

OSTEOGENIC MEDIUM IS SUPERIOR TO GROWTH FACTORS IN DIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS TOWARDS BONE-FORMING CELLS IN 3D CULTURE

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Abstract

Human adipose stem cells (hASCs) have been recently used to treat bone defects in clinical practice. Yet there is a need for more optimal scaffolds and cost-effective approaches to induce osteogenic differentiation of hASCs. Therefore, we compared the efficiency of bone morphogenetic proteins (BMP-2 and BMP-7), vascular endothelial growth factor (VEGF), and osteogenic medium (OM) for the osteo-induction of hASCs in 3D culture. In addition, growth factors were tested in combination with OM. Commercially available bioactive glass scaffolds (BioRestore) and biphasic calcium phosphate granules (BoneCeramic) were evaluated as prospective carriers for hASCs. Both biomaterials supported hASC-viability, but BioRestore resulted in higher cell number than BoneCeramic, whereas BoneCeramic supported more significant collagen production. The most efficient osteo-induction was achieved with plain OM, promoting higher alkaline phosphatase activity and collagen production than growth factors. In fact, treatment with BMP-2 or VEGF did not increase osteogenic differentiation or cell number significantly more than maintenance medium with either biomaterial. Moreover, BMP-7 treatment consistently inhibited proliferation and osteogenic differentiation of hASCs. Interestingly, there was no benefit from growth factors added to OM. This is the first study to demonstrate that OM enhances hASC-differentiation towards bone-forming cells significantly more than growth factors in 3D culture.

Keywords: Adipose stem cells; mesenchymal stem cells; bone tissue engineering; bioactive glass; biphasic calcium phosphate; growth factors; osteogenic differentiation; *in vitro* culture; 3D scaffolds.

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Introduction

Tissue engineered bone has emerged as a potential alternative to meet the increasing need for bone replacements in clinical medicine and to overcome the limitations of autologous grafts as well as the risks related to allogeneic bone grafts (Betz, 2002). Specifically, bone regeneration *via* autologous stem cell transplantation is a promising approach to treat large bone defects. For example, patient's own multipotent mesenchymal stem cells (MSCs) can be isolated from adipose tissue, expanded *ex vivo*, and transplanted to the defect site using biomaterial scaffold as a carrier (Mesimaki *et al.*, 2009; Thesleff *et al.*, 2011). Among adult stem cells, human adipose stem cells (hASCs) are readily available multipotent cells having the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma (Zuk *et al.*, 2001; Zuk *et al.*, 2002; Park *et al.*, 2010; Choi *et al.*, 2012). The osteogenic capacity of ASCs has been demonstrated in both *in vitro* (Zuk *et al.*, 2002; De Girolamo *et al.*, 2007) and *in vivo* studies (Cowan *et al.*, 2004; Hicok *et al.*, 2004; Hattori *et al.*, 2006; Di Bella *et al.*, 2008; Behr *et al.*, 2011). In addition, our group has reported several clinical cases, where autologous hASCs combined with biomaterials have been used to repair cranio-maxillofacial defects (Mesimaki *et al.*, 2009; Thesleff *et al.*, 2011).

In order to enhance the bone formation capacity, a number of inducing factors have been tested in combination with biomaterials and cells. Based on the fact that bone formation is modulated by a number of osteogenic factors released from the bone and surrounding tissues during the repair process, recombinant bone morphogenetic protein (BMP)-2 and BMP-7 have been utilised in clinical applications in order to stimulate bone healing (Samartzis *et al.*, 2005; Garrison *et al.*, 2007; Clokie and Sandor, 2008). Similar to BMPs, vascular endothelial growth factor A (VEGF-A) has been used to promote angiogenesis and osteogenesis of MSCs *in vitro* (Behr *et al.*, 2011; D'Alimonte *et al.*, 2011) and *in vivo* (Roldan *et al.*, 2010; Behr *et al.*, 2011). However, critical views on the use of BMPs have been emerging lately due to their short half-lives, expensiveness and ineffectiveness (Garrison *et al.*, 2007; Garrison *et al.*, 2010; Zuk *et al.*,

Table 1. The compositions of different media used in the study.

	Medium	Supplements
	Maintenance medium (MM)	DMEM/F-12, 10 % FBS, 1 % L-glutamine, 1 % antibiotics
	Osteogenic medium (OM)	5 nM dexamethasone, 250 µM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma-Aldrich, MO, USA) in MM
Part I	MM + BMP-2	50 ng/mL bone morphogenetic protein-2 (Genscript, NJ, USA) in MM
	MM + BMP-7	100 ng/mL bone morphogenetic protein-7 (Sigma-Aldrich) in MM
	MM + VEGF	20 ng/mL vascular endothelial growth factor (R&D Systems Inc, MN, USA) in MM
Part II	OM + BMP-2	50 ng/mL BMP-2 (Genscript, NJ, USA) in OM
	OM + BMP-7	100 ng/mL BMP-7 (Sigma-Aldrich) in OM
	OM + VEGF	20 ng/mL VEGF (R&D Systems Inc, MN, USA) in OM

2011). The clinical use of growth factors has been hindered by the significantly large, and hence costly, doses needed in humans. Large doses of exogenous growth factors may cause unexpected physiological effects ranging from bone resorption (Giannoudis *et al.*, 2007) to heterotopic ossification (Wysocki and Cohen, 2007; Axelrad *et al.*, 2008). Hence, alternative methods for effective osteo-induction of MSCs are under investigation (Kim *et al.*, 2003; Kim *et al.*, 2005; McCullen *et al.*, 2010; Tirkkonen *et al.*, 2011).

For example, osteogenic medium (OM) supplemented with L-ascorbic acid 2-phosphate (AsA2-P), dexamethasone (Dex) and β-glycerophosphate (β-GP) has been commonly used for the osteogenic differentiation of MSCs *in vitro* (Zuk *et al.*, 2001; Ogawa *et al.*, 2004; Giusta *et al.*, 2010; Rada *et al.*, 2011). The *in vivo* studies have also shown the advantage of OM-induction in ASC-based bone regeneration; significantly greater bone healing was detected with the OM-induced ASCs compared to the non-induced ASCs (Dudas *et al.*, 2006; Yoon *et al.*, 2007; Di Bella *et al.*, 2008; Schubert *et al.*, 2011). However, the osteo-induction efficiency of OM has not been systematically compared to BMP-2, BMP-7 and VEGF with hASCs *in vivo* or *in vitro*. Although *in vivo* studies are required in the future to demonstrate the utility of OM over growth factors, obtaining sufficient *in vitro* data is of fundamental importance before it is reasonable to initiate *in vivo* studies.

In addition to effective osteo-induction, suitable biomaterial carriers for supporting the hASC proliferation and differentiation are required for successful bone regeneration. Bioactive glass and biphasic calcium phosphate, both biomaterials currently used in cranio-maxillofacial (Peltola *et al.*, 2006; Peltola *et al.*, 2008; Frenken *et al.*, 2010; Clozza *et al.*, 2012b) and orthopaedic surgery (Lindfors *et al.*, 2010; Garrido *et al.*, 2011), were compared as prospective carriers for hASCs in the current study. Synthetic bioceramics are among the most promising biomaterials for the use of bone tissue engineering largely due to their capacity to form bone-like apatite layers in contact with physiological fluids, a reaction which facilitates their strong bonding to bone (Hench, 1998; Kokubo *et al.*, 2003). Furthermore, bioactive glass and calcium phosphate biomaterials are capable of promoting

bone formation through the dissolution of Ca and P ions (Xynos *et al.*, 2000; E *et al.*, 2010). Essential characteristics for silicate bioactive glasses include high Na₂O and CaO content, high CaO/P₂O₅ ratio, and SiO₂ content less than 60 mol%. Biphasic calcium phosphates, in turn, consist of varying ratios of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP). With slowly resorbing HA and more soluble β-TCP, the ratio 60/40 wt% of HA/β-TCP has been widely used as it offers controlled degradation and optimal osseous substitution (Frenken *et al.*, 2010; Ghanaati *et al.*, 2012).

While searching for an optimal approach to treat patients with adipose stem cells, the aim of the present study was to compare the efficiency of BMP-2, BMP-7, VEGF and OM for the osteogenic differentiation of hASCs in clinically relevant 3D environment. In addition, BMP-2, BMP-7 and VEGF were tested in combination with OM.

Materials and Methods

Biomaterial characterisation and preparation

Two commercially available biomaterials, Straumann® BoneCeramic granules (Straumann AG, Basel, Switzerland) and Inion BioRestore™ (Inion Oy, Tampere, Finland) bioactive glass scaffolds were compared as 3D carriers for hASCs in this study. The morphology and surface topography of both materials was examined using scanning electron microscopy (SEM; Philips XL-30; Philips, Eindhoven, The Netherlands). The biomaterial samples were air-dried and sputter coated with gold prior to analysis with SEM.

The fully synthetic, granular form Straumann® BoneCeramic composed of biphasic calcium phosphate with 60 % hydroxyapatite (HA; 100 % crystalline) and 40 % β-tricalcium phosphate (β-TCP) was used in this study. BoneCeramic has a porosity of 90 %, and a granule size between 0.5 and 1.0 mm with interconnected pores of 100-500 µm in diameter. For cell culturing, 0.25 g aliquots of sterile BoneCeramic granules were transferred onto 24-well plates (Nunc, Roskilde, Denmark) using an analytical balance that was placed inside a laminar hood. The granules were incubated in 1 mL of corresponding medium (Table 1) for 48 h prior to cell seeding.

The Inion BioRestore™ bioactive glass scaffolds used in this study had a nominal composition of 11.1-12.0 wt% Na₂O, 15.0-17.1 wt% K₂O, 2.8-3.3 wt% MgO, 12.7-15.2 wt% CaO, 2.7-3.8 wt% P₂O₅, 1.0-1.4 wt% B₂O₃, 0.0-0.6 wt% TiO₂ and 48.5-52 wt% SiO₂. The porous bioactive glass scaffolds were manufactured from fibres as described previously (Moimas *et al.*, 2006). Briefly, bioactive glass fibres of 75 µm diameter and 3 mm length were produced by melt spinning. The fibres were sintered under defined conditions to obtain desired structural and mechanical properties. Scaffolds with porosity of 70 % and dimensions of 7 x 7 x 3 mm were used in this study.

Before cell seeding, the bioactive glass scaffolds were sterilised with 70 % ethanol followed by several steps of washing with Dulbecco's phosphate-buffered saline (DPBS; Lonza Biowhittaker; Verviers, Belgium), and incubated in 1 mL of corresponding medium (Table 1) for 48 h.

Cell isolation, characterisation and culture

Adipose tissue samples were acquired from surgical procedures in the Department of Plastic Surgery, Tampere University Hospital with the patients' written consent. The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058). The adipose tissue samples were obtained from 11 donors (mean age 50 ± 14 years).

The adipose tissue was manually chopped into small fragments and digested with collagenase type I (1.5 mg/mL; Invitrogen/Life Technologies, Carlsbad, CA, USA) in a water bath at 37 °C for 60 min, and the hASCs were isolated by centrifugation. Following isolation, the hASCs were expanded in T75 cm² polystyrene flasks (Nunc) in maintenance medium (MM) consisting of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12 1:1) (Invitrogen, Paisley, UK), 10 % foetal bovine serum (PAA Laboratories, Pasching, Austria), 1 % antibiotics (100 U/mL penicillin; 100 U/mL streptomycin; Lonza Biowhittaker, Basel, Switzerland) and 1 % L-glutamine (GlutaMAX I; Invitrogen). After expansion hASCs were cryo-preserved in gas phase nitrogen in freezing solution consisting of 10 % dimethyl sulphoxide (Hybri-Max; Sigma-Aldrich, St. Louis, MO, USA) in foetal bovine serum (PAA Laboratories, Pasching, Austria).

After primary cell culture (at passage 1-2) the hASCs were characterised by a fluorescent-activated cell sorter (FACSria; BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies against CD14-PE, CD19-PE, CD49d-PE, CD73-PE, CD90-APC, CD106-PE, (BD Biosciences); CD45-FITC (Miltenyi Biotech, Bergisch Gladbach, Germany); CD34-APC, HLA-ABC-PE, HLA-DR-PE (Immunotools, Friesoythe, Germany); and CD105-PE (R&D Systems, Minneapolis, MN, USA) were used. The analysis was performed on 10,000 cells per sample and unstained cell samples were used to compensate for the background autofluorescence levels.

The hASCs needed for the study were thawed and expanded in maintenance medium in T75 cm² polystyrene flasks (Nunc). At passage 2-4, the hASCs were seeded onto

BoneCeramic granules at a density of 9.7 x 10⁴ cells per well, and similarly to the BioRestore scaffolds at a density of 9.7 x 10⁴ cells per scaffold, using a small volume (50 µL) of MM. The cells were let to attach at +37 °C for 3 h before adding 1 mL of corresponding medium (Table 1). Fresh medium was changed 3 times per week. During each medium change the growth factors were freshly added to the medium from frozen aliquots avoiding multiple freeze-thaw cycles in order to keep the growth factors active. The experiments were repeated 3-6 times using one donor cell line per each repeat.

In the first part of this study, recombinant human BMP-2 (rhBMP-2; Genscript, Pisataway, NJ, USA), BMP-7 (rhBMP-7; Sigma-Aldrich), and VEGF-A (rhVEGF₁₆₅; R&D Systems) were added to MM and compared with osteogenic medium (OM). In the second part of the study, the growth factors were combined with OM. The compositions of the different media used in the study are described in Table 1. The concentrations of BMP-2 (Barr *et al.*, 2010), BMP-7 (Shen *et al.*, 2010) and VEGF (Behr *et al.*, 2011) used in this study were based on literature.

Cell viability

Cell attachment and viability were evaluated qualitatively using Live/dead-staining probes (Molecular Probes/Invitrogen, Eugene, OR, USA) at day 3, 7 and 14. The hASCs were incubated for 45 min at room temperature with a mixture of 0.5 µM calcein acetoxymethyl ester (Molecular Probes) and 0.25 µM ethidium homodimer-1 (Molecular Probes). Images of the viable cells (green fluorescence) and dead cells (red fluorescence) were obtained using an Olympus IX51 phase contrast microscope with fluorescence optics and Olympus DP30BW camera (Olympus, Tokyo, Japan).

Cell number

The cell number of the samples was studied at 3-, 7- and 14-day time points by determining the amount of total DNA by CyQUANT Cell Proliferation Assay Kit (CyQUANT; Molecular Probes) according to the manufacturer's protocol. CyQUANT GR dye expresses fluorescence when bound to cellular nucleic acids.

The cells were washed with DPBS and lysed with 500 µL of 0.1 % Triton-X 100 buffer (Sigma-Aldrich). The Triton cell lysates were frozen and stored at -70 °C until analysis. After thawing three parallel 20 µL samples of each cell lysate were pipetted on a 96-well plate (Nunc) and mixed with 180 µL of working solution containing CyQUANT GR dye. Fluorescence was measured at 480/520 nm with a microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was analysed at 3-, 7- and 14-day time points. ALP cleaves phosphate groups from *p*-nitrophenol phosphates at pH 10.4 liberating yellow-coloured *p*-nitrophenol and phosphate. The rate of *p*-nitrophenol formation is proportional to the catalytic concentration of ALP in the sample. The ALP activity was determined from the same Triton-X 100 cell lysates as the cell number.

Table 2. Surface marker expression of undifferentiated hASCs at passage 1-2. The results are displayed as mean percentage of the surface marker expression ($n = 11$).

Antigen	Surface protein	Mean	SD	Expression
CD14	serum lipopolysaccharide binding protein	1.8	1.1	negative
CD19	B lymphocyte-lineage differentiation antigen	0.8	0.7	negative
CD34	sialomucin-like adhesion molecule	17.4	8.3	moderate
CD45	leukocyte common antigen	1.4	1.1	negative
CD49d	integrin $\alpha 2$, VLA-4	41.7	20.0	moderate
CD73	ecto-50-nucleotidase	90.4	9.4	positive
CD90	Thy-1 (T cell surface glycoprotein)	99.3	0.6	positive
CD105	SH-2, endoglin	95.7	4.4	positive
CD106	VCAM-1 (vascular cell adhesion molecule)	1.1	1.1	negative
HLA-ABC	major histocompatibility class I antigens	42.0	24.6	moderate
HLA-DR	major histocompatibility class II antigens	0.9	0.8	negative

Three parallel 20 μL samples were pipetted on a MicroAmp™ Optical 96-well plate (Applied Biosystems, CA, USA). The reaction was started by pipetting 90 μL of working solution per well, containing 50 % of alkaline buffer solution (2-amino-2-methyl propanol; 1.5 M; pH 10.3; Sigma-Aldrich) and 50 % of stock substrate solution (*p*-nitrophenol phosphate; Sigma-Aldrich) and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μL of NaOH (1 M, Sigma-Aldrich) and the colour intensity was determined with a microplate reader (Victor 1420) at 405 nm.

Collagen assay

The amount of collagen was determined at 7- and 14-day time points by Sircol Soluble Collagen Assay (Biocolor, Carrickfergus, Northern Ireland). The basis of the assay was binding of Sirius red dye to [Gly-X-Y] peptide sequence of mammalian collagen types I-V. Production of collagenous matrix was measured, as it was not possible to analyse mineralisation due to the calcium containing 3D-scaffolds.

For the measurement, acid-soluble collagen was extracted from the cultures by 0.5 M acetic acid (Merck, Darmstadt, Germany) containing 0.1 mg/mL pepsin (Sigma-Aldrich) for 2 h at 4 °C. The collagen content was determined from 100 μL samples, with 1 mL of Sircol Dye reagent (Sirius Red in picric acid; Biocolor) added to each aliquot and incubated with gentle shaking for 30 min. The samples were centrifuged at 12,000 rpm for 10 min, and the collagen-dye pellet was washed once to remove unbound dye with 750 μL of ice-cold Acid-Salt Wash Reagent (acetic acid, sodium chloride and surfactants; Biocolor). After centrifugation, the supernatant was discarded and the collagen-bound dye was released by adding 250 μL of Alkali Reagent (0.5 M sodium hydroxide; Biocolor). The dye intensity was measured from two parallel 100 μL samples on a 96-well plate (Nunc) with a microplate reader (Victor 1420) at 540 nm.

Statistical analysis

Statistical analyses were performed with SPSS version 19 (IBM, Armonk, NY, USA). Data were reported as mean and standard deviation (SD). The effects of different culture conditions on cell number, ALP activity, and collagen

content were compared using a one-way analysis of variance (ANOVA) with Tukey *post hoc* test. The results were considered significant when $p < 0.05$. The effect of culture duration was analysed using a Student's *t*-test for independent samples for collagen content (day 7 *versus* day 14), and one-way ANOVA for cell number and ALP activity for comparing 3-, 7- and 14-day time points. In the first part of this study growth factors were added to control medium and compared with OM, the experiments for cell number and ALP activity were repeated using 6 donor lines ($n = 6$), and the analysis of collagen content was repeated with 3 donor lines ($n = 3$). In the second part, where growth factors were tested in combination with OM, all experiments were repeated using 3 donor lines ($n = 3$).

Results

Scanning electron microscopy

The scanning electron microscopy (SEM) images (Fig. 1) illustrated the macro structure (Fig. 1a,b) and surface roughness (Fig. 1e,f) of the biomaterials. Based on the SEM images a larger surface area may be expected for BioRestore that is composed of small sized fibres (Fig. 1, left side). Regarding surface topography, BoneCeramic exhibited more surface roughness than BioRestore (Fig. 1e,f). Overall, the SEM images confirmed the high porosity of both biomaterials. More detailed information on material characteristics of BioRestore scaffold has been provided previously (Haimi *et al.*, 2009a).

Flow cytometric surface marker expression analysis

The flow cytometric analysis (Table 2) demonstrated that the hASCs used in this study expressed the surface markers CD73, CD90, CD105, and lacked the expression of the CD14, CD19, HLA-DR, the haematopoietic marker CD45, and the vascular cell adhesion molecule CD106. The expression of CD34, CD49d and HLA-ABC, was moderate. Overall, the surface marker analysis confirmed the mesenchymal origin of the cells in agreement with literature (Dominici *et al.*, 2006; McIntosh *et al.*, 2006; Lindroos *et al.*, 2010).

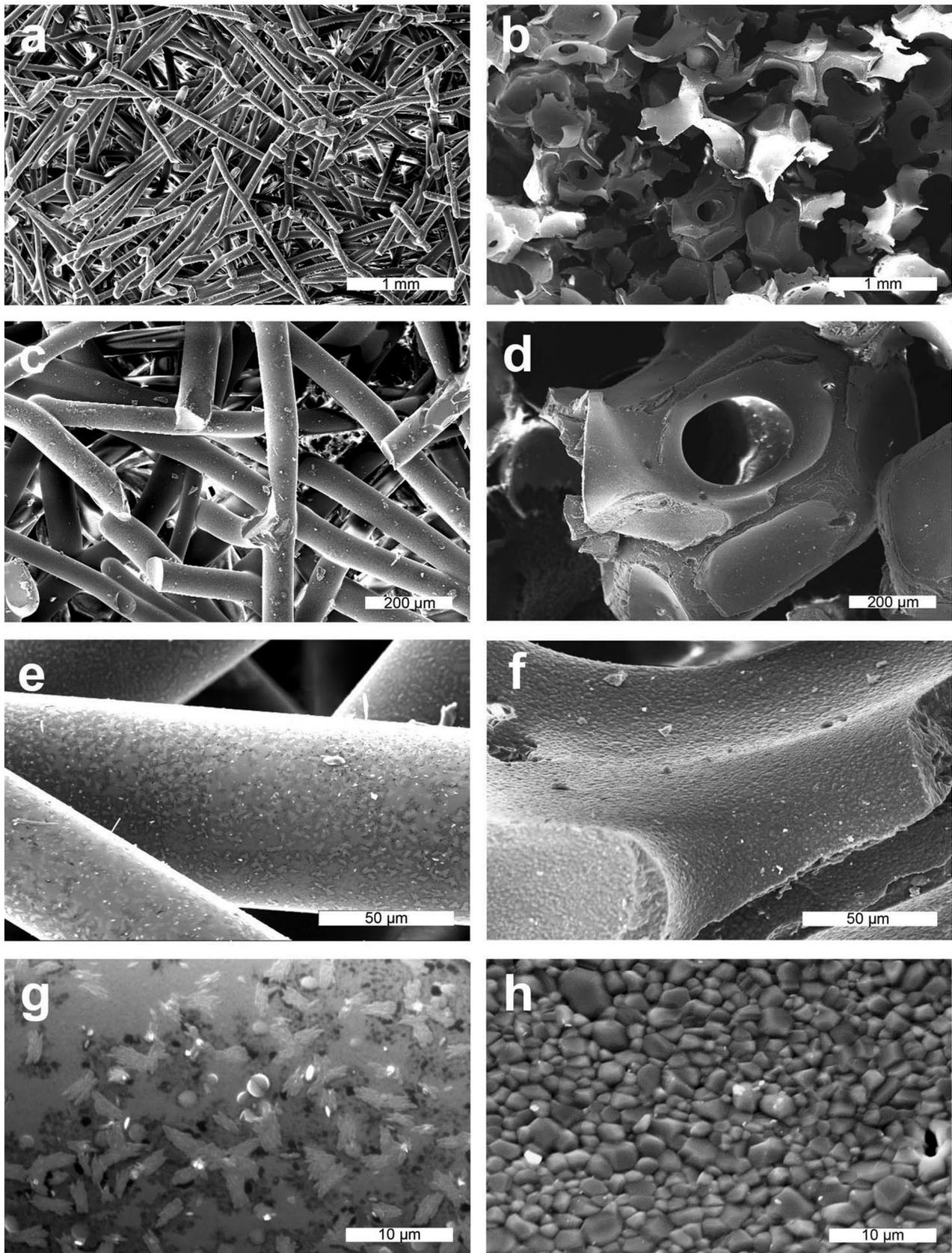


Fig. 1. Scanning electron micrographs of BioRestore (left; **a, c, e, g**) and BoneCeramic (right; **b, d, f, h**) at magnification of 20x (**a, b**), 80x (**c, d**), 500x (**e, f**), and 2000x (**g, h**). Scale bars in the lower right corner of the micrographs.

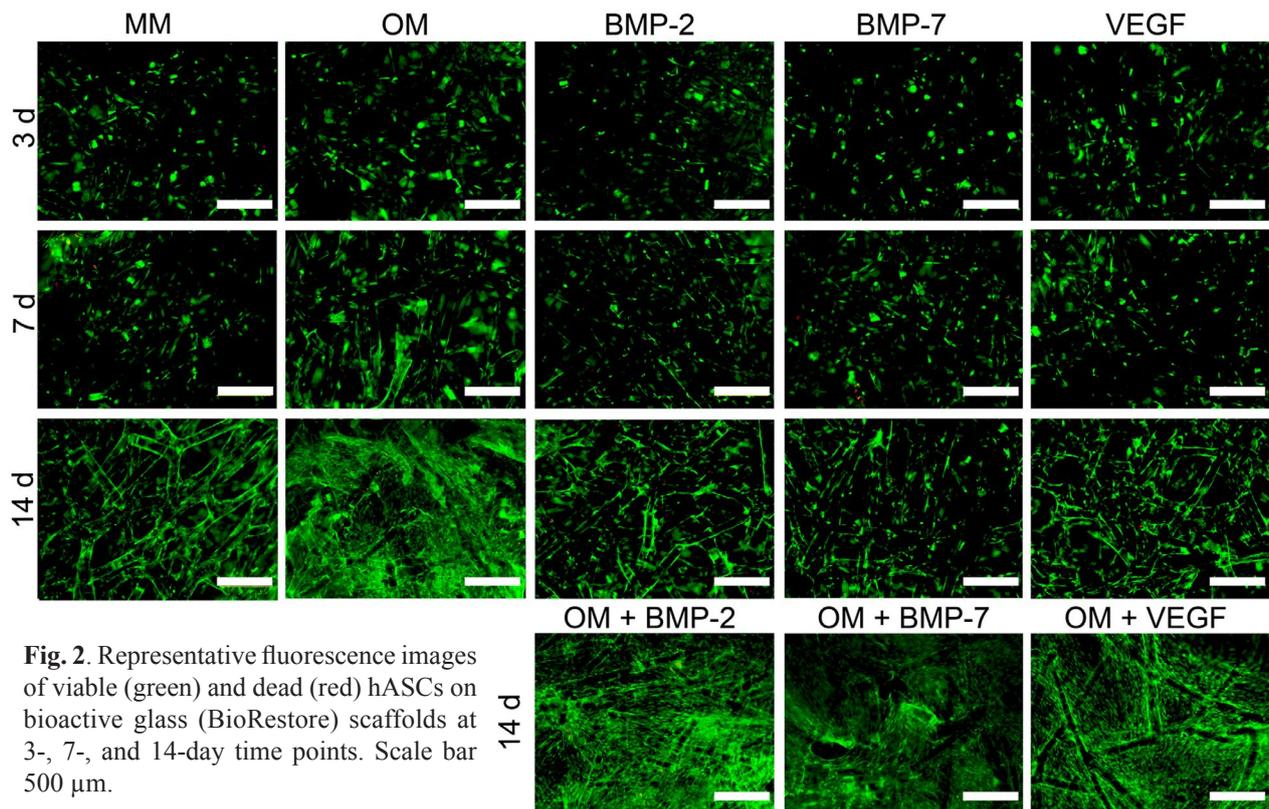


Fig. 2. Representative fluorescence images of viable (green) and dead (red) hASCs on bioactive glass (BioRestore) scaffolds at 3-, 7-, and 14-day time points. Scale bar 500 μm .

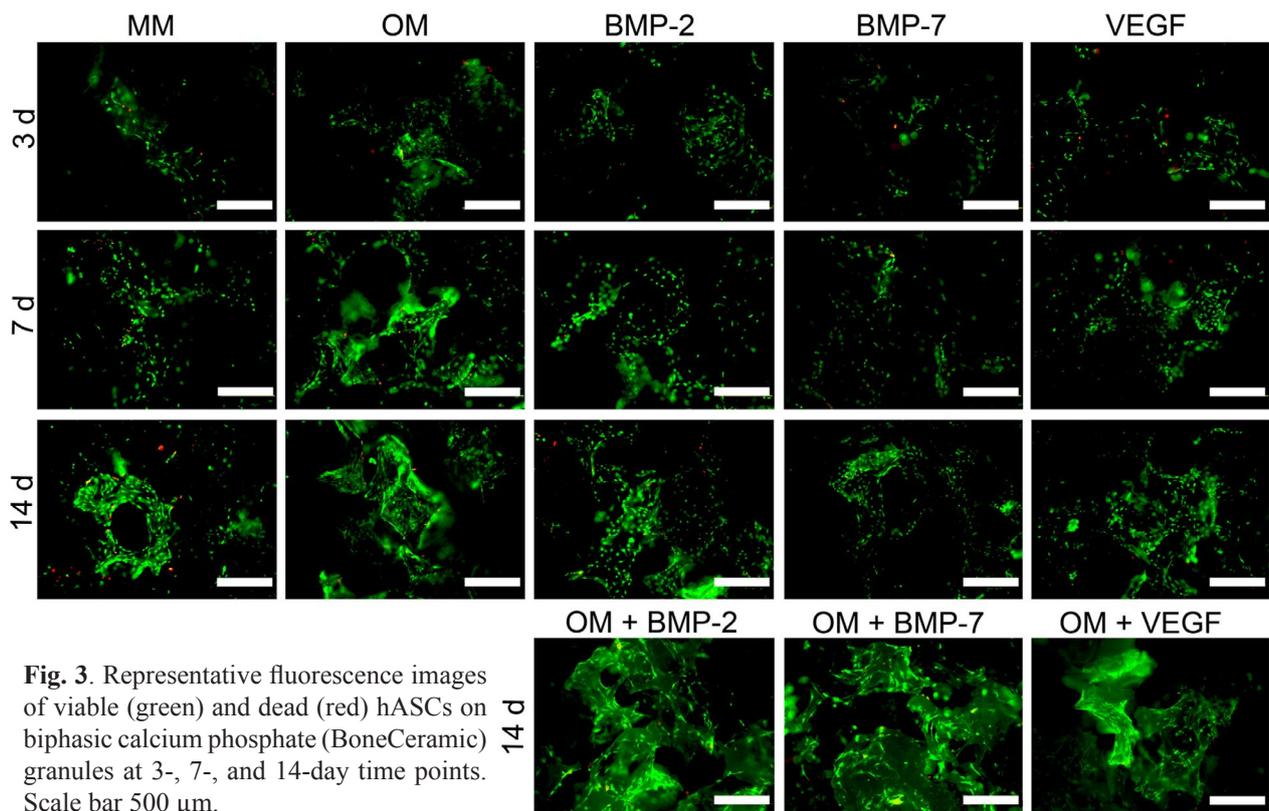


Fig. 3. Representative fluorescence images of viable (green) and dead (red) hASCs on biphasic calcium phosphate (BoneCeramic) granules at 3-, 7-, and 14-day time points. Scale bar 500 μm .

Comparison of BioRestore and BoneCeramic biomaterials

The hASCs seeded on both BioRestore (Fig. 2) and BoneCeramic (Fig. 3) were viable and proliferated steadily under control conditions during the whole 14-day culturing period as monitored by qualitative Live/dead Cell Viability (Fig. 2 and 3) and quantitative CyQuant Cell Proliferation

(Fig. 4) assays. According to Live/dead assay, BioRestore supported cell proliferation more than BoneCeramic. This was confirmed by the quantitative analysis of cell number (Fig. 4a), which showed 1.4-fold higher cell number with BioRestore than BoneCeramic in MM ($p = 0.026$), and 1.3-fold in OM ($p = 0.002$) at day 14.

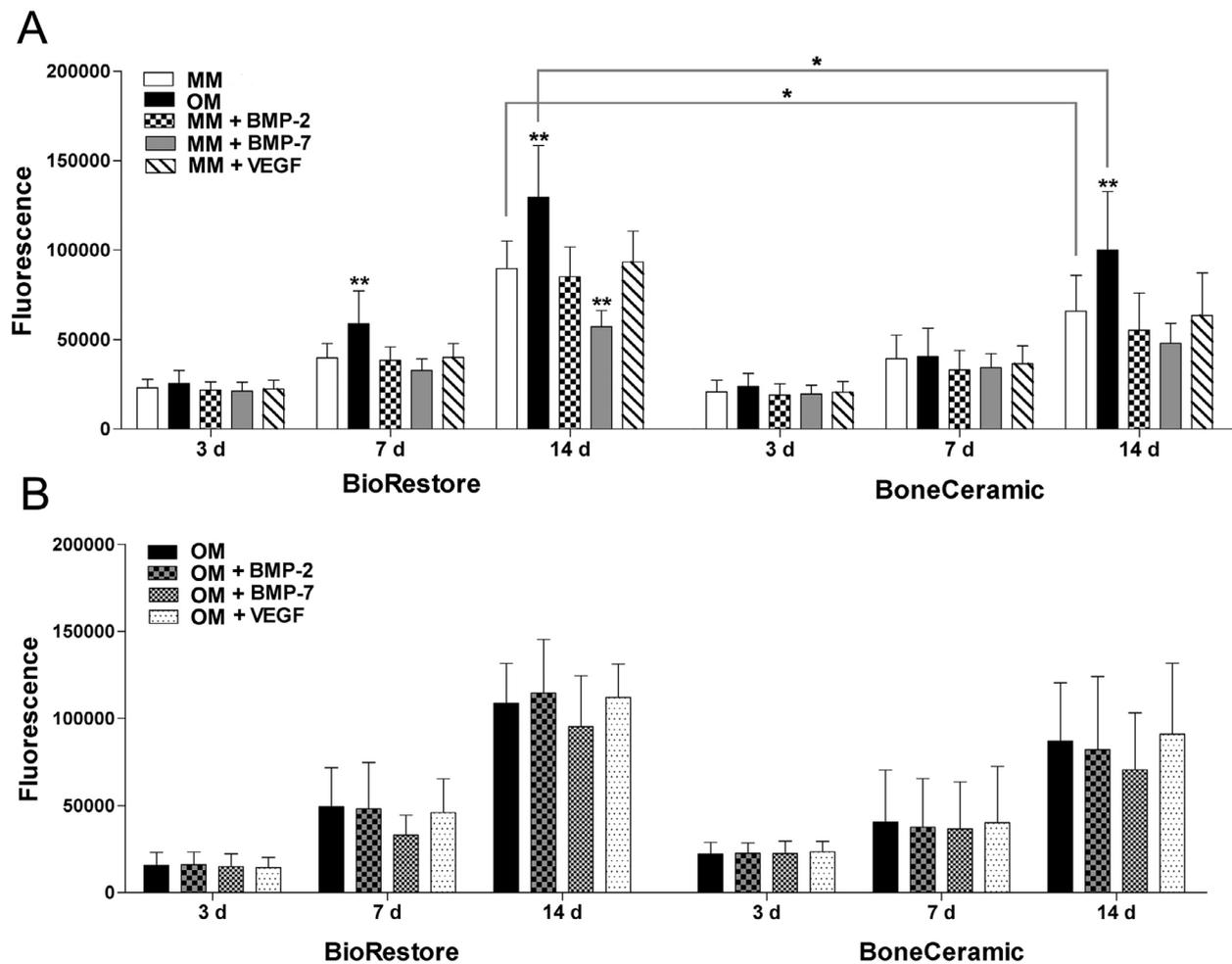


Fig. 4. Cell number at 3-, 7-, and 14-day time points on BioRestore or BoneCeramic. **(A)** Growth factors in MM: Number of hASCs cultured with MM, OM or growth factors in MM. * $p < 0.05$ between the indicated groups. ** $p < 0.05$ with respect to other groups within that time point. **(B)** Growth factors in OM: Number of hASCs cultured with OM or growth factors in OM. No significant differences in panel B. Results are expressed as mean fluorescence + SD.

The ALP activity of hASCs (Fig. 5) was supported equally by both biomaterials in MM, but the induction by OM was more significant and consistent in BoneCeramic. For example, with BoneCeramic the ALP activity was enhanced by OM 2-fold when compared to MM, 3-fold to BMP-2, 8-fold to BMP-7, and 2-fold to VEGF ($p < 0.05$ in all). In BioRestore, the ALP activity induced by OM was higher than with BMP-2 (1.7-fold) and BMP-7 (4.5-fold), but not when compared to MM.

Furthermore, BoneCeramic supported the collagen production of hASCs significantly more than BioRestore (Fig. 6a). Collagen production by hASCs grown on BoneCeramic in MM or MM + growth factors was significantly higher when compared to BioRestore both at day 7 and day 14. At day 14, the amount of collagen was 5-fold higher in BoneCeramic MM than in BioRestore MM ($p < 0.05$). In fact, BoneCeramic combined with MM produced comparable collagen levels to that made by osteo-induced hASCs grown on BioRestore. Induction by OM resulted in increased collagen production on both biomaterials at day 14 with the highest levels of collagen being produced by hASCs induced in OM on

BoneCeramic. However, the enhancement of collagen production was more intense in the case of BioRestore; OM resulted in 5 times more collagen than MM in BioRestore, but only a 1.9-fold induction in BoneCeramic – due to the high level of collagen present under control conditions. When compared to MM control, growth factor treatment did not increase collagen production on either biomaterial.

Comparison of OM induction to MM

Overall, OM resulted in higher cell numbers and collagen production than MM with both biomaterials. When comparing the effect of OM to MM in BioRestore, a significant stimulation was detected on proliferation of hASCs (Fig. 4a). OM increased the cell number 1.5-fold on day 7 and 14 when compared to MM ($p < 0.05$). Live/dead analysis confirmed the high growth rate of OM-cultured hASCs in BioRestore (Fig. 2). In addition, OM induced significantly higher collagen production (5-fold) than MM at day 14 in BioRestore (Fig. 6a). In turn, the ALP activity of hASCs seeded in BioRestore was not significantly higher in OM than in MM (Fig. 5a).

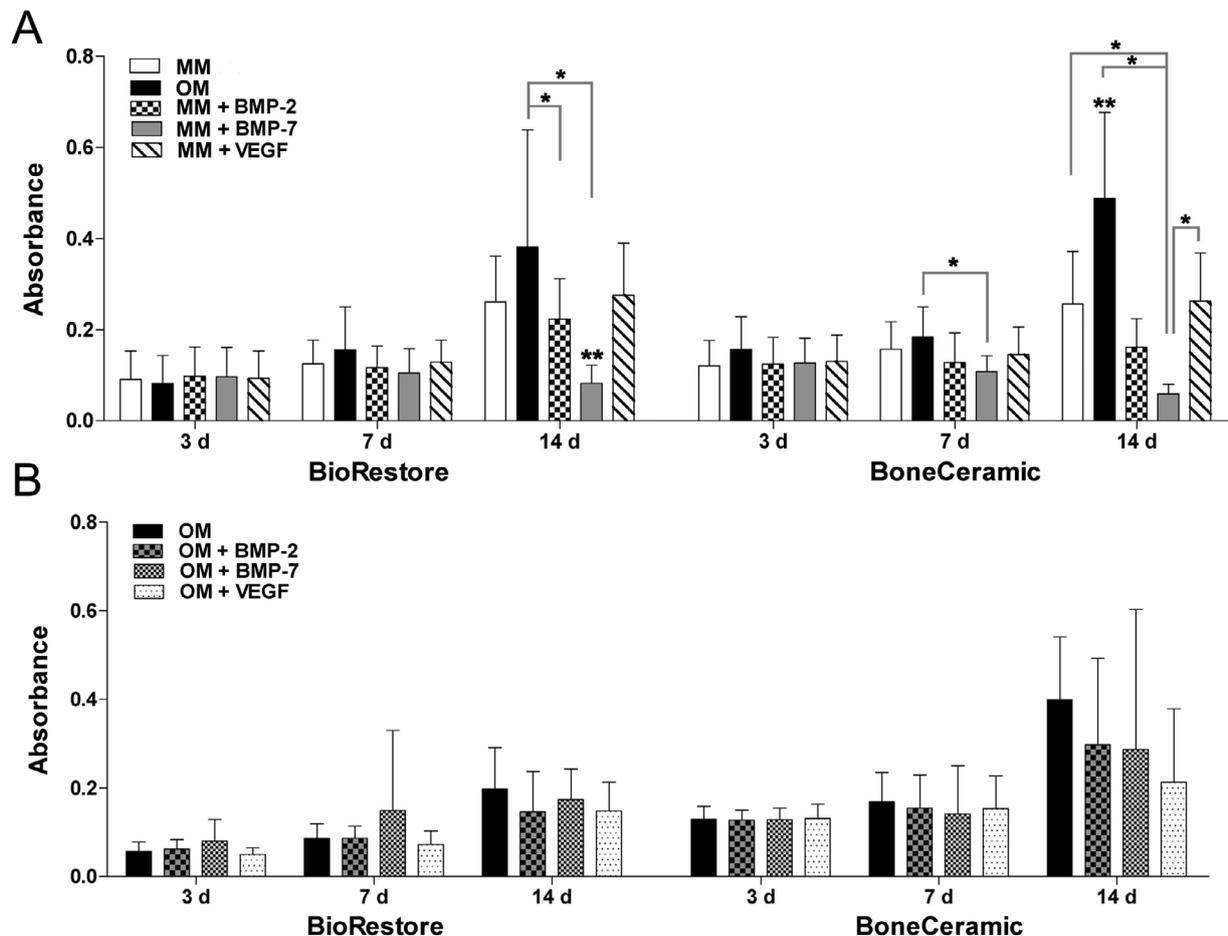


Fig. 5. ALP activity of hASCs at 3-, 7-, and 14-day time points on BioRestore or BoneCeramic. **(A)** Growth factors in MM: ALP activity of hASCs cultured with MM, OM or growth factors in MM. * $p < 0.05$ between the indicated groups. ** $p < 0.05$ with respect to other groups within that time point. **(B)** Growth factors in OM: ALP activity of hASCs cultured with OM or growth factors in OM. No significant differences in panel B. Results are expressed as mean absorbance + SD.

In BoneCeramic, the cell number (Fig. 4a) was significantly higher in OM than in MM cultures (1.5-fold) on day 14. In contrast, Live/dead analysis showed slightly higher cell number in OM than MM on day 7, but no notable difference between OM and MM on day 14 in BoneCeramic (Fig. 3). The ALP activity (Fig. 5a) and collagen production (Fig. 6a) were induced 1.9-fold more with OM than MM in BoneCeramic at day 14 ($p < 0.05$).

Comparison of growth factor induction to MM

In general, all the growth factors tested in the present study supported the viability (Fig. 2 and 3) and proliferation of hASCs (Fig. 4a). BMP-2 and VEGF did not increase but rather supported similar levels of proliferation as MM (Fig. 4a). The result was similar with ALP activity (Fig. 5a) and collagen production (Fig. 6a), where BMP-2 and VEGF resulted in comparable levels as MM in both biomaterials. In contrast to BMP-2 and VEGF, BMP-7 resulted in two times lower cell number than MM in BioRestore ($p < 0.001$). Consistently, ALP activity (Fig. 5a) was three times lower in BMP-7 group than in MM in BioRestore ($p = 0.002$). Equally low amounts of collagen

(Fig. 6a) were produced by all growth factors and MM in hASCs cultured in BioRestore.

The effects of growth factors in BoneCeramic were mostly similar to BioRestore, i.e., no enhancement in comparison to MM. However, in BoneCeramic BMP-7 did not significantly inhibit proliferation. In turn, the adverse effect of BMP-7 was significant in ALP activity; four times lower values than with MM were detected on day 14 (Fig. 5a). Some differences in collagen production (Fig. 6a) were detected between the growth factor groups in BoneCeramic. On day 7, BMP-2 exhibited significantly higher collagen amount than MM and OM ($p < 0.05$), although there was no difference from MM on day 14. Furthermore, VEGF exhibited significantly higher ALP activity and collagen content than BMP-7 (4.4-fold and 1.8-fold, respectively), but not higher in comparison to MM.

Comparison of OM induction to growth factors

Overall, OM showed superior capacity to induce osteogenic differentiation and proliferation of hASCs than the growth factors tested in the study. In BioRestore,

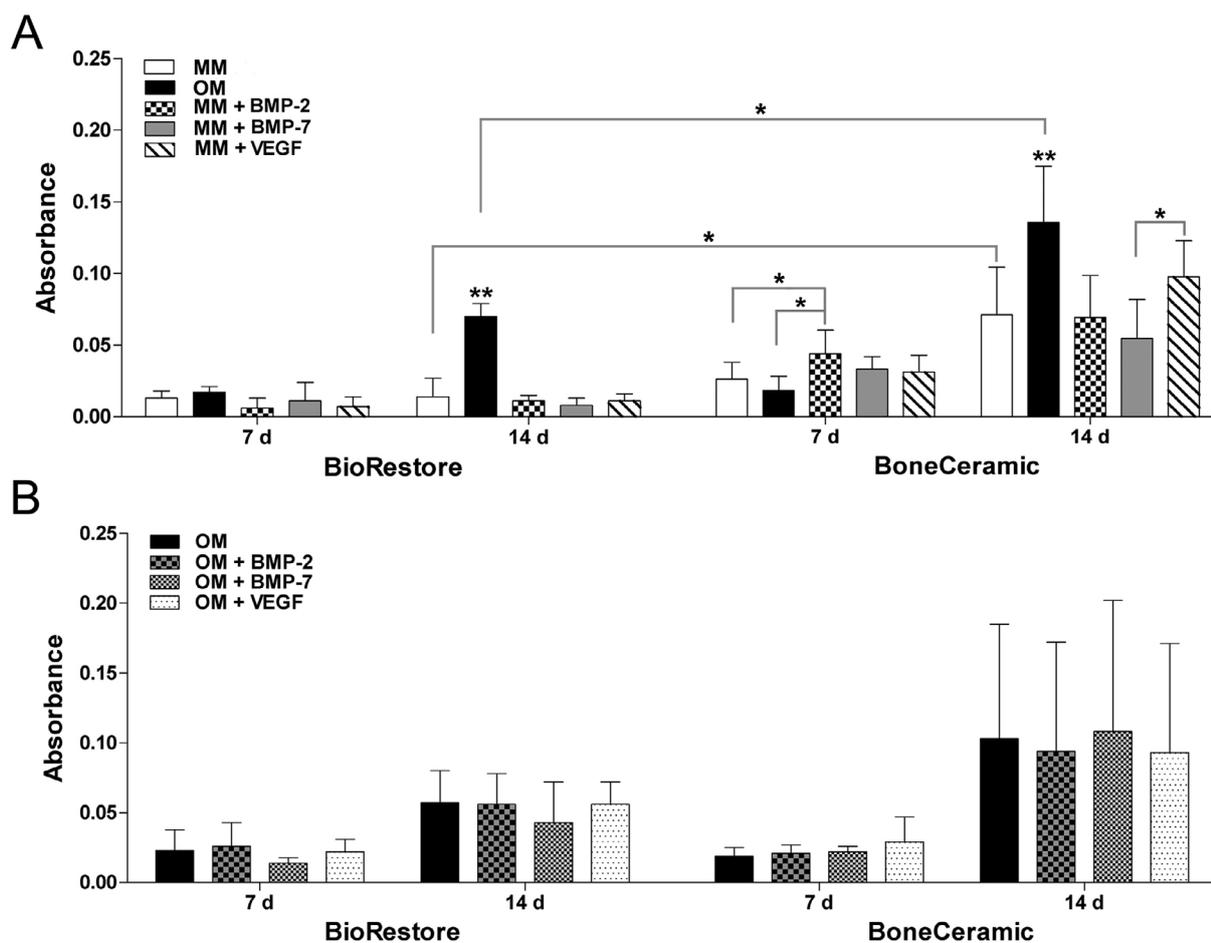


Fig. 6. Collagen production of hASCs at 7- and 14-day time points on BioRestore or BoneCeramic. **(A)** Growth factors in MM: Collagen production of hASCs cultured with MM, OM or growth factors in MM. * $p < 0.05$ between the indicated groups. ** $p < 0.05$ with respect to other groups within that time point. **(B)** Growth factors in OM: Collagen production of hASCs cultured with OM or growth factors in OM. No significant differences in panel B. Results are expressed as mean absorbance + SD.

Live/dead analysis (Fig. 2) showed increased viability and cell number by the OM group when compared with growth factor groups at day 7 and 14. This was confirmed by the quantitative analysis of cell numbers (Fig. 4a); OM induced significantly higher cell numbers than growth factors (1.4- to 2.2-fold) in BioRestore. In a similar way, the collagen production was enhanced 6- to 9-fold by OM when compared to growth factors in BioRestore (Fig. 6a). OM also induced significantly higher ALP activity than BMP-2 (1.7-fold) and BMP-7 (4.6-fold) in BioRestore (Fig. 5a). VEGF resulted in comparable level of ALP activity to MM and OM in BioRestore. The OM-induced ALP activity was donor dependent, and hence was not as consistent as the OM-induced collagen production.

In BoneCeramic, the growth factors supported comparable levels of viability to OM and MM (Fig. 3). However, according to the quantitative analysis of cell number (Fig. 4a), OM did result in significantly higher numbers of cells on day 14 than the growth factor groups ($p < 0.05$). The effect of OM on ALP activity (Fig. 5a) and collagen production (Fig. 6a) in BoneCeramic was significantly higher than with any of the growth factors.

In ALP activity, the effects of OM were 2- to 8-fold, and in collagen production 1.4- to 2.5-fold in comparison to growth factors. In particular, the negative effect of BMP-7 was evident in ALP activity of hASCs seeded on BoneCeramic (Fig. 5a), as the ALP activity of BMP-7 group decreased with time ($p = 0.006$, day 7 versus day 14).

Combination of growth factors with OM

As osteogenic differentiation was detected mostly with OM, in the second part of the study growth factors were added to OM to determine whether greater osteo-induction could be achieved by their combination. The viability (Fig. 2 and 3) and cell number (Fig. 4b) of OM + growth factors were comparable to that of plain OM, although slightly higher than with MM + growth factors. In BioRestore the inhibitory effect of BMP-7 on cell number could be detected even when combined with OM, but the difference was not significant to other groups.

Overall, combining growth factors with OM did not result in enhanced ALP activity or collagen production when compared to plain OM. However, in ALP activity,

the negative effect detected by BMP-7 (Fig. 5a) was rescued when combined with OM (Fig. 5b); in some donor cells OM + BMP-7 increased ALP activity on day 7 in BioRestore and on day 14 in BoneCeramic, although the difference to plain OM was not significant.

When comparing the effects of OM + growth factors between the two biomaterials, the results were comparable to those obtained with MM + growth factors; BioRestore stimulated higher cell number than BoneCeramic, whereas BoneCeramic induced higher ALP activity and collagen production.

Discussion

Every year, over 2 million bone graft operations are carried-out world-wide (Giannoudis *et al.*, 2005). Due to the limitations of the traditional bone-grafting, alternative approaches such as tissue engineering are emerging in order to meet the increasing need for bone substitutes (Betz, 2002). To date, much reliance has been put on the osteo-inductive effect of BMPs (Garrison *et al.*, 2007; Clokie and Sandor, 2008; Mesimaki *et al.*, 2009). However, the cost, safety and effectiveness of growth factors have been the subject of controversy lately (Alarmo *et al.*, 2009; Garrison *et al.*, 2010; Zuk *et al.*, 2011). Due to the several risks and disadvantages related to the use of growth factors, we studied OM as an alternative method to enhance osteogenic differentiation of hASCs. OM containing AsA2-P, Dex and β -GP is a cost-effective osteo-inducer of hASCs *in vitro* as well as *in vivo*. In several *in vivo* studies, the osteogenic commitment of ASCs has been enhanced by OM during the *in vitro* culture prior to implantation (Cui *et al.*, 2007; Di Bella *et al.*, 2008; Bohnenblust *et al.*, 2009; Schubert *et al.*, 2011). For clinical bone tissue engineering it is critical to reduce the total time required to regenerate functional bone tissue. The pre-implantation expansion of hASCs takes several days to weeks, and bone formation after implantation of the construct takes from several weeks to months. In this respect, induction by OM would be a highly cost-effective way to enhance both osteogenic differentiation and proliferation of hASCs during the *in vitro* culture preceding implantation of the construct. Although the feasibility of OM-induction has been illustrated in several *in vivo* studies, the effect of OM has not been systematically compared to BMP-2, BMP-7 and VEGF *in vitro*.

Consequently, the current study demonstrated significantly greater osteogenic differentiation of hASCs by OM over BMP-2, BMP-7 and VEGF treatment in 3D culture, when the growth factors were added to MM. In fact, MM + BMP-2 or MM + VEGF treatment did not enhance the osteogenic differentiation of hASC more than MM alone with either biomaterial. In addition, it was discovered that BMP-7 significantly decreases proliferation and ALP activity of hASCs, an adverse effect that was detected consistently with both biomaterials. As osteogenic differentiation was detected solely with OM, in the second part of the study we tested whether OM could sensitise hASCs to growth factors in order to yield greater osteo-induction. However, the combination of BMP-2, BMP-7 or

VEGF with OM resulted in similar level of osteo-induction to OM alone, suggesting that OM induction is sufficient, and that differentiating hASCs may not respond to growth factor stimulation. Interestingly, the inhibitory effect of BMP-7 was rescued when BMP-7 was combined with OM. Hence, OM showed superiority over BMP-2, BMP-7 and VEGF added to MM, and these growth factors added to OM hold no advantage over plain OM induction *in vitro*.

Some studies have suggested that exogenously added BMP-2 (Knippenberg *et al.*, 2006; Song *et al.*, 2011), BMP-7 (Al-Salleeh *et al.*, 2008) and VEGF (Behr *et al.*, 2011) could have an osteo-inductive effect on ASCs, but in the current study these growth factors did not enhance osteogenic differentiation of hASC more than MM with either biomaterial. Out of these three growth factors, BMP-2 is the most studied with ASCs (Knippenberg *et al.*, 2006; E *et al.*, 2010; Song *et al.*, 2011; Zuk *et al.*, 2011; Mehrkens *et al.*, 2012; Overman *et al.*, 2012). Although BMP-2 concentrations from 50 to 100 ng/mL have successfully induced osteogenic differentiation of ASCs in some studies (Song *et al.*, 2011; Knippenberg *et al.*, 2006), Zuk and co-workers found no effect with doses in the range of 10–100 ng/mL (Zuk *et al.*, 2011). Similar to the findings of the present study and those reported by Zuk and co-workers, a recent study showed no effect by BMP-2 with 50 ng/mL (Mehrkens *et al.*, 2012). However, Mehrkens and co-workers suggested that a 10 times higher concentration, 500 ng/mL, is able to stimulate osteogenic differentiation of hASCs *in vitro* (Mehrkens *et al.*, 2012). In turn, this is in contrast to two studies, where a short, only 15 min treatment with 10 ng/mL BMP-2 was able to support osteo-induction of ASCs for 14–21 days (Knippenberg *et al.*, 2006; Overman *et al.*, 2012). Hence, there is no consensus on the effect of BMP-2 on osteo-induction of ASCs, and further systematic *in vitro* and *in vivo* studies are required.

Several factors may explain the great variation in the outcomes of BMP-2 studies, including use of animal-derived ASCs (Knippenberg *et al.*, 2006; E *et al.*, 2010), differential effects of 3D biomaterials (E *et al.*, 2010; Overman *et al.*, 2012) to 2D culture, and the use of various cell densities and culture media. Nevertheless, various explanations have been suggested for the failure of BMP induction in ASCs and bone marrow stromal cells (BMSCs), including up-regulation of BMP antagonists such as noggin and gremlin (Pereira *et al.*, 2000; Diefenderfer *et al.*, 2003; Sutherland *et al.*, 2004; Zuk *et al.*, 2011). On the other hand, the problem may originate from an insufficient activation of downstream signalling related to phosphorylation and nuclear translocation of certain Smads, the intracellular target proteins of *e.g.* BMP-2 signalling, as proposed by Zuk and co-workers (Zuk *et al.*, 2011). It is obvious that several aspects of cell signalling including timing, dosage, and complex interplay between different effectors and inhibitors need further elucidation to master the effect of growth factors.

The expanding literature on *in vivo* experiments conducted with ASCs suggests two critical requirements for successful bone formation; firstly, the osteogenic commitment of ASCs, and secondly, the presence of a mineral component in a scaffold (Scherberich *et al.*, 2010). The biomaterials used in the current study were

chosen because they represent prospective candidates for hASC-based bone tissue engineering approaches. As such, osteoconductivity and ability to heal bony defects has been reported in clinical and *in vivo* studies for both BioRestore (Moimas *et al.*, 2006; Clozza *et al.*, 2012a; Clozza *et al.*, 2012b) and BoneCeramic (Jensen *et al.*, 2007; Jensen *et al.*, 2009; Frenken *et al.*, 2010; Rokn *et al.*, 2011). Although capable of orthotopic bone formation, BoneCeramic failed to form ectopic bone together with BMSCs in subcutaneous rodent models (Zimmermann *et al.*, 2011; Mrozik *et al.*, 2012) indicating a need for enhanced osteo-induction. Recently, a similar biomaterial to BoneCeramic, except in a porous block format, was shown to increase bone formation in segmental femoral defects significantly more when seeded with hASCs than without cells (Choi *et al.*, 2011). In a clinical study of 13 patients, BioRestore alone was able to induce formation of woven bone in tooth extraction sockets, although no mature lamellar bone was detected within a 6 months healing period (Clozza *et al.*, 2012b). However, the bone formation capacity of BioRestore could be enhanced significantly by hASC transplantation in the future. Promising *in vitro* results have been reported previously for culturing hASCs in BioRestore scaffolds under control conditions (Haimi *et al.*, 2009b). Previous *in vitro* studies with BoneCeramic have been conducted with fibroblasts (Kauschke *et al.*, 2006), periodontal ligament fibroblasts (Mrozik *et al.*, 2012) and BMSCs (Mrozik *et al.*, 2012), but this is the first study to report hASC response on BoneCeramic and BioRestore when combined with osteo-induction by growth factors or OM.

The aim of the current study was to compare the efficiency of OM and growth factors for the osteo-induction of hASCs using two clinically prospective biomaterial carriers. Importantly, our findings indicate that the 3D biomaterial plays a notable role in hASC behaviour. The most significant difference between the 3D hASC-carriers, BioRestore and BoneCeramic, was detected in their ability to stimulate collagen production by hASCs. BoneCeramic induced a significantly larger amount of collagenous matrix than BioRestore under control conditions, and the effect was further enhanced with OM. Although different types of collagens are present in several tissues, the onset of osteoblastic differentiation is strongly related to the formation of organic matrix, mainly consisting of collagen type I, which will be subsequently mineralised – a process that is in turn facilitated by the accumulation of phosphate as a function of ALP enzyme. The differential behaviour of hASCs may be dependent on differences between the chemistry, topography, or 3D structure of the biomaterials.

The other major difference between the biomaterials was detected in their capacity to support cell proliferation, as BioRestore resulted in a significantly higher hASC number than BoneCeramic, an effect likely related to the 3D structure of the biomaterials. The fibre structure of BioRestore provides large surface area and allows the cells to spread more easily throughout the scaffold, whereas the granular format in BoneCeramic provides less contact area between the granules, hindering the spreading of the cells. This hypothesis was further supported by the viability assay, as some BoneCeramic granules were fully populated by hASCs whereas other granules in the same well had no

or very few cells. Based on these results, a larger initial cell seeding concentration would be recommended for BoneCeramic when considering clinical approaches to guarantee sufficient and evenly distributed cell population over the biomaterial. Although the comparison of these two biomaterials is challenging, due to the major differences in their 3D structures, based on our results both carriers represent viable alternatives for hASC-based bone tissue engineering as such, although combined stimulation with OM is suggested for efficient osteo-induction.

Based on the valuable *in vitro* findings of the present study, a corresponding *in vivo* study to demonstrate the utility of OM over growth factors has been planned. In order to reduce the number of animal experiments to be performed, it was critical to evaluate the hASCs response to growth factors and OM *in vitro*. For example, based on our results, there is no point in testing growth factors with OM, because there was no extra benefit from OM + growth factors in comparison to OM alone. Therefore, it is not necessary to test all the groups used in the current study *in vivo*. However, in the future *in vivo* study, effectiveness of OM over growth factors in MM should be demonstrated.

Conclusions

To conclude, this is the first study to demonstrate that OM enhances hASC differentiation towards bone-forming cells in 3D culture significantly more than the traditionally used growth factors BMP-2, BMP-7 and VEGF. Both biomaterials tested in this study, BioRestore and BoneCeramic, supported the hASC viability and proliferation during 14 days of culture. The most significant osteogenic differentiation of hASCs was achieved by BoneCeramic combined with OM. In contrast, combining growth factors with either biomaterial did not increase osteogenic differentiation or proliferation when compared to MM. Moreover, BMP-7 consistently inhibited proliferation and osteogenic differentiation of hASCs in 3D culture. Combining OM with BMP-7 eliminated the adverse effect of BMP-7. Otherwise, there was no significant benefit from adding growth factors to OM. Our results indicate that instead of BMP-2, BMP-7 or VEGF, OM should be used to obtain successful osteogenic commitment of hASCs *in vitro*.

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Discussion with Reviewers

Reviewer I: Fig. 6 compares collagen content among different groups, what is the collagen normalised to among different groups?

Authors: Fig. 6 shows the absolute values obtained as absorbance; hence the results have not been normalised. Absorbance is directly proportional to the amount of collagen in the samples; therefore, a reliable comparison among different groups can be obtained using absolute values. Unfortunately, with the collagen assay, it was not possible to determine cell number from the same samples.

Reviewer I: Authors need to provide *in vivo* data to demonstrate that ADSC preconditioned in osteogenic medium are better at forming bone *in vivo* than ADSCs exposed to BMP-2 or any other growth factors. Results from these studies would enhance the data being reported.

Authors: The authors agree that *in vivo* study is important to further demonstrate the efficiency of OM over BMP-2, BMP-7 and VEGF. However, the authors feel that the present study in its current form provides valuable information on the effects of BMP-2, BMP-7, VEGF and OM on hASC proliferation and osteogenic differentiation in 3D scaffolds, and behaviour of hASCs in two clinically relevant biomaterials. These valuable findings can be utilised to better plan the corresponding *in vivo* study. In order to reduce the number of animal experiments to be performed, it is critical to evaluate the hASCs response to growth factors and OM *in vitro*. For example, based on our results, there is no point in testing growth factors with OM, because there was no extra benefit from OM + growth factors in comparison to OM alone. Therefore, it is not necessary to test all of the groups used in the current study *in vivo*. However, in the future *in vivo* study, effectiveness of OM over growth factors in MM should be demonstrated. It is clear that systematic *in vitro* data is of fundamental importance before it is reasonable to conduct *in vivo* studies. The *in vitro* results also facilitate the correct interpretation of the *in vivo* results.

Reviewer I: The present report concludes that osteogenic medium is superior in enhancing adipose derived stem cells differentiation toward osteogenic lineage than bone morphogenetic proteins. Although, the data reported is valid, use of osteogenic medium to assess osteogenic differentiation is an established protocol. There are controversies regarding response of adipose derived stem cells to BMP-2 to induce osteoblasts differentiation; some reports have shown positive response others have shown reduced response. The findings reported in this manuscript thus confirm original observations. Please comment.

Authors: We would like to note that the OM used in our study is not that traditionally used, but specifically optimised for hASCs. The modified OM with low Dex (5 nM) and high AsA2-P (250 µM) concentration used in this study may have contributed to the superior effect of the OM over the tested growth factors. Because the traditional OM with high Dex (100 nM) and low AsA2-P (50 µM) was originally developed based on studies with BMSCs (Jaiswal *et al.*, 1997, additional reference), it has been reported that OM with lower Dex and higher AsA2-P is more optimal for ASCs (De Girolamo *et al.*, 2007, text reference; Kyllönen *et al.*, unpublished results). However, our study, where the efficiency of the modified OM over the traditional OM has been reported, is under submission (Kyllönen *et al.*, unpublished results). This modified OM has been used successfully for the efficient osteo-induction of hASCs previously (Tirkkonen *et al.*, 2011, text reference). Although several studies have been conducted with BMP-2, there is still no consensus on the effect of BMP-2 on osteo-induction of hASCs. In addition, the effects of BMP-7 and VEGF have been even less reported on hASCs than BMP-2. Furthermore, in the present study, these growth factors were systematically compared in control medium and OM.

Additional Reference

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