J Mater Sci: Mater Med (2010) 21:353–363
DOI 10.1007/s10856-009-3840-z

β-Tricalcium phosphate 3D scaffold promote alone osteogenic differentiation of human adipose stem cells: in vitro study

Gerardo Marino · Francesco Rosso · Gennaro Cafiero · Carla Tortora · Marco Moraci · Manlio Barbarisi · Alfonso Barbarisi

Received: 7 May 2009 / Accepted: 24 July 2009 / Published online: 5 August 2009

Abstract Human adipose tissues surgically resected from the subcutaneous abdominal region were enzymatically processed to obtain Human Adipose Stem cells (fibroblast-like adipose tissue-derived stromal cells—ADSC-FL) that were immunophenotypically characterized using a panel of mesenchymal markers by flow cytometry. The formation of new hydroxyapatite crystals in culture dishes, by differentiating cells, further demonstrate the osteogenic potential of purified cells. The aim of this study was to evaluate the osteogenic differentiation potential of ADSC-FL seeded onto a porous β-tricalcium phosphate (β-TCP) matrix. ADSC-FL was cultured on the β-TCP matrix in medium with or without osteogenic differentiation additives. Time-dependent cell differentiation was monitored using osteogenic markers such as alkaline phosphatase (activity assay), osteocalcin and ostopontin (ELISA method) expression. Our results reveal that β-TCP triggers the differentiation of ADSC-FL toward an osteoblastic phenotype irrespective of whether the cells are grown in a proliferative or a differentiative medium. Hence, a β-TCP matrix is sufficient to promote osteoblastic differentiation of ADSC-FL. However, in proliferative medium, alkaline phosphatase activity was detected at lower level respect to differentiative medium and osteocalcin and osteopontin showed an expression delay in cells cultured in proliferative medium respect to differentiative one. Moreover, we observed an increase in FAK phosphorylation at level of tyrosine residue in position 397 (Western-blot) that indicates a good cell adhesion to β-TCP scaffold. In conclusion, our paper demonstrates that a three-dimensional β-TCP scaffold in vitro triggers on its own the differentiation of ADSC-FL toward an osteoblastic phenotype without the need to use differentiative media.

G. Marino (✉) · F. Rosso · G. Cafiero · C. Tortora · M. Moraci · M. Barbarisi · A. Barbarisi
Laboratory of Applied Biotechnology, Department of Anaesthesiological, Surgical and Emergency Sciences, Second University of Naples, via Costantinopoli 16, 80138 Naples, Italy
e-mail: gerardo.marino@unina2.it

1 Introduction

Among the various sources currently available for hMSCs, adipose tissue appears to be one of the most promising candidates for cell therapy and tissue engineering. hMSCs isolated from adipose tissue have been differentiated into adipogenic, chondrogenic, myogenic, and osteogenic cells when cultured under appropriate conditions [1]. In this study, we use fibroblast-like cells obtained from human adipose tissue after collagenase digestion. These cells were immunophenotyped with a panel of mesenchymal differentiation markers according to current literature [2, 3]. Phenotypically, hMSCs have been defined as $CD44^+$, $CD105^+$, and negative for hematopoietic lineage markers [4]. Fibroblast-like adipose tissue-derived mesenchymal stem cells (ADSC-FL) are morphologically similar to hMSCs obtained from other tissues by isolation and culturing [2]. ADSC-FL has the capacity to differentiate into cells of mesenchymal origin, such as adipocytes, myocytes, chondrocytes, and osteocytes [5]. As reported in literature, several factors can influence the isolation and growth of ADSC-FL, such as age of the donor, adipose tissue type (white or brown), harvest location (subcutaneous or visceral fat tissue), culture conditions, plating density, and media formulation [5].

In regenerative medicine, scaffolds with different calcium/phosphate stoichiometric ratio are widely used for permanent implants [6]. These materials have been shown

to have osteoinductive properties and may be useful in bone tissue engineering. Although little is known yet about the mechanisms that regulate the differentiation of MSCs through their interactions with the ExtraCellular Matrix (ECM), the physical structure and the chemistry of a biomaterial surface are known to modulate the signals from the ECM directing proliferation and differentiation of osteogenic progenitor cells [7].

The sites of cellular adhesion are called focal contacts or adhesion plaques [8], and in the most general case the external faces of focal contacts present specific receptor proteins such as integrins; on the internal face, instead, proteins like talin, paxillin, vinculin, tensin are present, known for mediating the interactions between actin filaments and membrane receptor proteins [9].

Focal adhesion kinase (FAK or pp125FAK) was found to be a ubiquitous nonreceptor protein tyrosine kinase [10], co-localizing with integrins at focal adhesions in adherent cells [11]. This enzyme mediate integrin-dependent regulation of the Erk family of MAP kinases through several pathways [12]; which may act synergistically with mitogenic signaling pathways to regulate cell growth and differentiation [13–15].

In particular, tyrosine residue in position 397 is the major site of autophosphoryation and Y397 a site of interaction with the SH2 domain of Src.

In general, the Y397 FAK phosphorylation represent the first event for the kinase activation [16], and the presence of high phosphorylation level indicates a good cell adhesion to the substrate [11, 17].

Efforts to demonstrate the ability of β-TCP matrix to promote Humane Bone Marrow Mesenchymal Stem Cell and ADSC-FL cells differentiation towards osteogenic phenotype were present in literature [7, 18–21].

Muller et al., demonstrated that calcium phosphate matrix seeded with Human Bone Marrow Mesenchymal Stem Cell (bMSC) not only promote osteoblast-specific marker expression compared with cells on culture dishes, but stimulate osteoblast differentiation of bMSC without addition of osteogenic additive in culture medium [7].

Moreover, Birk et al. demonstrated the ability of marine calcium carbonate matrix to support, alone, differentiation of preadipocytes to osteoblasts, supporting the idea that osteogenic and adipogenic phenotypes have a high interconversion potential [18].

Hicok et al. showed that HA-TCP matrix containing osteogenic predifferentiated ADSC-FL cells, implanted subcutaneously in severe combined immunodeficiency (SCID) mice, form osteoid in higher percentage respect to the same cells seeded on Collagraft (a HA-TCP composite covered with collagen) [19]. Justesen et al. used extramedullary adipocytes, predifferentiated both in osteogenic and in adipogenic culture media mixed with β-TPC powder, for ectopic implant in immunodeficient mice. The authors demonstrated that β-TCP matrix promote osteogenic differentiation of extramedullary adipocytes in vivo [20].

Finally, Hattori et al. demonstrated that β-TCP matrix seeded with predifferentiated bone marrow-derived mesenchymal stem cells and adipose tissue-derived stromal cell in ectopic implants in nude mice, have a similar ability to induce osteogenic differentiation and bone formation in vivo [21].

The same paper also showed that bone marrow-derived mesenchymal stem cells and adipose tissue-derived stromal cells had, in vitro, the same ability to induce osteogenic differentiation and bone formation when cultured in osteogenic medium [21]. Hence, using literature data, it was observed that ADSC-FL cells seeded on β-TCP matrix differentiate in vivo towards osteogenic phenotype. In particular, Hattori et al. showed that the β-TCP matrix mixed with ADSC-FL cells results, after 8 weeks of ectopic implantation, in osteoid formation.

However, since those studies focused on in vivo experiments, where cells are exposed to a variety of other molecules possibly promoting osteogenic differentiation, the role of β-TCP on its own in promoting ADSC-FL osteogenic differentiation cannot be easily singled out.

Recently, same authors have investigated "in vitro" the osteogenic potential of a β-TCP surface seeded with ADSC-FL cells. In particular, Liu et al. [22] studied the osteogenic differentiation of ADSC-FL cultured in proliferative and differentiative media on two-dimensional TCP surfaces.

Moreover, a lot of papers concern the study of ADSC-FL seeded on TCP matrix blended with various organic polymers [23–25] at the aim to improve the mechanical properties and biological degradation.

For these reasons, with the aim to study the effect of β-TCP matrix on the osteogenic potential of ADSC-FL cells, we choose an in vitro three-dimensional (3D) model able to avoid the interference of in vivo environment (e.g., presence of soluble differentiative factors coming by blood circulation or locally produced).

2 Materials and methods

2.1 Scaffold

Porous β-tricalcium phosphate (β-TCP) scaffolds (Synhapor^{TM}) were obtained from Hi-Por Ceramics Ltd (Sheffield, UK). The pore size was in the range 200–500 μm (diameter), and the average void volume was 82%. Round β-TCP dishes (diameter 10 mm, thickness 2 mm) were used in this study. Cells were seeded at density of 2×10^5 cells/β-TCP scaffold.

Table 1 The data on age and body mass index of five subjects

No.	Sex	Age	IMC Kg/m^2
1	Male	32	23
2	Male	35	23
3	Male	40	22
4	Female	38	20
5	Female	35	21

2.2 Procedure for selection of subjects of the study

Adipose tissue samples were obtained from the subcutaneous abdominal region of three male and two female adults in the age range between 30 and 40 years undergoing elective abdominal surgery. Patients suffering from an inflammatory or malignant disease were excluded. The operations were carried out for the following reasons: two for herniotomy, two for cholecystectomy, and one for tubal ligation. All subjects were of normal weight as defined by guidance of the World Health Organization. Body mass index (IMC) for male and females was between 18.5 and 24.9 Kg/m^2. The data on age and body mass index of the individual subjects are given in Table 1. All patients had a normal physical examination and routine laboratory tests were within normal limits. None of the subjects was under a reduction diet at the time of study. The procedure followed in this investigation has been approved by the Ethical Committee of the Second University of Naples.

2.3 ADSCs isolation, expansion and osteogenic differentiation

Five grams of tissue were washed four to five times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS supplemented with 1% bovine serum and 0.1% collagenase typeI (Worthington Biochemical Corporation, Lakewood, NJ) and incubated at 37°C under continuous shacking for 60 min. The samples were then centrifuged for 5 min at 300 g at room temperature. The supernatant, containing mature adipocytes, was discarded and the pellet washed with PBS and centrifuged at 150 g for 5 min at room temperature. The pellet were resuspended in DMEM-Ham's F-12 medium supplemented with 5% FBS [Gibco, Invitrogen], 100U penicillin/100 μg, streptomycin/0.25 μg, and fungizone (defined as proliferative medium) and lives nucleated cell were counted using Trypan Blue exclusion Test using a chamber counting. Nucleated cells were plated immediately in T25 flasks at a density of 1×10^5 cells\cm^2. After 48 h of incubation at 37°C and 5% CO_2, the cultures were washed with PBS to eliminate the non-adhesive cells and the adherent cells were maintained in proliferative medium until they achieved confluence (about 4 days). For culture expansion, cells were harvested with 0.05% trypsin-0.53 mM EDTA (Gibco-Invitrogen) and replated at a density of 5000 cells/cm^{2}until semiconfluence about 3 days.

For osteogenic differentiation we used DMEM-Ham's F12 medium supplemented with 5% FBS, 100 U penicillin/100 Lg, streptomycin/0.25 Lg, fungizone, 10 mM β-glycerophosphate, 20 nM dexamethasone, and 50 mg/ml sodium 2-phosphate ascorbate (defined as differentiative medium). ADSCs obtained from each subject were submitted to experimental procedures, statistical data were achieved calculating media and standard deviation.

2.4 Immunephenotype

ADSC-FL was cultured in proliferative medium for 21 days prior to analysis (five passages) [26]. Cells were detached using 10 mM EDTA in phosphate-buffered saline (PBS), counted by Trypan Blue exclusion Test and washed in 0.1% BSA in PBS. At least 200,000 cells (in 150 (l PBS/0.5% BSA) were incubated with fluorescent-labelled monoclonal antibodies (1/15 diluted 4C° for 30 min in the dark).

Cells were labeled with the following anti-human antibodies: CD3-APC, CD13-APC, CD14-FITC, CD33-APC, CD34-PE, CD44-FITC, CD45-FITC, (all from Becton Dickinson [BD], San Jose, CA, USA); CD105 PE (Serotech, Kidlington, UK); CD133 PE (Miltenyi, Bergisch Gladbach, Germany); mouse isotype antibodies served as respective controls (BD). After washing steps, more than 5,000 labelled cells were acquired and analyzed by flow cytometry using a BD FACS Calibur equipped with ModFit LT software.

2.5 Cell viability and proliferation

Cell viability and proliferation was evaluated by a modified MTT method according to the instructions of the manufacturer (Dojindo Molecular Technologies-USA). Briefly, quantification of mitochondrial dehydrogenase activity was achieved via the enzymatic conversion of MTT tetrazolium water soluble salt to a coloured formazan product. Since reduction of MTT occurs only in metabolically active cells, the level of activity was a measure of cell viability. ADSC-FL were seeded on β-TCP scaffolds at density of 2×10^5 cells/β-TCP scaffold and incubated in either proliferative and osteogenic differentiative medium. Cell viability was detected at 7, 14, 21 and 28 days at 37°C and 5% CO_2, each experimental point was performed in quintuplicate.

2.6 Environmental scanning electron microscopy (ESEM)

ADSC-FL were plated on β-TCP scaffolds (diameter 10 mm, thickness 2 mm) at a density of 2×10^5 cells/scaffold and incubated in either proliferative or osteogenic differentiative medium for 7 and 28 days at 37°C under 5% CO_2 95% air. At the end of the experimental times, cultures were rinsed gently three times in PBS, fixed with 2.5% wt/wt glutaraldehyde/PBS, then rapidly dipped in deionized water to avoid crystal precipitation. Samples were immediately mounted onto holders, and observations were performed with an ESEM Quanta 200 FEI electron microscope equipped with Electron Dispersion Spectroscopy (EDS) by EDAX. Environmental conditions in ESEM modality were 75% relative humidity, 5°C, and 4.90 Torr.

2.7 EDS analysis

During ADSC-FL cells incubation in osteoblastic differentiating medium in dish culture, new crystals formation was observed by light microscopy**.** We collected them by washing in bidistilled water. Since the mineral matrix of human bone is constituted by Hydroxyapatite, secreted by mature osteoblasts, we used Electron Dispersion Spectroscopy (EDS) to evaluate the chemical nature of these newly formed crystals. In particular, we studied the Calcium/Phosphate atomic ratio that is a specific chemical parameter able to discriminate between various hydroxyapatite isoforms.

2.8 Alkaline phosphatase activity assay

The alkaline phosphatase enzyme (ALP) is expressed in the early stages of differentiation and is a marker of osteoblastic phenotype. ADSC-FL cells were seeded onto β-TCP matrix (2×10^5 cells/scaffold) and onto culture dishes (5000 cells/cm^2) and incubated either in proliferative and osteogenic differentiative media for 3, 7, 14, 21, and 28 days at 37C° under 5% CO_2 95% air. At each experimental point, cells were harvested from β-TCP scaffolds and culture dishes using 0.05% trypsin-0.53 mM EDTA for 10 min at 37°C and washed with PBS, lysed in 0.5 ml of 0.2% Triton X-100 aqueous solution under shaking for 20 min at room temperature. The specific activity of ALP was quantified with an ALP Assay Kit (BioAssay System Kit, Hayward, CA USA) based on the transformation of p-nitrophenylphosphate to p-nitrophenol (PNP). Optical density was spectrophotometrically measured at 405 nm. ALP activity was normalized to total protein content. Total protein was determined using the Bradford method (BioRad protein assay kit, Munich, Germany).

Statistical data analysis was achieved calculating media and standard deviation, each experimental point was performed in triplicate.

2.9 Quantitative estimation of osteocalcin

ADSC-FL were seeded onto β-TCP (2×10^5 cells/scaffold) and culture dishes (5000 cells/cm^2).Cells were incubated either in proliferative and in osteogenic differentiative media for 7, 14, 21 and 28 days at 37°C, under 5% CO_2 95% air. The culture media was changed every 3 days. 24 h before the test, the culture media was changed to DMEM containing only antibiotics. After incubation for 24 h, the secreted osteocalcin was quantified with the GLA-type Osteocalcin EIA Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's indications. Osteocalcin secretion was normalized to total secreted proteins. Total protein was determined using the Bradford method (BioRad protein assay kit, Munich, Germany).

Statistical data analysis was achieved calculating media and standard deviation, each experimental point was performed in triplicate.

2.10 Quantitative estimation of osteopontin

ADSC-FL were seeded onto β-TCP (2×10^5/scaffold) and culture dishes (5000 cells/cm^2).Cells were incubated either in proliferative and in osteogenic differentiative media for 7, 14, 21 and 28 days at 37°C, under 5% CO_2 95% air. The culture media was changed every 3 days. 24 h before the test, the culture media was changed to DMEM containing only antibiotics. After incubation for 24 h, the secreted osteopontin was quantified with Osteopontin EIA Kit (Osteopontin (human) TiterZyme® EIA kit, Assay Designs) according to the manufacturer's indications. Osteopontin secretion was normalized to total secreted proteins. Total protein was determined using the Bradford method (BioRad protein assay kit, Munich, Germany).

Statistical data analysis was achieved calculating media and standard deviation, each experimental point was performed in triplicate.

2.11 Immunoprecipitation

Cells were seeded on culture dishes (5000 cells/cm^2) and on β-TCP (2×10^5/scaffold) in proliferative and osteogenic differentiative medium. After 3, 7 and 14 days cells were detached from culture dishes and β-TCP scaffolds with

trypsin 0.05%, EDTA 0.02%, NaCl 0.085%, centrifuged at 800 g for 10 min and resuspended and lysated in 0.2 ml of ice-cold RIPA buffer with 50 mM HEPES, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, and 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail from SIGMA as suggest by the supplier. Lysates were clarified by centrifugation at 15,000 g for 10 min and the pellets discarded. After centrifugation, supernatants were transferred to fresh tubes, then to protein assay (Bio-Rad). Successively, samples were normalized for protein concentration and immunoprecipitated (4°C, 2 h) with protein-A Sepharose linked to polyclonal anti-FAK antibody [27]. Immunoprecipitates were rinsed three times with RIPA buffer and extracted in 2% SDS–PAGE sample buffer (200 mM Tris-HCl, pH 6.8, 1 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, 10% glycerol), by boiling 5 min and resolved by 7% SDS–PAGE.

2.12 Western blotting

After SDS–PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, membranes were blocked for 1 h with 5% nonfat dried milk in Tris buffer saline Tween 20 0.05% pH 7.2 (TBST) and incubated for 2.5 h at 4°C with anti-phosphoFAK (tyr 397) monoclonal antibody (0.1 mg/ml). The membranes were rinsed three times with TBS–0.05% Tween 20, incubated with secondary antibodies (horseradish peroxidase conjugated goat antimouse IgG antibody) diluted 1:5,000 in phosphate buffer solution (PBS), for 1 h at room temperature. After rinsing three times with TBS Tween 20 0.05%, the immunoreactive bands were visualized with enhanced chemiluminescence detection reagents (ECL, Bio-Rad, Hercules CA). Protein signals on PVDF membranes were assessed with the ChemiDocTM XRS imaging densitometer (Bio-Rad), using the Quantity One software program (Bio-Rad). For detecting total FAK signals, PVDF membranes were stripped with 0.25 M Tris-HCl pH 6.8, 2% SDS and 100 mM b-Mercaptoethanol, and reprobed with anti-FAK antibody (incubated overnight at 4°C). Visualization and quantification were performed with the same procedure reported above for phosphoFAK (tyr 397).

FAK activation per sample was calculated as the densitometric intensity ratio between the phosphoFAK (tyr 397) and the total FAK signals as described by Walsh et al. [28].

For western blotting analysis we used a pool of ADSC-FL cells derived from five donors. Statistical data analysis was achieved calculating media and standard deviation, each experimental point was performed in triplicate.

3 Results

3.1 ADSC-FL harvesting and immunophenotype

Five grams of adipose tissue from the subcutaneous abdominal region of each donor was used to isolate ADSC-FL cells. After collagenase digestion and 48 h of incubation in expansion media adhered fibroblast-like ADSC cells were obtained (Fig. 1). A cell aliquot was cultured for 21 days (five passages) to achieve immunophenotype stabilization [26]. Moreover, cells were observed daily to assess the morphological homogeneity. Characterization using a panel of surface markers revealed that ADSC-FL were negative for CD3, CD14, CD33, CD34, CD45, and CD133 while they were positive for CD13, CD44, and CD105 (Fig. 2).

3.2 Cell viability and proliferation

Figure 3 showed the increasing proliferation rate of ADSC-FL cells seeded, on β-TCP scaffold indicating that cells were metabolically active. At each experimental point no statistically difference was observed between proliferative and differentiative media.

3.3 Environmental scanning electron microscopy

ESEM is an electron optical instrument which enables the examination of the surface of soft, hydrated, unfixed, uncoated, and electrically insulated specimens with a depth of field and magnifications that typically achieved by Conventional SEM.

Using ESEM technology, we studied the interactions of ADSC-FL with the β-TCP matrix. Electron micrographs (Fig. 4) showed that ADSC-FL cells were spread out and

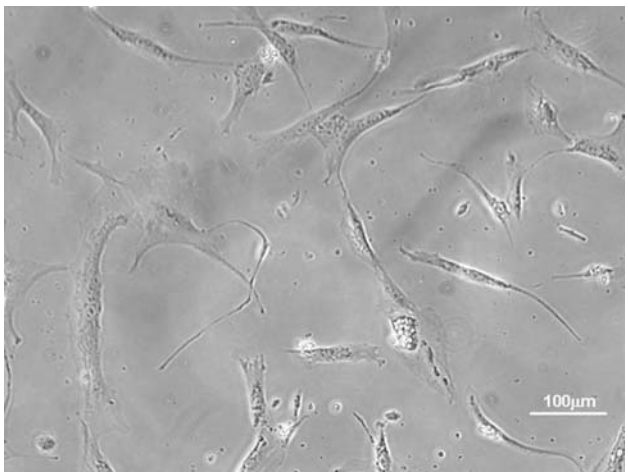

Fig. 1 ADSC-FL harvested from human adipose tissue after 48 h of incubation in proliferative medium

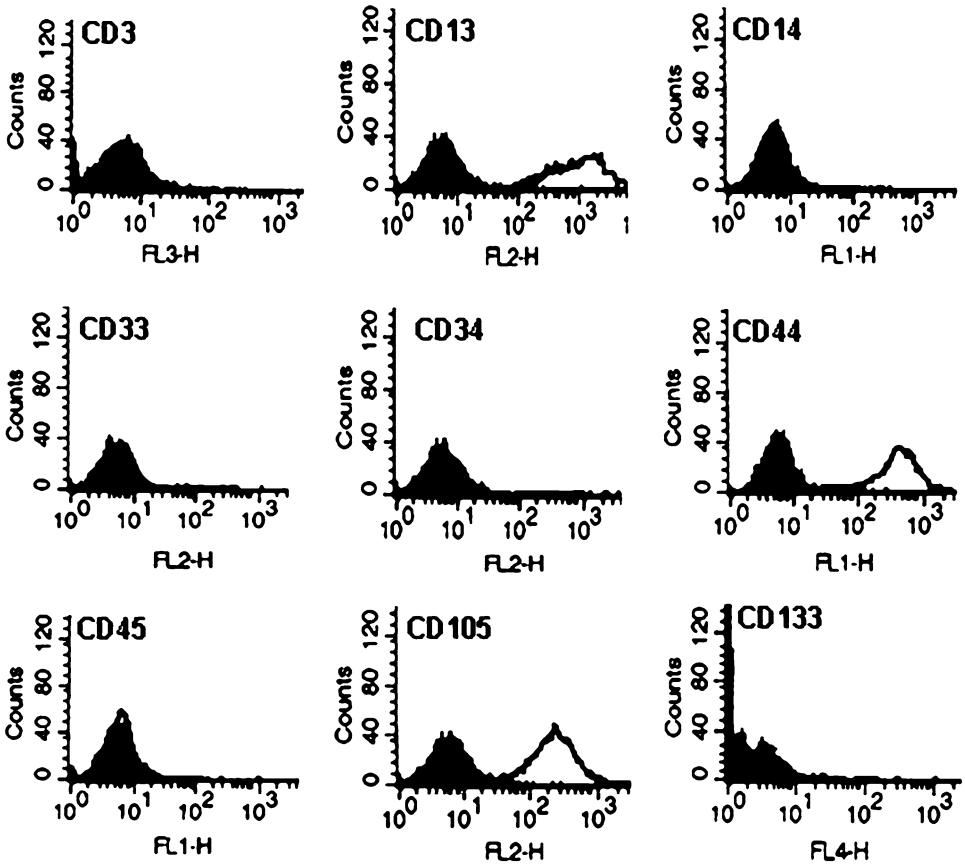

Fig. 2 Flow cytometry histogram of ADSC-FL. The open histogram indicates the positive stained cells, whereas the filled histogram indicates the isotype-matched monoclonal antibody control

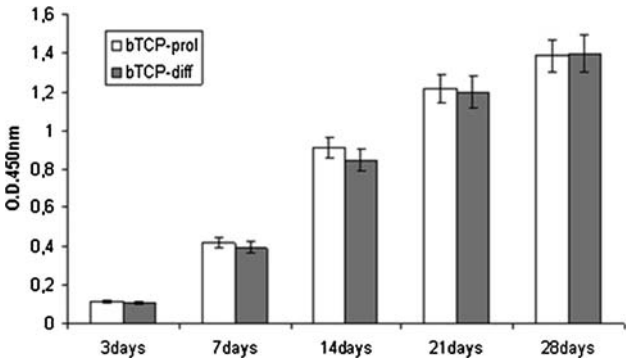

Fig. 3 Vitality of ADSC-FL cells. *β-TCP prol* ADSC-FL cells seeded on β-TCP in proliferative medium. *β-TCP diff* ADSC-FL cells seeded on β-TCP in osteogenic medium

interacted with the β-TCP surface after seven (panels a and c) and 28 (panels b and d) days of incubation in both proliferative and differentiative media, showing a plumped morphology, characteristic of osteoblasts.

3.4 Electron dispersion spectroscopy analysis

To study the differentiation of ADSC-FL cells (culture dishes) toward osteogenic phenotype, we evaluated the chemical nature of newly formed crystals, harvested from culture media, by EDS. The Ca/P atomic ratio was about 1.63 ± 0.03, characteristic of human bone hydroxyapatite (Figs. 5, 6) [29, 30].

3.5 Alkaline phosphatase activity assay

ALP activity of ADSC-FL cells cultured in dishes and on β-TCP scaffolds in proliferative and osteogenic differentiative media at various time points are given in Fig. 7. Obviously, no ALP activity was observed for cells seeded on dishes with proliferative medium; conversely, in osteoblastic differentiative medium, cells had an ALP expression peak at around 14 days of culture, followed by a decrease at 21 and 28 days.

Cells seeded on β-TCP scaffolds with osteogenic differentiative medium followed a similar ALP activity profile, but each experimental point was more intense with respect to dish-cultured cells.

Surprisingly, also cells seeded onto β-TCP scaffolds and cultured in proliferative medium had an ALP activity profile similar to those cultured in the osteogenic differentiative environment (Fig. 7).

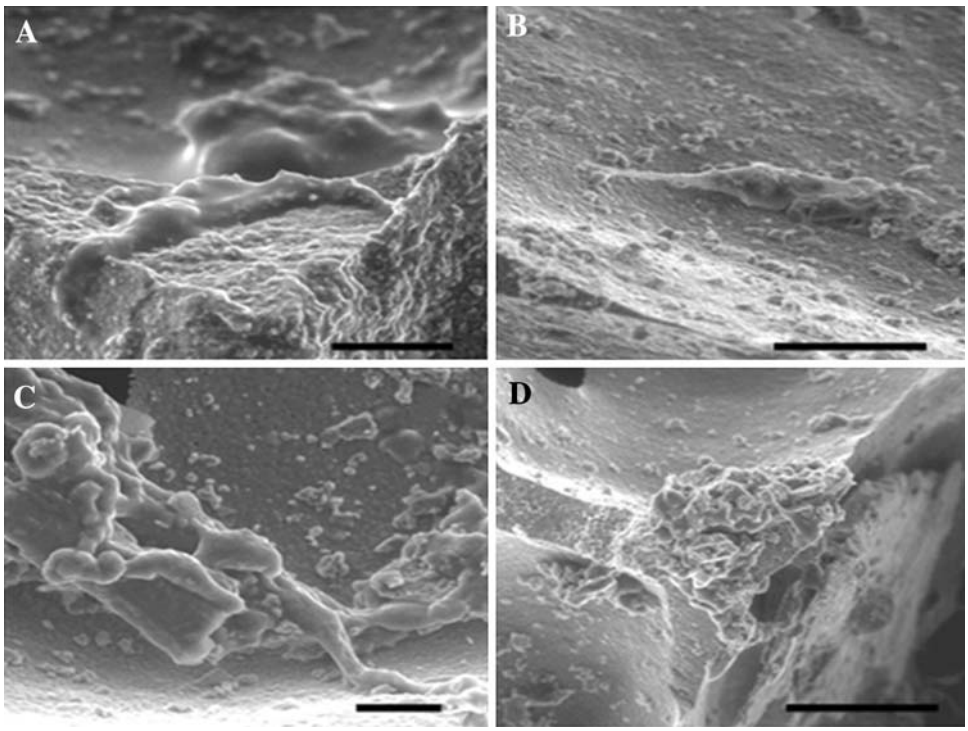

Fig. 4 ESEM micrographs of ADSC-FL seeded onto β-TCP scaffolds. Panels **a** and **c**, cells after 7 and 28 days of culture in proliferative medium. Panels **b** and **d**, cells after 7 and 28 days of culture in differentiative medium. **a–b** Bars = 20 μm; **c–d** Bars = 200 μm

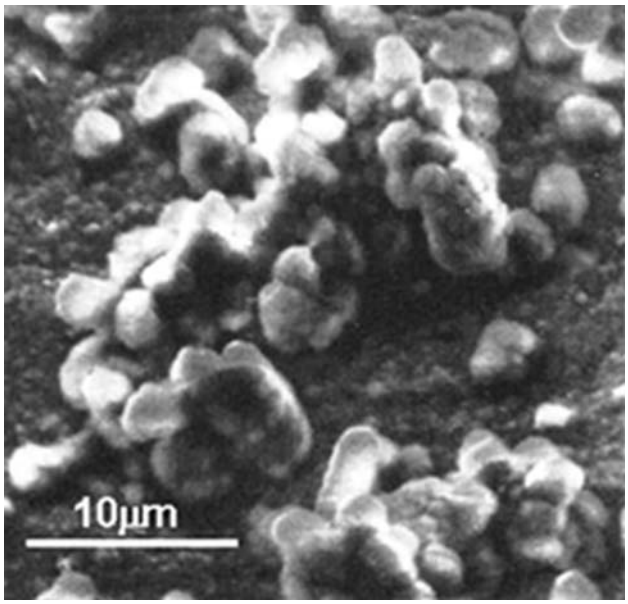

Fig. 5 ESEM micrograph of hydroxyapatite crystals harvested from culture medium of ADSC-FL cells in osteogenic conditions (dish culture)

3.6 Evaluation of osteocalcin and osteopontin secretion

Quantification of secreted active osteocalcin (OC) and Osteopontin (OP) from ADSC-FL cells cultured in proliferative and osteogenic differentiative media both in dishes and on β-TCP scaffolds are given in Figs 8 and 9. No OC and OP secretion were observed for cells seeded in culture dishes with proliferative medium; however, in osteoblastic differentiative medium, cells had an increased OC and OP expression. In particular, from days 14 to 28 higher levels of OC and OP were found, indicating the presence of a high percentage of osteoblasts in the cell population.

Cells seeded onto β-TCP scaffolds in osteogenic differentiative medium shaved an intense and earlier OC and OP expression profile with respect to cells seeded in culture dish.

Surprisingly, also cells seeded onto β-TCP scaffolds and cultured in proliferative medium had an OC and OP expression profile similar to those cultured in the osteogenic differentiative environment (Fig. 8, 9).

3.7 Biochemical evaluation of FAK activation

In Fig. 10, we showed a typical result from FAK immunoprecipitation and Western blot procedure after 3, 7 and 14 days of ADSC-FL adhesion on dish culture and β-TCP matrix with and without osteogenic differentiative medium. All bands were subjected to densitometric analysis, as described in the Sect. 2. Levels of FAK activation were monitored through the quantification of the ratio between the phosphorylated form (Y397) and the total enzyme [28]. As evident from Figs. 10 and 11, the levels of FAK activation after 3, 7 and 14 days were found lower for cells adhering on dish culture respect to cells seeded on β-TCP matrix.

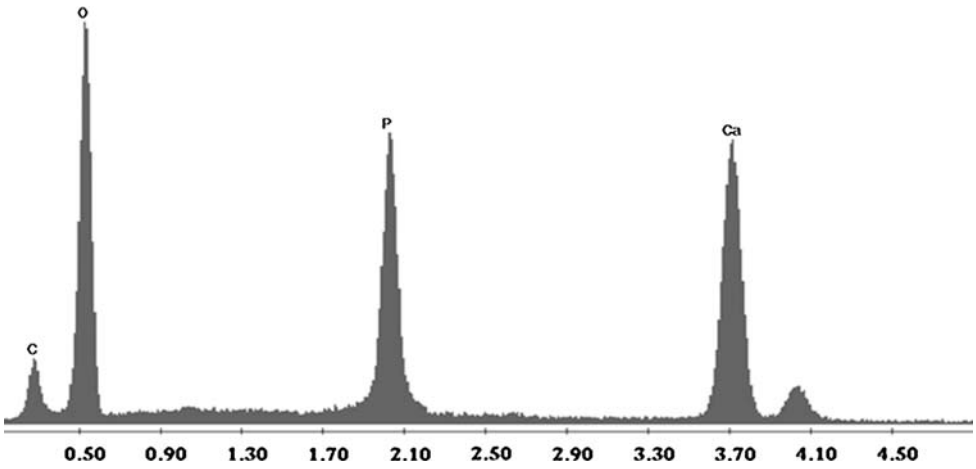

Fig. 6 Microanalysis EDS spectra of newly formed hydroxyapatite crystals on differentiating cells

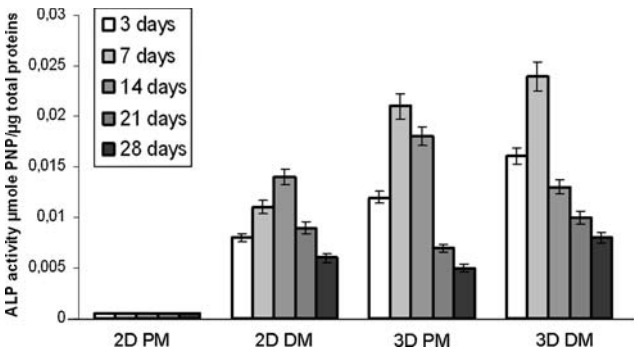

Fig. 7 Alkaline phosphatase activity assay (mean ± standard deviation of five independent determinations). *2D* = dish cultures; *3D* = β-TCP scaffolds; *PM* proliferative medium, *DM* differentiative medium. ALP activity was normalized to sample total protein content

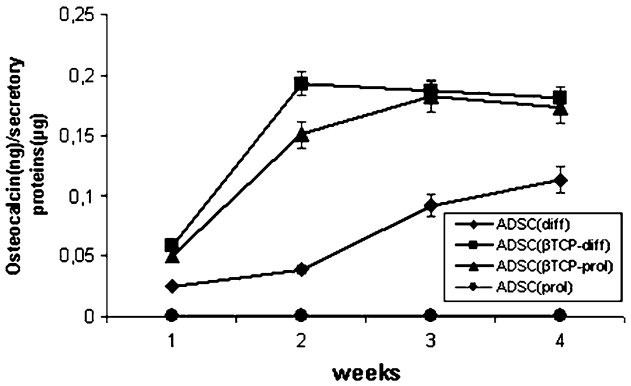

Fig. 8 Osteocalcin secretion (mean ± standard deviation of five independent determinations); *Prol* proliferative medium, *Diff* differentiative medium. Osteocalcin activity was normalized to sample total secreted proteins

4 Discussion

First, we tried to optimize the starting concentration of ADSC-FL cells seeded on β-TCP scaffold to achieve a good cell concentration and to avoid confluence at the end of experimental time. As showed by viability study (Fig. 3) the starting concentration of 2×10^5 cells/scaffold was an appropriate choice, since cell vitality was retained along all the experimental time (28 days). Moreover, also ESEM micrographs (Fig. 4) showed, that after 28 days of incubation, β-TCP scaffold was colonized by cells, with large spaces still available for the cell growth.

ADSC-FL cells were obtained from human adipose tissue harvested from the subcutaneous abdominal region of patients. Immunophenotypical characterization clearly revealed the stem origin of the purified cells: in fact, these cells were strongly $CD13^+/CD44^+/CD105^+$ and $CD3^-/CD14^-/CD33^-/CD34^-/CD45^-/CD133^-$ in agreement with the literature [2–4]. However, surface markers should not be

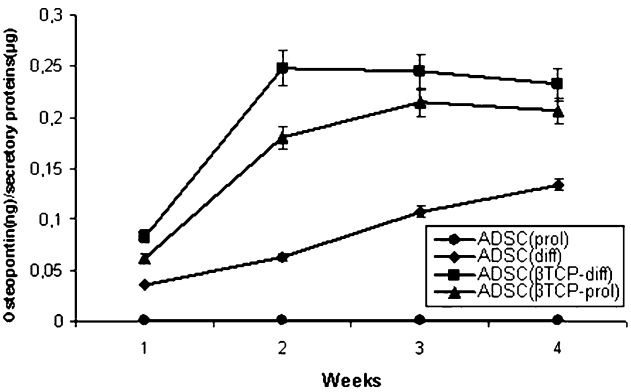

Fig. 9 Osteopontin secretion (mean ± standard deviation of five independent determinations); *Prol* proliferative medium, *Diff* differentiative medium. Osteopontin activity was normalized to sample total secreted proteins

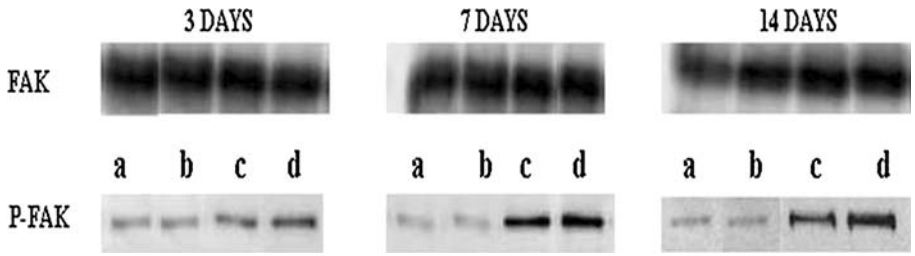

Fig. 10 A typical Western blot of ADSC-FL cells seeded on culture dish and β-TCP matrix. a cell cultured in dishes in proliferative medium. b cell cultured in dishes in osteogenic differentiative medium. c cell cultured on β-TCP in proliferative medium. d cell cultured on β-TCP in osteogenic differentiative medium. *FAK* total enzyme, *P-FAK* phosphoFAK (tyr 397)

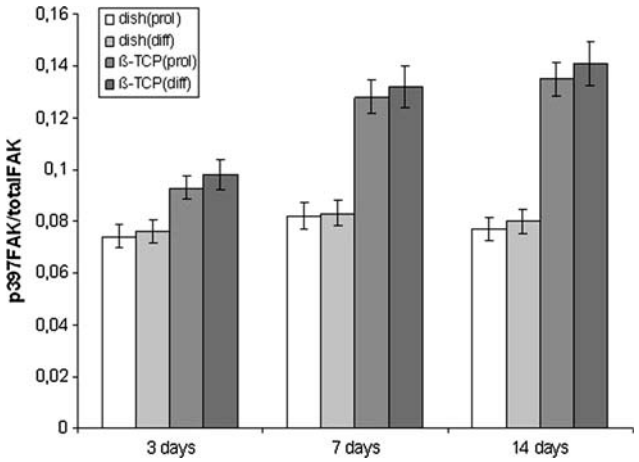

Fig. 11 Levels of FAK activation after 3, 7 and 14 days in ADSC-FL cells growing on 2D culture dish and β-TCP matrix with proliferative medium (*Prol*) and differentiative medium (*Diff*)

considered by itself a condition sufficient for the identification or definition of MSC. Hence, we evaluated the capacity of these isolated cells to differentiate toward a specific phenotype (a characteristic of stem cells). Indeed, the formation of bone-like hydroxyapatite crystals by differentiating cells in dishes with osteogenic medium further demonstrated the mesenchymal origin of purified cells (Figs. 3, 4).

The main purpose of this study was the evaluation of the osteogenic potential of ADSC-FL seeded onto β-TCP scaffolds. Firstly, we verified the osteogenic potential of ADSC-FL in culture dishes either with proliferative or differentiative media. In proliferative medium, no osteoblastic differentiation was observed during the entire experimental time (28 days) while, in differentiative medium, biochemical evaluation of ALP activity (an early osteoblastic marker) (Fig. 7), active OC and OP secretion (a late osteoblastic markers) (Figs. 8, 9) strongly confirmed the high osteogenic potential of these cells. Surprisingly, ADSC-FL grown on β-TCP scaffolds express high levels of differentiative markers (OC and OP) and ALP activity, also when cultured with proliferative medium. The expression profiles of these osteogenic markers, in our experimental conditions, were in agree with literature data concerning mesenchymal cells osteogenic differentiation on bone-like mineral matrix [7, 18, 21, 31–34]. All these authors, considered an experimental time of 28 days, sufficient to demonstrate a stable expression of osteoblastic markers.

In particular, a previous study demonstrated the ability of human bone marrow hMSCs seeded on β-TCP scaffolds to differentiate to osteoblasts also in proliferative media [7]. In this paper, the ALP activity, OC and OP expression were similar to that observed in our work using ADSC-FL cells. Another interesting work reported that a human preadipocyte cell line (3T3F442A) differentiated into osteoblasts when seeded on calcium carbonate scaffolds in proliferative media [18].

Interestingly, our current report also supports the notion that a 3D β-TCP matrix can cue the triggering of the differentiatative process of ADSC-FL toward a specific phenotype. Our finding that surface properties specifically trigger osteogenic differentiation in the absence of osteogenesis stimulating factors in the medium is supported by the literature. For example, elastin-like polypeptide surface supported Human adipose derived adult stem cells (hADAS or ADSC-FL) differentiation into chondrocytes without chondrogenic supplements [31]. Other reports showed that the presence of $-NH_2$ and –SH chemical groups on biomaterial surfaces promoted osteogenesis, whereas –OH and –COOH chemical groups supported chondrogenesis also in the absence of chondrogenic supplements in the medium [34]. In particular, in a recent paper Liu et al. [22] studied the ADSC-FL cells osteoblastic differentiation on two-dimensional TCP surface, but in this study the timing of cell culture were too short and not in agree with literature data [7, 18, 21, 31–34] that recommended an experimental time of 28 days to demonstrate a stable expression of osteoblastic markers. Moreover, our data showed that ADSC-FL cells seeded on 3D β-TCP matrix had an intense markers expression respect to two-dimensional TCP matrix, demonstrating that cells preferred the 3D environment respect to 2D.

Modulation of MSC differentiation to osteoblasts by surface chemistry implies that intracellular signal transduction is driven by changes in the cell–ECM interaction and could replace soluble factors that stimulate cell differentiation.

The chemical properties of a surface could induce selective absorption of matrix proteins [35]. Secreted proteins, like osteocalcin and bone sialoprotein have the capacity to bind to calcium and hydroxyapatite and could preferentially be absorbed by a calcium phosphate surface. A selective effect of matrix proteins on osteogenic differentiation of MSCs has been demonstrated [36, 37].

Hence, our results support these data, consolidating the idea that on calcium scaffolds (i.e., hydroxyapatite and β-TCP) the binding of osteogenic secreted proteins triggers and enhances differentiation of MSCs toward an osteoblastic phenotype.

At the aim to study the interaction between β-TCP matrix and ADSC-FL cells, we evaluated the activation levels of Focal Adhesion Kinase (FAK) a non receptor tyrosine kinase, that is phosphorylated at level of tyrosine residues in position 397 when a cellular system interact with its substrate. Hence this molecule is a key enzyme that translates the external stimuli to the cell biochemical machinery [7, 11].

Very likely, an increase in FAK activation was detected in cells seeded on the β-TCP surfaces respect to dish cultures, irrespective of whether the cells are grown in a proliferative or a differentiative medium (Figs. 10, 11), hence, these behavior was due to the different chemical composition of β-TCP matrix respect to culture dish.

However, in the present work we focalize our attention on FAK activation to demonstrate the good adhesion of ADSC-FL cells on β-TCP matrix, that represent the first step to evaluate the β-TCP matrix ability to support ADSC-FL cells adhesion and osteoblastic differentiation.

These experimental results demonstrated that ADSC-FL cells strongly interact with β-TCP matrix and that cells are metabolically active as further demonstrated by vitality assay (Fig. 3).

Finally, cell-scaffold interactions were studied using ESEM technology. ESEM is an electron optical instrument which permits the examination of the surfaces of soft, hydrated, unfixed, uncoated, and electrically insulating specimens with a depth of field and with magnifications that comparable to conventional SEM [38]. The high water vapor pressure in the ESEM analysis chamber allows high resolution imaging of wet or hydrated specimens, obviating the need for sample drying and the associated artifacts [39, 40]. Using this technology, we demonstrate that ADSC-FL cells interact with the β-TCP surface when grown either in proliferative or in differentiative media, and that cells have a plumped morphology typical of metabolically active osteoblasts.

In conclusion, our paper demonstrates that a 3D β-TCP scaffold on its own is sufficient to trigger the differentiation of Human Adipose Stem Cells toward an osteoblastic phenotype.

References

1. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211–8.
2. Kern S, Eichler H, Stoeve J. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006;24:1294–301.
3. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol. 2001;189(1):54–63.
4. Tocci A, Forte L. Mesenchymal stem cell: use and perspectives. Hematol J. 2003;4:92–6.
5. Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells-basic and clinical implications for novel cell-based therapies. Stem Cells. 2007;25:818–27.
6. El-Ghannam A. Bone reconstruction: from bioceramics to tissue engineering. Expert Rev Med Devices. 2005;2:87–101.
7. Müller P, Bulnheim U, Diener A, Lüthen F, Teller M, Klinkenberg ED, et al. Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. J Cell Mol Med. 2008;12(1):281–91.
8. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 1992;70(3):389–99.
9. Parsons JT. Integrin-mediated signaling: regulation by protein tyrosine kinases and small GTP-binding proteins. Curr Opin Cell Biol. 1996;11:146–52.
10. Schwartz M, Schaller M, Ginsberg MH. Integrins: emerging paradigms of signal transduction. Annu Rev Cell Dev Biol. 1995;11:549–99.
11. Hynes RO. Cell adhesion: old and new questions. Trends Cell Biol. 1999;9(12):M33–7.
12. Schlaepfer DD, Jones KC, Hunter T. Multiple Grb2-mediated integrinstimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol Cell Biol. 1998;18:2571–85.
13. Short SM, Talbott GA, Juliano RL. Integrin-mediated signalling events in human endothelial cells. Mol Biol Cell. 1998;9:1969–80.
14. Hanks SK, Polte TR. Signalling through focal adhesion kinase. Bioessays. 1997;19:137–45.
15. Zhao J-H, Reiske H, Guan J-L. Regulation of the cell cycle by focal adhesion kinase. J Cell Biol. 1998;143:1997–2008.
16. Thomas Parsons J. Focal adhesion kinase: the first ten years. J Cell Sci. 2003;116:1409–16.
17. Rosso F, Marino G, Muscariello L, Cafiero G, Favia P, D'Aloia E, et al. Adhesion, proliferation of fibroblasts on RF plasma-deposited nanostructured fluorocarbon coatings: evidence of FAK activation. J Cell Physiol. 2006;207(3):636–43.
18. Birk RZ, Abramovitch-Gottlib L, Margalit I, Aviv M, Forti E, Geresh S, et al. Conversion of adipogenic to osteogenic

phenotype using crystalline porous biomatrices of marine origin. Tissue Eng. 2006;12:21–31.
19. Hicok KC, Du Laney TV, Zhou YS, Halvorsen YD, Hitt DC, Cooper LF, et al. Human adipose-derived adult stem cells produce osteoid in vivo. Tissue Eng. 2004;10(3–4):371–80.
20. Justesen J, Pedersen SB, Stenderup K, Kassem M. Subcutaneous adipocytes can differentiate into bone-forming cells in vitro, in vivo. Tissue Eng. 2004;10(3–4):381–91.
21. Hattori H, Masuoka K, Sato M, Ishihara M, Asazuma T, Takase B, et al. Bone formation using human adipose tissue-derived stromal cells and a biodegradable scaffold. J Biomed Mater Res B: Appl Biomater. 2006;76(1):230–9.
22. Liu Q, Cen L, Yin S, Chen L, Liu G, Chang J, et al. A comparative study of proliferation and osteogenic differentiation of adipose-derived stem cells on akermanite and beta-TCP ceramics. Biomaterials. 2008;29(36):4792–9. Epub 2008 Sep 26.
23. Leong DT, Nah WK, Gupta A, Hutmacher DW, Woodruff MA. The osteogenic differentiation of adipose tissue-derived precursor cells in a 3D scaffold/matrix environment. Curr Drug Discov Technol. 2008;5(4):319–27.
24. McCullen SD, Zhu Y, Bernacki SH, Narayan RJ, Pourdeyhimi B, Gorga RE, Loboa EG. Electrospun composite poly(L-lactic acid)/tricalcium phosphate scaffolds induce proliferation and osteogenic differentiation of human adipose-derived stem cells. Biomed Mater. 2009;4(3):35002. Epub 2009 Apr 24.
25. Hao W, Hu YY, Wei YY, Pang L, Lv R, Bai JP, et al. Collagen I gel can facilitate homogenous bone formation of adipose-derived stem cells in PLGA-beta-TCP scaffold. Cells Tissues Organs. 2008;187(2):89–102. Epub 2007 Oct 15.
26. Mitchell J, Micintosh k, Zavonic S, Garrett S, Floyd E, Loster A, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell–associated markers. Stem Cells. 2006;24:376–85.
27. Salazar EP, Hunger-Glaser I, Rozengurt E. Dissociation of focal adhesion kinase and paxillin tyrosine phosphorylation induced by Bombesin and Lysophosphatidic acid from epidermal growth factor receptor transactivation in Swiss 3T3 Cells. J Cell Physiol. 2003;194:314–24.
28. Walsh MF, Thamilselvana V, Grotelueschena R, Farhanab L, Bassona MD. Absence of adhesion triggers differential FAK and SAPKp38 signals in SW620 human colon cancer cells that may inhibit adhesiveness and lead to cell death. Cell Physiol Biochem. 2003;13:135–46.
29. Kim H-M, Kishimoto K, Miyaji F, Kokubo T, et al. Composition and structure of apatite formed on organic polymer in simulated body fluid with a high content of carbonate ion. J Mater Sci: Mater Med. 2000;11:421–6.
30. Legeros RZ, Legeros JP. In: Hench LL, Wilson J, editors. An introduction to bioceramics. Singapore: World Scientific; 1993. p. 139.
31. Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA. Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. Biomaterials. 2006;27:91–9.
32. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL, et al. Extracellular matrix mineralization, osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng. 2001;7(6):729–41.
33. Kawaguchi J, Mee PJ, Smith AG. Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. Bone. 2005;36(5):758–69. Epub 2005 Mar 24.
34. Curran JM, Chen R, Hunt JA. The guidance of human mesenchymal stem cell differentiation in vitro by controlled modifications to the cell substrate. Biomaterials. 2006;27:4783–93.
35. Keselowsky BG, Collard DM, Garcia AJ. Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. J Biomed Mater Res A. 2003;66:247–59.
36. Wollenweber M, Domaschke H, Hanke T, Boxberger S, Schmack G, Gliesche K. Scharnweber et al. Mimicked bioartificial matrix containing chondroitin sulphate on a textile scaffold of poly(3-hydroxybutyrate) alters the differentiation of adult human mesenchymal stem cells. Tissue Eng. 2006;12:345–59.
37. Datta N, Pham QP, Sharma U, Sikavitsas VI, Jansen JA, Mikos AG. In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. Proc Natl Acad Sci USA. 2006;103:2488–93.
38. Muscariello L, Rosso F, Marino G, Cafiero G, Barbarisi A. A critical overview of ESEM applications in the biological field. J Cell Physiol. 2005;205:328–34.
39. Baker FS, et al. Secondary electron imaging at gas pressures in excess of 1 KPa. Appl Phys Lett. 2007;91:053122/1–3.
40. Iliescu M, et al. Ultrastructure of hybrid chitosan-glycerol phosphate blood clots by environmental scanning electron microscopy. Microsc Res Tech. 2008;71:236–47.