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New Strategies in Cartilage Tissue Engineering for Osteoarthritic Patients: Infrapatellar Fat Pad as an Alternative Source of Progenitor Cells

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Novel tissue engineering strategies have proposed the combined use of articular condrocytes (ACs) and mesenchymal stem cells (MSCs) for the treatment of chondral lesions to overcome limitations related to the reduced cellular yield and low chondrogenic potential of ACs derived from subjects affected by osteoarthritis (OA). The aim of this study was to evaluate the multidifferentiative ability of MSCs derived from the knee infrapatellar fat pad (IFP-MSCs) by determining their osteogenic, adipogenic and chondrogenic potential as well as performing pellet coculture experiments using IFP-MSCs and ACs derived from the same donor. IFP-MSCs showed a marked clonogenic ability and the typical surface markers pattern of MSCs and efficiently differentiated towards adipogenic and osteogenic lineages. No significant difference in chondrogenic potential was observed between IFP-MSCs and ACs from OA patients. In coculture experiments, histological analysis showed an increase in glycosamminoglycans production correlated to the percentage of ACs and a significant up-regulation of aggrecan gene expression was observed in pellets containing 25% and 50% ACs respect to 100% IFP-MSCs pellets. Our data demonstrate that IFP-MSCs possess a considerable multidifferentiative potential and may be a suitable candidate to be used alone or combined with ACs for cell-based chondral treatments.

Keywords:

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1. INTRODUCTION

Cartilage is a tissue with a very self-regenerative potential due to the lack of vascularization; as a consequence, untreated cartilage lesions lead to a progressive tissue degeneration, which usually results in osteoarthritis (OA), a highly invalidating pathology, characterized by the loss of articular functionality.[1] It is therefore important to promptly treat isolated cartilage defects, to avoid the worsening of the joint situation leading to OA. Autologous chondrocytes transplantation (ACT)[2] is one of the most used cell-based therapy approaches to treat chondral lesions, but patients suffering from degenerative articular pathologies were usually excluded from receiving cell-based therapies, usually undergoing partial joint replacement.

Most of the patients affected by early OA are still active persons whereby a prosthetic solution can be too radical and definitive. For this reason, researchers are trying to extend the tissue engineering approach involving the use of chondrogenic cells for the treatment of lesions also in these patients to prevent or at least posticipate the joint replacement.[3–4] The use of articular chondrocytes (ACs) presents some limitations related to the need to harvest a defined region of healthy cartilage for ACs isolation and to the subsequent need to expand ACs, leading to dedifferentiation with the progressive loss of chondrogenic potential.[5–7] It is still not clear if OA cartilage degeneration affects the differentiative features of ACs and their ability to re-build a stable cartilage-like tissue,[8–10] but the observed correlation between donor-age and reduction in chondrocytes differentiation[11–12] suggests that an alternative cell source should be preferentially used when treating older patients.

In this context, adult mesenchymal stem cells (MSCs), which are able to differentiate towards the chondrogenic lineage, represent a suitable candidate for cell-based articular cartilage repair technologies.[13–16]

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MSCs are present in many adult tissues[17–21] and play an important role in the physiological cellular turnover and in repair processes following damages induced by trauma, disease or aging. Even if bone marrow still remains the most common source of mesenchymal progenitor cells for clinical applications, controversial results have been obtained regarding the multidifferentiative and chondrogenic potential of bone marrow MSCs (BMSCs) derived from patients affected by OA.[22–23]

The presence of a population of MSCs in the infrapatellar fat pad (IFP-MSCs) of the knee has been recently demonstrated.[24–26] These cells are able to differentiate towards different mesodermic lineages[24–26]and have been proposed for the treatment of OA in a rabbit model.[27] Moreover this cellular source is particularly appealing for the orthopaedic surgeon due to the easy accessibility of the infrapatellar fat pad during knee surgery procedures that could be associated to ACT, such as ligament reconstructions, meniscectomy, meniscal suture and osteotomies.

The aim of this study was to assess the multidifferentiave potential of IFP-MSCs, with a particular focus on their chondrogenic ability. Since it is well-known that strong inductive signals are required to induce cultured MSCs to chondrogenesis, cocolturing them with ACs may represent a valid strategy to promote and to stabilize MSCs chondrogenic phenotype.[28–30]

In particular, to better mimic possible clinical applications, as compared to other previously published studies,[28–32] we uniquely used IFP-MSCs and ACs derived from the same patients to perform pellet coculture experiments. The combination of ACs and IFP-MSCs could potentially allow to overcome known limitations of current clinical applications, such as timely achievement of required number of cells, dedifferentiation of ACs following expansion and chondrogenic potential of OA affected ACs, when applying cell-based therapies to articular cartilage degenerative pathologies.

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2. MATERIALS AND METHODS

2.1. Cell Isolation and Expansion

Samples of infrapatellar fat pad and articular cartilage were harvested from 15 patients (3 males and 12 females, mean age 65 ± 5 years) affected by osteoarthritis. Samples were taken during a routine unicompartmental knee arthroplasty procedure. All patients signed a written consent form before undergoing operation.

To isolate IFP-MSCs, infrapatellar fat pad was minced and washed with cold phosphate-buffered saline (PBS, Gibco, Italy). Extracellular matrix was enzymatically digested with 0.075% type I collagenase (Worthington Biochemical Co, USA) at 37 °C with continuous agitation for 30 min and the sample was then centrifuged (1200 g, 10 min) and filtered through a cell strainer to remove undigested tissue. Mononucleated cells were counted and plated in control medium consisting of HG-DMEM (High Glucose, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Lonza, Switzerland), 0.029 mg/ml L-glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 10 mM hepes (Gibco), 1 mM sodium pyruvate (Gibco) at approximately 10^4 cells/cm^2. The cells were cultured at 37 °C in a humidity controlled atmosphere (5% CO_2) and after 3 days non adherent cells were removed. During the culture period, medium was replaced twice a week.

ACs were isolated from cartilage by enzymatic digestion using 0.15% type II collagenase (Worthington Biochemical Co) for 22 h at 37 °C according to a previously established protocol.[12] The cells were counted, plated at a density of 10^4 cells/cm^2 and cultured in control medium supplemented with 1 ng/ml of transforming growth factor-β1 (TGF- β1, PeproTech Inc, Germany) and 5 ng/ml fibroblast growth factor-2 (FGF-2, PeproTech Inc).

When IFP-MSCs and ACs were about 90% confluent, the cells were detached using 0.05% trypsin/0.53 mM EDTA (Gibco) and replated at 3×10^3 cells/cm^2 and 5×10^3 cells/cm^2 respectively.

2.2. IFP-MSCs Clonogenic Ability

A colony-forming unit-fibroblast (CFU-F) assay was performed as described previously,[33] with minor modifications, to assess the clonogenic ability of IFP-MSCs. Cells were plated in six-well plates at low density by limiting dilution (starting dilution 48 cell/cm^2, ending dilution 1 cell/cm^2) and cultured in control medium supplemented with 20% FBS. After 10 days cells were fixed with 10% neutral buffered formalin and stained with Gram's crystal violet (Sigma-Aldrich, Italy). The frequency of CFU-F was established by scoring the individual colonies and expressing them as a percentage relative to the total number of seeded cells.

2.3. IFP-MSCs Surface Markers Expression by Flow Cytometry Analysis

After 2 months of culture the expression of specific surface markers was evaluated; IFP-MSCs were detached using 0.05% trypsin/0.53 mM EDTA and washed twice in cold FACS Buffer (PBS containing 0.1% FBS and 0.1% NaN_3) to remove the culture medium. 3×10^5 cells for each sample were incubated with the following anti-human primary monoclonal antibodies: CD13-FITC, CD-14-FITC, CD45-FITC, CD54-biotinilated, CD29-biotinilated, CD71-biotinilated, CD105-biotinilated (all from Ancell Corporation, USA) and purified CD90 (ThermoFisher Scientific Inc, USA). After incubation, the cells were washed and incubated with secondary antibodies: Streptavidin-PE (Ancell Corporation) or sheep anti-mouse conjugated with FITC (Boheringer, Germany).

Samples were then washed with FACS Buffer and analyzed by FACSCalibur using the software CellQuest (Becton Dickinson, USA).

2.4. IFP-MSCs Cell Lineage Differentiation and Evaluation of Differentiation Markers

2.4.1. Adipogenic Differentiation

To evaluate their adipogenic potential, human IFP-MSCs were plated at 3×10^3 cells/cm^2 and differentiated for 14 days in adipogenic medium using a pulsed induction[34] with 3 days of induction in control medium supplemented with 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), 500 μM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 200 μM Indomethacin (Sigma-Aldrich), followed by 3 days of maintenance in control medium supplemented only with 10 μg/ml insulin.

After 14 days, samples were washed with PBS and fixed in 10% neutral buffered formalin for 1 h and stained with 8.5 mM Oil Red O (Sigma-Aldrich) in 100% isopropanol, for 15 min. To quantify the lipid vacuole content, the dye was extracted with 100% isopropanol and absorbance was read at 490 nm using a Perkin Elmer Victor X3 microplate reader (Perkin Elmer, USA).

2.4.2. Osteogenic Differentiation

Human IFP-MSCs were plated at 3×10^3 cells/cm^2 and differentiated for 14 days in osteogenic medium[34] consisting of control medium supplemented with 10 mM glicerol-2-phosphate (Sigma-Aldrich), 10 nM dexamethasone, 150 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 nm cholecalciferol (Sigma-Aldrich).

To evaluate Alkaline Phosphatase activity (ALP) cells were washed 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).[35] Enzymatic reaction was stopped with NaOH 1 N and absorbance was read at 410 nm using a Perkin Elmer Victor X3 microplate reader). ALP activity was normalized on total protein content, determined by BCA Protein Assay Kit (Pierce Biotechnology, USA) and expressed as ALP Units for μg of proteins.

In order to quantify calcified matrix deposition, the 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, Fluka) for 15 min. After washing, each sample was destained for 30 min with 10% cetylpyridinium chloride monohydrate (CPC, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.0) and absorbance was read at 570 nm using a Perkin Elmer Victor X3 microplate reader.[36]

2.4.3. Chondrogenic Differentiation and Coculture Experiments

At passage 4, 4×10^5 IFP-MSCs were centrifuged (250 g, 5 min) to obtain cell pellets that were then cultured in chondrogenic medium consisting of HG-DMEM (4.5 mg/ml Glucose) 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 (HSA) (Sigma-Aldrich), 1% ITS + 1 (containing 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. To compare the chondrogenic potential of IFP-MSCs with the one of ACs isolated from the same donors, ACs were expanded until passage 2, detached and used to prepare cell pellets (4×10^5 cells/pellet). To evaluate the effect of cocultures between IFP-MSCs and ACs isolated from the same donor, we used cells derived from 3 female donors (mean age 71 ± 3 years); IFP-MSCs were expanded until passage 4, while ACs were frozen at passage 1 and then thawed and expanded to prepare pellets for the experiments. Cocolture pellets were prepared mixing IFP-MSCs and ACs in 2 different proportion: 75%–25% and 50%–50%, respectively; the chondrogenic differentiation of coculture pellets was compared with that of pellets consisting of 100% IFP-MSCs.

All the pellets were maintained in chondrogenic medium for 14 days and culture medium was changed twice a week. After 14 days pellets were processed either for histological analysis or glycosamminoglycans (GAGs) and DNA quantification.

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

For GAGs and DNA quantification, the pellets were washed with PBS and incubated in 500 μl of PBE buffer (100 mM Na_2HPO_4, 10 mM NaEDTA, pH = 6.8) containing 1.75 mg/ml L-cystein (Sigma-Aldrich) and 14.2 U/ml papain (Worthington) for 16 h at 60 °C.

The quantity of GAGs was spectrophotometrically measured using 16 mg/ml dimethylmethylene blue (Sigma-Aldrich), with chondroitin sulfate as standard, and DNA content was quantified using the CyQUANT Kit (Molecular Probes, USA).

2.4.4. Gene Expression Analysis

Real time PCR was used to evaluate the expression of characteristic matrix protein coding genes after 14 days

in monolayer or pellet culture, supplemented or not with adipogenic, osteogenic or chondrogenic medium.

Total RNA was isolated and purified from cell lysates using the RNeasy Mini kit (Qiagen, The Netherlands). During RNA purification, the RNase-Free DNase Set (Qiagen) was used for residual genomic DNA digestion and the RNA isolated was quantified with a spectophotometer (Nanodrop, Thermo Scientific, USA). 100 ng of the isolated RNA was reverse-transcribed to cDNA employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). The final volume of 20 μl included a 5× reaction mix containing oligo (dT), random hexamer primers and reverse transcriptase preblended with RNase inhibitor. The reaction mix was incubated for 5 min at 25 °C, 30 minutes at 42 °C and 5 min at 85 °C.

20 ng of total RNA was used as template for real-time PCR performed using a Rotor Gene RG3000 system (Corbett Life Science, Australia). PCR mixture included TaqMan Universal PCR Master Mix and TaqMan® Assays-on-Demand™ Gene expression probes (Applied Biosystems, USA) in a final volume of 20 μl. Amplification and real-time data acquisition were performed using the following cycle conditions: 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. Genes examined were those for glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), peroxisome proliferator-activated receptor gamma (*PPARG*) and leptin (*LEP*) to assess adipogenic differentiation, collagen-I (*COL1A1*) to assess osteogenic differentiation, collagen-II (*COL2A1*) and aggrecan (*ACAN*) to assess chondrogenic differentiation. Relative expression was calculated by using the $2^{-\Delta\Delta CT}$, where CT is the cycle threshold. The fold change in expression of the different genes was normalized to the expression of the housekeeping GAPDH gene. The $\Delta\Delta$CT was calculated by subtracting CT for *GAPDH* from CT for each target gene.

2.5. Statistical Analysis

Data are expressed as mean ± SEM and statistical analysis was performed using Student's *T*-test for paired data and One-Way ANOVA (GraphPad Prism v5.00, GraphPad Software USA). Level of significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. IFP-MSCs and ACs Isolation and Culture

The employed protocol applied to the isolation of IFP-MSCs was straightforward, whereby biopsies yielded an average of $1.2 \times 10^5 \pm 1.3 \times 10^4$ cells for each ml of tissue. One week after isolation, IFP-MSCs started to proliferate and assumed the typical fibroblastoid morphology that was maintained for the whole expansion period (Fig. 1(a)).

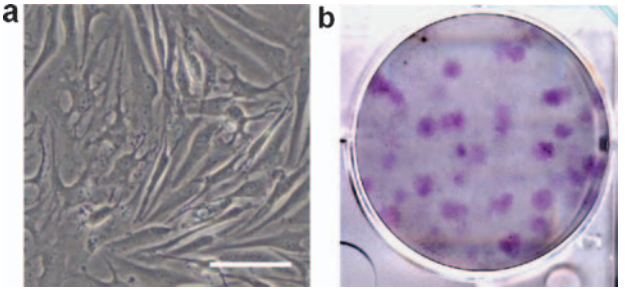

Fig. 1. Morphology and clonogenic potential of IFP-MSCs. (a) Micrographs of IFP-MSCs 10 days after isolation (scale bar 100 μm). (b) CFU-F assay performed after 4 passages in culture (30 CFU-F counted on 58 seeded cells).

IFP-MSCs maintained their proliferation potential with an average of 0.12 ± 0.02 population doublings/day until passage 4, showing just a slight decrease in their proliferation rate along with passaging. The clonogenic ability of IFP-MSCS was very high, with about 40% of cells able to form a colony, and it was maintained throughout subsequent passages (Fig. 1(b)). After 2 months of culture, the evaluation of cell surface markers by FACS analysis confirmed the maintenance of the profile described for IFP-MSCs[24] with the expression of CD90, CD105, CD13, CD29, CD54, CD166 and the absence of CD45, CD14 and CD71, demonstrating the ability of IFP-MSCs to retain their phenotype even after prolonged periods of culture. This pattern is similar to the profile of surface markers that we observed in previous studies on MSCs isolated from adipose tissue (ASCs),[34,37] whereas IFP-MSCs cellular yield and proliferation rate was lower when compared to previously published data on ASCs.[18,34] Differently from ASCs where clonogenic ability decreased along with passaging, in IFP-MSCs clonogenica potential was maintained without any decrease during the period of culture.[34]

For ACs the average cellular yield was $2.2 \times 10^6 \pm 6.5 \times 10^5$ cells for each gram of digested cartilage. After an initial lag phase, ACs started to proliferate with 0.18 ± 0.02 population doublings/day until passage 1 and increased their proliferation rate from passage 1 to passage 2 (0.53 ± 0.06 population doublings/day). Immediately after isolation ACs lost their rounded shape to assume a fibroblastoid morphology with prolonged cellular protrusion (Fig. 2(a)). During culture we observed a progressive modification of ACs phenotype, with the cells becoming smaller and assuming a cuboidal shape around the nucleus (Fig. 2(b)), that was similar to the morphology observed by Schnabel et al.[5] and is known to be one of the evidences of chondrocytes dedifferentiation process.

Our data on ACs cellular yield and proliferation rate closely resemble data published by Barbero et al.[12] confirming a significant reduction in older donors (more than 60 years) in comparison with cells isolated from younger patients.

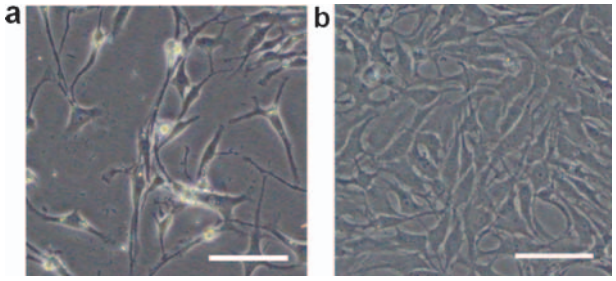

Fig. 2. Morphology of ACs after different periods of culture. (a–b) Micrographs of ACs in culture 5 days (a) and 15 days (b) after isolation (scale bar 100 μm).

3.2. IFP-MSCs Adipogenic Differentiation

IFP-MSCs which had been induced to differentiate for 14 days towards the adipogenic lineage showed significant morphological changes compared to undifferentiated cells: during differentiation adipo-induced IFP-MSCs progressively lost the fibroblastoid-like shape and produced a great number of cytoplasmatic lipid vacuoles (Fig. 3(a)). This observation was confirmed by Oil Red O staining and extraction that revealed a significant increase in lipid vacuoles production in differentiated cells in comparison to cells maintained in control medium (+30%, $p < 0.001$, Fig. 3(b)). A significant increase (+432%, $p < 0.05$) of peroxisome proliferator-activated receptor gamma was observed in IFP-MSCs cultured in adipogenic medium compared with undifferentiated cells. Adipogenic differentiated IFP-MSCs also showed an up-regulation of leptin, even if the increase was not statistically significant with respect to undifferentiated cells due to the great inter-donor variability (Fig. 3(c)).

In agreement with literature data on IFP-MSCs,[24–26] our data demonstrate that these cells are able to efficiently differentiate towards adipogenic lineage, when cultured in the presence of suitable factors, showing both morphological changes and the upregulation of genes involved in the adipogenic process.

3.3. IFP-MSCs Osteogenic Differentiation

IFP-MSCs showed a marked osteogenic potential, as demonstrated by the increase in differentiative markers after 14 days of culture in osteogenic medium. Osteo-induced IFP-MSCs partially lost their initial fibroblastoid shape, assuming a more cuboidal morphology and showing a significant increase in ALP activity (+287%, $p < 0.05$, Fig. 4(a)). Moreover, we observed a significant increase in the deposition of calcified extracellular matrix (+346%, $p < 0.05$, Fig. 4(b)), as revealed by Alizarin Red-S that stained the abundant deposits of calcium as shown in Figure 4(c). Gene expression of IFP-MSCs following osteogenic differentiation showed increased levels

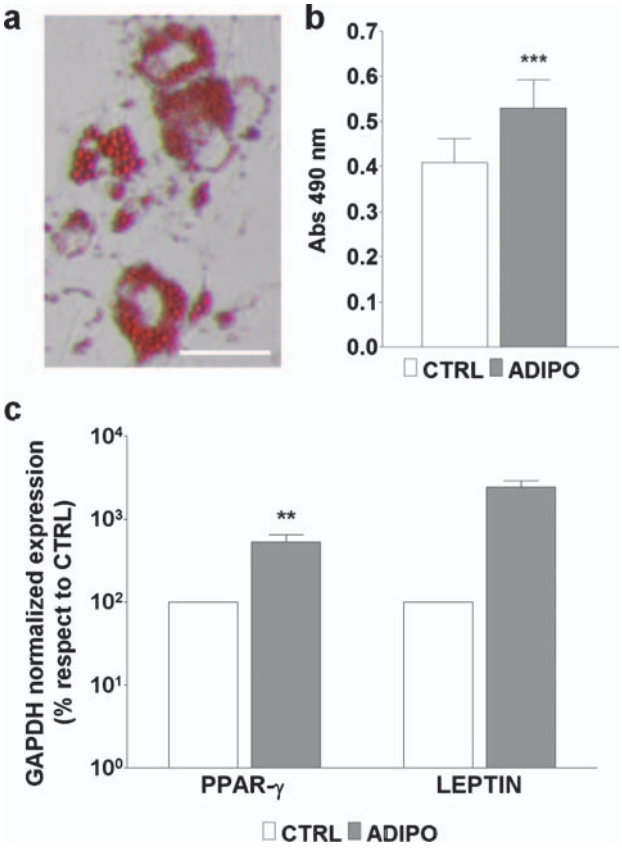

Fig. 3. Adipogenic differentiation of IFP-MSCs. (a) Micrographs of IFP-MSCs differentiated for 14 days and stained with Oil Red O (scale bar 50 μm). (b) Quantification of lipid vacuoles production in undifferentiated (CTRL, white bar) and adipogenic-differentiated IFP-MSCs (ADIPO, grey bar) after 14 days of culture ($n = 9$, ADIPO vs. CTRL $p < 0.001$) (c) Gene expression of PPAR-γ and leptin determined using real-time PCR. Levels of gene expression were normalized to house-keeping gene *GAPDH* and showed as% respect to undifferentiated cells (CTRL set as 100%, $n = 5$, logarithmic scale, ADIPO vs. CTRL $p < 0.01$).

of collagen-I with respect to undifferentiated cells, however this increase was not statistically significant due to the great inter-donor variability (Fig. 4(d)).

Our data on osteogenic potential of IFP-MSCs confirmed previous studies reporting the ability of this cellular population to respond to osteoinductive stimuli leading to the expression of osteogenic markers as collagen-I, osteocalcin and osteopontin as well as to the deposition of calcified extracellular matrix.[24–26]

3.4. IFP-MSCs Chondrogenic Differentiation

The chondrogenic potential of IFP-MSCs cultured in pellets for 14 days was determined in terms of GAGs production and gene expression of aggrecan and collagen-II. Response to the serum free medium containing chondrogenic factors such as TGF-β1 was evaluated by comparison with pellets made from ACs isolated from the same donors.

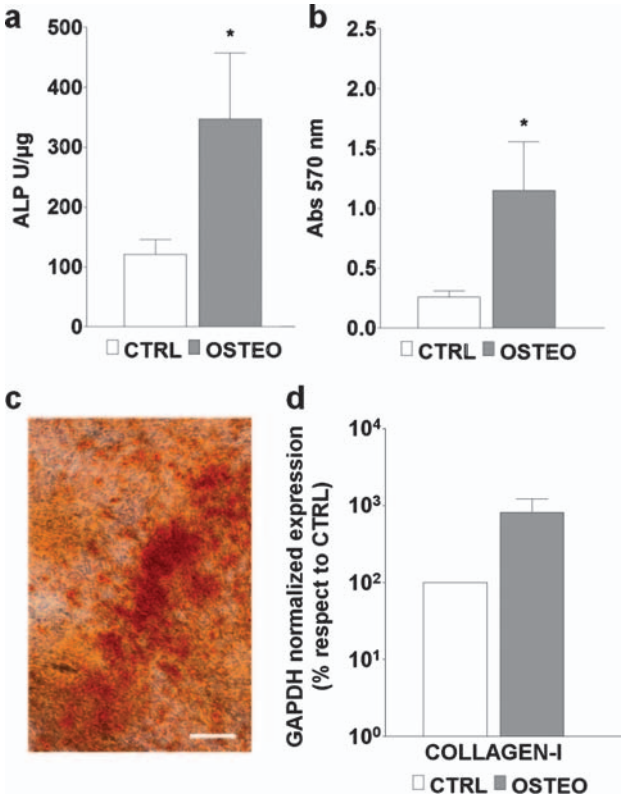

Fig. 4. Osteogenic differentiation of IFP-MSCs. (a–b) ALP activity normalized on total protein content (a) and quantification of calcified matrix (b) in undifferentiated (CTRL, white bar) and osteogenic-differentiated IFP-MSCS (OSTEO, grey bar) after 14 days of culture ($n = 10$, OSTEO vs. CTRL $p < 0.05$). (c) Micrographs of IFP-MSCs differentiated for 14 days and stained with Alizarin Red-S (scale bar 500 μm). (d) Gene expression of collagen-I determined using real-time PCR. Levels of gene expression were normalized to house-keeping gene *GAPDH* and showed as % respect to undifferentiated cells (CTRL set as 100%, $n = 4$, logarithmic scale).

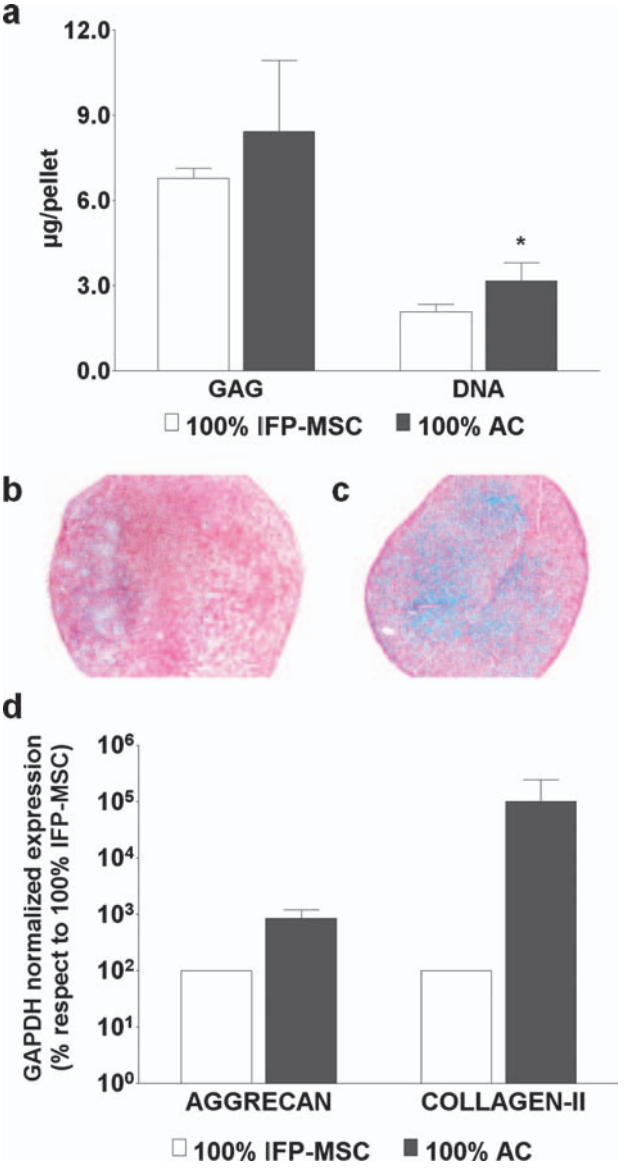

Fig. 5. Comparison between chondrogenic potential of IFP-MSCs and ACs. (a) Quantification of GAGs and DNA content in 100% IFP-MSCs (white bar) and 100% ACs (grey bar) pellets after 14 days in chondrogenic medium ($n = 7$, 100% ACs vs. 100% IFP-MSCs $p < 0.05$). (b–c) Histological evaluation by Alcian Blue staining of cartilage-like extra-cellular matrix in 100% IFP-MSCs (b) and 100% ACs pellets (c). (d) Gene expression of aggrecan and collagen-II determined using real-time PCR. Levels of gene expression were normalized to the house-keeping gene *GAPDH* and showed as % with respect to 100% IFP-MSCs (100% IFP-MSCs set as 100%, $n = 3$, logarithmic scale).

Surprisingly, we did not observe any significant difference in terms of GAGs content (μg GAGs/pellet) between pellets containing 100% IFP-MSCs and 100% ACs, even if GAGs values were slightly higher for 100% AC pellets (+24%, Fig. 5(a)). On the other hand, the difference in DNA content observed in 100% ACs pellets in comparison to 100% IFP-MSCs pellets was statistically significant (+53%, $p < 0.05$, Fig. 5(a)), with ACs showing a greater survival and proliferative ability in these culture conditions.

Both in 100% IFP-MSCs and in 100% ACs pellets the presence of extra-cellular matrix was observed and histological staining with Alcian Blue revealed a higher content of GAGs in 100% ACs pellets (Figs. 5(b–c)).

Following differentiation in chondrogenic medium, as expected, higher levels of aggrecan and collagen-II gene expression were observed in 100% ACs pellets with respect to 100% IFP-MSCs pellets, even if differences between the two cellular populations were not statistically significant (Fig. 5(d)).

We did not observe any significant difference between 100% IFP-MSCs and 100% ACs pellets, which is in accordance with data published by English et al.[25] who reported a similar production of GAGs by IFP-MSCs and OA chondrocytes. This may be due to both the degenerative state of the cartilage used for ACs isolation and the donor age that affects the ability of ACs to respond to chondrogenic factors used during expansion to prevent or reduce their

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dedifferentiation.[12] Histological and gene expression analysis partially confirmed the biochemical data, showing an increase in GAGs production and in expression of specific matrix proteins genes in 100% ACs pellets (Figs. 5(c–d)).

The higher DNA content in 100% ACs pellets demonstrates that chondrocytes are able to survive and proliferate in pellet culture, whereas IFP-MSCs alone show a growth inhibition that is probably due to a lower ability to adapt to the change from monolayer expansion phase to pellet culture conditions. This observation finds a confirmation in a recent study where Giovannini et al.[31] showed a similar decrease in DNA content comparing 100% ACs and 100% BMSCs pellets.

3.5. Cocultures Between IFP-MSCs and ACs Isolated from the Same Donor

In order to better mimic a future autologous therapy for the treatment of chondral lesions using a combination of autologous IFP-MSCs and ACs, we performed each coculture experiment isolating both cell types from the same donor. Pellets were prepared with different percentages of IFP-MSCs and ACs to evaluate the influence of ACs on the chondrogenic differentiation of IFP-MSCs. The expression of chondrogenic markers was evaluated in pellets composed of 75% IFP-MSCs −25% ACs (75:25) and 50% IFP-MSCs −50% ACs (50:50). 100% IFP-MSCs pellets (100:0) were used as a control.

The coculture with ACs led only to a slight increase in GAGs content and, probably due to the greater chondrocytes percentage, it was higher in 50:50 pellets, with no significant difference between 100:0 pellets and pellets containing either 25% or 50% ACs. Pellets combinations of 75:25 and 50:50 showed an increased content of DNA, corresponding to a greater number of cells and demonstrating a higher survival and proliferation rate of ACs with respect to IFP-MSCs. In particular, the increase in DNA content was correlated to the percentage of ACs, with a statistically significant difference for 50:50 pellets in comparison with 100:0 pellets (+32%, $p < 0.05$).

Alcian Blue staining showed an increase in GAGs deposition within the extracellular matrix correlating with the percentage of ACs in cocolture with IFP-MSCs (Figs. 6(b–d)). A significant up-regulation of aggrecan gene expression was observed in 75:25 and 50:50 pellets (+214% and +486% respectively, $p < 0.05$) with respect to 100% IFP-MSCs pellets; also the transcriptional expression of collagen-II was positively regulated in the presence of ACs, even if increases were not statistically significant with respect to 100% IFP-MSCs pellets.

The comparison with published data on GAGs content in pellets containing 100% ACs obtained from younger healthy subjects highlighted a striking difference with our data: in their experiments, Giovannini et al.[31] measured more than 100 μg GAGs/pellet after 3 weeks of pellet culture, which is 10-fold higher than the values that we observed in our tests for 100% ACs pellets. This observation may explain the limited increase in GAGs content in coculture pellets, which is probably due to the reduced quality of ACs derived from cartilage from older patients affected by severe OA degeneration. Even in coculture experiments we observed a partial discrepancy between

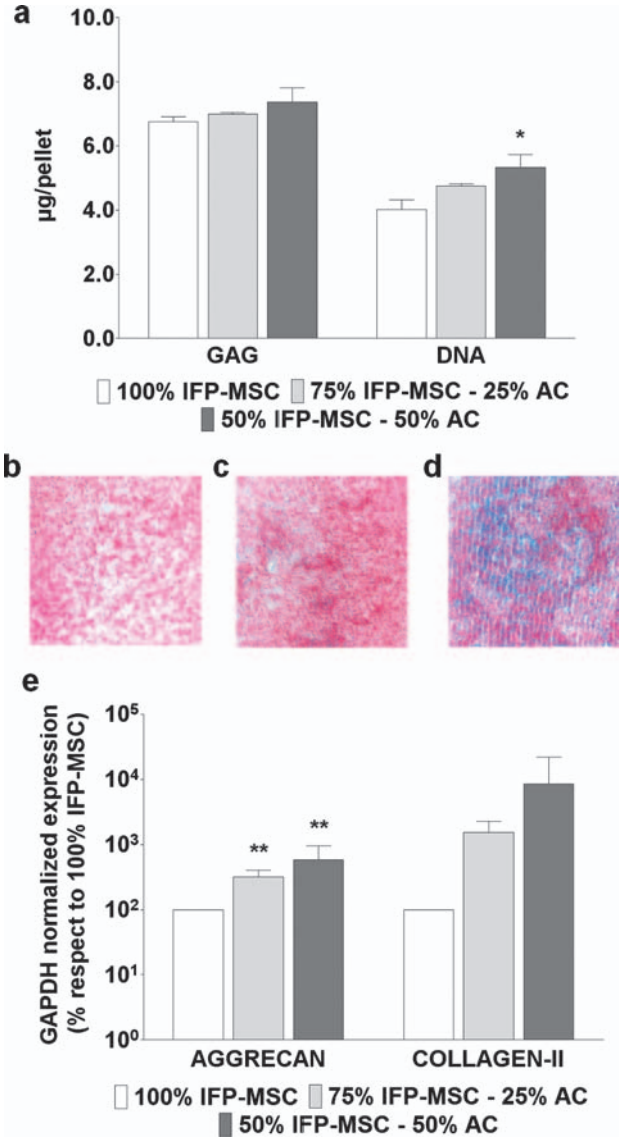

Fig. 6. Cocolture between IFP-MSCs and ACs isolated from the same donor. (a) Quantification of GAGs and DNA content in 100% IFP-MSCs (white bar, 100:0), 75% IFP-MSCs – 25% ACs (light grey bar, 75:25) and 50% IFP-MSCs – 50% ACs (dark grey bar, 50:50) pellets after 14 days of culture in chondrogenic medium ($n = 3$, 50:50 vs. 100:0 $p < 0.05$). (b–d) Histological evaluation by Alcian Blue staining of cartilage-like extracellular matrix in 100:0 (b), 75:25 (c) and 50:50 (d) pellets. (e) Gene expression of aggrecan and collagen-II determined using real-time PCR. Levels of gene expression were normalized to the house-keeping gene *GAPDH* and showed as % with respect to 100% IFP-MSCS (100% IFP-MSCS set as 100%, $n = 3$, logarithmic scale, 75:25 vs. 100:0 $p < 0.05$, 50:50 vs. 100:0 $p < 0.01$).

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biochemical data versus histological and gene expression results, with an evident increase, related to ACs percentage, both in specific matrix production and aggrecan expression. Nevertheless, cocultured cells did not seem to profit from a synergistic effect as compared to the sum of the estimated single contribution of each cellular type. This observation is in contrast with other studies reporting a contribution in chondrogenesis of ASCs cocultured with ACs[29] and a significant influence of soluble factors secreted by ACs on chondrogenic differentiation of ASCs.[30] As for the limited increase in GAGs production, this discrepancy may depend on the reduced chondrogenic potential of ACs used in our experiments, depending on donor age, on the pathological state of cartilage and on the dedifferentiation following the expansion phase.

The difference in DNA content observed between 100% IFP-MSCs and 100% ACs was reflected in the increase in DNA in 75:25 and 50:50 pellets and, as we already hypothesized, that may be probably due to the greater ability of ACs to survive and proliferate in pellet culture conditions.

4. CONCLUSIONS

Our results confirm the presence of a population of MSCs within the knee infrapatellar fat pad. The multidifferentiave potential of IFP-MSCs efficiently granted both osteogenic and chondrogenic commitment and indicates a promising use of IFP-MSCs for osteochondral applications.

Given the reduced yield and chondrogenic potential of ACs obtained from OA joints and the simple infrapatellar fat pad accessibility during knee surgical procedures, IFP-MSCs represent a cellular candidate that may integrate, or substitute, autologous chondrocytes in cellular therapies applications in early OA patients. Furthermore, the fast isolation protocol employed for IFP-MSCs isolation could result advantageous for future one-step surgical cell delivery procedures, preventing MSCs isolation from other sites with higher morbidity as well as the harvesting of ACs from healthy cartilage regions.

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