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Osteoblast Maturation and New Bone Formation in Response to Titanium Implant Surface Features are Reduced with Age

Rene Olivares-Navarrete^{1,^}, Andrew L. Raines^{1,2,^}, Sharon L. Hyzy¹, Jung Hwa Park¹, Daphne L. Hutton¹, David L. Cochran³, Barbara D. Boyan^{1,*}, and Zvi Schwartz^{1,3}

¹Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA 30332, USA

²St. Joseph's Translational Research Institute, Atlanta, GA 30342, USA

³University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

Abstract

The surface properties of materials contribute to host cellular response and play a significant role in determining the overall success or failure of an implanted biomaterial. Rough titanium (Ti) surface microtopography and high surface free energy have been shown to enhance osteoblast maturation *in vitro* and increase bone formation *in vivo*. While the surface properties of Ti are known to affect osteoblast response, host bone quality also plays a significant role in determining successful osseointegration. One factor affecting host bone quality is patient age. We examined both *in vitro* and *in vivo* whether response to Ti surface features was affected by animal age. Calvarial osteoblasts isolated from 1-, 3-, and 11-month-old rats all displayed a reduction in cell number and increases in alkaline phosphatase specific activity and osteocalcin in response to increasing Ti surface microtopography and surface energy. Further, osteoblasts from the three ages examined displayed increased production of osteocalcin and local factors osteoprotegerin, VEGF-A, and active TGF- β 1 in response to increasing Ti surface roughness and surface energy. Latent TGF- β 1 only increased in cultures of osteoblasts from 1- and 3-month-old rats. Treatment with the systemic osteotropic hormone 1 α ,25(OH) $_2$ D $_3$ further enhanced the response of osteoblasts to Ti surface features for all three age groups. However, osteoblasts derived from 11-month-old animals had a reduced response to 1 α ,25(OH) $_2$ D $_3$ as compared to osteoblasts derived from 1- or 3-month-old animals. These results were confirmed *in vivo*. Ti implants placed in the femoral intramedullary canal of old (9-month) mice yielded lower bone-to-implant contact and neovascularization in response to Ti surface roughness and energy compared to younger (2-month) mice. These results show that rodent osteoblast maturation *in vitro* as well as new bone formation *in vivo* is reduced with age. Whether comparable age differences exist in humans needs to be determined.

INTRODUCTION

The surface properties of materials and the quality of the bone contribute to host cellular response and ultimately determine the overall success or failure of an implanted biomaterial. Cells interact with the surface of a material through an adsorbed layer of proteins, ions, sugars, and lipids present in the blood and tissue fluid. Surface properties including

Address for Correspondence: Barbara D. Boyan, PhD, Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, 315 Ferst Drive NW, Atlanta, GA 30332-0363, Phone: 404-385-4108, Fax: 404-894-2291, barbara.boyan@bme.gatech.edu.

[^]RON and ALR are co-first authors of this paper.

topography, surface energy, chemistry, and surface charge are largely responsible for this interaction (1–3).

In vitro studies show that modifications to titanium (Ti) substrate micro-architecture have effects on the attachment and differentiation of osteoblast-like cells, including MG63 and MC3T3-E1 cell lines, as well as fetal rat calvarial cells and normal human osteoblasts (4). On micron- and submicron-scale rough Ti substrates, cell proliferation is decreased and differentiation is increased, as these cells display changes in alkaline phosphatase specific activity and increased osteocalcin production (5). Further, MG63 osteoblast-like cells cultured on these rough Ti surfaces release increased levels of local bone regulatory factors, including osteoprotegerin (OPG), prostaglandin E₂ (PGE₂), and transforming growth factor β 1 (TGF- β 1). These surface dependent effects are mediated by α -2, β -1 (α 2 β 1) integrin signaling (6). Osteoblastic differentiation of human mesenchymal stem cells (HMSCs) is also increased on Ti surfaces with micron- and submicron scale rough topographies, and this is mediated by α 2 β 1 integrin signaling (7). Taken together, these results indicate that rough Ti substrates promote osteoblast differentiation and maturation more than smooth Ti surfaces.

In addition to surface roughness, surface free energy of Ti substrates also plays a role in regulating bone cell responsiveness. Ti implant surfaces have a low surface free energy due to the adsorption of hydrocarbons and carbonates from the ambient atmosphere onto the TiO₂ surface layer. This adsorption also makes the Ti surfaces hydrophobic. Clean TiO₂ surfaces are hydrophilic with a high surface free energy (8), and this can be maintained by limiting exposure to ambient air conditions. MG63 cells cultured on high surface free energy substrates show an enhanced response to Ti surface microtopography. On microrough, high surface free energy Ti substrates, these cells show increases in alkaline phosphatase specific activity, osteocalcin production, and levels of TGF- β 1 and OPG compared to rough Ti surfaces alone (9).

Human osteoblasts and HMSCs also exhibit enhanced differentiation and osteogenic factor production on the high-energy surfaces (10,11). Importantly, factors secreted into the conditioned media of osteoblasts grown on the high-energy microtextured Ti surfaces can act on HMSCs cultured on tissue culture polystyrene to induce their osteoblastic differentiation (7). These results suggest that peri-implant bone formation *in vivo* may be modulated by events associated with surface design and that the cells populating the surface will contribute to the peri-implant milieu.

In vivo, Ti substrates with a rough microtopography support greater bone-to-implant contact than smooth surfaces do, resulting in greater removal torque strength (12–14). Preliminary studies have indicated that high surface free energy Ti surfaces have the potential to improve the early stages of soft and hard tissue integration of implants (15–17). One contributing factor to this may be an increase in vascularization. Both osteoblasts and HMSCs produce increased levels of angiogenic growth factors including vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (FGF-2), and endothelial growth factor (EGF) (7,10,18). Whether angiogenesis is increased *in vivo*, however, is not known.

Host bone quality also plays a significant role in determining successful osseointegration. Bone quality and subsequent bone formation can be affected by several factors including smoking, diabetes, cancer and other diseases (19,20). Another factor that is known to play a role in bone formation is age. Several studies have shown that both natural bone formation and bone fracture healing in response to osteoinductive factors is impaired in an aged population (21,22). The results of these studies suggest that new bone formation surrounding Ti implants may also be affected by patient age.

In the present study, osteoblast maturation in response to Ti surface microtopography and surface free energy was assessed with respect to donor age. To do this, osteoblasts were isolated from 1-month, 3-month, and 11-month-old Sprague-Dawley rats, representing young, adult, and old age groups, respectively, and were cultured on Ti substrates with two different surface morphologies and identical morphology but different surface hydrophilicity. In addition, we investigated whether donor age had an effect on the surface-dependent responses to the systemic regulatory hormone $1,25\text{-(OH)}_2\text{D}_3$ [$1,25\text{-(OH)}_2\text{D}_3$]. To investigate potential mechanisms underlying age-specific differences in cell response, the expression of mRNAs for integrin subunits and receptors for $1,25\text{-(OH)}_2\text{D}_3$ in osteoblasts derived from young and old populations in response to Ti substrate surface properties was also examined. Finally, the effects of surface microstructure and surface free energy on bone formation and vascularity *in vivo* were examined using a novel peri-implant bone formation model in which implants were placed in the femoral intramedullary canal of young and old C57BL/6 mice.

MATERIALS & METHODS

In Vitro Studies

Titanium Disk Preparation—Ti disks (15mm diameter) were prepared from 1mm thick sheets of grade 2 unalloyed Ti as described previously (23) and were supplied by Institut Straumann AG (Basel, Switzerland). Fabrication methods and resulting morphology of the surfaces have been reported previously (8,11,24). Pretreatment (PT) surfaces are smooth, with a mean peak to valley roughness (R_a) of 600 nm. Sandblasted with large grit and acid etched (SLA) surfaces were created from PT disks that were coarse grit-blasted with 0.25–0.50 mm corundum until the surface reached a uniform gray tone, followed by acid etching, resulting in a combination of micron and sub-micron scale features ($R_a = 3.2\text{ }\mu\text{m}$). Modified SLA (modSLA) surfaces were created in the same manner as SLA but they were processed with limited exposure to the ambient atmosphere, resulting in a hydrophilic substrate with the same topographical characteristics. modSLA surfaces were rinsed under nitrogen during fabrication and stored in isotonic sodium chloride until use. PT, SLA and modSLA Ti disks all have a TiO_2 surface layer, with the PT and SLA surfaces being hydrophobic due to the adsorption of atmospheric hydrocarbons while the modSLA surface is hydrophilic. Advancing contact angles were used to calculate the hydrophilicity of the surfaces as PT (95.8°), SLA (139.80°), and modSLA ($\sim 0^\circ$). Surface free energy for PT, SLA, and modSLA surfaces was calculated according to Zisman (critical surface tension), equation of state (EOS), and geometric mean approaches and is described in detail elsewhere (8). Terminal sterilization of all Ti disks was by overnight gamma irradiation at 25 kGy. Control cultures were grown on standard tissue culture polystyrene (TCPS).

Cell Culture—Osteoblasts were isolated from the calvaria (frontal and parietal) bones of 1-month, 3-month, and 11-month-old male Sprague-Dawley rats using an explant technique (10,25). Briefly, bones were cleaned of soft tissue and periosteum. Bones pieces were digested in 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA) for 15 minutes at 37°C to avoid fibroblast contamination. They were then cultured in 100 cm $\times$ 20 cm Petri dishes (BD Biosciences, Franklin Lakes, NJ) in DMEM (Cellgro®, Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT) and 1% penicillin-streptomycin (Invitrogen) at 37°C , 5% CO_2 , and 100% humidity. The resulting cultures were subpassaged and seeded on tissue culture polystyrene (TCPS), PT, SLA, or modSLA substrates at an initial cell density of 20,000 cells/cm².

Biochemical Assays—When cultures reached confluence on TCPS, cells were incubated with media containing either vehicle (0.01% ethanol) or 10^{-7}M $1,25\text{-(OH)}_2\text{D}_3$ (Enzo Life

Sciences, Plymouth Meeting, PA) for 24 hours. At harvest, cells were released by two sequential 10-minute incubations in 0.25% trypsin-EDTA at 37°C. Cell number was determined using a Z2 Cell Counter (Beckman Coulter, Fullerton, CA). Cells were lysed in Triton X-100 (Sigma Aldrich, St. Louis, MO) and cellular alkaline phosphatase specific activity measured by assaying the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2. Alkaline phosphatase specific activity was normalized to total protein content of the cell lysates (BCA Protein Assay, Thermo Fisher Scientific).

Levels of secreted factors in the conditioned media were normalized to cell number. Osteocalcin was measured using a commercially available radioimmunoassay following manufacturer's instructions (Biomedical Technologies, Stoughton, MA). Osteoprotegerin (OPG) and vascular endothelial growth factor A (VEGF-A) were measured using commercially available enzyme-linked immunosorbent assays (ELISA, DuoSet, R&D Systems, Minneapolis, MN). Active TGF- β 1 was measured in the conditioned media using an ELISA kit (DuoSet, R&D Systems). Media was acidified and total TGF- β 1 measured. Latent TGF- β 1 was determined by subtracting active TGF- β 1 from total TGF- β 1.

Gene Expression—Osteoblasts isolated from 3-month and 11-month-old animals were cultured on TCPS or Ti substrates. When cells reached confluence on TCPS, cells were incubated with fresh media for 12 hours. RNA was harvested using a TRIzol® (Invitrogen) extraction method. The resulting RNA was quantified (Nanodrop Spectrophotometer, Thermo Scientific) and 1 µg RNA reversed transcribed to cDNA (OmniScript Reverse Transcription Kit, Qiagen, Valencia, CA) using random oligomers (Promega, Madison, WI). Starting mRNA quantities were determined using SybrGreen (BioRad, Hercules, CA) in an iQ5 Real-time PCR machine (BioRad) and a standard curve method. Expression of mRNA was measured for the following integrin subunits: alpha 2 (ITGA2, F: 5'-ACTGTTCAAGGAGGAGAC-3'; R: 5'-GGTCAAAGGCTTGTTTAGG-3'); alpha 5 (ITGA5, F: 5'-ATCTGTGTGCCTGACCTG-3'; R: 5'-AAGTTCCTGGGTGTCTG-3'); and beta 1 (ITGB1, F: 5'-ATTACTCAGATCCAACCAC-3'; R: 5'-TCCTCCTCATTTTCATTCATC-3'); as well as for the membrane associated receptor for 1, 25(OH) $_2$ D $_3$, protein disulfide isomerase family A, member 3 (PDIA3) (26,27) (F: 5'-CCAATGATGTGCCTTCTC-3'; R: 5'-TGTGCCTTCTTCTTCTTC-3'); and the classical vitamin D receptor (VDR) (F: 5'-CATCTTGGCAGTGAGTGAGC-3'; R: 5'-ACACAACCTACCCATCATCCC-3'). All genes are presented as normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, F: 5'-CATACTCAGCACCAGCATCACC-3'; R: 5'-AAGTTCAACGGCACAGTCAAGG-3').

In Vivo Study

Titanium Implant Fabrication and Characterization—Bone formation and osseointegration of PT, SLA, and modSLA *in vivo* were examined using a femoral intramedullary bone formation model. Micro-computed tomography (microCT) imaging of 6-week-old mice was done in order to determine the size of the femoral medullary canal (Fig. 1A, 1B) and custom designed Ti implants were manufactured to fit into the femoral medullary space of C57BL/6 mice (Fig. 1C, 1D). The implants were fabricated from ASTM grade 4 Ti using the same processing methods used for commercially available dental implants. Surface treatments for Ti implants for *in vivo* studies were prepared in a manner similar to those for Ti surfaces described above. Ti implants consisted of two parts: a handling device and the Ti implant rod (Fig. 1C, 1D, 1E). The cylindrical Ti implants were manufactured to be 5 mm in length and 0.9 mm in diameter. All implants were sterilized by gamma irradiation at 25 kGy prior to use.

To characterize the surface morphology of Ti implants, surfaces that were not coated with gold were examined using an Ultra 60 field emission (FE) scanning electron microscope (SEM, Carl Zeiss SMT Ltd., Cambridge, UK). Images were recorded using a 5 kV accelerating voltage.

Surface wettability of Ti implants was determined with a Ramé-Hart goniometer (model 250-F1, Ramé-Hart Instrument Co., Mountain Lakes, NJ). Because the Ti implants had a cylindrical shape, it was not possible to measure the surface contact angle directly. Therefore, Ti implant surface wettability was qualitatively determined by immersing Ti implants into ultra pure water while recording the process. Images were recorded with DROPimage CA software package (Ramé-Hart Instrument Co.).

Surface chemistry was analyzed by using X-ray photoelectron spectroscopy (XPS) on a Thermo K-Alpha (Thermo Fisher Scientific, Waltham, MA). XPS was performed using monochromatic Al K X-ray source ($h\nu = 1486.6$ eV photons) at a 90° take-off angle in an analysis chamber evacuated to a pressure of 10^{-9} Torr or lower. Data analysis was performed using the Thermo Advantage 4.43 software package (Thermo Fisher Scientific, Inc).

Surface roughness of Ti implants was characterized by using a LEXT 3D Material Confocal Laser Microscope (CLM, Olympus America Inc., Center Valley, PA). Roughness was evaluated using the LEXT OLS4000 software provided by Olympus. Three measurements were made on two implants per surface topography. All measurements were performed in an organic clean room.

Animals—All animal handling and procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC), and were conducted in accordance with NIH guidelines. Custom-made Ti implants were inserted into the femoral medullary canal of mice via a medial parapatellar arthrotomy. Briefly, male C57BL/6 mice (aged 2-months or 9-months) were anesthetized with 5% isoflurane gas inhalation. The right hind limb was prepared by shaving and cleaning using ethanol and chlorhexidine. Anesthesia was maintained with 2% isoflurane gas inhalation for the duration of the surgical procedure. Cleaned, anesthetized animals were placed in a supine position and were covered with a sterile surgical drape. Using a scalpel, an 8 mm incision was made over the distal side of the knee. Blunt dissection was used to move aside the ligament and patella to expose the intercondylar notch of the distal femur. Once the femoral cartilage was exposed, a 1 mm round dental bur was used to penetrate the distal intercondylar notch of the femur to access the medullary cavity. A 22-gauge needle was gently pushed into the femur to confirm penetration of the medullary cavity. Cylindrical Ti implants were inserted into the femoral medullary canal and then were broken from the handling device. To confirm successful insertion of the implant into the femoral medullary canal, x-ray imaging was done prior to closure of the surgical incision (Fig. 1F, 1G). If the Ti implant was not inserted into the medullary canal or if insertion of the Ti implant resulted in a broken femur, the mouse was withdrawn from the study and euthanized using CO₂ inhalation. This was done to ensure an appropriate number of successful surgeries for each Ti implant type. Six successful implants were performed for each surface type to ensure adequate sample sizes for statistical analysis as determined by power analysis. Following successful implant insertion, periosteal tissue was closed using resorbable sutures and the surgical incision was closed with wound clips. After recovery from anesthesia, mice were injected with 0.03 mg/kg buprenorphine to relieve post-operative pain. Animals were monitored every 12 hours for 48 hours post-surgery and once per day thereafter for the duration of the study. Wound clips were removed 10–14 days post-surgery. All animals had access to food and water *ad libitum* for the duration of the study.

Implant Harvest and Vascular Perfusion—Limbs from the 2-month-old mice were harvested 35 days post-implantation. The limbs containing Ti implants were stored in 10% neutral buffered formalin for histological processing. Limbs from the 9-month-old animals were harvested at 28 days post-implantation for blood vessel quantification and histological analysis. Mice were euthanized via CO₂ inhalation. Immediately after death, the thoracic cavity was opened and a 22G butterfly needle was inserted into the left ventricle. The inferior vena cava was severed to allow the vasculature to be flushed. Heparinized saline (100 U/mL) was used to flush the vasculature followed by perfusion with 10% neutral buffered formalin to fix the tissue specimens. The vasculature was perfused using a radio-opaque silicone rubber compound (Microfil, Flow Tech, Inc., Carver, MA) mixed in a 9:1 v/v ratio with Microfil curing agent. Following vascular perfusion, the injected Microfil compound was allowed to polymerize overnight at 4°C before histological processing.

Histology—Fixed limbs were embedded in plastic. Sagittal tissue sections were cut and polished to a final thickness of 10–20 µm and were stained with haematoxylin and eosin for histological analysis. Using commercially available image analysis software (Image-Pro Plus, Media Cybernetics, Bethesda, MD), stained tissue sections were analyzed for bone-to-implant contact (BIC) using two different methods. First, the BIC where the implant surface was in direct contact with the femoral cortical bone surface was calculated for each animal for each implant type and the average total BIC was determined. We also measured the BIC where new bone had formed within the femoral intramedullary space for each animal for each implant surface type.

Statistical Analysis—For each *in vitro* experiment, data presented are mean ± SEM for six independent cultures per variable. Experiments were repeated to ensure validity. Data are presented from one representative experiment of two experiments. For each *in vivo* study, six implants were harvested for each implant type. *In vitro* and *in vivo* data were analyzed by ANOVA and when statistical differences were detected, Student's *t*-test for multiple comparisons using Bonferroni's modification was used. P-values < 0.05 were considered significant.

RESULTS

In Vitro Results

Total osteoblast cell number was reduced on all three Ti surfaces compared to TCPS control surfaces for all three donor ages examined (Fig. 2A). On TCPS and PT surfaces, osteoblasts derived from 1-month-old rats had a significantly higher cell number compared to osteoblasts from both 3- and 11-month-old rats while total cell number on SLA surfaces were similar amongst cells derived from all three age groups. 1,25(OH)₂D₃ treatment did not affect cell number on any of the surfaces for any of the ages examined (Fig. 2B).

Alkaline phosphatase specific activity was higher in osteoblasts of all three age groups on all three Ti surfaces compared to TCPS (Fig. 2C). The addition of 1,25(OH)₂D₃ increased enzyme activity on all surfaces but the magnitude of the stimulatory effect was donor age dependent (Fig. 2D). Osteoblasts from 11-month-old animals did not respond to treatment to the same extent as cells from 1-month or 3-month-old animals. Secreted levels of osteocalcin, which is a late marker of osteoblast maturation, were higher with increased surface roughness and energy for all three age groups examined (Fig. 2E). The increase on SLA and modSLA was significantly less robust in cells from aged animals. 1,25(OH)₂D₃ caused a further increase in osteocalcin levels in cultures grown on PT but this increase was less robust in cells from 11-month-old donors (Fig. 2F).

Local factor production was also sensitive to donor age. In cells from younger animals, osteoprotegerin (OPG) levels were higher on all three Ti surfaces compared to TCPS control surfaces. In cells from 11-month-old animals, OPG levels were increased only in cultures grown on microstructured Ti surfaces (Fig. 3A). The addition of $1,25(\text{OH})_2\text{D}_3$ significantly increased OPG secretion on all surfaces examined, however the effect was donor age dependent (Fig. 3B). Cells derived from younger donors responded to $1,25(\text{OH})_2\text{D}_3$ to a greater extent than cells derived from the older donors. Similar to OPG production, VEGF-A levels were higher in cultures grown on Ti surfaces compared to TCPS control surfaces in cells from young animals, whereas, secreted levels of VEGF-A in cells from 11-month-old animals were only increased on microstructured Ti surfaces (Fig. 3C). $1,25(\text{OH})_2\text{D}_3$ enhanced the stimulated VEGF-A secretion on all surfaces in cultures from 1- and 3-month-old animals but did not have a stimulatory effect in cultures from 11-month-old animals (Fig. 3D). VEGF-A levels were highest in cells from 3-month-old animals. Active TGF- β levels were increased on all three Ti surfaces compared to TCPS in all three age groups. $1,25(\text{OH})_2\text{D}_3$ increased levels of active TGF- β in osteoblasts derived from all donor age groups with levels being higher on all three Ti surfaces compared to TCPS control surfaces (Fig. 3F). Latent TGF- β levels were higher only in response to growth on microtextured Ti surfaces in cells from 1- and 3-month-old animals while levels in cells from 11-month-old animals were unaffected by either Ti surface roughness or energy (Fig. 3G). $1,25(\text{OH})_2\text{D}_3$ increased latent TGF- β levels on all surfaces examined and this effect was greatest in cells from younger animals (Fig. 3H).

Expression of integrin receptor subunits α_2 and α_5 were higher on all three Ti surfaces examined compared to TCPS control surfaces for both 3- and 11-month-old animals, however expression of both integrins α_2 and α_5 were lower in cells derived from 11-month-old animals on all substrates examined when compared to 3-month-old donor cells (Fig. 4A, 4B). Levels of α_1 integrin mRNA were higher for cells on microrough SLA and microrough, hydrophilic modSLA Ti surfaces compared to TCPS substrates for cells from both 3- and 11-month-old animals (Fig. 4C). Expression of the α_1 integrin was similar between cells from 3- and 11-month-old animals except on modSLA, where cells from 11-month-old animals displayed a reduction in α_1 expression.

Receptors for $1,25(\text{OH})_2\text{D}_3$ were expressed in osteoblasts isolated from both young and old donors. Levels of expression were specific to receptor type and varied with the surface and donor age. mRNAs for PDIA3, the membrane receptor for $1,25(\text{OH})_2\text{D}_3$, were reduced in response to increasing Ti surface roughness and surface hydrophilicity, but no differences in expression between osteoblasts from the different aged animals were observed (Fig. 4D). In contrast, mRNAs for VDR, the nuclear receptor for $1,25(\text{OH})_2\text{D}_3$, increased on SLA and modSLA surfaces compared to TCPS for osteoblasts from both age groups (Fig. 4E). In cell cultures from 11-month-old animals, expression of VDR was significantly higher than that observed in cell cultures from 3-month-old animals on all surfaces examined.

Implant Characterization

The surface morphology of PT, SLA, and modSLA implants was obtained at low and high magnification with SEM (Fig. 5A). The surfaces of PT implants were relatively smooth with more irregular surface features than SLA and modSLA implants. Both SLA and modSLA surfaces exhibit complex features such as shallow micron-scale craters (10–60 μm) and submicron-scale pits (1–3 μm). There was no clear boundary between craters and pits. Unmodified areas characterized by flat and smooth sections were also found on SLA and modSLA surfaces. However, there were no observed topographical differences between SLA and modSLA surfaces.

The modSLA surface showed excellent wettability compared to the SLA surface with poor wettability (Fig. 5B). Titanium (Ti2p), oxygen (O1s), and carbon (C1s) were detected on PT, SLA, and modSLA surfaces (Table 1). Trace nitrogen (N1s) was detected on PT and SLA surfaces. Atomic percentages for the PT, SLA, and modSLA surfaces are summarized in Table 1. More oxygen and titanium with less carbon was found on modSLA surfaces compared to PT and SLA surfaces.

Surface roughness determined by CLM was significantly different between PT ($R_a = 0.21 \mu\text{m}$) and SLA ($R_a = 3.39 \mu\text{m}$). In contrast, there was no difference between SLA ($R_a = 3.39 \mu\text{m}$) and modSLA ($R_a = 3.47 \mu\text{m}$) (Table 1). SEM and CLM suggest that no morphological difference was observed between SLA and modSLA surfaces. However, higher surface wettability of the modSLA surface than the SLA surface was induced by different surface chemistry.

In Vivo Results

Implant osseointegration was quantified by evaluating transverse histology sections. An example of such a cut is presented in Figure 6. The amount of bone and the BIC was different when the implant was in contact with cortical bone compared to bone in the marrow compartment. Accordingly, the morphometric analysis was performed separately on the areas contacting cortical bone versus the bone contact in the marrow area. In both age groups, neither Ti implant surface microstructure nor surface energy had an effect on BIC where the implant surface came in direct contact with the femoral cortical bone surface (Table 2). Neither Ti surface microstructure nor surface energy had an effect on total marrow to implant contact in young (2-month-old) male C57BL/6 mice (Fig. 7A). In 9-month-old male C57BL/6 mice, surface microtopography alone had no significance effect on total BIC within the femoral marrow cavity, however the BIC was significantly increased in response to the surface microtopography and high surface energy seen on modSLA Ti implants compared to smooth PT Ti implants (Fig. 7A). The total number of blood vessels within the marrow space surrounding Ti implants within the femoral medullary space was also increased in the modSLA implant group relative to the smoother PT controls (Fig. 7B).

DISCUSSION

The success of implants in compromised patients in general, and in older patients specifically, has attracted research attention. Several studies have reported conflicting results on whether the healing capacity diminishes with increasing age (21,22). Endosteal bone formation and marrow regeneration following tibial marrow ablation are delayed with increasing age in immunocompromised rats (22). Healing of segmental fracture defects was delayed in older animals with demineralized bone matrix (DBM) treatment compared to younger animals (21). Diminished healing capabilities with increasing age have been reported in other organ systems as well. Bone marrow derived mesenchymal stem cell homing to sites of burn injury was significantly reduced in older animals (28). In contrast, compensatory bone formation during tooth movement is maintained at similar levels in both young and old rats (29). The results of the present study indicate that responses of osteoblasts from aged animals to surface roughness and the addition of $1,25(\text{OH})_2\text{D}_3$ as well as *in vivo* osseointegration in older animals were reduced when compared to younger animals.

Ti implant surface properties are known to affect osteoblast maturation *in vitro* and new bone formation *in vivo*. The response of osteoblasts to microstructured Ti surfaces is similar to their response to bone wafer surfaces that have been pre-conditioned by osteoclasts to create microtextured resorption pits (30), suggesting that the results of this study may have broader application to our understanding of the role aging in bone physiology. Consistent

with previous results, the present study found that osteoblasts display reduced proliferation and increased maturation in response to increasing surface roughness and surface free energy (3,5,15,31–33). It was also observed that treatment with the systemic osteotropic hormone $1,25(\text{OH})_2\text{D}_3$ further enhanced osteoblast response to Ti substrate features as has been reported previously (34). While osteoblasts isolated from 1-, 3-, and 11-month-old Sprague-Dawley rats showed surface roughness and energy dependent increases in maturation and local factor production, osteoblasts isolated from 11-month-old animals had a less robust response to both Ti surface roughness properties. Cells from older animals also had a diminished response to treatment with 10^{-7}M $1,25(\text{OH})_2\text{D}_3$ compared to osteoblasts isolated from 1- and 3-month-old animals.

Our results suggest that integrin signaling may play a role in the reduced response observed in cells from aged rats. We have previously shown that $\alpha_5\beta_1$ is required for osteoblast attachment and proliferation, but $\alpha_2\beta_1$ is required for osteoblast differentiation of HMSCs and osteoblasts (6,7,35). Expression levels of α_2 and α_5 as well as β_1 were lower in osteoblasts derived from older animals.

Age-dependent differences in expression of PDIA3 were not observed, suggesting that signaling via PDIA3-dependent pathways may not mediate the age-dependent differences in response to $1,25(\text{OH})_2\text{D}_3$. In contrast, osteoblasts from older animals had higher expression of mRNAs for VDR compared to osteoblasts from younger animals. This suggests that the diminished responsiveness of osteoblasts from older animals to treatment with $1,25(\text{OH})_2\text{D}_3$ is not caused by a lack of receptors in these cells but rather by some other mechanism. Alternatively, increased VDR may be a compensatory mechanism in the older animals.

mRNAs for PDIA3 were reduced in osteoblasts cultured on the microtextured surfaces whereas mRNAs for VDR were increased, regardless of the age of the donor rats. We have observed similar changes in expression of these two receptors during osteogenic differentiation of mouse embryoid bodies or cultures of HMSCs (36). Embryonic stem cells (ESCs) expressed mRNA and protein for both receptors for $1,25(\text{OH})_2\text{D}_3$ and they continued to be present as the ESCs differentiated in the form of embryoid bodies. Interestingly, the expression of *Vdr* increased in embryoid bodies after incubation in osteogenic medium, while *Pdia3* expression decreased. In adult human MSCs, osteogenic induction increased VDR mRNA whereas levels of PDIA3 mRNA remained stable after osteogenic induction. Taken together with the results of the present study, these findings suggest that expression levels of these receptors are modulated during the course of osteogenic differentiation but it is not known if changes in mRNA are required to initiate a change in osteoblast differentiation state or if they are a consequence of that state.

The novel murine intramedullary bone formation model used in this study enabled us to compare BIC and vasculogenesis as a function of age in two different conditions. Injury to the medullary canal caused by insertion of the implant induced endosteal bone formation and with it new blood vessel formation (37). In the absence of a biomaterial within the canal, the endosteal bone will be remodeled, restoring the contours of the canal within 28 days. The rate and extent of remodeling and the quality of the regenerated marrow are sensitive to biomaterial properties (38) and vary with animal age (22).

The results of the present study show that both age as well as biomaterial surface features affect osseointegration and the neovasculature, not only in the peri-implant cortical bone but also in the endosteal bone in the medullary canal. In young, skeletally mature C57BL/6 mice, neither Ti surface roughness nor wettability had a significant effect on osseointegration as determined by histological examination of total bone to implant contact

at 35 days post-implantation. However, in adult animals smooth and SLA surfaces had less BIC and new blood vessels compared to mod SLA. Moreover, BIC and neovascularity in older animals with modSLA implants were comparable to levels found in young animals. modSLA had higher BIC after 4 weeks of implantation compared to SLA.

Age-dependent differences in peri-implant bone formation may be resolved over time. Similar to our results, modSLA implants supported significantly greater BIC compared to SLA implants in minipigs at 14 days post-implantation (16). It may also be possible that in the young mice, new bone formation around modSLA implants occurs more rapidly than around PT or SLA implants, however at our harvest time of 35 days post-implantation, these differences may not have been observed.

Our results are consistent with results found previously demonstrating that new bone formation capacity is diminished with age (22) and support the hypothesis that implant surface properties can enhance osseointegration in sites of compromised bone. In 9-month-old C57BL/6 mice, which are an aged model representing compromised bone, the combination of a rough surface microtopography and high surface energy on modSLA substrates resulted in a significant increase in total bone to implant contact surrounding these implants. In addition, when the vasculature of these animals was perfused and the total number of blood vessels surrounding the implants within the marrow space determined, it was found that modSLA implants had significantly increased total blood vessel number. These results are in agreement with our previous findings *in vitro*, which indicated that surface roughness enhanced production of angiogenic and vasculogenic factors (18).

In a clinical setting, establishment of a vasculature preceding or concomitant with bone formation allows for the delivery of oxygen, systemic hormones, and nutrients to the injury site, as well as the migration of mesenchymal stem cells. In the absence of neovascularization, the implant may be surrounded by a fibrous capsule, resulting in implant loosening and ultimately failure, demonstrating the importance of the initial reaction of the first cells to encounter an implanted material. The results from this study have shown that microstructured, hydrophilic Ti surfaces can improve neovascularization and subsequently implant osseointegration in sites of compromised bone formation, such as in an older patient population.

In addition to the data presented here, this novel mouse model of intramedullary bone formation provides several advantages to evaluate the numerous factors involved in peri-implant bone formation. The numerous genetically modified mouse strains readily available, such as vitamin D receptor knockout mice will make it easier to isolate and examine the specific roles of these factors in influencing implant osseointegration, particularly in an aged population.

CONCLUSIONS

In the present study, it was determined that osteoblast maturation in response to Ti implant surface roughness and surface free energy diminishes with age. Furthermore, osteoblast responsiveness to the systemic osteotropic hormone $1,25(\text{OH})_2\text{D}_3$ was shown to be reduced in osteoblasts derived from aged animals. However, VDR expression was significantly higher in osteoblasts derived from older animals compared to osteoblasts derived from a younger population, indicating that the diminished responsiveness of cells to treatment with $1,25(\text{OH})_2\text{D}_3$ was not caused by a lack of these vitamin D receptors in these cells. Further, we describe here the development of a novel mouse intramedullary bone formation model and show that both new bone formation and neovascularization are reduced in response to Ti implants in older animals. This effect can be avoided by titanium implants

with roughened and high-energy surfaces. Taken together, these results indicate that osteoblast maturation *in vitro* and new bone formation *in vivo* are diminished with age, suggesting that these factors should be taken into consideration clinically when selecting appropriate biomaterials.

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RON and ZS designed the *in vitro* studies. RON, SLH, and DLH performed the *in vitro* studies. ALR and ZS designed the *in vivo* studies. JHP performed implant surface characterization. ALR, SLH, and ZS performed *in vivo* studies. RON, DLC, BDB, and ZS performed data analysis and interpretation. RON and ALR drafted the manuscript, and all authors have critically revised it. BDB accepts responsibility for the integrity of the data analysis for the work described.

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Figure 1.

Titanium implant design. Three-dimensional microCT reconstructions of the mouse femoral medullary canal (A) and microCT images of mouse femurs (B) were used to inform implant design. (C) Three-dimensional CAD representation and (D) mechanical drawing showing dimensions of Ti implant with handling device. (E) Image of custom manufactured Ti implant. (F) X-ray image showing an implant that has been inserted into the femoral medullary space without breaking of the bone. (G) Gross morphology of a mouse femur with an implant (arrow).

Figure 2.

Response of osteoblasts derived from 1-month, 3-month, and 11-month-old rats to Ti surface roughness and hydrophilicity and treatment with $10^{-7}\text{M } 1,25(\text{OH})_2\text{D}_3$. Cells were cultured on TCPS, PT, SLA, and modSLA Ti surfaces with or without $10^{-7}\text{M } 1,25(\text{OH})_2\text{D}_3$. (A,D) Cell number, (B,E) alkaline phosphatase specific activity in the cell lysate, and (C,F) osteocalcin levels were determined. Values presented are mean $\pm$ SEM of six independent cultures. * $p < 0.05$ vs. TCPS, ^ $p < 0.05$ vs. 1-month-old rats.

Figure 3.

Response of osteoblasts derived from 1-month, 3-month, and 11-month-old rats to Ti surface roughness and hydrophilicity and treatment with $1,25(\text{OH})_2\text{D}_3$. Cells were cultured on TCPS, PT, SLA, and modSLA Ti surfaces with or without $10^{-7}\text{M } 1,25(\text{OH})_2\text{D}_3$. (A,E) Osteoprotegerin, (B,F) VEGF-A, (C,G) active TGF- β 1, and (D,H) latent TGF- β 1 levels were determined. Values presented are mean $\pm$ SEM of six independent cultures. * $p < 0.05$ vs. TCPS, ^ $p < 0.05$ vs. 1-month-old rats.

Figure 4.

Gene expression in adult (3-month) and old (11-month) rats. Osteoblasts isolated from 3- and 11-month-old rats were cultured on TCPS and PT, SLA, and modSLA Ti surfaces and expression of Integrin α_2 (ITGA2, A), Integrin α_5 (ITGA5, B), and Integrin β_1 (ITGB1, C) and vitamin D receptors PDIA3 (D) and VDR (E) measured. Values presented are mean $\pm$ SEM of six independent cultures. * $p < 0.05$ vs. TCPS, • $p < 0.05$ vs. 3-month-old rats.

Figure 5. Representative histology sections. Representative histology sections PT, SLA, and modSLA titanium implants placed in of 2-month-old (A, B, C) or 9-month-old mice (D, E, F).

Figure 6.

In vivo bone formation. Bone formation and neovascularization surrounding PT, SLA, and modSLA Ti implants was examined using a novel murine intramedullary bone formation model. The percent bone to implant contact (% BIC) was measured for both 2-month-old and 9-month-old animals (A). * $p < 0.05$ vs. PT. (B) The total number of blood vessels within the marrow space of 9-month-old animals. * $p < 0.05$ vs. PT. Values presented are the mean $\pm$ SEM of six implants per group.

Fig. 7.

In vivo bone formation. Bone formation and neovascularization surrounding PT, SLA, and modSLA Ti implants was examined using a novel murine intramedullary bone formation model. (A) The percent bone to implant contact (% BIC) was measured for both 2-month-old and 9-month-old animals. $p < 0.05$ versus PT. (B) The total number of blood vessels within the marrow space of 9-month-old animals. $p < 0.05$ versus PT. Values presented are the mean SEM of six implants per group.

Table 1

Surface chemical composition and roughness parameters of titanium cylinder surfaces (mean value $\pm$ SD).

	Concentration (atomic % $\pm$ std.)				Roughness parameter (mean $\mu\text{m} \pm$ std.)		
	Cl _{1s}	N _{1s}	O _{1s}	Ti _{2p}	Ra	Rt	
PT	31.7 $\pm$ 2.3	2.4 $\pm$ 0.1	49.5 $\pm$ 1.2	16.4 $\pm$ 1.0	0.21 $\pm$ 0.03	1.39 $\pm$ 0.32	
SLA	40.3 $\pm$ 1.1	3.0 $\pm$ 0.4	43.5 $\pm$ 0.7	13.3 $\pm$ 0.7	3.39 $\pm$ 0.63	19.71 $\pm$ 5.09	
modSLA	24.8 $\pm$ 2.1		54.5 $\pm$ 1.5	20.6 $\pm$ 0.6	3.47 $\pm$ 0.71	21.15 $\pm$ 4.96	

Table 2

Bone-to-implant contact for 8-week and 9-month-old mice for PT, SLA, and modSLA Ti implants.

Cortical Bone to Implant Contact		
<i>Surface Type</i>	<i>8 Week</i>	<i>9 Month</i>
PT	0.88 ± 0.06	0.89 ± 0.06
SLA	0.87 ± 0.05	0.89 ± 0.07
modSLA	0.92 ± 0.07	0.93 ± 0.07