

Investigative study

## XENOGRAFT ACTS ON STEM CELLS

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### ABSTRACT

Xenogeneic bone substitute derived from bovine cancellous bone has become a widely used biomaterial in dentistry and maxillofacial surgery for bone augmentation and regeneration procedures. It is composed of deproteinized bovine bone minerals with organic components removed to minimize immunogenicity and enhance biocompatibility. Xenogeneic bone substitute exhibits excellent osteoconductivity, allowing for the ingrowth of host bone and facilitating long-term stability and integration with surrounding tissues. For this reason, we investigated how xenogeneic bone substitutes act on dental pulp stem cells to differentiate them into osteoblasts, measuring the expression levels of bone-related genes and stem cell markers by Real-Time Polymerase Chain Reaction (real-time RT-PCR). The results indicated that RUNKS and FOSL1 strongly increased gene expression after 4 days of treatment. Although its role in bone biology is still being elucidated, FOSL1 and MMP XII appear to exert effects on osteoblasts and osteoclasts, modulating their activity and contributing to bone homeostasis and disease pathogenesis. Xenograft bone substitutes act on dental stem cells and promote osteoblast differentiation.

**KEYWORDS:** *graft, bone, osteoblasts, stem, expression*

### INTRODUCTION

Xenogeneic bone substitute (XBS) derived from bovine cancellous bone has become a widely used biomaterial in dentistry and maxillofacial surgery for bone augmentation and regeneration procedures. It is composed of deproteinized bovine bone mineral, with the organic components removed to minimize immunogenicity and enhance biocompatibility.

It closely resembles human bone in terms of its structure and composition, providing an ideal scaffold for new bone formation (1, 2). XBS exhibits excellent osteoconductivity, allowing for the ingrowth of the host bone and facilitating long-term stability and integration with surrounding tissues. XPS is used in various dental and maxillofacial procedures, including ridge augmentation, sinus floor elevation, socket preservation, and guided bone regeneration (3, 4).

It serves as a filler material in bone defects and provides support for dental implants, thereby enhancing their stability and success rates. XBS is compatible with autogenous bone grafts and other biomaterials, allowing versatile treatment approaches tailored to individual patient needs (5, 6). XBS offers several advantages for bone augmentation procedures, including biocompatibility, osteoconductivity, and predictable clinical outcomes. Its natural origin and structure minimize the risk of adverse reactions or rejection, making it suitable for a wide range of patients. XBS eliminates the need for additional donor-site surgery and reduces patient morbidity and surgical complexity. Moreover, its availability in various particle sizes and formulations enables customized treatment strategies for different clinical

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scenarios. Despite its widespread use and clinical efficacy, XBS presents challenges, including limited resorption and remodeling capacity. XBS may elicit immune responses in some patients, necessitating careful selection and pre-operative evaluation. Among XBS, Bio-Oss (Geistlich, Germany) is one of the most commonly used, so we decided to investigate the impact of Bio-Oss on dental pulp stem cells (DPSC).

Dental pulp stem cells (DPSCs) residing within the dental pulp tissue of teeth have garnered significant attention in regenerative medicine and tissue engineering because of their multipotent differentiation potential and accessibility.

DPSCs are a heterogeneous population of cells with self-renewal capacity and multi-lineage differentiation potential. They can differentiate into various cell types, including odontoblasts, osteoblasts, adipocytes, and neural-like cells, making them promising candidates for tissue regeneration and repair. DPSCs can be isolated from the dental pulp of deciduous and permanent teeth, exfoliated deciduous teeth (SHED), and third molars through minimally invasive procedures (7, 8).

DPSC has immense therapeutic potential for various medical and dental applications. They have been investigated for their ability to regenerate dental tissues, such as dentin, pulp, and the periodontal ligament, in cases of dental caries, trauma, and pulpitis. Additionally, DPSCs have shown promise in regenerating non-dental tissues, including bone, cartilage, nerve, and cardiac tissues, making them versatile tools for tissue engineering and regenerative medicine therapies. Therefore, we investigated how Bio-Oss act on DPSCs to differentiate them into osteoblasts.

## MATERIALS AND METHODS

### *Dental pulp stem cells (DPSCs) isolation*

Dental pulp was extracted from the third molars of healthy subjects and digested for 1 h at 37°C in a solution containing 1 mg/ml collagenase type I and 1 mg/ml dispase dissolved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin 100 µg/ml streptomycin, and 500 µg/ml clarithromycin. The solution was then filtered using 70 µm Falcon strainers (Sigma Aldrich, St Louis, Mo, U.S.A.) to separate mesenchymal stem cells from fibroblasts. Stem cells were cultivated in  $\alpha$ -MEM culture medium (Sigma Aldrich, St Louis, Mo, U.S.A.) supplemented with 20% Fetal Bovine Serum (FBS), 100 µM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich, St Louis, Mo, U.S.A.). The flasks were incubated at 37°C with 5% CO<sub>2</sub>, and the medium was changed twice weekly.

DPSCs were characterized by immunofluorescence for the cytoskeletal component vimentin, positive mesenchymal stem cell markers CD90 and CD73, and the negative marker CD34, as described in Sollazzo et al. (9).

### *Cell treatment*

DPSCs were seeded at a concentration of  $1.0 \times 10^5$  cells/ml in 9 cm<sup>2</sup> (3 ml) wells containing DMEM supplemented with 10% serum and antibiotics, and Bio-Oss (Geistlich, Wolhusen, Switzerland) was added at a concentration of 10 mg/ml. Another set of wells containing untreated cells was used as the control. The treatment was performed at two time points: 24 h and 4 days.

Cells were maintained in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. At the end of the treatment period, cells were lysed and processed for total RNA extraction.

### *RNA isolation, reverse transcription, and quantitative real-time RT-PCR*

Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The pure RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis was performed using 500 ng of total RNA and PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan). The reaction mixture was incubated at 37°C for 15 min and inactivated by heating at 70°C for 10 s. cDNA was amplified by real-time quantitative PCR using ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA).

All PCR reactions were performed in a 20 µL volume. Each reaction contained 10 µl of 2x qPCRBIO SYGreen Mix Lo-ROX (PCR Biosystems, Ltd., London, UK), 400 nM of each primer, and cDNA.

Custom primers belonging to the “extracellular matrix, adhesion molecule” pathway, “osteoblast differentiation” and “inflammation” pathway were purchased from Sigma-Aldrich. The selected genes grouped by functional pathways are listed in Table I.

All the experiments were performed using non-template controls to prevent reagent contamination. PCR was performed using two analytical replicates.

The amplification profile was initiated by incubation for 10 min at 95 °C, followed by a two-step amplification for 15 s at 95°C and 60 s at 60°C for 40 cycles. In the final step, melt curve dissociation analysis was performed.

**Table I.** Selected genes used in Real-Time PCR grouped by functional pathway.

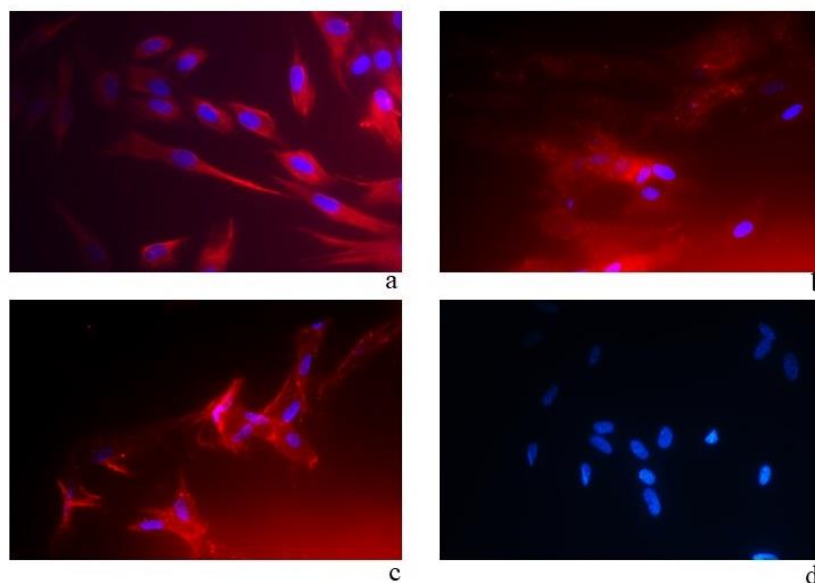
Pathway	Gene
Osteoblast differentiation	SPP1 (Osteopontin) SPARC (Osteonectin) RUNX2 (Runt-related transcription factor 2) ALP (Alkaline phosphatase) FOSL1 (FOS-like antigen 1) SP7 (Osterix) ENG (Endoglin)
Extracellular matrix, adhesion molecule	COL1A1 (Collagen type I alpha1) COL3A1 (Collagen, type III, alpha 1) COL4A1 (Collagen, type IV, alpha 1) MMP7 (Matrix Metalloproteinase 7) MMP12 (Matrix Metalloproteinase 12) MMP14 (Matrix Metalloproteinase 12)
Inflammation	IL1 $\alpha$ (Interleukin 1 Alpha) IL1R (Interleukin 1 Receptor Type 1) IL6 (Interleukin 6) IL6R (Interleukin 6 Receptor)
Reference gene	RPL13 (Ribosomal protein L13)

#### Statistical analysis

The gene expression levels were normalized to the expression of the reference gene (RPL13) and expressed as fold changes relative to the expression in untreated cells. Quantification was performed using the delta-delta Ct method (10).

## RESULTS

The DPCSs were phenotypically characterized using immunofluorescence. Fig. 1a shows cytoskeletal filaments stained with vimentin. The cell surfaces were positive for mesenchymal stem cell markers CD90 (Fig. 1b) and CD73 (Fig. 1c) and negative for markers of hematopoietic origin CD34 (Fig. 1d).



**Fig. 1.** DPSCs by indirect immunofluorescence (Rhodamine). Immunofluorescence staining of vimentin (a), mesenchymal stem cell marker CD73 (b), CD90 (c), and hematopoietic markers CD34 (d). Nuclei were stained with DAPI. Original magnification  $\times 40$ .

Bio-Oss treatment in DPSCs was analyzed using quantitative real-time PCR after 24 h and 4 days of treatment, and the expression levels of osteoblast-related genes, extracellular matrix, and inflammation pathways were measured.

Table II reports the significant fold changes obtained after 24 h and 4 days.

**Table II.** Gene expression in DPSCs after 24h and 4 days of treatment. Numbers express the fold changes of the de-regulated genes in treated cells vs. untreated cells. In bold significant gene expression level.

	24 h	4 DAYS
<b>SPP1</b>	nd	nd
<b>SPARC</b>	0.9	0.67
<b>RUNX2</b>	1.16	<b>2.38</b>
<b>ALP</b>	0.59	0.36
<b>FOSL1</b>	0.84	<b>4.31</b>
<b>SP7</b>	<b>5.18</b>	0.23
<b>ENG</b>	0.91	1.47
<b>COL1A1</b>	0.67	0.63
<b>COL3A1</b>	1.5	0.4
<b>COL4A1</b>	1.3	1.1
<b>MMP7</b>	1.8	0.2
<b>MMP12</b>	<b>3.8</b>	0.2
<b>MMP14</b>	<b>2.1</b>	1.8
<b>IL1<math>\alpha</math></b>	1.8	0.4
<b>IL1R</b>	1	0.4
<b>IL6</b>	<b>10.6</b>	<b>6.2</b>
<b>IL6 R</b>	0.6	1.2

Significantly upregulated genes showed  $\geq 2$ -fold change in expression ( $P$  value  $\leq 0.05$ ), while significantly downregulated genes showed  $\leq 0.5$ -fold change in expression ( $P$  value  $\leq 0.05$ ).

In DPSCs, after 24 h of treatment, SP7 was strongly upregulated, as were MMP12, MMP14, and IL6 (Table II). After 4 days, IL6 was still upregulated, whereas SP7, MMP12, and MMP14 decreased. RUNX2 and FOSL1 strongly increased gene expression after four days of treatment.

## DISCUSSION

Dental pulp stem cells (DPSCs) have emerged as a significant focus in regenerative medicine due to their unique properties and versatile applications. Isolated from the dental pulp of deciduous and permanent teeth, DPSCs are mesenchymal stem cells capable of differentiating into various cell types.

DPSCs exhibit several key characteristics that make them valuable in regenerative medicine. DPSCs can differentiate into multiple cell lineages, including osteoblasts (bone cells), chondrocytes (cartilage cells), adipocytes (fat cells), and neurons (nerve cells). This multipotency is crucial for their application in tissue engineering and repair. DPSCs have a high proliferative rate, meaning they can rapidly multiply to generate sufficient cells for therapeutic use.

DPSCs secrete various bioactive molecules that modulate immune response, reduce inflammation, and promote tissue repair. DPSCs can be easily obtained from extracted teeth, which is a common and minimally invasive procedure, making them an accessible source of stem cells.

DPSCs have significant potential for various applications in regenerative medicine. DPSCs can be used to regenerate dental tissues, including dentin, pulp, and the periodontal ligament, offering potential treatments for tooth decay, pulpitis, and periodontal disease. DPSCs can differentiate into osteoblasts, making them suitable for bone tissue engineering and treatment of bone defects and fractures. DPSCs have the ability to differentiate into neural cells, presenting potential therapeutic options for neurodegenerative diseases and spinal cord injuries. DPSCs can contribute to the regeneration of cardiac tissues and offer promise for the treatment of myocardial infarction and other heart conditions.

DPSCs can enhance wound healing and skin regeneration, thereby providing new approaches for treating burns and chronic wounds (10, 11).

The use of DPSCs has several advantages. The collection of DPSCs from extracted teeth is a minimally invasive procedure that reduces the risk and discomfort associated with stem cell harvesting. DPSCs avoid ethical issues related to embryonic stem cells as they are derived from discarded dental tissues. DPSCs can be used in autologous therapies, where the patient's own cells are used for treatment, thus minimizing the risk of immune rejection. The ability of DPSCs to differentiate into multiple cell types renders them suitable for a wide range of regenerative applications.

For all the above-mentioned reasons, we decided to verify how Bio-Oss act on DPSCs to stimulate their differentiation into osteoblasts.

Both FOSL1 and MMP12 were activated by Bio-Oss (Table II).

FOSL1, a member of the FOS family of transcription factors, plays a significant role in regulating cellular processes, such as proliferation, differentiation, and apoptosis. Its involvement in osteogenesis, which is the process of bone formation, has garnered considerable interest in recent years.

FOSL1 belongs to the AP-1 (Activator Protein-1) family of transcription factors and is characterized by a basic leucine zipper (bZIP) domain that mediates dimerization and DNA binding. FOSL1 forms heterodimers with members of the Jun family (c-Jun, JunB, and JunD) constituting the AP-1 transcription complex. Its transcriptional activity is modulated by various signaling pathways, including the mitogen-activated protein kinase (MAPK) and Wnt/ $\beta$ -catenin pathways, which regulate FOSL1 expression and activity during osteogenesis. Post-translational modifications such as phosphorylation and acetylation also regulate FOSL1 function, influencing its stability, subcellular localization, and interaction with co-regulatory proteins. These regulatory mechanisms fine-tune FOSL1 activity, allowing the precise control of gene expression during osteogenesis (12).

FOSL1 plays diverse roles in osteogenesis, influencing both osteoblast differentiation and bone matrix mineralization. During the early stages of osteoblast differentiation, FOSL1 cooperates with other transcription factors such as RUNX2 and Osterix to activate the expression of osteogenic genes, including alkaline phosphatase (ALP), osteocalcin (OCN), and collagen type I (COL1A1). FOSL1 also promotes cell cycle progression and proliferation, facilitating expansion of the osteoblast progenitor pool.

In addition to its role in osteoblast differentiation, FOSL1 regulates bone matrix mineralization by modulating the expression of genes involved in extracellular matrix (ECM) synthesis and remodeling. FOSL1 promotes the expression of matrix metalloproteinases (MMPs) and other proteases that degrade ECM components, thereby facilitating the deposition of mineralized matrices. Moreover, FOSL1 interacts with signaling pathways involved in calcium homeostasis and phosphate metabolism, thereby influencing bone mineralization processes (13, 14).

Matrix Metalloproteinase 12 (MMP12), also known as macrophage metalloelastase, is a member of the matrix metalloproteinase family involved in the degradation of extracellular matrix components. Although traditionally studied in the context of inflammation and tissue remodeling, emerging evidence suggests that MMP12 may also play a role in osteogenesis (15, 16).

MMP12 is a zinc-dependent endopeptidase characterized by a catalytic domain, prodomain, and hemopexin-like domain. It is primarily produced by macrophages and is involved in the degradation of elastin and other components of the extracellular matrix. MMP12 expression and activity are regulated at multiple levels, including transcriptional regulation by cytokines, growth factors, and inflammatory mediators as well as post-translational modifications such as proteolytic cleavage and inhibition by tissue inhibitors of metalloproteinases (TIMPs).

Although the role of MMP12 in osteogenesis is less well characterized than that of other MMPs, emerging evidence suggests that MMP12 may influence bone formation through its effects on bone cells. In vitro studies have shown that MMP12 is expressed by osteoblasts and osteoclasts and can modulate their activity. MMP12 may promote osteoblast differentiation and mineralization by facilitating turnover of the extracellular matrix and releasing bioactive factors that regulate bone cell function (17, 18).

Conversely, MMP12 may contribute to bone resorption by enhancing osteoclast activity and bone matrix degradation. MMP12 degrades collagen and other components of the bone matrix, leading to the release of growth factors and cytokines that stimulate osteoclast formation and activation. Additionally, MMP12 may indirectly influence osteogenesis by modulating the inflammatory microenvironment, which plays a critical role in bone remodeling and repair.

## CONCLUSIONS

Bio-Oss is one of the most widely used xenografted bone substitutes. It acts on DPSCs stimulates the differentiation of DPSCs into osteoblasts. DPSCs represent a versatile and promising resource in regenerative medicine. Their ability to differentiate into various cell types, coupled with their accessibility and ethical advantages, makes them an attractive option for tissue engineering and therapeutic applications. Among the investigated genes, we focused on FOSL1 and MMP12. FOSL1 is a key regulator of osteogenesis and influences osteoblast differentiation, bone matrix mineralization, and bone homeostasis. MMP12 is emerging as a potential regulator of osteogenesis and influences the bone formation and remodeling processes. Although its role in bone biology is still being elucidated, MMP12 appears to exert effects on both osteoblasts and osteoclasts, modulating their activity and contributing to bone homeostasis and disease pathogenesis. Further research is required to better understand how xenografts stimulate stem cell differentiation into osteoblasts.

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