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Rahul D. Prabha, Bindu P. Nair, Nicholas Ditzel, Jorgen Kjems, Prabha D. Nair, Moustapha Kassem

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Rahul D Prabha\textsuperscript{1,4}, Bindu P Nair \textsuperscript{2}, Nicholas Ditzel\textsuperscript{1}, Jorgen Kjems\textsuperscript{3}, Prabha D Nair \textsuperscript{2}, Moustapha Kassem\textsuperscript{1}

\textsuperscript{1}Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital & University of Southern Denmark, Odense, Denmark

\textsuperscript{2}Division of Tissue Engineering and Regeneration Technologies (DTERT), Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, India

\textsuperscript{3}Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Aarhus C, 8000, Denmark

\textsuperscript{4}Department of Orthodontics and Dentofacial Orthopaedics, Amrita School of Dentistry, Amrita Vishwa Vidyapeetham, Kochi, Kerala-682041, India.

* Corresponding Author: Rahul Damodaran Prabha, MDS, PhD, Laboratory of Molecular Endocrinology (KMEB), Department of Endocrinology, University of Southern Denmark and University Hospital of Odense, J.B. Winslow vej 25, 1st Floor, DK-5000 Odense C, Denmark. Telephone: +45–65504084; Fax: +45–65919653;
E-mail: rahuldp6@gmail.com

Present Address: Rahul Damodaran Prabha, MDS, PhD, Department of Orthodontics and Dentofacial Orthopaedics, Amrita School of Dentistry, Amrita Vishwa Vidyapeetham, Kochi, Kerala-682041, India.

Email: rahuldp6@gmail.com
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Highlights
- Poly (ε) caprolactone – Laponite – Strontium ranelate composite scaffold (PLS3) scaffolds supports proliferation of Human Bone marrow derived stem cells (hMSC)
- In vitro studies shows PLS3 scaffold upregulates hMSC osteogenic gene expression and mineralization
- In vivo implantation of hMSC seeded PLS3 scaffold promotes ectopic vascularized bone formation in immunocompromised mice model

ABSTRACT
Drug functionalized scaffolds are currently being employed to improve local delivery of osteoprotective drugs with the aim of reducing their loading dose as well as unwanted systemic complications. In this study we tested a poly-(ε) caprolactone (PCL) -laponite-strontium ranelate (SRA) composite scaffold (PLS3) for its abilities to support growth and osteogenic differentiation of human marrow derived stromal stem cells (hMSC). The in vitro experiments showed the PLS3 scaffold supported cell growth and osteogenic differentiation. The in vivo implantation of hMSC seeded PLS3 scaffold in immunocompromised mice revealed vascularized ectopic bone formation. PLS3 scaffolds can be useful in bone regenerative applications in the fields of orthopedics and dentistry.

Key words: Laponite, Strontium, Bone, Stem cells, Drug delivery

1. Introduction
Repair of bone defects arising from trauma, tumors resection and need for repair of developmental disorders is a common clinical problem[1]. Small critical sized defects can be repaired with limited autologous bone grafts [2]. Treatments of bone defects of larger dimensions need some alternative bone graft techniques due to limitations of autologous bone grafting [3, 4]. Autologous bone harvest may lead to development of donor site morbidity [4]. Moreover, more successful bone grafting technique, like free vascularized fibular grafts are complicated and technically challenging [5]. As a result, there exists an increasing demand for bone graft substitutes [6]. Synthetically developed bone
graft substitutes would also alleviate the limitations of tissue derived reconstructive materials, like batch variations and immunogenic potential [6].

For successful bone tissue engineering, the bone graft substitutes should perform as bioactive material with osteoconductive and osteoinductive properties. Combinations of stromal derived stem cells (also known as skeletal stem cells or mesenchymal stem cells) (MSCs) with bioactive scaffolds have shown promising results with respect to bone formation, in vitro and in vivo. The craniofacial region, a complex region with complex anatomy, high vascularization and increased bone turnover needs a material that would integrate with the recipient site and vascularize [7]. Sufficient vascularization of newly formed bone is a key factor for ensuring the maintenance of the benefits of bone tissue engineering in the craniofacial region. Many anatomically challenging areas of craniofacial region would also require a material that could be implanted by less invasive procedures [8].

Recently, Hybrid materials and inorganic materials are emerging as upcoming materials for gene delivery, therapy, sensors, etc [9-17]. Subsequently many new hybrid biomaterials in the form of polymers, bioceramics, and hydrogels have been successfully tested as scaffolds for craniofacial reconstruction [18, 19]. Functionalization of scaffolds with osteoinductive drugs has also shown some promising results [20]. Nevertheless some of these systems may have drawbacks like lack of bioactivity, poor vascularization induction and delayed degradation [21]. Poly (ε) caprolactone (PCL) is a biocompatible polymer with sufficient mechanical properties [22]. Recently, laponite clays, (a synthetic silicate) (empirical formula of Na⁺₀.₇[(Si₈₋₅.₅Mg₅.₅Li₀.₃)O₂₀(OH)₄]₀.₇) have gained considerable attention for its bioactive as well as drug carrying properties [23, 24]. Laponite clays functionalized with vascular endothelial growth (VEGF) have been reported to support bone vascularization [25]. In the present study, we hypothesize that a composite of PCL and laponite would form a bioactive scaffold to be suitable for bone reconstruction in the craniofacial region. We also tested the possibility of functionalization of laponite with an anti-osteoporotic drug e.g. strontium ranelate (SRA), that together with hMSC can provide an engineered scaffold clinically relevant for repair of bone defects. The local delivery of drugs from scaffolds against osteoporosis has been reported to enhance osteoporotic fracture healing [26, 27]. The PCL – laponite – strontium ranelate composite scaffold (PLS3) would hence perform as a bioactive construct to address the biological demands of the
craniofacial region. In this study we have evaluated the PLS3 scaffold for its ability to support MSC growth and osteogenic differentiation both in vitro and in vivo.

2. Materials and Methods

2.1 Scaffold

The scaffold tested in this study is a PLS3 scaffold. The fabrication and characterization of PCL - LS composite scaffolds has been reported elsewhere [28]. The specific PLS3 scaffold was selected for its porous three dimensional structure and sustained strontium release properties [28]. Briefly, the PLS3 scaffolds of this study were prepared through solution blending method. PCL (Sigma Aldrich, Mw=70000 -90000 g/mol) was dissolved in 1, 4 dioxane (Merck specialties private. Ltd) to obtain a 7 wt. % solution, to which the required amount of LS complex was, added (3 wt. % of PCL) and the mixture was blended manually. The PCL-LS blend obtained was frozen at - 80 °C and lyophilized for about 8 hr. to obtain a three dimensional porous and composite scaffold denoted as PLS3.

2. In vitro experiments

Cell culture experiments on the PLS3 scaffolds were conducted with a human telomerase immortalized bone marrow derived skeletal stem cell line: hMSC-TERT that has been created in our laboratory and exhibit all characteristics of primary hMSC including in vivo bone formation[29, 30]. For simplicity, we will refer to these cells hereafter as hMSC. Cell seeded scaffolds were cultured at 37 °C, 5% CO₂ in culture medium (CM) comprised of Minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) and 10000 IU ml⁻¹ penicillin and 10 mg ml⁻¹ streptomycin (Gibco, Life Technologies). The culture, upon attaining 80% confluence was trypsinised with 0.05% Trypsin with EDTA (Gibco, Life Technologies). All experiments included have a control group supplemented with CM and an osteogenic differentiation group (OB). OB consisted of culture medium supplemented with L-ascorbic acid 2-phosphate (50 μg ml⁻¹, Wako Chemicals, Neuss, Germany), β-glycerol phosphate (10 mM, Sigma, MO), 1α,25-dihydroxyvitamin D₃ (10⁻⁸ M, Sigma), dexamethasone (10⁻⁸M, Sigma) . CM and OB were replaced twice a week.

2.3.1 Cell Seeding, Attachment and Proliferation
The PLS3 scaffolds of 3mm diameter were punched out using biopsy punch (Kai Europe, GmbH, Germany). Scaffolds were sterilized in 70% ethanol for 30 min, followed by washing thrice in sterile water. Prior to seeding, the scaffolds were conditioned by wetting with CM for 1 hr., to obtain uniform seeding. The conditioned scaffolds placed in ultra-low adhesion 24 well tissue culture plates (Costar, Corning) were seeded with cell suspension of $5 \times 10^4$ cells in 5 µl CM. The seeding technique used was sessile drop technique. Scaffolds were then incubated at 37 °C, 5% CO$_2$ for 45 min to allow cell attachment. The CM was supplemented immediately after the cell attachment. Osteogenic induction was initiated at 24 hr. post seeding. Both CM and OB were changed twice a week.

Cell attachments on the scaffold were visualized by Scanning Electron Microscopy (SEM). Cell seeded PLS3 scaffolds were fixed in 2.5% glutaraldehyde for one hour, washed in PBS and dehydrated in graded series of alcohol. The dried scaffolds were further gold sputter coated and imaged under LEO 435VP SEM.

Cell proliferation on the PLS3 scaffold was visualized by DAPI/Phalloidin staining at Day 1 and Day 7 and quantified by Cell Titer- Blue (Promega, Madison, USA) assay respectively for time points 1, 2, 5 and 7 days. For DAPI/Phalloidin staining, cells seeded on PLS3 scaffold were fixed in 4% paraformaldehyde solution for one hour after washing in PBS. Fixed cell seeded constructs were stained with Rhodamine labeled Phalloidin (Sigma–Aldrich, Germany) and 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, Germany) for actin cytoskeleton and nucleus respectively as per protocol[31]. The stained cell seeded scaffolds were imaged under Olympus FV1000MPE Confocal microscope for DAPI (359nm) and Phalloidin (550nm) respectively. The proliferation of cells seeded on the scaffolds was estimated by number of viable cells present at time points 1, 2, 5 and 7 days. Cell Titer-Blue reagent (Promega, Madison, USA) was added to culture medium, and fluorescent intensity (560EX/590EM) measured after incubating at 37°C for one hr. The absorbance obtained was normalized to the standard linear curve established to obtain cell number. The assumption made was cells are not metabolically active until 24 hr.

2.3.2 ALP activity

Alkaline phosphatase (ALP) activity was measured by using enzymatic $p$-nitrophenyl phosphate (Sigma-Aldrich) substrate reduction and further, normalized against the cell number. Cell number was quantified by the addition of Cell Titer-Blue reagent to culture medium, incubating at 37 °C for 1 hr.,
and measuring fluorescent intensity (560<sub>EX</sub>/590<sub>EM</sub>). Samples were then washed with PBS and Tris-buffered saline, fixed with 3.7% formaldehyde in 90% ethanol for 30 s at room temperature, incubated with substrate (1 mg/ml of p-nitro phenyl phosphate in 50 mM NaHCO<sub>3</sub>, pH 9.6, and 1 mM MgCl<sub>2</sub>) at 37 °C for 20 min, and the absorbance measured at 405 nm [30]. ALP activity was normalized to cell number. Cell seeded scaffolds were also stained to visualize ALP activity. The staining was performed post-fixation, 0.10 mM citrate buffer pH 4.2/acetone fix (ratio 3:2) for 5 min at room temperature (ALP). ALP staining was carried out with a (ratio 1:1) solution mix of 0.2 mg/ml Napthol AS-TR phosphate substrate (Sigma-Aldrich, Denmark) in water and 0.417 mg/ml of Fast red (Sigma-Aldrich, Denmark) in 0.1 M Tris (pH 9.5) for 1 hr. at room temperature.

2.3.3 Osteogenic gene expression

Cells seeded on PLS3 were lysed for total RNA extraction using Trizol (Invitrogen, Denmark); according to manufacturer's instructions. The phase separation and RNA pellet precipitation was performed using chloroform and isopropanol respectively. The RNA pellets obtained were washed in 70% ice cold ethanol, before air drying and resuspension in RNase free water (Sigma Aldrich, U.S.A) for quantification using NanoDrop1000 spectrophotometer v3.7 instrument (Thermo Fisher Scientific, U.S.A). cDNA was constructed using a revertAid H minus first strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions after DNAse treatment (Sigma Life sciences, USA). RT-qPCR analysis was performed with StepOnePlus™ system (Applied Biosystems, Denmark) in a 10 μl volume with 20 pmol of each primer, 2× SYBERgreen Master Mix (Applied Biosystems, Denmark) and 10ng cDNA. Using the Step one v2.3 software (Applied Biosystems) and Microsoft Excel, relative gene expression data was calculated. Following normalization to the geometric mean (GM) of reference genes β-Actin, B2M, UBC and HPRT, quantification of gene expression was carried out using a comparative CT method where ΔCT is the difference between the CT values of the target and GM of reference genes. At day 15 after osteogenic induction, samples were taken for RT-qPCR for expression of osteogenic markers RUNX2, alkaline phosphatase (ALP), Collagen 1α1 (COL1a1) and Osteocalcin (BGLAP). The sequence of primers (Eurofins MSW Operon, Germany) used for RT-qPCR reactions are depicted in supplementary information Table S1.
2.3.4 *In vitro* mineralisation

*In vitro* mineralisation was visualized on Day 15 samples using µCT imaging. Using a VivaCT40 scanner (Scanco Medical AG, Bassersdorf, Switzerland), a two-dimensional image was acquired from the *in vitro* cultured cell seeded constructs cultured under CM and OB. The 3-dimensional reconstructions with a cubic voxel-size of 38 μm were generated from the 2-dimensional images. Images were analyzed using the built-in software.

Alizarin red staining [32] (AZR) (Sigma-Aldrich, Denmark) for osteogenic mineralization was also performed post-fixation using either ice cold 70% ethanol for 1 hr. (AZR). Samples at Day 15 for AZR staining were incubated in 40 mM AZR at pH 4.2 for 10 min at room temperature followed by washing in distilled water and PBS, before examination for the presence of mineralized matrix.

2.4 *In vivo* implantation for ectopic bone formation

All *in vivo* experiments were performed under the permission from Danish National Ethical committee on Animal experiments. Danish regulations for care and use of laboratory animals were maintained throughout the experiments. Ectopic bone formations on cell seeded scaffolds were tested by implantation of cell laden scaffolds subcutaneously in NOD.CB17-*Prkdc<sup>scid</sup>/J mice. 1×10<sup>6</sup> cells were seeded on 6 mm diameter PLS3 scaffolds *in vitro* and were implanted subcutaneously after 24hrs of culture in CM. Each mouse had four implants, two were the cell laden PLS3 and the other two were control implants. 5×10<sup>5</sup> cells were seeded on test PLS3 scaffolds; whereas, non–cell seeded PLS3, served as controls. Three hMSC seeded PLS3 scaffolds, for baseline imaging were fixed in 4 % paraformaldehyde after 24 hrs. Eight weeks after implantation, the scaffolds and control implants were removed after termination of the experiment and fixed in 4% paraformaldehyde for one day. Followed by, decalcification with 0.38 M EDTA, pH 7.2 and embedding in paraffin for histology. Sections were stained with Haematoxylin and Eosin (H&E), human Vimentin antibody (VM) (1:400, clone SP20, Thermo Scientific), Collagen Type I antibody (Col Type I) (LF-67 kindly provided by Dr. Larry Fisher, the National Institute of Dental and Craniofacial Research, National Institutes of Health) and Sirius red in picric acid (Sirius red F3BA).
2.5 Statistical Analysis

All in vitro experiments were performed at least in triplicates. The data represented are mean ± standard error of the mean. Statistical analysis was performed using Graph Pad Prism (version 6.00, Graph Pad Software, La Jolla California USA). * P values < 0.05 were considered significant.

3. Results

Cell Attachment and Proliferation

SEM images (Figure 1A) of non-cell seeded scaffolds showed open porous scaffolds. At Day 1 (Figure 1B), post seeding, the cells were seen to extend filopodia like structures across the pore walls and scaffold surface pores were seen filled with ECM like deposition (Figure 1C). Dapi/Phalloidin staining (Figure 2A) of the cell seeded region showed densely packed cell mass at Day 1. At day 7 (Figure 2B), the cells exhibited elongated morphology and elongated actin filaments (red). The cells were also observed to be uniformly distributed over the scaffold surface. Cell proliferation on the PLS3 scaffolds was quantified by Cell-titer Blue assay (Figure 3). The assay quantifies number of viable cells that transform a resazurin dye into a fluorescent end product (resorufin). At day 1, more than 80% of cells attached to the scaffold were viable cells. Cells proliferated on the scaffold and increased significantly (p < 0.05) at Day 5 and Day 7.

ALP activity

ALP activity (Figure 4A) and ALP staining (Figure 4B) demonstrated the osteoblastic differentiation of hMSC when cultured on PLS3 scaffolds. hMSC seeded on PLS3 scaffold showed a significant increase (p < 0.05) in ALP activity from day 10 and day 15 when cultured under OB media compared to CM. ALP stained samples also demonstrated a uniform intense red ALP stain from day 10 on the PLS3 scaffold when cultured under OB media compared to CM.

Osteogenic Gene expression

Osteogenic gene expressions (Figure 5) were quantitated at day 15 post seeding. The mRNA levels of osteogenic marker genes (RUNX2, ALP, Col1a1, and BGLAP) of hMSC seeded on PLS3 scaffolds
and cultured under OB were compared to CM. Osteogenic gene expression were seen in both CM and OB. ALP gene expression was significantly ($p<0.05$) upregulated in OB.

**In vitro** mineralization

The **in vitro** mineralization testing was studied by µCT imaging and AZR staining at day 15 post osteoblastic induction. Three dimensional reconstructions of µCT images (**Figure. 6**) showed formation of uniformly distributed radiopaque mineralized nodules at day 15 when cultured in OB media. AZR staining (**Figure. 6**) performed at day 15, revealed intense red staining of presence of calcium deposition denoting the mineralized areas.

**In vivo** implantation

The experiment was terminated at eight weeks. The animals were healthy at termination. The harvested PLS3 scaffolds were seen highly vascularized (**Figure. 7A**). The H&E staining of baseline collected prior to implantation confirmed cell integration and cells migrating into deeper layers of the scaffold (**Figure. 7B**). The control, non-cell seeded scaffolds showed fewer host cells migrating into the scaffolds (**Figure. 7 C**). After eight weeks scaffold sections from implanted cell seeded scaffold stained with H&E, revealed areas of vascularized ectopic bone matrix formation (**Figure. 7 D, E**). The matrix was rich in Type I collagen fibers as visualized by immunohistochemical staining (**Figure. 7F**). Sirius red staining demonstrated presence of organized collagen bundle with characteristic birefringence under polarized light (**Figure. 7G**). The Vimentin (VM) staining of adjacent serial sections confirmed that the cells embedded in the matrix are of human origin (**Figure. 7 H**) . The VM staining also confirms that the cells visualized within the scaffold on the cell seeded scaffolds were hMSC cells when compared to control non-cell seeded scaffolds.

4. Discussion

The craniofacial region consists of complex architecture of soft cancellous bone and dense cortical bone. The complex architecture makes it a challenging area to reconstruct. The cancellous areas get vascularized more easily compared to cortical area. Thus, there is a need for biomaterial that supports MSC growth, bone formation and vascularization to allow vascular reconstruction of craniofacial bone grafts. Larger defects in the craniofacial region need sizeable quantities of bone grafts for restoration. Moreover, with increasing requirement of bone graft and donor site morbidity;
bone tissue engineering remains the most appropriate therapeutic approach. Bone tissue engineering requires autologous MSC and biomaterial scaffolds that would mimic the native tissue. In this study, a PCL - laponite-SRA composite scaffold (PLS3) was prepared for the controlled release of SRA [28]. Further we have evaluated the new composite PLS3 scaffold for its ability to support stromal derived skeletal stem cell growth and osteogenic differentiation both in vitro and in vivo, so as to explore its relevance for craniofacial bone tissue engineering.

The composite scaffold PLS3 of our study consists of biocompatible materials like PCL, laponite clay and its complex with the drug SRA. PCL is an FDA approved biomaterial known for biocompatibility and biomechanical properties. Laponite clays belongs to family of FDA approved clay type materials that is generally regarded as safe products and have previously reported for drug delivery purpose [33]. Laponite has also reported to promote osteogenic differentiation of MSC as its degradation products of have been shown to promote cell attachment, extra cellular matrix deposition and osteogenesis [24]. SRA is a drug used for osteoporosis treatment due to its effects on osteoclastic bone resorption and osteoblastic bone formation [34]. The incorporation of SRA was aimed to develop a system capable of local sustained drug delivery that promotes bone formation and enhance the bone quality of the new formed bone at the implanted defect site.

The highly porous structure of PLS3 scaffolds as revealed by SEM is known to enhance cell infiltration, exchange of nutrients and vascular ingrowth [35, 36]. However, porosity would have some unwanted effects like cell seeding loss that may compromise the ability of scaffold to in vivo bone formation [37, 38]. In PLS3 scaffold more than 80 % of seeded cells were viable cells at day 1. It is assumed that cell seeded on scaffolds do not replicate within 24 hrs. following seeding [39]. While previous studies reported that scaffold coating e.g. with collagen can enhance cell attachment [37], the PLS3 scaffold seems to provide a suitable environment for proper cell attachment which may be related to its content of magnesium ions. Magnesium ions have been reported to regulate integrin protein leading to cell adhesion on scaffold biomaterial [40].

The ALP activity of osteogenic differentiating cells was influenced by cell confluence and interactions of osteoinductive elements on the scaffold. Even though, ALP is an early marker of osteogenesis, their expression at later stages indicate extended osteoinductive potential of PLS3 scaffold.
Furthermore, the interactions of SRA and magnesium ions have also been reported to trigger ALP activity [41].

The PLS3 scaffold contains active osteoinductive elements like strontium ranelate and laponite clay. The dissolution of PLS3 generates many ions that may interact and upregulate osteogenic factors in culture. Nevertheless to be mentioned, osteogenic gene expression is also influenced by cell confluence and cell-scaffold interactions. The *in vitro* osteogenic phenotype transformation of the hMSC when cultured on PLS3 scaffolds were confirmed by Alizarin matrix mineralization. Appearance mineralized nodules seen at day 15 post osteogenic induction indicated initiation of functional bone.

In our *in vivo* implantation hMSC cells were seeded on PLS3 scaffolds without prior osteoblast differentiation induction. Some studies have reported the benefits of osteoinduction of MSCs prior to seeding on scaffolds for enhancing *in vivo* bone formation [42, 43]. However, our previous experience with scaffolds does not support the necessity of this procedure [32]. In addition we wanted to examine the ability of PLS3 scaffold to induce osteoblast differentiation and bone formation in vivo. We observed that PLS3 scaffold can support the formation of organized matrix rich in collagen suggesting osteoinductive abilities [44]. MSC were also reported to perform with limited *in vivo* bone formation, due to lack of proliferation upon prolonged *in vitro* differentiation [42]. The VM (brown) stained cells of human origin around the bone matrix zone indicate proliferating and differentiating hMSC over a period of eight weeks. The *in vivo* PLS3 implantation was tested for ectopic bone formation. The ectopic bone formation confirmed the bone formation was from the hMSC differentiation and not from neighboring osteogenic cell recruitment as in a calvarial defect model [43]. Cranial bones are devoid of inherent vasculature [42]. The vascularization of repaired craniofacial bones is essential to maintain the implanted tissue engineered grafts. We observed that PLS3 scaffolds were highly vascularized. Laponite are silicates clays have some dissolution products similar to that of dissolution products of bioactive glasses [45]. These dissolution products stimulate local production of VEGF [46]. The laponite clays incorporated would also allow surface adsorption of local VEGF, which in turn also directs vascularization of the PLS3[47]. Vascularization of *in vivo* implanted scaffolds determines the successful integration of scaffold into the host tissues. Thus histological evidence supported that hMSC seeded PLS3 scaffold is suitable for clinical situations where bone formation is needed.

5. Conclusions
The *in vitro* and *in vivo* results confirm the osteoinductive properties of PLS3 scaffold. MSC survival on the PLS3 scaffold, confirmed the strontium ranelate incorporation does not have local cell toxicity. Based on the enhancement of osteoblast differentiation of hMSC observed at an ectopic site; hMSC seeded PLS3 scaffold engineered construct is a promising candidate for craniofacial bone regeneration.

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Figure 3

![Graph showing the average cell number over different days.](image)

- Days 1, 2, 5, and 7 are marked with asterisks, indicating significance.

The graph plots Average Cell Number against Days.
Figure 5
Figure 6

µCT

Alizarin Red Stain

CM

OB