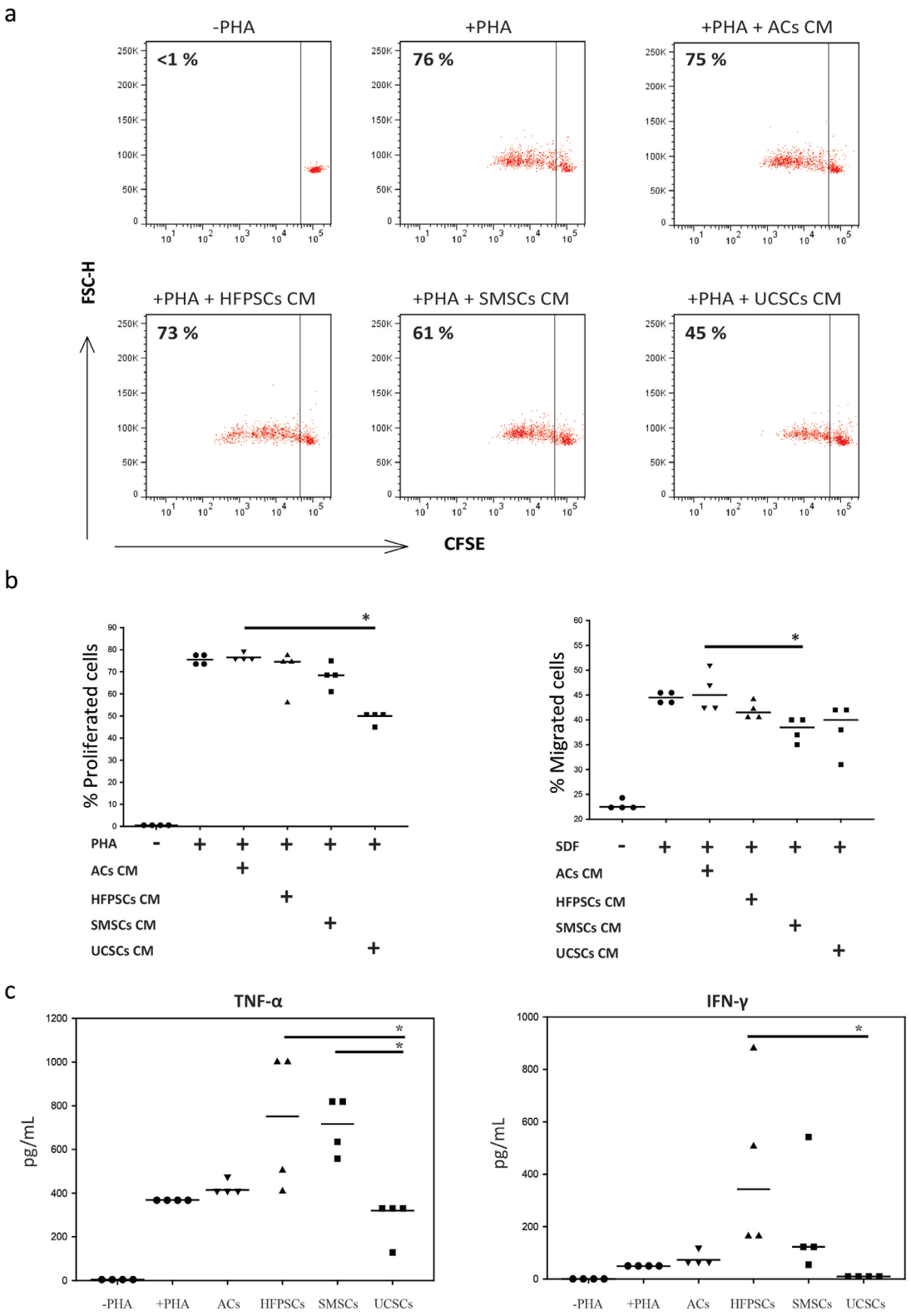

Fig. 7. Inhibition of PBMC activation by CM. (**a**) PBMC proliferation assay: representative flow cytometry dot plots depict the percentage of proliferating PBMCs stimulated with 10 μg/mL of PHA in the presence and absence of CM from the four different stromal cell types. (**b**) Quantitative analyses of PBMCs proliferation and migration in the presence and absence of CM from four different stromal cell types. 100 ng/mL SDF-1 was used for chemo-attraction in migration assays. (**c**) Total concentration of TNF-α and IFN-γ detected in 10^6 cells/well PBMCs-CM after incubation with PHA and CM from four different stromal cell types. * $p < 0.05$; $n = 4$ donors/cell sources.

however, the superior anabolic phenotype of UCSCs, including the highest expression of TGF-β1 among the compared cell types, is also observed by Dabrowski *et al.* (2017). Data from the multiplex protein analyses revealed a significant reduction of MMPs and increased expression of TGF-β1 by UCSCs, thus reasserting observations made in the large-scale proteomic approach. Altogether, these observations highlighted the less catabolic phenotype of UCSCs when compared to the three other adult MSCs.

The immunomodulatory profile of the different MSCs was also investigated by proteomics and multiplex arrays. Secretome analyses revealed comparable expression of complement components, heat-shock proteins, galectins and immunoregulators, such as CSF-1, MCP-1, MIF and TGF-β1, among the different cell sources. The data from multiplex protein arrays showed enhanced expression of the immunomodulators IL-6, MCP-1 and PGE2 and reduced expression of IL-17 and MIP-1α in UCSCs. Notably, stimulation of stromal cells with pro-inflammatory cytokines increased the expression of PGE2 in all cell types. However, a limitation of the study was the relatively low number of biological replicates, where the inclusion of more biological replicates could prove to be statistically significant for most of the analysed cytokines. IL-6 has an omnidirectional role in maintaining biological functions, whereas it has deleterious effects in the joint (Poree *et al.*, 2008; Sui *et al.*, 2009). However, selective depletion of IL-6 in animals is associated with accelerated joint degeneration upon ageing (de Hooge *et al.*, 2005). Bouffi *et al.* (2010) demonstrate IL-6-dependent inhibition of local inflammation in experimental arthritis. MCP-1 (also called CCL2), MIP-1α (CCL3) and IL-17 are all potent inflammatory factors mediating recruitment and activation of myeloid cells. Their presence is associated with cartilage degeneration and progression of OA (Appleton *et al.*, 2015; Snelling *et al.*, 2017; Wang *et al.*, 2017; Xu *et al.*, 2015; Zhao *et al.*, 2015). IL-17 inhibits chondrogenesis and promotes MMPs in chondrocytes (Benderdour *et al.*, 2002; Kondo *et al.*, 2013). PGE2 regulates phenotype and functions of pro-inflammatory macrophages and natural killer (NK) cells (Manferdini *et al.*, 2017), however, the overall role of this factor in OA progression and

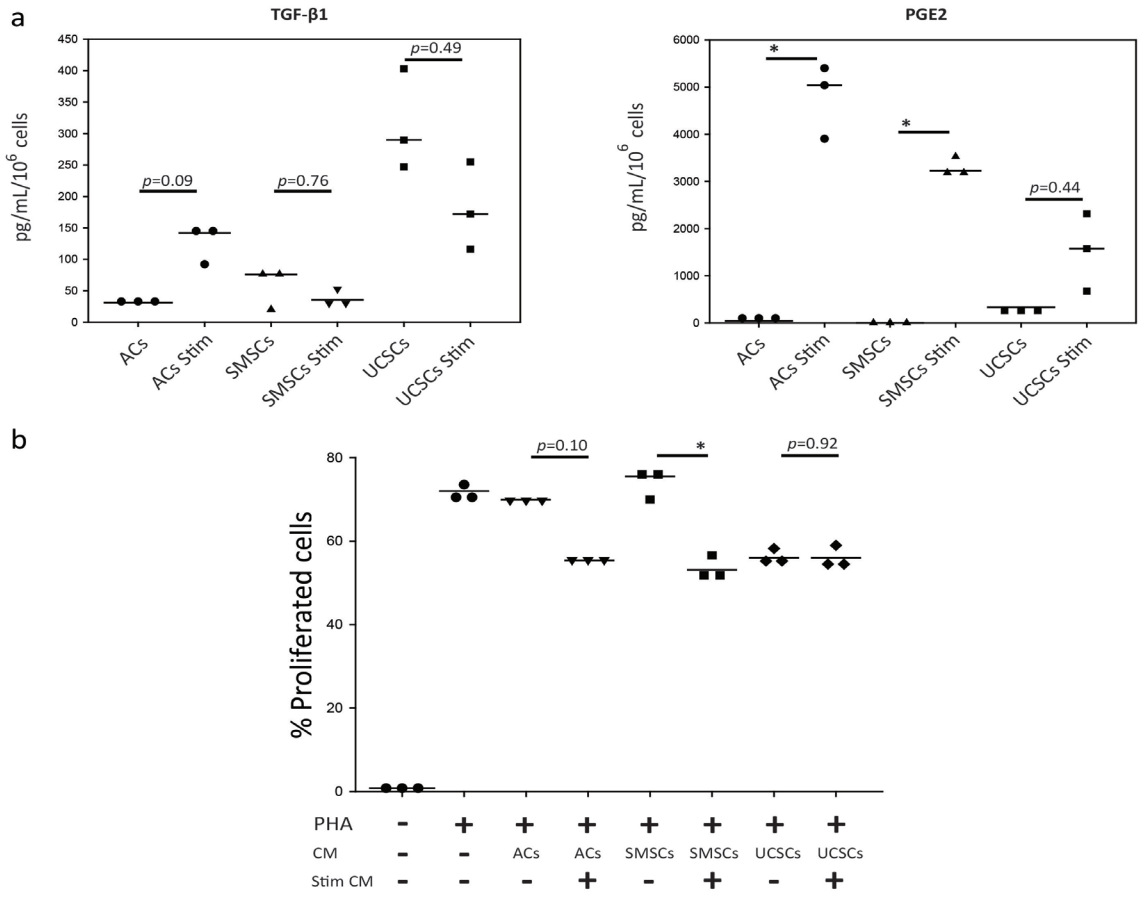

Fig. 8. Immunomodulatory properties of ACs, SMSCs and UCSCs after stimulation with TNF-α and IFN-γ. Cells in monolayer culture (70-80 % confluence) were stimulated for 48 h with 50 ng/mL each of TNF-α and IFN-γ. Data were analysed using Mann-Whitney U comparison between non-stimulated and stimulated CM of each cell type. (**a**) Dot density shows the concentration of TGF-β1 and PGE2 detected in supernatants of non-stimulated and stimulated cell types. (**b**) PBMC proliferation assays in the presence of non-stimulated and stimulated CM of ACs, SMSCs and UCSCs. * $p < 0.05$; $n = 3$ donors/cell sources.

cartilage homeostasis is still controversial (Bouffi *et al.*, 2010; Miwa *et al.*, 2000; Otsuka *et al.*, 2009). Despite the difficulty to reach general conclusions due to the pleiotropic nature of many chemokines and cytokines, the observations based on the global expression of released factors indicated that UCSCs displayed a more immunosuppressive and anti-inflammatory phenotype than their adult counterparts.

To investigate further the paracrine potential of the different MSCs on immunomodulation, functional assays were conducted on lymphocytes and macrophages. Results using functional assays

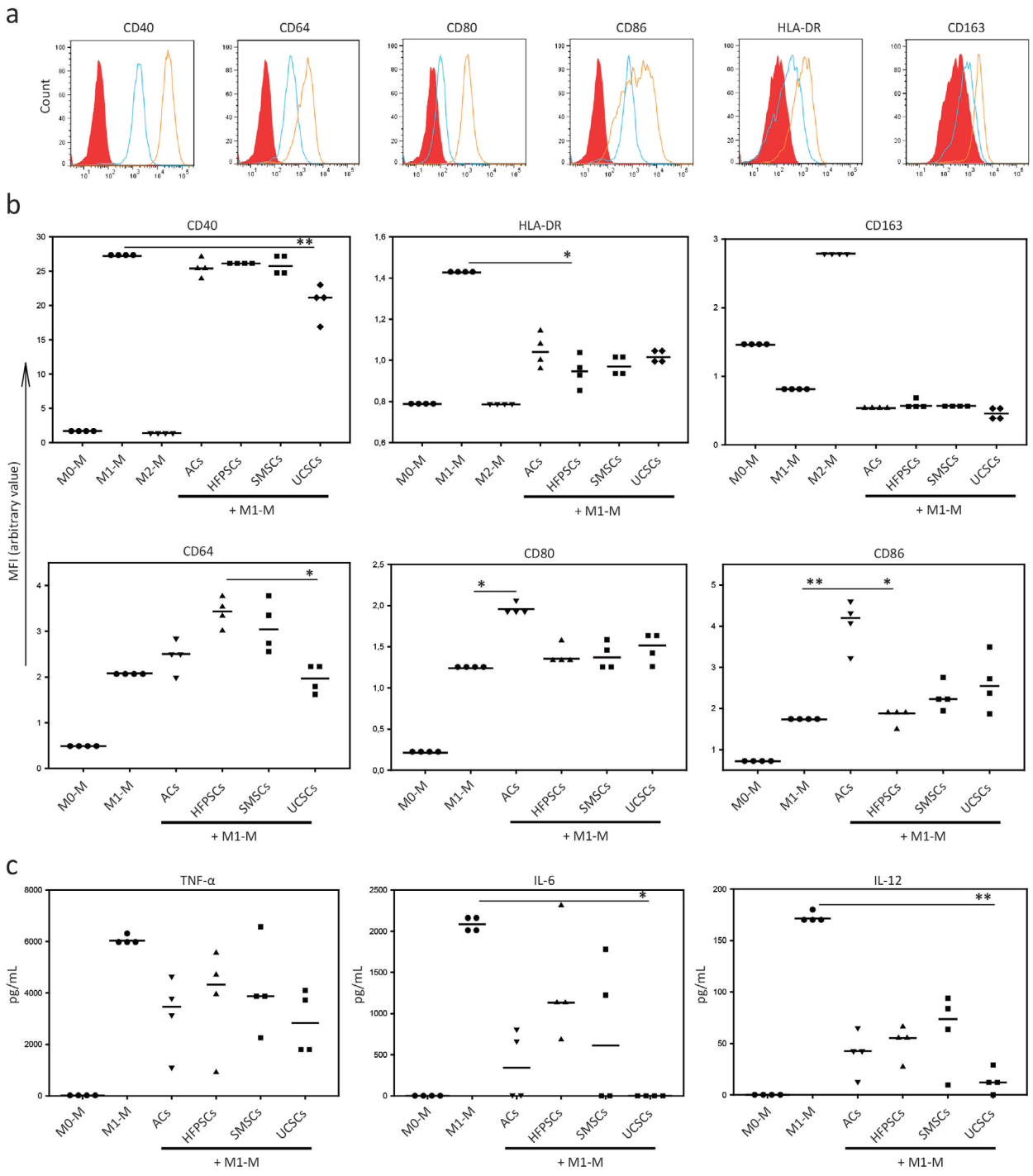

Fig. 9. UCSC secretomes could modulate macrophage-mediated inflammation. (**a**) Characterisation of surface molecules during polarisation of M0-M into M1-M (CD40, CD64, CD80, CD86 and HLA-DR) and M2-M (CD163) by flow cytometry. Red, blue and orange peaks represent isotype control, M0-M and activated macrophages, respectively. (**b**) Dot density depicts M1-M activation and distinct blocking of M1-M activation by CM from ACs, HFPSCs, SMSCs and UCSCs. M2-M polarisation was used as a negative control for surface expression of CD40 and HLA-DR. (**c**) Levels of TNF-α, IL-6 and IL-12 detected in macrophage culture CM (2.5×10^6 cells/well) after incubation with CM from four different stromal cell types. * $p < 0.05$; ** $p < 0.005$; $n = 4$ donors/cell sources.

harmonised with protein profiles, highlighting the superior immunosuppressive phenotype of UCSCs as compared to the other MSCs. The MSC ability to regulate inflammation and immunity has been the focus of intense research over recent years (Donders *et al.*, 2018; von Bahr *et al.*, 2012). Many *in vitro* studies show that mesenchymal cells from multiple sources, including differentiated connective tissue cells, such as chondrocytes and fibroblasts, have the potential to regulate inflammation and T-cell functions to some extent (Bouffi *et al.*, 2011; Lohan *et al.*, 2016). Still, there is no consensus on which cell source is the most powerful in this respect. Most published studies compare bone marrow, adipose tissue and foetal tissues with different outcomes. Some comparative studies demonstrate that bone marrow MSCs have slightly superior immunosuppressive capacity than other MSCs (Heo *et al.*, 2016; Karaoz *et al.*, 2017). In line with the observations of the present study, some groups observe superior immunosuppressive abilities by MSCs from cords (Jin *et al.*, 2013; Najar *et al.*, 2012). The chondro-inductive potential of UCSC supernatants on dedifferentiated chondrocytes was also tested in the present study. Whilst cartilage reference genes were upregulated after incubation with UCSCs-CM, no clear differences where observed in 3D neo-tissue formation *in vitro*. These results put forward the anabolic potential of the UCSC secretome; however, the concentration of anabolic factors, such as TGF-β1, in supernatants remained insufficient to trigger proper chondrogenesis from dedifferentiated chondrocytes.

Priming of MSCs is extensively documented and is recommended as a mandatory step to unleash the full immunosuppressive potential of MSCs (Gomez-Aristizabal *et al.*, 2017; Najar *et al.*, 2012; van Buul *et al.*, 2012). In the present study, stimulation of adult stromal cells with pro-inflammatory cytokines altered the expression of some immunoregulatory factors and increased the blocking effects over PBMCs by adult MSCs (Fig. 8). On the other hand, the effects exerted by primed UCSCs on PBMC proliferation were unchanged. These differences could be due to early embryonic differentiation of the cell phenotypes. Notably, constitutive immunoregulation by non-stimulated UCSCs is observed in an OA rabbit model (Saulnier *et al.*, 2015). In this context, *ex vivo* priming of MSCs is related to few controversial outcomes *in vivo*, describing increased immunogenicity of pre-stimulated MSCs (Papadopoulou *et al.*, 2012; Treacy *et al.*, 2014).

In clinical settings, MSCs from different sources are investigated for the treatment of focal cartilage lesions and OA (Lee and Wang, 2017). Articular

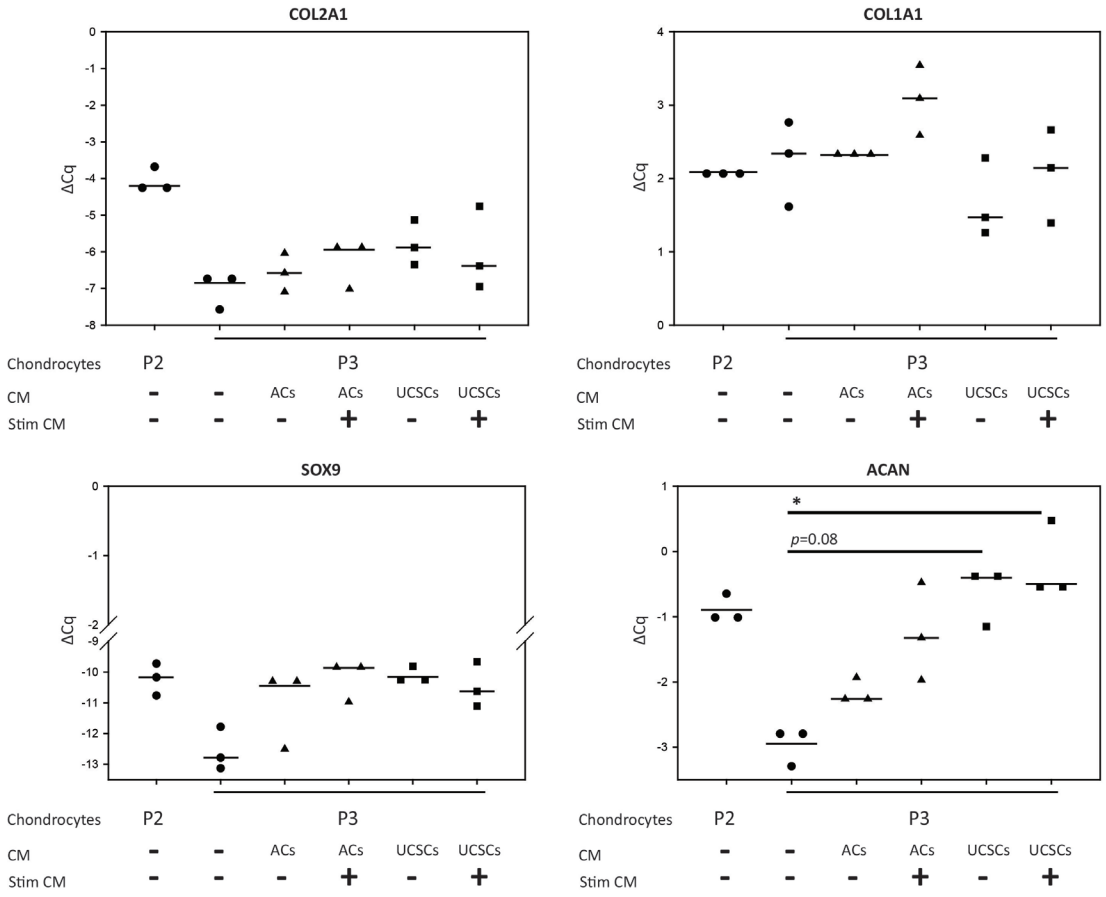

Fig. 10. Effects of UCSCs-CM on cartilage signature gene expression of dedifferentiated chondrocytes in monolayer cultures. Relative expression of COL2A1, COL1A1, SOX9 and ACAN by chondrocytes in the presence of CM from stimulated and non-stimulated ACs and UCSCs. Dedifferentiated chondrocytes at P2 and P3 in the absence of CM were used as controls. $p < 0.05$; $n = 3$ donors.

chondrocytes, bone marrow and adipose tissue stromal cells are the most commonly used sources for cartilage repair (Vonk *et al.*, 2015). These studies mostly assess safety and efficacy of used MSCs for specific clinical implications. A comparative study in humans argues that autologous SMSCs exert superior healing outcomes (Akgun *et al.*, 2015). On the other hand, in preclinical models, MSCs from cords exert immunosuppression and disease regression in experimental models of OA and autoimmune disorders (Donders *et al.*, 2015; Saulnier *et al.*, 2015; Yang *et al.*, 2015). In contrast to MSCs from autologous sources, there are only few ongoing trials exploring the potential of allogeneic UCSCs for both OA management and focal cartilage repair in humans (NCT02580695, NCT02291926, NCT03166865 and NCT03358654), without published outcomes hitherto. Confirming the results of the present comparative study in suitable animal models would provide more insight into the use of UCSCs for cartilage disorders in the clinics.

Conclusions

Traditionally, the regenerative potential of MSCs has been directly linked to their multipotent differentiation and tissue-forming capabilities. Nowadays, increasing attention is given to their role as paracrine modulators. In the field of articular cartilage lesions and degenerative joint diseases, there is no consensus on the best cell source for treatment. Considering the relevance of paracrine signalling, the present study compared the secretome among MSCs from different sources. Both the molecular analyses and the functional assays indicated that UCSCs displayed superior anti-inflammatory and trophic effects when compared to MSCs from adult

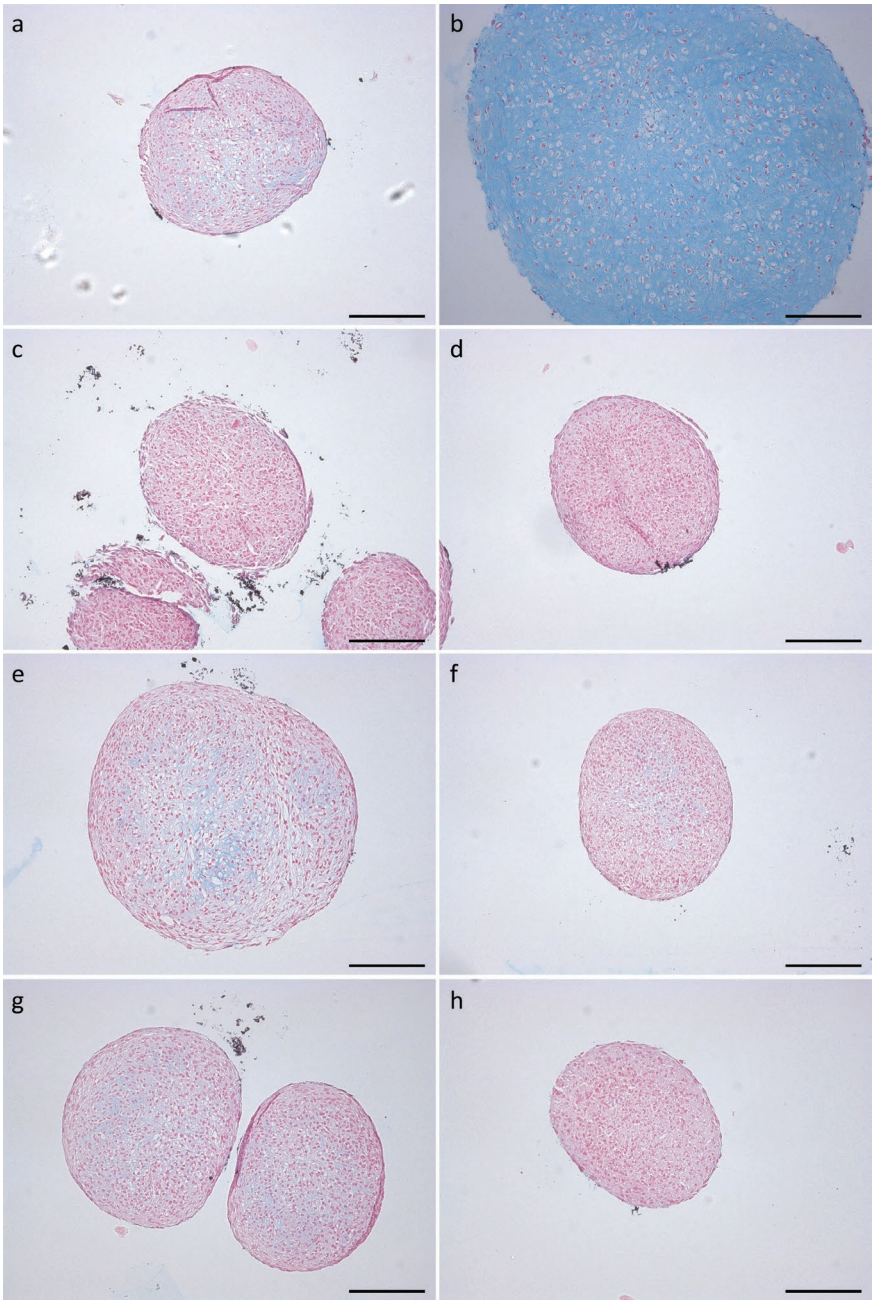

Fig. 11. Effects of CM on *in vitro* chondrogenesis of dedifferentiated chondrocytes. Spheroids (n = 2 donors) were generated by pellet culture method and harvested after 21 d of chondrogenesis for histological evaluation. Bright light microscopy images of alcian-blue-stained spheroids (4 μm-thick sections) show (**a**) no growth factor, (**b**) TGF-β1 and BMP-2, (**c**) AC CM, (**d**) primed AC CM, (**e**) SMSC CM, (**f**) primed SMSC CM, (**g**) UCSC CM and (**h**) primed UCSC CM. Scale bar: 200 μm.

tissues. The hypoimmunogenic nature of UCSCs, along with their high abundance, simple isolation and favourable protein profiles make this cell source an attractive tool for off-the-shelf allogeneic adjuvant therapy.

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AI primarily conducted the laboratory work, planned the study and prepared the manuscript. IMZ participated in the conception of the study, evaluated the data, edited and approved the final draft of the manuscript. IU and JAB performed the LC-MS/MS. All authors contributed to the data interpretation and provided direction and comments on the manuscript. The datasets used in the study are available upon request to the corresponding author.

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Web References

1. https://www.uniprot.org/proteomes/UP000005640 [19-02-2019]

Discussion with Reviewers

Andrea Barbero: The authors have analysed the secretome of different MSCs after their *in vitro* culture. However, the secretion profile of cells can drastically change after their injection in the joint (*i.e.* an environment that is extremely more complex that

the *in vitro* one). Can the authors state their opinion on this issue?
Authors: We agree with the reviewer on the likelihood of cells displaying different secretome *in vitro* and in joint conditions. Unfortunately, the technology to assess the secretory profile of the implanted MSCs *in vivo* was not available. To shed some light on this point, cells were stimulated *in vitro* with a cocktail of inflammatory cytokines. Results showed that adult MSCs became more immunosuppressive in inflammatory environments. This point has also been discussed in the manuscript.

Lucienne Vonk: For future purposes, how do you envision to produce a well-characterised and quality-controlled product from the secretome of stromal cells?
Authors: To produce a well-characterised and quality-controlled product from the secretome of stromal cells is almost as complicated as to produce a quality-controlled and well-characterised product from stromal cells *per se*. As shown in the proteomic analyses, secretomes from stromal cells contained (at least) more than 500 different proteins and it was difficult to speculate which individual factor or cocktail of factors might play a role in inflammation or tissue repair. Most probably different paracrine signal molecules act concomitantly. A well-characterised product would require knowing exactly which molecules are present in the product and what would be the precise effect of those bioactive factors. Actions that could help to move in that direction would include characterisation of samples by fractionation (for example molecular size). Specific blockade of distinct factors would also help to ascertain which factors from the entire secretome are more relevant to induce repair. One possibility is that the effects of CM would come from the extracellular vesicles. In this case, the complexity of the sample preparation would be reduced. Still, a well-characterised product would require a thorough characterisation of the extracellular vesicles and the determination of the effector molecule(s) which could include miRNA or other non-protein molecules. Most probably the effects from stromal cells secretome are multifactorial and not reduced to one or a handful of factors, so that the manufacture of a well-characterised and quality-controlled product for its safe use in clinics is close to an oxymoron.

Yuk Wai Lee: How did the authors ensure they were detecting secretory cytokines but not residual cytokines from the serum or cytokines released from the cells due to cell damage/death?
Authors: Residual amounts of serum proteins could be present in the experimental samples. However, after extensive washings, only very abundant serum proteins, such as albumin or immunoglobulins, can be detected by MS. In any case, this aspect would be equal for all experimental samples and would not have an impact on the outcomes of the study, which focussed on differences between experimental groups. The same argument applied to proteins derived from dead cells. Notably, the extent of cell death in cultures at the time of supernatants collection (from all cell sources) was negligible/undetectable by trypan blue exclusion.

Katie Lee: Why did the authors choose to culture the cells in normoxic conditions, rather than more physiologically relevant hypoxic conditions?
Authors: It could be interesting to check hypoxic culture conditions. Surface markers characterisation of MSCs is routinely performed by the authors, showing no significant differences in surface markers expression during monolayer culture in high or low oxygen tensions. In our experience, hypoxic environments play a more important role in 3D cultures and chondrogenesis. Therefore, normoxic conditions were used in the study.

Katie Lee: How did the authors confirm the cell types isolated in the study were actually MSCs and not fibroblasts or other forms of progenitor cell?
Authors: MSCs were referred to as mesenchymal stromal cells (not stem cells), emphasising on the stromal origin more than the stem cell or progenitor nature. This was consciously done taking into consideration that it is very difficult to differentiate between progenitor cells, fibroblasts or other type of stromal cells based on phenotypic markers or even on their multi-differentiation. By using the term mesenchymal stromal cells, the potential problem with semantics was circumvented.

Editor's note: The Scientific Editor responsible for this paper was Christine Hartmann.