EFFECTS OF STRONTIUM-DOPED BIOACTIVE GLASS ON THE DIFFERENTIATION OF CULTURED OSTEOGENIC CELLS

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Abstract

There is accumulating evidence that strontium-containing biomaterials have positive effects on bone tissue repair. We investigated the *in vitro* effect of a new Sr-doped bioactive glass manufactured by the sol-gel method on osteoblast viability and differentiation. Osteoblasts isolated from foetal mouse calvaria were cultured in the presence of bioactive glass particles; particles were undoped (B75) or Sr-doped with 1 wt.% (B75-Sr1) and 5 wt.% (B75-Sr5). Morphological analysis was carried out by contrast-phase microscopy and scanning electron microscopy (SEM). Cell viability was evaluated by the MTS assay at 24 h, 48 h and 72 h. At 24 h, day 6 and day 12, osteoblast differentiation was evaluated by assaying alkaline phosphatase (ALP) activity, osteocalcin (OC) secretion and gene expression of various bone markers, using Real-Time-PCR. Alizarin Red staining and ALP histoenzymatic localisation were performed on day 12. Microscopic observations and MTS showed an absence of cytotoxicity in the three investigated bioactive glasses. B75-Sr5 particles in cell cultures, in comparison with those of B75 and B75-Sr1, resulted in a significant up-regulation of Runx2, Osterix, Dlx5, collagen I, ALP, bone sialoprotein (BSP) and OC mRNA levels on day 12, which was associated with an increase of ALP activity on day 6 and OC secretion on day 12. In conclusion, osteoblast differentiation of foetal mouse calvarial cells was enhanced in the presence of bioactive glass particles containing 5 wt.% strontium. Thus, B75-Sr5 may represent a promising bone-grafting material for bone regeneration procedures.

Keywords: Bioactive glass, strontium, osteoblast, cell differentiation, *in vitro*.

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Introduction

Various grafting materials are being utilised today in an attempt to repair osseous defects. The use of these materials is based on the assumption that they possess osteogenic potential, and are osteoconductive (able to support bone formation) or osteoinductive (able to induce bone formation) (Wang *et al*., 2005). Although autogenous bone is considered the gold standard for bone regeneration, as it is the only material that possesses these three properties, some factors may limit its use (Becker *et al*., 1994). In fact, associated donor site morbidity and limited intraoral supply have encouraged the development of alternative materials (Nkenke *et al*., 2001; Clavero and Lundgren, 2003). Synthetic grafts, allografts and xenografts are essentially osteoconductive materials that act as a scaffold for revascularization and new bone ingrowth. Among these materials, bioactive glasses are bone substitutes that are thought to have not only osteoconductivity, but also to be responsible for osteoproduction by stimulating proliferation and differentiation of osteoprogenitor cells through a direct genetic control (Hench *et al.*, 1971; Xynos *et al.*, 2000). The discovery of these new materials led Hench and Wilson to propose the concept of "osteostimulation" or "osteopromotion" to define this class of bioactive materials and their effects on the genetic activation of bone cells (Hench and Wilson, 1984). Bioactive glasses are surface reactive biomaterials that, when in contact with physiological fluids, release soluble ionic products that have been suggested to stimulate *in vitro* osteogenesis (Xynos *et al*., 2000; Tsigkou *et al.*, 2009). In addition, *in vivo* studies have demonstrated beneficial results from their use in various clinical situations (Froum *et al.*, 1998; Anderegg *et al.*, 1999; Froum *et al.*, 2002; Peltola *et al.*, 2006). After implantation, interaction with surrounding tissues results in a time-dependent alteration of the material's surface and the formation of a hydroxyl carbonate apatite layer that is very similar to the mineral phase of bone (Hench and Paschall, 1973). More recently, a new category of sol-gel glasses has been manufactured with enhanced bioactivity and open pores enclosed in a mesoporous matrix (Xiaoxia *et al.*, 2004; Izquierdo-Barba *et al.*, 2008). Furthermore, bioactive glass manufactured *via* the sol-gel technique permits the synthesis of materials

with higher purity and homogeneity at low temperatures (Li *et al.*, 1991). Additives can be easily introduced during the sol-gel process to improve the bioactivity of such glasses. Indeed, improvement of the biological properties of bioactive materials can be achieved by the incorporation of ions that positively affect osteoblast behaviour and consequently enhance *de novo* bone formation. For example, niobium incorporation in biphasic calcium phosphate enhances the alkaline phosphatase (ALP) activity of normal human osteoblasts (NHOst), and increases the amount of calcium that is deposited with a dose-dependent effect (Tamai *et al.*, 2007). Using zinc-containing calcium phosphate ceramics with a maintained (Ca+Zn)/P molar ratio and varying zinc contents, Ikeuchi *et al.* also reported enhanced ALP activity with increasing zinc content in the ceramics in both rat and human bone marrow cells (BMCs), suggesting the superiority of zinc-containing ceramics in supporting osteogenic differentiation (Ikeuchi *et al.*, 2003).

Strontium (Sr) is a naturally occurring trace element in the human body. The total amount of Sr in the body of a 70 kg "standard" man represents 0.32 g; a normal diet provides a daily intake of 2-4 mg Sr per day, most of it derived from vegetables and cereals. Sr often acts similarly to Ca in the human body; both have strong bone-seeking properties, and Sr can be substituted with Ca in the apatitic phase of bone mineral (Vaughan, 1981). Some detrimental effects of Sr deficiency have been previously investigated. For example, caries prevalence is inversely related to Sr levels in water, plaque, and enamel (Curzon *et al.*, 1982). Administration of Sr in moderate doses prevented caries in rats (Curzon, 1985). Among the trace metals present in human bone, Sr was the only one that was correlated with bone compression strength (Jensen *et al.*, 1997). Furthermore, over the past few years Sr has attracted attention through its beneficial effects on bone healing. Indeed, both *in vitro* and *in vivo* studies have demonstrated stimulatory effects of Sr on osteoblasts and an inhibitory effect on osteoclasts, associated with an increase in bone density and resistance (Marie *et al.*, 1993; Canalis *et al.*, 1996; Baron and Tsouderos, 2002; Bonnelye *et al.*, 2008). Nowadays, strontium ranelate is used as a commercial anti-osteoporotic oral drug that has been proven to reduce the incidence of fractures in osteoporotic patients (Meunier *et al.*, 2004; Reginster *et al.*, 2008). The growing evidence of the beneficial effects of strontium on bone justifies the increasing interest in Sr incorporation into biomaterials for hard tissue repair. Thus, strontium-doped bioactive glasses have been recently developed *via* a sol-gel method that enables a better control of the reaction kinetics (Lao *et al.*, 2008; Lao *et al.*, 2009).

The objective of this study was to investigate the *in vitro* effect of strontium-doped bioactive glasses manufactured *via* the sol-gel process on biological events associated with osteoblast viability, proliferation and differentiation. For this purpose, we studied bone cell behaviour in the presence of three types of bioactive glasses, an undoped control (B75) and two Sr-doped bioactive glasses (B75-Sr1 and B75-Sr5), using a cell culture system from foetal mouse calvaria that closely mimics the cellular events of *in vivo* intramembranous bone formation process, from the stage of osteoprogenitor cell proliferation through to the stages of matrix formation and mineralisation.

Materials and Methods

Preparation of the bioactive glass particles

Glasses in the SiO_2-CaO-SrO system were manufactured using the sol-gel process (Jallot *et al.*, 2009). Samples were labelled using the following convention: glasses named B75-SrX were composed of SiO_2(75 wt.%)-CaO(25-X wt.%)-SrO(X wt.%), respectively, with X= 0, 1, and 5 wt.%. Tetraethylorthosilicate ($Si(OC_2H_5)_4$), calcium nitrate $Ca(NO_3)_2,4H_2O$, strontium nitrate $Sr(NO_3)_2$ (Sigma-Aldrich, St. Louis, MO, USA) were mixed in ethanol in the presence of water and HCl under ambient pressure. The prepared sols were then transferred to an oven at 60°C for gelification and ageing. Four hours later, the gels obtained were heated at 125°C for 24 h, then grinded to powder and stabilised at 700°C to achieve nitrate elimination and further densification. The corresponding heating schedule was as follows: a furnace was programmed to heat up from room temperature to 700°C at a rate of 10°C/min, then the samples were heated at 700°C for 24 h, and finally they were allowed to cool down to room temperature. For cell cultures, bioactive glass granules were sterilised by dry heat at 180°C for 3 h in a furnace and were pre-incubated for 24 h in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, San Diego, CA, USA) at a concentration of 4 mg/mL prior to the experiments.

Characterisation of bioactive glasses

The compositions (expressed in molar and weight %) of the bioactive glass powders under study were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Table 1). The ICP-AES can be applied to both liquid and solid samples. The technique allows the direct analysis of liquids. In case of a solid sample, it is first dissolved using the following procedure: the sample powder is mixed with $LiBO_3$ and then fused in graphite crucibles at 1000°C. A small spherical bead is

Table 1: Oxide composition (molar and weight %) of B75, B75-Sr1 and B75-Sr5 glasses. Theoretical composition in weight are given into brackets.

mol %	B75	B75-Sr1	B75-Sr5
SiO_2	73.7	74	75.5
CaO	26.3	25.4	21.6
SrO	-	0.6	2.9
wt %			
SiO_2	72.2 (75)	74.2 (75)	74.1 (75)
CaO	24.5 (25)	23.6 (24)	19.0 (20)
SrO	-	0.83 (1)	3.83 (5)

obtained which is finally dissolved in an HNO_3 2N acid solution. The sample solution is then introduced into the apparatus where it is transformed into an aerosol by a so-called nebulizer. An inductively-coupled plasma is used to dissociate the sample droplets into their constituent atoms or ions, exciting them to a higher energy level. They return to their ground state by emitting electromagnetic radiation of characteristic wavelengths. This light is recorded by an optical spectrometer. When calibrated against standards the technique provides a highly sensitive quantitative analysis of the original sample.

Nitrogen gas sorption analyses were performed to characterise the textural properties of the glasses. The samples were vacuum outgassed at 120°C for 12 h to remove physically adsorbed molecules such as moisture from the pores. The adsorption/desorption isotherms were recorded on a Quantachrom Autosorb-1 apparatus. The instrument determined isotherms volumetrically by a discontinuous static method at 77 K. The surface areas were obtained by applying the Barrett-Emmett-Teller (BET) method to the N_2 isotherm. The pore size distribution was determined by the Barrett-Joyner-Halanda (BJH) method on the desorption branch. Total pore volume was measured at a relative pressure $P/P_0 = 0.995$.

Dissolution assays were also conducted on the three glasses, in order to evaluate the ion leaching capacity of each material in the absence of cells. The bioactive glasses were first put in contact with the basal DMEM medium for 24 h, then with the supplemented cell culture media (see Culture model and cell cultivation) at a concentration of 4 mg/mL. The experiments were conducted at 37°C and the culture media was renewed every 72 h. After 24 h and 2, 3, 4, and 6 days of interaction, Si, Ca and Sr concentrations in the liquid media were determined by ICP-AES analyses.

Cell culture and analyses

Culture model and cell cultivation
Osteoblastic cells were enzymatically isolated from calvaria of Swiss OF1 mouse foetuses on embryonic day 18. All procedures for the care and use of animals were conducted after approval from the regional animal experimentation ethics committee (p3/2008/043) and the French Ministry for the Environment and Research (no. 5195). Briefly, frontal and parietal bones of calvaria were aseptically dissected and bone fragments were digested with 0.25% collagenase type I (Sigma-Aldrich) in PBS for 2 h at 37°C. Then cells dissociated from bone fragments were plated at a density of 2×10^4 cells/cm^2 in culture dishes, and grown in osteogenic medium composed of DMEM (Invitrogen), supplemented with 10% foetal calf serum (FCS) (Invitrogen), 50 µg/mL ascorbic acid (Sigma-Aldrich), 10 mM β-glycerophosphate (Sigma-Aldrich), and 50 UI/mL penicillin-streptomycin (Invitrogen). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO_2 in air and culture medium was changed at 48 h intervals. The cells were allowed to proliferate in culture until subconfluence and were passaged once before use in experiments. After one day of culture, the cells reached

80% confluence and the pre-incubated granules were added to the cultures at the concentration of 4 mg/mL of culture medium. The experiments were conducted in triplicate to minimize errors caused by the uneven distribution of particles.

Cell morphology - scanning electron microscopy (SEM)
Calvarial bone cells were plated at a density of 2×10^4 cells/cm^2 on Thermanox Plastic coverslips (NUNC, Rochester, NY, USA) placed in a 12-well plate. At 80% confluence, B75-Sr5 glass particles were added to cell cultures at a concentration of 4 mg/mL of culture medium. 48 h later, cells cultured on the plastic coverslips were fixed for 1 h in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) and rinsed three times with 0.2 M sodium cacodylate buffer at pH 7.4. Samples were then dehydrated through a graded series of ethanol and amyl acetate solutions before critical point drying in CO_2. Finally, the samples were mounted on specimen stubs and sputter-coated with gold palladium. Samples were examined on a JEOL JSM 6100 SEM (Jeol France, Croissy-Sur-Seine, France) operating at a 15 kV accelerating voltage.

Cell viability-MTS test
Cell viability at 24 h, 48 h and 72 h was measured using The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). This procedure determines formazan production in living cells that are capable of reducing the MTS tetrazolium compound to coloured formazan derivatives. This intracellular bioreduction system is mostly located in the mitochondria and the amount of coloured formazan derivatives excreted into the culture medium can be measured with a microplate reader. The optical density (OD) of the cell supernatant was measured with a spectrophotometer (Precision Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 490 nm. Cells grown without bioactive glass served as a control.

Evaluation of gene expression – real-time (RT)-PCR
Calvarial bone cells grown in the presence of B75, B75-Sr1 or B75-Sr5 glass particles were recovered at 24 h, day 6 and day 12, and total RNA was extracted using the TriReagent kit (Euromedex, Souffelweyersheim, France), following supplier's instructions. In brief, total RNA was precipitated with isopropanol and centrifuged at 12000g at 4°C. Then, the RNA pellet was washed with 75% ethanol and resuspended in RNase-free water. The concentration and the purity of total RNA in each sample were determined by light absorbance at 260 nm and by calculating the A_{260}/A_{280} ratio, respectively. The integrity of RNA was confirmed by electrophoresis on an agarose ethidium bromide gel. One microgram of total RNA of every sample was reverse transcribed into cDNA using 200 units of Superscript II (Invitrogen) and 250 ng of random primers, according to the manufacturer's instructions. Real-time PCR reactions were performed using a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratory, Hercules, CA, USA). According to manufacturer's recommendations, a 15 µl volume, containing 7.5 µl of

Table 2: Primers used in real-time quantification of selected gene transcripts.

Gene transcript	Code	Forward primer sequence	Reverse primer sequence
GAPDH	XM_001473623.1	ACCCCTTCATTGACCTCAACTAC	AGTTGTCATGGATGACCTTG
Dlx5 (Distal-less homeobox 5)	NM_198854.1	CTGGCCGCTTTACAGAGAAG	CTGGTGACTGTGGCGAGTTA
Runx2 (Runt-related transcription factor 2)	NM_009820.2	GGACGAGGCAAGAGTTTCAC	TGCCTGCCTGGGATCTGTAA
Osterix (Trans-acting transcription factor 7)	NM_130458.2	CCTAGGTTATCTCCTTGCATGTCT	ATTGGGAAGCAGAAAGATTAGATG
Collagen I (Collagen, type I, alpha 1)	NM_007742.3	AAGATGTGCCACTCTGACTG	ATAGGTGATGTTCTGGGAGG
ALP (Alkaline phosphatase)	NM_007431	CCAGCAGGTTTCTCTCTTGG	CTGGGAGTCTCATCCTGAGC
BSP (Integrin binding sialoprotein)	NM_008318.1	CTTACCGAGCTTATGAGGATGAATA	AAATGGTAGCCAGATGATAAGACAG
Osteocalcin (BGLAP)	NM_031368.3	CTTGGTGCACACCTAGCAGA	ACCTTATTGCCCTCCTGCTT

IQ SYBR Green Supermix (Bio-Rad), 50 ng of cDNA as template and 0.3 µM of the appropriate primer-pairs (Eurogentec, Liège Belgium), was used. The final mixture was incubated in the MiniOpticon under the following conditions: 98°C for 10 s as the denaturation program, followed by 40 amplification cycles (95°C for 10 s, 60°C for 20 s, and 72°C for 20 s). Melting curve analysis was done to ensure that all transcripts under investigation were represented by a single peak, indicating specificity.

Each target gene (Table 2) was run in triplicate on a single 48-well plate, which included samples from all treatments, a no-template control, and 5 dilutions (1/10-1/810) of a control cDNA sample. The amplification curve of the target gene was created using these various dilutions to estimate gene expression of each sample. The gene expression levels were normalised to the expression of the housekeeping gene GAPDH and were expressed as fold changes relative to the expression of cells grown in the presence of B75 after 24h. Cells grown in the absence of bioactive glass served as a control.

ALP histoenzymatic localisation
Calvarial bone cells were cultured as described above, and, on day 12, cells were fixed in fixative solution (60% citrate buffered acetone) at room temperature and gently rinsed in deionized water for 45 s. An alkaline-dye mixture (solution of fast blue salt RR and naphthol phosphate (Sigma-Aldrich)) was added and cell samples were incubated at room temperature and protected from direct light for 30 min. After the histoenzymatic reaction, cells

were rinsed thoroughly in deionized water for 2 min and examined under a phase contrast light.

ALP activity assay
Before the biochemical assay, cells cultured in the presence of B75, B75-Sr1 and B75-Sr5 were prepared at different times (24 h, day 6 and day 12), washed with DMEM (0% FCS) on ice, and incubated in sodium-carbonate-bicarbonate buffer (0.1 M $NaHCO_3$-Na_2CO_3, pH 10.2). Samples were stored at -80°C. For all assays (in triplicate), osteoblastic cell cultures were unfrozen and incubated in an extraction buffer (0.1 M $NaHCO_3$-Na_2CO_3, pH 10.2, 1 M $MgCl_2$ and 0.2% Igepal CA-630 (Sigma-Aldrich)) for 10 min and removed from their substrate with a rubber policeman. Cell extracts were sonicated before the enzyme assay to dissociate the extracellular matrix and liberate membranous ALP. The protein content was estimated using the Pierce BCA Protein Assay Kit (Pierce Chemical. Rockford, IL, USA). The specific activity of ALP was assayed in cell layers by the release of *p*-nitrophenol (pnp) from *p*-nitrophenolphosphate (Sigma-Aldrich). The optical density was read at 410 nm in a spectrophotometer and the enzyme activity was expressed as nmol pnp/µg protein/hour. Cells grown in the absence of bioactive glass served as a control.

Osteocalcin protein production
Osteocalcin (OC) protein levels in cell culture supernatants were determined by an enzyme immunoassay (EIA) kit for intact mouse OC (Biomedical Technologies, Stoughton,

MA, USA). Cells were cultured in the presence of various bioactive glasses for periods of 6 and 12 days. Cells grown in the absence of bioactive glass served as a control. Culture medium was replaced by a serum-free medium 48 h prior to assaying. Supernatants were collected and assayed according to the manufacturer's instructions, and the OC level was normalised to the total cell number.

Calcium deposit staining
Calvarial bone cells were cultured in the presence of B75, B75-Sr1 and B75-Sr5 up to day 12, then fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature and stained for 15 min with 2% (w/v) Alizarin Red S solution (Sigma-Aldrich) at pH 4.2. Cells grown in the absence of bioactive glass served as a control. The cells were washed extensively with distilled water and viewed under a phase contrast light microscope.

Statistical analysis
All experiments were performed three times using independent cell cultures, and the measurements for each biomarker at each time point were performed in triplicate. Results of the three experiments are shown, expressed as the mean ± standard error of the mean (m ± sem). Means for biomarkers were compared within the 3 bioactive glass groups, except for the MTS test, in which control data were also included.

For each biomarker, the statistical analyses were performed using a two-way analysis of variance (bioactive glass and time point) followed by multiple pairwise comparisons with the Holm-Bonferroni adjustment to investigate the evolution of each group of materials during the culture period (3 time-points) and for each time-point, the differences between the different groups of materials. To take into account heteroscedasticity of data, these analyses were performed on log-transformed data. The overall risk was fixed at $p < 0.05$.

Results

Textural properties of bioactive glasses and dissolution assays
The physicochemical properties of bioactive glass particles with different compositions were summarised in Table 3. N_2 sorption analyses showed that bioactive glasses with different compositions possessed similar BET surface area

(24-30 m^2/g), BJH average pore (8.4-11.8 nm), BJH modal pore diameter (4.6-6.1 nm), and total pore volume (0.062-0.069). These results indicated that no significant textural changes occurred when doping B75 with 1 and 5 wt.% of strontium. Additionally, particle size distribution of bioactive glasses was determined by laser granulometry on a Malvern Mastersizer S instrument (Malvern, Worcestershire, UK). Our results showed that all samples exhibit the same morphology independently of the presence of strontium, with a wide distribution spreading from a few microns up to 100 μm with an average around 40 μm (data not shown).

The dissolution experiments revealed that Si and Ca were released in a similar way for the three materials (Fig. 1). As demonstrated in previous studies (Ikeuchi *et al.*, 2003), Ca was quickly released during the first 24 h of immersion in the basal medium (dealkalinisation stage, first step of the bioactive process) reaching 94 ppm for B75, compared to 57 and 69 ppm for B75-Sr5 and B75-Sr1 respectively, because B75 initially contains more Ca. After the first 24 h, the glasses were put in supplemented cell culture medium and Ca concentration decreased very slowly, and about 60 ppm Ca were present in the cell culture media after 24 h of interaction. Furthermore, after 144 h the Ca concentration became nearly equal to 50 ppm. Si was progressively released from the glasses to the medium, in a similar way for the three materials. Indeed, Si concentration continuously increased with incubation time (the "sharp decreases" observed in the Si curves corresponded to the renewing of the media after 72 h of interaction). Finally, in line with previous studies (Lao *et al.*, 2008), only one significant difference was observed in the dissolution behaviour of the materials: the quantity of Sr released by B75-Sr5 was about five time greater than those released by B75-Sr1.

Microscopic observations
Observations of cultures by phase contrast microscopy allowed a step-by-step investigation of the morphological changes associated with foetal mouse osteoblast maturation culminating in bone nodule formation (Fig. 2). Throughout the observation period, no noticeable differences were observed between the experimental and control cultures, including morphological changes associated with bone nodule formation. Four hours after plating, cells started to attach and spread out on the culture dishes, exhibiting polygonal morphology (data not shown). At 80%

Table 3: N_2 sorption analyses of the gel-glass particles. The surface area (S) was determined from the linear portion of the BET plot, the total pore volume (V) was estimated from the amount of N_2 adsorbed at $P/P_0 = 0.995$, the pore size distribution and the modal pore diameter were calculated by applying the BJH method to the N_2 desorption branches, and the average pore diameter was calculated as r = 2V/S.

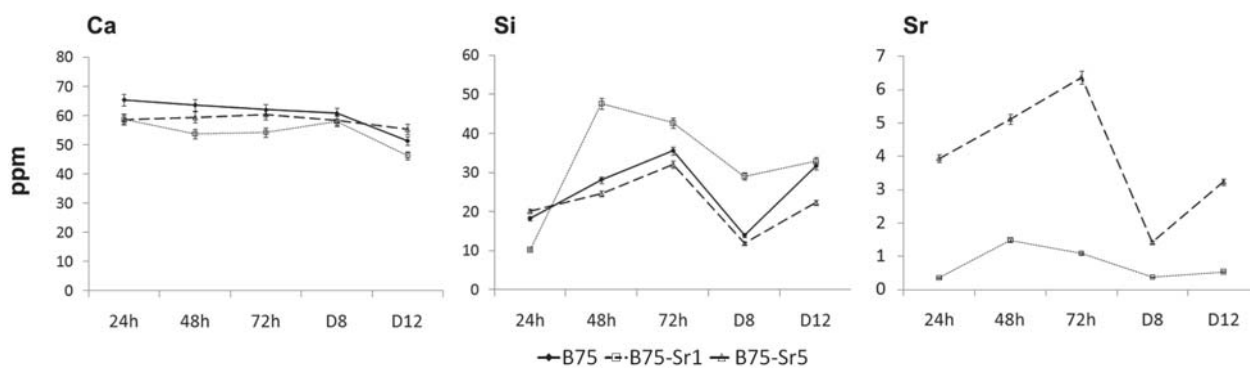|	*B75*	*B75-Sr1*	*B75-Sr5*
BET surface area (m^2/g)	30	24	28
BJH average pore diameter (nm)	8.4	11.8	9.3
BJH modal pore diameter (nm)	4.6	6.1	4.7
Total pore volume (cm^3/g)	0.062	0.069	0.066

Fig. 1. ICP analyses of the evolution of Ca, Si and Sr concentration (ppm) in the supplemented cell culture medium after 24 h, 48 h, 72 h, day 6, and day 12 in contact with B75, B75-Sr1 and B75-Sr5 glasses (*NB: the curves break observed after 72 h is due to renewing of the medium*). Note: initially, the cell culture medium is composed of 58 ppm Ca, and contains neither Si nor Sr.
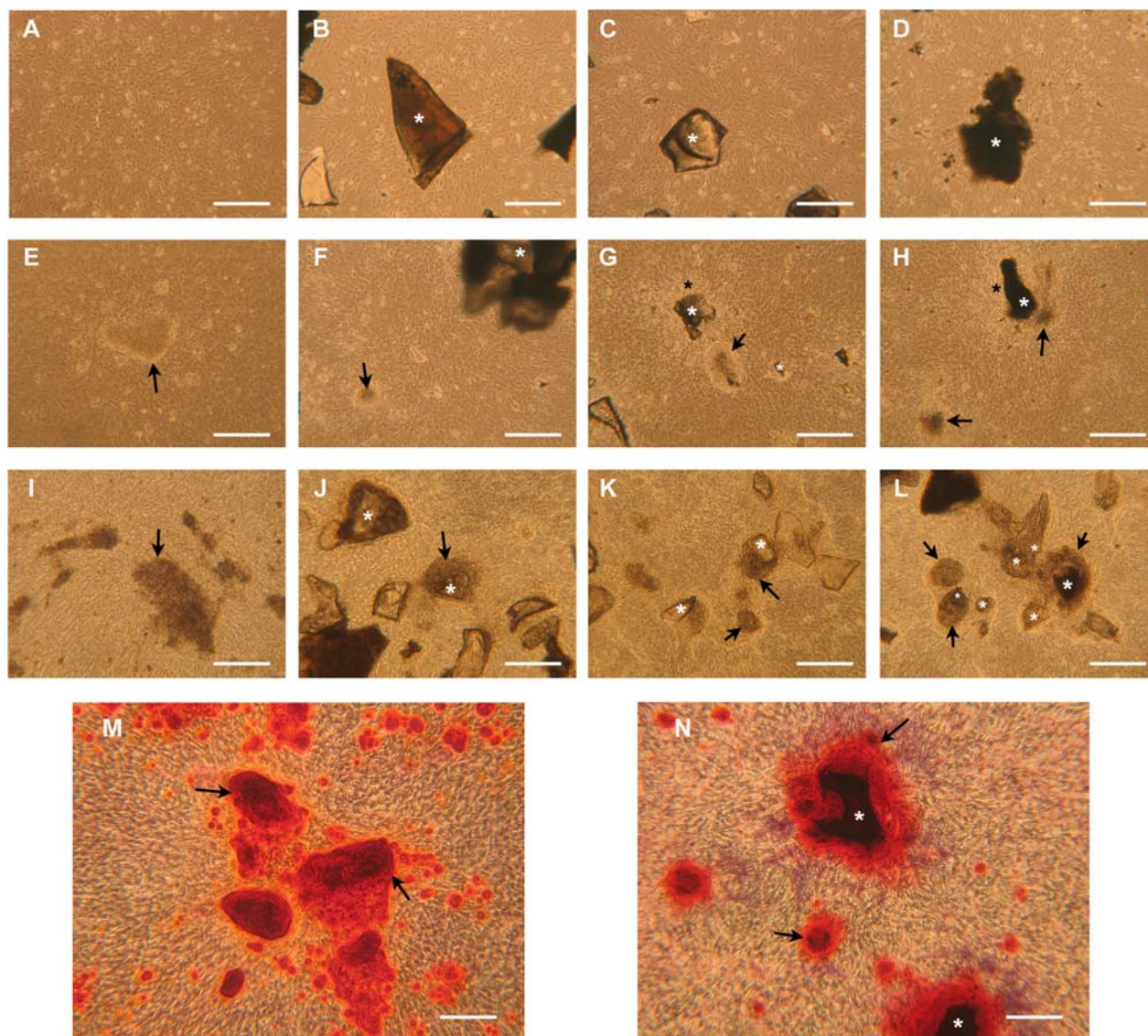

Fig. 2. Observations by phase contrast microscopy of osteoblast cultures at 24 h **(A-D)**, day 6 **(E-H)** and day 12 **(I-L)**. Cells were grown in the absence of bioactive glass **(A, E, I)**, or with particles (white asterix) of B75 **(B, F, J)**, B75-Sr1 **(C, G, K)** and B75-Sr5 **(D, H, L)**. (Bar =250 µm). At 24 h, particles were immobilised in the confluent cell layer **(B-D)**. On day 6, differentiated areas were characterised by a refringent matrix around bioactive glass particles (black asterix) and some darker condensation zones (arrows) **(F-H)**. On day 12, the differentiating areas had enlarged and dark nodules were observed (arrows) **(I-L)**. On day 12, staining for calcium accumulation with Alizarin Red dye **(M, N)** showed the presence of mineralised bone nodules (arrows) in the control condition **(M)** and surrounding B75-Sr5 particles **(N)**. (Bar = 170 µm).
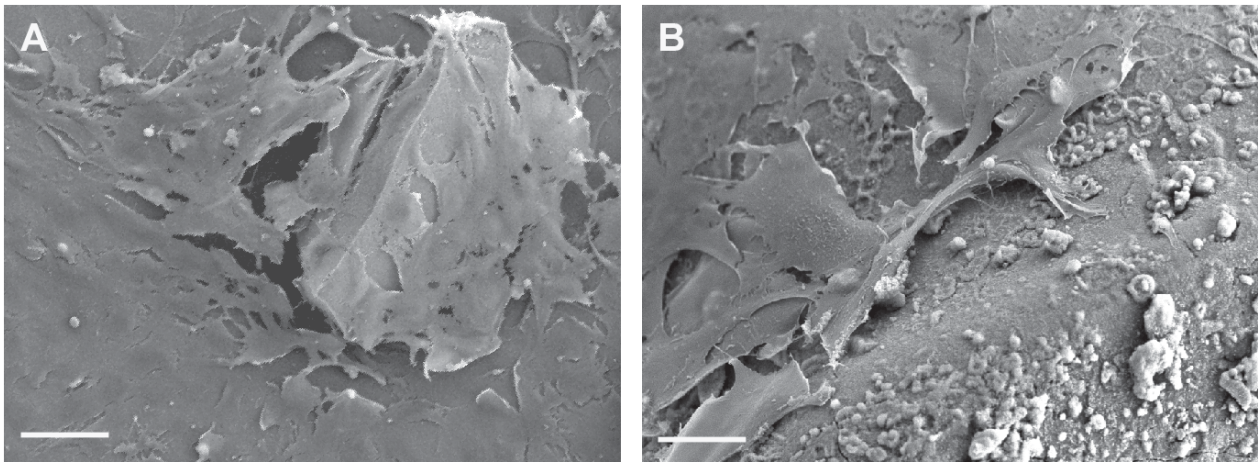
Fig. 3. Scanning electron microscopic analysis of osteoblastic cells in the presence of bioactive glass B75-Sr5 after 48 h of culture. Low magnification micrograph (**A**) showing the bound cell layer covering part of the bioactive glass particle surface (Bar = 40 μm). Higher magnification of the bioactive glass particle surface (**B**) showing osteoblast attached to the substrate by means of cytoplasmic digitations (Bar = 5 μm).
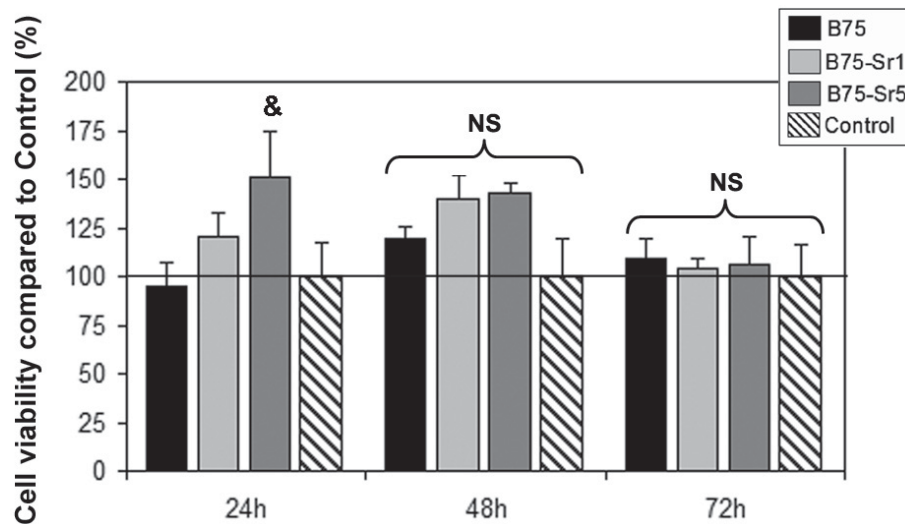

Fig. 4. MTS assay of osteoblastic cells grown with B75, B75-Sr1 and B75-Sr5 or without bioactive glass (Control) at 24 h, 48 h and 72 h. Measurements in the control are set at 100% at each time point. Bioactive glass data were expressed in percentages compared against the control. At the same time # indicates significant difference compared to control ($p<0.05$); & indicates significant difference compared to B75 and * indicates significant difference compared to B75-Sr1. NS, no significant difference.

confluence, bioactive glass particles were added. After 24 h, cells proliferated and reached confluence, thus immobilizing the granules of glass in the cell layer (Fig. 2, A-D). Thereafter, the cells formed a multilayer, and a refringent collar was observed at the periphery of the granules on day 6 (Fig. 2, E-H). On day 12 in culture, dark areas corresponding to bone nodules were observed at the periphery of the granules (Fig. 2, J-L). Some bioactive glass particles were completely surrounded by bone nodules. Alizarin Red staining revealed the presence of calcium in the bone nodules, indicating matrix mineralisation (Fig. 2, M-N).

SEM observations after 2 days of culture revealed that bone cells also colonised B75-Sr5 particle surfaces (Fig. 3A). Similar observations were obtained with the various glass particles (data not shown). Higher magnification micrographs showed that cells were anchored to particle surfaces by cytoplasmic digitations (Fig. 3B). Globular deposits were observed on the bioactive glass surface, probably resulting from ion precipitation and interaction with the culture medium.

Viability assay
Cell viability and metabolic activity were determined using the MTS assay. Foetal calvarial cells were cultured in the presence of bioactive glass particles or in control conditions, and MTS conversion was assessed after 24 h, 48 h and 72 h (Fig. 4). At each time point, no significant differences were found between control and B75 or B75-Sr1 conditions. However, significantly higher viability was observed at 24 h in the presence of B75-Sr5 particles than with B75 particles, reaching 159% of values. Nonetheless, at 48 h and 72 h, no significant differences were found between B75-Sr5 and the other conditions.
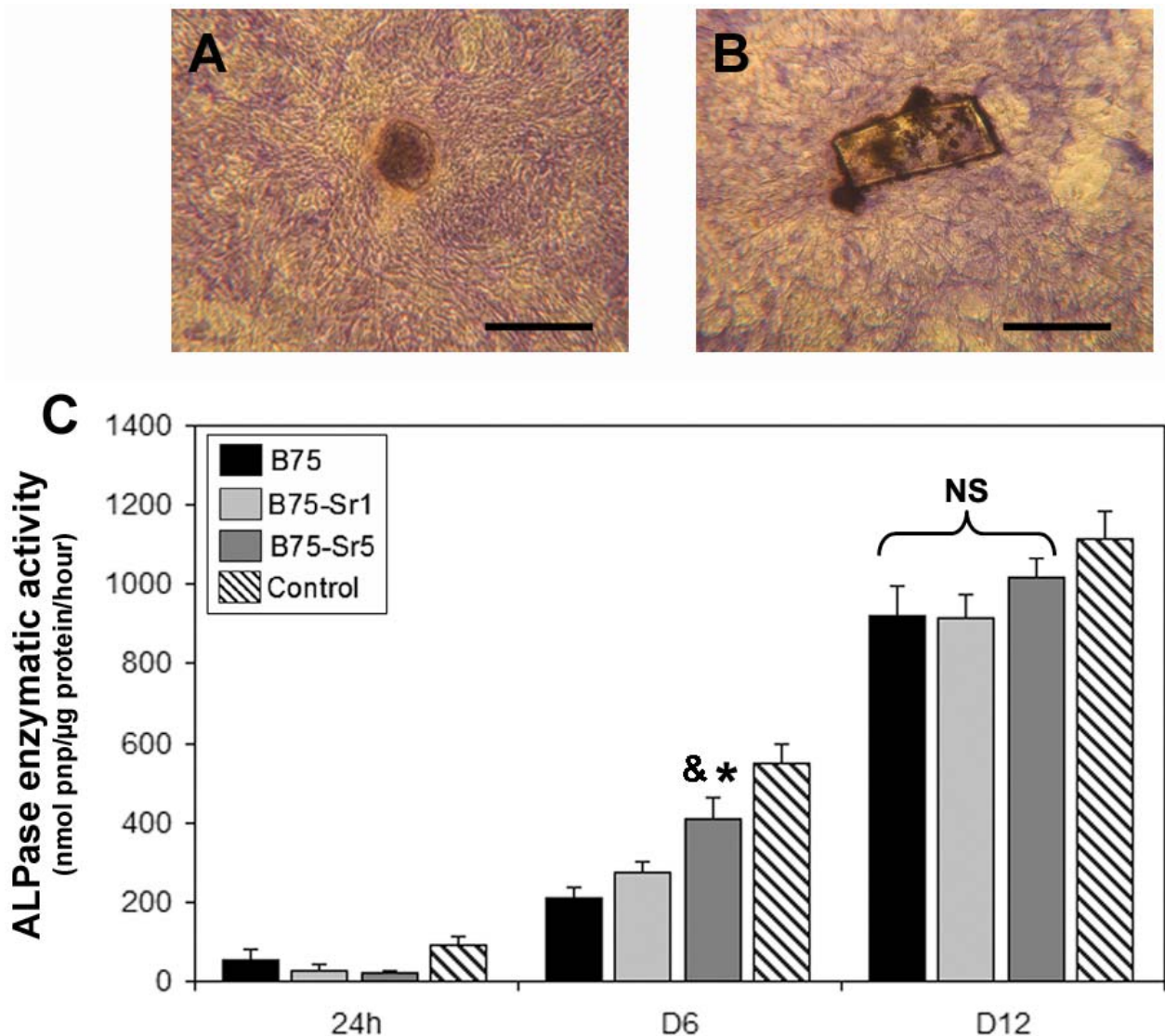
Fig. 5. Histoenzymatic localisation of alkaline phosphatase on day 12 in the control culture (A), and in the presence of B75-Sr5 particles (B) (Bar = 350 μm). Time course of ALP-specific activity over 12 days of culture, (C) in the presence or absence (Control) of bioactive glass particles. At the same time point, & indicates significant difference compared to B75 ($p<0.05$) and * indicates significant difference compared to B75-Sr1. NS, no significant difference.

ALP activity

On day 12, most osteoblastic cells were positively stained for ALP under both control and B75-Sr5 conditions (Fig. 5A,B). Furthermore, an intense staining was observed around B75-Sr5 particles (Fig. 5B). Similar staining was seen in the presence of B75 and B75-Sr1 particles (data not shown). ALP activity showed gradual and significant increases over the culture period, for all tested conditions ($p<0.05$) (Fig. 5C). No significant differences were observed among the 3 bioactive glass groups for each time point, except on day 6 where ALP activity was significantly greater in the presence of B75-Sr5 particles than in the presence of the other particles.

RT-PCR gene expression levels of specific osteogenic markers

To investigate osteoblastic differentiation of foetal calvarial bone cells in the presence of bioactive glass particles, gene expression levels of selected osteoblast markers, including bone protein markers (Fig. 6) and osteoblastic transcription factors (Fig. 7), were determined by RT-PCR at 24 h, day 6 and day 12. We found that selected genes were expressed throughout the culture period.

Genes related to extracellular bone matrix proteins had similar expression patterns, with a significant increase over time for all studied conditions ($p<0.05$) (Fig. 6). However, the magnitude of the observed increase was highly dependent on the bioactive glass being investigated. Indeed, on day 12, the expression of type I alpha 1 Collagen (Collagen I) was significantly higher in the presence of B75-Sr5 particles than in the presence of B75 and B75-Sr1 particles. ALP mRNA levels were also significantly greater in the presence of B75-Sr5 at day 6 than in the presence of B75 and B75-Sr1; this was similarly observed at day 12 in comparison with B75. Finally, in the presence of B75-Sr5 particles, cells expressed significantly higher amounts of both BSP and OC mRNA at all time points than those in the presence of B75 and B75-Sr1. BSP mRNA levels were also greater in the presence of B75-Sr1 at day 6 than in the presence of B75.
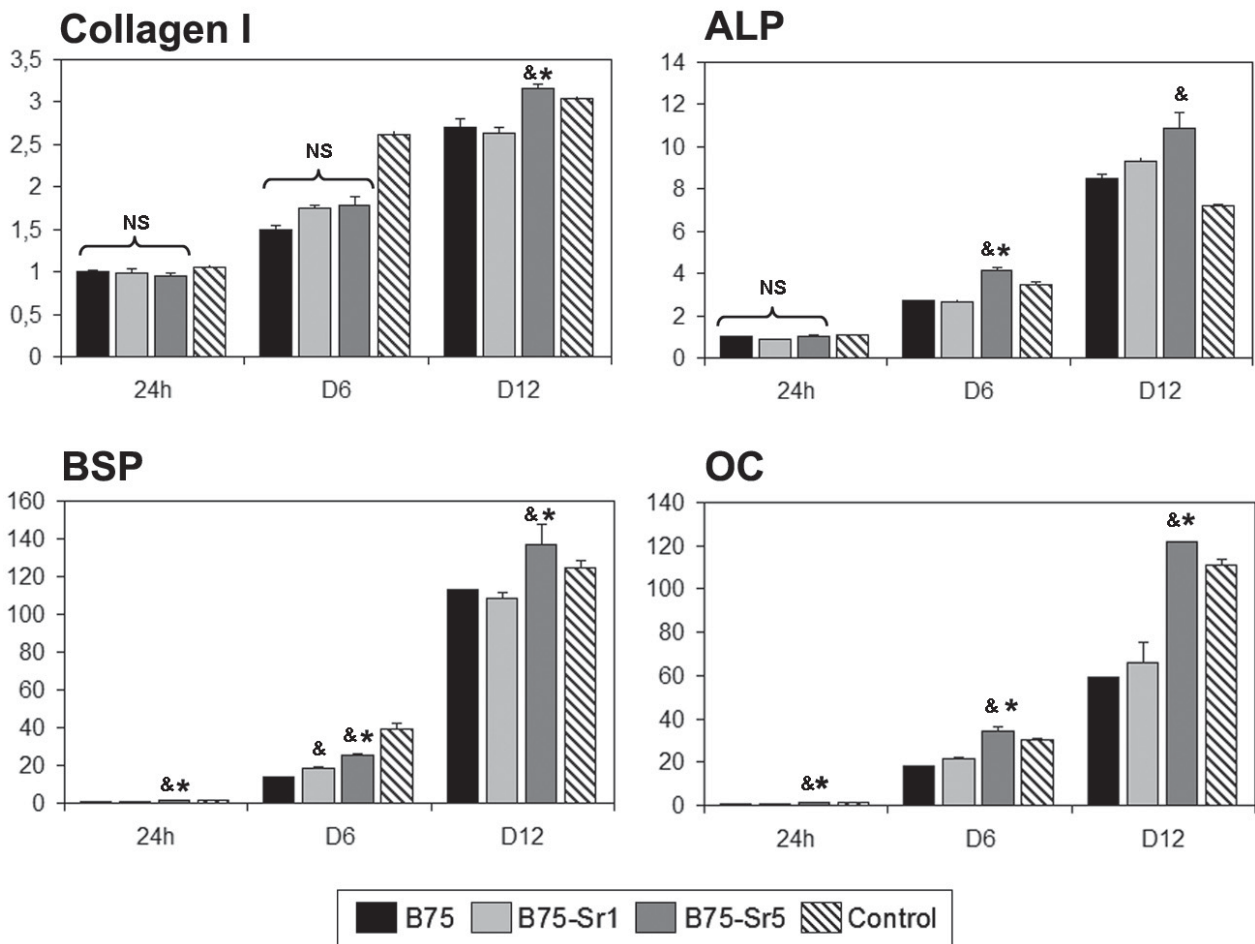
Fig. 6. Real-time PCR analysis of the gene expression of selected bone protein markers at 24 h, day 6 and day 12 by osteoblastic cells grown with B75, B75-Sr1 and B75-Sr5 particles or without bioactive glass (Control). mRNA levels of the genes of interest were normalised against the expression of the housekeeping gene GAPDH, and are represented as fold differences relative to B75 at 24h. At the same time point, & indicates significant difference compared to B75 ($p<0.05$) and * indicates significant difference compared to B75-Sr1. NS, no significant difference.
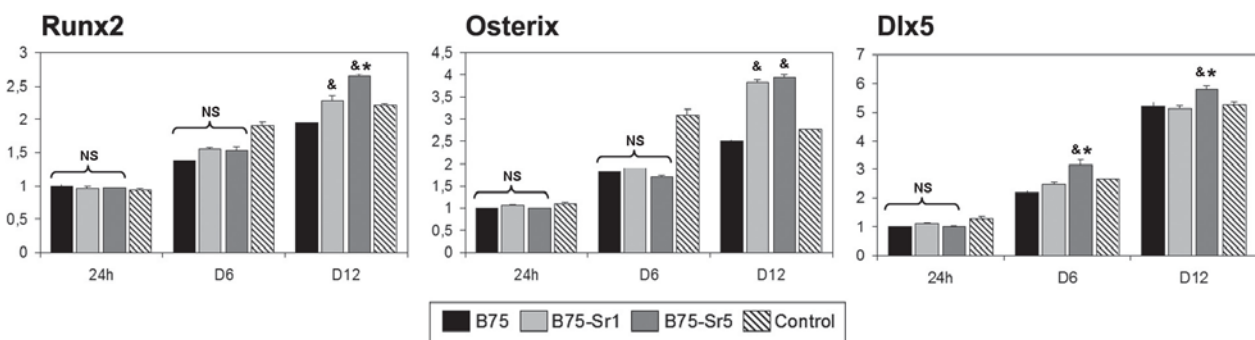

Fig. 7. Real-time PCR analysis of the expression of selected osteoblastic transcription factors at 24 h, day 6 and day 12 by osteoblastic cells grown with B75, B75-Sr1 and B75-Sr5 particles or without bioactive glass (Control). mRNA levels of the genes were normalised against the expression of the housekeeping gene GAPDH and are represented as fold differences relative to B75 at 24 h. At the same time point, & indicates significant difference compared to B75 ($p<0.05$) and * indicates significant difference compared to B75-Sr1. NS, no significant difference.

To investigate osteoblastic differentiation of calvarial bone cells further, we also determined the expression of genes related to selected transcription factors (Fig. 7). Overall, Runx2, Osterix (Osx) and Dlx5 had similar expression profiles with all the tested materials, with mRNA levels gradually and significantly increasing up to day 12 ($p<0.05$). Runx2 and Osx expressions remained comparable for all groups at 24 h and day 6 but on day 12 were significantly enhanced with both strontium-doped bioactive glasses. Moreover, on day 12, cells grown in the presence of B75-Sr5 had higher Runx2 mRNA levels than those with B75-Sr1. Culture in the presence of B75-Sr5 particles resulted also in higher Dlx5 mRNA levels on days 6 and 12 than B75 and B75-Sr1.
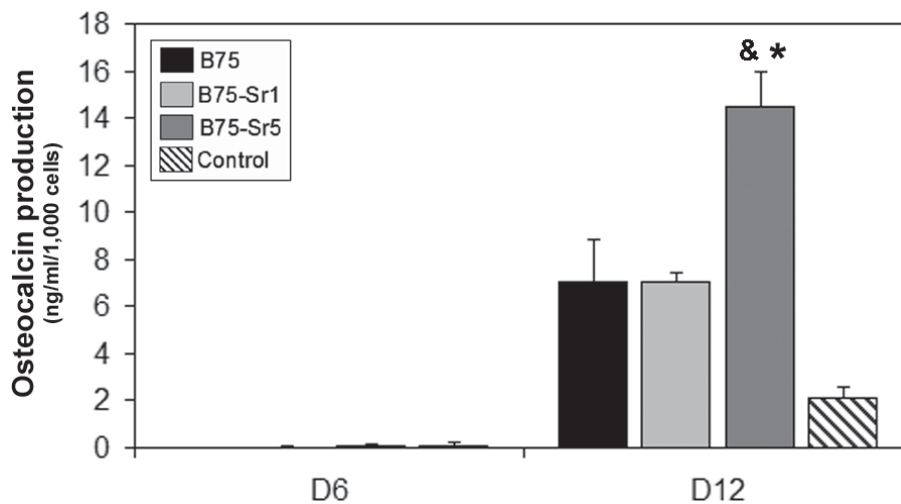
Fig. 8. Osteocalcin (OC) protein production by osteoblastic cells in the presence of bioactive glass particles (B75, B75-Sr1 or B75-Sr5) or in control condition performed by ELISA assay at days 6 and 12. Data are expressed as OC (ng/mL) secreted during 48 h in the serum-free medium, and normalised to the total cell count. At the same time point, & indicates significant difference compared to B75 ($p<0.05$) and * indicates significant difference compared to B75-Sr1.

Osteocalcin secretion

Osteocalcin protein production was assayed using an ELISA test on days 6 and 12 (Fig. 8). There was a slight secretion of OC on day 6, which significantly increased on day 12 for all tested conditions ($p<0.05$). Furthermore, although there were no significant differences observed in cells grown in the presence of B75 and B75-Sr1 on day 12, OC secretion was significantly greater in the presence to B75-Sr5 particles than in the presence of B75 and B75-Sr1.

Discussion

Bioactive glasses are a group of synthetic silica-based bioactive materials with bone-bonding properties first discovered by Larry Hench (Hench *et al*., 1971). Although bioactive glasses have been used for over 15 years with success in oral and maxillo-facial clinical applications (Froum *et al*., 1998; Peltola *et al*., 2006), limited benefits have been reported (Turunen *et al*., 2004; Wang *et al*., 2005). Recently, due to the growing evidence showing that strontium influences bone cells and bone metabolism *in vitro* and *in vivo* (Marie, 2006), melt-derived strontium-doped bioactive glasses prepared from a base 45S5 bioglass have been developed and implanted in rat tibia bone marrow (Gorustovich *et al*., 2010). However, the affinity indices of the strontium-doped bioactive glass, *i.e.*, the percentage of bone at the material-tissue interface, did not differ significantly with respect to 45S5. New bioactive glasses have been developed using the sol-gel process to improve the biological properties of these biomaterials (Li *et al*., 1991; Pereira *et al*., 1994; Hamadouche *et al.*, 2001; Olmo *et al.*, 2003; Gil-Albarova *et al.*, 2004; Yan *et al.*, 2006). The objective of this study was to evaluate the effect of new strontium-doped bioactive glasses manufactured by means of a sol-gel method on biological events associated with osteoblast differentiation. For this purpose,

we used foetal mouse calvaria cells grown in the presence of sol-gel binary SiO_2-CaO glasses that were undoped (B75) or doped with 1 or 5 wt.% of strontium (B75-Sr1 and B75-Sr5), as described by Lao *et al*. (2009).

In vivo, bone healing can be divided into three different biological phases: osteoconduction, *de novo* bone formation and bone remodelling (Davies, 1998). In this study, primary foetal mouse osteoblasts were enzymatically isolated from calvaria and then cultured with bioactive glass particles with the aim of mimicking *de novo* bone formation occurring in the presence of bone substitutes. Cell lines often exhibit incomplete patterns of osteoblastic differentiation (Cooper *et al.*, 1998) and various other mechanisms of attachment (Kilpadi *et al.*, 2004); thus, cell cultures obtained through the enzymatic digestion of bones are considered to be more relevant for investigating phenotypic changes occurring during the process of osteoblastic differentiation. Indeed, if cultured in osteogenic medium, osteogenic cells from these cultures support the proliferation and govern the commitment of osteoprogenitor cells to mature osteoblasts (Nefussi *et al.*, 1985; Owen *et al.*, 1990).

In the present study, microscopic observations of cultures and the MTS viability assay revealed that the presence of bioactive glass particles did not affect viability of bone cells, when compared with control cultures without biomaterials. SEM also revealed that calvarial bone cells colonised the surface of all bioactive glass particles tested. Taken together, these results suggested an absence of cytotoxicity in the three investigated bioactive glass compositions.

Osteoblast differentiation and subsequent bone formation is a gradual and well orchestrated process associated with characteristic temporal modifications in gene expression and characterised *in vitro* by three developmental stages: 1) proliferation, 2) extracellular matrix production and maturation, and 3) matrix mineralisation (Owen *et al.*, 1990; Garcia *et al.*, 2002).

We used this paradigm to investigate osteoblast differentiation occurring in the presence of bioactive glass particles, by monitoring bone gene expression patterns using real-time RT-PCR and extracellular matrix protein production.

We first investigated gene expression of two early osteoblast differentiation markers: Collagen I, the major organic component of the bone extracellular matrix, and ALP, a cell membrane-bound enzyme that is thought to be associated with osteoblast differentiation and phosphate metabolism (Bellows *et al.*, 1991). Both Collagen I and ALP mRNA levels gradually and significantly increased over the culture period in the various cultures. These observations, in addition to the gradual increase of ALP activity throughout the culture period and the presence of several positively stained ALP cells, demonstrated the commitment toward osteoblast differentiation for all culture conditions. These observations were further confirmed by the gene expression levels of Runx2, Osterix (Osx) and Dlx5, three transcription factors strongly involved in skeletal formation and bone repair (Ferguson *et al.*, 1999; Devlin and Sloan, 2002). Runx2 (cbfa1) and Osx are transcription factors required for osteoblast differentiation and bone formation. Thus, Runx2 or Osx gene invalidation in mice results in the absence of a bone skeleton (Komori *et al.*, 1997; Nakashima *et al.*, 2002), whereas forced expression stimulates transcription of both early and late bone-marker genes (Ducy *et al.*, 1997; Tu *et al.*, 2006). Furthermore, Runx2 is expressed in Osx-deficient mice, indicating that Osx acts downstream from Runx2 (Nakashima *et al.*, 2002). Dlx5, a member of the Dlx family (Distal Less homeobox), is a homeobox-containing transcription factor, acting upstream from Runx2 and Osterix during osteoblast differentiation (Ryoo *et al.*, 2006). Dlx5 levels gradually increased during osteoblast differentiation (Hassan *et al.*, 2004) and its overexpression stimulates the transcription of several bone-marker genes, such as Collagen I, ALP, osteopontin and OC (Tadic *et al.*, 2002). In this study, the Runx2, Osterix and Dlx5 genes were expressed throughout the culture period, and the expression of which significantly increased over time.

In the ordered hierarchical sequence of events occurring during osteoblast differentiation and maturation, collagen I biosynthesis and ALP activity are followed by the deposition of BSP, an RGD-containing glycoprotein; this is followed by osteocalcin synthesis, a protein considered to be the most specific marker for mature osteoblasts (Lian *et al.*, 1989). In this study, both BSP and OC showed similar gene expression patterns, with a significant increased expression throughout the 12 days of culture, for all investigated conditions. In addition, the secretion of osteocalcin in culture medium had a similar profile to that of OC mRNA levels, in which a significant increase was observed over the culture period.

Interestingly, quantitative analysis also revealed that all the investigated biomarkers were significantly affected by bioactive glass strontium content. Indeed, the MTS assay showed a significant increase in the presence of B75-Sr5 particles at 24 h suggesting a positive effect on cell

viability. The presence of B75-Sr5 particles was also associated with a significant stimulation of both early and late osteoblast markers at the mRNA and protein levels in comparison with undoped B75 particles. Indeed, gene expression of Runx2, Osx, Dlx5, Collagen I, ALP, BSP and OC was up-regulated on day 12, whereas, at the protein level, ALP activity was increased on day 6 and OC secretion, on day 12. Thus, calvarial bone cells cultured in the presence of B75-Sr5 particles showed greater osteoblast differentiation than those cultured with undoped bioactive glasses.

We suggested that the stimulation effect on calvarial bone cell differentiation observed in the presence of B75-Sr5 particles is due to strontium ions released from bioactive glass particles into the culture media. Indeed, according to ICP analyses, the quantities of Si and Ca released in the culture media during the first six days of culture were similar for the three tested bioactive glass particles. On the contrary, Sr concentration in cell culture media was strongly affected during the first three days of culture by bioactive glass strontium content. Indeed, strontium concentration in the cell culture media is equal to 1 and 6 ppm after 72 h of interaction with B75-Sr1 and B75-Sr5, respectively. Moreover, mRNA levels of some bone markers were also greater in the presence of B75-Sr1 particles than in the presence of undoped bioactive glass, but our overall findings showed that B75-Sr1 particles have a limited effect on osteoblast differentiation when compared to B75-Sr5. These findings suggested a strontium dose-dependent effect on osteoblast differentiation. This hypothesis were in line with our ICP analyses and previous ones performed by Lao *et al.* (Lao *et al.*, 2009), showing that the release of strontium ions during the first days of cell culture by B75-Sr5 particles was about 5-fold greater than that by B75Sr1.

Our results are in agreement with previous studies showing that strontium ions enhance bone cell replication and osteoblast differentiation *in vitro* (Canalis *et al.*, 1996) with a dose-dependent effect, both when directly administrated in osteoblastic cell culture (Atkins *et al.*, 2009; Peng *et al.*, 2009) and when incorporated into calcium phosphate biomaterials (Qiu *et al.*, 2006; Xue *et al.*, 2006; Panzavolta *et al.*, 2008; Capuccini *et al.*, 2009; Gentleman *et al.*, 2010).

Conclusions

Our findings indicated that calcium being substituted for strontium in a 5wt.% composition in binary sol-gel bioactive glasses had a beneficial effect on foetal mouse calvarial bone cells. Indeed, osteoblast differentiation was enhanced in the presence of B75-Sr5, as shown by greater ALP activity and OC secretion and the up-regulation of Runx2, Osx, Dlx5, collagen I, ALP, BSP and OC mRNA levels, with respect to undoped B75 and the bioactive glass with a lower Sr content (B75-Sr1). Thus, B75-Sr5 bioactive glass may be a promising bone-graft material for bone regeneration procedures in oral and maxillo-facial clinical applications. Indeed, this bioactive glass combines

strontium-induced biological stimulation in osteoblastic cells with the advantages of the sol-gel process, such as increased porosity and bioactivity.

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Discussion with Reviewer

Reviewer I: In this study, strontium is used for doping of bioglasses in order to affect their biological performance. A number of other ions have also been suggested to be involved in processes related to bone formation and remodelling. Please comment whether the system presented here would be suitable for other cations, and how it should be adjusted in order to be applicable to anions?

Authors: A key advantage of the sol-gel process over the classical melting route is the glass that can be obtained in a wide variety of composition, while preserving or even enhancing the initial bioactive properties. The high versatility inherent to the sol-gel process allows easy variation of the nature of the doping ion and of its concentration, combined with a good chemical homogeneity. Doping the glasses with trace elements that are naturally present in bone and/or of biological interest can thus be achieved conveniently with the sol-gel technique; the systems studied in this article are easily tunable and other ions can be introduced in the glass matrix simply by adding the right salt/component during the mixing of the sol. In our research group we are currently studying glasses doped with Mg, or Zn. Other ions such as Ag^+, Mn^{2+}, and F^- are of interest and could be incorporated without any difficulty.