DONOR-MATCHED MESENCHYMAL STEM CELLS FROM KNEE INFRAPATELLAR AND SUBCUTANEOUS ADIPOSE TISSUE OF OSTEOARTHRITIC DONORS DISPLAY DIFFERENTIAL CHONDROGENIC AND OSTEOGENIC COMMITMENT

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Abstract

Cell-based therapies have recently been proposed for the treatment of degenerative articular pathologies, such as early osteoarthritis, with an emphasis on autologous mesenchymal stem cells (MSCs), as an alternative to terminally differentiated cells. In this study, we performed a donor-matched comparison between infrapatellar fat pad MSCs (IFP-MSCs) and knee subcutaneous adipose tissue stem cells (ASCs), as appealing candidates for cell-based therapies that are easily accessible during surgery.

IFP-MSCs and ASCs were obtained from 25 osteoarthritic patients undergoing total knee replacement and compared for their immunophenotype and differentiative potential.

Undifferentiated IFP-MSCs and ASCs displayed the same immunophenotype, typical of MSCs ($CD13^+/CD29^+/CD44^+/CD73^+/CD90^+/CD105^+/CD166^+/CD31^-/CD45^-$). IFP-MSCs and ASCs showed similar adipogenic potential, though undifferentiated ASCs had higher *LEP* expression compared to IFP-MSCs ($p < 0.01$). Higher levels of calcified matrix ($p < 0.05$) and alkaline phosphatase ($p < 0.05$) in ASCs highlighted their superior osteogenic commitment compared to IFP-MSCs. Conversely, IFP-MSCs pellets showed greater amounts of glycosaminoglycans ($p < 0.01$) and superior expression of *ACAN* ($p < 0.001$), *SOX9*, *COMP* ($p < 0.001$) and *COL2A1* ($p < 0.05$) compared to ASCs pellets, revealing a superior chondrogenic potential. This was also supported by lower *COL10A1* ($p < 0.05$) and *COL1A1* ($p < 0.01$) expression and lower alkaline phosphatase release ($p < 0.05$) by IFP-MSCs compared to ASCs.

The observed dissimilarities between IFP-MSCs and ASCs show that, despite expressing similar surface markers, MSCs deriving from different fat depots in the same surgical site possess specific features. Furthermore, the *in vitro* peculiar commitment of IFP-MSCs and ASCs from osteoarthritic donors towards the chondrogenic or osteogenic lineage may suggest a preferential use for cartilage and bone cell-based treatments, respectively.

Keywords: Mesenchymal stem cell, cartilage, bone, infrapatellar fat pad, adipose tissue, cell-based therapy, osteoarthritis.

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Introduction

The low self-repair ability of articular cartilage and the lack of treatments that can reproducibly restore defects at the bone-cartilage interface provide a major reason for the development of cell-based therapies for bone and cartilage repair (Beane and Darling, 2012; Mohal *et al.*, 2012; Szpalski *et al.*, 2012). Joint replacement, considered a successful and standard procedure in the treatment of elderly patients affected by osteoarthritis (OA), is controversial in patients considered too young or active for conventional arthroplasty, who experience higher implant failure rates and earlier revisions (Harrysson *et al.*, 2004; Julin *et al.*, 2010). Thus, cell-based therapies have been recently proposed also for the treatment of middle-aged early OA patients to alter the progression of this degenerative disease, with the hope of obviating or at least delaying the need for joint replacement (Gomoll *et al.*, 2012).

Mesenchymal stem cells (MSCs) have been investigated as an alternative to terminally differentiated cells to develop novel treatments for bone and cartilage defects, since they can be easily harvested from several adult tissues and are able to differentiate towards the osteogenic and chondrogenic lineages (Johnstone *et al.*, 1998; Pittenger *et al.*, 1999). Beside bone marrow MSCs (BMSCs), more recently adipose derived mesenchymal stem cells have been successfully used for bone and cartilage applications (Rada *et al.*, 2009; Jung *et al.*, 2010; Rhee *et al.*, 2011; Kang *et al.*, 2012; Choi *et al.*, 2014). In particular, MSCs resident in the infrapatellar fat pad (IFP-MSCs) and knee subcutaneous adipose tissue (ASCs) can be considered appealing alternative cell sources for articular cell-based therapies, thanks to their differentiative potential and ease of harvesting during knee surgery, which causes minimal additional morbidity to patients. Furthermore, the fast protocol employed in their isolation is advantageous in view of a future one-step surgical cell-based treatment.

Beneficial effects have been observed when treating animals with induced OA with MSCs derived from different adipose depots, with cells playing a key role in the inhibition of OA progression and in the restoration of damaged cartilage (Toghraie FS *et al.*, 2011; ter Huurne

et al., 2012; Toghraie F *et al.*, 2012; Desando *et al.*, 2013). Moreover, it has been demonstrated that intra-articular injection of IFP-MSCs leads to relief of pain symptoms and to improvement in knee functions in patients suffering from knee OA (Koh *et al.*, 2013).

Several studies have characterised the multi-lineage potential and immunophenotype of IFP-MSCs (Dragoo *et al.*, 2003; Wickham *et al.*, 2003; English *et al.*, 2007; Khan *et al.*, 2009; Liu *et al.*, 2012; Lopez-Ruiz *et al.*, 2013), but – to our knowledge – only few studies have reported a preliminary comparison of mesenchymal stem cells from infrapatellar fat pad and knee subcutaneous adipose tissue (Mochizuki *et al.*, 2006; Alegre-Aguaron *et al.*, 2012; Pires de Carvalho *et al.*, 2012). Nevertheless, in these few comparative studies, the evaluation of osteogenic and chondrogenic potential has been performed mainly by qualitative histological stainings, without investigating gene expression profiles (Alegre-Aguaron *et al.*, 2012; Pires de Carvalho *et al.*, 2012), and the comparison was restricted to very few donors (Mochizuki *et al.*, 2006), limiting the clinical relevance of the findings.

In the present study, to evaluate the different and specific relevance of IFP-MSCs and ASCs for orthopaedic cell-based therapies, we compared the features and the multi-differentiative potential of cells derived from 25 OA patients undergoing total knee replacement. To eliminate the common issue of inter-donor variability, we performed a donor-matched evaluation, harvesting and comparing both IFP-MSCs and ASCs from the same patient and focusing on their osteogenic and chondrogenic potential through multiple, quantitative analyses, and evaluating also the expression of hypertrophic and fibrocartilaginous markers in chondrogenic differentiated cells.

Materials and Methods

Cell isolation and expansion

Infrapatellar fat pad and subcutaneous adipose tissue were harvested from the knee of 25 patients affected by OA (mean age 70 ± 8 years, range 54-85 years) during total knee replacement, with patients' informed consent and with the approval of the Institutional Review Board.

Inclusion criteria were: male and female gender, age between 50 and 85 years, grade III and IV knee osteoarthritis according to the Kellgren-Lawrence grading scale, indication for total knee replacement. Exclusion criteria were: rheumatoid arthritis, autoimmune diseases, systemic diseases, tumours, previous implant at the same knee and medical contraindication to elective surgery.

The infrapatellar fat pad appeared as a mixture of yellow adipose and white fibrous tissue. Based on the different colour and tissue consistence, we carefully separated the adipose fraction to proceed with IFP-MSCs isolation. The subcutaneous adipose tissue appeared as yellow tissue. No macroscopic signs of hypertrophy were observed in the samples.

IFP-MSCs were isolated from infrapatellar fat pad as previously described (Lopa *et al.*, 2011). Briefly, adipose tissue was carefully separated from the synovium and minced into small pieces. The sample was then enzymatically digested (37 °C, 30 min) by 0.075 % type I collagenase (Worthington Biochemical Co, Lakewood, NJ, USA). After digestion, the sample was centrifuged (1200 g, 10 min) and filtered through a cell strainer (100 μm pores) to remove undigested tissue. Cells were counted by Trypan blue exclusion and plated in control medium consisting of high glucose DMEM (HG-DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum (FBS, Lonza, Basel, Switzerland), 0.029 mg/mL L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate (all from Life Technologies) at approximately 10^4 cells/cm^2 (37 °C, 5 % CO_2). The same protocol was used to isolate ASCs from knee subcutaneous adipose tissue. Non-adherent cells were removed with the first medium refreshment. During culture, medium was changed twice a week. When IFP-MSCs and ASCs were about 90 % confluent, they were detached using 0.05 % trypsin/0.053 mM EDTA (Life Technologies) and plated at 3 x 10^3 cells/cm^2 for the following passages.

Clonogenic ability assay

A colony-forming unit-fibroblast (CFU-F) assay was performed to assess the clonogenic ability of IFP-MSCs and ASCs (Staszkiewicz *et al*., 2010). Cells were plated at different low densities (range, 48-1 cells/cm^2) and cultured in control medium with 20 % FBS. After 10 days, cells were fixed with 10 % neutral buffered formalin and stained with Gram's crystal violet (Sigma-Aldrich, St. Louis, MO, USA). CFU-F frequency was established by scoring the individual colonies and expressing them as a percentage relative to the number of seeded cells.

Flow cytometry for the assessment of typical MSCs surface markers

At passage 4 (45-50 days of expansion, corresponding to 5-6 doublings from P1 to P4 with a doubling time ranging from 0.10 to 0.18 doublings/day), surface marker expression was evaluated by flow cytometry. IFP-MSCs and ASCs were detached using 0.05 % trypsin/0.53 mM EDTA and washed twice in cold Fluorescence-Activated Cell Sorting (FACS) Buffer (phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} containing 2 % foetal bovine serum (FBS) and 0.1 % NaN_3). For each sample, 2.5 x 10^5 cells were incubated for 30 min with the following anti-human primary monoclonal antibodies: CD13-FITC, CD29-biotinylated, CD31-FITC, CD34-biotinylated, CD44-FITC, CD45-FITC, CD105-biotinylated, CD106-FITC, CD166-FITC (all from Ancell Corporation, Bayport, MN, USA), CD90-FITC and CD73-PE (from Miltenyi Biotec, Bergisch Gladbach, Germany), and CD151 (R&D Systems, Minneapolis, MN, USA). After incubation, cells were washed with FACS buffer to remove the excess of primary antibody. Cells stained with biotinylated antibodies were incubated for 20 min with streptavidin-PE (Ancell Corporation), whereas samples stained with anti-CD151 primary antibody were incubated with a FITC-conjugated goat anti-mouse secondary antibody (Ancell Corporation). After incubation, cells were washed with FACS Buffer and suspended in 500 μL of FACS buffer for analysis. Background fluorescence was established by negative

controls and data were acquired using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) collecting a minimum of 10,000 events. Analysis was performed using CellQuest software (BD Biosciences).

Adipogenic differentiation

IFP-MSCs and ASCs at passage 4 were plated at 3 x 10^3 cells/cm^2 and differentiated for 14 days in adipogenic medium using a repeated pulsed induction (de Girolamo *et al.*, 2009) with 3 days of induction in control medium supplemented with 1 µM dexamethasone, 10 µg/mL insulin, 500 µM 3-isobutyl-1-methylxanthine and 200 µM indomethacin (all from Sigma-Aldrich), followed by 3 days of maintenance in control medium supplemented with 10 µg/mL insulin.

The production of lipid vacuoles was quantified by Oil Red O staining. Cells were fixed in 10 % neutral buffered formalin for 10 min, washed with 60 % isopropanol, and stained with 8.5 mM Oil Red O (Sigma-Aldrich) for 15 min. After rinsing with ddH_2O, Oil Red O was unstained with 100 % isopropanol and absorbance was read at 490 nm (Perkin Elmer Victor X3 microplate reader; Perkin Elmer, Waltham, MA, USA).

Osteogenic differentiation

IFP-MSCs and ASCs at passage 4 were plated at 3 x 10^3 cells/cm^2 and differentiated for 14 or 21 days in osteogenic medium (de Girolamo *et al.*, 2009) consisting of control medium supplemented with 10 mM glycerol-2-phosphate, 10 nM dexamethasone, 150 µM L-ascorbic acid-2-phosphate and 10 nm cholecalciferol (all from Sigma-Aldrich).

Calcified matrix deposition was measured using Alizarin Red-S staining. Cells were rinsed with PBS, fixed with ice-cold 70 % ethanol for 1 h and stained with 40 mM Alizarin Red-S (pH 4.1, Sigma-Aldrich) for 15 min. After washing with ddH_2O, samples were unstained for 30 min with 10 % cetylpyridinium chloride monohydrate (CPC, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0). Absorbance was read at 570 nm (Perkin Elmer Victor X3 microplate reader).

Alkaline phosphatase activity (ALP) was quantified by enzymatic assay (Bodo *et al.*, 2002). Cells were rinsed with PBS and lysed in 0.1 % Triton X-100 (Sigma-Aldrich). ALP was quantified by incubating cellular lysates at 37 °C with 1 mM p-nitrophenylphosphate (Sigma-Aldrich) in alkaline buffer (100 mM diethanolamine and 0.5 mM $MgCl_2$, pH 10.5). The enzymatic reaction was stopped with 1 N NaOH and absorbance was read at 410 nm (Perkin Elmer Victor X3 microplate reader). ALP activity was normalised on total protein content, determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), and expressed as ALP Units per mg of proteins.

Chondrogenic differentiation

Chondrogenic differentiation was performed by pellet culture. At passage 4, 4 x 10^5 IFP-MSCs and ASCs were centrifuged (250 g, 5 min) to obtain cell pellets. Pellets were cultured for 14 days in chondrogenic medium (Barbero *et al.*, 2004) consisting of HG-DMEM supplemented with 0.029 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1.25 mg/mL human serum albumin (all from Sigma-Aldrich), 1 % ITS+1 (1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, 0.5 µg/mL sodium selenite, 50 mg/mL bovine serum albumin and 470 µg/mL linoleic acid, Sigma-Aldrich), 0.1 µM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate and 10 ng/mL TGF-β1 (Peprotech, Rocky Hill, New Jersey, USA).

For histological analysis, pellets were fixed for 24 h in 10 % neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Sections were stained with haematoxylin-eosin (Sigma-Aldrich) and Alcian Blue (pH 2.5, Sigma-Aldrich) to evaluate extracellular matrix and glycosaminoglycans (GAGs) deposition.

For GAGs quantification, pellets were digested (16 h, 60 °C) in 500 µL of PBE buffer (100 mM Na_2HPO_4, 10 mM NaEDTA, pH 6.8) containing 1.75 mg/mL L-cysteine (Sigma-Aldrich) and 14.2 U/mL papain (Worthington). Samples were incubated with 16 mg/L dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm (Perkin Elmer Victor X3 microplate reader). The same samples were used for DNA quantification by CyQUANT Kit (Life Technologies).

Soluble ALP activity released in culture supernatants was measured by enzymatic assay. At the moment of medium refresh, culture supernatant was collected from each pellet and 50 µL of medium were incubated with 1 mM p-nitrophenylphosphate. The release of ALP was determined at early phase (day 0 to day 3) and late phase (day 11 to day 14) of chondrogenic differentiation.

Gene expression analysis

After 14 days of adipogenic, osteogenic or chondrogenic differentiation, gene expression was evaluated by real time PCR (Rotor Gene RG3000 system, Qiagen, Venlo, Netherlands). Total RNA was purified from cell lysates using the RNeasy Mini kit (Qiagen) and reverse-transcribed to cDNA (5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C) using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). 20 ng of cDNA were incubated with a PCR mix (2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C) including TaqMan Universal PCR Master Mix and TaqMan® Assays-on-Demand™ Gene Expression probes (Life Technologies) using the following assays: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1), peroxisome proliferator-activated receptor gamma (*PPARG*, Hs01115513_m1), leptin (*LEP*, Hs00174877_m1), runt-related transcription factor 2 (*RUNX2*, Hs00231692_m1), collagen type I alpha 1(*COL1A1*, Hs01076777_m1), aggrecan (*ACAN*, Hs00153936_m1), SRY (Sex determining region Y)-box9 (*SOX9*, Hs00165814_m1), cartilage oligomeric matrix protein (*COMP*, Hs00164359_m1), collagen type II alpha I (*COL2A1*, Hs01060345_m1), collagen type X alpha 1 (COL10A1, Hs00166657_m1). The fold change in the expression of the different genes was normalised on the housekeeping *GAPDH*.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Normal distribution of values was assessed by the Kolmogorov-Smirnov normality test. Statistical analysis was performed using Student's *t*-test and Wilcoxon test for paired data since IFP-MSCs and ASCs were obtained from the same donors; Student's *t*-test was used for data with a Gaussian distribution and Wilcoxon test for data with a non-Gaussian distribution (GraphPad Prism v5.00; GraphPad Software, San Diego, CA, USA). Level of significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The number of data used for the statistical analyses, indicated in figure legends as "*n*", corresponds to independent experiments performed with IFP-MSCs and ASCs isolated from "*n*" individual donors, according to Ranstam (Ranstam, 2012).

Results

Undifferentiated IFP-MSCs and ASCs share common features and express a similar set of cell surface markers

IFP-MSCs and ASCs displayed a fibroblastoid shape, which was maintained throughout the entire culture period, without any difference between the two cell types (Fig. 1a). From passage 1 to 4, both cell populations displayed a high clonogenic ability (Fig. 1b,c), with a significantly higher clonogenic ability observed in IFP-MSCs compared to ASCs at passage 1 ($p < 0.05$).

As reported in Fig. 2a, both IFP-MSCs and ASCs analysed at passage 4 showed a similar immunophenotype, being positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166 and negative for CD31 and CD45. For all the tested markers no difference was observed between IFP-MSCs and ASCs (Fig. 2b). A variable expression of CD34 and CD106 was found for IFP-MSCs and ASCs depending on the donor (10-35 % of positive cells). Both cell types expressed CD29, CD90, CD105, CD151 and CD166 (35-90 % of positive cells). A consistent expression of CD13, CD44 and CD73 was measured on IFP-MSCs and ASCs (90-100 % of positive cells).

Adipogenic differentiation of IFP-MSCs and ASCs induces a similar upregulation of adipogenic markers except for leptin gene expression

IFP-MSCs and ASCs cultured for 14 days in adipogenic medium showed the progressive loss of the fibroblastoid-like shape and the production of cytoplasmic lipid vacuoles, as evidenced by Oil Red O staining (Fig. 3a,b). The dye extraction revealed a significant increase in lipid vacuoles content in differentiated cells in comparison with cells in control medium ($p < 0.001$ for IFP-MSCs and $p < 0.05$ for ASCs) (Fig. 3c).

Gene expression analysis confirmed the ability of both cell types to respond to adipogenic induction. Indeed, a significant increase of *PPARG* expression was observed in IFP-MSCs ($p < 0.05$) and ASCs ($p < 0.01$) cultured in adipogenic medium in comparison with undifferentiated cells (Fig. 3d). Differentiated IFP-MSCs and ASCs showed also a significant up-regulation of *LEP* expression, ($p < 0.05$ and $p < 0.01$, respectively) compared with undifferentiated cells. Finally, a significantly higher expression of *LEP* was observed in differentiated ASCs in comparison with differentiated IFP-MSCs (+295 %, $p < 0.01$).

ASCs respond more efficiently to osteogenic induction in respect to IFP-MSCs

Both IFP-MSCs and ASCs were able to differentiate towards the osteogenic lineage when cultured in osteo-inductive medium, as demonstrated by the upregulation of specific markers such as calcified matrix, ALP activity, and transcriptional expression of *RUNX2* and *COL1A1* (Fig. 4).

Our results demonstrated that ASCs were more committed towards the osteogenic lineage compared with IFP-MSCs. Indeed, after 14 days of culture in osteogenic medium the deposition of calcified matrix was more consistent in differentiated ASCs (Fig. 4a) and the quantification of calcified matrix revealed a significant difference between the two cell populations (+75 % in ASCs, $p < 0.05$, Fig. 4b). Furthermore, after 14 days of differentiation, ASCs produced significantly higher levels of ALP in comparison with IFP-MSCs (+66 %, $p < 0.05$, Fig. 4c).

After 21 days of osteogenic differentiation calcified matrix deposition and ALP levels were significantly increased in both cell populations compared to their correspondent controls. Comparing 14 and 21 days, we observed a significant increase of calcified matrix deposition in IFP-MSCs ($p < 0.05$) and ASCs ($p < 0.01$) over time. ALP levels were increased from 14 to 21 days in differentiated IFP-MSCs ($p < 0.05$), whereas this marker remained stable in ASCs. After 21 days of induction, we did not observe any significant difference between IFP-MSCs and ASCs in terms of calcified matrix deposition and ALP activity.

Higher expression of *RUNX2* and *COL1A1* was observed both in undifferentiated and differentiated ASCs in comparison to their IFP-MSCs counterparts (Fig. 4d,e), with a significant difference in the expression of *COL1A1* in undifferentiated cells (+53 % in ASCs, $p < 0.05$, Fig. 4e).

IFP-MSCs are more committed towards the chondrogenic lineage and express lower levels of hypertrophic and fibrocartilaginous markers as compared to ASCs

After 14 days of chondrogenic induction in pellet culture conditions, histological evaluation revealed a superior and more homogeneous deposition of GAGs in IFP-MSCs compared to ASCs pellets. As shown in Fig. 5, this difference was found in donors with different age, with some inter-donor variability.

No significant difference was observed in terms of DNA content, related to cell proliferation, between IFP-MSCs and ASCs pellets, even if slightly higher values were measured in IFP-MSCs pellets (Fig. 6a**).** According to the histological results, a significantly higher amount of GAGs was measured in IFP-MSCs pellets, as demonstrated by

the biochemical quantification (+16 %, $p < 0.01$, Fig. 6b). No significant difference was observed between the two populations in terms of GAGs/DNA ratio.

Accordingly with data obtained by histological and biochemical analyses, significantly higher levels of *ACAN* gene expression were observed in IFP-MSCs pellets compared to ASCs pellets (+474 %, $p < 0.001$, Fig. 6c). Moreover, all the tested chondrogenic markers showed higher expression in IFP-MSCs **(**Fig. 6d-f**)**, with significant differences for *COMP* (+127 %, $p < 0.001$) and *COL2A1* (+728 %, $p < 0.05$) in comparison with ASCs.

The superior chondrogenic potential of IFP-MSCs compared to ASCs was confirmed also by data on hypertrophic and fibrocartilaginous markers (Fig. 6g,h): indeed, expression of *COL10A1* and *COL1A1* was significantly higher in ASCs in respect to IFP-MSCs (+132 %, $p < 0.05$ for *COL10A1*; +75 %, $p < 0.01$ for *COL1A1*). Furthermore, in the early phase of chondrogenic differentiation (day 0 to day 3) ALP released from ASCs pellets was significantly higher in comparison with IFP-MSCs pellets (+119 %, $p < 0.05$, Fig. 6i).

Discussion

The main finding of our study was the significantly different commitment of IFP-MSCs and ASCs in terms of differentiation ability; in particular, undifferentiated ASCs showed higher osteogenic potential in comparison with IFP-MSCs, whereas IFP-MSCs were characterised by superior chondrogenic ability. In our study both cell populations were isolated from the same knee of each patient, thus eliminating the bias of inter-donor variability and allowing a strict comparison of cell features, giving more consistent results. Furthermore, all the cells were derived from OA donors, in order not to neglect a possible impact of the pathological origin on cell features, in view of the possible future application of IFP-MSCs and ASCs in cell-based therapies designed to treat early OA patients. Indeed, in the quest to identify cells able to regenerate bone and cartilage damages and to develop new treatments for early OA patients, a major issue is to establish the ideal anatomical sites for autologous MSCs isolation. Both the fat depots investigated in this study are easily accessible during orthopaedic surgery and their harvesting causes a minimal discomfort and donor site morbidity, thus being ideal sources for the isolation of MSCs.

Undifferentiated IFP-MSCs and ASCs displayed similar features in terms of cell morphology, typically fibroblastoid. Both populations presented high clonogenic ability, with percentage of clonogenic cells ranging between 30 and 40 %, with IFP-MSCs forming a higher number of colonies compared to ASCs at passage 1, in accordance with previously published data (Pires de Carvalho *et al.*, 2012).

After *in vitro* expansion, the immunophenotype was coherent with the one reported in previous studies for IFP-MSCs and ASCs (Gronthos *et al.*, 2001; Lee *et al.*, 2004; Mochizuki *et al.*, 2006; English *et al.*, 2007; Alegre-Aguaron *et al.*, 2012) and, in accordance with literature,

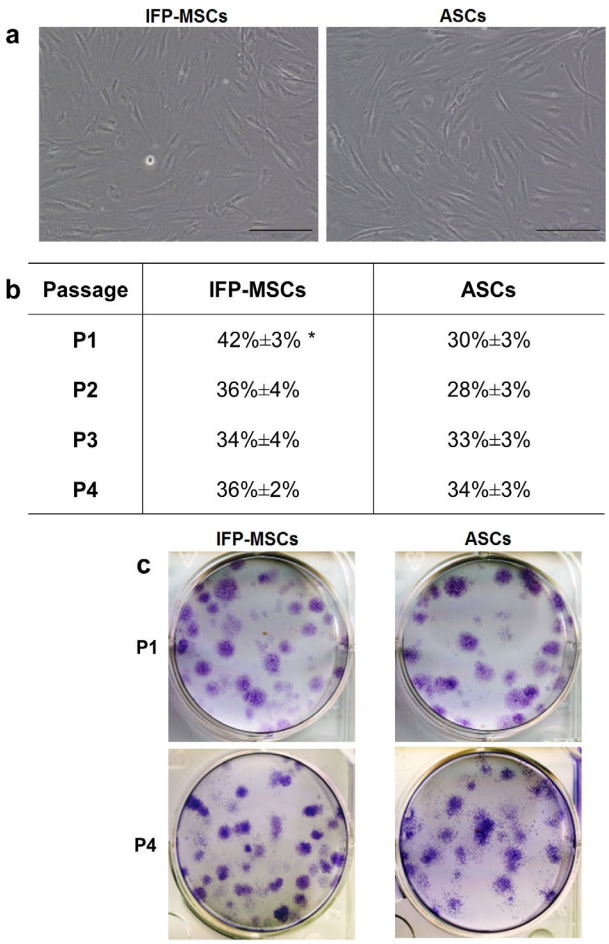
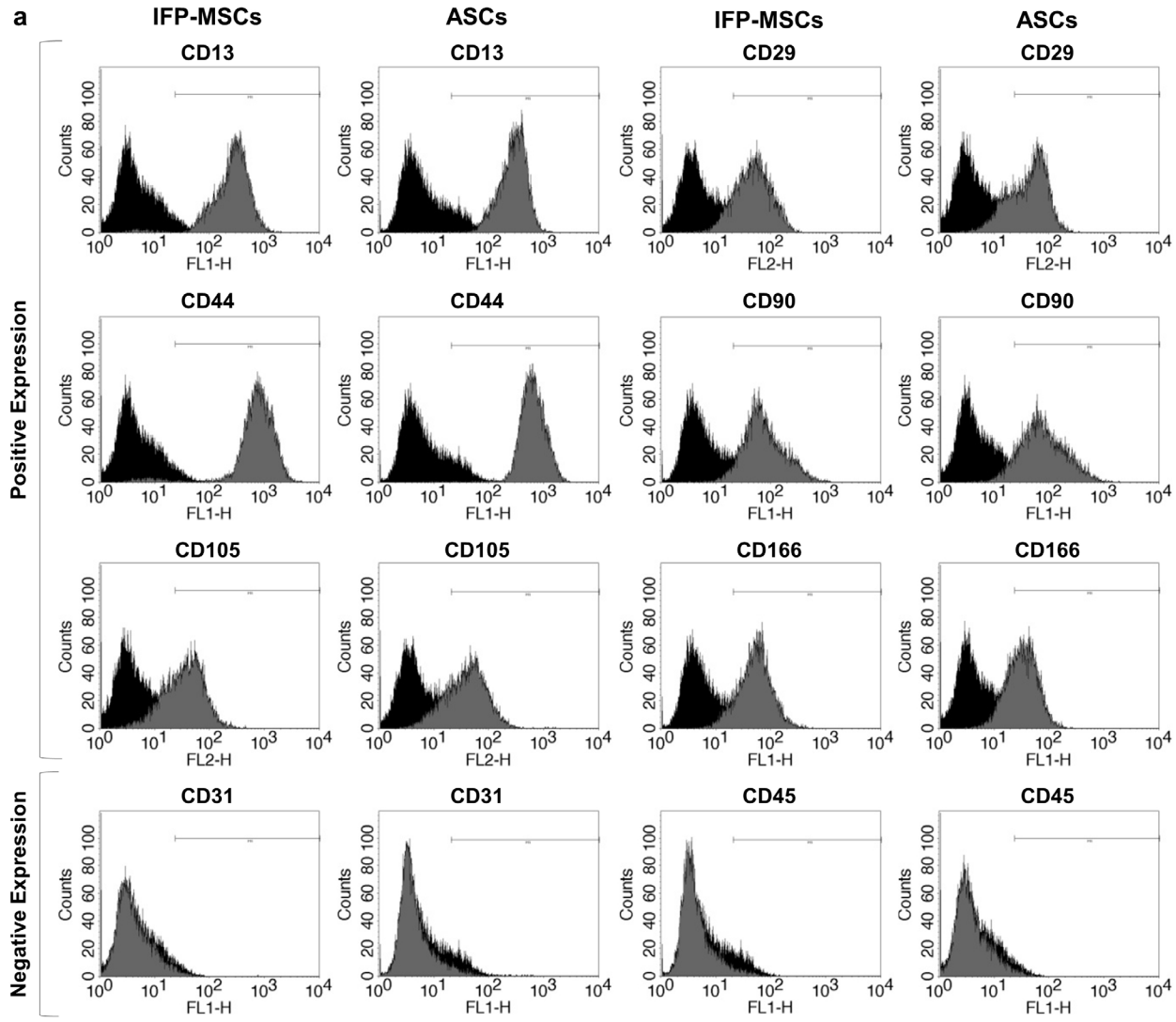


Fig. 1. Morphology and clonogenic potential of IFP-MSCs and ASCs. (**a**) Cells in culture at passage 4 (scale bars 200 μm). **(b)** Percentage of clonogenic cells from passage 1 to passage 4 (scored colonies were normalised on number of seeded cells, $n = 7$, $p < 0.05$) **(c)** Representative pictures of stained CFU-F at P1 and P4.

no difference was observed between the two populations (Alegre-Aguaron *et al.*, 2012; Mochizuki *et al.*, 2006).

Regarding the multilineage potential, both IFP-MSCs and ASCs were able to respond to the adipogenic stimuli, showing a similar induction of the characteristic adipogenic markers, but a significantly higher expression of *LEP* was observed in undifferentiated ASCs in comparison with undifferentiated IFP-MSCs. Leptin is an inflammatory mediator which is mainly produced and secreted by adipocytes (Dumond *et al.*, 2003; Fain, 2006). This adipokine stimulates IL-1β production, increases the effect of pro-inflammatory cytokines and induces the expression of matrix metalloproteinases which participate to cartilage matrix degradation in OA cartilage (Toussirot *et al.*, 2007; Vuolteenaho *et al.*, 2009). The significantly superior basal expression of *LEP* in ASCs in respect to IFP-MSCs is coherent published data, reporting a significantly higher production and secretion of this adipokine in the subcutaneous adipose tissue of OA patients compared to their infrapatellar fat pad (Distel *et al.*, 2009). However, the same study also reported a superior secretion of IL-6



b

Surface Markers	IFP-MSCs		ASCs	
CD13	98.8%±0.2%	+++	96.2%±2.1%	+++
CD29	65.3%±3.1%	++	78.5%±11.9%	++
CD31	5.3%±1.1%	-	9.4%±2.4%	-
CD34	24.5%±9.2%	+/-	19.8%±3.6%	+/-
CD44	99.2%±0.5%	+++	98.4%±1.2%	+++
CD45	4.9%±1.2%	-	8.6%±2.2%	-

Surface Markers	IFP-MSCs		ASCs	
CD73	98.2%±1.2%	+++	91.2%±6.7%	+++
CD90	88.6%±4.5%	++	90.2%±3.8%	++
CD105	71.1%±11.4%	++	60.1%±8.7%	++
CD106	18.3%±8.0%	+/-	10.2%±2.5%	+/-
CD151	50.4%±6.9%	+	45.3%±7.9%	+
CD166	65.3%±3.1%	++	61.4%±14.3%	++

Fig. 2. Cell surface markers expression. (**a**) Representative expression of the typical MSCs surface markers pattern found in IFP-MSCs and ASCs from the same donor at passage 4 (markers are represented as grey histograms and isotype control antibodies are represented as black histograms). (**b**) Percentage of positive cells for the whole panel of surface markers tested in cells at passage 4 (n = 6). Ranking in table was established as: - for 0-10 %, +/- for 10-35 %, + for 35-50 %, ++ for 50-90 %, and +++ for 90-100 % positive cells.

and its soluble receptor in infrapatellar fat pad compared to subcutaneous adipose tissue. The inflammatory profile of MSCs derived from both adipose depots would hence need further investigations to better define how these cell populations could react to the inflammatory state of OA joints, in view of their possible application in a future cell-based therapy.

Several studies have demonstrated the osteogenic and chondrogenic potential of IFP-MSCs (Dragoo *et al.*, 2003; Wickham *et al.*, 2003; English *et al.*, 2007; Khan *et al.*, 2009; Liu *et al.*, 2012) and ASCs (Zuk *et al.*, 2002; Gimble and Guilak, 2003; Guilak *et al.*, 2004), but without comparing these cell populations.

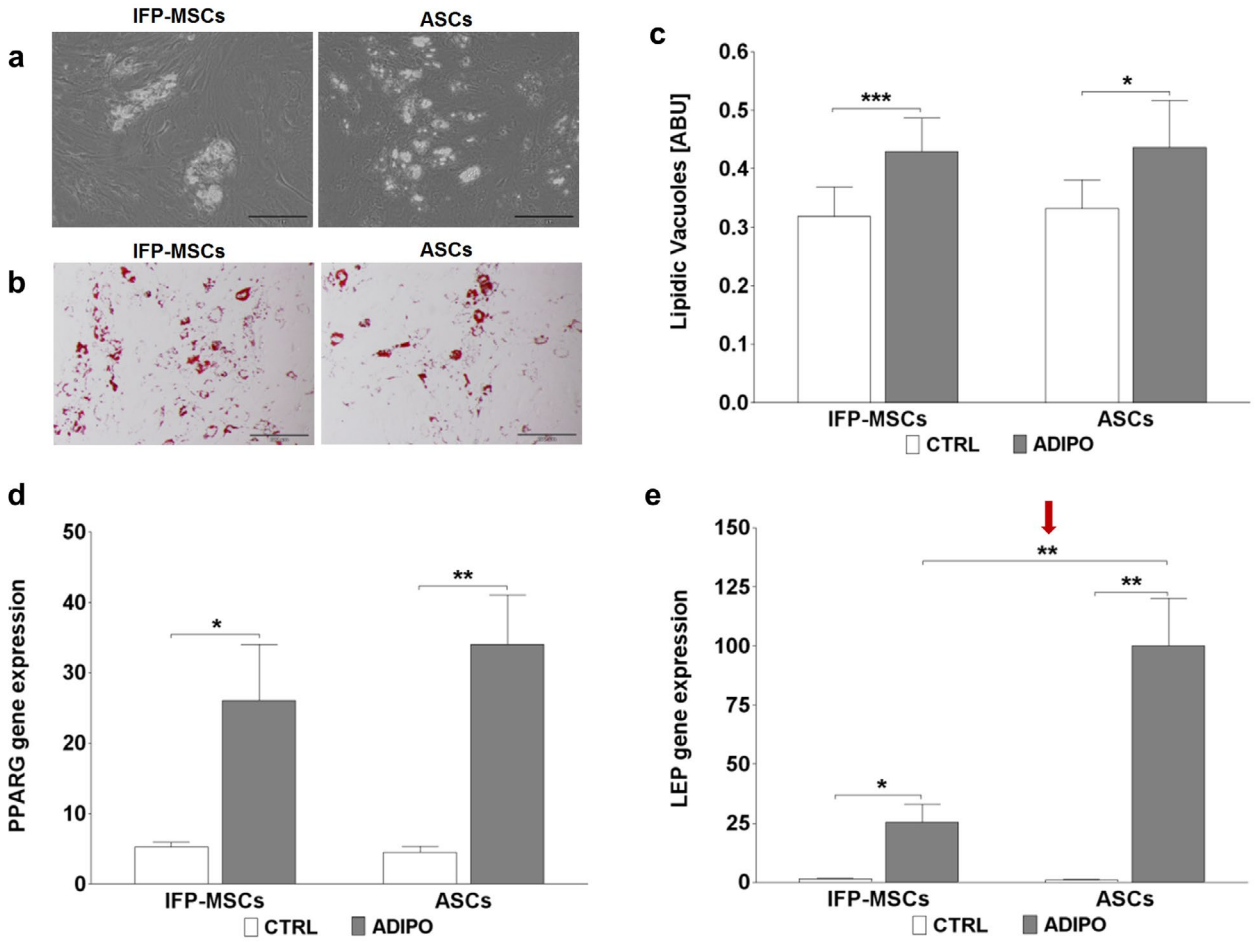


Fig. 3. Adipogenic differentiation. (**a**,**b**) Micrographs of IFP-MSCs and ASCs differentiated towards the adipogenic lineage during culture and after Oil Red O staining (scale bars 200 µm). **(c)** Quantification of lipid vacuoles in undifferentiated (CTRL) and adipogenic-differentiated (ADIPO) cells (n = 13). (**d**,**e**) Gene expression of *PPARG* and *LEP* normalised to *GAPDH* (n = 7). Red arrow indicates a significant difference between IFP-MSCs and ASCs.

In our study, the direct donor-matched comparison between IFP-MSCs and ASCs through quantitative analyses allowed to observe important differences in their osteogenic and chondrogenic commitment. Significantly higher levels of ALP and calcified matrix deposition in osteo-induced ASCs compared to IFP-MSCs, together with the superior expression of *COL1A1* in undifferentiated ASCs, demonstrated that ASCs are more committed towards the osteogenic lineage with respect to IFP-MSCs. These results differ from those described in previous studies whereby, on the basis of qualitative stainings, it has been reported that IFP-MSCs possess a similar (Pires de Carvalho *et al.*, 2012) or higher (Mochizuki *et al.*, 2006) osteogenic potential compared to ASCs. We believe that quantitative analyses and the inclusion of a more appropriate number of donors can give a higher sensitivity compared to qualitative staining, that could have led to such contrasting results compared to the current reported literature. Finally, differently from Mochizuki *et al.* (Mochizuki *et al.*, 2006) that used as tissue source a mixture of synovium and adipose subsynovium, we carefully selected only the adipose fraction of infrapatellar fat pad prior to cell isolation to evaluate solely the differentiation potential of IFP-MSCs, which could also partially explain the different results obtained in our study.

On the other hand, a more pronounced chondrogenic commitment was found in IFP-MSCs compared to ASCs, as revealed by the significantly higher transcriptional expression of chondrogenic markers such as *ACAN*, *COL2A1* and *COMP* in IFP-MSCs pellets. This result is in accordance with previously published results (Alegre-Aguaron *et al.*, 2012), where the histological evaluation of pellets revealed that ASCs were characterised by lower chondrogenic ability compared to IFP-MSCs that displayed a chondrogenic phenotype more similar to the one of BMSCs. A superior chondrogenic potential in IFP-MSCs has also been described by Mochizuki *et al.* (2006) who, however, analysed expression of chondrogenic genes in pellets obtained from IFP-MSCs and ASCs from a single young non-OA donor. Our data, comprising GAGs quantification and gene expression analysis of cells derived from a larger number of old OA donors, support these literature data, providing an effective demonstration of the superior chondrogenic commitment of IFP-MSCs compared to ASCs, even when cells are obtained from osteoarthritic donors.

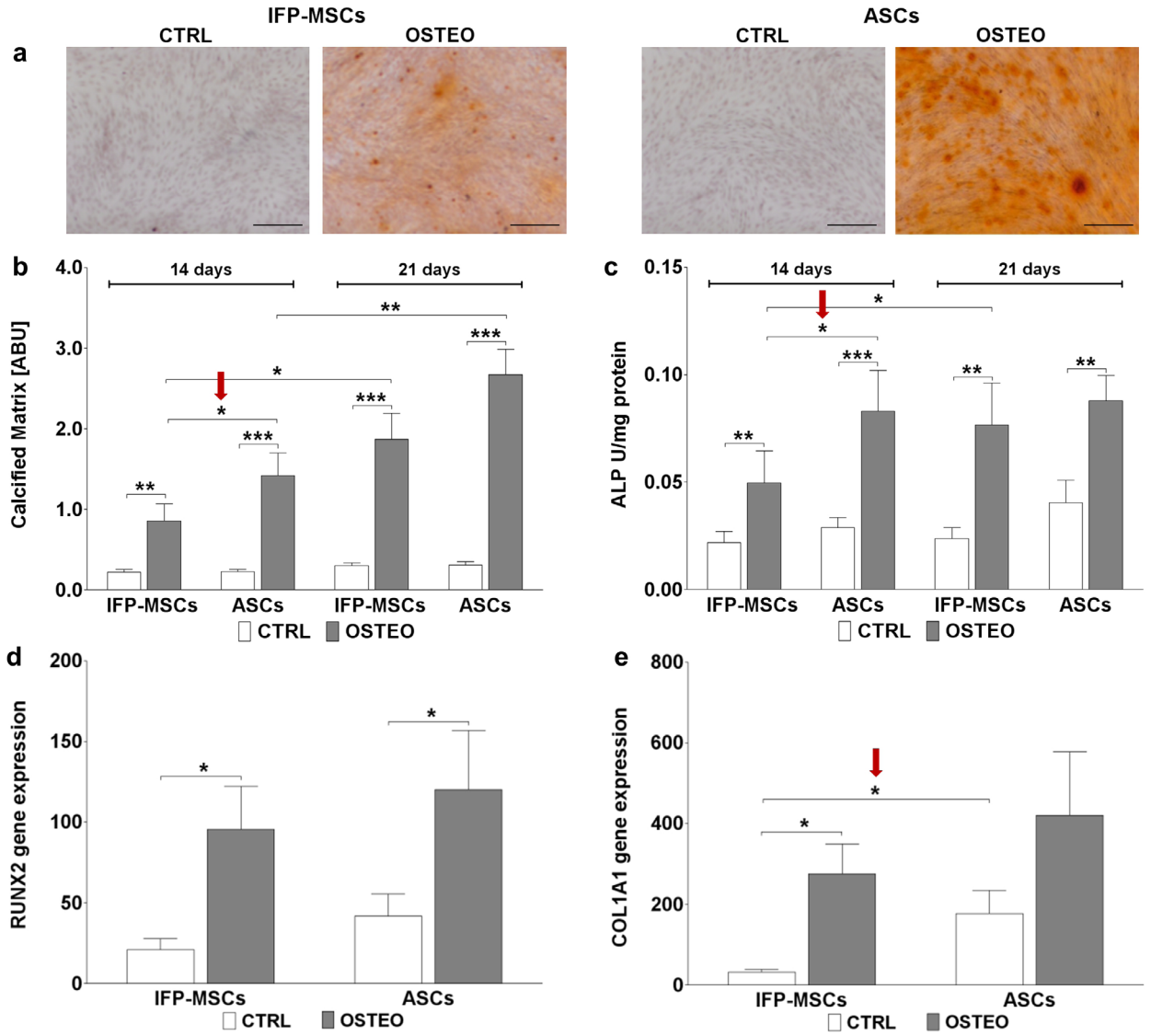


Fig. 4. Osteogenic differentiation. (**a**) Micrographs of IFP-MSCs and ASCs differentiated for 14 days and stained by AR-S (scale bars 500 µm). (**b**) Quantification of calcified matrix by AR-S staining and extraction in undifferentiated (CTRL) and osteogenic-differentiated (OSTEO) IFP-MSCs and ASCs (n = 14). (**c**) ALP activity determined by enzymatic assay and normalised by protein content (n = 9). (**d**,**e**) Gene expression of *RUNX2* and *COL1A1* normalised to *GAPDH* (n = 7). Red arrows indicate significant differences between IFP-MSCs and ASCs.

Furthermore, we investigated the expression of collagen X and alkaline phosphatase which are usually associated with the premature development of a hypertrophic phenotype during MSCs chondrogenesis (Ichinose *et al.*, 2005; Mueller and Tuan, 2008; Pelttari *et al.*, 2006; Winter *et al.*, 2003). We found that, in the early phase of chondrogenic differentiation, release of ALP was significantly higher in ASCs pellets and that, after 14 days of chondrogenic differentiation, levels of *COL10A1* were significantly lower in IFP-MSCs compared to ASCs, which may indicate a lower propensity of IFP-MSCs to develop a hypertrophic phenotype. Furthermore, we found that levels of *COL1A1* were 50-fold higher compared to transcriptional levels of *COL10A1*, which may indicate that both IFP-MSCs and ASCs are more prone to develop a fibrotic rather than a hypertrophic phenotype, as demonstrated for IFP-MSCs in a recent study (Vinardell *et al.*, 2012).

The dissimilarities found between IFP-MSCs and ASCs may depend on the different anatomical localisation of infrapatellar fat pad and knee subcutaneous adipose tissue, considering that infrapatellar fat pad is in direct contact with synovial membrane and that the local tissue microenvironment could affect the commitment of MSCs. Differences in the expression of hypertrophic and fibrocartilaginous markers suggest that IFP-MSCs could be more suitable for cartilage applications aiming at the generation of specific cartilage-like matrix. However, further investigations after a longer period of chondrogenic differentiation are required to confirm the different trend between IFP-MSCs and ASCs in developing a hypertrophic phenotype.

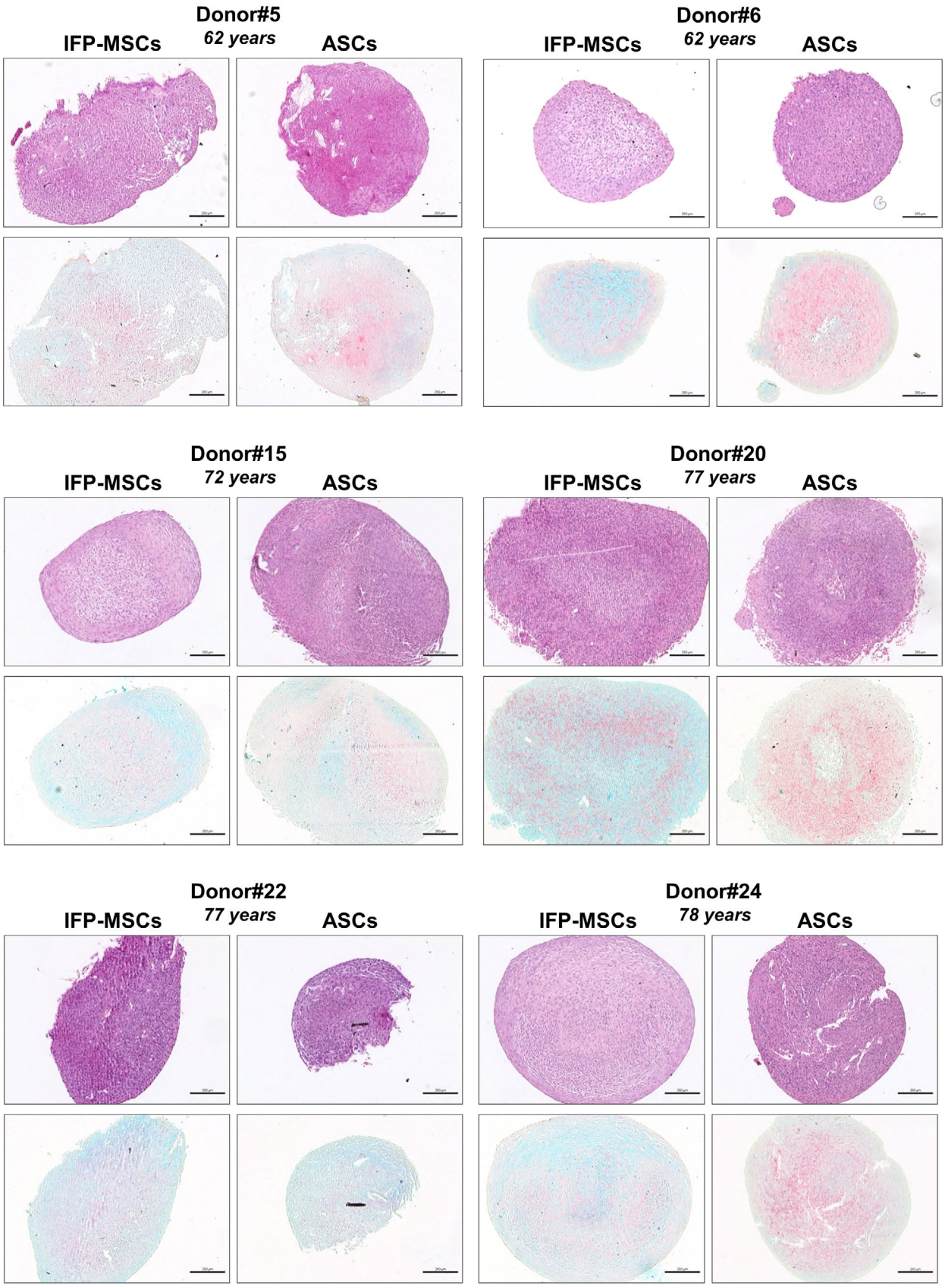


Fig. 5. Histological evaluation of pellets from different donors. Haematoxylin-eosin and alcian blue staining of pellets prepared with IFP-MSCs and ASCs derived from younger and older donors (scale bars, 200 µm).

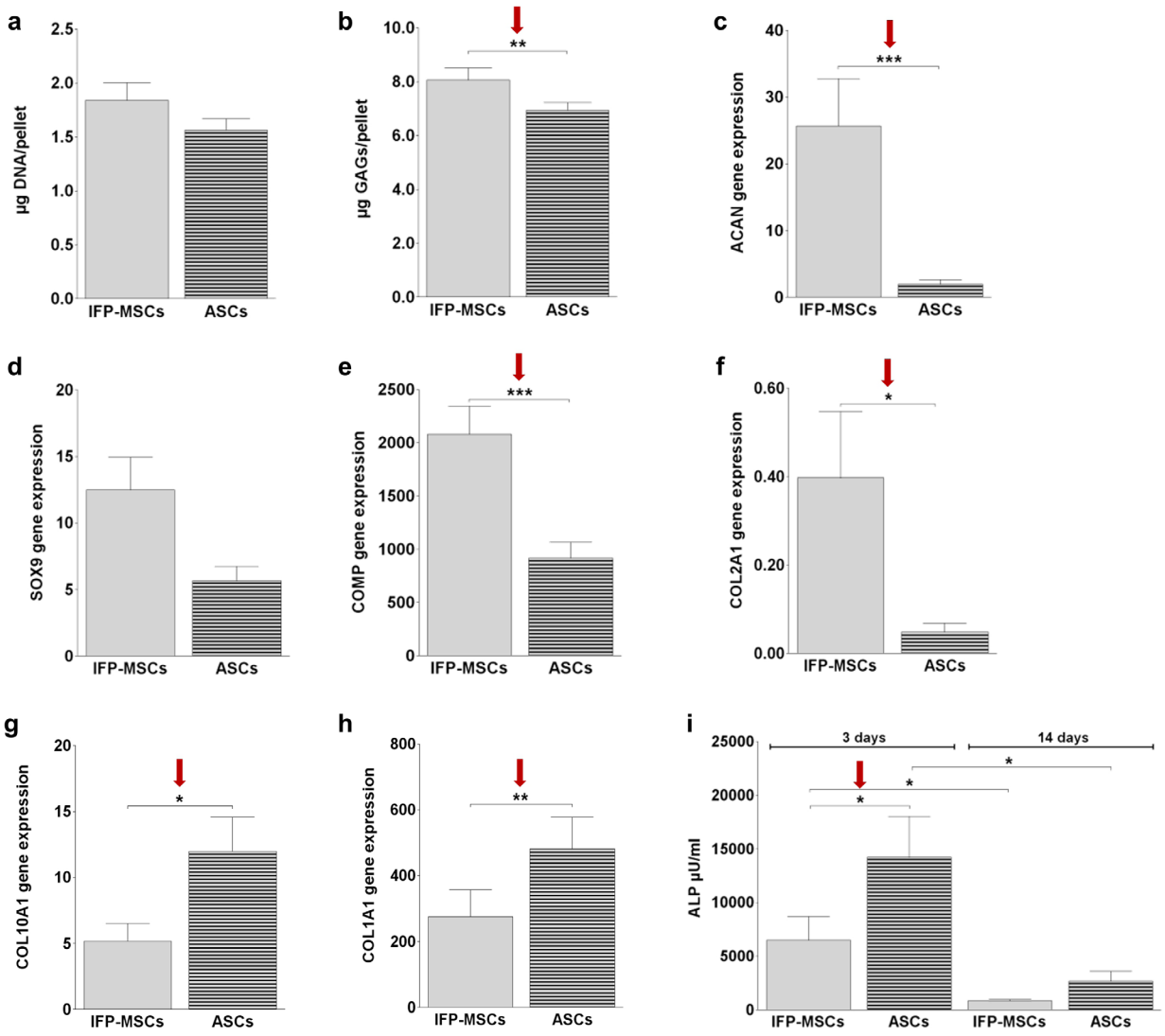


Fig. 6. Chondrogenic differentiation**.** (**a**,**b**) DNA and GAGs content in IFP-MSCs and ASCs pellets (n = 15). (**c-h**) Gene expression of *ACAN, SOX9, COMP, COL2A1, COL10A1* and *COL1A1* normalised to *GAPDH* (n = 10). (**i**) Soluble ALP activity released in culture supernatants from day 0 to day 3 (indicated as 3 days) and from day 11 to day 14 (indicated as 14 days) (n = 8). Red arrows indicate significant differences between IFP-MSCs and ASCs.

Despite similar surface markers expression in the undifferentiated state, different populations of MSCs can display significantly different commitment towards specific cell lineages. This supports the importance of introducing more specific and predictive markers to identify which population of MSCs could be more suitable for a specific clinical application. as proposed by recent studies that have investigated the differentiation potential of specific subsets of MSCs (Jiang *et al.*, 2010; Quirici *et al.*, 2010; Kim *et al.*, 2012).

It should be highlighted that the differential commitment of IFP-MSCs and ASCs emerged from *in vitro* experiments and should be further confirmed through *in vivo* studies to better validate the clinical relevance of our data. However, our results contribute to show how quantitative analyses on larger donor populations are fundamental for a more reliable characterisation of primary human cells, contributing to build a more consistent base for cell-based therapies.

Conclusions

To the best of our knowledge, this is the first study to compare donor-matched IFPs and ASCs from such a large number of OA donors, characterising their osteogenic and chondrogenic potential through several quantitative analyses. This provided novel and interesting insights on their specific differentiation capabilities, opening to a more rational selection as preferential sources for regenerating cartilage and bone tissues or developing osteochondral treatments, aimed at future clinical cell-based treatments, also in OA patients.

Acknowledgements

The authors would like to thank L. J. Turner and C. Bonora for their contribution in the experiments and in the harvesting of human biopsies. This study was supported

by the Italian Ministry of Health that contributed solely in terms of financial support to the study.

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Discussion with Reviewers

A. Barbero: The authors showed that IFP-MSCs cultured with medium containing TGFβ produced better cartilaginous tissues than ASCs. It is, however, known that adipose derived MSC (as compared to bone marrow-derived MSC) required BMP-6 in order to chondro-differentiate. Did the authors try to culture ASCs with different chondrogenic cocktails?

Authors: In this study, we observed superior deposition of GAGs and superior expression of chondrogenic markers in IFP-MSCs pellets compared to donor-matched ASCs pellets. Due to the limited amount of cells that can be obtained from each patient, we were able to test only a single formulation of chondrogenic medium. However, in a previous study comparing donor-matched IFP-MSCs and ASCs from OA patients (Alegre-Aguaron *et al.*, 2012, text reference) the superior chondrogenic potential of IFP-MSCs in comparison with ASCs was observed even when combining TGFβ and BMP-6 to induce chondrogenic differentiation, as revealed by the histological scoring. Our results confirm this finding, suggesting that the differences observed between the two cell populations are independent from the presence of BMP-6 in the chondrogenic medium formulation.

A. Barbero: Due to its important role in the joint homeostasis, IFP tissue cannot be harvested in large quantity. What is the amount of IFP tissue that can be harvested from a knee in order to get enough MSC for possible clinical application?

Authors: In our experience it is possible to harvest up to 40 % of the entire infrapatellar fat pad volume, which in total measures about 20-25 cm^3 (Chuckpaiwong *et al.*, 2010, additional reference), without impairing joint function. In our experiments, samples ranged from 3 to 10 cm^3 (average volume 5.8 cm^3) and cell isolation yielded about 1 x 10^5 - 1.5 x 10^5 cells/mL, with some donor-dependent variations. Our data are in accordance with results from a recent study where IFP-MSCs were isolated from infrapatellar fat pad (average weight 9.4 g; range, 6.9-11.2 g; average number of cells/sample 1.89 × 10^6; range 1.2 × 10^6 - 2.3 × 10^6 cells/sample) and immediately injected back in the patients in combination with Platelet Rich Plasma (PRP) for the treatment of knee OA, showing an improvement in symptoms compared to patients treated with PRP only (Koh *et al.*, 2012, additional reference). This study, together with a follow-up study (Koh *et al.*, 2013, additional reference), has demonstrated that even a limited number of IFP-MSCs can improve knee OA symptoms, reporting no adverse effect deriving from the infrapatellar fat pad harvesting. Hence, we believe that this adipose depot represents a promising cell source for future cell-based applications, even considering the possibility to use IFP-MSCs in combination with autologous articular chondrocytes thus eliminating, or at least reducing, the duration of *in vitro* cell expansion prior to re-implantation.

D. Kelly: BMP-6 has been shown to be critical for chondrogenesis of ASCs (Estes *et al.*, 2006; Hennig *et al.*, 2007). Do the authors believe that the enhanced chondrogenesis observed here in IFP-MSCs compared to ASCs would be replicated in alternative media formulations additionally supplemented with BMP-6? Is there evidence from the literature to suggest that the chondrogenic capacity of IFP-MSCs is also enhanced in the presence of BMP-6?

Authors: In a previous study comparing donor-matched IFP-MSCs and ASCs from OA patients (Alegre-Aguaron *et al.*, 2012, text reference) the superior chondrogenic potential of IFP-MSCs was observed even using a chondrogenic medium containing both TGFβ and BMP-6, suggesting that the differences between the two cell populations are independent of the presence of BMP-6. We found in the literature only few studies reporting the use of BMP-6 for the chondrogenic induction of human IFP-MSCs (Alegre-Aguaron *et al.*, 2012, text reference; Felimban *et al.*, 2014, additional reference). However, in these studies a comparison between medium formulations with TGFβ only and TGFβ combined with BMP6 is not present, which does not allow us to verify whether BMP-6 enhances the chondrogenic ability of this cell population. A recent study, performed using porcine IFP-MSCs, has shown that the simultaneous use of TGFβ and BMP-6 is able to improve the chondrogenic differentiation of IFP-MSCs, compared to cells differentiated in the presence of TGFβ alone (He *et al.*, 2013, additional reference). However, this improvement was not observed in cells differentiated after several passages in culture, suggesting that the chondrogenic effect of BMP-6 may be influenced by the duration of IFP-MSCs expansion phase prior to chondrogenic differentiation.

D. Kelly: The authors use differences in ALP and collagen X expression to suggest that IFP-MSCs may have a lower tendency towards hypertrophy. It has been shown that *in vivo*, cartilage tissues engineered using IFP derived stem cells undergo fibro-cartilaginous or fibrous dedifferentiation rather than progressing along an endochondral route (Vinardell *et al.*, 2012). This raises a question as to whether type X collagen expression and ALP are appropriate markers for accessing the ability of IFP-MSCs to generate stable cartilage tissue. Can the authors comment on this and whether they believe their cartilage pellets are proceeding along an endochondral or an alternative pathway.

Authors: In our experiments we found that the transcriptional expression of *COL1A1* was 50-fold higher compared to transcriptional levels of *COL10A1*, which may suggest that cells are developing a fibrous phenotype rather than progressing towards an endochondral pathway. However, in our study a short time point was chosen to evaluate the chondrogenic differentiation of IFP-MSCs and ASCs and we did not monitor the kinetic of *COL1A1* and *COL10A1* expression over time to confirm this hypothesis.

In the study by Vinardell and co-workers (Vinardell *et al.*, 2012, text reference), one of the main differences between porcine bone marrow MSCs and IFP-MSCs seem to be the ability to generate calcified matrix after 49 days of *in vitro* culture. Hence, this parameter could be evaluated in cells differentiated over a long period of time to verify this hypothesis in human cells and to highlight

possible differences between IFP-MSCs and ASCs in the development of a fibrous rather than a hypertrophic phenotype.

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