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Laurent-Emmanuel Monfoulet, Pierre Becquart, David Marchat, Katleen Vandamme, Marianne Bourguignon, et al.. The pH in the Microenvironment of Human Mesenchymal Stem Cells Is a Critical Factor for Optimal Osteogenesis in Tissue-Engineered Constructs. *Tissue Engineering: Parts A, B, and C*, 2014, 20 (13-14), pp.1827-1840. 10.1089/ten.tea.2013.0500 . hal-01629762

HAL Id: hal-01629762

<https://u-paris.hal.science/hal-01629762v1>

Submitted on 6 Nov 2017

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The pH in the microenvironment of human mesenchymal stem cells is a critical factor for optimal osteogenesis in tissue engineered constructs

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Abstract: The present study aimed at elucidating the effect of local pH in the extracellular microenvironment of tissue engineered (TE) constructs on bone cell functions pertinent to new tissue formation. To this aim, we evaluated the osteogenicity process associated with bone constructs prepared from human, bone marrow-derived stromal cells (hBMSC) combined with 45S5 bioactive glass (BG), a material that induces alkalization of the external medium. The pH measured in cell-containing BG constructs was around 8.0, i.e., 0.5 unit more alkaline than that in two other cell-containing material (hydroxyapatite/tricalcium phosphate (HA/TCP) and coral) constructs tested. When implanted ectopically in mice, there was no de novo bone tissue in the BG cell-containing constructs, in contrast to results obtained with either HA/TCP or coral ceramics which consistently promoted formation of ectopic bone. In addition, the implanted 50:50 composites of both HA/TCP:BG and coral:BG constructs, that displayed a pH of around 7.8, promoted 20-30 fold less amount of bone tissue. Interestingly, hBMSC viability in BG constructs was not affected compared to the other two types of material constructs tested both in vitro and in vivo. Osteogenic differentiation (specifically, ALP activity and gene expression of RUNX2, ALP and BSP) was not affected when hBMSC were maintained in moderate alkaline pH (7.90) external milieu in vitro, but was dramatically inhibited at higher pH values. Formation of mineralized nodules in the extracellular matrix of hBMSC was fully inhibited at alkaline (> 7.54) pH values. Most importantly, there is a pH range (specifically, 7.9-8.27) at which hBMSC proliferation was not affected but the osteogenic differentiation of these cells was inhibited. Altogether, these findings provided evidence that excessive alkalization in the microenvironment of TE constructs (resulting, for example, from material degradation) affects adversely the osteogenic differentiation of osteoprogenitor cells.

Introduction:

Bone marrow stromal cells (BMSC), which are able of osteodifferentiation and, thus, promote bone formation (1, 2), support the repair and/or regeneration of osseous defects when they are distributed in appropriate biomaterial scaffolds (3-6). Such scaffolds are designed as temporary matrices for supporting new tissue development and bone growth. The ideal scaffolds should provide a framework, as well as a specific environment, for cell attachment, proliferation, differentiation and formation of tissue-specific extracellular matrix (ECM) (7). Such scaffolds should also be absorbable with rates of resorption that are commensurate to those of bone formation.

Understanding, and controlling, pertinent cell functions by modulating the local engineered extracellular environment is a critical issue in the development of suitable material scaffolds for bone tissue engineering (TE) applications. In this respect, an extensive number of studies have reported the significant effect of scaffold properties, such as chemical composition, surface topography and chemistry, pore size, porosity, and specimen size on cell functions and, therefore, on the osteogenic outcome of engineered scaffolds (8, 9). However, the microenvironment or milieu where the cells reside has received little attention. This issue is even more critical because such local environments can be affected by the resorption of the scaffold material, especially by solutes arising from the material dissolution and by concomitant pH changes (10).

Among the biomaterials tested for bone TE application to date, calcium-based materials such as hydroxyapatite (HA), tricalcium phosphate (TCP), calcium carbonate (coral) ceramics and bioactive glass (BG) are the most promising ones. These calcium-containing materials are appropriate for bone-related applications because of similarities of their chemical and mechanical properties with the mineral phase of natural bone. In addition, these materials are considered osteoconductive and bioactive because of both their bonding capacity to surrounding osseous tissue and ability to promote new bone tissue formation (11, 12). When combined with human BMSC (hBMSC), HA, TCP, biphasic HA/TCP and coral ceramics induced *de novo* bone tissue in the subcutis of immunocompromised mice (13-15) (16-18). In contrast, and despite the expanding application of BG (such as 45S5 BG) and glass-ceramics as bone substitutes (19), information regarding the osteogenic potential of hBMSC in combination with BG scaffolds is strikingly lacking in the bone TE field.

While the biocompatibility and osteoconductivity of BG has long been established (19, 20), such material is also known for the release of ion dissolution products as well as for inducing alkalization of the external medium upon exposure to either physiological solutions or body fluids (21-23). Changes in the extracellular fluid pH of the local biological microenvironment can profoundly affect cell metabolism and function (22, 24) as well as the processes of bone tissue formation and mineralization (25). Chronic systemic acidosis promotes bone resorption, whereas alkalosis promotes bone formation by stimulating pertinent osteoblast functions (26-28). More recently, Shen et al. reported the beneficial role of local alkalization mediated by borosilicate BG on the viability of preosteoblasts (10). However, the positive or negative effect of pH in cell constructs has never been investigated in the bone TE field. This aspect is crucial since the implanted hBMSC are in a confined space within tissue constructs and are, therefore, especially vulnerable to chemical changes within the local biological microenvironment.

For these reasons, the present study focused on examining the impact of pH, specifically alkalization, in the engineered extracellular microenvironment on hBMSC-mediated osteogenesis. To this aim, we evaluated both, *in vitro* and *in vivo*, the osteogenicity of bone constructs prepared from hBMSC combined with 45S5 BG (a material that induces alkalization of the external medium) in comparison with results obtained using two other clinically-available, calcium-based scaffolds, specifically biphasic HA/TCP and coral ceramics. Biomaterial granules of equivalent size (~400 μm in diameter), similar construct preparation, and similar implant dimensions were used to ensure an equivalent material surface area (at the cell scale) as well as an equivalent inter-granule volume available to cells within 3D material constructs. The *in vivo* osteogenic outcomes of TE constructs were correlated with their local pH. In addition, the *in vitro* effect of pH on the proliferative and osteogenic differentiation of hBMSC was determined.

Materials and Methods:

Material scaffolds

Three calcium-based materials in the form of granules were studied. These granules differed in terms of their chemical composition as well as shape and surface area but all had sizes in the 300-690 μm range. The biphasic calcium phosphate granules (HA/TCP), composed of 60% HA and 40% TCP, were produced

using the spray drying process followed by high temperature calcination; these granules (300-570 μm ; mean size: 417 μm) were donated by Zimmer, Inc (Saint-Priest, France). Natural coral granules (Porites species; Biocoral®) consisted of 99% calcium carbonate in the form of aragonite (29, 30); these materials (300-600 μm ; mean size: 426 μm) were donated by Inotek, Inc. (Levallois-Perret, France). The non-porous 45S5 silicate BG (designated BG hereafter) granules of nominal 45% SiO₂, 24.5% CaO, 24.5% Na₂O and 6% P₂O₅ in wt% composition (300-690 μm ; mean size: 457 μm) were donated by Noraker (Villeurbanne, France) and were preconditioned in laboratory-made simulated body fluid (SBF) as recommended by the supplier. Briefly, BG granules were immersed in a SBF solution (0.1g of BG per mL of solution) and were maintained under mild stirring by rotation at 37°C for 4 weeks. The SBF solution was renewed twice a week. Then, granules were rinsed with double distilled water and dried before sterilization. SBF pretreatment promotes the formation of a bone-like carbonated HA (cHA) layer as well as calcite on its surface (31-34).

The surface of the granules used in the present study was examined using scanning electronic microscopy (SEM) (SEM-FEG, JEOL JSM-6500F, USA). The specific surface areas were determined according to the BET method 8 points using N₂ adsorption isotherms (Micromeritics ASAP 2010) as previously described (35). The particle size distribution was determined by using a laser diffractometer with a Hydro 2000S module (Mastersizer 2000 from Malvern Instruments Ltd, UK).

hBMSC isolation, expansion and labeling with Luc gene

Human mesenchymal stem cells (hBMSC) were harvested from bone marrow obtained as discarded tissue (intramedullary reamings) from 4 female and 1 male patients 17, 31, 31, 49 and 63 years old (mean age: 38.8 +/-17.7 years) who were operated for traumatic orthopedic indications in the absence of any detected chronic pathologies (such as diabetes, cancer, arthritis, etc) at the Lariboisière Hospital (Paris, France). The tissues were collected with the respective patient's consent in agreement with Lariboisière Hospital (Paris, France) regulations. The hBMSC were isolated using a procedure adapted from literature reports (1). Briefly, cells were harvested by gently flushing the collected bone-marrow samples through decreasing (from 16G to 21G) needle-sizes. These cells were then cultured in standard cell culture medium, that is, alpha-modified Minimum Essential Medium (MEM) containing 10% fetal calf serum (FCS; PAA Laboratories) and 1% of an antibiotic/anti-mycotic solution (PAA Laboratories), in a humidified, 37°C, 5% CO₂/95% air environment. When the hBMSC reached 60-70% confluence, they were trypsinized and subcultured at a density of 1,000-5,000 cells cm⁻². hBMSC obtained from the 5 aforementioned patients were pooled at the same density, expanded, and used up to passage 4-5 for the experiments described in the sections that follow.

Genetically modified hBMSC were obtained following their transduction with a lentiviral vector encoding firefly luciferase (fLuc) (pTMLW-MND-Luc; Vector platform/ INSERM U876) as previously described (36). Transduced cells (designated as LuchBMSC hereafter) were expanded and used to monitor the hBMSC viability in cell-containing constructs both *in vitro* and *in vivo*.

Preparation of cell-containing constructs

For all cell-related experiments in the present study, aliquots of 40 mg of granules of the materials tested were sterilized by

autoclaving, then sequentially rinsed twice in phosphate buffer saline (PBS), and also rinsed once in serum-free MEM (Sigma-Aldrich) cell culture medium.

Aliquots of hBMSC (or LuchBMSC when so specified) were delivered (suspended in 100 μ l of standard cell culture medium) to each material granules tested; the final cell density was 106 cells per cell-containing construct for *in vivo* studies or 105 cells per construct for *in vitro* studies. Unseeded scaffolds served as controls. After 4 hours of incubation at 37°C, the supernatant was discarded and the granules were embedded in a fibrin gel (Tissucol®, Baxter) prepared by mixing 100 μ l of fibrinogen (9 mg ml⁻¹), with 5 μ l of thrombin (100 UI ml⁻¹) as previously described (37). These cell-containing constructs were maintained in 2 ml of standard cell culture medium at 37°C overnight before use in experiments.

In vivo studies

Ten-week-old, female, nude mice were obtained from Janvier (Legenest-Saint-Isle, France) and handled according to the European Guidelines for Care and Use of Laboratory Animals (EEC Directives 86/609/CEE of 24.11.1986). All experimental animal procedures conducted in the present study were approved by the Ethics Committee on Animal Research of the Lariboisiere/Villemin, Paris, France. The hBMSC-containing constructs (n=7 per group for the HA/TCP, coral, and BG constructs; n=6 per group for both the HA/TCP-BG and coral-BG composite constructs) were subcutaneously implanted in mice as previously described (36). Briefly, each mouse was preoperatively given analgesics (0.4 mg of buprenorphine per kg animal weight; Axience), anesthetized by intra-peritoneal injection of 100 mg kg⁻¹ ketamine (Ketalar®; Virbach) and 10 mg kg⁻¹ xylazine (Rompun® 2%; Bayer), and its skin was disinfected. Symmetrical incisions were made on the back of each mouse on both sides of the spine and subcutaneous pouches were created. The cell-containing constructs to be tested were inserted into each pouch (4 constructs per mouse). The soft tissues at the implantation sites were then closed with interrupted non-resorbable sutures. Eight weeks post-implantation, the animals were sacrificed via injection of lethal doses of pentobarbital (Dolethal®; Vetoquinol). At that time, the constructs were retrieved en-bloc and fixed in 10% (v/v) phosphate-buffered formalin before analysis as described in the next section.

For monitoring the implanted cell survival *in vivo*, special cell-containing constructs were prepared using LuchBMSC. Following implantation of these cell constructs, the mice were imaged twice a week during the 8-weeks post-implantation period as previously described (38). Briefly, the mice were anesthetized by inhaling isoflurane followed by local injection of 100 μ l of D-luciferin (15 mg ml⁻¹ in PBS) at each implantation site. The animals were then imaged using a bioluminescence imaging system (Ivis Lumina II, Caliper Life Sciences, USA). Standard regions of interest (ROI) surrounding each implant were delineated on the bioluminescence images and the photon flux emitted by each construct was quantified using the Living Image 3.1 software (Caliper Life Science). To compare cell survival in each construct tested, photon fluxes were normalized with respect to the signal acquired at the beginning (day 1) of the respective *in vivo* experiment.

Histology and immunohistochemistry

Six out of 7 retrieved constructs for each material tested were processed for undecalcified histology. After fixation, the explanted samples were rinsed in water, dehydrated in ethanol, cleared in

xylene, and embedded in methyl methacrylate as previously described (29). Each sample was then cut into either 6 or 7 sections (each 500 μ m thick) using a diamond circular saw (Leica 1600, Leica). Each such specimen section was then ground to a thickness of 100 μ m and stained using Stevenel's blue and Van Gieson Picrofuchsin red for subsequent histological analysis. Three non-adjacent sections from each specimen were selected for histomorphometrical analysis. Histological examination was performed using an optical microscope (Nikon Eclipse TE2000-U; Nikon France) equipped with a numeric camera (DXM1200F; Nikon). Bone was quantified using the NIS-Elements BR 2.30 software (Nikon). The surface area of bone (stained in red) was measured in each specimen section and normalized over the whole surface area delineated around each section.

The remaining seventh retrieved construct (from the HA/TCP, coral, and BG constructs groups) was fixed in 4% paraformaldehyde (pH 7.4) for 36 hours, decalcified in Decalcifier II (Surgipath) at 4°C for 48 hours, and embedded in paraffin. Sequential sections were processed for human beta-microglobulin (a membrane protein that enables tracking human cells) immunodetection. Briefly, tissue sections were pre-treated in a proteolytic enzyme solution (Dako, Glostrup, Denmark) at 37°C for 20 min. Cell membranes were permeabilized using 0.3% Triton X-100 for 15 min; the endogenous peroxidase activity was inhibited by addition of peroxidase block (Kit Envision+, Dako), and the non-specific binding sites were blocked using Protein Block (Dako). The tissue sections thus prepared were sequentially incubated in primary antibody, polyclonal rabbit anti-beta-2-microglobulin (1/1000; Novocastra) for 1 hour, incubated with Labelled Polymer-HRP Anti-Rabbit antibody (Envision+ Kit, Dako K4011) for 30 min, and visualized using the DAB chromogen (Envision+ Kit, Dako K4011) for 5 min.

In vitro hBMSC adhesion and proliferation onto material scaffolds

Cell adhesion was assessed using SEM. Briefly, hBMSC were seeded onto granules (103 cells per mg of material granules) and cultured under standard cell culture conditions for 24 hours. At that time, the specimens were washed once with cacodylate 0.1M (pH 7.4) and fixed in 2.5% glutaraldehyde (in cacodylate 0.1M; pH7.4) at 4°C for 1 hour. After two washings in cacodylate, the specimens were dehydrated in successively increasing concentrations of alcohol (from 30 to 100%). For SEM examination, the specimens of interest were treated in hexamethylsilazane baths (HMDS, Delta microscopy), dried over-night, and coated using a sputter coater equipped with a gold target (EMSCOPE SC 500, Elexience, Verrières le Buisson). These specimens were examined using a 505 Philips SEM (FEI Company, Eindhoven) at 10 keV.

Proliferation of hBMSC onto the material granules tested was monitored *in vitro* both in 2D (i.e., granules deposited on the bottom of a tissue culture polystyrene dish) and in 3D (i.e., granules dispersed within fibrin gel constructs). Cell proliferation in 2D conditions was determined by DNA quantification. Specifically, aliquots of 40 mg of material granules were transferred in each well of 48-well plates; hBMSC (3x10⁴ cells) in standard cell culture medium were seeded. At different times of culture (specifically, at 0, 14, 21 and 28 days), cells were lysed using lysis buffer (1 mM MgCl₂, 0.1M Na₂CO₃, 0.1M NaHCO₃, 0.1% Triton X100 (v/v); pH 10.2) and frozen/thawed three times (to disrupt the cell membranes). Cell DNA content was determined

using the CyQuant® cell proliferation assay kit (Molecular Probes) according to the manufacturer's instructions. Cell proliferation in the 3D constructs was assessed using bioluminescent imaging. For this purpose, the cell-constructs were prepared using LuchBMSC (105 cells per construct), transferred into 24 well-plates, and cultured in standard cell culture medium for the prescribed time periods (up to 28 days). The bioluminescent (BLI) signal from each cell-containing construct was determined non-destructively 3-times a week by adding D-luciferin (at a final concentration of 300 ng mL⁻¹ PBS) into each cell-construct-containing well; BLI images were acquired using a bioluminescence imaging system. To compare the time course of cell proliferation in each construct tested, photon fluxes were normalized with respect to the signal acquired at the beginning (day 1) of the respective cell culture period.

Determination of the osteogenic differentiation of hBMSC in vitro

In order to assess the osteogenic differentiation of hBMSC cultured onto the material granules of interest to the present study, cells were seeded at a density of 3x10³ cells cm⁻² in each well of 6-well plates in the presence of 40 mg of granules of each one of the three materials tested. These cells were cultured in osteogenic medium, that is standard cell culture medium containing 10⁻⁷ M dexamethasone, 150 M ascorbic acid-2 phosphate and 2 mM -glycerophosphate (all chemicals from Sigma-Aldrich), for up to 28 days. The supernatant medium was replaced every 2-3 days for the duration of the study. In order to assess the impact of the cell culture medium pH on the osteogenic differentiation of hBMSC, the cells (3x10³ cells cm⁻²) were cultured in osteogenic medium buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) the pH adjusted at either 7.47, 7.90, 8.27, 8.85 or 9.37, and cultured in a humidified, 37°C, atmospheric air environment (in the absence of supplementary CO₂ inlet) for up to 28 days. Cells in culture medium buffered using the physiological CO₂/HCO₃ buffer system at pH 7.54 were the respective controls. In this experiment, the initial pH value (pH_{ini}) of the osteogenic culture medium (which was added fresh to the cells), and the final pH value (pH_{fin}) of the supernatant cell culture medium (which was removed from the cells) were specifically checked at each culture medium change (i.e., every 2-3 days) during the cell culture period.

At the end of each prescribed cell culture period, the osteogenic differentiation of these cells was assessed by quantifying expression of a panel of osteoblastic markers (as described in the next section) as well as by determination of the activity of alkaline phosphatase (ALP) and mineralization. ALP activity was determined both by staining the cell cultures in situ using a commercially-available kit according to the manufacturer's (Sigma-Aldrich) instructions and by quantifying ALP activity in cell lysates as previously described (39). The presence of calcium-containing deposits in the extracellular matrix was detected by staining the cells using the Alizarin red (AR) method. All these experiments were performed in triplicate and repeated at two separate times.

Quantitative gene expression analysis by Real Time Polymerase Chain Reaction (q-PCR)

Total ribonucleic acid (RNA) was extracted from hBMSC using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany).

RNA concentration was measured using a NanoDrop ND-1000 (LabTech, Ringmer, UK) spectrophotometer. One µg of extracted RNA was used as template for single-strand cDNA synthesis using the SuperScript™ II Reverse transcriptase Kit (Invitrogen). Five µl of cDNA (diluted at a 1:20 ratio) were used to program amplifications through real-time PCR reactions using specific primers of genes encoding for runt-related transcription factor 2 (RUNX2) (Hs00231692_m1), collagen type 1 1 (Col1a1)(Hs00164004_m1), osteopontin (OPN) (Hs00960942_m1), osteocalcin (OC) (Hs00609452_g1) and for bone sialoprotein (BSP) (Hs00173720_m1) (all from Applied Biosystems Inc.), combined with TaqMan Universal PCR Master Mix using the MyiQ™ Single-Color Real-Time PCR thermocycler (Bio-Rad Laboratories) according to the protocol described previously (40). All samples, including specimens from experiments and standards (n=3 for each condition), were run in triplicate and in parallel. Data were analyzed using the iCycler IQ software (Biorad) and were compared by the Ct method. The results from hBMSC cultured onto the material granules tested in the present study were normalized to the amount of 18S transcript and were expressed relative to the results obtained from cells at day 0. The results from hBMSC cultured at different pH values were normalized to the amount of 18S transcript, and were expressed relative to the results obtained from hBMSC cultured in non-osteogenic medium for the same time period.

pH in the 3D constructs

In order to determine the pH in the core environment of the material constructs containing the tested granules (without cells), a microelectrode (PerpHecT@ROSS; Thermo Scientific) was inserted in the material granules before the fibrin gelation. After gelation, the constructs were maintained in 2 ml of a weakly buffered solution, specifically PBS (1 mM sodium phosphate at pH 7.4) in order to minimize the buffering impact on the pH determination. The pH was monitored at room temperature for 24 consecutive hours. The global pH of the constructs seeded with 106 hBMSC was also determined before (day 0) and after implantation in mice for either 3 or 7 days. After animal sacrifice, the cell constructs were excised, chopped into small pieces and transferred in 1 ml of 1 mM PBS solution (pH 7.4). After mixing, the pH of each such solution was measured using a calibrated pH-meter (Star Orion).

Statistical analyses

Numerical results were reported as mean +/- standard deviation. The nonparametric Mann-Whitney U-test was used to analyze data from two independent conditions. When comparing data of more than two conditions, the Kruskal-Wallis one-way analysis test was used. Quantitative data from in vivo BLI signals were analyzed using the two-way analysis of variance (ANOVA). For all analyses, differences were considered to be statistically significant at p < 0.05.

Results

Physical properties of the granule surfaces

Visualization of the three materials tested using SEM enabled analysis of their macro- and microstructure (Fig. 1 A-F). The HA/TCP granules exhibited round shape and rough surface texture (Fig. 1 A). At higher magnification (Fig. 1 B), the round HA/TCP granules contained dense aggregates (1-10 µm) of round elemental

particles/grains of the 100 nm size. The shapeless aggregates, which formed agglomerates were lightly bonded in contrast to the elemental grains which were highly sintered (i.e., more strongly bonded). The coral granules had branching shapes (Fig. 1 C). At higher magnification (Fig. 1 D), the coral granules exhibited a rough and granular surface as that observed with HA/TCP granules. The main coral granules were composed of dense aggregates of small, round and acicular particles a few nanometers in size (Fig. 1 D). The coral appeared denser than the HA/TCP granules (Fig. 1B). The BG granules, preconditioned with SBF, displayed sharp angular shapes (Fig. 1 E). At higher magnification (Fig. 1 F), the cHA layer coating the surface of the BG granules contained round aggregates ranging between 200 nm to 1 μ m in diameter, which were composed of smaller particles of about 100 nm in diameter. The specific surface area of the coral, HA/TCP and BG granules was 0.06 m²/g, 0.33 m²/g and 1.09 m²/g, respectively. Altogether, these observations indicated that, at the nanometer scale, the surface topography and roughness of the three materials tested were different, but at the hBMSC scale (20-50 μ m), they were similar.

In vivo bone formation by hBMSC contained in the constructs tested

De novo bone formation was assessed when hBMSC-containing constructs (prepared with either HA/TCP, coral, BG or composite of either HA/TCP:BG or Coral:BG) were implanted ectopically in a mouse model. After 8 weeks of subcutaneous implantation, there was no bone formation in conjunction with material scaffolds without cells; these scaffolds, however, were infiltrated with fibrous tissue (data not shown). At the same time point, new bone tissue was observed both in the hBMSC-containing HA/TCP and coral constructs as shown in the representative histology results from such explants (Fig. 2A). The osteogenic outcome of hBMSC contained within the tested coral- and HA/TCP- implants was similar and was characterized by high prevalence of bone formation and by similar amount of deposited new bone tissue (Fig. 2B). In contrast, no bone tissue was detected in any of the hBMSC-containing BG constructs (Fig. 2A and 2B). When hBMSC were contained within the 50:50 composites of either HA/TCP:BG or coral:BG constructs, newly-formed bone was found at the periphery of the constructs as well as consistently in contact with the coral and HA/TCP material surfaces. Very small amount of bone tissue (< 0.2 % compared to 4.2 and 3.8 % for HA/TCP and coral cell-containing constructs, respectively) was observed (Fig 2A) and the prevalence of bone formation was reduced (Fig 2B) in composite constructs. Altogether, the present results provided evidence that, in contrast to the HA/TCP and coral materials tested, hBMSC cultured on BG did not induce bone formation; moreover, the presence of BG in the composite constructs tested had a negative effect on osteogenesis.

Viability of hBMSC onto the materials tested

The issue of potential toxicity of BG on hBMSC was addressed. For this purpose, adhesion and proliferation of hBMSC on the material granules was determined. SEM examination at 24 h confirmed that the hBMSC adhered and spread in a similar fashion onto the three substrates tested (Fig. 3A). Cell proliferation was determined when hBMSC were cultured onto granules of each one of the three materials tested that had been placed on the bottom of individual wells of tissue culture dishes (Fig. 3B). DNA quantification at different time points over a 4-week period of

culture provided evidence that, under these conditions, hBMSC proliferation was similar for hBMSC cultured on the three materials tested as well as on tissue culture polystyrene (TCPS), the reference substrate (Fig. 3B).

Cell viability was also assessed by determining hBMSC proliferation within 3D cell-containing constructs composed of either HA/TCP, coral, or BG granules embedded in fibrin gel (Fig. 3C). Proliferation of hBMSC (previously labeled with the luciferase gene reporter) was monitored non-destructively in each construct using bioluminescence imaging. Similar profiles of hBMSC proliferation kinetics were observed for the three types of constructs of interest to the present study. Compared to the initial seeding, the number of cells increased by 2- to 3-fold after 15 days of culture; the highest cell proliferation was observed in the BG constructs (3.4 fold at day 14; $p < 0.05$ compared to the other two material constructs) (Fig. 3C). Furthermore, hBMSC survival was also assessed *in vivo* after subcutaneous implantation of the cell-containing constructs in nude mice for 2 months (Fig. 3D). For the cases tested, the number of viable implanted cells decreased over this time period. The decrease in cell survival was significantly ($p < 0.05$) slower in the BG constructs compared to that obtained on the HA/TCP and coral ones up to 14 days post-implantation. After that time point, a steady decrease in the luminescence signal from all implanted cell-containing constructs was observed (Fig. 3D). As a result, only 10 to 30% of the initial cell numbers remained for all constructs tested at 8 weeks post-implantation. These results indicated loss of viable hBMSC, which occurred upon implantation independent of the type of material tested; more importantly, the implanted hBMSC did not die faster in the BG than in the other two material constructs.

Immunostaining of human α -2-microglobulin enabled visualization of the hBMSC still present in sections of constructs explanted 8 weeks post-implantation (Fig. 3E). Human α -2-microglobulin-positive cells were detected at different locations in the tissue surrounding the granules, but were most frequently found on the material surface of all three types of constructs tested (Fig. 3E). These results confirmed the cytocompatibility of the three materials tested over the 8-weeks period of implantation. It should be noted that hBMSC were detected not only in fibroblastic connective tissue but also in newly-formed bone as well as around blood vessels in the HA/TCP- and coral-cell-containing constructs (data not shown). In contrast, positive-stained hBMSC were only detected in the fibroblastic connective tissue in the BG-cell-containing constructs. Overall, cell spreading on the granules surfaces (Fig. 3A), cell proliferation *in vitro* (Fig. 3B-C), as well as the scaffold material cytocompatibility *in vivo* (Fig. 3 D-E) provided evidence that the observed absence of de novo bone formation in the hBMSC-containing BG constructs is not the result of lack of cytocompatibility of the BG material.

Osteogenic differentiation of hBMSC cultured onto the materials tested

Another aspect addressed in the present study was the potential for osteogenic differentiation of hBMSC cultured on BG granules as compared to results obtained on either the HA/TCP or coral substrates. For this purpose, the ALP activity of hBMSC cultured in osteogenic differentiation medium on the three material-granules tested was assessed. ALP activity of hBMSC cultured on either HA/TCP- or coral-granules peaked at 14 days (Fig. 4A), indicating osteogenic differentiation of hBMSC on these materials.

hBMSC exhibited a significant ($p < 0.05$) greater ALP activity on HA/TCP and coral than on BG granules at 14 and 21 days of culture (Fig 4A). In addition, the ALP activity of hBMSC cultured on TCPS (reference substrate) was 3-, 3-, and 7-fold higher than that obtained when the cells were cultured onto the HA/TCP, coral and BG granules, respectively (data not shown). The results were confirmed by the enhanced positive ALP-staining of cells cultured on HA/TCP and on coral granules compared to BG ones for 14 and 28 days (Fig. 4B).

A panel of genes indicative of osteogenic differentiation was also investigated using qPCR (Supplementary Fig 1). Expressions of all markers (specifically, RUNX2, Osterix, Colla1, OC and OPN) tested by hBMSC were similar when cultured on the three types of granules at both 7 and 14 days of culture.

Determination of the pH in the material constructs tested

The pH in the core environment of the cell-free BG constructs was determined to assess the alkalinization induced by the material itself (in the absence of cell-mediated acidosis). Starting from 7.4, the pH increased during the first hours post-gelation and reached a plateau at later (>10 h) times (Fig 5A). At 24 h post-gelation, the pH values in the HA/TCP-, coral- and BG-containing constructs were 8.6, 8.3 and 9.1 \pm 0.1, respectively. The pH of the BG constructs was 0.5 and 0.8 units more alkaline than the pH determined in the HA/TCP- and coral-constructs, respectively.

Because these cell-free conditions did not reflect those of the cell-containing constructs implanted in vivo, the pH of constructs seeded with 106 cells was also measured before their implantation (day 0) and immediately upon their removal from mice after 3 and 7 days of implantation. The pH in the cell-containing BG constructs was 8.03 \pm 0.03 at day 0 and remained similar at the prescribed post-implantation time points (Fig. 5B). The pH value in these BG constructs was 0.4-0.5 unit more alkaline ($p < 0.05$) than that measured in the other two types of cell-containing material constructs. The pH from both cell-containing HA/TCP:BG and coral:BG composite constructs that contained increasing ratios of BG was also measured in vitro. The data showed a strong correlation ($r^2 = 0.88$ and 0.99 for the HA/TCP:BG and the coral: BG composites, respectively) between the pH and the content of BG in the scaffolds of cell-containing constructs (Fig. 5C). Altogether, these results confirmed the alkalinization of the environment of the BG cell-containing constructs.

Osteogenic differentiation of hBMSC as a function of the pH of the cell culture medium

The effect of the pH of the cell culture medium (ranging from 7.47 to 9.37 using HEPES buffer) on both the proliferation and the osteoblastic differentiation potential of hBMSC was also investigated. It is worth to note that a decrease of both pHini and pHfin values ranging from 0.1 unit of pH, for the lowest pHini (7.47), to 0.8 unit for the highest pHini (9.37) value was noticed after 2-3 days of cell culture (Table in Fig. 6D).

Under these conditions, DNA quantification provided evidence that hBMSC proliferation was unaffected at alkaline pH up to 8.27; there was no cell proliferation at pH 8.85 and the cells died at pH 9.37 (Fig. 6A). In order to assess the impact of the medium pH on the osteoblastic differentiation potential of hBMSC, a panel of genes indicative of osteogenic differentiation was investigated

using qPCR (Fig. 6B). hBMSC were cultured in non-osteogenic medium as negative control. At either physiological pHini (7.47) or moderate alkaline pHini (7.90), expression of the osteoblastic (RUNX2, ALP and BSP) markers was higher when the cells were cultured in osteogenic than in non-osteogenic media indicating osteogenic differentiation of the hBMSC in the range of these pH values (7.47 \leq pHini \leq 7.90). At higher (> 7.90) pHini, gene expression of the three markers monitored dramatically decreased and reached similar, or even lower, levels of expression as the ones obtained when the cells were cultured in non-osteogenic medium.

The extent of both the ALP activity and mineralization of the hBMSC extracellular matrix were also strongly dependent on the pH of the cell culture medium (Figs. 6C and 6D). The highest level of ALP activity occurred at day 14 when the cells were cultured at pHini 7.47 (Fig. 6C). Similar results were obtained when the cell culture medium was buffered (using a CO₂/HCO₃ system) either at pHini 7.54 or at a moderate alkaline pHini (7.90) level (Fig. 6C). At pHini 8.27, the ALP activity significantly decreased while at pHini 8.85, minimal ALP production occurred at a later time, specifically, at 28 days of culture (Fig. 6C). ALP staining of cells confirmed these results (Fig. 6D). Mineralization of the hBMSC extracellular matrix occurred only at physiologic pHini (pHini < 7.54) and was visualized using Alizarin red staining (Fig. 6D). It is worth to note, however, that the cell culture medium buffered with HEPES affected the mineralization process; at similar pHini, the kinetics of mineral accumulation was delayed and the amount of mineral deposited was lower in the HEPES-buffered system compared to the results obtained using CO₂/HCO₃ system (Fig. 6D).

These results provided evidence that the alkaline pH (specifically pH > 7.9) affected adversely both the hBMSC osteogenic differentiation and, more strongly, the mineralization process of the extracellular matrix. Interestingly, while hBMSC proliferation (Fig. 6A) was not affected at pH 8.27 (the pH value in the vicinity of the BG granules in the cell constructs tested), their osteogenic differentiation was inhibited (Fig 6B-C).

Discussion:

The present study is the first to address the impact of pH in the microenvironment of bone TE constructs on osteogenesis. The results provided evidence that constructs of hBMSC contained in a material, specifically, BG, that induces alkalinization of the microenvironment did not promote de novo bone formation; in contrast, cell-containing constructs prepared with either HA/TCP or coral, the two other materials tested, which did not change significantly the microenvironment pH but consistently induced bone tissue.

BG is well known to alkalinize surrounding fluids (22, 23) due to the consumption of protons (H₃O⁺ ions) during the ion exchange reactions between the glass network and the aqueous media (41). In the present study, the pH within the cell-free BG constructs was around 9.0, and decreased to 8.0 in the cell-containing BG constructs both prior and after implantation in mice. This decrease in pH value, albeit without reaching the physiological value (7.4), is explained by the acidosis that accompanies cell metabolism. In addition, the strong correlation established between the pH and the proportion (in weight) of BG granules within the cell-containing composite constructs confirmed the effect of BG material in the alkalization process.

The hBMSC-mediated osteogenic potential was assessed *in vivo* after subcutaneous implantation of the cell-containing material constructs in mice. This is a standard *in vivo* model that excludes and/or reduces the effects of endogenous bone-forming cells and of bone-stimulating mechanotransduction on the process of new bone formation (13, 42). Interestingly, the *in vivo* results evidenced that the implanted 50:50 composites of both HA/TCP:BG and coral:BG constructs, whose pH was around 7.8, had very little amount of bone tissue after 8 weeks post-implantation. Altogether, these results evidenced a correlation between loss of the bone forming potential of the cell-containing constructs and the degree of alkalization within these constructs: osteogenesis decreased 20-30 fold when the pH rose from 7.5 to 7.8, and it was totally inhibited when the pH reached 8.

BG and glassoceramics are widely used in bone repair applications and are considered appropriate materials for bone TE applications (43). However, information regarding the performance of BG as scaffolds for the delivery of osteocompetent cells in TE applications has been lacking. The absence of osteoinductivity observed with BG-containing constructs in the present study is in agreement with the results from other groups who reported that various formulations of silicate glasses (such as 45S5 BG, 13-93 BG and BG/polymer composites) seeded with stem/precursor cells promoted abundant fibrous tissue infiltration upon implantation in ectopic sites in rodents. These studies reported the presence of sporadic osteoid or bone-like tissue within constructs but none of them evidenced bone tissue formation unequivocally (44-46).

In the present study, histological analysis of the composite constructs tested showed that the bone tissue was located onto the surface of either HA/TCP or coral, but not on BG. Such observation suggests that the reduced osteogenic potential in BG-containing constructs *in vivo* was closely related to the BG material surface and/or to its local microenvironment.

In respect to the physical properties of the material surface, the SEM images showed that, at the scale of hBMSC, i.e., 20-50 μm , the topography, roughness and available material surface for cell attachment was equivalent for the three materials tested. In respect to the material microenvironment, it is well-documented that dissolution of BG material mediates the release of ions such as Na^+ , Ca^{2+} and Si products (presumably in the form of silicic acid $\text{Si}(\text{OH})_4$) into the surrounding aqueous media; continuous hydrolysis of silica groups results in increased local pH (19).

The impact of both material surface physico-chemistry and dissolution products (released in the microenvironment) on the hBMSC cytocompatibility was evaluated *in vitro* and *in vivo*. *In vitro*, both adhesion and proliferation of hBMSC cultured directly onto the BG surface were not affected compared to the other materials tested. When hBMSC were seeded within 3D BG constructs, and, therefore, exposed locally to this material dissolution products, cell viability was even higher compared to respective results obtained from the other two types of material constructs tested both *in vitro* and *in vivo*. Implanted hBMSC still remained on the surface of all types of materials tested 8 weeks post-implantation.

Among the hypotheses that could explain the observed higher viability of hBMSC in BG constructs is the possible impact of BG on the metabolic activity of cells. Indeed, Silver et al. reported that BG enhanced glycolysis in osteoblasts *in vitro* and, thus, their cellular ATP production; those authors stated that these metabolic

effects are most likely consequences of external and internal alkalization in the cell milieu (22). If BG has similar effects on the hBMSC metabolism *in vivo*, as it does on osteoblasts *in vitro* (22), stimulation of ATP generation may be beneficial for cell viability inside the TE constructs tested. All in all, these data confirmed that BG materials are cytocompatible both *in vitro* and *in vivo* in agreement with previous studies (47, 48).

On one hand, viability of hBMSC was not affected by BG, on the other hand, however, its potential for osteogenic differentiation was diminished *in vitro*. Such result obtained *in vitro*, as well as lack of osteogenic evidence in the cell-containing BG constructs *in vivo*, seems to be affected by the ionic exchange occurring at the surface of the material and/or by its side effects (such as pH changes). This hypothesis is strengthened by the minimal bone formation obtained from the 50:50 composites of both HA/TCP:BG and coral:BG constructs: osteogenesis decreased 20-30-fold when half (in weight) of either the HA/TCP or coral granules was replaced by BG granules; this result suggests that one or several diffusible chemicals, such as ion complexes (concomitant to pH changes), impede the hBMSC-mediated osteogenesis.

The aforementioned results are in striking contrast with the numerous literature reports regarding the beneficial effects of products released from BG (without taking into account concomitant pH changes) on various functions (such as proliferation, differentiation, extracellular matrix production, mineralization and even angiogenesis) of human progenitors pertinent to new tissue formation (49-52). Especially, Si-products were reported to enhance osteogenic markers in osteoprogenitor cells (53, 54). Nevertheless, within the microenvironment of the BG constructs *in vivo*, cells may have been exposed locally to higher concentrations of Si-products in the present study than those used in the published studies. Thus, one cannot exclude that release of ions/species (such as Na^+ , Ca^{2+} or more particularly $\text{Si}(\text{OH})_4$) from the BG granules into their microenvironment in the present study, may have contributed to the inhibition of bone tissue formation *in vivo*.

The release of Si products from the BG material and the subsequent alkalization of the surrounding medium are two chemical events that could not be separated in the present study because they are closely interlinked (19). However, the observed considerable (0.5 unit) rise in the pH of the BG constructs (compared to the other two types of material constructs tested) raised questions regarding how the biological, in particular osteogenic, responses of hBMSC were affected in an alkaline milieu. It should be noted that alkalization of the external medium was likely accompanied by a shift in the intracellular pH (pH_{int}) in the same direction. However, the magnitude of change in the pH_{int} is usually smaller than that in the external/extracellular pH (pH_{ext}) because of the control mechanisms (such as ion-transport systems) in eukaryotic cells (22, 55).

The present results provided evidence that the *in vitro* hBMSC proliferation and osteogenic differentiation were not significantly affected at moderate alkaline pH_{ext} (up to 7.90) but were dramatically inhibited when the pH_{ext} increased further. Formation of mineralized nodules in the extracellular matrix of hBMSC proved more sensitive to the pH_{ext} since it was fully inhibited at alkaline (> 7.54) pH_{ext} values. Most importantly, these findings indicated that there is a pH_{ext} range (specifically, 7.9-8.27) at which hBMSC proliferation was not affected but the

osteogenic differentiation of these cells was inhibited. Such pHext range encompassed the one determined in the cell-containing BG granules (pHext ~8) and could explain the observed proliferation of hBMSC in BG constructs in vitro and the cell viability in vivo but also, the absence of bone tissue formation in vivo.

The effect of the pH of the tissue microenvironment on bone mineralization and repair has been reported previously (56-58). During the early healing phase in bone, Chakkalakal et al. determined that the tissue pH was lower than the physiologic (7.4) pH; when, subsequently this pH increased to more alkaline values (specifically, to pH 7.56), it was accompanied by mineral deposition (25). On a cellular level, during metabolic acidosis, osteoblast functions decline whereas during metabolic alkalosis osteoblast functions (specifically, cell viability, ALP activity and mineral deposition) increase (10, 26-28). With respect to bone TE using hBMSC, Kohn et al. reported that short-term (specifically, 48 h) exposure of hBMSC at decreasing (specifically, from 7.8 to 6.5) pH led to decrease of osteogenic phenotype markers, specifically, ALP activity and collagen synthesis (59). Some of the aforementioned studies, however, reported decrease of the osteoblastic activity at pH around 7.8 (27, 60), but have not tested a pH range that encompasses the excessive alkalosis (pH~8) observed in the microenvironment of the cell-containing BG constructs tested in the present study. Therefore, it is possible that the high local alkalization induced by the BG constructs had adverse effects on the osteogenic differentiation of stem cells in contrast to the beneficial effects obtained with moderate alkalosis; in fact, such a condition was deleterious to hBMSC metabolism and function in vitro and, therefore, likely to their bone-formation ability in vivo.

In conclusion, the results of the present study evidenced a close relationship between inhibition of the hBMSC-mediated osteogenesis observed in vivo and the local alkalization within the TE constructs tested. Excessive alkalization in the TE construct microenvironment (resulting, for example, from material degradation) affected adversely the osteogenic differentiation of osteoprogenitor cells and may have consequently inhibited the osteogenicity of the constructs in vivo. Thorough characterization and control of the local, engineered, extracellular environment (specifically, changes of pH) are critical issues, which must be addressed in the development of bioactive scaffolds for successful TE, bone-related applications.

Acknowledgments

The authors gratefully acknowledge Inoteb Inc. (Levallois- Perret, France) for donating the coral scaffolds and Baxter, Inc., for donating the Tissuocol used in the present study. We are grateful to Brigitte Gaillard (INRA, Unité de Microbiologie, Clermont-Ferrand, France) for conducting the SEM analysis for cell adhesion. We also thank Professor R. Bizios for critically reading the manuscript. This project was supported by the French National Research Agency (ANR) through the TecSan program (project GLASSBONE n°08-TECS-004), and by the Centre National de la Recherche Scientifique (CNRS) of France..

Disclosure Statement

The authors declare no competing financial interests

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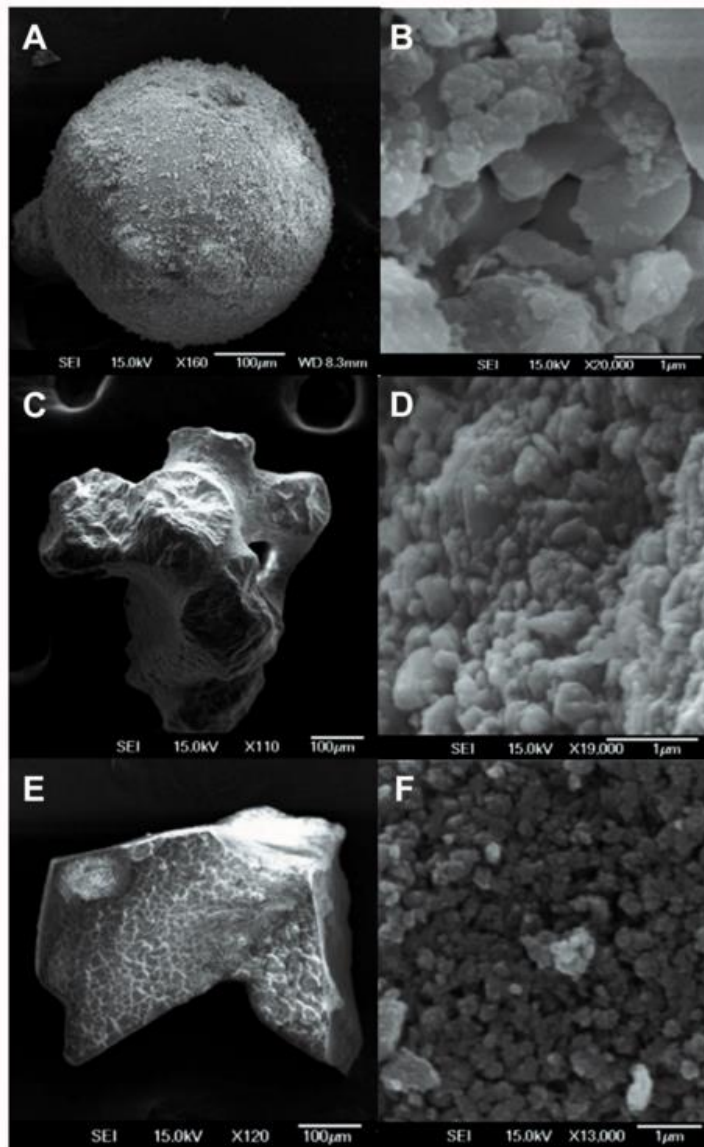
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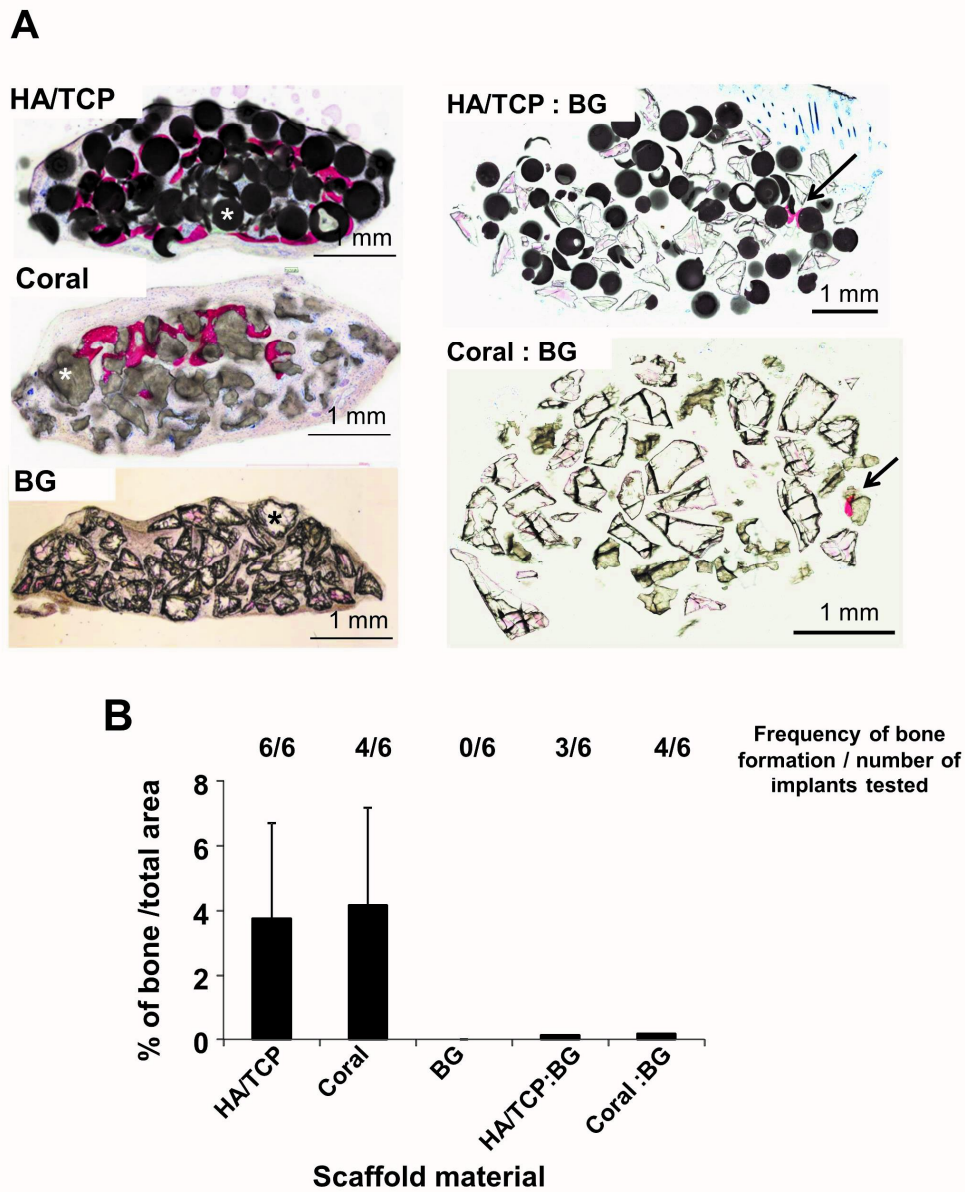
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Figure 1



Scanning electron micrographs of HA/TCP (A-B), coral (C-D) and BG (E-F) granules at low (A-C-E) and high (B-D-F) magnification.

Figure 2



Ectopic bone formation after 8 weeks of subcutaneous implantation of scaffolds containing hBMSC. (A) Representative, undecalcified, histological cross-sections of hBMSC-containing constructs composed of either HA/TCP, coral, BG granules or composites of either HA/TCP:BG or coral: BG (50:50 w/w). Stains: Stevenel's blue and Van Gieson Picrofuchsin red. (B) Amount of new bone tissue determined by histomorphometric analysis. The frequency of bone formation per implant material tested is also indicated. (*) material scaffold; the red color provided evidence for mineralized bone tissue.

Figure 3

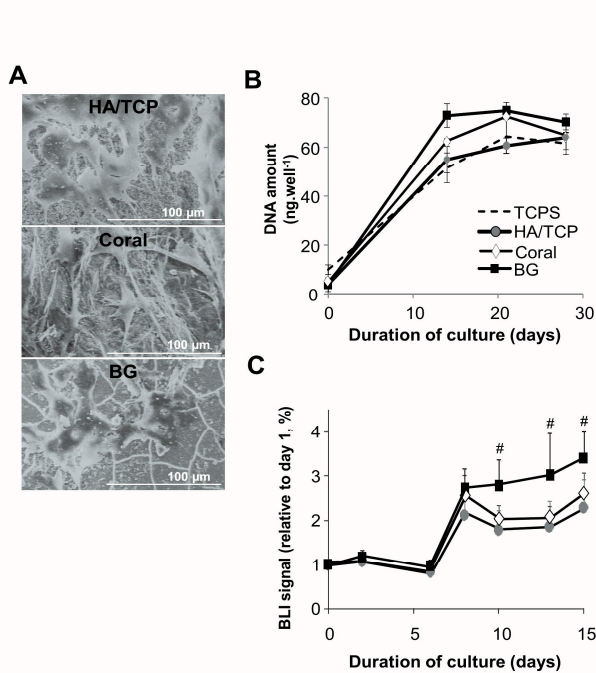
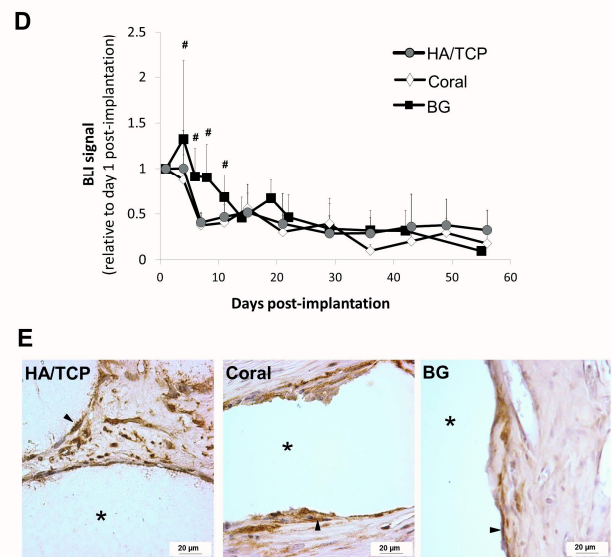
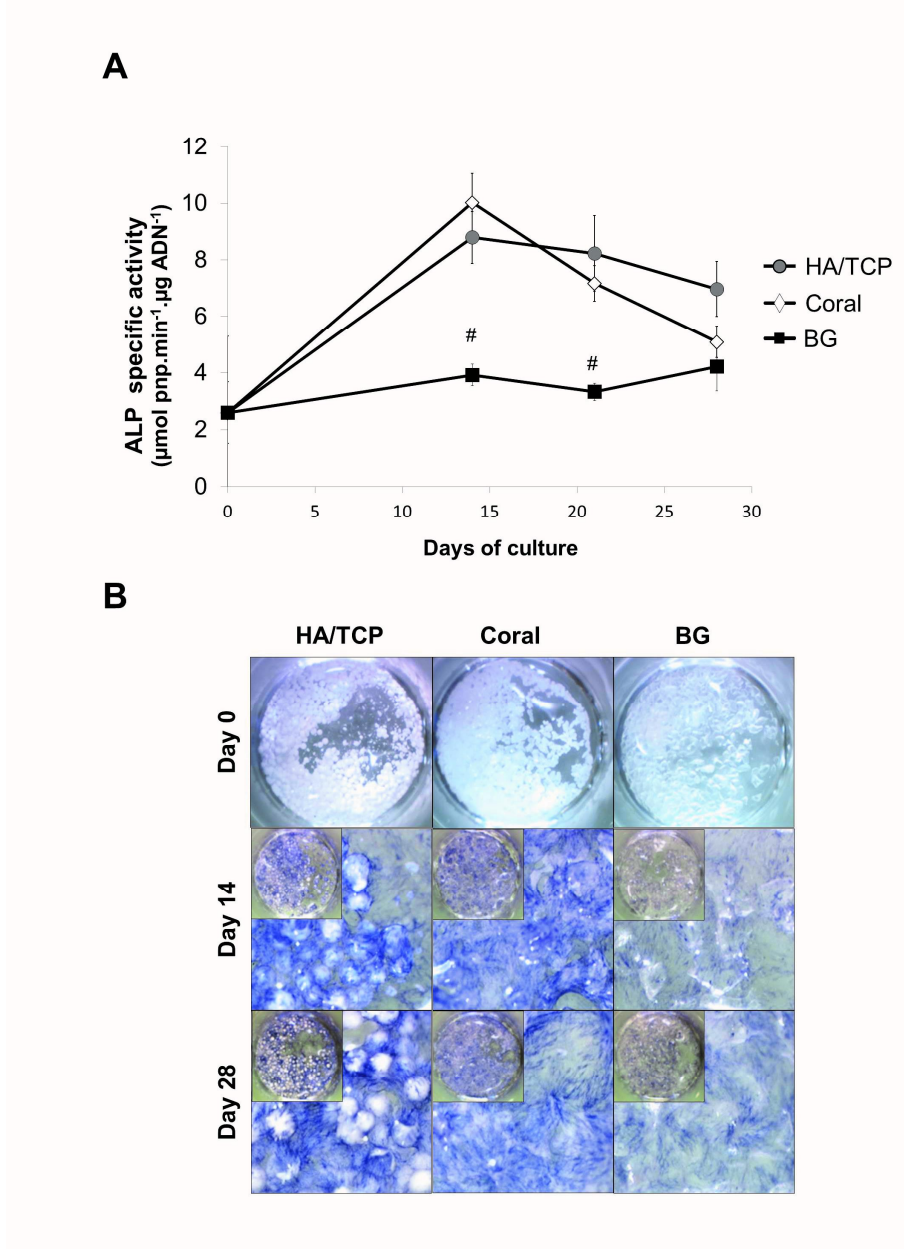


Figure 3 continued



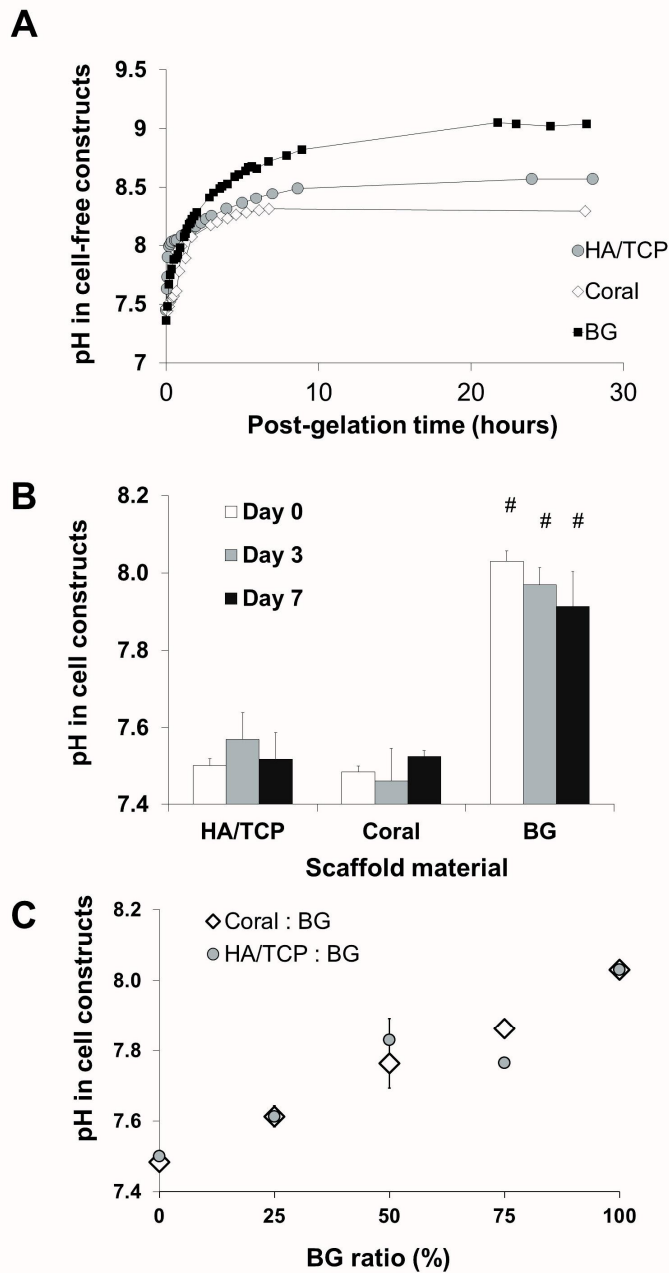
Viability of hBMSC onto material scaffolds either cultured in vitro (A-C) or implanted in vivo in nude mice (D-E). (A) In vitro hBMSC adhesion evaluated by SEM 24h post-cell-seeding. (B) In vitro cell proliferation assessed when hBMSC were cultured either directly on tissue culture polystyrene (TCPS; TPP®) alone or onto granules placed on the bottom of individual wells of TCPS dishes and determined by DNA quantification; (C) In vitro cell proliferation assessed when LuchBMSC were cultured within 3D constructs (composed of material granules embedded in fibrin gel) and determined by BLI monitoring. Photon fluxes (normalized to those obtained at day 1 after cell seeding) were plotted versus the number of days of cell culture. $p < 0.001$ (two-ways analysis of variance) ; (#) $p < 0.05$ for the BG constructs compared to the other two material constructs tested (Mann–Whitney test). (D) In vivo cell viability was determined by monitoring BLI over 58 days of implantation: Photon fluxes (normalized to those obtained at day 1 post-implantation) were plotted versus duration of implantation; $p < 0.001$ (two-ways analysis of variance); (#) $p < 0.05$ (Mann–Whitney test) for the BG constructs compared to the other two material constructs. (E) hBMSC, still present in the constructs explanted 2 months post-implantation, were detected on paraffin-treated sections following immunostaining against human β -2-microglobulin. (*) scaffold material (either HA/TCP, coral or BG); (black arrow head) cells stained positive for human β -2-microglobulin..

Figure 4



Osteogenic differentiation of hBMSC cultured onto granules of HA/TCP, coral and BG. (A) Kinetics of the ALP activity expressed by hBMSC; $p < 0.001$ (two-ways analysis of variance) and (#) $p < 0.05$ (Mann–Whitney test) compared to values obtained with either HA/TCP or coral granules at the same time point. (B) hBMSC stained in situ for ALP after 0, 14 and 28 days of cell culture.

Figure 5



pH determination within the material constructs. (A) Time-course of pH values at the core of the material granule constructs (without cells) using a microelectrode inserted before fibrin gelation. (B) pH measurements from cell constructs before (day 0) and after in vivo subcutaneous implantation for either 3 or 7 days. (C) In vitro pH determination as a function of the ratio (from 0 to 100 % in weight) of BG granules in either HA/TCP: BG or coral: BG cell-containing composite constructs. (#) $p < 0.05$ (Mann-Whitney test) compared to values obtained with either HA/TCP or coral materials at the same time point.

Figure 6

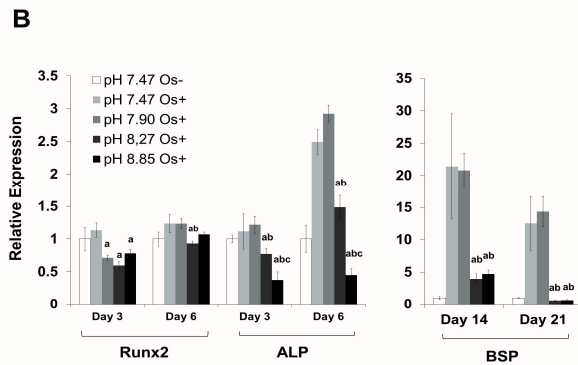
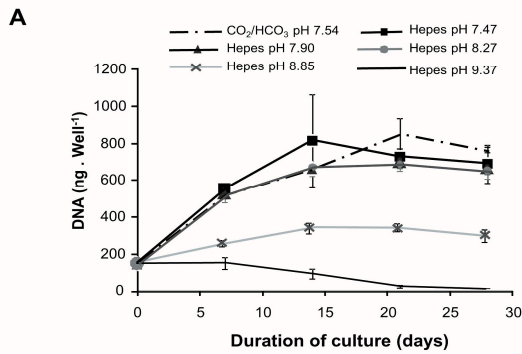
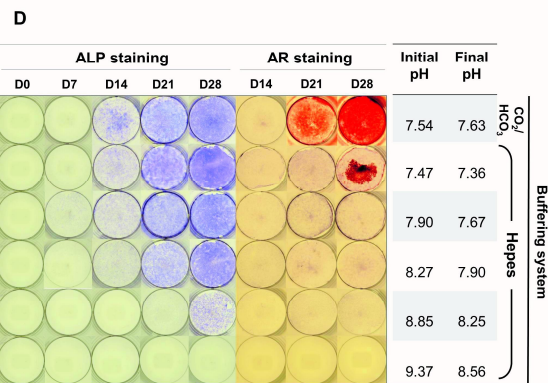
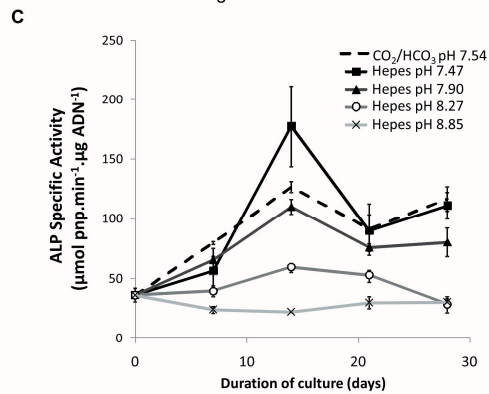
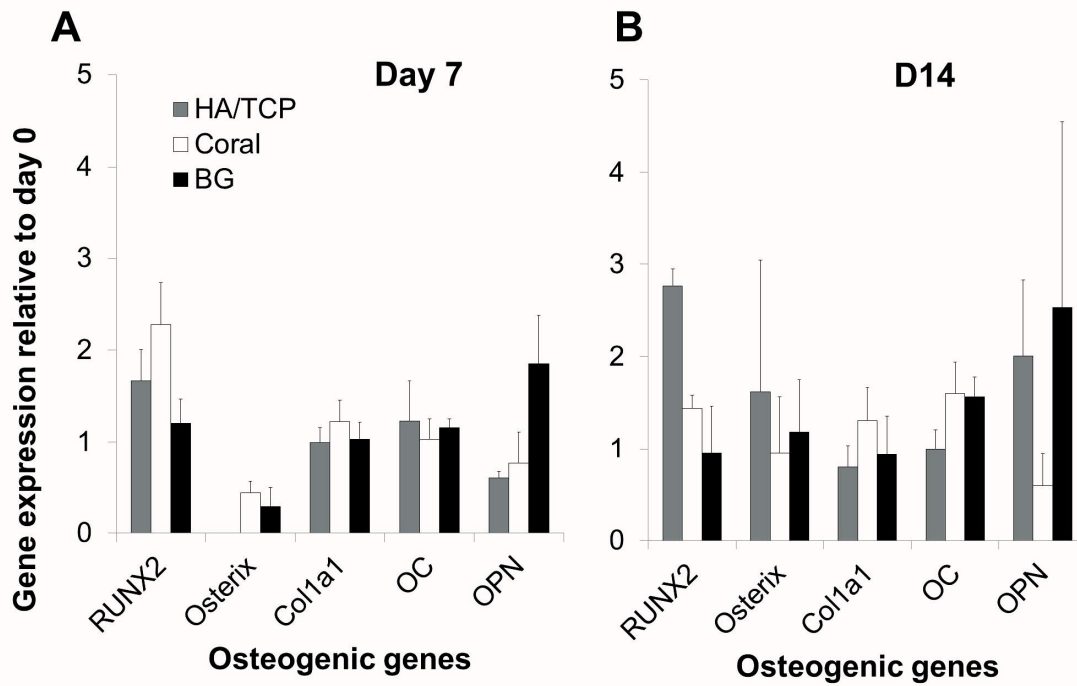


Figure 6 continued



Osteogenic differentiation of hBMSC as a function of the pH of the culture medium. (A) Proliferation of hBMSC cultured in buffered osteogenic medium either using a standard CO₂/HCO₃ system or adjusted to increasing alkaline pH values using 25 mM HEPES; $p < 0.001$ (two-way ANOVA) for the cell cultures at either pH 8.85 or 9.37; these results were compared to those obtained from cell cultures at other pH values (specifically, 7.47, 7.54, 7.90 and 8.27). (B) Gene expression of RUNX2, ALP and BSP by hBMSC cultured in osteogenic medium (Os+); gene expression was normalized first to that of the respective 18S (internal standard), and then to results obtained when cells were cultured in the non-osteogenic culture medium (Os-); (a) $p < 0.05$ compared to pH 7.47 Os+ group; (b) $p < 0.05$ compared to pH 7.90 Os+ group; (c) $p < 0.05$ compared to pH 8.27 Os+ group (Mann-Whitney tests). (C) Kinetics of the ALP activity expressed by hBMSC cultured in osteogenic medium; $p < 0.001$ (two-way ANOVA) for the cell cultures performed at either pH 8.27 or 8.85 compared to cultures at other pH (specifically, 7.47, 7.54 and 7.90). (D) ALP and Alizarin Red (AR) in situ stainings of hBMSC. Average values of both the initial and final pH of the supernatant culture medium which were determined at each medium change during the course of the experiment are indicated in the Table included on this frame.

Supplementary figure 1



Supplementary Figure 1: Osteogenic gene expression by hBMSC cultured directly onto material granules in osteogenic medium for 7 (A) and 14 (B) days. Expression of the osteoblastic (RUNX2, Osterix, type 1 collagen (Col1a1), osteocalcin (OC) and osteopontin (OPN)) genes by these hBMSC was normalized first to that of the respective 18S (internal standard), and then to the respective gene expression results obtained from these cells at day 0.