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ORIGINAL RESEARCH

Proof of Concept on Functionality Improvement of Mesenchymal Stem-Cells, in Postmenopausal Osteoporotic Women Treated with Teriparatide (PTH1-34), After Suffering Atypical Fractures

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Abstract

Osteoporosis long-term treatment with nitrogen-containing bisphosphonates, has been associated with uncommon adverse effects, as atypical femoral fractures (AFF). Thus, treatment with teriparatide (TPTD; fragment of human parathyroid hormone; PTH_{1-34}) has been proposed for such patients. Besides its anabolizing effect on bone, TPTD may affect stem-cell mobilization and expansion. Bone marrow mononuclear cells (BMMNC) were isolated from five women that had suffered AFF associated to bisphosphonate treatment, before and after 6 months of TPTD therapy. The presence of mesenchymal stromal cells (CD73, CD90 and CD105 positive cells), gene expression of *NANOG, SOX2* and *OCT4*, proliferation, senescence and capacity to differentiate into osteoblasts and adipocytes were analyzed. After TPTD treatment, BMMNC positive cells for CD73, CD90 and CD105 increased from 6.5 to 37.5% ($p < 0.05$); *NANOG, SOX2* and *OCT4* were upregulated, being statistically significant for *NANOG* ($p < 0.05$), and cells increased proliferative capacity more than 50% at day 7 ($p < 0.05$). Senescence was reduced 2.5-fold ($p < 0.05$), increasing differentiation capacity into osteoblasts and adipocytes, with more than twice mineralization capacity of extracellular matrix or fat-droplet formation ($p < 0.05$), respectively. Results show that TPTD treatment caused BMMNC "rejuvenation", increasing the number of cells in a more undifferentiated stage, with higher differentiation potency. This effect may favor TPTD anabolic action on bone in such patients with AFF, increasing osteoblast precursor cells. Such response could also arise in other osteoporotic patients treated with TPTD, without previous AFF. Furthermore, our data suggest that TPTD effect on stromal cells may have clinical implications for bone-regenerative medicine. Further studies may deepen on this potential.

Keywords Osteoporosis · Regenerative medicine · Parathormone · Cellular differentiation · Atypical femoral fractures

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Introduction

Osteoporosis is a systemic skeletal-disorder characterized by bone loss, leading to increased risk of fractures [1]. Osteoporosis-related fragility fractures contribute to morbidity and mortality associated with aging, being the cause of a serious public health problem [2]. Nitrogen-containing bisphosphonates (N-BP) have proven efficacy for preventing bone loss and fractures in women with postmenopausal osteoporosis, and women and men with glucocorticoid-induced osteoporosis [3]. In fact, N-BP are the first-line and most commonly prescribed drugs worldwide for the treatment of osteoporosis, in view of their proven efficacy, safety and cost effectiveness [4].

Nevertheless, a number of uncommon adverse effects, potentially associated with prolonged use of N-BP, have been described, including atypical femoral fractures (AFF) [5]. AFF may be devastating, leading to reduction in mobility and high rates of re-operation, because of cumbersome and delayed fracture healing [6]. Given the low absolute incidence of AFF, there may be underlying rare genetic causes that might interact with N-BP, triggering occurrence of these fractures [7].

In 2014, the task force report from the American Society for Bone and Mineral Research (ASBMR) reviewed published clinical data, suggesting treatment recommendations for patients with AFF. Thus, they should be managed by surgical stabilization, N-BP treatment should cease and patients should receive daily calcium and vitamin D supplementation. Anabolic agents, such as teriparatide (TPTD; human PTH_{1-34}) and abaloparatide, have been considered as a potential treatment option for such patients [6], even though available evidence may not be convincing [8].

Teriparatide has been used to accelerate healing of different types of fractured bones, including AFF [9, 10]. Indeed, such peptide has a bone-anabolic effect related to enhanced osteoblast viability and differentiation capacity [11]. This includes conversion of lining cells into active osteoblasts [12]. The latter cells are in charge of bone formation, being derived from mesenchymal stromal cells (MSC) of bone marrow. Such cells can differentiate into different cellular types, including osteoblasts, adipocytes, chondrocytes or myocytes. Therefore, they are considered as an important biological source for tissue regeneration [13].

However, proliferation and differentiation capability of MSC decline with age [14]. Aging may also cause an unwanted shift of MSC commitment into adipocyte instead of osteoblast lineage in bone [15], resulting in an increase in bone marrow adipogenesis [16]. Interestingly, intermittent TPTD administration increases differentiation of MSC towards osteoblastic lineage. It also decreases senescence and apoptosis of mature osteoblasts [11], inhibiting its differentiation into adipocytes [17]. Moreover, an increase in circulating osteoblastic precursor cells has been demonstrated in postmenopausal women treated with teriparatide [18].

Recently, it has been reported that PTH has a positive effect on stem cells in general, with capacity to mobilize MSC and hematopoietic stem cells (HSC) from bone marrow. Such cells may be involved in different regenerative processes [19]. In particular, PTH increases MSC proliferation, reducing senescence and apoptosis [20]. In this context, several facts should be taken into account: (i) Need to deepen knowledge about TPTD effect on patients with AFF [21]; (ii) TPTD is currently recommended as rescue antiosteoporotic agent for AFF treatment; and (iii) TPTD potential effects on stem cells. Therefore, the main objective of this study was to ascertain in vivo the effect of treatment with such peptide, related to quality and differentiation capacity of MSC from bone marrow of patients with AFF. Therefore, MSC were isolated from bone marrow (donated by patients with atypical fracture treated with TPTD), before and after 6-month treatment, as described below. That should allow to better understand the positive effect of TPTD on AFF healing, as well as other bone pathologies, with potential implications for healthcare systems worldwide, mostly on aging populations.

Methods

Patients and Isolation of Mononuclear Cells from Bone Marrow

Patients were recruited, after written consent, from "Unidad de Metabolismo Mineral del Servicio de Endocrinología" at "Hospital Universitario Reina Sofía" in Córdoba (Spain). They were five women of ages 65, 74, 75 (two) and 77 years, with atypical femoral fracture, treated with bisphosphonates for more than 5 years. Diagnoses were carried out according to the criteria of ASBMR task force [6]. Patients signed informed consent, in accordance with regulations of the Clinical Research Ethics Committee of Parc de Salut Mar, which approved the study. This was carried out in accordance with terms of the Declaration of Helsinki.

All patients had normal serum levels of vitamin D (25-hydroxyvitamin D or calcifediol) and adequate inhibition of bone remodeling markers [22]. One of them had a bilateral atypical femur fracture. Bone quality of all patients, as measured by bone microindentation, was deeply deteriorated, as we have previously reported [23]. According to recommendations of the ASBMR task force, treatment with N-BP was withdrawn. Instead, they received

0.266 mg calcifediol (Hidroferol)/month from Faes Farma (Madrid, Spain) [24] and 20 mg TPTD/day from Forteo Eli Lilly&Company (Indianapolis, IN, USA), via subcutaneous injections (SCI), for 6 months.

Four milliliter of bone marrow aspirates were obtained from patients, both before and after 6 months of treatment. Bone marrow mononuclear cells (BMMNC), with capacity to both adhere to plastic flasks and proliferate, were isolated, as we have previously described [17]. In short, cells were grown in Minimum Essential-Medium Alpha (MEMα) from Cambrex Bio Science—Lonza (Basel, Switzerland), supplemented with 15% fetal bovine serum (FBS) from Gibco—Life Technologies—Thermo Fisher Scientific (Waltham, MA, USA), 2 mM UltraGlutamine (Cambrex Bio Science—Lonza), 100 U ampicillin, 0.1 mg streptomycin/ml and 1 ng basic fibroblast growth factor (bFGF)/ml from Sigma-Aldrich (St Louis, MO, USA). Cells from first passage were cryopreserved until used. All pre- and post-treatment BMMNC were cryopreserved and maintained in the same way, to equalize experimental conditions. BMMNC of healthy donors from Hematology Service under Bone Marrow Transplantation Program at "Reina Sofía University Hospital" [17] were used as controls.

Flow Cytometry

Cryopreserved BMMNC were thawed and expanded in 75 cm^2 culture flasks from Nalgene-Nunc—Thermo Fisher Scientific, in MEMα plus 15% FBS medium. Cultures with 70–80% confluence were detached and incubated with human monoclonal antibodies (Ab): anti-CD34, anti-CD73, anti-CD90 and anti-CD105, from Becton Dickinson (BD) Biosciences (Franklin Lakes, NJ, USA) for 30 min, and then washed with phosphate-buffered saline (PBS) solution. Cells were resuspended in 500 µl CellFIX solution, and 10^4 events were collected and analyzed with a FACSCalibur flow cytometer (all from the same manufacturer).

Proliferation and Senescence Quantification

BMMNC were seeded in P12 plates from Nalgene-Nunc—Thermo Fisher Scientific at a density of 500 cells/cm^2, in MEMα plus 15% FBS and one ng bFGF/ml. Cells were detached at days 4 and 7. Proliferation was quantified counting total cells with hemocytometer. Senescence Cells Histochemical Staining Kit from Sigma-Aldrich was used to quantify senescence. Proportions of cells positive for beta-galactosidase, among total, were determined by counting both in at least nine different and randomly chosen well fields. Micrographs were taken with an Eclipse Ti inverted microscope from Nikon (Shinagawa, Tokyo, Japan) and analyzed by ImageJ software from the National Institutes of Health (NIH; Bethesda, MD, USA).

Gene-Expression Quantification

Total mRNA was isolated using High Pure RNA Isolation Kit from Roche Applied Science (Penzberg, Germany), following the manufacturers' directions. RNA integrity was determined by agarose gel electrophoresis (AGE). Nucleic acids were quantified with a ND 1000 spectrophotometer from NanoDrop—Thermo Fisher Scientific 1 µg of total RNA was retrotranscribed using iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA).

Quantitative real-time polymerase chain reaction (QRT-PCR) was carried out in a LightCycler 480 from Roche

Table 1 Primer sequences and amplicon sizes

Gene	Forward and reverse primer sequence (5′ → 3′)	Product size (bp)
NANOG homeobox *(NANOG)*	GTGATTTGTGGGCCTGAAGAA AAGTGGGTTGTTTGCCTTTG	157
Sex-determining region Y (SRY) box 2 (*SOX2*)	AGCCCATGCACCGCTACGAC CTGGCCTCGGACTTGACCACC	163
Octamer-binding transcription factor 4 (*OCT4*)	CTGCAGCAGATCAGCCACATCG CCCAGCAGCCTCAAAATCCTCTCG	138
Runt-related transcription factor 2 (*RUNX2*)	TGGTTAATCTCCGCAGGTCAC ACTGTGCTGAAGAGGCTGTTTG	143
Integrin-binding sialoprotein (*BSP*)	AGGGCAGTAGTGACTCATCCG CGTCCTCTCCATAGCCCAGTGTTG	171
Lipoprotein lipase (*LPL*)	AAGAAGCAGCAAAATGTACCTGAAG CCTGATTGGTATGGGTTTCACTC	113
Fatty acid-binding protein 4 (*FABP4*)	TCAGTGTGAATGGGGATGTGAT TCTGCACATGTACCAGGACACC	162
Polymerase (RNA; DNA directed) II polypeptide A *(POLR2A)*	TTTTGGTGACGACTTGAACTGC CCATCTTGTCCACCACCTCTTC	125
Ribosomal protein L13a (*RPL13A*)	CTCTGGACCGTCTCAAGGTGT CTGGTACTTCCAGCCAACCTC	158

Applied Science. Each reaction contained one µl cDNA, 10 pmol of each primer pair (Table 1) and LightCycler 480 SYBR Green I Master mix from the same manufacturer. PCR amplification profile included one cycle at 95 °C for 10 min (DNA denaturation and DNA-polymerase activation) and 45 amplification cycles: 94 °C for 10 s (DNA denaturation), 65 °C for 15 s (primer annealing) and 72 °C for 15 s (primer extension). Results were analyzed with LightCycler 1.5.0 software from the same manufacturer, using the second derivative method for cycle threshold (Ct) calculations. Polymerase (RNA; DNA directed) II polypeptide A (*RPL13A*) and ribosomal protein L13a (*POLR2A*) were used as housekeeping genes.

BMMNC Differentiation into Osteoblasts or Adipocytes

Cells were induced to differentiate into osteoblasts, supplementing culture media (without bFGF) with 10^{-8} M dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerolphosphate. On the other hand, differentiation into adipocytes was accomplished supplementing media with $5{\cdot}10^{-7}$ M dexamethasone, 0.5 mM isobutylmethylxanthine and 50 µM indomethacin. All reagents were from Sigma-Aldrich. Cultures induced into adipocytes or osteoblasts were grown for 17 or 21 days, respectively.

Mineralization in cultures induced to differentiate into osteoblasts was evaluated by alizarin-red staining. Thus, cultures were fixed with 3.7% formaldehyde for 10 min and stained for 45 min, using a mixture of 10 ml solution made of 1% alizarin red (w/v, in distilled water) with 1 ml of 1% ammonium hydroxide. All reagents were from Sigma–Aldrich. Then, wells were washed with distilled water, dried and visualized under the microscope. Alizarin-red deposits were eluted with 10% acetic acid and neutralized with 10% ammonium hydroxide. Eluates were quantified by absorbance at 405 nm, using a PowerWave XS microplate spectrophotometer from BioTek Instruments (Winooski, VT, USA), as described elsewhere [25].

Fat-droplet formation in cultures induced to differentiate into adipocytes was evaluated by oil-red staining. Thus, cultures were fixed with 3.7% formaldehyde for 20 min and stained with a solution made by mixing 8.2 ml of 0.3% oil red (w/v, in isopropanol) with 6.8 ml of distilled water. After 15 to 20 min of incubation, cells were washed with distilled water, stained with hematoxylin and visualized by optical microscopy. Stains were eluted with isopropanol at room temperature for 10 min, and quantified at 510 nm by spectrophotometry. Values of oil-red staining were normalized, taking into account total number of cells/well. After oil-red staining, cells were further stained with 0.1% crystal violet in 10% ethanol for 20 min. Then, after five washes with distilled water, stains were eluted with 10% acetic acid for 20 min. Eluates were quantified by absorbance at 590 nm by spectrophotometry. Fat-droplet values in cell cultures were expressed as A510 nm/A590 nm.

Statistics

Since sample size was lower than 30, medians were compared using a nonparametric statistical test. Thus, different parameters were compared on BMMNC isolated from patients before and after treatment. Being related samples, nonparametric Wilcoxon signed-rank test was used. It was applied using Statgraphics Centurion software XV from Statpoint Technologies (The Plains, VA, USA). Results were considered statistically significant when $p < 0.05$.

Results

Teriparatide Treatment Increased MSC Yield from Bone Marrows of AFF Patients

BMMNC from patients with AFF were subjected to culture expansion before and after TPTD treatment. Flow cytometry was used in cultures, to identify triple-positive cells for MSC biochemical markers (CD73, CD90 and CD105). Results showed a significant increase of CD73 + CD90 + CD105 + cells after TPTD treatment ($p < 0.05$) (Fig. 1a). This increase was between 6.5 and 37%, depending on the patient.

Additionally, expression of genes encoding transcription factors involved in immortality of embryonic stem-cells was quantified. They included: (i) Homeobox protein NANOG (*NANOG*; named after the "Tír na nÓg" Irish immortality legend); (ii) Sex-determining region Y (SRY) box 2 (*SOX2*); and (iii) Octamer-binding transcription factor 4 (*OCT4*), also known as "Pituitary-specific Pit1—Octamer Oct1 and Oct2—neural Unc-86 (POU) domain, class 5, homeodomain transcription factor 1" (*POU5F1*). Gene expression increased, in general, in BMMNC post-TPTD treatment, being statistically significant for *NANOG* gene ($p < 0.05$) (Fig. 1b).

Teriparatide Effects on BMMNC Proliferation and Senescence

BMMNC proliferation, after 4 and 7 days in culture, was significantly higher in cells obtained in post-TPTD treatment of AFF patients (Fig. 2a). Such proliferation increase was 38.2 and 51.7% at days 4 and 7, respectively ($p < 0.05$). On the other hand, BMMNC obtained before and after teriparatide treatment were stained for beta-galactosidase activity at day seven to study senescence. BMMNC obtained after TPTD

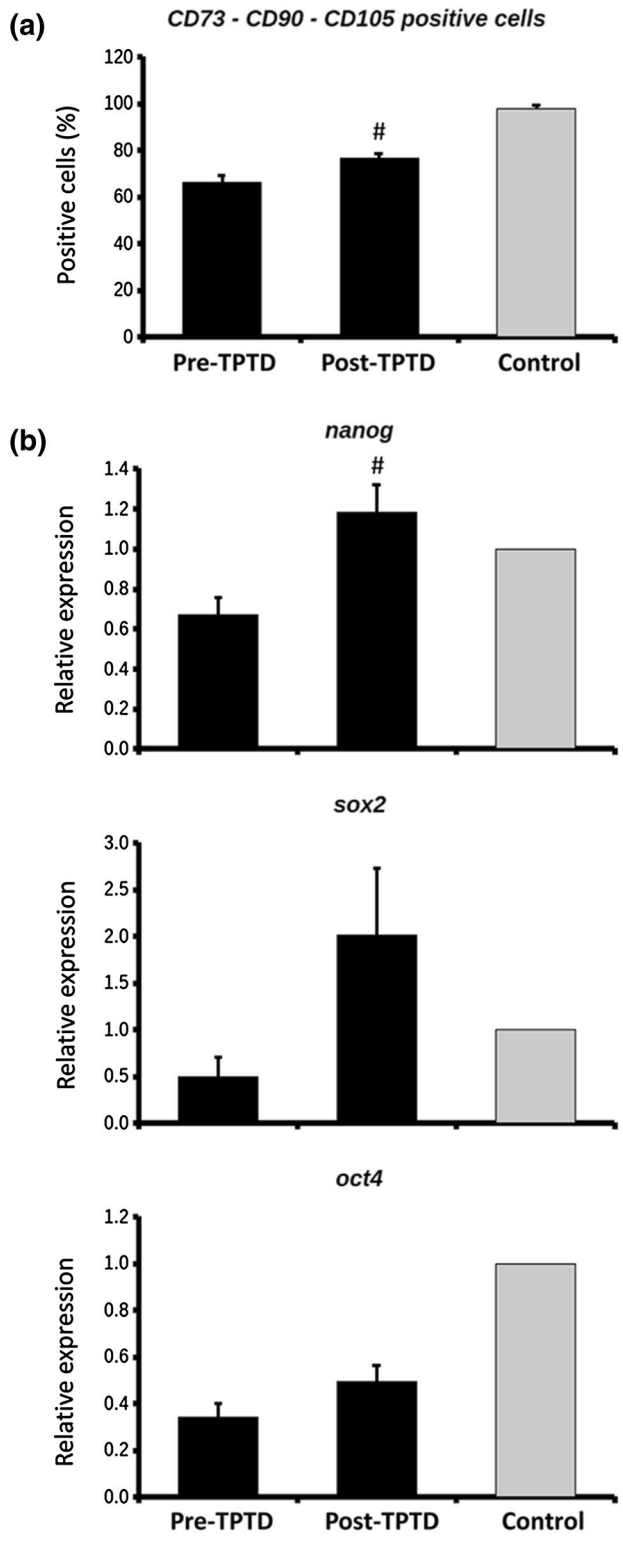

Fig. 1 Biochemical and molecular markers. MSC from BMMNC were obtained from patients with AFF before (Pre-TPTD) and after (Post-TPTD) teriparatide treatment. **a** Triple-positive cells for CD73, CD90 and CD105 were identified by flow cytometry. **b** Expression of *NANOG, SOX2* and *OCT4* genes. #: Significance level versus Pre-TPTD: $p < 0.05$

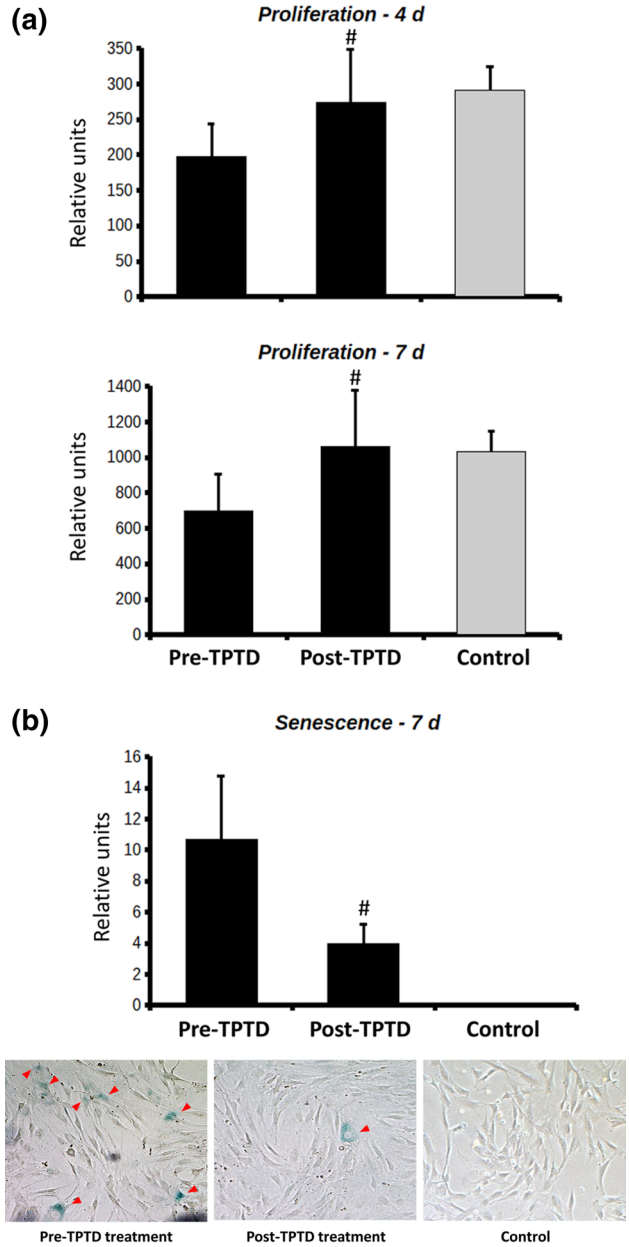

Fig. 2 Proliferation and senescence. **a** Proliferation comparisons at days 4 and 7 of cell culture. **b** Cellular senescence determined by measuring beta-galactosidase activity. Inverted-microscopy pictures are shown below, after staining for beta-galactosidase activity. Red arrowheads point to cells positive for such enzymatic activity. #: Significance level: see legend of Fig. 1

treatment showed a significantly ($p < 0.05$) lower proportion of senescent cells (Fig. 2b).

Teriparatide Treatment Improved Differentiation Capacity of BMMNC from AFF Patients

MSC differentiation into adipocytes or osteoblasts was analyzed in BMMNC obtained from AFF patients, before and

after TPTD treatment. BMMNC mineralization of cells from all patients was heterogeneous after osteoblastic induction, albeit with significant increase ($p < 0.05$) after its quantification (Fig. 3a). Interestingly, mineralization images showed that patients 3 and 5 improved from no mineralization, in pre-TPTD treatment, to a light mineralization after post-treatment. Such results represent a remarkable improvement in cell capacity to differentiate into osteoblasts. (Fig. 3a).

Gene expression was studied in cultures induced to differentiate into osteoblasts at day 17 after induction. That included runt-related transcription factor 2 (*RUNX2*) and integrin-binding sialoprotein (*IBSP*) osteoblastic genes. They encode a transcription factor that induces osteoblastogenesis, as well as an extracellular matrix protein involved in bone mineralization, respectively. Expression of both genes significantly increased ($p < 0.05$) in BMMNC after TPTD treatment (Fig. 3b).

On the other hand, fat-droplet formation was quantified with oil-red staining. BMMNC differentiation into adipocytes showed a significant increase of more than three-fold ($p < 0.05$), at day 17 after TPTD treatment (Fig. 4a). It is also relevant to highlight morphological differences between cells before and after TPTD treatment, as revealed by micrographs of cultures induced to differentiate into adipocytes (Fig. 4). On the other hand, genes encoding lipoprotein (*LPL*) and fatty-acid-binding protein 4 (*FABP4*) were used as late molecular markers for adipogenesis. Thus, their expression was quantified for cells differentiating into adipocytes at day 10 after induction. Both genes were significantly upregulated ($p < 0.05$), in BMMNC cultures derived from post-TPTD treatment (Fig. 4b).

Discussion

Interestingly, treatment with TPTD subcutaneously administered every day during 6 months improved capacity to produce stem cells with higher functionality, as seen in the bone marrow of patients with atypical fractures. Thus, patients improved their bone health after 6 months of TPTD treatment. Besides, BMMNC obtained from them showed an increased proportion of positives for CD73, CD90 and CD105. Presence of such biochemical markers, together with adherence capacity in culture, proliferation and differentiation, are the minimum criteria defining MSC, after the Mesenchymal and Tissue Stem-Cell Committee of the International Society for Cellular Therapy [26].

Thus, increase of triple-positive ones for CD73, CD90 and CD105 indicated that TPTD favored expansion of such populations in bone marrow, after treatment in such patients. Besides, increase of MSC populations was in parallel to upregulation of *NANOG, SOX2* and *OCT4* genes in BMMNC, after TPTD treatment. Such genes are mainly

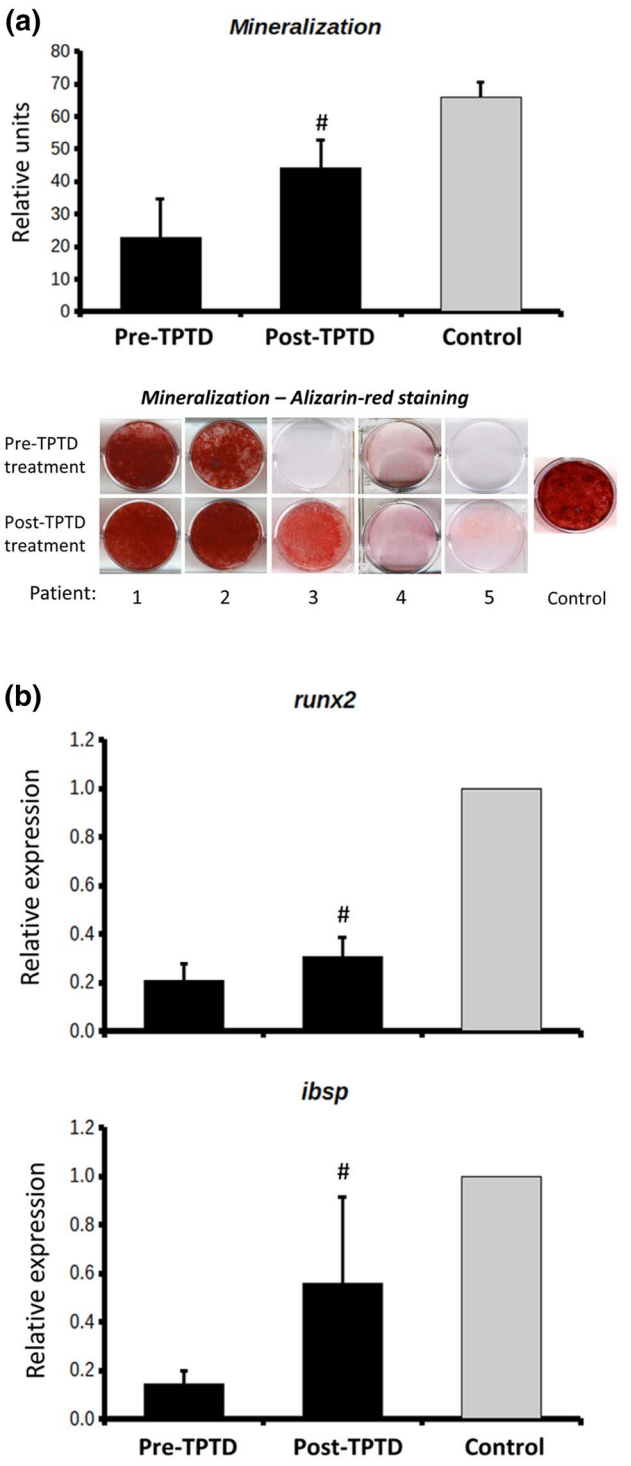

Fig. 3 Osteogenic differentiation. **a** Mineralization measurements by alizarin-red staining, elution and spectrophotometric quantification in BMMNC cultures, at day 21 after induction of osteoblastic differentiation. Micrographs show stained cultures before elution, representative of each patient. **b** Expression of *RUNX2* and *BSP* osteoblastic genes at day 17 after osteoblastic differentiation. #: Significance level: see legend of Fig. 1

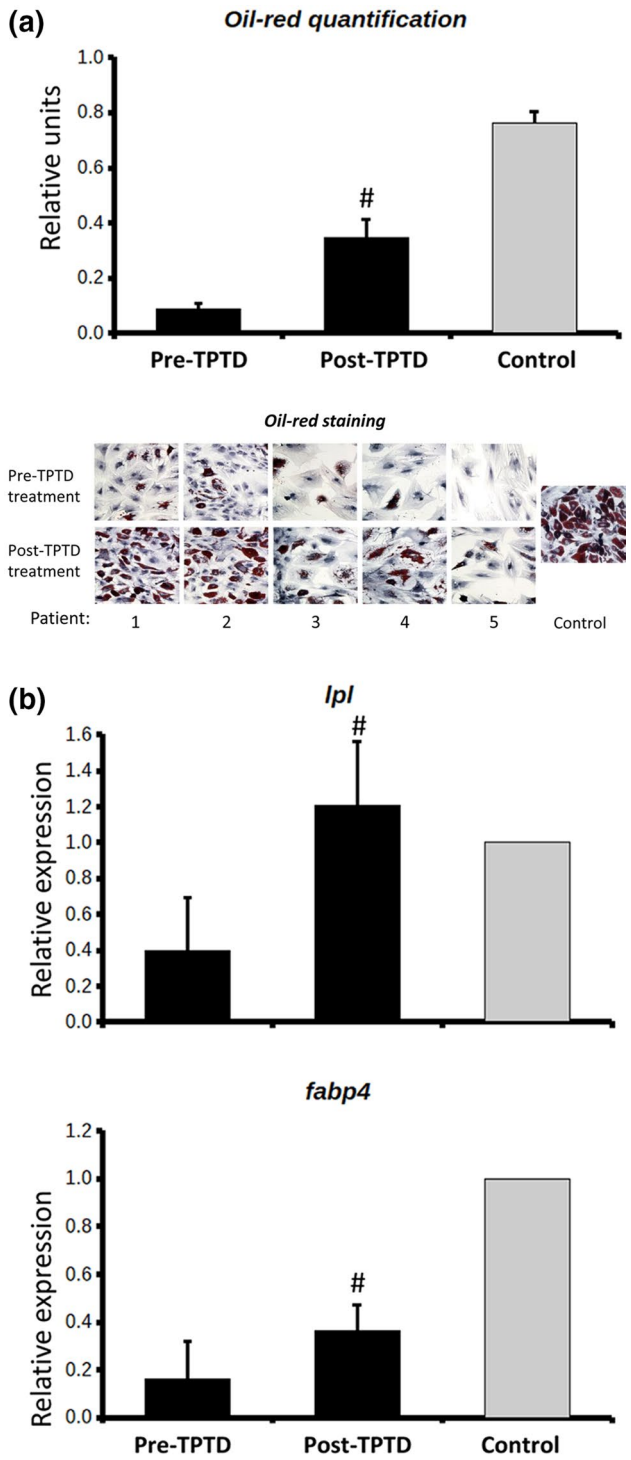

Fig. 4 Adipogenic differentiation. **a** Quantification of fat-droplet formation by oil-red staining, elution and spectrophotometric quantification in BMMNC cultures, at day 17 after induction of adipogenic differentiation. Micrographs show stained cultures with oil red and hematoxylin before elution, representative of each patient. **b** Expression of *LPL* and *FABP4* adipogenic genes at day 10 after adipogenic differentiation. #: Significance level: see legend of Fig. 1

expressed in embryonic stem cells. They encode transcription factors regulating cellular cycle, and their expression is associated to highly proliferative cells and pluripotency maintenance. They are considered additional molecular markers in MSC from adult tissues. Indeed, such genes are repressed after differentiation of totipotent or pluripotent cells [27]. Furthermore, regulation of their gene expression is carried out in a coordinated reciprocal way, maintaining cellular pluripotency [28].

Not surprisingly, *NANOG* overexpression in mouse MSC protected against cellular senescence, maintaining cells in an undifferentiated proliferative stage. Its silencing induced differentiation into osteoblasts, although, later on, during bone remodeling, there was bone loss and increase in adipose tissue [29]. It has also been described that *NANOG* expression can recover proliferative and mitogenic-differentiation capacities in MSC from adults, where they may be otherwise reduced. Therefore, *NANOG* may reverse the effects of organismal aging, "rejuvenating" such cells [30]. On the other hand, *OCT4* and *SOX2* overexpression in MSC derived from human adipose tissue also increased their proliferation and capacity, to differentiate into osteoblasts and adipocytes [31].

Likewise, it has been demonstrated that *SOX2* is essential for proliferation and differentiation of MSC (derived from bone marrow) into osteoblasts [32]. *NANOG, SOX2* and *OCT4* were upregulated in BMMNC from studied patients, after TPTD treatment. That can be correlated to observed increase in proliferation and differentiation capacities of such cultures. On the other hand, there is no direct evidence indicating that PTH could regulate expression of such genes. Yet, silencing the gene encoding parathyroid hormone-related protein (PTHrP), in preimplantation during embryonic development in mouse, produced downregulation of *NANOG* and *OCT4* [33]. Interestingly, 1–36 N-terminal fragment of PTHrP binds the same receptor as PTH, known as PTH1R [34]. Therefore, activation of such receptor could modulate expression of related genes.

Not surprisingly, it has been found that proliferation reduction and senescence increase of MSC are correlated with age of source individuals [14, 35]. Patients analyzed in the present work were between 65 and 77 years old. Thus, as expected, BMMNC isolated from them before TPTD treatment had very low proliferation capacity, quickly becoming senescent. These results are in agreement with the ones obtained by other authors in different animals, including humans, comparing proliferation and senescence of MSC from individuals of different ages [14, 36].

MSC used in the present work showed extremely low expansion capacities. Yet, interestingly, TPTD treatment increased their proliferation capacity, reducing senescence of cells obtained from all patients. These data, together with the increase of MSC and upregulation of *NANOG, SOX2*

and *OCT4* genes, indicate that TPTD produced a rejuvenating effect on MSC isolated from studied patients. Besides, such cells also exhibited an increase in their differentiation capacity. All those results represent positive aspects, effectively improving regenerative functionality of MSC from such women, after teriparatide treatment.

In relation to pluripotency, BMMNC were inefficient to differentiate into osteoblasts or adipocytes before teriparatide treatment. That is in agreement with the low proportion of MSC observed. As indicated, TPTD treatment increased the number of such cells, but also—and most significantly—produced a higher potential to differentiate into different cellular types. For instance, morphological differences between cells obtained before and after TPTD treatment were evident in relation to differentiation into adipocytes. Thus, the former showed larger cells, with expanded cytoplasm, being characteristic of senescent cells. On the contrary, the latter included smaller cells, with higher density, mainly for patients 1 and 2 (Fig. 4a). Interestingly, they were also the ones more efficiently differentiated. That suggests a positive correlation between increased BMMNC viability due to TPTD treatment with increased differentiation capacity.

The low differentiation capacity of BMMNC from some patients, before TPTD treatment, could somehow be positively correlated to their age, as shown above for proliferative capacity. Yet, some authors have not found differences in differentiation capacity between MSC from individuals with different ages [37, 38]. Nevertheless, others have described MSC differentiation-capacity loss with aging [14, 35]. Results of the present work are in agreement with the latter. Yet, the reduced differentiation capacity of MSC, from studied patients, suggests the involvement of other physiological or genetic factors, besides age, which may be partially corrected with TPTD treatment. That could explain the higher probability of atypical fractures in such women, in circumstances of low bone remodeling, induced by long-term bisphosphonate treatments [6].

Teriparatide treatment showed a positive effect on MSC populations of patients with atypical fractures. That could also explain healing improvement of such fractures and bone-mass increase after TPTD injections [9, 11]. It has been described that PTH anabolic role on bone is due, to a large extent, on its positive effects on osteoblast maturation and survival [11]. Yet, PTH might have also other important effects on bone marrow, favoring MSC expansion, effectively increasing the number of available osteoprogenitors. It should be also taken into account that: (i) MSC tend to differentiate into adipocytes instead of osteoblasts with aging, reducing the number of the latter [39]; and (ii) PTH inhibits MSC differentiation into adipocytes [17, 40]. Therefore, TPTD treatment might be involved in at least three effects, favoring bone regeneration. Namely, increasing the number and functional activity of osteoprogenitor MSC, in addition to the most known effects favoring osteoblastogenesis and inhibiting adipogenesis.

Results of the present work about TPTD effects on MSC are in agreement with reports of other authors, in relation to positive PTH effects on stem cells. Thus, it has been described that such recombinant peptide form of parathyroid hormone increased MSC proliferation, reducing culture senescence [20]. Actually, treatment with PTH1-34 in rats with irradiated tibiae (microcomputed tomography; μCT) allowed to maintain MSC and osteoblast populations, whereas the former was reduced up to 75% in controls treated with placebo [41]. Moreover, it has also been demonstrated that PTH1-34 induced cytochrome P450 family-27 subfamily-B member 1 (*CYP27B1*) gene expression, increasing enzymatic activity of its encoded protein in human MSC from elderly. CYP27B1 enzyme catalyzes hydroxylation of calcidiol ($25OHD_3$) into calcitriol [$1,25\ (OH)_2D_3$], which favors MSC differentiation into osteoblasts, via autocrine/paracrine pathways. Yet, such effect is reduced with aging. Therefore, such authors highlighted the relevance of maintaining an appropriate vitamin D status in teriparatide treatments [42].

PTH anabolic effect on elderly bone is well defined. The present work suggests a response of MSC populations to PTH treatment. Yet, it is interesting to highlight that PTH effect on stem cells is not just limited to MSC. Thus, it has been suggested that such hormone stimulates hematopoiesis and HSC mobilization [43, 44], as well as mobilization of endothelial precursor cells (EPC). Indeed, such hormone has been suggested for treatment of cardiac or cerebral ischemia [45]. These data show that PTH has clearly pleiotropic effects, and therefore, it may be useful for treatment of different diseases.

Results from the present work show that TPTD had a positive effect on MSC populations of tested elderly patients. Thus, teriparatide effectively increased cell number and overall functionality, including pluripotency and regenerative capacity, further favoring mobilization of other stem cells, as other authors have described. That, together with the effects of PTH on other stem-cell types, as described by other authors, may help to explain the overall improvement observed in clinical practice of osteoporotic patients treated with TPTD. This way, teriparatide not only reduced the risk of new fractures in patients, but also increased their overall quality of life [46], which is of special relevance for modern aging populations.

Available data further suggest an interesting teriparatide potential for treatment of other pathologies, as activator agent of different regenerative processes. This could be particularly relevant for healthcare systems worldwide, especially on aging populations. We are aware of the reduced number of patients analyzed in the present study. Indeed, it is difficult to find voluntary patients with

atypical fractures, induced by long-term treatment with bisphosphonates, since prevalence ranges just between 3.22 and 113.1 cases per 100,000 patients [6]. Besides, very few of them are willing to donate bone marrow for scientific purposes, twice in 6 months, mostly being elderly people between 65 and 77 years. Actually, to the best of our knowledge, this is the first report of MSC functionality improvement in postmenopausal osteoporotic women treated with teriparatide, after suffering atypical fractures. Moreover, until now, most studies have focused on demonstrating the positive effect of TPTD on osteogenic differentiation of MSC, but not on how bone marrow MSC populations of osteoporotic patients are affected by this treatment. On the other hand, although our results were obtained on osteoporotic patients with atypical fractures, observed improvements on bone marrow MSC populations could also arise in other osteoporotic patients treated with TPTD, without previous AFF. Thus, our results suggest an important TPTD positive potency, which is also supported by other studies. Therefore, comprehensive in vitro and in vivo studies are required to better understand its mechanisms of action, further to the present proof of concept that represents this work. That should shed light on the relevance of TPTD effects on MSC and other stem-cell types, with potential and implications for regenerative medicine.

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Author Contributions ACD, GD and JMQG conceived and designed the experiments. ACD, MG, MJM and CNV performed the experiments. ACD, ADP and JMQG contributed with analysis tools and patients data. ACD, GD and JMQG wrote the paper. All authors analyzed and interpreted the data. JMQG is guarantor.

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Compliance with Ethical Standards

Conflict of interest Antonio Casado-Díaz, Gabriel Dorado, Mercè Giner, María José Montoya, Cristina Navarro-Valverde, Adolfo Díez-Pérez, José Manuel Quesada-Gómez declare no conflict of interest.

Ethics Approval Patients signed informed consent, in accordance with regulations of the Clinical Research Ethics Committee of Parc de Salut Mar, which approved the study. This was carried out in accordance with the terms of the Declaration of Helsinki.

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