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PARACRINE EFFECTS OF LIVING HUMAN BONE PARTICLES ON THE OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Abstract

Bone autografting remains the clinical treatment of choice for resolving problematic fractures. The precise mechanisms through which the autograft promotes bone healing are unknown. The present study examined the hypothesis that cells within the autograft secrete osteogenic factors promoting the differentiation of mesenchymal stem cells (MSCs) into osteoblasts. Particles of human bone ("chips") were recovered at the time of joint replacement surgery and placed in culture. Then, conditioned media were added to cultures of human, adipose-derived MSCs under both basal and osteogenic conditions. Contrary to expectation, medium conditioned by bone chips reduced the expression of alkaline phosphatase and strongly inhibited mineral deposition by MSCs cultured in osteogenic medium. Real time PCR revealed the inhibition of collagen type I alpha 1 chain (*Col1A1*) and osteopontin (*OPN*) expression. These data indicated that the factors secreted by bone chips inhibited the osteogenic differentiation of MSCs. However, in late cultures, bone morphogenetic protein-2 (*BMP-2*) expression was stimulated, suggesting the possibility of a delayed, secondary osteogenic effect.

Keywords: Autograft, bone chips, osteogenesis, mesenchymal stem cell, fracture healing.

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List of abbreviations

ALP	alkaline phosphatase
BCPs-BM	bone chip products from culture in basal medium
BCPs-OM	bone chip products from culture in osteogenic medium
BM	basal medium
BMP-2	bone morphogenetic protein 2
Col1A1	collagen type I alpha 1 chain
DKK-1	dickkopf 1
DMEM	Dulbecco's modified Eagle medium
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
IgG	immunoglobulin G
IL-1β	interleukin-1 beta
IL-6	interleukin-6
MHC	major histocompatibility complex
MSCs	mesenchymal stem cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OA	osteoarthritis
OCN	osteocalcin
OM	osteogenic medium
ON	osteonectin
OPG	osteoprotegerin
OPN	osteopontin

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RANKL	receptor activator of NF-κB ligand
TNF-α	tumour necrosis factor alpha

Introduction

Autologous bone grafts (autografts) are frequently used in clinical practice to induce osteogenesis in areas of bone loss (Atasoy and Kose, 2016; Egol *et al.*, 2015). Living cells within the autograft presumably contribute to the osteogenic process because allografted bone, whose processing kills endogenous cells, is far less effective (Costain and Crawford, 2009). In many cases, the autograft material is introduced into the lesion in the form of small, osseous particles ("chips") (Sen and Miclau, 2007). The investigation described in the present study rested on the hypothesis that living cells within autografted bone contribute to the formation of new bone by secreting soluble, diffusible factors that promote local osteogenesis at adjacent anatomic locations in a paracrine fashion. Such a mechanism is also likely to be important in the early stages of fracture healing.

New bone is deposited by osteoblasts formed by the differentiation of mesenchymal precursors (Knight and Hankenson, 2013). If the above hypothesis is correct, soluble bone-secreted factors would be predicted to promote the differentiation of mesenchymal precursors into osteoblasts. Periosteum (Wang *et al.*, 2017), bone marrow (Park *et al.*, 2012) and skeletal muscle (Owston *et al.*, 2016) are physiologically relevant sources of such precursors. Cultures of mesenchymal cells from these sources share the ability of MSCs to undergo tri-lineage differentiation under the appropriate culture conditions and, also, display a common cell surface immuno-phenotype (Caplan, 2005). MSCs derived from adipose tissue also share these properties and, even though cells from this source are not thought to contribute to bone formation physiologically, they are easy to obtain and manipulate and serve as experimentally useful surrogates for *in vitro* experiments (Minteer *et al.*, 2013).

Relatively little is known about the cell biology of bone healing using autograft bone (Goldberg *et al* 1991; Khan *et al* 2005). Data from animal models suggest that cancellous bone grafts initiate a local inflammatory reaction at the implantation site, which precedes early neovascularisation and osteogenic differentiation of host and donor MSCs. This early response is followed by bone formation and subsequent resorption as the osseous regenerate remodels. In contrast, when cortical bone is used as the autograft material, the initial response is osteoclastic (Goldberg *et al* 1991; Khan *et al*, 2005). However, there has been very little *in vitro* study of the osteogenic properties of autograft material. The present study addressed this matter.

To test the hypothesis that living bone particles influence the osteoblastic differentiation of mesenchymal progenitors, monolayers of MSCs derived from human adipose tissue were exposed or not to the secreted products of human bone chips under basal and osteogenic conditions.

Materials and Methods

Isolation and culture of human, adipose-derived MSCs

With the approval of the local ethics committee, lipoaspirates were obtained from 3 different donors, 2 female and 1 male, age 20-45 years. Samples (~ 200 mL) were mixed with an equal volume of saline and digested at 37 °C for 1 h with 0.075 % (w/v) collagenase (Sigma-Aldrich) in PBS. Digestion was stopped by the addition of an equal volume of DMEM/Ham's F12 (Gibco) containing 10 % (v/v) FBS (Gibco) and the digested material centrifuged. Supernatants were discarded and cells re-suspended in erythrocyte lysis buffer, after which the nucleated cells were resuspended in DMEM/Ham's F12 (Gibco) containing 10 % (v/v) FBS (Gibco), 100 μg/mL primocin (InvivoGen, San Diego, CA, USA) and 1 ng/mL basic fibroblast growth factor (Sigma-Aldrich). Medium was changed every other day and cells sub-cultured when confluent. Cell growth was measured by MTS assay. Cells at passages 2-4 were used for experiments.

Characterisation of adipose-derived MSCs

Flow cytometry was performed using a FACSCalibur instrument (Becton Dickinson) to determine expression of the following cell surface markers: CD90, CD44, CD73, CD81, CD117, CD34, CD14 and MHC class I and II. Appropriate isotype controls of conjugated antibodies IgG_1 and IgG_{2a} were used to control for non-specific binding. Data were analysed by Cell Quest software (Becton Dickinson).

Tri-lineage differentiation was assessed by culture in established adipogenic, chondrogenic and osteogenic media. After 10 and 21 d of culture, cells were fixed in 5 % formaldehyde for 30 min. Adipocytes were identified by staining with 0.3 % oil red O solution for 10 min. Chondrogenesis was confirmed by staining with alcian blue. Osteogenesis was evaluated by several assays, including ALP induction, mineral deposition and expression of bone-related transcripts, as described below.

Bone chips

Bone chips were harvested from the lateral portions of the anterior and posterior chamfer cuts obtained during total knee arthroplasty, as approved by the local ethics committee. 15 patients, both male and female, 55-80 years old, provided bone chips. Samples from patients with secondary knee arthritis, including rheumatoid disease, avascular necrosis and post-traumatic arthritis, were excluded.

Table 1. Primer pairs used for real-time PCR. F: forward. R: reverse.

Primers		Sequence (5′-3′)	Product length (bp)
β-actin	F	GACAGGATGCAGAAGGAGATCACT	142
	R	TGATCCACATCTGCTGGAAGGT	
Sclerostin	F	AAATCACATCCGCCCCAACT	173
	R	GGCGGTGTCTCAAAAGGGAT	
Dickkopf-1	F	GTGCAAATCTGTCTCGCCTG	284
	R	GCACAACACAATCCTGAGGC	
Osteocalcin	F	CAGATTCCCCCTAGACCCGC	299
	R	GCCTGGGTCTCTTCACTACC	
Osteonectin	F	AACCCTCCCCTTCGTGTTTC	274
	R	TTTAAGGCAGAGCCCAGCAG	
Osteopontin	F	TCCTAGCCCCACAGACCCTT	259
	R	TCTACTGTGGGGACAACTGGA	
Bone morphogenetic protein-2	F	TGGCTGGGGACTTCTTGAAC	364
	R	CAGCAACGCTAGAAGACAGC	
Collagen type I alpha 1 chain	F	ATGGGGAAGCTGGAAAACCT	286
	R	GCACCATCATTTCCACGAGC	
Osteoprotegerin	F	AAATGGCGACCAAGACACCT	290
	R	CACTGAAAGCCTCAAGTGCC	

As soon as the bone chips (1-2 mm) were collected from the operating theatre, they were suspended in high-glucose DMEM (Gibco) supplemented with 1 % penicillin/streptomycin (Gibco) and transferred to the laboratory within 1 h. Upon receipt, they were immediately weighed and placed into BM or OM, as described below.

Chips were suspended in either an equal volume of low-glucose DMEM (Gibco) supplemented with 10 % (v/v) foetal bovine serum and 100 μg/mL primocin (InvivoGen), BM or OM containing 10 nM dexamethasone (Sigma-Aldrich), 50 μg/mL ascorbic acid-2-phosphate and 10 mM β-glycerol phosphate. Conditioned media were collected after 72 h-incubation at 37 °C, 5 % CO_2, 90 % humidity and filtered through a 70 μm cell strainer. BM and OM, including the products of bone chips, were added to cultures of human adipose-derived MSCs. Conditioned media from 2 or 3 patients were pooled for each set of assays.

Co-culture experiments

Adipose-derived MSCs were plated at a density of 1×10^4 cells/well in 12-well Transwell plates (Life Sciences, New York, NY, USA) and cultured either in BM or OM. 500 μL conditioned media from bone chips cultured in either basal media (BCPs-BM) or osteogenic media (BCPs-OM) were loaded in the apical side of the Transwell inserts, separated from the MSCs by a polycarbonate membrane of pore size 400 nm (Life Sciences). The same volume (500 μL) of OM or BM was added to the well beneath the insert. As controls, human, adipose-derived MSCs were cultured in standard OM or BM without conditioned medium inserts. All media were changed every other day. MSCs and media were harvested at days 1, 3, 5, 7, 14 and 21.

ALP activity assay

Monolayers were washed 3 times with PBS, lysed with 0.1 M Tris buffer pH 9 containing 0.1 % Triton-X 100 and freeze-thawed 3 times. Then, samples were sonicated, centrifuged and ALP activity determined using a commercial kit (ALP kit, Randox, London, UK) using p-nitrophenol as substrate. Optical density was measured at 405 nm and activity normalised to total protein content (BCA protein assay kit, Intron Biotechnology, Seongnam, South Korea).

Mineralisation assays

Calcium content was measured by a QuantiChrome Calcium Assay kit (Bioassay Systems, Hayward, CA, USA), according to the manufacturer's instructions and normalised to total protein content. von Kossa staining was used to detect mineral deposits histochemically, with nuclear fast red as counter staining.

Real-time PCR

Total RNA was extracted with an RNA extraction kit (GeneJet RNA purification kit, ThermoFisher Scientific), according to the manufacturer's instructions. Synthesis of cDNA was performed using the iScript cDNA synthesis system (Bio-Rad). Real-time PCR assay was performed with a CFX96 touch sequence detection system (Bio-Rad) and real-time PCR analysis was performed with a Maxima SYBR Green/ROX qPCR master mix (ThermoFisher Scientific). Actin was used as an internal control for normalisation. Primer pairs used for amplification are given in Table 1.

Cytokine measurements

IL-1β, IL-6 and TNF-α were measured by AssayMax human ELISA kits (AssayPro, St. Charles, CA, USA).

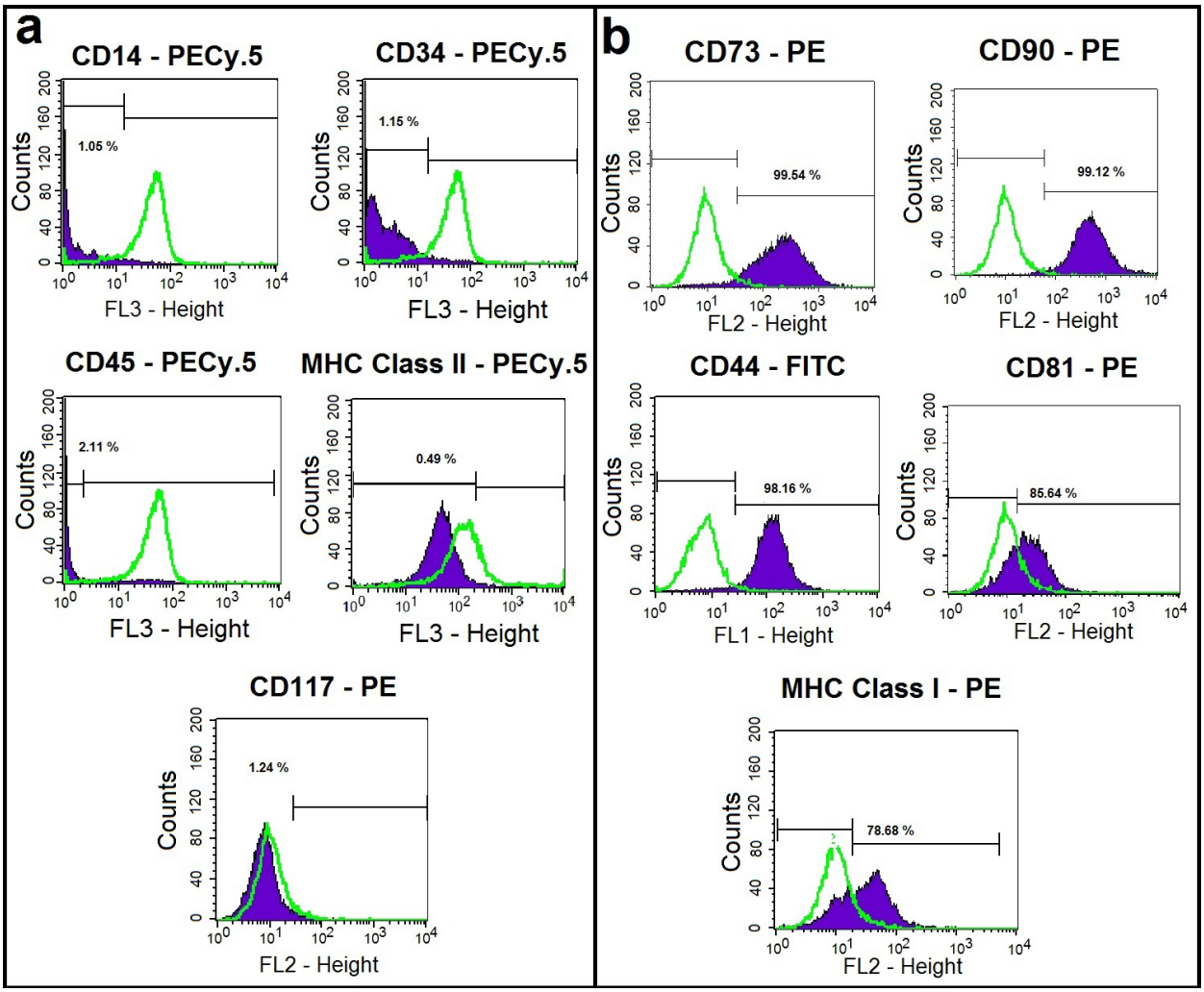

Fig. 1. Expression of cell surface markers on human adipose-derived MSCs. (**a**) CD14-PECy.5, CD34-PECy.5, CD45-PECy.5, CD117-PE, MHC class II-PECy.5 as negative surface markers, (**b**) CD73-PE, CD90-PE, CD44-FITC, CD81-PE and MHC class I-PE as positive surface markers; values are the means of three independent experiments. Isotype controls of each antibody, IgG_1 and IgG_{2a} are shown in green.

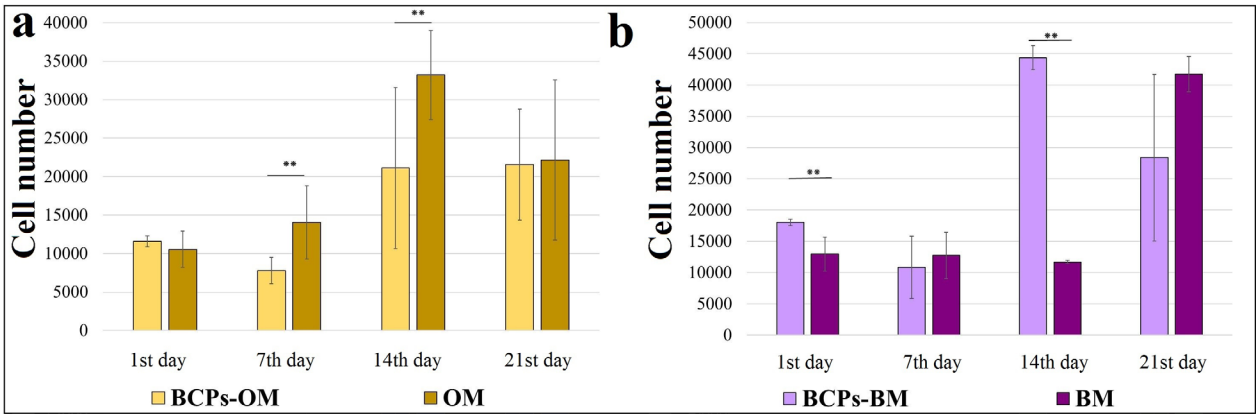

Fig. 2. Growth of human adipose-derived MSCs in BM or OM with or without products of bone chips throughout 21 d of incubation. Cell growth was determined by MTS assay. Initial cell seeding number was 1×10^4 cells/well. Results represent means ± SD calculated from at least 3 independent experiments in triplicate. ** $p < 0.01$ between the indicated groups.

Statistical analysis

Statistical analyses were performed using a statistical package for the social sciences, Version 23.0 (IBM-SPSS). Normality was determined by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Paired sample *t*-test and two related samples followed by Wilcoxon test were employed for comparing any significant change between groups. Heterogeneity of group variances of different time points was analysed using one-way ANOVA following Tukey *post-hoc* multi-comparison tests for parametric tests; two independent samples followed by Mann Whitney-U tests were carried out for non-parametric data. Statistical significance is represented as ** $p < 0.01$ and * $p < 0.05$. Representative data were derived from at least two independent experiments in duplicate and showed as mean ± standard deviation (SD).

Results

Human, adipose-derived MSCs

Consistent with the MSC phenotype, cells were positive for CD44, 73, 81, 90 and MHC class I, while negative for CD14, 34, 45, 117 and MHC class II (Fig. 1). Cells also underwent tri-lineage differentiation (data not shown).

Effects of bone chips on MSC growth and osteogenic differentiation

Bone chips had a modest inhibitory effect on the growth of MSCs under osteogenic conditions (Fig. 2). In contrast, under basal conditions, there was a marked increase in cell number at day 14.

OM induced a large increase in ALP expression by day 7 (Fig. 3) that was partially inhibited by bone chips at days 1, 7 and 14. OM induced a large deposition of calcium by day 21 (Fig. 4), which was absent from cultures maintained in BM. This was dramatically inhibited in the presence of bone chips. Quantitative calcium data were consistent with the results of the von Kossa staining (Fig. 4**a**,**b**).

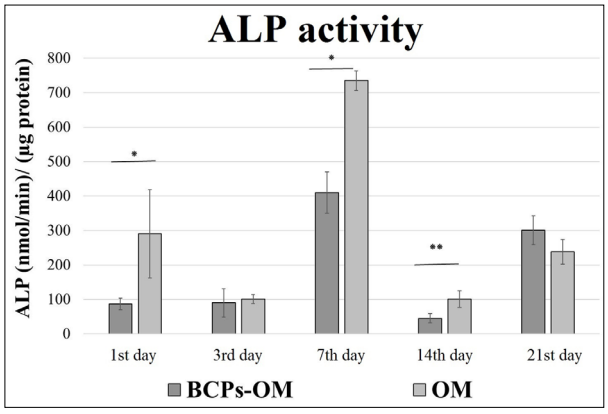

Fig. 3. ALP activity in human adipose-derived MSCs cultured in OM with or without products of bone chips for 21 d. ALP activity significantly decreased in cells cultured in osteogenic medium with products of bone chips. Values represent mean ± SD. Statistically significant differences are indicated as * $p < 0.05$ and ** $p < 0.01$.

Analysis of transcript expression revealed that, in OM, bone chips progressively inhibited the expression of *Col1A1* and *OPN* (Fig. 5), reaching approximately 90 % inhibition at day 21. *ON* expression was strongly inhibited at day 14 but stimulated at day 21. *OCN* expression was stimulated at day 7 but was little altered thereafter. Only minor effects were noted for the expression of *DKK1*, but *BMP-2* expression was elevated by bone chips, especially at day 21. Sclerostin gene expression was strongly elevated at day 14. Expression of *OPG* was inhibited strongly at days 7 and 21, but not at day 14.

Under basal culture conditions, bone chips strongly suppressed *Col1A1*, *ON* and *OPG* expression while stimulating the expression of sclerostin and *BMP-2* (Fig. 6). *OCN* and *OPN* expression were increased but *DKK* expression was modestly inhibited at days 7 and 21.

Media conditioned by bone chips contained no IL-1β, TNF-α or IL-6 that were detectable by ELISA. However, these media strongly induced IL-6, where bone chips stimulated a 3-4-fold increase at each time-point under both basal and osteogenic conditions (Fig. 7). In contrast, production of IL-1β and TNF-α was little affected by the presence of bone chips.

Discussion

The study was based upon the hypothesis that fragments of living bone secrete soluble, diffusible factors that promote the osteogenic differentiation of adjacent mesenchymal precursors. However, available data suggested otherwise. Particles of living human bone recovered at surgery were found to secrete substances that strongly inhibited mineral deposition by osteogenically stimulated adipose-derived MSCs, while reducing expression of *ALP* and certain other genes associated with osteogenesis. Similar results have been observed with human, bone marrow-derived MSCs (unpublished data), suggesting that this result was not an anomaly of adipose-derived cells.

Inhibition of *Col1A1* and *OPN* was particularly strong. Inhibition of *ALP* and collagen expression may well explain the inhibition of mineral deposition, as mineralisation is known to be dependent on the co-expression of these two osteoblast products (Murshed *et al.*, 2005). Bone particles induced the production of a large amount of IL-6, a cytokine that promotes the *in vitro* osteoblastic differentiation of adipose-derived MSCs (Fukuyo *et al.*, 2014). However, this property was not sufficient to overcome the inhibitory effects of the bone particles.

It is remarkable that the bone particles induced the expression of large amounts of BMP-2 under both basal and osteogenic media, especially in late cultures. In animal models, living bone particles

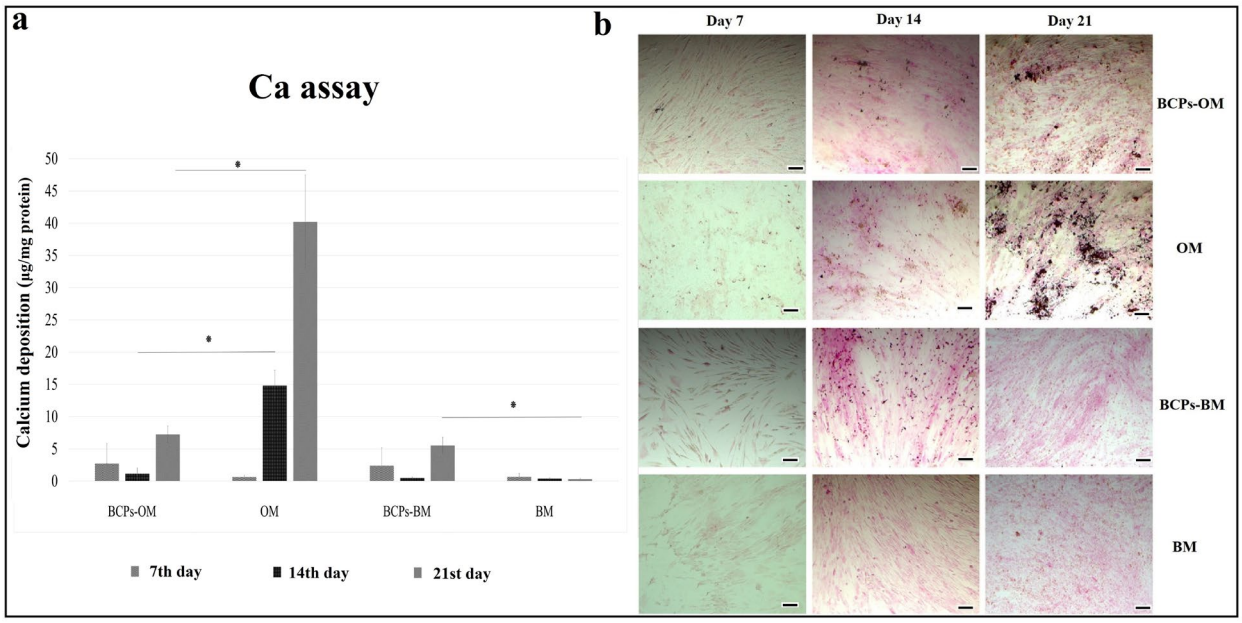

Fig.4. Effect of bone chip conditioned media on mineralisation. (**a**) Calcium content of cells cultured in OM or BM under the influence of bone chips' products throughout 21 d of incubation. Calcium concentrations of human adipose-derived MSCs incubated with products of bone chips were lower than their controls after 14 and 21 d of incubation. Bars represent means ± SD, * $p < 0.05$. (**b**) von Kossa staining of human adipose-derived MSCs in BM or OM with or without bone chip secretion at days 7, 14 and 21. Calcification showed a significant decrease in the presence of bone chips products as compared to control group after 21 d. Scale bars: 100 µm. Images were acquired at a magnification of 10×.

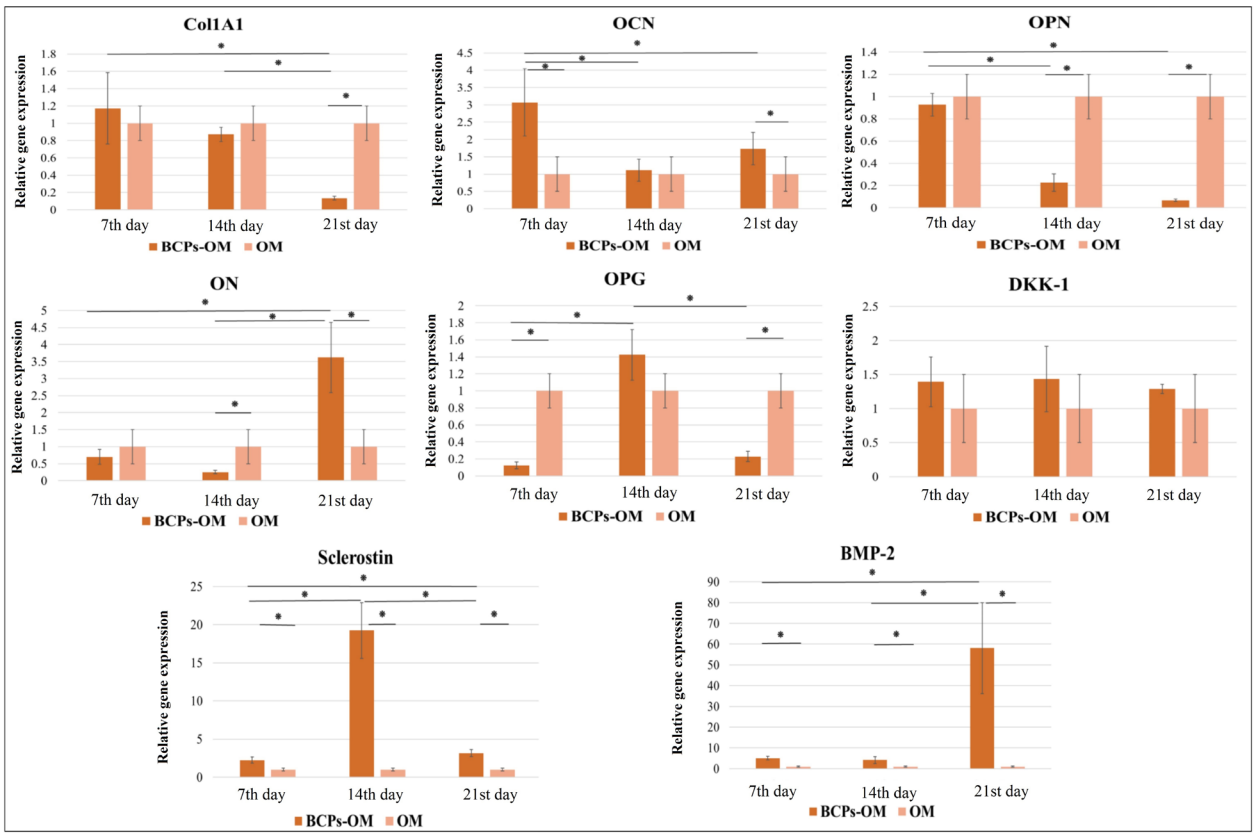

Fig. 5. Expression of the bone specific genes sclerostin, *DKK-1*, *BMP-2*, *OPG*, *Col1A1*, *OCN*, *ON* and *OP* in human adipose-derived MSCs with or without bone-chip-conditioned medium in OM at days 7, 14 and 21. Values are expressed relative to control cultures that did not receive bone chips. Means of at least 2 independent experiments in duplicate were plotted. Error bars represent means ± SD. Relative gene expression data were calculated according to the Pfaffl's method (Pfaffl, 2001). * $p < 0.05$ represents statistically significant differences between groups.

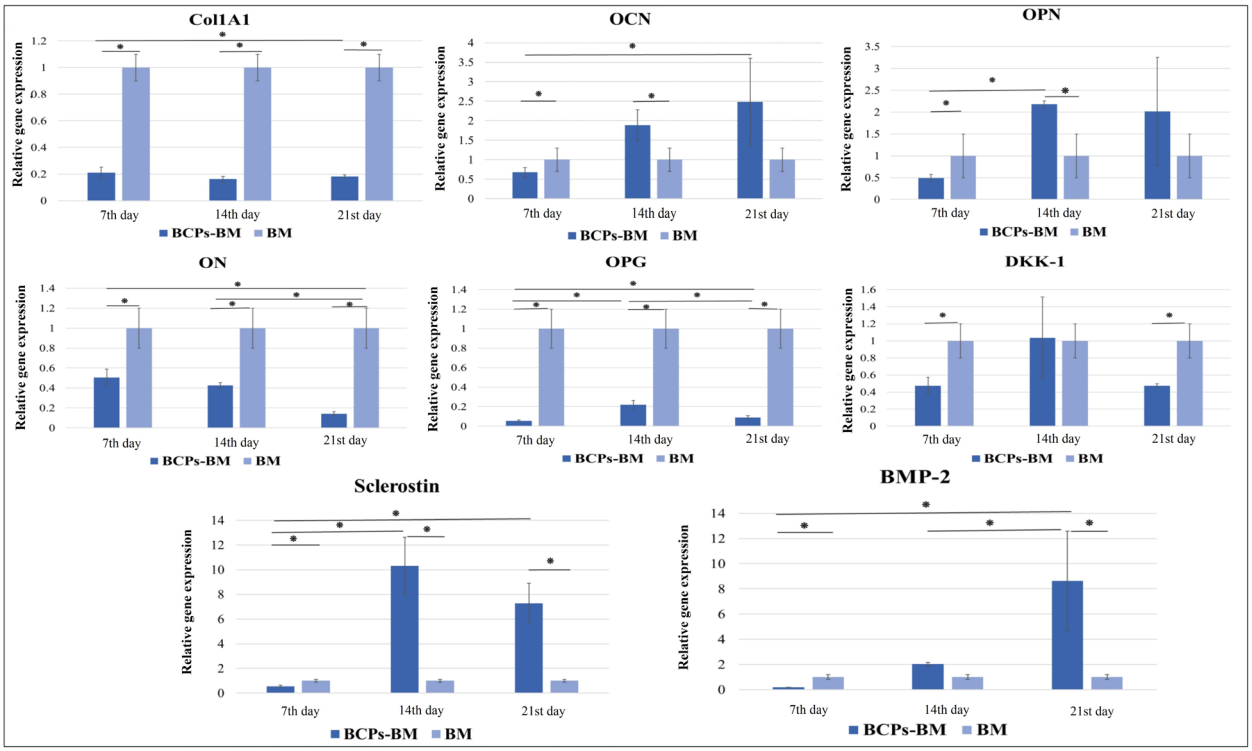

Fig. 6. Expression of the bone specific genes sclerostin, *DKK-1*, *BMP-2*, *OPG*, *Col1A1*, *OCN*, *ON* and *OP* in human adipose-derived MSCs with or without bone chip conditioned medium in BM at days 7, 14 and 21. Values are expressed relative to control cultures that did not receive bone chips. Means of at least 2 independent experiments in duplicate were plotted. Error bars represent means ± SD. Relative gene expression data were calculated according to the Pfaffl's method (Pfaffl, 2001). * $p < 0.05$ represents statistically significant differences between groups.

synergise with BMP-2 to form new bone (Liu *et al.*, 2016). This raises the possibility of a delayed, secondary osteogenic influence that would supersede any initial delay in mineralisation after an allograft implantation. It is possible that the early delay in osteogenic differentiation under the influence of the bone particles prepares the defect site for subsequent repair. This may be reflected in the expression profile of sclerostin, an inhibitor of osteogenesis that was strongly induced by the bone chips by day 14, but whose expression had fallen to nearly normal levels by day 21. Although sclerostin is primarily considered a product of osteocytes, observation of robust expression by osteoblastically induced MSCs agreed with the literature on this subject (Sutherland *et al.*, 2004). The inhibition of *OPG* by bone particles might signal an increased production of osteoclasts *in vivo*, stimulating the bone turnover necessary for efficient bone repair and remodelling. This suggestion is interesting in view of the observation that osteogenesis using cortical bone autografts, unlike cancellous autografts, begins with osteoclastic, rather than osteoblastic, activity (Goldberg *et al* 1991; Khan *et al* 2005). However, the main conclusion from the present investigation, namely that bone particles inhibited osteogenesis by MSCs, needs to be qualified in view of the static, *in vitro* cultures that were used. Under *in vivo* conditions, bone chips would be exposed to mechanical forces and fluid flow shear stresses that would influence their physiological behaviour.

The study did not address the degree to which the recovery and processing of the bone chips altered the behaviour of the endogenous cells contained within them. In this context it would be valuable to know the viability of these cells but, as described by Stoddart *et al.* (2006), standard methods for identifying live and dead cells in tissue do not perform well in cancellous bone.

It is also important to note that the bone chips used were recovered from patients with OA, whose bone metabolism may be abnormal. There is a growing literature, reviewed by Maruotti *et al.* (2017), describing metabolic changes in osteoblasts of the sub-chondral bone in OA. These changes include increased expression of prostaglandin E2, IL-6 and OPG and decreased expression of RANKL. Whether such changes extend to the area of bone that served as the source of the bone chips is unknown. Also, the chips were derived from an area of bone that is not a normal harvest site for autograft, which is typically recovered from the ilium. Nevertheless, data indicated that bone cells had a powerful paracrine influence on the osteogenic differentiation of MSCs. Such interactions may be important in fracture healing and osteogenic properties of autografted bone.

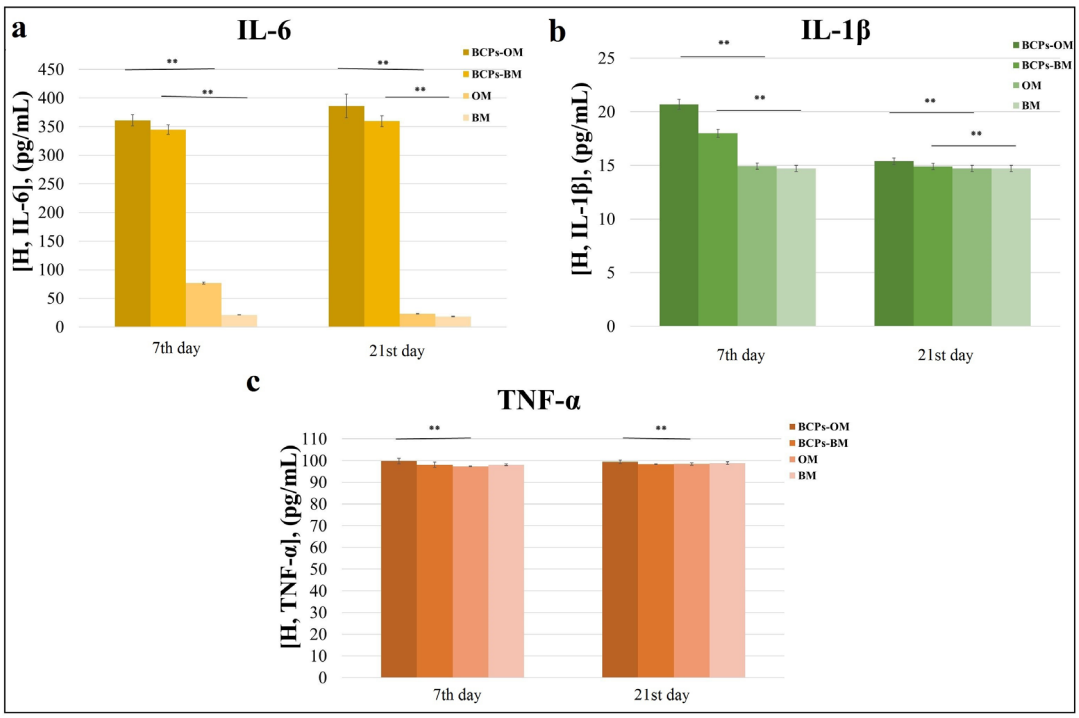

Fig. 7. Effect of bone chip products on the production of cytokines by MSCs. Production of (**a**) IL-6, (**b**) IL-1β and (**c**) TNF-α by human adipose-derived MSCs was measured by ELISA. Results represent means ± SD calculated from at least 3 independent experiments in triplicate. ** $p < 0.01$.

Conclusion

Freshly isolated bone chips from patients undergoing total knee joint replacement secreted soluble factors that inhibited the proliferation and osteogenic differentiation of human MSCs derived from lipoaspirates. As well as reducing ALP activity and mineral deposition, these factors strongly reduced the expression of *Col1A1* and *OPN*. These responses might be related to an induction of sclerostin noted in these experiments. However, the induction of *BMP-2* in late cultures suggested that while inhibiting bone formation in the short term, the bone chips could promote a subsequent, delayed osteogenic response.

These data are relevant to the mechanism of action of autograft in bone healing, although the bone chips used might be unrepresentative because they were harvested from patients with OA and not taken from the iliac crest, the normal harvest site for autograft. Moreover, the cultures were performed under static conditions and cell viability within the bone particles was not assessed.

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All authors state that they have no conflicts of interests.

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Editor's note: All comments/questions by the reviewers were answered by making changes in the text. Hence, there is no Discussion with Reviewers section.

The Scientific Editor responsible for this paper was Martin Stoddart.