



## Bioactive glass ions as strong enhancers of osteogenic differentiation in human adipose stem cells



Miina Ojansivu<sup>a,b,c,\*,1,2,3</sup>, Sari Vanhatupa<sup>a,b,c,1,2,3</sup>, Leena Björkvik<sup>d,4</sup>, Heikki Häkkänen<sup>e,5</sup>,  
Minna Kellomäki<sup>b,f,2,6</sup>, Reija Autio<sup>g,7</sup>, Janne A. Ihalainen<sup>e,5</sup>, Leena Hupa<sup>d,4</sup>, Susanna Miettinen<sup>a,b,c,1,2,c</sup>

<sup>a</sup> Adult Stem Cell Research Group, University of Tampere, Tampere, Finland

<sup>b</sup> BioMediTech, University of Tampere and Tampere University of Technology, Tampere, Finland

<sup>c</sup> Science Centre, Tampere University Hospital, Tampere, Finland

<sup>d</sup> Johan Gadolin Process Chemistry Centre, Åbo Akademi University, Turku, Finland

<sup>e</sup> Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland

<sup>f</sup> Biomaterials and Tissue Engineering Group, Department of Electronics and Communications Engineering, Tampere University of Technology, Tampere, Finland

<sup>g</sup> School of Health Sciences, University of Tampere, Tampere, Finland

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### ABSTRACT

Bioactive glasses are known for their ability to induce osteogenic differentiation of stem cells. To elucidate the mechanism of the osteoinductivity in more detail, we studied whether ionic extracts prepared from a commercial glass S53P4 and from three experimental glasses (2-06, 1-06 and 3-06) are alone sufficient to induce osteogenic differentiation of human adipose stem cells. Cells were cultured using basic medium or osteogenic medium as extract basis. Our results indicate that cells stay viable in all the glass extracts for the whole culturing period, 14 days. At 14 days the mineralization in osteogenic medium extracts was excessive compared to the control. Parallel to the increased mineralization we observed a decrease in the cell amount. Raman and Laser Induced Breakdown Spectroscopy analyses confirmed that the mineral consisted of calcium phosphates. Consistently, the osteogenic medium extracts also increased osteocalcin production and collagen Type-I accumulation in the extracellular matrix at 13 days. Of the four osteogenic medium extracts, 2-06 and 3-06 induced the best responses of osteogenesis. However, regardless of the enhanced mineral formation, alkaline phosphatase activity was not promoted by the extracts. The osteogenic medium extracts could potentially provide a fast and effective way to differentiate human adipose stem cells *in vitro*.

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### 1. Introduction

With the aging population in the Western countries the amount of bone injuries is constantly growing highlighting the need for

novel tissue engineering-based treatment solutions which could circumvent the drawbacks of the traditionally used autologous bone grafts (e.g., donor site morbidity, lack of adequate amount and quality of bone). Of the various biomaterials tested for bone tissue engineering applications, bioactive glasses (BaGs), originally described by Hench and coworkers [1], have proven to be especially advantageous due to their strong bonding to bone, biocompatibility and biodegradation (for thorough reviews of BaGs, see Jones et al. [2] and Rahaman et al. [3]). Importantly, BaGs are osteoinductive materials able to stimulate the osteogenic differentiation of stem and progenitor cells without any added chemical supplements [4–7].

Recently, the mechanism of BaG osteoinductivity has evoked interest. It has been observed that also when cells are cultured in media containing ions released from BaGs and having no contact to the BaG surface, osteogenic differentiation is enhanced, implying that the ions from BaG are alone capable of inducing osteogenic

\* Corresponding author at: University of Tampere, FM5, BMT, Regenerative Medicine, Adult Stem Cell Group, 33014 University of Tampere, Finland. Tel.: +358 50 494 7925; fax: +358 3 3551 8498.

E-mail address: [miina.ojansivu@uta.fi](mailto:miina.ojansivu@uta.fi) (M. Ojansivu).

<sup>1</sup> Postal address: University of Tampere, FM5, BMT, Regenerative Medicine, Adult Stem Cell Group, FI-33014 University of Tampere, Finland.

<sup>2</sup> Postal address: Biokatu 10, FI-33520 Tampere, Finland.

<sup>3</sup> Postal address: Pirkanmaa Hospital District, Science Centre, P.O. Box 2000, FI-33521 Tampere, Finland.

<sup>4</sup> Postal address: Biskopsgatan 8, FI-20500 Åbo, Finland.

<sup>5</sup> Postal address: P.O. Box 35, FI-40014 University of Jyväskylä, Finland.

<sup>6</sup> Postal address: P.O. Box 692, FI-33101 Tampere, Finland.

<sup>7</sup> Postal address: School of Health Sciences, FI-33014 University of Tampere, Finland.

differentiation [8–14]. However, the majority of the studies investigating the effect of BaG ions on osteogenic differentiation have used either osteosarcoma cell lines [15–17] or osteoblasts [10–14,18–21] and currently no knowledge exists about the response of mesenchymal stem cells to the ions dissolved from BaGs. Furthermore, of the various BaG compositions designed for biological applications, only those named as 45S5 [9–13,22], 58S [8,9,18,19], 6P53-b [11,13,22], MBG85 [15] and BG60S [20] have been studied in the context of ionic dissolution products in cell culture. Since the glass composition and dissolution kinetics between the different BaG types vary, the results obtained with a few glasses can by no means be applied to the other BaG compositions. To promote the utilization of the BaG materials in bone tissue engineering, a better understanding of the mechanisms of BaG induced osteogenic differentiation as well as the role of BaG ions in cell responses are required.

S53P4 glass, commercially available as BonAlive®, is known to induce osteogenic differentiation of human ASCs (hASCs) cultured in direct contact with the surface [7] and it has also proven to perform well in clinical settings [23–25]. We therefore wanted to test the performance of this otherwise well-characterized glass type in the context of ionic dissolution. The other three BaG types used in this study (2-06, 1-06 and 3-06) are experimental silica-based glass compositions which have not been characterized in cell culture experiments. However, 1-06 and 3-06 glasses have shown good bone and soft tissue bonding properties in *in vivo* studies [26,27] raising interest in their mechanism of action in cellular level.

In the present study, we hypothesized that ionic extracts dissolved from four different BaG compositions, S53P4, 2-06, 1-06 and 3-06, could stimulate the osteogenic differentiation of hASCs. To examine the validity of our hypothesis, we analyzed the viability, proliferation and osteogenic differentiation of hASCs after culturing the cells in BaG extract media prepared from each glass type using either basic medium (BM) or osteogenic medium (OM) as a base composition. This is the first study in which the BaG ion supplemented BM and OM are systematically compared in their ability to induce osteogenic differentiation. Unlike the other studies conducted thus far with ionic species, we carried out all the experiments in HS supplemented media which represents the natural growth environment of human-originated cells. The use of animal-origin free culture conditions enables the extrapolation of our results to the development of clinical-oriented bone tissue engineering applications.

## 2. Materials and methods

### 2.1. Ethics statement

This study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058). The hASCs were isolated from adipose tissue samples obtained from surgical procedures conducted in the Department of Plastic Surgery, Tampere University Hospital. There were five women donors of age  $52 \pm 12$  years. All the donors gave a written informed consent for the utilization of the adipose tissue samples in research settings.

### 2.2. Manufacturing bioactive glass granules

Bioactive glasses 2-06, 1-06, 3-06 and S53P4 were prepared from batches of analytical grade reagents  $\text{Na}_2\text{CO}_3$ ,  $\text{K}_2\text{CO}_3$ ,  $\text{CaCO}_3$ ,  $\text{MgO}$ ,  $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , and Belgian quartz sand (Sigma–Aldrich, MO, USA). The batches giving 300 g glass were melted in a platinum crucible for 3 h at  $1360^\circ\text{C}$ , cast, annealed, crushed and

remelted to ensure homogeneity. The oxide compositions of the glasses S53P4, 2-06, 1-06 and 3-06 are depicted in Table 1. Annealed glass blocks were crushed and sieved to give a 500–1000  $\mu\text{m}$  size range fraction. The crushing was done according to the ISO 719 procedure without milling. After crushing, the granules were washed with acetone in an ultrasound bath at least five times to minimize the fine grained particles attached on their surface. Finally, the acetone was evaporated and the particles were dried at  $120^\circ\text{C}$ .

### 2.3. Preparation of bioactive glass extracts

The BaG granules (500–1000  $\mu\text{m}$ ) to be used in the extract preparation were first disinfected with ethanol (10 min in absolute ethanol +10 min in 70% ethanol) after which they were let to dry at room temperature for 2 h. In order to dissolve ions from the BaG granules 87.5 mg/ml granules were incubated for 24 h at  $+37^\circ\text{C}$  in cell culture dishes (diameter 10 cm). The extraction medium contained Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12 1:1; Life Technologies, Gibco, Carlsbad, CA, USA) supplemented with 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; Lonza, BioWhittaker, Verviers, Belgium) and 1% L-glutamine (GlutaMAX I; Life Technologies, Gibco). After incubation the extracts were sterile filtered (0.2  $\mu\text{m}$ ) and human serum (HS; PAA Laboratories, Pasching, Austria) was added to the concentration of 5%. This medium composition is referred to as basic medium extract (BM extract). In order to obtain osteomeidum extracts (OM extracts), BM extracts were supplemented with osteogenic factors (10 mM  $\beta$ -glycerophosphate, 250  $\mu\text{M}$  L-ascorbic acid 2-phosphate and 5 nM dexamethasone). The BaG extracts were freshly made for each 2 week experiment so the maximum storage time of the extracts was 14 days at  $+4^\circ\text{C}$ . No visible precipitate was formed during this time. A schematic representation of the BaG extract preparation is shown in Fig. 1.

### 2.4. Determination of the ion concentrations of the bioactive glass extracts

The ion concentrations of the BaG extracts after 24 h of extraction were determined using inductively coupled plasma optical emission spectrometer (ICP-OES; Optima 5300 DV, Perkin Elmer, Waltham, MA, USA). The extract samples containing 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin, Lonza) and 1% L-glutamine (GlutaMAX, Life Technologies) were sterile filtered (0.2  $\mu\text{m}$ ) prior to the analysis but neither serum nor the osteogenic supplements were added. The elements analyzed by ICP-OES were sodium ( $\lambda = 589.592$  nm), potassium ( $\lambda = 766.490$  nm), magnesium ( $\lambda = 285.213$  nm), calcium ( $\lambda = 317.933$  nm), phosphorus ( $\lambda = 213.617$  nm), boron ( $\lambda = 249.667$  nm) and silicon ( $\lambda = 251.611$  nm). The results of the analysis are depicted in Table 2.

### 2.5. Adipose stem cell isolation, expansion and culture

The isolation of hASCs was conducted using a mechanical and enzymatic procedure described previously [28,29]. The isolated hASCs were maintained in T-75 polystyrene flasks (Nunc, Roskilde, Denmark) in DMEM/F-12 (Life Technologies) supplemented with 5% HS (PAA Laboratories), 1% L-glutamine (GlutaMAX I, Life Technologies) and 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin; Lonza, BioWhittaker). This medium composition will be denoted by control basic medium (BM). When 80–100% confluence was reached hASCs were cryo-preserved in gas-phase nitrogen in freezing solution (HS supplemented with 10% dimethyl sulfoxide; DMSO HybriMax®, Sigma–Aldrich) and thawed when needed for the experiments. The hASCs used in the experiments

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