Published in final edited form as:

J Biomed Mater Res A. 2019 June ; 107(6): 1303–1313. doi:10.1002/jbm.a.36643.

Participation of Integrin $\beta 3$ in Osteoblast Differentiation Induced by Titanium with Nano or Microtopography

Helena B. Lopes¹, Gileade P. Freitas¹, Carlos N. Elias², Coralee Tye³, Janet L. Stein³, Gary S. Stein³, Jane B. Lian³, Adalberto L. Rosa¹, Marcio M. Beloti¹

¹Cell Culture Laboratory, School of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

²Biomaterials Laboratory, Instituto Militar de Engenharia, Rio de Janeiro, RJ, Brazil

³Department of Biochemistry, University of Vermont Cancer Center, University of Vermont, Burlington, VT, USA

Abstract

The major role of integrins is to mediate cell adhesion but some of them are involved in the osteoblasts-titanium (Ti) interactions. In this study, we investigated the participation of integrins in osteoblast differentiation induced by Ti with nanotopography (Ti-Nano) and with microtopography (Ti-Micro). By using a PCR array, we observed that, compared with Ti-Micro, Ti-Nano upregulated the expression of five integrins in mesenchymal stem cells, including integrin $\beta 3$, which increases osteoblast differentiation. Silencing integrin $\beta 3$, using CRISPR-Cas9, in MC3T3-E1 cells significantly reduced the osteoblast differentiation induced by Ti-Nano in contrast to the effect on Ti-Micro. Concomitantly, integrin $\beta 3$ silencing downregulated the expression of integrin αv , the parent chain that combines with other integrins and several components of the Wnt/ β -catenin and BMP/Smad signaling pathways, all involved in osteoblast differentiation, only in cells cultured on Ti-Nano. Taken together, our results showed the key role of integrin $\beta 3$ in the osteogenic potential of Ti-Nano but not of Ti-Micro. Additionally, we propose a novel mechanism to explain the higher osteoblast differentiation induced by Ti-Nano that involves an intricate regulatory network triggered by integrin $\beta 3$ upregulation, which activates the Wnt and BMP signal transductions.

Keywords

CRISPR; integrin; nanotopography; osteoblast; titanium

Correspondence to: Marcio M. Beloti, School of Dentistry of Ribeirão Preto, University of São Paulo, Av. do Café s/n, 14040-904, Ribeirão Preto, SP, Brazil, Tel.: +55 16 3315 4785, mmbeloti@usp.br.

DISCLOSURES

The authors have nothing to declare.

All authors agree with the submission. The material submitted for publication has not been previously reported and is not under consideration for publication elsewhere.

INTRODUCTION

The surface characteristics of titanium (Ti) implants affect cell adhesion, proliferation and osteoblast differentiation, which ultimately dictate the interfacial tissue formation to achieve implant fixation to the host and determine the success rate of dental implants.^{1–3} Modifications of Ti topography may affect osteoblast differentiation by acting on several cell mechanisms and signaling pathways, including those triggered by integrins.^{4–8}

Integrins are proteins that mediate cell interactions with components of the extracellular matrix and with other plasma membrane proteins.⁹ They are comprised of two subunits, α and β , with eighteen α and eight β subunits already identified, which are bonded by covalent unions to form twenty-four possible combinations between α and β , each one with specific characteristics of ligand-binders.^{10,11} In osteoblast lineage integrins regulate cell adhesion and contribute to the progressive differentiation, initiation of matrix mineralization, and consequently, to bone formation.^{6,12–14}

A unique nanotopography can be generated on Ti surfaces by a controlled chemical oxidation using a solution of $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$. This surface exhibits nanopits with an average diameter of 22 nm, a thicker TiO_2 layer and lower amounts of contaminants such as N and Si compared with an untreated Ti surface.¹⁵ The higher osteogenic potential of this nanotopography has been demonstrated using different cell culture and animal models.^{6,8,16} In addition, we have shown that the signaling pathway triggered by integrin $\alpha 1\beta 1$ complex has a key role in the osseointegrative capacity of Ti with nanotopography since the use of an antagonist of the heterodimer $\alpha 1\beta 1$, obtustatin, prevented the osteoblast differentiation induced by nanotopography.⁶

Surface modifications at the microscale levels may also modulate integrin signaling pathway and consequently affect osteoblast differentiation of cells cultured on Ti. It has been shown that the osteoblast responses to microstructured Ti are mediated by integrin $\alpha 2\beta 1$ heterodimer.^{17,18} Also, it was observed that integrin $\alpha 1$ silencing decreased osteoblast maturation of cells cultured on Ti with microtopography and the association of both micro and nanotopography promotes the osteoblast differentiation by increasing the expression of integrins $\beta 1$ and $\beta 3$.^{2,19}

Based on these findings, which have highlighted the striking role of surface topography on the modulation of integrin signaling pathway in cells cultured on Ti, we hypothesized that other integrins are involved in osteoblast responses to Ti with different surface topographies. In order to test this hypothesis, we used a gene array analysis and a novel and powerful tool of gene editing, the clustered regularly interspaced short palindromic repeats/associated nuclease 9 (CRISPR-Cas9). To the best of our knowledge, this is the first report of a CRISPR-Cas9 edited deletion of integrin $\beta 3$ in the context of Ti osseointegration and our results have shown that integrin $\beta 3$ exhibits distinct effects on osteoblast/Ti interactions depending on surface topography either with nano or microtopography.

MATERIALS AND METHODS

Preparation of Ti surfaces

The Ti discs (12×2 mm) of commercially pure (grade II) were polished and cleaned as previously described.⁸ To obtain Ti with nanotopography (Ti-Nano), discs were conditioned for 4 h with a solution of 10 N H₂SO₄ and 30% aqueous H₂O₂ (1:1) at room temperature under agitation and cleaned by sonication. The Ti with microtopography (Ti-Micro) was prepared as a commercially available surface under industrial secret, using an acidic solution containing HNO₃, H₂SO₄, and HCl (Conexão Sistemas de Prótese LTDA, Arujá, SP, Brazil).

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Characterization of Ti surfaces

Qualitative analysis of Ti-Nano and with Ti-Micro was made applying a field emission scanning electron microscope (Inspect S50, FEI, Hillsboro, OR, USA) operated with a voltage of 5 kV. The roughness parameters were quantified using a New View 7100 Profilometer (Zygo Co, Middlefield, CT, USA) on five discs of both Ti surfaces with three areas randomly selected in each disc (n=15).

Cell culture

Mesenchymal stem cells (MSCs) were obtained from bone marrow of Wistar male rat weighing approximately 150 g following the research protocols approved by the Animal Ethics Committee (School of Dentistry of Ribeirão Preto, University of São Paulo, # 20141796587). Cells were cultured in growth medium constituted by α minimum essential medium (α -MEM, Invitrogen-Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco-Life Technologies, Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco-Life Technologies) until subconfluence. At first passage, cells were cultured in 24-well culture plates (Corning Life Sciences, Corning, NY, USA) on both Ti-Nano and Ti-Micro at a cell density of 2×10^4 cells per disc in growth medium, for up to 3 days. Cultures were kept at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Expression of genes related to the integrin signaling pathway

On day 3, cells cultured on both Ti-Nano and Ti-Micro were submitted to total RNA extraction with Trizol reagent (Invitrogen) following the manufacturer's instructions. An equal amount of each sample (1 μ g) was used for reverse transcription reaction (Kit High Capacity, Invitrogen). Custom array plates were used to detect the expression of 87 genes related to integrin signaling pathway and focal adhesion (RT2 ProfilerTM PCR Array Rat Integrin Signaling Pathway, Qiagen, Hilden, NRW, DE). The assays were done using RT² Real-Time SYBR Green/ROX PCR master mix (Qiagen). The relative gene expression was analyzed by the RT2 Profiler PCR Array Data Analysis Software (Qiagen) using 6 housekeeping genes, β -actin (*Actb*), 18S ribosomal (*18s*), Beta-2-microglobulin (*B2m*), hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), lactate dehydrogenase A (*Ldha*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and calibrated in relation to cells cultured on Ti-Micro (Table S1).

CRISPR-Cas9 construction

MC3T3-E1 cells expressing a constitutive dCas9-KRAB (KRAB)—For a stable cellular expression, MC3T3-E1 cells (sub-clone 14, ATCC, Manassas, VA, USA) were infected with lentiviral particles containing a constitutive dCas9-KRAB lentiviral vector followed by puromycin selection. Briefly, to generate the lentiviral particles, 293-FT cells (Thermo Fischer Scientific, Waltham, MA, USA) were transfected with lentiviral vector pHAGE EF1 α dCas9-KRAB (Addgene #50919, gift from Rene Maehr & Scot Wolfe), envelope encoder pMD2.G and packer vector pspAX2 (ratio 3:2:1) using transfection reagent X-tremeGENE HP DNA (Sigma-Aldrich, St. Louis, MO, USA). The virus-containing medium was collected 48 and 72 hours post-transfection and the virus particles were concentrated using Lenti-X Concentrator (Clontech, Mountain View, CA, USA). Thus, the MC3T3-E1 cells were seeded in 6-well culture plates at a density of 1×10^5 cells per well and maintained in growth medium, which is α -MEM (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 24 h, the culture medium was replaced by a solution containing growth medium with 8 μ g/ml of Polybrene (Sigma-Aldrich) and the virus previously prepared and maintained overnight at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 24 h, the lentiviral solution was replaced by fresh growth medium and 48 h post-infection, the selection using puromycin (1.5 μ g/mL) was initiated (Thermo Fischer Scientific) and kept for 7 days. The presence of dCas9-KRAB was confirmed by qualitative PCR and Western blot to detect dCas9 (Fig. S1A and B, respectively), as described below. These KRAB-MC3T3-E1 (KRAB) cells were used as scrambled cells (Control).

Qualitative PCR—To isolate genomic DNA (gDNA), 1×10^5 of both MC3T3-E1 and KRAB-MC3T3-E1 cells were resuspended in 50 μ L of QuickExtract Solution (Lucigen - Epicenter, Middleton, WI, USA). The PCR reaction mixture was prepared with 10 μ L of KAPA HiFi HotStart ReadyMix (2 $\times$) (Kapa Biosystems - Roche Applied Science Penzberg, DE-BY, DE), 0.5 μ L of forward and reverse primers (10 μ M) (F- ACTGATAAGGCTGACTTGCGGT R-CGAAGTTCCAGGGAGTGATGGT), 100 ng of gDNA and H₂O up to 20 μ L. The temperature cycling condition included an initial denaturation at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1min and a final extension for 10 min at 72 °C. The PCR products were mixed with 4 μ L of Orange DNA Loading Dye (6 $\times$) (Thermo Fischer Scientific) and separated by 1% gel electrophoresis. The bands were recorded in the GelDoc UV (Bio-Rad Laboratories, Hercules, CA, USA) gel documentation system.

2.5.3 Western blot—To detect dCas9 protein, the MC3T3-E1 and KRAB-MC3T3-E1 cells were treated, and the protein of each cell were transferred to PVDF membrane as previous described.⁸ For membrane blocking, it was used Tris-buffered saline, 0.1% Tween-20 (Sigma-Aldrich) with 5% nonfat milk (Bio-Rad Laboratories), for 1 h at room temperature. The membrane was incubated with primary antibody anti-CRISPR (Cas9) protein (1:500, Biolegend, San Diego, CA, USA) overnight at 4°C and with secondary antibody goat-anti-rabbit IgG HPR conjugated (1:3000, Santa Cruz Biotechnology, Santa

Cruz, CA, USA) for 1 h at room temperature. Then, the membrane was stripped and reprobed with a primary antibody anti-tubulin (1:1000, Santa Cruz Biotechnology), followed by secondary antibody goat-anti-mouse (1:3000, Sigma-Aldrich). The protein bands were analyzed with Western Lightning Plus Kit (PerkinElmer - Life Sciences, Waltham, MA, USA). The protein expression was quantified in triplicate (n=3) by counting pixels.

Single guide RNA design—In order to silence integrin $\beta 3$ expression, three single guide RNAs (sgRNA) were designed and evaluated through the Benchling platform (<https://benchling.com>). The target region was –300 to +100 base pairs of the transcriptional start site. The sgRNA, with 20 base pairs, was selected considering the best score for “on target” and “off target”. The oligonucleotide sequences were purchased from Invitrogen (Invitrogen-Life Technologies).

Transduction of KRAB-MC3T3-E1 cells with lentiviral particles containing the single guide RNA to integrin $\beta 3$ (sgITGB3)—To produce lentivirus containing sgRNA, the oligonucleotides were annealed and subjected to a digestion reaction and ligation with the plasmid pLK5.sgRNA.EFS.tRFP (Addgene #57823, gift from Benjamin Ebert). Next, the plasmid was transformed into Chemically Competent *E. coli* cells (One Shot®, Stbl3™ - Thermo Fischer Scientific) and amplified in LB medium with ampicillin (50 $\mu\text{g/mL}$). The plasmid extraction and purification were performed by ZR Plasmid Maxiprep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer’s instructions and the concentration assessed by optical density at 260 nm. The lentiviral particles were generated as described above and the KRAB-MC3T3-E1 cells (Control) were infected with the virus for each sgRNA to generate sgITGB3-MC3T3-E1 cells. Then, cells were submitted to cell sorting for red fluorescent protein. To test the efficiency of sgITGB3, the gene and protein expression of integrin $\beta 3$ of cells cultured on polystyrene were analyzed at day 3 after sorting. The integrin $\beta 3$ gene expression was assessed by Real-time PCR as described below and protein expression was evaluated by Western blot, as described above using the primary antibody anti-integrin $\beta 3$ (1:1000, Abcam, Cambridge, UK) and secondary goat-anti-mouse IgG HRP conjugate (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The integrin $\beta 3$ protein expression in sgITGB3-MC3T3-E1 cells was normalized to β -actin protein and calibrated by KRAB-MC3T3-E1 cells (n=3). The β -actin protein was detected by using a primary antibody anti-ACTB (1:1000, Cell Signaling, Danvers, MA, USA), followed by secondary antibody goat-anti-mouse (1:3000, Sigma-Aldrich).

Real-time PCR—To detect integrin $\beta 3$ gene expression, the total RNA of sgITGB3-MC3T3-E1 and KRAB-MC3T3-E1 cells were extracted with Trizol reagent (Invitrogen) following the manufacturer’s instructions. An equal amount of each sample (1 μg) was used for reverse transcription reaction (Kit High Capacity, Invitrogen-Life Technologies). Real-time PCR was performed in a Step One Plus Real-Time PCR (Thermo Fischer Scientific) with TaqMan® PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reactions were done in triplicates (n=3), using 5 μL of TaqMan® Gene Expression Master Mix, 0.5 μL of TaqMan® probes and 4.5 μL of cDNA (11.25 ng). The relative integrin $\beta 3$ gene expression in sgITGB3-MC3T3-E1 cells was normalized to β -actin expression and the changes were relative to the expression of KRAB-MC3T3-E1 cells.

Effect of integrin $\beta 3$ silencing on osteoblast responses to Ti-Nano and Ti-Micro

Based on the analyses of each sgRNA, we selected the cells with the most efficient sgITGB3 to be seeded on both Ti surfaces. Then, KRAB-MC3T3-E1 and sgITGB3-MC3T3-E1 cells were counted, seeded at a cell density of 2×10^4 cells per disc in 24-well culture plates (Corning Incorporated) containing Ti-Nano and Ti-Micro discs and cultured in growth medium for up to 10 days. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. On day 7, it was evaluated the gene expression of runt-related transcription factor 2 (*Runx2*), Osterix (*Sp7*), alkaline phosphatase (*Alp*), osteocalcin (*Oc*), bone sialoprotein (*Bsp*) and osteopontin (*Opn*), by real-time PCR (n=3), and the protein expression of RUNX2, by Western blot (n=3), as described above. The RUNX2 protein was detected with a primary antibody anti-RUNX2 (1:1000, MBL, Woburn, MA, USA) and a secondary antibody goat-anti-mouse IgG HRP conjugate (1:2000, Santa Cruz Biotechnology). The RUNX2 protein expression in sgITGB3-MC3T3-E1 cells was normalized to β -actin protein and calibrated by KRAB-MC3T3-E1 cells (n=3). The β -actin protein was detected by using a primary antibody anti- β -actin (1:1000, Cell Signaling, Danvers, MA, USA), followed by secondary antibody goat-anti-mouse (1:3000, Sigma-Aldrich). In addition, the *in situ* ALP activity was evaluated by Fast red assay on day 10, as described below.

ALP histochemistry

In order to detect *in situ* ALP activity, 0.8 ml of a solution of 1.8 mM Fast Red-TR 1,5-naphthalenedisulfonate salt (Sigma-Aldrich) and 0.9 mM Naphtol AS-MX phosphate (Sigma-Aldrich) was added in the wells with cells cultured on Ti-Nano and Ti-Micro. The plates were kept at 37°C for 30 min, the solution was removed and the Ti discs were dried overnight before examining by fluorescence in a microscope Axio Imager M2 (Carl Zeiss, Göttingen, GO, DE) equipped with an Axiocam MRm digital camera (Carl Zeiss). The images were processed using Adobe Photoshop CS5 Extended software (version 12.1 - Adobe Systems, San Jose, CA, USA). The *in situ* ALP was quantified by counting pixels on six discs of both Ti-Nano and Ti-Micro within three areas randomly selected in each disc (n=18).

Effect of integrin $\beta 3$ silencing on the integrin αv gene expression of osteoblasts cultured on Ti-Nano and Ti-Micro

On day 7, the gene expression of integrin $\beta 3$ and integrin αv was evaluated in KRAB-MC3T3-E1 and sgITGB3-MC3T3-E1 cells cultured on Ti-Nano and Ti-Micro, by real-time PCR (n=3), as described above

Effect of integrin $\beta 3$ silencing on the gene expression of Wnt and BMP signaling pathway components of osteoblasts cultured on Ti-Nano and Ti-Micro

On day 7, the gene expression of some components of Wnt signaling pathway, adenomatous polyposis coli (*Apc*), *Axin1*, β -Catenin, *Cke1*, Frizzled 5 (*Fzd5*), glycogen synthase kinase 3β (*Gsk3b*) and protein phosphatase 2A (*Pp2a*), and BMP signaling pathway, activin receptor-like kinase 2 (*Alk2*), bone morphogenetic protein receptor type 1A (*Bmpr1a*), bone morphogenetic protein receptor type 2A (*Bmpr2a*), *Smad1* and *Smad4*, was evaluated in

KRAB-MC3T3-E1 and sgITGB3-MC3T3-E1 cells cultured on Ti-Nano and Ti-Micro, by real-time PCR (n=3), as described above.

Statistical analysis

All the data were analyzed using Student's t-test and the results were expressed as mean $\pm$ standard deviation. The level of significance was established at $p = 0.05$.

RESULTS

Surface characterization

Ti-Nano and Ti-Micro displayed different topographical features. While Ti-Nano exhibited a surface with a network of nanocavities (Fig. 1A and B), Ti-Micro showed numerous cavities at the micrometer level (Fig. 1C and D). Both the Ra and Rz values were lower ($p=0.001$ for both) for Ti-Nano in comparison with Ti-Micro (Fig. 1E and F).

Gene expression related to the integrin signaling pathway

From 87 evaluated genes (Table S1), compared with Ti-Micro, Ti-Nano upregulated (modulation 1.9-fold, $p = 0.05$) the expression of 10 genes, 5 integrins, 1 involved in G protein signaling, 1 involved in AKT/PI-3-kinase signaling, 2 members of the focal adhesion kinase signaling and 1 cell surface receptor (Table 1).

CRISPR-Cas9 efficiency

Because Ti-Nano upregulated the expression of integrin $\beta 3$, a subunit that favors cell differentiation in osteoblast,²¹ we hypothesized that the signaling pathway downstream to integrin $\beta 3$ could be involved in the osteogenic potential of this nanotopography and to investigate it, we knockdown the integrin $\beta 3$ using CRISPR-Cas9. On day 3 after cell sorting, the gene and protein expression of integrin $\beta 3$ were evaluated in cultures of KRAB-MC3T3-E1 and sgITGB3-MC3T3-E1 cells. The most efficient sgRNA reduced nearly 82% of the integrin $\beta 3$ gene expression ($p=0.001$, Fig. 2A) and 83% at protein level ($p=0.013$, Fig. 2B).

Effect of integrin $\beta 3$ silencing on osteoblast responses to Ti-Nano and Ti-Micro

On Ti-Nano the gene expression of *Runx2* (Fig. 3A, $p=0.001$), *Sp7* (Fig. 3B, $p=0.001$), *Alp* (Fig. 3C, $p=0.001$), *Oc* (Fig. 3D, $p=0.002$), *Bsp* (Fig. 3E, $p=0.001$) and *Opn* (Fig. 3F, $p=0.005$) as well as the protein expression of RUNX2 was lower after integrin $\beta 3$ silencing (Fig. 4A, $p=0.001$). Also, the *in situ* ALP activity reduced 81% after integrin $\beta 3$ silencing (Fig. 4B, $p=0.018$). On Ti-Micro the gene expression of *Runx2* (Fig. 5A, $p=0.013$) and *Bsp* (Fig. 5E, $p=0.001$) was higher while *Sp7* (Fig. 5B, $p=0.001$) and *Alp* (Fig. 5C, $p=0.044$) were lower after integrin $\beta 3$ silencing. In addition, the gene expression of *Oc* (Fig. 5D, $p=0.200$) and *Opn* (Fig. 5F, $p=0.116$) was not significantly affected by integrin $\beta 3$ silencing. The integrin $\beta 3$ silencing did not affect the protein expression of RUNX2 (Fig. 6A, $p=0.730$) but reduced 60% the *in situ* ALP activity (Fig. 6B, $p=0.001$).

Effect of integrin $\beta 3$ silencing on the integrin αv gene expression of osteoblasts cultured on Ti-Nano and Ti-Micro

Based on the fact that the integrin $\alpha v/\beta 3$ heterodimer is of relevance to the process of osteoblast differentiation,^{22,23} we evaluated the impact of integrin $\beta 3$ silencing on integrin αv gene expression in osteoblasts cultured on Ti-Nano and Ti-Micro. As expected, the integrin $\beta 3$ silencing reduced the integrin $\beta 3$ gene expression on Ti-Nano (Fig. 7A, $p=0.001$) and on Ti-Micro (Fig. 7C, $p=0.026$). The integrin $\beta 3$ silencing inhibited the integrin αv gene expression (Fig. 7B, $p=0.008$) on Ti-Nano but not on Ti-Micro (Fig. 7D, $p=0.293$).

Effect of integrin $\beta 3$ silencing on the gene expression of Wnt and BMP signaling pathway components of osteoblasts cultured on Ti-Nano and Ti-Micro

Because a crosstalk among integrins, Wnt and BMP signaling pathways has been described in the literature,^{24,25} we investigated if the integrin $\beta 3$ silencing could affect the gene expression of Wnt and BMP signaling pathway components in osteoblasts cultured on both Ti surfaces. For the Wnt components, on Ti-Nano, the gene expression of *Apc* ($p=0.002$), *Axin1* ($p=0.007$), *β -catenin* ($p=0.006$), *Cke1* ($p=0.002$), *Fzd5* ($p=0.020$), *Gsk3b* ($p=0.001$) and *Pp2a* ($p=0.022$) was lower after integrin $\beta 3$ silencing (Fig. 8A) while on Ti-Micro the gene expression of *Apc* ($p=0.504$), *Axin1* ($p=0.505$), *β -catenin* ($p=0.091$), *Cke1* ($p=0.828$), *Fzd5* ($p=0.572$), *Gsk3b* ($p=0.465$) and *Pp2a* ($p=0.052$) was not significantly affected by integrin $\beta 3$ silencing (Fig. 8C). A similar outcome was observed for BMP components where, on Ti-Nano, the gene expression of *Alk2* ($p=0.004$), *Bmpr1a* ($p=0.006$), *Bmpr2a* ($p=0.001$), *Smad1* ($p=0.004$) and *Smad4* ($p=0.027$) was lower after integrin $\beta 3$ silencing (Fig. 8B). However, on Ti-Micro the gene expression of *Bmpr2a* ($p=0.045$) was lower while *Alk2* ($p=0.794$), *Bmpr1a* ($p=0.664$), *Smad1* ($p=0.623$) and *Smad4* ($p=0.261$) were not significantly affected by integrin $\beta 3$ silencing (Fig. 8D).

DISCUSSION

The Ti may modulate cell and tissue functions and modifications of its surface topography represent an excellent strategy to achieve better osseointegration.^{26–28} In this study, we have demonstrated that integrin $\beta 3$ regulates the interactions between osteoblasts and Ti surfaces depending on their topographies either micro or nanotopography in different ways, with more pronounced effects on nanotopography. Silencing integrin $\beta 3$ with CRISPR-Cas9 significantly reduced the osteoblast differentiation induced by Ti-Nano but this effect was not evident on T-Micro. In addition, only in cells cultured on Ti-Nano integrin $\beta 3$ silencing downregulated the expression of integrin αv and several components of the Wnt/ β -catenin and BMP/Smad signaling pathways, all involved in osteoblast differentiation (Fig. 9). Our results suggest that the higher impact of integrin $\beta 3$ silencing on the osteogenic potential of nanotopography may be due a crosstalk among integrin/Wnt/BMP signaling pathways promoted by this Ti surface.

The chemical and physical characteristics of this Ti-Nano have already been described and here we confirmed its differences in terms of topography and roughness compared with a commercially available surface with microtopography.¹⁵ The osseoinductive capacity makes this nanotopography an appealing model of investigation of the signaling pathways triggered

by an osteogenic microenvironment that drive the process of osseointegration.^{1,8,29} In this context, we have already shown that this surface regulates several cell machinery components, including integrins, in order to induce osteoblast differentiation.^{5,6,8,30}

The role of integrin signaling in cell responses to Ti with different topographies has been investigated showing the participation of some integrins in the osteoblast differentiation induced by these surfaces.^{2,6,31,32} In a previous study, we have shown that by blocking the signaling downstream to the integrin $\alpha 1\beta 1$ heterodimer, the osteoblast differentiation of MSCs induced by nanotopography is dramatically reduced.⁶ Here, we used a customized PCR array and observed that the nanotopography upregulates the expression of integrins $\alpha 10$, $\alpha 11$, αL , αM and $\beta 3$ compared with microtopography. In general, the integrins $\alpha 10$, $\alpha 11$, αL and αM mediate several mechanisms of cell-cell and cell-substrate adhesion in different cells including leucocytes, fibroblasts and osteoblasts, but there are no evidences of their participation in osteoblast differentiation.^{33–37} On the other hand, the involvement of integrin $\beta 3$ in bone formation and homeostasis has been demonstrated in several *in vitro* and *in vivo* models.^{21,38–40}

The knockdown of integrin $\beta 3$ decreased the ALP activity and extracellular matrix mineralization in cultures of MC3T3-E1 cells.²¹ In keeping with these findings, here, we demonstrated that the integrin $\beta 3$ silencing through the use of a novel gene editing tool, the CRISPR-Cas9, inhibited osteoblast differentiation of cells cultured on nanotopographic surface as evidenced by reduced gene expression of several bone markers as well as RUNX2 protein expression and ALP activity. Such negative effect on osteoblast differentiation was not so clear in cells cultured on microtopographic surface since the expression of the evaluated osteoblast genotypic and phenotypic markers was increased, reduced or not affected by integrin $\beta 3$ silencing. Together, these results suggest that the signaling triggered by integrin $\beta 3$ is of relevance to the osteogenic potential of nanotopography but not to the microtopography. Our hypothesis is supported by the upregulation of integrin $\beta 3$ gene expression induced by a nanostructured Ti and the lack of effect of a microstructured Ti on its expression as reported elsewhere.^{41,42} However, different from those studies that only evaluated the expression of integrin $\beta 3$ in cells cultured on Ti surfaces, here we genetically modified the cells to silence the expression of integrin $\beta 3$ in order to investigate its participation in osteoblast/Ti interactions.

The integrin $\alpha v\beta 3$ complex mediates osteoblast differentiation and extracellular matrix mineralization since the downregulation or upregulation of its downstream signaling inhibits and stimulates these events, respectively.^{22,23} In addition, the integrin $\alpha V\beta 3$ is crucial for the MSCs commitment to the osteoblast lineage through its interactions with extracellular matrix ligands, such as vitronectin and osteopontin.⁴³ Here, the integrin $\beta 3$ silencing resulted in a concomitant decrease of the integrin αv gene expression only in cells cultured on nanotopography. Corroborating this finding and our observation of the key role of integrin $\beta 3$ in the osteogenic induction of Ti with nanotopography, it has been shown that the integrin $\alpha v\beta 3$ complex is upregulated on nanostructured surfaces.⁴⁴

The integrins may establish a signaling network with Wnt and BMP in order to mediate osteoblast differentiation.²⁴ The knockout of integrin-linked kinase, a downstream effector

of integrins, reduced the Wnt/ β -catenin and BMP/Smad signaling, which resulted in reduced trabecular bone mass *in vivo* and inhibition of both collagen matrix production and mineralization *in vitro*.²⁵ Here, we have shown that the integrin β 3 silencing reduced the expression of several genes that encode proteins involved in both Wnt/ β -catenin and BMP/Smad signaling pathways of cells cultured only on Ti with nanotopography. Thus, it suggests that the higher osteogenic potential of Ti-Nano is due to its ability to upregulate the integrin β 3 expression and consequently activating Wnt and BMP signaling pathways in order to create a favorable microenvironment to drive osteoblast differentiation. In agreement with this, the association of nanomaterials with exogenous BMP-2 increased osteoblast differentiation through a mechanism of activating integrin β 1 and induction of Wnt-3a signaling pathway.²⁴

In conclusion, our results showed that the signaling pathway triggered by integrin β 3 exerts a striking role in the osteogenic potential of Ti-Nano but not of Ti-Micro. By using CRISPR-Cas9 to silence integrin β 3, we observed that the osteoblast differentiation induced by nanotopography was reduced concomitantly with a downregulation of the expression of several components of the Wnt/ β -catenin and BMP/Smad signaling pathways. Taken together, these findings uncover a novel mechanism to explain, at least in part, the higher osteoblast differentiation induced by Ti-Nano that involves a complex regulatory network triggered by integrin β 3 upregulation, which activates the Wnt and BMP signal transductions. The exploration of this mechanism may pave the way for developing smart nanomaterials capable of switching on and/or off specific signaling pathways to control the process of osseointegration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Fabiola S. de Oliveira, Natalie A. Page and Roger R. Fernandes are acknowledged for technical assistance during the experiments.

FUNDING INFORMATION

This study was supported by the State of São Paulo Research Foundation (FAPESP, Brazil, # 2013/05181-3, 2014/08443-1 and 2016/21116-5), National Council for Scientific and Technological Development (CNPq, Brazil, # 303464/2016-0) and Coordination of Improvement of Higher Education Personnel (CAPES, Brazil).

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Figure 1. Surface characterization of Ti with nanotopography (Ti-Nano) and Ti with microtopography (Ti-Micro). High resolution scanning electron micrographs of Ti-Nano (A and B) and Ti-Micro (C and D) and roughness parameters, average roughness (Ra, E) and average peak-to-valley roughness (Rz, F). The data are presented as mean $\pm$ standard deviation (n=15) and the asterisks (*) indicate statistically significant difference (p < 0.05).

Figure 2.

Efficiency of CRISPR-Cas9 to silence integrin β 3. Gene (A) and protein expression (B) of integrin β 3 of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells on day 3 after cell sorting. The data are presented as mean $\pm$ standard deviation (n=3) and the asterisks (*) indicate statistically significant difference (p 0.05).

Figure 3.

Effect of integrin $\beta 3$ silencing on gene expression of some osteoblast markers of cells cultured on Ti with nanotopography (Ti-Nano). Gene expression of runt-related transcription factor 2 (*Runx2*, A), Osterix (*Sp7*, B), alkaline phosphatase (*Alp*, C), osteocalcin (*Oc*, D), bone sialoprotein (*Bsp*, E) and osteopontin (*Opn*, F) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells on day 7. The data are presented as mean $\pm$ standard deviation (n=3) and the asterisks (*) indicate statistically significant difference (p < 0.05).

Figure 4.

Effect of integrin $\beta 3$ silencing on some markers of osteoblast phenotype of cells cultured on Ti with nanotopography (Ti-Nano). Protein expression of runt-related transcription factor 2 (RUNX2), on day 7, (A) and *in situ* alkaline phosphatase (ALP) activity, on day 10, (B) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells. The data RUNX2 protein expression (n=3) and of *in situ* ALP Activity (n=18) are presented as mean $\pm$ standard deviation and the asterisks (*) indicate statistically significant difference (p 0.05).

Figure 5.

Effect of integrin $\beta 3$ silencing on gene expression of some osteoblast markers of cells cultured on Ti with microtopography (Ti-Micro). Gene expression of runt-related transcription factor 2 (*Runx2*, A), Osterix (*Sp7*, B), alkaline phosphatase (*Alp*, C), osteocalcin (*Oc*, D), bone sialoprotein (*Bsp*, E) and osteopontin (*Opn*, F) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells on day 7. The data are presented as mean $\pm$ standard deviation (n=3) and the asterisks (*) indicate statistically significant difference (p < 0.05).

Figure 6.

Effect of integrin $\beta 3$ silencing on some markers of osteoblast phenotype of cells cultured on Ti with microtopography (Ti-Micro). Protein expression of runt-related transcription factor 2 (RUNX2), on day 7, (A) and *in situ* alkaline phosphatase (ALP) activity, on day 10, (B) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells. The data RUNX2 protein expression (n=3) and of *in situ* ALP Activity (n=18) are presented as mean $\pm$ standard deviation and the asterisk (*) indicates statistically significant difference (p 0.05).

Figure 7.

Effect of integrin $\beta 3$ silencing on the integrin αv (*Itgav*) gene expression of osteoblasts grown on Ti with nanotopography (Ti-Nano) and on Ti with microtopography (Ti-Micro). Gene expression of *Itgb3* (A,C) and *Itgav* (B,D) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells, on day 7. The data are presented as mean $\pm$ standard deviation (n=3) and the asterisks (*) indicate statistically significant difference (p 0.05).

Figure 8.

Effect of integrin $\beta 3$ silencing on the gene expression of Wnt and BMP signaling pathway components of osteoblasts cultured on Ti with nanotopography (Ti-Nano) and Ti with microtopography (Ti-Micro). Gene expression of adenomatous polyposis coli (*Apc*), *Axin1*, β -Catenin, *Cke1*, Frizzled 5 (*Fzd5*), glycogen synthase kinase 3 β (*Gsk3b*) and protein phosphatase 2A (*Pp2a*) (A,C) and of activin receptor-like kinase 2 (*Alk2*), bone morphogenetic protein receptor type 1A (*Bmpr1a*), bone morphogenetic protein receptor type 2A (*Bmpr2a*), *Smad1* and *Smad4* (B,D) of sgITGB3-MC3T3-E1 (sgITGB3) and KRAB-MC3T3-E1 (KRAB) cells, on day 7. The data are presented as mean $\pm$ standard deviation (n=3) and the asterisks (*) indicate statistically significant difference (p 0.05).

Figure 9.

Schematic representation of the participation of integrin $\beta 3$ on osteoblast differentiation induced by Ti with nanotopography (Ti-Nano) and Ti with microtopography (Ti-Micro).

Table 1.

Expression of genes related to integrin signaling pathway upregulated (≥ 1.9 -fold, $p \leq 0.05$) in MSCs grown on Ti with nanotopography (Ti-Nano) compared with Ti with microtopography (Ti-Micro) on day 3

Gene	Ti-Micro	Ti-Nano	p Value
<i>Actb</i> (β -actin)	1	1	1
Integrins			
<i>Itga10</i> (Integrin $\alpha 10$)	1	1.98	0.001
<i>Itga11</i> (Integrin $\alpha 11$)	1	2.02	0.001
<i>Itgal</i> (Integrin αL)	1	1.98	0.001
<i>Itgam</i> (Integrin αM)	1	2.01	0.001
<i>Itgb3</i> (Integrin $\beta 3$)	1	2.02	0.001
G Protein Signaling			
<i>Pak3</i> (Serine/threonine-protein kinase 3)	1	2.03	0.001
AKT / PI-3-Kinase Signaling			
<i>Vav1</i> (Proto-oncogene vav)	1	1.99	0.001
Focal Adhesion Kinase Signaling			
<i>Shc2</i> (SHC-transforming protein 2)	1	2.02	0.001
<i>Prkcb</i> (Protein kinase C beta type)	1	4.01	0.001
Other Cell Surface Receptors (Caveolins)			
<i>Cav3</i> (Caveolin 3)	1	2.00	0.001