Estrogen deficiency does not decrease the in vitro osteogenic potential of rat adipose-derived mesenchymal stem cells

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Abstract Osteoporosis due to estrogen deficiency is an increasing bone health issue worldwide: new strategies are being studied for regenerative medicine of bone pathologies in these patients. The most commonly used cells for tissue engineering therapy are the bone marrow mesenchymal stem cells (BMSCs), but they might be negatively affected by aging and estrogen deficiency. Besides the general advantages of adipose-derived mesenchymal stem cells (ADSCs) over BMSCs, ADSCs also seem to be less affected by aging than BMSCs, but in the literature, little is known about ADSCs in estrogen deficiency. The present study investigated the in vitro behavior of ADSCs, isolated from healthy (SHAM) and estrogen-deficient (OVX) rats. Phenotype, clonogenicity, viability, and osteogenic differentiation, at both cellular and molecular levels, were evaluated with or without osteogenic stimuli. Pro-inflammatory cytokines, growth factors, and adipogenic differentiation markers were also analyzed. There were no significant differences between OVX and SHAM ADSCs in some analyzed parameters. In addition, clonogenicity, osteopontin (*Spp1*) gene expression, alkaline phosphatase (ALP) activity at 2 weeks

of culture, total collagen (COLL), osteocalcin (*Bglap*) gene expression and production, and matrix mineralization were significantly higher in OVX than in SHAM ADSCs. Besides the increase in some osteogenic markers, peroxisome proliferator-activated receptor gamma (*Pparg*) gene was also more expressed in OVX in osteogenic medium, with a concomitant estrogen receptor 1 (*Esr1*) gene expression decrease. These results underlined that ADSCs were not affected by estrogen deficiency in an osteogenic microenvironment.

Keywords Adipose stem cells · Adult stem cells · Cell biology · Cell culture · Gene expression · Osteoporosis

Introduction

Osteoporosis is usually caused by several factors related to genetics, aging, hormone levels, drug assumption, malabsorption or chronic kidney disease, and harmful lifestyles (e.g., smoking and alcoholism) (Giavaresi et al. 2011). Among them, the most common cause, in women, is estrogen deficiency, which not only leads to bone weakness, increased fracture risk, and implant failure (Kim et al. 2012), but also “fragility syndrome,” with manifestations and implications also on soft tissues (Torricelli et al. 2012; Calleja-Agius and Brincat 2009). The management of complications due to bone alterations, related to estrogen deficiency, represents one of the most important challenges in the orthopedic field. Nowadays, bone grafting procedure limitations have shifted the focus of preclinical research to tissue

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engineering strategies, which are strictly dependent on the proliferation and differentiation ability of mesenchymal stem cells (MSCs) (Zhu et al. 2009).

MSCs have the ability to self-renew, proliferate, and differentiate toward different lineages (osteoblasts, adipocytes, chondroblasts, myoblasts, etc.), but MSCs from bone marrow (BMSCs), which are the most commonly employed cells in the clinical setting for different orthopedic diseases (Aldahmash et al. 2012), are reported to be negatively influenced by various factors, most of which are also responsible for osteoporosis such as systemic diseases, lifestyles, drug assumption, and aging. In particular, estrogen levels may be critical for clinical applications of BMSCs (Veronesi et al. 2011) since some authors observed an *in vitro* decrease in number, stemness, proliferation, osteogenic differentiation, and mineralization of BMSCs from postmenopausal patients in comparison with cells from normal donors (Dalle Carbonare et al. 2009; Valenti et al. 2011; Rodriguez et al. 2004, 2008). BMSCs are the physiological source of osteoblasts (OBs) and also of other cells of mesodermal lineage such as adipocytes, chondrocytes, and fibroblasts (Benisch et al. 2012), depending on hormonal and local factors (paracrine/autocrine), that regulate the expression of master differentiation genes. The adipocytes are inversely related to OBs, with a prevalence of osteoblastogenesis in healthy conditions (Boyce et al. 2012) and an increase in BM adipocytes in estrogen deficiency (Benisch et al. 2012; Pino et al. 2012). This imbalance not only plays a central role in osteoporosis development, but also limits the therapeutic use of autologous BMSCs in osteoporotic patients (Pino et al. 2012).

In recent years, the osteogenic potential of MSCs from adipose tissue (ADSCs) has been investigated to identify alternative stem cell sources (Tobita et al. 2011). Interest in ADSCs is also growing because of the development of adipose tissue concentrators, which allow the one-step surgical application of ADSCs, thus limiting the disadvantages of MSC culture expansion (Hoogendoorn et al. 2008). ADSCs not only share similar characteristics with BMSCs (morphology, phenotype, colony frequency, multilineage differentiation ability, and culture stability) (Pachon-Pena et al. 2010), but also have numerous advantages because they (a) are easier to harvest with repeatable surgical accesses and less discomfort (morbidity and wound infection); (b) are isolated in a larger quantity (ADSCs are 5 % of nucleated cells, whereas BMSCs account for 0.0001–0.01 %); (c) are easier to

expand *in vitro*; and (d) show higher proliferation rate, paracrine signaling, and angiogenic properties with respect to BMSCs (Fraser et al. 2006). ADSCs seem to be less affected than BMSCs by aging and maintain their number, self-renewal, viability, proliferation, and osteogenic potential in the elderly (Wu et al. 2013; Mojallal et al. 2011; Khan et al. 2009; Shi et al. 2005; Fei et al. 2013). Until now, to the authors' knowledge, only two papers have evaluated ADSCs in estrogen deficiency and observed that proliferation rate and osteogenic differentiation, mesenchymal surface markers, and telomere length were not affected by estrogen deficiency in both patients and mice (Chen et al. 2012; Mirsaidi et al. 2012).

Starting from these results and to improve knowledge about ADSCs in regenerative medicine in bone pathologies, the present study aimed to establish whether ADSCs show any alterations in their capability to differentiate toward the osteogenic lineage when derived from estrogen-deficient animals. The osteogenic differentiation of ADSCs was evaluated in ADSCs derived from ovariectomized (OVX) and sham age-matched healthy (SHAM) rats cultured with an osteogenic medium, by taking into account different biologic aspects such as viability, early and late bone marker expression, as well as estrogen receptor gene expression and matrix molecules synthesis. Other factors related to adipogenic and osteoclastogenic differentiation processes and release of pro-inflammatory cytokines and growth factors (GFs) were also investigated to better understand the role of estrogen deficiency in ADSC commitment.

Materials and methods

ADSC isolation

The study protocol was in the scope of other projects on biomaterial osteointegration in estrogen deficiency and not affecting ADSCs. These studies were approved by the Rizzoli Orthopedic Institute Ethical Committee and by the Italian Ministry of Health and performed strictly following national laws on animal experiments (Law by Decree 116/92).

Immediately after euthanasia, the adipose tissue was harvested from three "healthy" (SHAM) female Sprague Dawley rats (10 months old) and three ovariectomized (OVX) rats (13 months old: 10 months at ovariectomy and further 3 months of estrogen deficiency). Adipose tissue was first washed with phosphate-buffered saline

(PBS) and then cut into small pieces and digested with 0.075 % collagenase II (Sigma Aldrich, St. Louis, MO, USA) in agitation (T-Shaker EuroClone EMSC-100, Italy) for 1 h at 37 °C. The enzymatic reaction was stopped by the addition of complete medium (CM): Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich), 10 % fetal bovine serum (FBS; Lonza, Verviers, Belgium), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA), and 5 µg/ml plasmocin (InvivoGen, San Diego, CA, USA). The obtained pellet was resuspended in 160 mM NH₄Cl (Sigma Aldrich) and incubated at room temperature (RT) for 10 min to remove red blood cells. The nucleated cells were seeded in T75 flasks in CM, maintained in standard conditions at 37 °C, 5 % CO₂ in a humidified atmosphere (GALAXY 170S incubator, New Brunswick, USA), and refed with fresh medium twice a week. At subconfluence, the cells were detached and seeded in 24-well plates to perform the experiments.

ADSC surface epitope characterization

Before cell seeding and to test the surface antigen expression, $0.5\text{--}1 \times 10^5$ ADSCs for each antigen were washed with PBS, centrifuged at 260g for 5 min, and incubated at 4 °C for 30 min in flow cytometry buffer (FCB, 2 % FBS in PBS) adding 0.5 µg/ml of fluorescein isothiocyanate (FITC)-conjugated antibody against CD31, CD45, CD34, CD44, CD73, CD90, and CD105. FITC-conjugated nonspecific IgG was used as isotype control (BioLegend, San Diego, CA, USA). The cell fluorescence was evaluated with FACSCanto II instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed by FACSDiva software (Becton Dickinson).

CFU-F assay

At first confluence, 200 ADSCs/cm² in CM were seeded in T25 flasks and cultured for 10 days. At the endpoint, the cells were fixed with 10 % formalin for 20 min and stained with 0.1 % toluidine blue in 1 % paraformaldehyde (PFA) for 1 h. The aggregates with ≥ 20 cells were visually scored as colonies and counted. The clonogenicity of ADSCs was expressed as percentage of colony-forming units (CFUs) with respect to the seeded ADSCs.

ADSC cultures

At P1, the expanded cultures were detached from the T75 flasks with 0.05 % trypsin/EDTA (Gibco, Invitrogen), counted with vital dye trypan blue (Sigma Aldrich), and seeded in 24-well plates at a density of 5×10^3 cells/cm² with CM or osteoblast differentiating medium (OM): CM enriched with 50 µg/ml ascorbic acid 2P, 7 mM β -glycerophosphate, and 10^{-7} M dexamethasone (Sigma Aldrich).

The experimental groups were defined as follows according to SHAM and OVX ADSCs and culture media (CM and OM):

- SHAM CM: ADSCs from healthy rats, cultured in CM
- SHAM OM: ADSCs from healthy rats, cultured in OM
- OVX CM: ADSCs from ovariectomized rats, cultured in CM
- OVX OM: ADSCs from ovariectomized rats, cultured in OM

All cultures were maintained for 1 and 2 weeks, replacing the medium every 2 days. At the time points, the supernatants were collected and stored at -80 °C for ELISA tests, while the cells were evaluated for viability (WST-1 test), cellular alkaline phosphatase (ALP) activity, and gene expression profile.

ADSC viability (WST-1 test)

At both experimental times and for both culture conditions (CM and OM), the WST-1 colorimetric reagent (Roche Diagnostics GmbH, Mannheim, Germany) was used to evaluate cell viability. The reagent, mixed to CM at a final dilution of 1:10, was added to the cell monolayer in each well. After an incubation of 3 h and 30 min, the colorimetric reaction, which reveals mitochondrial activity in viable cells, was quantified spectrophotometrically at 450 nm (Bio-Rad Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA) with a reference wavelength at 625 nm, and the results were reported as optical density (OD). In other wells, after detachment using trypsin, the number of cells was calculated by a light microscope with a Neubauer chamber. Finally, the WST-1 values were normalized to the corresponding cell number.

Analysis of osteogenic and adipogenic marker gene expression (RNA isolation and RT-qPCR)

The relative expression of genes reported in Table 1 was assessed for ADSCs from SHAM and OVX rats in both culture conditions. Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed with Superscript VILO cDNA Synthesis kit (Invitrogen), following the manufacturer's instructions. Ten nanograms of each cDNA sample was tested in duplicate. Semiquantitative PCR analysis was performed in a LightCycler Instrument (Roche Diagnostics) using QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). The following protocol was pursued: initial denaturation at 94 °C for 15 min and 40 cycles of amplification (15 s at 94 °C, 20 s at the appropriate annealing temperature for each target—as detailed in Table 1—and 20 s at 72 °C). The protocol was concluded by melting curve analysis to check for amplicon specificity. The threshold cycles (Ct) were determined for each sample, and these values were used for comparative gene expression analysis employing the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) using glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) expression as reference. The results were expressed as fold variation relative to the value of control condition at 1 week.

ADSC protein release

To assess the osteogenic differentiation, ADSC monolayers were first lysed with 100 µl of 1 % SDS in distilled water at 37 °C for 15 min, and then an equal volume of 20 mM *p*-nitrophenylphosphate (*p*-NNP, Sigma Aldrich) in assay buffer (0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4; Sigma) was added to each well and incubated at 37 °C for a further 15 min. The amount of *p*-nitrophenol, as stained product of this colorimetric reaction, was measured spectrophotometrically at 405 nm. The ALP activity was obtained by referring to a standard curve performed with serial dilution of 100 µM *p*-nitrophenol, and the ALP values were normalized to cellular total proteins (TPs) content (Total Protein micro-Lowry kit, Sigma Aldrich).

The supernatants from all culture conditions, previously collected from culture wells, were assayed for total collagen (total COLL, collagen assay; Sircol S1000, Biocolor, Life Science assays, UK), osteoprotegerin (OPG, rat; USCN Life Science Inc, Wuhan, China), receptor activator of nuclear factor kappa B ligand (RANKL, rat; USCN Life Science), osteocalcin (OC, rat; USCN, Life Science Inc, Wuhan, China), interleukin-1 β (IL-1β, rat; USCN Life Science), interleukin-6 (IL-6, rat; eBioscience, Bender

Table 1 Gene primers specific for osteogenic and adipogenic differentiation or involved in the differentiating process. Their expression was normalized to the GAPDH reference gene

Gene	Primer forward	Primer reverse	Amplicon length (bp)	Annealing temperature (°C)
<i>Gapdh</i>	5'-CTCTACCCACGGCAAGTTCAAC-3'	5'-GACATACTCAGCACCAGCATCAC-3'	136	60
<i>Runx2</i>	Hs_RUNX2_1_SG ^{a,b}		101	55
<i>Col1a1</i>	5'-GCAAGAGGCGAGAGAGGTTT-3'	5'-GGACCAATGGGACCAGTCAG-3'	290	57
<i>Alpl</i>	5'-GCCAGCTACACCACAACACG-3'	5'-CGCCACACAAGTAGGCAGTG-3'	138	57
<i>Bglap</i>	5'-CCGTTTAGGGCATGTGTTGC-3'	5'-CCGTCCATACTTTCGAGGCA-3'	108	57
<i>Spp1</i>	5'-CCGAGGTGATAGCTTGGCTT-3'	5'-TCGGACTCCTGGCTCTTCAT-3'	123	55
<i>Bmp2</i>	5'-CACAGGGACACACCAACCAT-3'	5'-GCCACGATCCAGTCATTCCA-3'	295	55
<i>Pparγ</i>	Rn_Pparg_1_SG ^a		146	55
<i>Adipoq</i>	Rn_Adipoq_2_SG ^a		84	55
<i>Lpl1</i>	Rn_Lpl1_1_SG ^a		106	55
<i>Esr1</i>	5'-GACATGAGAGCTGCCAACCT-3'	5'-ACTTGACGTAGCCAGCAACA-3'	447	55
<i>Esr2</i>	5'-GCGACCCATTGCCAATCATC-3'	5'-TCACATGACCAACGCCGTA-3'	233	55
<i>Tgfb1</i>	Rn_Tgfb1_1_SG ^a		145	55
<i>Vegfa</i>	Rn_RGD:619991_1_SG ^a		68	55

^a QuantiTect Primer Assay (Qiagen)

^b Designed for human Runx2, also detects rat transcript

MedSystem GmbH, Vienna, Austria), transforming growth factor- β (TGF- β , rat; R&D Systems, MN, USA), and vascular endothelial growth factor (VEGF, rat; R&D Systems). The results were normalized by TPs taking into account the differences in cell growth, and the OPG/RANKL ratio was evaluated.

Matrix calcium deposition (Alizarin red staining)

At P1, 1×10^4 ADSCs/cm² were cultured in T25 flasks for 21 days in OM to observe the mineral deposition, whereas cultures in CM were prepared as reference. At the endpoint, the ADSCs were fixed with 4 % PFA in PBS for 15 min, washed with distilled water, incubated with 2 % Alizarin red solution (pH 4.2) (Sigma Aldrich) for 20 min, and carefully washed in distilled water. Calcium phosphate deposition was visualized as mineralized nodules using an inverted light microscope at $\times 4$ magnification (Olympus IX 71, Olympus Italia SRL, Milano, Italy) to obtain a global image of the culture. The quantification was performed by adding 10 %w/v cetylpyridinium chloride previously dissolved in sodium phosphate 10 mM (pH 7) to the cultures. The eluted Alizarin was evaluated spectrophotometrically at 570 nm. Finally, the Alizarin concentration was obtained by referring to a standard curve.

Statistical analysis

Statistical analysis was performed using the software package SPSS 12.1 (SPSS Inc., Chicago, IL, USA). Data are reported as median, upper and lower quartiles, and min and max (boxplot). After having verified the normal distribution (Kolmogorov-Smirnov test) and the homogeneity of the variance (Levene test), the general linear model (GLM) with adjusted Sidak multiple comparison test with “culture medium” (CM, OM), “cell origin” (SHAM and OVX), and “experimental time” (1 and 2 weeks) as fixed effects was performed to assess the differences between factors on the tested parameters. Student's *t* test was used to compare CD surface marker expression between OVX and SHAM ADSCs.

Results

ADSC surface epitope characterization

The isolated and expanded ADSCs at P1 from both SHAM and OVX rats appeared to be a homogenous

cell population expressing high CD44, CD73, CD90, and CD105 and very weak CD31, CD45, and CD34 percentages, with no significant differences between SHAM and OVX (Fig. 1).

ADSC clonogenicity

ADSCs from both SHAM and OVX animals were homogeneous with an elongated spindle-shaped morphology. After 10 days of culture in CM, the clonogenicity of ADSCs isolated from OVX animals was significantly ($p < 0.05$) higher than that of the SHAM ones (Fig. 2a).

ADSC viability

Normalizing the WST-1 values for the cell number (cell count), it was observed that at 1 week, the viability per cell of SHAM OM was significantly higher than that of OVX OM ($p < 0.05$) and SHAM CM ($p < 0.0005$). At 2 weeks, SHAM and OVX OM showed higher values than those of CM ($p < 0.0005$). Finally, for all the experimental groups, with the exception of OVX OM, this ratio decreased over time ($p < 0.0005$) (Fig. 2b).

Analysis of osteogenic and adipogenic marker gene expression

When comparing SHAM and OVX ADSCs, collagen 1 $\alpha 1$ (*Coll1*), osteocalcin (*Bglap*), osteopontin (*Spp1*), peroxisome proliferator-activated receptor gamma (*Pparg*), and estrogen receptor 1 (*Esr1*) gene expressions significantly changed. *Coll1* and *Bglap* were expressed significantly ($p < 0.05$) lower and higher in OVX than SHAM ADSCs, respectively, independent of the culture medium and experimental time (Fig. 3a, b). *Spp1* was significantly higher ($p < 0.0005$) in ADSCs from OVX OM than that of SHAM OM at both experimental times, in OM than in CM culture conditions at both experimental times ($p < 0.0005$), and at 1 week than at 2 weeks for OVX OM ($p < 0.0005$) (Fig. 3c). With regard to *Pparg* gene expression, significantly higher values ($p < 0.0005$) were achieved in OVX OM than in SHAM OM and OVX CM, independent of experimental time (Fig. 3d). *Esr1* gene expression was significantly higher in SHAM OM than that of OVX OM and SHAM CM ($p < 0.005$), independent of experimental time (Fig. 4a).

The expression of runt-related transcript (*Runx2*) and bone morphogenetic protein 2 (*Bmp2*) genes (Fig. 4b, c)

Fig. 1 Cytofluorimetric analysis of CD markers on SHAM and OVX ADSCs. Count histograms and boxplots of the most representative subjects in terms of median fluorescence intensity (MFI) of stained cells normalized to the MFI of the corresponding isotype control: **a** IgG (isotype control); **b** CD31; **c** CD34; **d** CD44; **e** CD45; **f** CD73; **g** CD90; **h** CD105

was not significantly different between SHAM and OVX ADSCs, but it was influenced by “culture medium” and “experimental time” factors. In particular, *Runx2* and *Bmp2* expressions were higher in ADSCs cultured in OM than in CM: for *Bmp2* at both experimental times ($p<0.0005$) and for *Runx2* at 1 week ($p<0.0005$) and 2 weeks ($p<0.005$). A significant decrease in *Runx2* ($p<0.005$) and *Bmp2* ($p<0.0005$) expression was observed in OM at 2 weeks.

Significant differences related only to the exposure of OM culture medium were found for the other genes of osteogenic and adipogenic differentiation: *Alpl* expression was higher in OM than in CM ($p<0.0005$) (Fig. 4d) as well as lipoprotein lipase (*Lpl*) ($p<0.05$) (Fig. 5a) and *Tgfb1* ($p<0.05$) (Fig. 5b) expressions. On the other

hand, *Vegfa* ($p<0.0005$) and estrogen receptor 2 (*Esr2*) ($p<0.005$) expressions were lower in OM than in CM (Fig. 5c, d). Adiponectin (*Adipoq*) expression was not detected (data not shown).

ADSC protein release

ALP activity and OC, total COLL, and VEGF protein releases were significantly different between SHAM and OVX cells. More precisely, the ALP activity in SHAM CM was significantly higher than that of OVX CM ($p<0.005$) and in SHAM OM than that of OVX OM ($p<0.05$) at 1 week, whereas at 2 weeks, it was significantly higher in OVX OM than that of SHAM OM ($p<0.0005$) (Fig. 6a). OC was released significantly more by OVX OM than by SHAM OM at both experimental times ($p<0.0005$), and at 2 weeks, it was released more by SHAM CM than by OVX CM ($p<0.0005$) (Fig. 6b). In addition, ALP activity and OC release were significantly higher in OM than those of CM for OVX ($p<0.0005$) and significantly higher in SHAM OM than those of SHAM CM at 1 week ($p<0.0005$) and 2 weeks ($p<0.05$) for OC. Total COLL release was significantly higher in OVX than that of SHAM ADSCs, independent of the culture medium and experimental time ($F=4.21$, $p<0.05$) (Fig. 6c). Finally, VEGF was significantly ($p<0.0005$) higher in OVX CM than that of SHAM CM, independent of experimental time. Moreover, OVX OM and SHAM OM released significantly less VEGF than OVX CM and SHAM CM, respectively ($p<0.0005$) (Fig. 6d).

Only the type of culture medium influenced the release of OPG/RANKL, IL-6, and IL-1 β . In particular, the OPG/RANKL ratio was significantly higher with OM in comparison to CM at 1 week ($p<0.05$) and 2 weeks ($p<0.0005$) (Fig. 7a), whereas IL-1 β ($p<0.05$) and IL-6 ($p<0.0005$) were released significantly less by cells in OM (Fig. 7b, c).

The duration of stimulation had a significant impact on the decrease in ALP activity for SHAM CM or SHAM OM and OPG/RANKL for both cell types in CM ($p<0.005$) (Figs. 6a and 7a), but a significant impact on the increase in ALP activity for OVX OM ($p<0.0005$) and OC synthesis for SHAM CM ($p<0.0005$) and OVX OM ($p<0.005$) (Fig. 6a, b). No significant differences were found for TGF- β 1 results (Fig. 7d).

Fig. 2 **a** Boxplots of colony-forming unit results, expressed as percentage; **b** cell viability normalized for cell count results, expressed as %WST-1/cell count ($n=3$ replicates). Sidak test: * $p<0.05$; *** $p<0.0005$. In **b**, the mean of the cell count was expressed for all the experimental groups

Fig. 3 Boxplots of osteogenic and adipogenic marker gene expression ($n=3$ replicates): **a** *Col1a1*; **b** *Bglap*; **c** *Spp1*; **d** *Pparg*. **a**, **b** Interaction of “cell origin” for *Col1a1* ($F=6.13$, $*p<0.05$) and *Bglap* ($F=6.39$, $*p<0.05$) expressions; **c** interaction of “cell origin,” “culture medium,” and “experimental time” factors for *Spp1*

expression ($F=16.93$, $p<0.005$, Sidak test: $***p<0.0005$); **d** interaction of “cell origin” and “culture medium” factors for *Pparg* expression ($F=19.32$, $p<0.005$); Sidak test: $***p<0.0005$, OVX OM versus SHAM OM; $*p<0.0005$, OVX OM versus OVX CM, independently of experimental time

Matrix calcium deposition

The evaluation of the mineral deposition by Alizarin red S staining at 21 days of culture showed that ADSCs derived from OVX rats exhibited higher ($p<0.05$) matrix mineralization than that derived from SHAM ones, without a significant effect or interaction of the “culture medium” factor (Fig. 8).

Discussion

The present in vitro study was carried out to investigate the behavior of OVX and SHAM ADSCs, stimulated or not with an osteogenic medium. It was conducted to find an alternative stem cell source to BMSCs for estrogen-deficient patients, who need regenerative medicine procedures. OVX rats are still employed in the literature as a standardized model of osteoporosis due to estrogen deficiency and already used in many studies to investigate the effects of hormones (Smith et al. 2014), drugs

(Cui et al. 2013), and biomaterials (Li et al. 2010) in the treatment of osteoporosis (Ray et al. 2008).

The current results showed that estrogen deficiency does not decrease the osteogenic potential of rat ADSCs. These findings underline the higher potentiality of OVX cells to differentiate toward mature lineages, also reflected by their higher expression and protein production of some bone-specific markers, as discussed subsequently.

Regarding surface antigen expression, both OVX and SHAM ADSCs showed almost the same high expression of mesenchymal markers (CD44, CD90, CD105, CD73) and low expression of hematopoietic and endothelial ones (CD34, CD31, CD45). This expression profile was obtained also by literature works in aged (Pachon-Pena et al. 2010; de Girolamo et al. 2009; Khan et al. 2009) women, but only Mirsaidi et al. (2012) observed the same CD expression in healthy and osteoporotic mice. The clonogenicity was higher in ADSCs derived from OVX than that from SHAM rats, whereas the ratio between viability and cell count was affected only by the culture medium and the experimental time

Fig. 4 Boxplots of osteogenic and adipogenic marker gene expression ($n=3$ replicates): **a** *Esr1*; **b** *Runx2*; **c** *Bmp2*; **d** *Alpl*. **a** Interaction of “cell origin” and “culture medium” factors for *Esr1* expression ($F=10.94$, $p=0.005$); Sidak test: $**p<0.005$, SHAM OM versus OVX OM; $^ap<0.005$, SHAM OM versus SHAM CM; **b** interaction of “culture medium” and “experimental time” factors on *Runx2* expression ($F=13.72$, $p<0.005$); Sidak test:

$***p<0.0005$, OM versus CM at 1 week; $**p<0.005$, OM versus CM at 2 weeks; $^ap<0.005$, 1 week versus 2 weeks for OM; **c** interaction of “culture medium” and “experimental time” factors on *Bmp2* expression ($F=11.69$, $p<0.005$); Sidak test: $***p<0.0005$, OM versus CM at both experimental times; $^ap<0.0005$, 1 week versus 2 weeks for OM; **d** effect of “culture medium” factor on *Alpl* expression ($F=45.08$, $***p<0.0005$)

factors, independent of estrogen deficiency, except for SHAM OM that showed higher values than those of OVX OM at 1 week. This can be explained by the higher cell number of OVX OM in comparison to that of SHAM OM, which reduced cell viability. The same explanation can be applied to the significantly increased viability/cell count observed in the OM conditions in comparison to those of CM at 2 weeks and to the significant reduction in time for all the groups, with the exception of OVX OM. At 2 weeks, the differentiating cells in OM proliferated less than the undifferentiated ones in CM.

The osteogenesis of MSC toward mature OBs is complex and driven by a stepwise succession of key transcription factors, gene expression, and protein synthesis that are dependent on the microenvironment (Hoshiba et al. 2012). In this study, the osteogenic development of ADSCs was evaluated by specific bone marker gene expression and protein production. It was observed that some of these genes or proteins were significantly differently expressed or synthesized by OVX ADSCs (*Colla1*, *Bglap*, *Spp1*, ALP activity, total

COLL, OC), whereas others seemed to be unaffected by estrogen deficiency (*Alpl*, *Runx2*, *Bmp2*, OPG/RANKL), but strongly conditioned by the presence of osteogenic medium. As expected, *Alpl*, *Runx2*, *Bmp2* (the earlier markers of osteoblast commitment), and OPG/RANKL results underlined that the osteogenic microenvironment seems to start ADSC osteogenesis more than the presence of estrogen.

A dual behavior pattern was observed between *Colla1* gene expression and total COLL protein production, underlining that the reduction of the major component of type I collagen expression in OVX cells does not influence the total collagen production. *Bglap* and *Spp1* gene expression and OC synthesis, which were higher in OVX cells and related to a late osteogenic differentiation phase, showed that ADSCs were able to proceed in this process without alteration of their commitment and future matrix mineralization. Similar to the present results, Chen et al. also found an increase in *Bglap* expression in osteoporotic patients with respect to healthy young ones, but they observed a concomitant decrease in *Alpl* gene expression (Chen et al. 2012).

Fig. 5 Boxplots of osteogenic and adipogenic marker gene expression ($n=3$ replicates): **a** *Lpl*; **b** *Tgfb1*; **c** *Vegfa*; **d** *Esr2*. Effect of “culture medium” factor on *Lpl* ($F=4.28$, $*p<0.05$); *Tgfb1*

($F=8.15$, $*p<0.05$); *Vegfa* ($F=48.26$, $***p<0.0005$); and *Esr2* ($F=15.37$, $**p<0.005$) expressions

Fig. 6 Boxplots of ALP activity and protein releases (ELISA), expressed as ratio with total protein ($n=3$ replicates): **a** ALP activity, **b** OC, **c** total COLL, and **d** VEGF protein releases. **a**, **b** Interaction of “cell origin,” “culture medium,” and “experimental time” for ALP activity ($F=7.95$, $p<0.05$) and OC ($F=13.16$, $p<0.005$) release; Sidak test: $*p<0.05$; $**p<0.005$;

$***p<0.0005$; **c** interaction of “cell origin” for total COLL release ($F=4.21$, $*p<0.05$); **d** interaction of “culture medium” and “cell origin” for VEGF release ($F=8.98$, $p<0.05$); Sidak test: $^{\circ\circ\circ}p<0.0005$, OVX CM versus SHAM CM; $***p<0.0005$, SHAM CM versus SHAM OM and OVX CM versus OVX OM

Fig. 7 Boxplots of protein release (ELISA), expressed as ratio with total protein ($n=3$ replicates): **a** OPG/RANKL, **b** IL-1 β , **c** IL-6, and **d** TGF- β 1. **a** Interaction of “culture medium” and “experimental time” factors on OPG/RANKL ratio ($F=9.96$, $p<0.005$);

Sidak test: $*p<0.05$, OM versus CM at 1 week; $***p<0.0005$, OM versus CM at 2 weeks; $^{\circ}p<0.005$, 1 week versus 2 weeks for CM; **b**, **c** effect of “culture medium” factor on IL-1 β ($F=7.41$, $*p<0.05$) and IL-6 ($F=27.52$, $***p<0.0005$) releases

Concerning *Alpl* gene expression and ALP activity, the osteogenic culture medium was responsible for the observed higher expression of *Alpl*, whereas estrogen deficiency was responsible for the higher activity of ALP, observed in OVX cells at 2 weeks in comparison to 1 week, when ALP activity was more pronounced in SHAM cells. This probably occurred because ADSCs from estrogen deficiency exhibit a delay in ALP activity in comparison to healthy conditions.

Finally, the matrix calcium deposition that corresponds to the final phase of osteogenic differentiation was higher in OVX than that of SHAM cells, in contrast to Mirsaidi et al. (2012), who observed a lateness in

mineralization in osteoporotic mice in comparison to age-matched healthy ones (Mirsaidi et al. 2012). This phenomenon probably reflects the higher OC and total COLL production, *Bglap* and *Spp1* gene expression observed in OVX ADSCs, especially under osteogenic stimuli, provided by OM.

Regarding other gene expressions, it was found that *Pparg* and *Esr1* were expressed at the highest and lowest levels in OVX ADSCs at 2 weeks, respectively. *Pparg* is the master regulator of adipogenesis and its high expression in MSC culture suggests that they are prone toward an adipogenic lineage (Wan 2010). Moreover, after a comparison between ADSCs and

Fig. 8 Boxplots of matrix calcium deposition after 21 days of culture expressed as OD_{570} . Interaction of “cell origin” factor ($F=9.92$, $*p<0.05$)

BMSCs, Kim et al. (2010) observed that ADSCs are more directed toward an adipogenic lineage than BMSCs (Kim et al. 2010). However, the observed higher *Pparg* expression in OVX ADSCs seemed surprising, because all data indicated a clear osteogenic differentiation process of these cells. Since it is well known that *Esr1* negatively influences *Pparg* activity (Bonofiglio et al. 2005) and adipogenesis and osteoblastogenesis (Gimble and Nuttall 2012) have an inverse relationship, it might be hypothesized that (a) the low expression of *Esr1* might have induced the overexpression of *Pparg* and (b) a subpopulation of ADSCs committed toward the adipogenic lineage was simultaneously present after 2 weeks of osteogenic stimulation. The other adipogenic gene markers (*Adipoq* and *Lpl*) were not influenced by estrogen deficiency and the former was not detected, whereas the latter was low under osteogenic stimuli.

To complete the description of what happens during the osteogenic process, GFs (TGF- β 1 and VEGF) and pro-inflammatory osteolytic cytokines (IL-1 β and IL-6), involved in osteoclast development (Moon et al. 2013), were also evaluated. *Vegfa* and *Tgfb1* gene expression and the production of IL-1 β and IL-6 were unaffected by estrogen deficiency, but were affected by osteogenic medium. Lower *Vegfa* expression and IL-1 β and IL-6 release values and higher *Tgfb1* expression were found with OM. The low levels in VEGF synthesis observed in OM-conditioned cultures are in agreement with the authors' previous study on healthy human ADSCs (*unpublished data*). These results underlined that both OVX and SHAM ADSCs differentiate toward the osteogenic lineage because VEGF is a typical angiogenic molecule that usually maintains the MSC undifferentiated status over the differentiated one (Hoch et al. 2012).

Since it is difficult to distinguish the effects of estrogen deficiency from those of the aging process itself, this study was focused on ADSCs from healthy and ovariectomized rats and did not compare the behavior of cells from donors of different ages. In the future, other in vitro and in vivo studies with human cells will be mandatory to confirm the current results, to further evaluate the relationship between estrogen deficiency and the osteogenic potential of ADSCs also in association with appropriate scaffolds and to test the ADSC therapeutic potential in the orthopedic field.

Conclusion

This study not only improves the knowledge on ADSCs in estrogen deficiency, but it also underlines the osteogenic potential of ADSCs taken from osteoporotic donors. It seems that the ADSC clonogenicity, gene expression, and subsequent protein production of some important osteogenic markers are unaffected by estrogen deficiency. Some of these markers were higher in OVX ADSCs, including matrix mineralization, and the production of pro-inflammatory cytokines (IL-1 β and IL-6) is similar to that of healthy cells. These results lay the foundation for the use of autologous ADSCs for bone pathologies in osteoporotic women, even if other in vitro and in vivo studies are needed. Finally, since the adipogenic *Pparg* gene seems to be concurrently more expressed by OVX cells, further analyses to shed light on this question are required.

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