Calcif Tissue Int (2000) 67:321–329
DOI: 10.1007/s002230001134

Calcified Tissue International

Bioglass ®45S5 Stimulates Osteoblast Turnover and Enhances Bone Formation *In Vitro:* Implications and Applications for Bone Tissue Engineering

I. D. Xynos,[1] M. V. J. Hukkanen,[1] J. J. Batten,[2] L. D. Buttery,[1] L. L. Hench,[3] J. M. Polak[1]

[1]Department of Histochemistry, Commonwealth Building, Imperial College School of Medicine, Hammersmith Campus, The Hammersmith Hospital, Ducane Rd, London W12 ONN, UK
[2]Department of Surgery, Imperial College School of Medicine, Hammersmith Campus, The Hammersmith hospital, Ducane Rd, London W12 ONN, UK
[3]Department of Materials, Imperial College School of Science and Technology, South Kensington Campus, Prince Consort Road, London SW1 2BP, UK

Received 28 June 1999 / Accepted: 22 March 2000

Abstract. We investigated the concept of using bioactive substrates as templates for *in vitro* synthesis of bone tissue for transplantation by assessing the osteogenic potential of a melt-derived bioactive glass ceramic (Bioglass® 45S5) *in vitro.* Bioactive glass ceramic and bioinert (plastic) substrates were seeded with human primary osteoblasts and evaluated after 2, 6, and 12 days. Flow cytometric analysis of the cell cycle suggested that the bioactive glass-ceramic substrate induced osteoblast proliferation, as indicated by increased cell populations in both S (DNA synthesis) and G2/M (mitosis) phases of the cell cycle. Biochemical analysis of the osteoblast differentiation markers alkaline phosphatase (ALP) and osteocalcin indicated that the bioactive glass-ceramic substrate augmented osteoblast commitment and selection of a mature osteoblastic phenotype. Scanning electron microscopic observations of discrete bone nodules over the surface of the bioactive material, from day 6 onward, further supported this notion. A combination of fluorescence, confocal, transmission electron microscopy, and X-ray microprobe (SEM-EDAX) examinations revealed that the nodules were made of cell aggregates which produced mineralized collagenous matrix. Control substrates did not exhibit mineralized nodule formation at any point studied up to 12 days. In conclusion, this study shows that Bioglass 45S5 has the ability to stimulate the growth and osteogenic differentiation of human primary osteoblasts. These findings have potential applications for tissue engineering where this bioactive glass substrate could be used as a template for the formation of bioengineered bone tissue.

Key words: Bioactive glass-ceramics — Tissue engineering — Osteoblasts — Osteogenesis.

An ever increasing number of people suffer every year from traumas or diseases that affect bone. In a large number of cases the resulting skeletal deficiencies require surgical intervention and repair [1]. The relative shortage of autograft tissue and the potential for disease transmission and adverse host immune reaction with allografts have increased the need for synthetic bone substitutes. Inert materials such as metals and bone cements have been used extensively for this purpose despite problems arising from shear-stress failure [2]. During the last decade, bioactive materials, including synthetic hydroxyapatite and bioactive glass ceramics, have been applied to repair bone defects and the results have been far superior [2–4]. Nevertheless, synthetic materials typically do not replace all the functions of a lost tissue and are incapable of adapting to the body's changing needs over time [5].

Because of these limitations, the search for new alternative strategies for repairing bone defects has been focused on tissue engineering [6–8]. Tissue engineering has been defined as a 'combination of the principles and methods of the life sciences with those of engineering to elucidate fundamental understanding of structure-function relationships in normal tissues, to develop materials and methods to repair damaged or diseased tissue, and to create entire tissue replacements' [9]. A number of strategies have been evolved over the past 15 years towards engineering tissue replacements, but the most common approach implies the use of synthetic material matrices as templates for tissue growth *in vitro.* An ideal synthetic matrix should be selected on the basis of a number of essential requirements including the ability to enhance cell proliferation while supporting tissue-specific differentiation [8]. The integration of an appropriate scaffold with living cells will optimally yield a differentiated, functional tissue for implantation purposes. It is anticipated that following implantation, the artificial tissue will integrate rapidly with the host and take up normal function.

It is possible that certain bioactive glass ceramics fulfill most of the criteria required for a suitable scaffold to support bone tissue growth, since they are known to enhance proliferation and prevent de-differentiation of osteoblasts *in vitro* [10–12]. Furthermore, an increasing body of evidence from animal studies suggests that they can also increase bone formation in implantation sites *in vivo* [13–16].

In the present study, we have investigated the concept of using bioactive glass ceramic materials as templates for synthesis of bone tissue *in vitro* for transplantation purposes [17]. We therefore investigated the ability of a glass ceramic

Correspondence to: J. M. Polak

material containing 45% SiO_2, 24.5% Na_2O, 24.5% CaO, and 6% P_2O_5 in weight percent to induce a sequence of biological events that lead to bone formation *in vitro*, and examined the mechanisms involved.

Methods

Cells and Culture Systems

Osteoblasts were isolated from trabecular bone of femoral heads taken during total hip arthroplasty using the method described by Beresford et al. [18]. Osteoblasts derived from different individuals were pooled together and used in the studies in order to minimize variations in osteoblast behavior. Cells were seeded on Bioglass® 45S5 discs and on control substrates at a seeding density of 22.5×10^3 cells/cm^2. Cultures were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin B, and 0.3 μg/ml amphotericin B at 37°C, in 95% air humidity and 5% CO_2.

Preparation of Materials for the Experiments

Bioglass® 45S5 test discs (supplied by USBiomaterials) were polished using 800 and 1200 grit emery paper, cleaned by sonication in acetone, and sterilized by dry heat at 180°C overnight. The discs measured 15 mm in diameter × 3 mm and snugly fitted the 24-well plate (Nunc, Naperville, USA). The discs were 'conditioned' by equilibrating in culture media for 72 hours and then placed onto 24-well plates. Control substrates (Thermanox®, 10 mm in diameter, Nunc, Naperville, USA) were sterilized in an autoclave and placed into 24-well plates. The use of Thermanox discs was indicated in experiments where parameters such as cell morphology or cell density were evaluated and therefore cultures had to be removed from the wells, fixed, stained (where indicated), and analyzed microscopically. In experiments where quantitative results needed to be obtained (flow cytometry, ALP, osteocalcin), it was important to compare substrates of equal size. Therefore, culture grade 24-well polystyrene plates (without any Bioglass 45S5 discs) served as control substrates, since the size of each well matched exactly the size of a Bioglass disc. Both Thermanox and culture grade polystyrene are bioinert polymers, optimally treated to support cell attachment and growth and they represent the most widely used culture grade plasticware.

Examination of Surface Reaction Layer on Bioglass 45S5 by X-ray Probe Analysis (SEM-EDAX)

Following conditioning and then after 2, 6, and 12 days in culture medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin B, and 0.3 μg/ml amphotericin B), the samples were critical-point dried and carbon-sputter coated. X-ray microanalysis of the surfaces was performed with a LINK AN 10000 analyzer connected to a Cambridge 360 Scanning Electron Microscope at 20 KV. All samples were analyzed under the same microscopical conditions.

Investigation of Cell Morphology and Adhesion Patterns on Different Substrates by Scanning Electron Microscopy (SEM)

Osteoblast cultures were observed after 2, 6, and 12 days in culture. Fixation with 2.5% v/v glutaraldehyde in 0.1% w/v phosphate buffer (pH 7.4) for 1 hour was followed by rinses in 0.1% w/v phosphate-buffered solution containing 0.1 M sucrose (3 × 5 minutes) and postfixing in 1% v/v osmium tetroxide in 0.1% w/v phosphate buffer for 1 hour. Cultures were dehydrated in graded ethanol series, critical point dried, and gold-sputtered prior to observation with a Cambridge 360 SEM at 10 KV.

Assessment of Cell Proliferation

Cell Cycle Analysis. Cells were seeded on Bioglass 45S5 and polystyrene control wells with media exchanged at 48-hour intervals and maintained for up to 12 days. After 2, 6, and 12 days of culture, floating dead cells were collected together with the trypsinized cells. Cells from each culture were washed by centrifugation with phosphate buffered saline (PBS). The pellets were then resuspended in 500 μl propidium iodide (0.5 μg/ml) and ribonuclease A (100 μg/ml) solution and incubated at 37°C for 30 minutes. Finally, the cells were run through a flow cytometer (EPICS XL MCL, Coulter Electronics) set to detect propidium iodide-induced fluorescence (620 ± 10 nm).

Cell Density Determination. Cells were seeded on Bioglass 45S5 and polystyrene control wells with media exchanged at 48-hour intervals and maintained for up to 12 days. On days 2, 6, and 12 media was removed and the cells were fixed in 2% paraformaldehyde for 20 minutes, stained with DNA binding fluorescent reagent DAPI (25 μg/ml), and counted using fluorescence microscopy Olympus BX60 (Olympus Optical Co Ltd, Tokyo, Japan), and image analysis (Seescan Symphony, Seescan, Cambridge, UK).

Investigation of Apoptosis by Hoechst 33342 Staining of the Cell Nuclei

Cells were seeded on Bioglass 45S5 and Thermanox substrates with media exchanged at 48-h intervals and maintained for up to 12 days. After 2, 6, and 12 days in culture, the cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 30 minutes at room temperature (RT). Cell cultures were labeled using the DNA-binding fluorophore Hoechst 33342 (10 μg/ml), mounted in PBS-glycerine, and nuclear morphology was visualized using an Olympus BX60 (Olympus Optical Co Ltd, Tokyo, Japan) microscope. Nonapoptotic cells showed dim nonhomogeneous nuclear staining. Apoptotic cells were brightly stained and the classic progression of chromatin condensation and nuclear fragmentation was visible. Only intact cells were counted. At least 500 cells were counted from each sample and percent total apoptotic cells was calculated [20].

Biochemical Analysis of Osteoblast Differentiation Markers

Alkaline Phosphatase Specific Activity. Cells were cultured on Bioglass 45S5 discs and polystyrene controls for 2, 6, and 12 days. The cells were washed with PBS (3 × 5 minutes) and then lysed in 250 μl lysis buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 30 μl/ml aprotinin, and 1 mM sodium orthovanadate) under continuous mixing at 4°C. The cell lysates were frozen and stored at −40°C until assayed. Alkaline buffer solution (50μl) and 50 μl substrate were added to 50 μl of the lysate or cell culture medium. The mixture was incubated at 37°C for 30 minutes, the reaction was stopped by adding 100 μl 0.5 M NaOH, and the absorbance was read at 405 nm using a Multiscan RC plate reader (Labsystems, Life Sciences International Ltd, Hampshire, UK). A standard curve was constructed using p-nitrophenol as a standard, and ALP activity in activity units was calculated. One unit of activity is defined as the amount of enzyme activity that liberates 1 μmol of p-nitrophenol per hour under the assay conditions. Units were referred to 10^3 cells.

Osteocalcin Synthesis. Cells were cultured on Bioglass 45S5 discs and on polystyrene in culture medium, which was not supplemented with FBS. The culture medium from each well was aspirated after 6 days and stored at −40°C until assayed. The major N-terminal fragment of ostecalcin was assayed using a human osteocalcin EIA kit (Biomedical Technologies Inc, Tyne & Wear, UK) following the manufacturer's protocol. All samples were assayed in duplicate and osteocalcin levels were referred to 10^3 cells.

Investigation of Extracellular Matrix Formation by Transmission Electron Microscopy (TEM) and Confocal Scanning Laser Microscopy

Osteoblast cultures were observed after 2, 6, and 12 days in culture and fixed as for SEM. After dehydration in graded ethanols, a final solution of 50% TAAB resin and 50% v/v absolute alcohol was applied to the culture-bearing substrates under continuous warming to evaporate all final traces of alcohol. Resin-filled capsules were then inverted over the substrates and together they were polymerized overnight at 60°C. Substrates were then removed after momentary immersion in liquid nitrogen, leaving the cultures intact on the surface of the resin block [19]. The blocks were sectioned with diamond or glass knives using an ultratome. Semithin sections for light microscopy were stained 1% w/v toluidine blue. Ultrathin sections were collected on nickel grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10 CR electron microscope.

Immunofluorescent Labeling of Collagen I. After 2, 6, and 12 days cells were fixed in freshly prepared 4% paraformaldehyde-PBS (pH 7.4) for 20 minutes, at RT. Following three washes in PBS, cells were permeabilized in 0.2% Triton-X in PBS for 20 minutes at RT. The preparations were incubated with normal goat serum for 30 minutes at RT and then incubated overnight with rabbit anti-collagen I polyclonal antibody (1:30 dilution; MONOSAN, Netherlands) in PBS containing 0.01% sodium azide at +4°C. Following three washings in PBS, cells were treated with goat anti-rabbit FITC-conjugated antibody (1:100 dilution; TCS Biologicals, UK) in PBS containing 0.1% bovine serum albumin and 1% normal human serum for 1 hour at room temperature. Finally, cell nuclei were labeled with 2 μg/ml propidium iodide-PBS for 5 minutes and mounted with 1:1 PBS-Glycerol. Fluorescent-labeled osteoblast cultures were observed under a Bio-Rad Microradiance confocal scanning laser microscope.

Assessment of Calcified Nodule Formation by Alizarin Complexone Staining

Osteoblasts were seeded on Bioglass 45S5 and polystyrene control wells. After 2, 6, and 12 days and following fixation in 4% w/v paraformaldehyde, the specimens were stained with the Ca-specific dye Alizarin-complexone (pH 7.2) for 10 minutes at RT, nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, 25 μg/ml), and specimens were mounted in PBS/glycerine. Microscopy was performed using an Olympus BX60 (Olympus Optical Co Ltd, Tokyo, Japan) fluorescence microscope.

Effect of Calcium on Alkaline Phosphatase Activity

In order to investigate the possibility that Ca, a structural component and a diffusable product of the Bioglass 45S5 [2, 22], could influence ALP-specific activity, subconfluent cultures of osteoblasts on polystyrene were incubated in DMEM/serum-free media supplemented with 0–10 mM $CaCl_2$ for 24 hours. Then cells were homogenized and ALP activity was assayed as described above, and the results were adjusted to total protein concentration and expressed as U/mg protein.

Protein Content Determination

Protein concentration of the cell lysates was assayed by the Bradford dye-binding method [21] using bovine serum albumin (BSA) as a standard.

Reagents

All reagents were obtained from Sigma (Sigma-Aldrich Chemical Co, Poole, UK) unless otherwise stated in the text.

Statistical Analysis

Statistical analysis was performed using a Prism 2.01 statistical package software with appropriate statistical tests such as analysis of one-way variance (ANOVA) with Dunnett's post-tests for intergroup analysis, or unpaired Student's *t*-test. Specifically, in Dunnett's post-test, the data set from the earliest time point was used as control and all other data sets from later time points were compared to it. A $P < 0.05$ was regarded as statistically significant. All data are expressed as mean ± SE.

Results

Surface Analysis of Bioglass 45S5 Following Immersion in Cell-Free Culture Medium

After immersion for 72 hours in cell culture medium ('conditioning'), Bioglass 45S5 surfaces were analyzed by SEM-EDAX in order to confirm the development of a surface reaction layer [22]. Indeed, SEM demonstrated the presence of the reaction layer on the surface of Bioglass 45S5, and subsequent X-ray microanalysis of the reaction layer (EDAX) confirmed the presence of Ca and phosphorus peaks at a count ratio of 2.4, plus silicon (data not shown). X-ray analysis of control substrates (Thermanox plastic) failed to show any characteristic peaks under similar experimental conditions (data not shown).

Investigation of Cell Morphology and Adhesion Patterns on Different Substrates by SEM

Scanning electron microscopy demonstrated significant differences in cell morphology and mode of adhesion for human primary osteoblasts cultured on bioactive and bioinert (Thermanox coverslips) substrates. Osteoblasts cultured on the bioactive substrate showed features suggestive of cell activation, including numerous dorsal membrane ruffles and microvilli. Over a period of 6 days, osteoblasts demonstrated morphological characteristics associated initially with an anchorage stage (Fig. 1a), and subsequently with an attachment phase (b). Finally, they spread on the substrate forming confluent cellular multilayers by day 12 (c). In addition, SEM demonstrated the presence of discrete three-dimensional cellular structures (bone nodules) throughout the surface of Bioglass 45S5 from day 6 onwards (d-e). In marked contrast, osteoblasts cultured on the inert control substrate spread faster and formed confluent monolayers by day 6, despite exhibiting only minimal signs of cell activation (1f). However, they never formed multicellular layers or bone nodules at any point studied up to 12 days.

Osteoblast Proliferation

Osteoblast proliferation on Bioglass 45S5 and polystyrene was evaluated by flow cytometric analysis of cell cycle. The results from this experiment suggested that the bioactive substrate induced a biphasic response, with two distinct proliferative phases, as indicated by increased cell cycling, occurring at days 2 and 12. More specifically, there was a significantly higher percentage of cells in the S phase (DNA synthesis) on days 2 and 12 ($P < 0.05$) (Fig. 2a) and additionally a higher percentage of cells in the G2/M phase (mitosis) on day 12 ($P < 0.01$) on the bioactive substrate relative to control (b). This was interspersed by a lag-phase

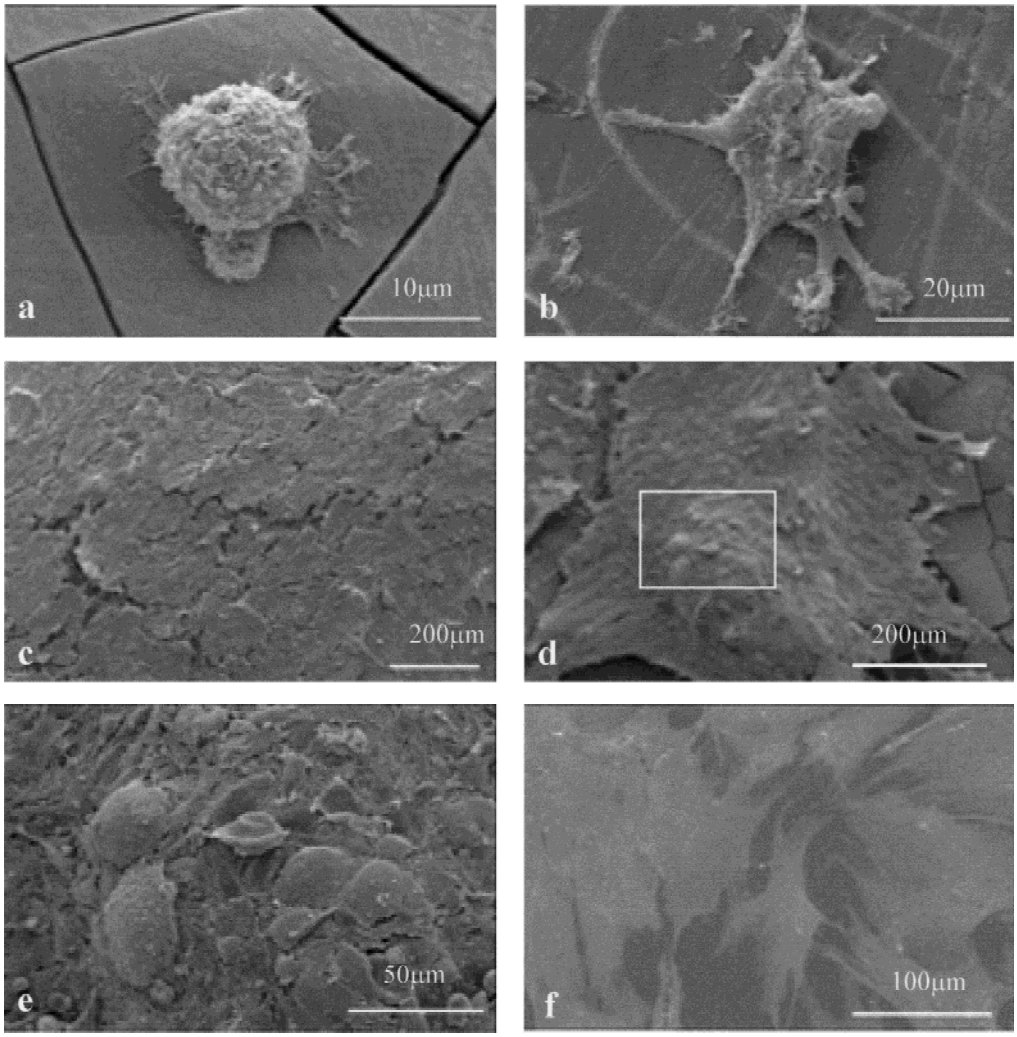

Fig. 1. SEM images of osteoblasts cultured on Bioglass 45S5. **(a)** Two days after seeding, spherical osteoblasts contacted the substrate by means of numerous filopodia and fiber-like processes. **(b)** After 6 days of culture, cells were anchored to the substrate by multiple lamellipodia. Note the dorsal ruffles and microvilli-type processes. **(c)** After 12 days of culture, cells appeared to be well spread and grew in multilayer fashion. **(d)** SEM image of a bone nodule present on Bioglass 45S5 on day 12. **(e)** Higher magnification of the central domed region of the bone nodule shown in **(d). (f)** After 6 days of culture, osteoblasts seeded on inert control substrate exhibited a smooth dorsal surface, adopted a flattened configuration, and formed confluent monolayers.

as suggested by lack of differences in cell cycling between the bioactive and control substrate on day 6. Figure 2c presents the results from the cell density determination on Bioglass 45S5 and polystyrene. A significant increase ($P < 0.001$) in cell density on the bioactive substrate compared with control did not become apparent until day 12 of culture. At earlier time points (days 2 and 6) cell densities on control (Thermanox) were significantly greater ($P < 0.001$) than on Bioglass 45S5.

Apoptosis

Microscopic evaluation of Hoescht stained cells revealed that apoptosis was markedly increased on the bioactive substrate at days 2 and 6 ($P < 0.001$) but declined to control levels by day 12 (Fig. 2d).

Osteoblast Differentiation

Alkaline phosphatase specific activity was higher ($P < 0.01$) in cultures growing on the bioactive glass on day 6, but declined by day 12 to levels significantly less ($P < 0.001$) than in the control cultures (Fig. 3a). Osteocalcin synthesis was found to be significantly higher ($P < 0.001$) in osteoblast cultures grown on the bioactive glass than on the inert substrate following 6 days in culture (b). Exogenous administration of Ca (10 mM) to osteoblast cultures grown on conventional polystyrene wells resulted in a significant decrease ($P < 0.01$) in ALP activity (c).

Investigation of Extracellular Matrix and Bone Nodule Formation by SEM, Confocal Scanning Laser Microscopy, Fluorescence Microscopy, and TEM

Detailed SEM examinations demonstrated that the nodular structures formed over the surface of Bioglass 45S5 were made by a network of cells exhibiting overlapping and superimposed borders and interconnected processes (Fig. 4a). These cells were producing extracellular matrix, visible un-

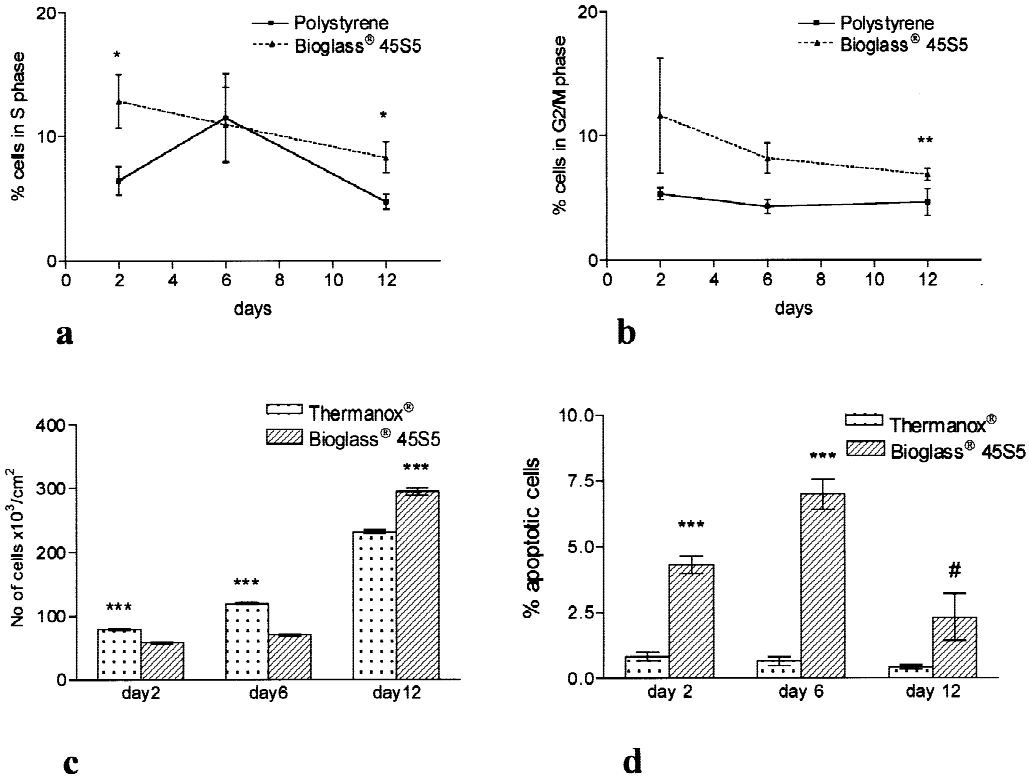

Fig. 2 (a) Flow cytometric analysis of the cell cycle. There was a significantly higher percentage of cells in the S phase after both 2 and 12 days, on the bioactive substrate compared with control, but not after 6 days in culture. Additionally **(b),** there was a significantly higher percentage of cells in the G2/M phase on the bioactive substrate compared with control after 12 days in culture. **(c)** Evaluation of cell densities on the bioactive glass and control bioinert substrate demonstrated significantly higher numbers of cells on the bioactive glass following 12 days in culture. **(d)** Microscopic enumeration of apoptotic cells following 2, 6, and 12 days in culture. Apoptosis was significantly higher during the initial 6 days in culture on the bioactive glass compared with control bioinert substrate but declined by day 12. Pairwise comparisons were performed using *t*-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Bioactive substrate-time comparisons were performed by ANOVA with Dunnet's post-test (#$P < 0.05$).

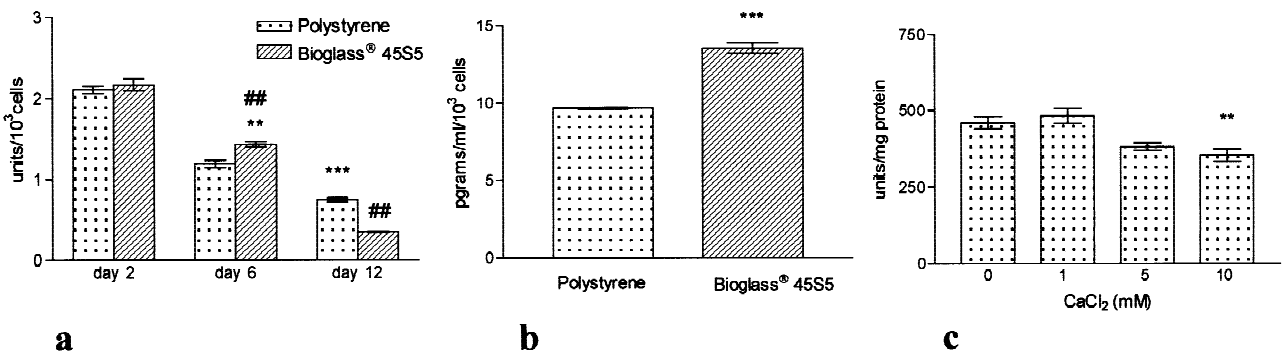

Fig. 3 (a) Akaline phosphatase specific activity of osteoblasts cultured on the bioactive glass was significantly higher compared with control on day 6, but declined to levels significantly lower than control by day 12. **(b)** Osteocalcin synthesis was significantly higher in cells cultured on Bioglass 45S5 than on polystyrene following 6 days in culture. **(c)** Alkaline phosphatase specific activity decreases as a response to a high calcium concentration. Pairwise comparisons were performed using *t*-test (**$P < 0.01$, ***$P < 0.001$). Bioglass 45S5-time comparisons were performed by ANOVA with Dunnet's post-test (#$P < 0.05$, ##$P < 0.01$).

der SEM (a) and TEM (b) as a fibrilar network, associated with matrix vesicles at various stages of calcification (b). Subsequent SEM-EDAX analysis also confirmed that the extracellular matrix was calcified (Fig. 5).

Additional information on the extracellular matrix produced by the cell cultures on the bioactive substrate was provided by confocal scanning laser microscopy. Investigation of a series of confocal images collected at different levels of collagen I stained nodules revealed the nodules to be three-dimensional cellular structures within a collagenous extracellular matrix (Fig. 6a–e). Furthermore, analysis by conventional fluorescence microscopy of alizarin-

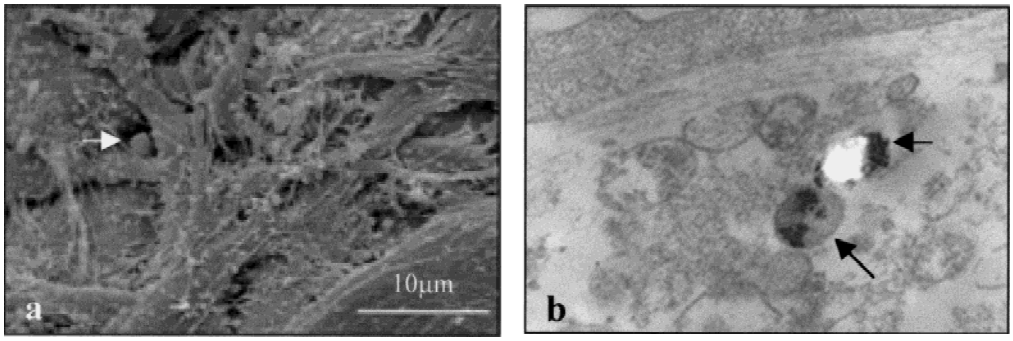

Fig. 4 (a) Scanning electron micrograph from a site within a bone nodule on Bioglass 45S5 showing the appearance of fibrillar extracellular matrix and matrix vesicles (arrow). **(b)** TEM image (×20,000) demonstrating foci of mineralisation in the form of calcified matrix vesicles (arrows).

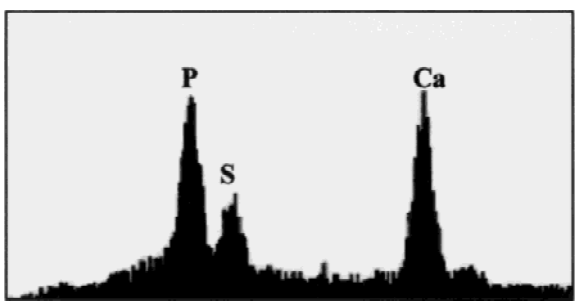

Fig. 5. EDAX analysis of a bone nodule demonstrated the presence of a mineral phase rich in calcium (Ca) and phosphorus (P). Linked to the presence of cells and extracellular organic matrix was the presence of a well-defined sulphur (S) peak. This peak is probably due to synthesis of sulfated proteoglycans by the cells.

complexone-stained nodules confirmed that the nodules also contained calcified matrix (f).

Discussion

A number of earlier studies have shown that certain bioactive glass ceramics can promote proliferation and prevent de-differentiation of osteoblasts (rat calvarial and human immortalized line) *in vitro* [10–12]. Furthermore, a line of evidence suggests that bioactive glass ceramics can increase bone formation at implantation sites *in vivo* [13–16]. The purpose of this study was to investigate the ability of bioactive glass ceramics to induce osteogenic differentiation in human primary osteoblast cultures and to investigate mechanisms related to osteogenesis *in vitro,* which previously have not been described. Specifically, this is the first attempt to correlate osteogenesis on a bioactive substrate, with a temporal sequence of biological events involving cell morphology, proliferation, and differentiation.

Initially, and in agreement with previous work, the study confirmed the surface reactivity of Bioglass 45S5, which is believed to endow the osteoproductive character of this material *in vivo* [2, 16, 22]. The surface reaction layer was visualized using SEM and subsequently analyzed with X-ray probe analysis and shown to be rich in calcium, phosphorus, and silicon. Therefore, the process of immersion of Bioglass in culture medium (conditioning), prior to seeding with cells, aimed to promote the deposition of this calcium-phosphate rich layer. Conditioning *in vitro* mimics a similar process occurring at implantation sites *in vivo,* once the implant becomes perfused with body fluids [16]. Additionally, conditioning attenuated variations in pH due to release of basic ions from the substrate and thereby minimized pH-dependent cell damage [12]. Finally, it is suggested that conditioning can promote adsorption of biological molecules from the culture medium onto the developing calcium-phosphate rich layer, which in turn may regulate biological events such as cell attachment [17].

It is well known that cell behavior in a culture system can be influenced by the physicochemical characteristics of the substrate [23–25]. Indeed, human primary osteoblasts cultured on bioactive and bioinert substrates demonstrated significant differences in cell morphology and mode of adhesion, as evaluated by SEM. Osteoblasts cultured on the bioactive substrate showed features suggestive of cell activation [26], including numerous dorsal membrane ruffles and microvilli. However, the temporal sequence of osteoblast adhesion [27] to the bioactive substrate was relatively much slower compared with that to the inert substrate, suggesting that the cells needed to adapt to the new environment.

Bone formation *in vivo* is dependent on recruitment of mesenchymal precursors that undergo several proliferative phases before differentiation to a phenotype that supports matrix deposition and mineralization [28]. Therefore, osteoblast proliferation was investigated by flow cytometric analysis of cell cycle. The results from this experiment suggested that the bioactive substrate induced a biphasic response, with two distinct proliferative phases, as indicated by increased cell cycling occurring at days 2 and 12. This was interspersed by a lag-phase, as suggested by lack of differences in cell cycling between the bioactive and control substrate on day 6.

Despite two distinct phases of increased activation of cell cycling and proliferation, significant differences in cell densities on bioactive substrate compared with control did not become apparent until day 12. This correlates with the formation of cellular multilayers on the bioactive substrate, which can be translated as more cells per unit area. However, the apparent disparity between stimulation of DNA synthesis, shortly after seeding on the bioactive substrate, and lack of progression into mitosis may be associated with the finding that apoptosis was markedly increased during this stage.

Recent studies have shown that apoptosis is a significant feature of osteoblast differentiation during the development

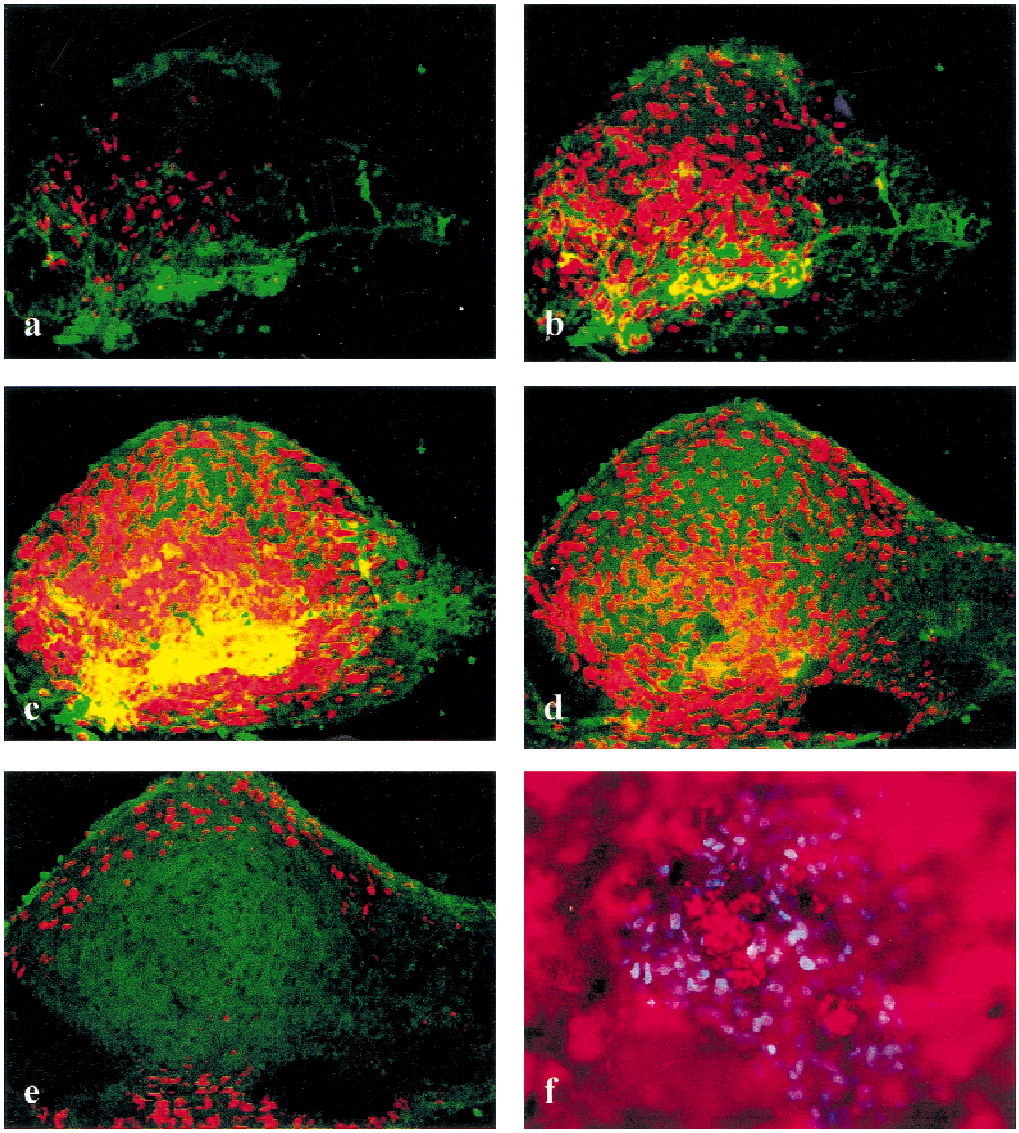

Fig. 6 (a–e). A series of confocal scanning laser microscopy images (z-series) collected at different levels through a bone nodule grown on Bioglass 45S5 and stained for collagen I (×250). The images, which are collected over a distance of 35 μm, demonstrate the three-dimensional form of the nodule and confirm the presence of collagenous matrix. Cell nuclei were counter-stained with propidium iodide. **(f)** Conventional fluorescence microscopy investigation of an Alizarin-complexone-stained nodule (×200) demonstrating diffuse calcium deposition. Cell nuclei were counter-stained with DAPI.

of bone-like tissue *in vitro* [29, 30]. Therefore, increase in apoptosis, during the initial days in culture, could suggest that the bioactive substrate induces remodelling within the osteoblast population in favor, possibly, of a subpopulation which can adapt to the substrate-specific microenvironment. This assumption is supported by SEM observations that demonstrate clear differences in cell morphology between bioactive and bioinert substrates. Further evidence to support this notion was obtained by investigating the expression of different osteoblast differentiation markers.

Parameters of osteoblast differentiation investigated included ALP specific activity and osteocalcin synthesis. Alkaline phosphatase, a cell membrane-associated enzyme, is known to be associated with osteoblast differentiation [31]. It has been suggested that ALP regulates phosphate metabolism and locally down-regulates inhibitors of apatite crystal growth [32]. It is a marker that appears early during osteoblast differentiation and both its expression and activity diminish with the onset of mineralization, a process that occurs during the later stages of osteoblast differentiation [33]. In contrast, osteocalcin is a noncollageneous matrix protein that contains two glutamic acid residues with high affinity for Ca [34] and is known to inhibit both *in vitro* [35] and *in vivo* [36] apatite crystal growth.

Many reports suggest that osteocalcin is a marker that appears late during osteoblast differentiation and characterizes mature cells of the osteoblastic lineage (e.g. osteocytes), actively producing mineralized tissue [33]. Results from the present study show that osteoblast ALP activity was higher in the cultures growing on the bioactive glass on day 6, but declined by day 12 to levels significantly less than in the control cultures. It might also be possible that the changes in ALP activity observed could have been a result of the calcium-rich environment of Bioglass 45S5. By con-

trast, osteocalcin synthesis was found to be significantly higher in osteoblast cultures grown on the bioactive glass than on the inert substrate following 6 days in culture. Taken together, these results suggest that the bioactive glass substrate enhanced the development of a mature osteoblast phenotype. This concept was further supported by the observation of discrete mineralized bone nodules in osteoblast cultures growing on the bioactive substrate.

Bone nodules consist of differentiated osteoblasts, extracellular matrix, and associated minerals, and their formation characterizes a late stage of osteoblast differentiation [28]. Several lines of evidence suggest that their presence is a good index of osteogenesis *in vitro* [28, 37, 38]. It has been reported that bone nodule formation occurs when human bone-derived cells are cultured for extended periods of time in the presence of ascorbate and/or β-glycerophosphate [28]. This study demonstrated that calcified bone nodule formation can be detected as early as day 6 in culture on the bioactive glass, without either of the above supplements in the culture medium.

In conclusion, these results provide new information regarding the biological action of bioactive glass ceramics. Taken together, the findings demonstrate the ability of these materials to stimulate cell cycling and subsequently enhance osteoblast turnover, resulting in the selection of a mature cell population capable of producing bone-like tissue *in vitro* in a relatively short period of time. These findings have potential implications and applications for tissue engineering where three-dimensional bioactive glass-ceramic substrates could be used as scaffolds for *in vitro* production of bioengineered bone.

Acknowledgments. This study was supported by USBiomaterials Inc, The Golden Charitable Trust, and The Julia Polak Lung Transplant fund. The authors thank Dr. T.A. Ryder, Mrs. M.A. Mobberley, and Mrs. C. Timpson for their expert assistance on electron microscopy.

References

1. Damien JC, Parson JR (1991) Bone graft and bone graft substitutes: a review of current technology and applications. J Appl Biomaterials 2:187–208
2. Hench LL, West JK (1996) Biological applications of bioactive glasses. Life Chem Rep 13:187–241
3. Cao W, Hench LL (1996). Bioactive materials. Ceramics Int 22:493–507
4. Kokubo T (1991) Bioactive glass-ceramics: properties and applications. Biomaterials 12:155–163
5. Putnam AJ, Mooney DJ (1996) Tissue engineering using synthetic extracellular matrices. Nat Med 2:824–826
6. Langer R, Vacanti JP (1993) Tissue engineering. Science 260: 920–926
7. Crane GM, Ishaug SL, Mikos A (1995) Bone tissue engineering. Nat Med 1:1322–1324
8. Minuth WW, Sittinger M, Kloth S (1998) Tissue engineering: generation of differentiated artificial tissues for biomedical applications. Cell Tissue Res 291:1–11
9. Nerem RM, Sambanis A (1995) Tissue engineering: from biology to biological substitutes. Tissue Eng 1:3–13
10. Matsuda T, Davies JE (1987) The in vitro response of osteoblasts to bioactive glass. Biomaterials 8:275–284
11. Vrouwenvelder WCA, Groot CG, de Groot K (1992) Behaviour of fetal rat osteoblasts cultured in vitro on bioactive glass and nonreactive glasses. Biomaterials 13:382–392
12. Price N, Bendall SP, Frondosa C, Jinnah RH, Hungerford DS (1997) Human osteoblast-like cells (MG63) proliferate on a bioactive glass surface. J Biomed Mater Res 37:394–400
13. Gross UM, Strunz V (1985) The interface of various glasses and glass-ceramics with a bony implantation bed. J Biomed Mater Res 19:251–271
14. Ito G, Matsuda T, Inoue N, Kamegai T (1987) A histological comparison of the tissue interface of bioglass and silica glass. J Biomed Mater Res 21:485–497
15. Schepers E, De Clercq M, Ducheyne P, Kempeneers R (1991) Bioactive glass particulate material as a filler for bone lesions. J Oral Rehab 18:439–452
16. Oonishi H, Kushitani S, Yasukawa E, Iwaki H, Hench LL, Wilson J, Tsuji E, Sugihara T (1997) Particulate Bioglass compared with hydroxyapatite as a bone graft substitute. Clin Orthop 334:316–325
17. El-Ghannam A, Ducheyne P, Shapiro IM (1995) Bioactive material template for in vitro synthesis of bone. J Biomed Mater Res 29:359–370
18. Beresford JN, Gallacher JA, Russell RG (1984) Production of osteocalcin by human bone cells in vitro. Effects of 1,25 $(OH)_2D_3$, 24,25$(OH)_2D_3$, parathyroid hormone and glycocorticoids. Metab Bone Dis Rel Res 5:229–234
19. Mitry RR, Sarraf CE, Wu CG, Pignatelli M, Habib NA (1997) Wild-type p53 induces apoptosis in Hep3B through up-regulation of Bax expression. Lab Invest 77:369–378
20. Maciorowski Z, Delic J, Padoy E, Klijanienko J, Dubray B, Cosset JM, Dumont J, Magdelenat H, Vielh P (1998) Comparative analysis of apoptosis measured by Hoechst and flow cytometry in non-Hodgkin's lymphomas. Cytometry 32:44–50
21. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principles of dye binding. Anal Biochem 72:248–254
22. Hench LL, Andersson ÖH, LaTorre GP (1991) The kinetics of bioactive ceramics part III: surface reactions for bioactive glasses compared with an inactive glass. In: Bonfield W, Hastings GW, Tanner KE (eds) Bioceramics 4:156–161
23. Ireland GW, Dopping-Hepenstal P, Jordan P, O'Neill C (1987) Effect of patterned surfaces of adhesive islands on the shape, cytoskeleton, adhesion and behaviour of swiss mouse 3T3 fibroblasts. J Cell Science S8:19–33
24. O'Neill C, Jordan P, Riddle P, Ireland G (1990) Narrow linear strips of adhesive substratum are powerful inducers of both growth and total focal contact area. J Cell Sci 95:577–586
25. Patel N, Padera R, Sanders GHW, Gannizzaro M, Davies MC, Langer R, Roberts CJ, Tendler SJB, Williams PM, Shakesheff KM (1998) Spatially controlled cell engineering on biodegradable polymer surfaces. FASEB 12:1447–1454
26. Wyckoff JB, Insel I, Khazaie K, Lichtner RB, Condeelis JS, Segall JE (1998) Suppression of ruffling by the EGF receptor in chemotactic cells. Exp Cell Res 242:100–109
27. Sautier JM, Kokubo T, Ohtsuki T, Nefussi JR, Boulekbache H, Oboeuf M, Loty S, Loty C, Forest N (1994) Bioactive glass-ceramic containing crystalline apatite and wollastonite initiates biomineralisation in bone cell cultures. Calcif Tissue Int 55:458–466
28. Beresford JN, Graves SE, Smoothy CA (1993) Formation of mineralised nodules by bone-derived cells in vitro: a model of bone formation? Am J Med Genet 45:163–178
29. Lynch MP, Capparelli C, Stein JL, Stein GS, Lian JB (1998) Apoptosis during bone-like tissue development in vitro. J Cell Biochem 68:31–49
30. Fratzl-Zelman N, Horandner H, Luegmayr E, Varga F, Ellinger A, Erlee MP, Klaushofer K (1997) Effects of triiodothyronine on the morphology of cells and matrix, the localization of alkaline phosphatase, and the frequency of apoptosis in long-term cultures of MC3T3-E1 cells. Bone 20:225–236
31. Aubin J, Turksen K, Heersche NM (1993) Osteoblastic cell lineage. In: Noda M (ed) Cellular and molecular biology of bone. Academic Press Inc. San Diego, CA p 2

32. Wuthier RE, Register TC (1985) Role of alkaline phosphatase, a polyfunctional enzyme, in mineralising tissues. In: Butler WT (ed) The chemistry and biology of mineralised tissues. Ebso Media, Birmingham, AL, p 113
33. Owen TA et al. (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430
34. Hauschka PV, Lian JB, Cole DEC, Gundberg CM (1989) Osteocalcin and matrix GLA protein: vitamin K-dependent proteins in bone. Physiol Rev 69:990–1047
35. Romberg RW, Werness PG, Riggs BL, Mann KG (1986) Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25:1176–1180
36. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsedy G (1996) Increased bone formation in osteocalcin-deficiency mice. Nature 382:448–452
37. Malaval L, Modrowski D, Gupta AK, Aubin JE (1994) Cellular expression of bone-related proteins during the in vitro osteogenesis in rat bone marrow stromal cell cultures. J Cell Physiol 158:555–572
38. Hughes FJ, Collyer J, Stanfield M, Goodman SA (1995) The effects of bone morphogenetic protein-2, -4, -6 on differentiation of rat osteoblast cells in vitro. Endocrinology 136: 2671–2677