Effects of αCGRP on the Adhesion, Proliferation and Differentiation of Osteoblasts Cultured on Titanium Surfaces

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Abstract: αCGRP, a neuropeptide widely distributed in bone tissue, has been demonstrated as a physiological activator of bone formation. However, the regulation of αCGRP on the osseointegration of dental implants is not well understood. In this study, mouse primary calvarial osteoblasts were obtained and cultured on smooth, rough (SLA), and chemically modified (SLActive) Ti surfaces to investigate its effect on the adhesion, proliferation, differentiation, and mineralization of osteoblasts, particularly on SLActive surfaces.

Key words: αCGRP, Osteoblasts, Titanium implant surfaces, Osteogenic differentiation

Introduction

Rigid osseointegration at the implant-bone interface is the functional foundation of dental implants. Bone around dental implants is continuously remodeled, and various factors have been demonstrated to regulate this process, including local cytokines, growth factors and systemic hormones1,2.

It is well known that bone exhibits abundant sensory nerve fiber innervations3. In addition to conducting pain, thermal, mechanical, and chemical stimuli information, and the sensory nervous system also regulates bone mass by releasing specific neuropeptides, such as the calcitonin gene-related peptide (CGRP)4. CGRP is a 37-amino-acid protein, resulting from the tissue-specific alternative splicing of mRNA derived from the calcitonin gene5. CGRP-immunoreactive nerve fibers are widely distributed in bone tissue, such as the periosteum, bone marrow, metaphysis, ligament, tendon insertions, and synovial membrane6. Bone tissue contains CGRP-immunoreactive nerve fibers whose concentrations increase during bone development and regeneration, suggesting that it is involved in the local regulation of bone metabolism7.

CGRP consists of two subtypes, αCGRP and βCGRP. Previous studies have shown that αCGRP, but not βCGRP, is a physiological activator of bone formation8. Our previous study has revealed that α-CGRP lenti-viral vector could be successfully transfected at peri-implant sites, which resulted in greater gains in osseointegration in α-CGRP-deficient mice9. In contrast, α-CGRP-deficient mice displayed osteopenia due to decreased bone formation. In addition, recent studies have shown that αCGRP maintained bone mass by directly stimulating bone marrow stromal cell (BMSC) osteoblast differentiation via binding with its receptor10.

Various modifications of the dental implant surface have been assessed to determine a reliable means to enhance osseointegration, particularly in patients with diabetes, osteoporosis, and bone metabolic diseases11. Because αCGRP plays a critical role in bone remodeling, we hypothesized that coating titanium implants with αCGRP may improve the growth and differentiation of osteoblasts around implants and subsequently accelerate the process of osseointegration. In this study, we coated αCGRP onto different titanium (Ti) surfaces, including smooth, rough (SLA), and chemically modified (SLActive) Ti surfaces to investigate its effect on the adhesion, proliferation, differentiation, and mineralization of mouse primary osteoblasts. In addition, we evaluated the role of different titanium surfaces and their combinatorial effect with αCGRP in osteoblast growth and differentiation.
Materials and Methods

Osteoblast isolation and culture

All animal experiments were performed in accordance with the international standards on animal welfare and approved (Approval Number: WCCSIRB-D-2014-052) by the Animal Research Committee of the West China Hospital Ethics Committees, Sichuan University.

Newborn mice (4-7 days old) from one litter of C57BL/6 mice (Sichuan University Experimental Animal Center, Chengdu, China) were euthanized by CO₂ inhalation, and the calvaria were dissected aseptically. After removal of soft tissues, the calvaria were subjected to four sequential 15-minute digestions in an enzyme mixture containing 0.05% trypsin (Gibco, BRL, Grand Island, NY, USA) and 0.1% collagenase type II (Sigma-Aldrich Inc., St Louis, Mo, USA) at 37°C on a rocking platform. Cell fractions 2-4 were collected and chilled by the addition of an equal volume of cold Dulbecco’s modified Eagle medium (DMEM; Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum and 100 µg/ml streptomycin (Hyclone, Logan, UT, USA). The fractions were pooled, centrifuged, resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Hyclone, Logan, UT, USA). The fractions were pooled, centrifuged, resuspended in DMEM containing 10% FBS, and filtered using a 70-µm cell strainer. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every other day. At 80% confluence, the cells were dissociated using 0.25% trypsin-0.01% EDTA and subcultured. Passage 2-5 cells were used in this study.

Titanium surfaces and αCGRP coating

Three different types of Ti surfaces, including smooth, SLA, and SLActive surfaces, were kindly provided by the Institut Straumann A.G. Briefly, 15 mm discs were punched from grade 2 unalloyed Ti sheets. Smooth surfaces were prepared using dilute nitric acid to clean the surface, followed by several washes with reverse osmosis purified water. The standard rough surface (SLA) was manufactured upon sandblasting with large grits of 0.25-0.5 mm and etched with HCl/H₂SO₄. Next, the SLActive surface was further rinsed under nitrogen protection to prevent exposure to air during the procedure and was stored in a sealed glass tube containing isotonic NaCl solution. All discs were sterilized using gamma irradiation at 25 kGy overnight and were then ready for use.

The αCGRP peptide was obtained from Phoenix Europe GmbH, Karlsruhe, Germany. Prior to use, it was diluted to 10⁻⁸ M in culture medium. One milliliter of αCGRP solution was poured onto each Ti disc, which was placed in a 24-well culture plate and incubated overnight at 4°C. Cells were then seeded and cultured in osteogenic induction medium (α-MEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml ascorbic acid and 4 mM β-glycerophosphate).

Cell adhesion assays

Osteoblasts were seeded onto Ti discs in 24-well plates coated with or without 10⁻⁸ M αCGRP at a density of 20,000 cells per well and cultured for 1, 4 and 8 hours. For the SEM assay, the cells were fixed in 3% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) at 4°C overnight and then dehydrated using a graded series of alcohol (30%, 50%, 75%, 85%, 95% and 100% ethanol), treated with hexamethyldisilazane and placed in a desiccator overnight. The surface of the Ti discs was observed using SEM (Inspect F, FEI, Netherlands). For 4′, 6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Inc., St Louis, Mo, USA) staining, Ti discs were placed into 4% paraformaldehyde for 10 min and then stained with DAPI. Cells were then rinsed three times in PBS and images were obtained using a fluorescent microscopy (Olympus IX71-F22FL, Tokyo, Japan). The nuclei were counted manually in five random (×100) microscopic fields per sample, and the average number of cells/field was determined. The experiment was repeated three times using independent osteoblast preparations.

Flow cytometry

Osteoblasts were cultured on Ti surfaces for 5 days as previously described, followed by trypsin digestion and terminated using α-MEM medium supplemented with 10% FBS. After centrifugation, the osteoblasts were fixed with 70% ethanol that was precooled at 4°C overnight. Next, the cells were washed with PBS and centrifuged at 1000 × g for 8 min prior to staining. Next, 100 µl of RNAase A was added at 37°C for 30 min, followed by 300 µl of propidium iodide (PI) staining solution (0.1% Triton X-100, 10 mg/ml PI in PBS) at 4°C for 30 min. The cells were then filtered through a 200-mesh steel wire screen. The cell cycle of the osteoblasts was detected using the EPICS Elite ESP flow cytometer (Beckman Coulter, Indianapolis, IN, USA). The S-phase fraction of the total cells (SPF) and cell proliferation index (PI) were calculated. The experiment was repeated three times using independent osteoblast preparations.

Total RNA extraction and quantitative real time RT-PCR

Total RNA was extracted from each sample using Trizol reagent according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 µg of RNA using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The expression levels of mRNA for RUNX2, ALP, OCN and GAPDH were quantified using the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, USA) and SYBR Prime-Script™ RT-PCR Kit II (TaKaRa, Dalian, China). The program used was 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 31 sec and annealing temperature for 1 min. The relative expression levels of the genes were analyzed using the 2⁻ΔΔCt method by normalizing against the GAPDH housekeeping gene expression. The experiment was repeated three times using independent osteoblast preparations.

Immunofluorescence staining

Osteoblasts were cultured in the conditions at a density of 10,000 cells per well for 7 days as previously described. Next, the cells were washed three times in PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After permeabilization in 0.25% Triton X-100 for 8 min, the cells were incubated with 1% BSA in PBS for 30 min to avoid nonspecific binding of the antibody and incubated overnight at 4°C.
4°C with rabbit anti-mouse ALP antibody (Santa Cruz Biotechnology, Inc., USA) diluted 1:50 in 1% BSA. After three washes, the cells were incubated for 1 hour with FITC-conjugated goat anti-rabbit secondary antibody diluted 1:50 (ZSGQ-Bio, Beijing, China). Next, the nuclei were stained with DAPI, and the cells were rinsed three times in PBS. Images were obtained using fluorescent microscopy.

Mineralization assay

Alizarin red staining (ARS) was performed to determine the mineralization of osteoblasts cultured on the Ti discs for 21 days as previously described. At the end of the incubation, the discs were washed with PBS, transferred into a new 24-well plate and fixed in ice-cold 70% ethanol for 1 hour, rinsed with dH2O, and stained with 40 mM Alizarin red solution (pH=4.1; Sigma-Aldrich Inc., St Louis, Mo, USA) at room temperature for 5 min. After aspiration of the overflow, the cells were washed three times with dH2O. Briefly, 300 µl of 10% acetic acid was added into each well. The plates were incubated at room temperature and shaken for 30 min. This solution was transferred to a microcentrifuge tube and after vortexing for 1 min; the slurry was overlaid with 100 ml of mineral oil (Sigma-Aldrich Inc., St Louis, Mo, USA), heated to precisely 85°C for 10 min, and transferred onto ice for 5 min. The slurry was then centrifuged at 20,000 × g for 15 min, and 100 µl of the supernatant was transferred into a new microcentrifuge tube. Next, 40 µl of 10% ammonium hydroxide was added to neutralize the acid, and this final solution was detected at 405 nm in 96-well plates. The data were expressed as the absorbance.

Statistical analysis

The significant difference was analyzed using one-way ANOVA followed by the SNK test. All data were presented as the mean ± SD, and P<0.05 was considered statistically significant. The experiment was repeated three times using independent preparations of osteoblasts. The

![Figure 1](image_url)

Figure 1. αCGRP promoted mouse primary osteoblast attachment to Ti surfaces. DAPI staining of cells on control smooth (A), control SLA (B), control SLActive (C), smooth coated with αCGRP (D), SLA with αCGRP (E) and SLActive with αCGRP (F) after 4 hours of culture. (G) Average number of attached osteoblasts. #: P<0.05, αCGRP-treated surface vs. respective control surfaces. The means and errors were obtained from three pooled experiments. Bar =100 µm
Figure 2. Representative SEM images of osteoblasts cultured on smooth, SLA and SLActive surfaces coated with or without αCGRP (A-L). Bar =200 μm

Figure 3. MTT colorimetric assay. αCGRP significantly upregulated osteoblast growth on smooth, SLA and SLActive surfaces. #: P<0.05, αCGRP-treated surface vs. respective control surfaces; a: P<0.05, SLA vs. smooth surfaces; b: P<0.05, SLActive vs. smooth surfaces; c: P<0.05, SLActive vs. SLA surfaces. The means and errors were obtained from three pooled experiments.
mean and errors shown in all figures were calculated from three pooled experiments.

**Results**

**Osteoblast attachment and morphology**

SEM and DAPI staining revealed that αCGRP coating promoted cell adhesion on three surfaces; the adhesion of osteoblasts on the control smooth, SLA and SLActive surfaces was similar at 1, 4 and 8 hours (Fig. 1). The Ti discs used in the study are shown in Fig. 1H. Four hours post-seeding, the osteoblasts were attached to all surfaces and began to spread, with the most prominent adhesions observed on the αCGRP-coated smooth and SLActive surfaces. After 8 hours, the osteoblasts seeded onto the smooth surface appeared well spread, whereas those grown on the SLA and SLActive surfaces formed numerous microspikes (Fig. 2). There were more cytoplasmic extensions and attachments between cells on the SLActive surface coated with αCGRP. However, no significant morphological differences were observed between cells grown on SLA or SLActive surfaces.

**Effect of αCGRP on osteoblast proliferation**

Results of the MTT test showed that αCGRP coating significantly upregulated osteoblasts growth on all three types of surfaces at 3, 5 and 7 days post-seeding (Fig. 3), indicating that αCGRP enhanced osteoblast proliferation independent of the Ti surface features. In addition, there was a significant increase in the cell number over time on all surfaces; however, the osteoblasts grew faster on the control smooth surface compared to the control SLA and SLActive surfaces at 3, 5 and 7 days (Fig. 3).

**Effect of αCGRP on osteoblast cell cycle progression**

Representative DNA content histograms for each group are shown in Fig. 4A. Results for the SPF (Fig. 4B) and PI (Fig. 4C) of the osteoblasts cultured on αCGRP-coated smooth, SLA and SLActive surfaces

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Figure 4. Cell cycle analysis of osteoblasts. (A) The representative DNA content histograms for each group. αCGRP-coating increased the SPF (B) and PI (C) of osteoblasts. #: P<0.05, αCGRP-treated surface vs. respective control surfaces; a: P<0.05, SLA vs. smooth surfaces; b: P<0.05, SLActive vs. smooth surfaces; c: P<0.05, SLActive vs. SLA surfaces. The means and errors were obtained from three pooled experiments.
were higher compared to their control surfaces. In addition, the SPF and PI of the SLA and SLActive surfaces were lower compared to the smooth surfaces with or without αCGRP coating. The results of the flow cytometry were consistent with that of the MTT assay, which confirmed the effect of αCGRP coating in promoting osteoblast proliferation on all three types of titanium discs.

**Effect of αCGRP on osteoblast differentiation**

Quantitative real-time RT-PCR was used to detect the expression levels of bone-associated mRNA (RUNX2, ALP, OCN) when the cells were cultured on smooth, SLA, and SLActive surfaces coated with or without αCGRP for 3, 7, 14 days. The relative gene expression was presented as the ratio of the respective PCR product to GAPDH PCR product. There was a decreasing trend for RUNX2 mRNA expression in osteoblasts over 14 days of cell culture, while the mRNA expression of ALP and OCN increased during cell culture. Moreover, αCGRP increased RUNX2 expression after 3 days post-seeding on all topographies (Fig. 5A). Although αCGRP decreased RUNX2 expression after 7 days, this difference was not statistically significant except for the SLActive surface on day 14. Moreover, the levels of ALP and OCN were upregulated when the surfaces were pre-coated with αCGRP, and the effect was more significant for SLA and SLActive surfaces (Fig. 5B, C). Overall, the changes in gene expression levels induced by αCGRP coating were relatively modest except for the increased OCN expression on day 14 on the SLA and SLActive surfaces.

Immunofluorescence results further confirmed that osteoblasts secreted more ALP when cultured on αCGRP-coated surfaces (Fig. 6D, E, and F) compared with control surfaces (Fig. 6A, B, and C) at 7 days post-seeding. The most remarkable ALP quantities were observed on the αCGRP-coated SLA and SLActive surfaces. In addition, osteoblasts preferred to form clusters on the SLA and SLActive surfaces compared to the control surfaces (Fig. 6B, C).

After culture for 21 days, Alizarin red-positive nodules formed on all discs. As shown in Fig. 7, the number of Alizarin red-positive nodules was significantly higher for osteoblasts cultured on the αCGRP-coated SLA and SLActive surfaces compared to the control surfaces, indicating that αCGRP coating enhanced mineralization. With respect to the effect of different Ti surfaces, the SLActive and SLA discs exhibited increased mineralization relative to the smooth surfaces, with the most prominent mineralization observed on the αCGRP-coated SLActive surface.
rate of αCGRP-induced osteoblast proliferation shown that activation of protein kinase C is involved in the increased adenylyl-cyclase have also reported that osteoblast αCGRP receptor activation stimulates onto titanium surfaces. The effect was also observed in the current study when αCGRP was coated expression marker expression on αCGRP-coated Ti surfaces. Gene expression (PKA) and phospholipase C served to express on osteoblasts in previous studies Ti surfaces within 8 hours post-seeding. In addition, the cells appeared to spread well sooner on αCGRP-coated surfaces compared to smooth surfaces. Wall et al. reported similar cellular responses in hMSCs and human primary osteoblasts when cultured on the three Ti surfaces. In contrast, cells on smooth surfaces were enlarged and rounded, which is indicative of slower spreading. Wall et al. also observed that osteoblast αCGRP receptor activation stimulates adenylyl-cyclase, which induces the activation of protein kinase A (PKA) and phospholipase C, thereby increasing intracellular free calcium with subsequent protein kinase C activation. It has also been shown that activation of protein kinase C is involved in the increased rate of αCGRP-induced osteoblast proliferation. A similar stimulatory effect was also observed in the current study when αCGRP was coated onto titanium surfaces.

Furthermore, we observed earlier and enhanced osteoblast differentiation marker expression on αCGRP-coated Ti surfaces. Gene expression of RUNX2 was upregulated when osteoblasts were cultured on Ti surfaces pre-coated with αCGRP for 3 days. There was a decreasing trend for RUNX2 mRNA expression in osteoblasts over 14 days of cell culture, while the mRNA expression of ALP and OCN increased during cell culture. This decline in RUNX2 expression was consistent with a previous report by Miron et al. describing a time-dependent decrease in RUNX2 mRNA levels in osteoblasts after 1 week of cell culture. RUNX2 is a master transcription factor that regulates bone formation and is necessary for early osteoblast differentiation, but at later time points, it inhibits immature osteoblasts from differentiating into mature osteoblasts and osteocytes. Moreover, ALP is an early marker for osteogenic differentiation. ALP gene expression and intracellular synthesis were enhanced on Ti surfaces coated with αCGRP. In addition, this effect was further enhanced on SLA and SLActive surfaces. We observed an increased in OCN mRNA expression on αCGRP-coated SLA and SLActive surfaces compared with control surfaces. Alizarin red staining was used to confirm the stimulatory effect on osteoblast mineralization on SLA and SLActive surfaces induced by αCGRP coating.

In this study, we evaluated the osteogenic effects of αCGRP on three types of titanium surfaces. It is an important clinical objective to improve osseointegration in patients with systemic conditions, such as diabetes, osteoporosis, and bone metabolic diseases, which can potentially compromise successful prosthetic implantation. Implant biocompatibility and its ability to osseointegrate can be modified by specific factors, such as surface composition, topography, the degree of roughness, surface free energy (SFE), hydrophilicity and wettability. The addition of bioactive molecules to the implant surface can enhance the desired cell response. Currently, most implants exhibit a rough surface. SLA (Sand-blasted, Large grit, Acid-etched) surfaces currently employed on Institut Straumann A.G. implants have been shown to improve bone formation. Chemically modified SLA surfaces (SLActive) have been produced by rinsing under a N₂ atmosphere. After acid etching, the metal is submerged in an isotonic NaCl solution to avoid contact with molecules from the atmosphere, thereby increasing hydrophilicity.

We observed no significant differences in the osteoblast attachment capabilities between the three types of Ti surfaces, although there was a slight decrease in the initial cell attachment when the cells were seeded onto SLA and SLActive surfaces compared to the smooth surface. These results were consistent with a previous study by Gu et al. demonstrating that there were no significant differences for MC3T3-E1 cell attachment among the three Ti surfaces. Wall et al. evaluated the cellular response of human mesenchymal stromal cells (hMSCs) cultured on smooth, SLA or SLActive surfaces and concluded that there were no differences in the initial attachment of hMSCs to the three surface types. However, there were apparent morphological differences in the osteoblast attachment onto different surfaces. Osteoblasts cultured on SLA and SLActive surfaces displayed more cytoplasmic extensions and attachments between cells. In contrast, cells on smooth surfaces were enlarged and rounded, which is indicative of slower spreading. Wall et al. and Vlacic-Zischke et al. reported similar cellular responses in hMSCs and human primary osteoblasts when cultured on the three Ti surfaces. These results indicated that neither surface roughness nor wettability affected the initial cellular number attached to the Ti surfaces tested; however, the cells still differed between the rough (SLA and SLActive) and smooth surfaces.

In addition, cells grew faster on smooth surfaces compared to SLA and SLActive surfaces. We also observed less cell proliferation on the SLActive surface compared with the SLA surface. Our results were consistent with previous studies; Zhao et al. reported a relative reduction in

Figure 7. Analysis of αCGRP pre-coating on mineralization as determined by Alizarin red staining after 21 days. #: P<0.05, αCGRP-treated surface vs. respective control surfaces; a: P<0.05, SLA vs. smooth surfaces; b: P<0.05, SLActive vs. smooth surfaces; c: P<0.05, SLActive vs. SLA surfaces. The means and errors were obtained from three pooled experiments.

Discussion

In the present study, we evaluated the effects of αCGRP coating on osteoblast adhesion, proliferation, differentiation, and mineralization on three types of dental titanium surfaces: smooth, rough (SLA), and chemically modified (SLActive). In addition, the effects of the different titanium topographies on osteoblasts cellular activities were also evaluated.

For the first time, we demonstrated that a Ti surface coated with αCGRP promoted the adhesion, proliferation, osteogenic differentiation, and mineralization of osteoblasts. In the adhesion assay, αCGRP pre-coating promoted mouse primary osteoblasts attachment on three types of Ti surfaces within 8 hours post-seeding. In addition, the cells appeared to spread well sooner on αCGRP-coated surfaces compared to control surfaces within the same time duration. These results confirmed that αCGRP coating enhanced the attachment capabilities of osteoblasts.

Cellular proliferation in the early period was analyzed using the MTT and cell cycle assays in this study. We observed significant upregulation of osteoblast growth on smooth, SLA and SLActive surfaces coated with αCGRP. Previous studies have suggested that αCGRP can directly activate bone-forming osteoblasts because these cells express the calcitonin receptor-like receptor and RAMP1 dimer complex, which serve as the binding site for αCGRP. Moreover, αCGRP was also observed to express on osteoblasts in previous studies. Other studies have also reported that osteoblast αCGRP receptor activation stimulates adenylyl-cyclase, which induces the activation of protein kinase A (PKA) and phospholipase C, thereby increasing intracellular free calcium with subsequent protein kinase C activation. It has also been shown that activation of protein kinase C is involved in the increased rate of αCGRP-induced osteoblast proliferation. A similar stimulatory effect was also observed in the current study when αCGRP was coated onto titanium surfaces.

Furthermore, we observed earlier and enhanced osteoblast differentiation marker expression on αCGRP-coated Ti surfaces. Gene expression...
the number of osteoblast-like cells grown on SLA and SLActive surfaces compared with the smooth surface. Vilacic-Zischke et al. observed a similar rough surface inhibitory effect on primary human osteoblast growth (smooth>SLA>SLActive).

The initial decline in osteoblast cell proliferation was followed by an enhanced osteogenic response on the SLA and SLActive surfaces compared with the smooth surface. These findings were consistent with previous studies by Vilacic-Zischke et al. and Gu et al. who demonstrated higher expression of osteoblast markers, such as OCN, OPN, and BSP, on the SLA and SLActive surfaces compared to smooth surfaces, which were particularly higher for the SLActive surface. The highest level of osteogenic marker expression in this study was observed on the SLActive surface coated with αCGRP. Compared with the SLA surface, αCGRP demonstrated a higher osteogenic effect on the SLActive surface, which may be due to its hydrophilicity.

Taken together, these novel data revealed that αCGRP coating enhances the adhesion, proliferation, differentiation and mineralization of osteoblasts cultured on Ti surfaces, demonstrating that αCGRP pre-coating may be a suitable modification to promote bone formation in dental implants. Due to its high hydrophilicity, SLActive surfaces demonstrated the highest synergistic effect on osteoblasts proliferation and differentiation when coated with αCGRP. With respect to the effect of different topographies, SLA and SLActive surfaces exhibited earlier and more robust osteoblast differentiation and mineralization, although initially, the osteoblasts grew slower on the rough surfaces compared to the smooth surface.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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