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Basic Science

Osteoblasts exhibit a more differentiated phenotype and increased bone morphogenetic protein production on titanium alloy substrates than on poly-ether-ether-ketone

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Abstract

BACKGROUND CONTEXT: Multiple biomaterials are clinically available to spine surgeons for performing interbody fusion. Poly-ether-ether-ketone (PEEK) is used frequently for lumbar spine interbody fusion, but alternative materials are also used, including titanium (Ti) alloys. Previously, we showed that osteoblasts exhibit a more differentiated phenotype when grown on machined or grit-blasted titanium aluminum vanadium (Ti6Al4V) alloys with micron-scale roughened surfaces than when grown on smoother Ti6Al4V surfaces or on tissue culture polystyrene (TCPS). We hypothesized that osteoblasts cultured on rough Ti alloy substrates would present a more mature osteoblast phenotype than cells cultured on PEEK, suggesting that textured Ti6Al4V implants may provide a more osteogenic surface for interbody fusion devices.

PURPOSE: The aim of the present study was to compare osteoblast response to smooth Ti6Al4V (sTiAlV) and roughened Ti6Al4V (rTiAlV) with their response to PEEK with respect to differentiation and production of factors associated with osteogenesis.

STUDY DESIGN: This in vitro study compared the phenotype of human MG63 osteoblast-like cells cultured on PEEK, sTiAlV, or rTiAlV surfaces and their production of bone morphogenetic proteins (BMPs).

METHODS: Surface properties of PEEK, sTiAlV, and rTiAlV discs were determined. Human MG63 cells were grown on TCPS and the discs. Confluent cultures were harvested, and cell number, alkaline phosphatase–specific activity, and osteocalcin were measured as indicators of osteoblast maturation. Expression of messenger RNA (mRNA) for BMP2 and BMP4 was measured

FDA device/drug status: Not applicable.

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by real-time polymerase chain reaction. Levels of BMP2, BMP4, and BMP7 proteins were also measured in the conditioned media of the cell cultures.

RESULTS: Although roughness measurements for sTiAlV (S_a=0.09±0.01), PEEK (S_a=0.43±0.07), and rTiAlV (S_a=1.81±0.51) varied, substrates had similar contact angles, indicating comparable wettability. Cell morphology differed depending on the surface. Cells cultured on Ti6Al4V had lower cell number and increased alkaline phosphatase specific activity, osteocalcin, BMP2, BMP4, and BMP7 levels in comparison to PEEK. In particular, roughness significantly increased the mRNA levels of BMP2 and BMP4 and secreted levels of BMP4.

CONCLUSIONS: These data demonstrate that rTiAlV substrates increase osteoblast maturation and produce an osteogenic environment that contains BMP2, BMP4, and BMP7. The results show that modifying surface structure is sufficient to create an osteogenic environment without addition of exogenous factors, which may induce better and faster bone during interbody fusion.

Keywords: Ti6Al4V; PEEK; Osteoblast; BMP; Roughness

Introduction

Currently, spine surgeons have multiple biomaterial choices when performing an interbody fusion. Recently, poly-ether-ether-ketone (PEEK) has gained significant popularity as the biomaterial of choice for interbody fusion, particularly in the lumbar spine because of its radiolucency and reports that it has a modulus similar to that of bone [1–3]. However, PEEK does not integrate well with the surrounding bone and may form a fibrous connective interface [3–5]. As a result, micromotion is possible, eventually leading to implant failure [6,7].

Implant osseointegration, or direct contact between the implant surface and surrounding bone under loading conditions [8,9], depends on both bone quality and the host environment. Osseointegration is slower in osteopenic bone than in normal bone [10] and has been shown to be 50% slower in osteoporotic animals than in normal animals [11,12]. Thus, it is important that spinal fusion devices present an osteogenic surface during the fusion process.

Titanium aluminum vanadium (Ti6Al4V) alloys have a well-established history of use as bone graft cages or spacers in lumbar spine fusion procedures. Previous studies have shown that these alloys support good bone to implant contact and are well osseointegrated with the surrounding bone [13–15]. In vitro experiments comparing the responses of immature osteoblasts to machined and smooth Ti6Al4V (sTiAlV) substrate surfaces indicate that the differentiation of the cells is greater when the surface has a texture with micron-scale roughness [16]. These observations were confirmed using Ti6Al4V that had been grit blasted to create micron-scale roughness [17]. Moreover, when the same surface treatment was applied to Ti6Al4V pedicle screws and tested in vivo in sheep spines, the force required to pull out the screws was doubled compared with screws that had a smooth surface [17].

In the body, osteoblasts mature in osteoclast-conditioned areas of bone that present a micron-scale roughness [18], suggesting that surface texture is an important variable in bone formation. Studies using commercially pure titanium (Ti) substrates have shown that surfaces with micron- and submicron-scale features promote greater osteoblast differentiation, matrix deposition, and production of osteogenic growth factors [19–21], which regulate the cells via autocrine and paracrine pathways [22–24], than do cells cultured on smooth surfaces. Similarly, microtextured Ti6Al4V surfaces support increased osteoblastic differentiation compared with sTiAlV surfaces [17]. Moreover, cells on Ti or Ti6Al4V are more differentiated than cells on traditional cell culture plastic [16,25,26]. These differences indicate that both surface chemistry and surface microtexture play a role and bring into question whether responses to materials typically used in interbody fusion, Ti6Al4V or PEEK, differ and if so, how.

The purpose of the present study was to compare the osteoblast phenotype of human osteoblast-like MG63 cells to smooth and microtextured Ti6Al4V surfaces with their phenotype on PEEK. MG63 cells are an immature osteoblast cell line used by many laboratories as a model to examine factors that promote osteoblast differentiation [27,28]. Of particular interest is whether cells grown on these biomaterials contribute to peri-implant bone formation by generating an osteogenic environment through production of osteoinductive factors. To test this, we assessed whether expression of bone morphogenetic proteins (BMPs) and their secretion into the medium were affected by the substrate surface. Because of the high doses used to induce bone formation and side effects derived from the clinical use of BMP2 for spine fusion [29–31], implant topographies that enhance cell-produced BMPs may enhance the osteogenic microenvironment and improve the stability of the interbody construct through bony on-growth to the interbody device [32].

Methods

Disc preparation and characterization

Surgical-grade Ti alloy (Ti6Al4V) and PEEK discs were used in this study (Titan Spine, LLC., Mequon, WI, USA). The discs were 15 mm in diameter and fit snuggly in a well of a 24-well culture plate. Smooth Ti6Al4V discs were machined, tumbled to remove any burs, and passivated

through an acid bath, which removes inorganic contaminants on the surface and forms a stable oxide layer that reduces the reactivity of the bulk material with the environment. To create a roughened surface texture (roughened Ti6Al4V [rTiAlV]), Ti6Al4V discs were machined, treated with a proprietary etch process, and passivated. Poly-ether-ether-ketone substrates were machined and subsequently tumbled. Before use in cell culture studies, all discs were ultrasonically cleaned, sonicated in ultrapure water (Millipore, Billerica, MA, USA), and sterilized by autoclave (Tuttnauer, Hauppauge, NY, USA) for 20 minutes at 121°C and 15 PSI.

The surface topography was evaluated qualitatively using a field emission gun scanning electron microscope (Ultra 60 FEG-SEM; Carl Zeiss SMT Ltd., Cambridge, UK). Scanning electron microscope images were recorded using a 5-kV accelerating voltage and 30-μm aperture. Poly-ether-ether-ketone samples were coated with a thin layer of gold-palladium (Au-Pd) to avoid charging. Surface roughness was quantitatively analyzed using a confocal laser microscope (CLM) (OLS4000 CLM, Lext; Olympus, Center Valley, PA, USA). Each CLM analysis was performed over an area of 644 μm×644 μm using a scan height step of 50 nm, a 20× objective, and a cutoff wavelength of 100 μm to determine the mean values of surface roughness (S_a). Qualitative atomic concentration close to the surface was obtained by energy-dispersive X-ray spectroscopy (INCAx-act EDX; Oxford Instruments, Concord, MA, USA) to determine if there were any gross compositional changes as a result of surface treatment of the Ti alloys. Energy-dispersive X-ray spectroscopy spectra were collected using a magnification of 500× and a working distance of 10 mm. Contact angle measurements were obtained using a goniometer (CAM 100; KSV, Helsinki, Finland) equipped with a digital camera and image analysis software. Ultrapure water was used as the wetting liquid, with a drop size of 5 μL.

Cell culture

Human MG63 osteoblast-like cells (American Type Culture Collection, Manassas, VA, USA) were cultured on tissue culture polystyrene (TCPS), PEEK, sTiAlV, or rTiAlV at an initial cell density of 10,000 cells/cm^2. Media were changed 24 hours after plating and then every 48 hours until cells reached confluence on TCPS. When MG63 cultures reached confluence on TCPS, all cultures were treated for 24 hours with fresh media. At harvest, cell number was determined. To ensure that cells were completely removed from surfaces, cells were released with two sequential 10-minute incubations in 0.25% trypsin at 37°C and counted (Z1 cell and particle counter; Beckman Coulter, Fullerton, CA, USA).

Biochemical analysis

Cells were lysed by freeze thawing in 0.05% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA). Specific activity of alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1], an early marker of osteoblast maturation that reaches a peak just before matrix mineralization [33], was assayed in the cell lysates by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2. Enzyme activity was normalized to total protein content (BCA Protein Assay; Thermo Fisher Pierce, Rockford, IL, USA) of the cell lysates.

Conditioned media were collected and assayed for secreted proteins and factors as described previously [34]. Osteocalcin, an extracellular matrix protein important in modulating hydroxyapatite crystal formation that is a later marker of osteoblast maturation [35], was measured using a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit; Biomedical Technologies, Stoughton, MA, USA). BMP2, BMP4, and BMP7 were assayed in the conditioned media using commercially available enzyme-linked immunosorbent assays (ELISA DuoSet; R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. Results of immunoassays were normalized to total cell number.

Molecular analysis

To determine the possible mechanism of increased osteogenic maturation of cells grown on Ti6Al4V substrates, we examined messenger RNA (mRNA) levels for BMP2, BMP4, and BMP7. When MG63 cultures reached confluence on TCPS, all cultures were treated for 12 hours with fresh media. RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription was performed with 125 ng of RNA using a High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Carlsbad, CA, USA) to create complementary DNA templates. Real-time quantitative polymerase chain reaction was then performed with gene-specific primers using the StepOnePlus Real-time PCR System and *Power* SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). Fluorescence values were quantified as starting quantities of mRNA using a standard curve created from known dilutions of MG63 cells cultured on TCPS. Genes are presented as normalized to glyceraldehyde 3-phosphate dehydrogenase (F: 5′-GCT CTC CAG AAC ATC ATC C-3′; R: 5′-TGC TTC ACC ACC TTC TTG-3′). Primers to BMP2 (F: 5′-GCG TGA AAA GAG AGA CTG C-3′; R: 5′-CCA TTG AAA GAG CGT CCA C-3′) and BMP4 (5′-ACG GTG GGA AAC TTT TGA TGT G-3′; 5′-CGA GTC TGA TGG AGG TGA GTC-3′) were designed using Beacon Designer software (PREMIER Biosoft, Palo Alto, CA, USA) and synthesized by Eurofins MWG Operon (Huntsville, AL, USA).

Statistical analysis

For each experiment, there were six independent cultures per experimental surface. Experiments were repeated

to ensure validity of the results. Data presented are from representative experiments. Data were first analyzed by analysis of variance; when statistical differences were detected, the Student *t* test was used with post hoc for multiple comparisons using Tukey method and p values less than .05 were considered significant.

Results

Materials characterization

Scanning electron microscopic images of the surfaces revealed different topographies of the samples (Fig. 1). The PEEK surface exhibited a machined surface finish with no distinct features except for parallel grooves along the entire surface because of processing (Fig. 1A). The high magnification images confirm the lack of smaller features (Fig. 1B). In a similar way, the sTiAlV surface also presented a machined finish with shallower grooves (Fig. 1C) and additional random scratches, evident at higher magnifications (Fig. 1D). The rTiAlV surface presented 100- to 300-μm craters with superimposed micron-scale features (Fig. 1E). At higher magnifications, micron-scale features were more easily distinguished, resembling small terraces with faint submicron-scale texture (Fig. 1F).

Confocal laser microscopy confirmed the qualitative evaluation by scanning electron microscope (Fig. 2). The roughness of the sTiAlV surface (S_a=0.09±0.01) was the lowest of all the samples followed by the PEEK surface (S_a=0.43±0.07). The rTiAlV surface had the highest roughness (S_a=1.81±0.51).

Energy-dispersive X-ray spectroscopy (Table 1) showed a clear distinction in chemical composition between the PEEK samples and the Ti alloy samples. Poly-ether-ether-ketone samples were composed of carbon (C) and oxygen. The spectra also recorded small traces of Au originating from the Au coatings used to avoid charging from the impeding electrons. In the case of the Ti alloy samples, both sTiAlV and rTiAlV were composed of Ti, aluminum, and vanadium with no significant compositional differences between the two. These analyses were qualitative and do not represent a quantitative assay of the alloy compositions.

Surface wettability assessed by contact angle measurements revealed that PEEK, sTiAlV, and rTiAlV substrates presented similar contact angles (Table 2).

Osteoblast maturation

Cell number was lower on PEEK substrates than on TCPS, but cell number on sTiAlV was 20% lower than on PEEK and cell number on rTiAlV was 65% of the number on PEEK (Fig. 3, Left). Alkaline phosphatase specific activity was the same in cells on TCPS and PEEK, but levels were higher on sTiAlV and highest on rTiAlV (Fig. 3, Middle). Osteocalcin was more than 55% higher on Ti6Al4V than on TCPS or PEEK, but there was no effect of roughness (Fig. 3, Right).

Bone morphogenetic protein expression

Both BMP2 and BMP4 mRNAs were lower on PEEK than on TCPS (Fig. 4). Cells on sTiAlV had more BMP2 and BMP4 mRNA than PEEK, but the highest expression was found on rTiAlV. Levels of BMP2, BMP4, and BMP7 in the conditioned media were the same in cells cultured on TCPS as on PEEK (Fig. 5). However, BMP2 was twofold higher on sTiAlV and rTiAlV than on PEEK (Fig. 5, Left). BMP4 was higher on sTiAlV than on PEEK and further increased in cells cultured on rTiAlV (Fig. 5, Middle). BMP7 was 50% higher in cells on sTiAlV and rTiAlV than on PEEK (Fig. 5, Right). The results show that culture on Ti surfaces stimulates production of BMPs at higher levels than cells cultured on PEEK.

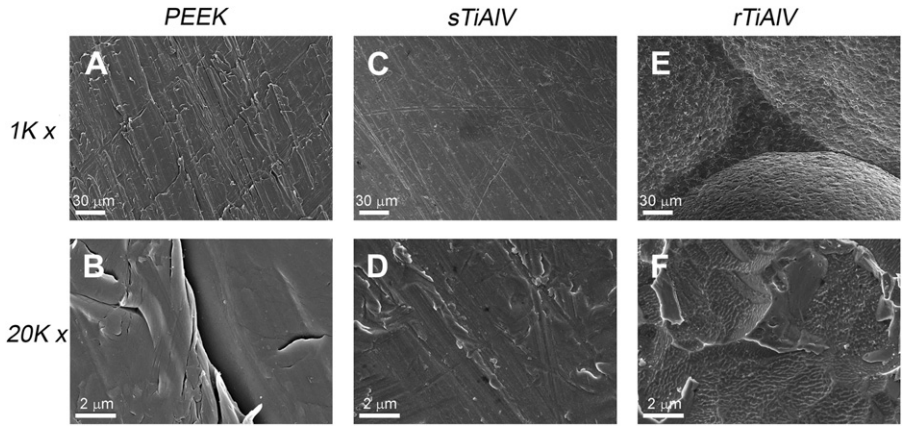

Fig. 1. Scanning electron microscope images of (A, B) PEEK, (C, D) sTiAlV, and (E, F) rTiAlV surfaces at low and high magnifications. The low magnification images show the high levels of roughness of the (E) rTiAlV samples when compared with both (A) PEEK and (C) sTiAlV. High magnification images reveal the presence of microgrooves on the (B) PEEK and (D) sTiAlV samples, whereas the (F) rTiAlV samples have micron- and submicron-scale terraces and ridges. PEEK, poly-ether-ether-ketone; sTiAlV, smooth Ti6Al4V; rTiAlV, roughened Ti6Al4V.

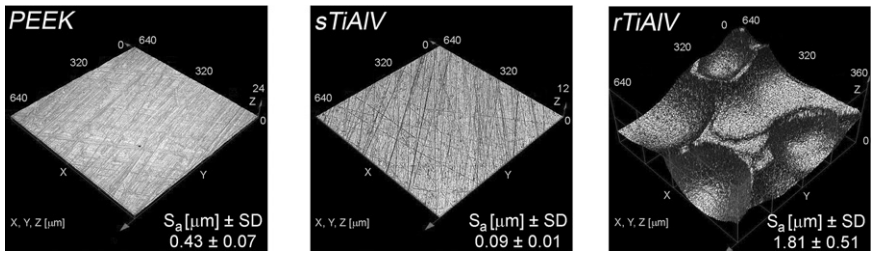

Fig. 2. Confocal laser microscopy images and average roughness (S_a) values of (Left) PEEK, (Middle) sTiAlV, and (Right) rTiAlV surfaces. The rTiAlV samples have the highest S_a values, evident from its surface topography image of a 644×644-µm^2 field. PEEK, poly-ether-ether-ketone; sTiAlV, smooth Ti6Al4V; rTiAlV, roughened Ti6Al4V; SD, standard deviation.

Discussion

Surface properties of implants have been recognized as one of the most important determinants of device success [36]. Scanning electron microscope analyses of the different samples showed that both PEEK and sTiAlV samples were relatively smooth at the micron and submicron levels when compared with rTiAlV samples. These results were confirmed quantitatively by CLM measurements, with rTiAlV samples having a significantly higher average roughness (S_a) than PEEK and sTiAlV samples. Increased surface roughness has been shown to enhance osteoblast differentiation and local factor production in vitro, as well as bone-to-implant contact and torque removal forces in vivo on both commercially pure Ti [37,38] and Ti alloys (ie, Ti6Al4V) [39]. A possible hypothesis is that the enhancement in osseointegration may occur through osteoblast activation and reduced osteoclast activity [39].

Energy-dispersive X-ray spectroscopy chemical analysis of the different samples revealed that PEEK samples were composed of C and oxygen, as its chemical formula suggests. Conversely, both sTiAlV and rTiAlV samples had very similar chemistries that included Ti, aluminum, and vanadium. Surface contact angle, which is an indirect way of measuring surface energy, was similar for all samples. Both surface chemistry [40,41] and surface energy [42,43] have been demonstrated to have a direct effect on osteoblast response and implant osseointegration. In this study, because the contact angles were similar, this suggests that the surface texture was the main reason for the difference in osteoblast behavior between the materials tested.

Surface roughness modifications can be applied to most of the biomaterials used in orthopedic applications. However, limitations in the physical, mechanical, and chemical properties of polymeric and ceramic biomaterials, and differences in the modification techniques, have limited their surface topographic modifications. Common spinal fusion biomaterials such as allograft and PEEK could be modified to present surface roughness and tested in vitro. Because of limitations in production, it would be difficult to manufacture these two types of materials to have the same surface roughness as Ti to decouple whether surface roughness or other surface properties are responsible for the enhanced osteogenic environment. However, this is partially examined in this study because we examine smooth PEEK and smooth Ti and found differential cell responses.

Although MG63 cells are human-derived osteosarcoma cells, they have been found to have comparable behavior to normal human osteoblasts, neonatal mouse calvarial osteoblasts, fetal rat calvarial osteoblasts, and adult rat calvarial osteoblasts when cultured on Ti surfaces [20,34,44–46] and are regarded as a valuable model for studying cell response to Ti [47]. In addition, their responses have been found to correlate well with quality of osseointegration in dental and orthopedic implants [16,17,48].

Osteoblast phenotype has been defined in three distinct periods: a growth period (proliferation), a period of matrix development, and a period of mineralization [33]. Our results showed that rTiAlV substrates promote

Table 1
Elemental composition of Ti6Al4V discs analyzed by energy-dispersive X-ray spectroscopy

	Concentration (atomic %±SD)				
Sample	C	O	Ti	Al	V
PEEK*	85.1±0.5	14.6±0.5	–	–	–
sTiAlV	–	–	84.6±3.2	10.5±0.6	3.4±0.1
rTiAlV	–	–	86.3±1.0	10.1±1.2	3.6±0.3

Ti, titanium; SD, standard deviation; C, carbon; O, oxygen; Al, aluminum; V, vanadium; PEEK, poly-ether-ether-ketone; sTiAlV, smooth titanium aluminum vanadium; rTiAlV, roughened titanium aluminum vanadium; Au, gold; Pd, palladium.

* PEEK samples were Au-Pd coated for analysis, with Au and Pd concentration less than 0.5%.

Table 2
Contact angle measurements of disc surfaces

Sample	Contact angle (°)±SD
PEEK	82±12
sTiAlV	82±22
rTiAlV	85±7

SD, standard deviation; PEEK, poly-ether-ether-ketone; sTiAlV, smooth titanium aluminum vanadium; rTiAlV, roughened titanium aluminum vanadium.

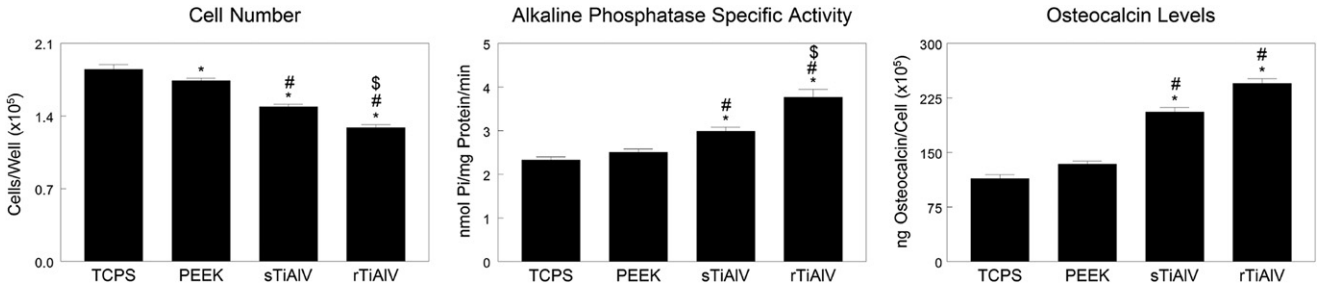

Fig. 3. Human MG63 osteoblast-like cells were harvested 24 hours after confluence on TCPS. (Left) Cell number, (Middle) alkaline phosphatase–specific activity in cell lysates, and (Right) levels of osteocalcin in the conditioned media were measured. *p<.05 versus TCPS; #p<.05 versus PEEK; $p<.05 versus sTiAlV. TCPS, tissue culture polystyrene; PEEK, poly-ether-ether-ketone; sTiAlV, smooth Ti6Al4V; rTiAlV, roughened Ti6Al4V.

osteoblast maturation, decreasing cell number but increasing alkaline phosphatase activity and osteocalcin levels. Alkaline phosphatase activity increases as a result of decreased proliferation and maturation of the extracellular matrix. This enzyme is responsible for the early osteogenic microenvironment that leads to the maturation and mineralization of the extracellular matrix. Osteocalcin, the second most common protein in bone, is known as a late marker of osteoblast phenotype because of its presence in mature or mineralizing extracellular matrix [49]. Osteocalcin binds with high affinity to calcium and hydroxyapatite and is a modulator of crystal formation. Our results confirm our previous findings that rough substrates promoted osteogenic maturation through the decrease of cell number and the increase of important markers of mature osteoblast phenotype. These findings suggest that this type of cellular response could enhance early bone formation.

Previous studies have shown that PEEK allows osteoblast and fibroblast attachment and cell proliferation and have emphasized the lack of a negative effect of this material on cell growth [50,51]. However, to support osteoblastic differentiation, groups have modified the PEEK surface with coatings such as hydroxyapatite, tricalcium phosphate, diamond-like carbon, and Ti [3,52]. All these coatings have shown a better osteoblastic response when compared with noncoated PEEK, suggesting that PEEK by itself does not provide an osteogenic surface.

Our group has shown previously that osteoblasts grown on rough substrates increase levels of autocrine and paracrine factors such as osteoprotegerin, vascular endothelial growth factor, prostaglandin E2, and transforming growth factor beta 1 [16,37,53,54], all of which contribute to increased osteoblast differentiation and reduced osteoclastic activity, both of which are needed to achieve net new peri-implant bone formation.

In this study, we examined the effect of rTiAlV surfaces on mRNA expression and protein levels of three BMPs associated with osteoinduction. BMP2 is currently commercially available, and several clinical and in vivo studies have shown the increase in bone formation in the BMP2-treated area [55,56]. However, there is also some concern about the high doses used, as well as some side effects derived from the use of BMP2 [29–31]. Our results showed that osteoblasts cultured on Ti6Al4V substrates increased BMP2, BMP4, and BMP7 mRNA and protein levels. This increase is most robust on rTiAlV substrates with a more than twofold increase. Interestingly, PEEK failed to increase these molecules, demonstrating similar levels to the control group.

Conclusions

Taken together, this study demonstrates that rTiAlV substrates increase osteoblast maturation and produce an osteogenic environment that contains BMP2, BMP4, and

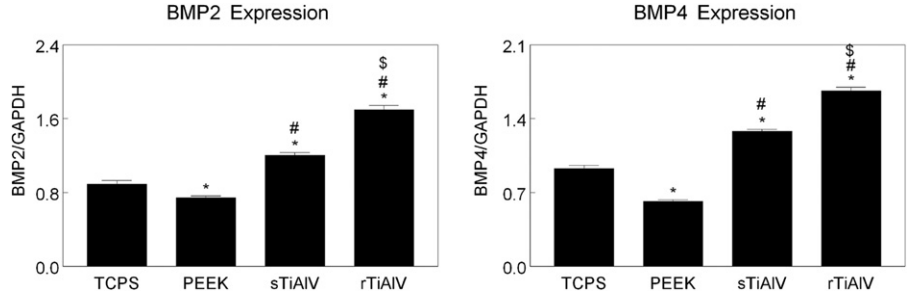

Fig. 4. Human MG63 osteoblast-like cells were harvested 12 hours after confluence on TCPS. Levels of messenger RNA for (Left) BMP2 and (Right) BMP4 were measured by real-time qPCR and normalized to GAPDH. *p<.05 versus TCPS; #p<.05 versus PEEK; $p<.05 versus sTiAlV. TCPS, tissue culture polystyrene; BMP, bone morphogenetic protein; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PEEK, poly-ether-ether-ketone; sTiAlV, smooth Ti6Al4V; rTiAlV, roughened Ti6Al4V.

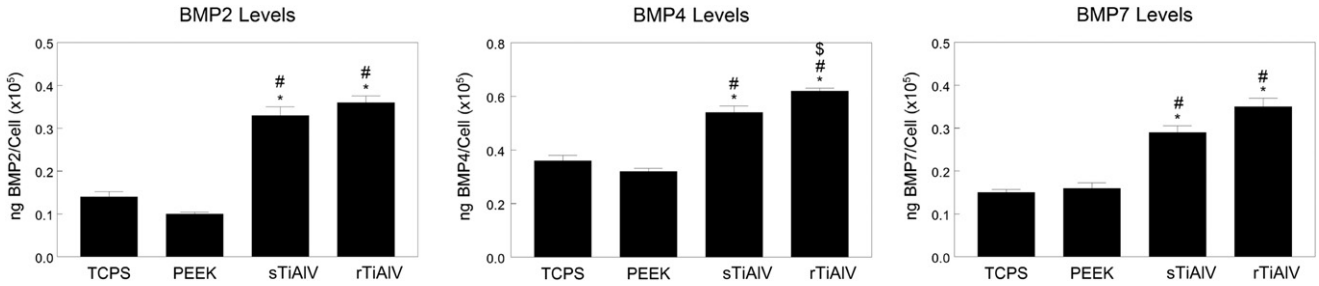

Fig. 5. Human MG63 osteoblast-like cells were harvested 24 hours after confluence on TCPS. (Left) Secreted BMP2, (Middle) BMP4, and (Right) BMP7 were measured in the conditioned media. Levels are normalized to total cell number. *p<.05 versus TCPS; #p<.05 versus PEEK; $p<.05 versus smooth Ti6Al4V. TCPS, tissue culture polystyrene; BMP, bone morphogenetic protein; PEEK, poly-ether-ether-ketone; sTiAlV, smooth Ti6Al4V; rTiAlV, roughened Ti6Al4V.

BMP7. The results show that modifying surface structure is sufficient to create an osteogenic environment that could enhance bone formation and implant stability, without addition of exogenous growth factors.

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