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# Effect of blasting treatment and Fn coating on MG63 adhesion and differentiation on titanium: a gene expression study using real-time RT-PCR

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**Abstract** Biomaterial surface properties, via alterations in the adsorbed protein layer, and the presence of specific functional groups can influence integrin binding specificity, thereby modulating cell adhesion and differentiation processes. The adsorption of fibronectin, a protein directly involved in osteoblast adhesion to the extracellular matrix, has been related to different physical and chemical properties of biomaterial surfaces. This study used blasting particles of different sizes and chemical compositions to evaluate the response of MG63 osteoblast-like cells on smooth and blasted titanium surfaces, with and without fibronectin coatings, by means of real-time reverse transcription-polymerase chain reaction (qRT-PCR) assays. This response included (a) expression of the $\alpha_5$, $\alpha_v$ and $\alpha_3$ integrin subunits, which can bind to fibronectin through the RGD binding site, and (b) expression of alkaline phosphatase (ALP) and osteocalcin (OC) as cell-differentiation markers. ALP activity and synthesis of OC were also tested. Cells on SiC-blasted Ti surfaces expressed higher amounts of the $\alpha_5$ mRNA gene than cells on $Al_2O_3$-blasted Ti surfaces. This may be related to the fact that SiC-blasted surfaces adsorbed higher amounts of fibronectin due to their higher surface free energy and therefore provided a higher number of specific cell-binding sites. Fn-coated Ti

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surfaces decreased $\alpha_5$ mRNA gene expression, by favoring the formation of other integrins involved in adhesion over $\alpha_5\beta_1$. The changes in $\alpha_5$ mRNA expression induced by the presence of fibronectin coatings may moreover influence the osteoblast differentiation pathway, as fibronectin coatings on Ti surfaces also decreased both ALP mRNA expression and ALP activity after 14 and 21 days of cell culture.

## 1 Introduction

The biological processes that take place when cells come into contact with a material surface include initial focal adhesion formation, cell adhesion, and extracellular matrix formation and reorganization. The quality of the cell–material interaction influences cells' capacity to proliferate and differentiate [1]. Cells indirectly bind to surfaces producing cytoskeletal associated transmembrane receptors that attach to specific extracellular proteins [2], such as fibronectin and vitronectin [3]. Integrins are the most important transmembrane receptors in cell binding processes [4, 5]. Biomaterial surface properties, via alterations in adsorbed protein structure, and the presence of specific functional groups may influence integrin binding specificity, thereby modulating signaling and expression of differentiated phenotypes [6–10].

The use of human cell lines such as MG63 offers a useful tool for investigating the effects of biomaterials and for identifying the mechanisms of cell response. To understand the mechanisms of bone formation on the implant surface, the material's effects on the surrounding cells must be analysed. In vitro studies support the hypothesis that implant surface may directly affect osteoblast migration, attachment, proliferation, and differentiation, as increased surface roughness has been shown to stimulate osteogenesis [11–13]. In addition to having cellular characteristics associated with osteogenic differentiation, surface roughness has also been shown to alter integrin and growth factor expression [14].

MG63 osteoblast-like osteosarcoma cells cultured on Ti of varying roughnesses have shown different osteoblastic differentiation expression depending on the degree of roughness, and $R_a$ values of micro-rough implant surfaces ranging from 3 to 5 μm increased in vitro and in vivo cell response [12, 15]. Cells cultured on Ti discs with an average roughness >6 μm showed a reduced capacity to differentiate compared with cells cultured on smooth (<0.1 μm) surfaces. This is because the increase in real surface area implies a lower cell confluence at the time of measurement, resulting in reduced ALP activity [16]. However, the effect of surface roughness on cells may be the result of the topographical/surface roughness characteristics of the surface itself, as well as of the layer of proteins and other biomolecules adsorbed on the surface. The latter is also influenced by the surface characteristics, which emerge as the material surface is conditioned by the media and serum. This initial interaction produces a layer of macromolecules that directly modulate cell response.

Fibronectin is an adhesive protein that mediates cell adhesion and the only protein that activates the $\alpha_5\beta_1$ integrins of cells [2, 17]. Thus, $\alpha_5$ is an integrin subunit specific for fibronectin protein [17]. Several studies have demonstrated that on Ti and Ti-alloy surfaces, osteoblasts express $\alpha_5$ integrin subunits, among others [17, 18]. The effect of fibronectin coating has been added as a variable in this study, since human fibronectin is a glycoprotein that promotes cell attachment [19] to the biomaterial surface through its central-binding domain arginine–glycine–aspartic acid (RGD) sequence. The RGD sequence is present in a variety of adhesive proteins (e.g. fibronectin and vitronectin) and recognized by several integrins. Specifically, integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ bind to and compete for the RGD site on Fn [20, 21]. Moreover, $\alpha_3\beta_1$ can also bind to fibronectin through the RGD binding site [21], and $\alpha_3$ is one of the most abundant $\alpha$ subunits expressed in unstimulated MG63 cells [22].

Previously in our group, an optimized shot-blasted titanium (c.p. Ti) dental implant was developed [23]. The optimal roughness and appropriate abrasive particles for a better in vitro response and earlier in vivo osseointegration were determined [11]. However, the first biological events leading to this successful response and the exact influence of the surface properties are still poorly understood. To address these questions, first, a thorough topographical and surface free energy characterization of the optimized surfaces was carried out [24]. Studies of fibronectin adsorption on the different roughened surfaces revealed differences in both the adsorbed fibronectin and the spatial organization of the extracellular matrix depending on specific topographical and physico-chemical properties of the tested surface [25].

As a further step, in this work we test the MG63 osteoblast-like cells response on smooth and blasted titanium surfaces—different sizes and chemical composition of the blasting particles were used with and without fibronectin coatings using real time reverse transcription-polymerase chain reaction (qRT-PCR). We have studied the cell adhesion response quantifying $\alpha_5$, $\alpha_3$, and $\alpha_v$ integrin-subunit gene expression; and the cell differentiation response quantifying alkaline phosphatase (ALP) and osteocalcin (OC) gene expression as well as ALP activity and synthesis of OC.

## 2 Materials and methods

### 2.1 Materials

#### *2.1.1 Surfaces*

Seven different series of commercially pure titanium grade 2 (c.p. Ti) disks, 8 mm in diameter and 2 mm thick, were obtained depending on the surface treatments applied to each sample:

Smooth: polished c.p. Ti. The samples were subsequently abraded with 400, 600 and 1200 grit silicon carbide abrasive paper and then polished with a water suspension of 1 μm alumina powder.

A3: c.p. Ti blasted with $Al_2O_3$ particles of 212–300 μm in size.
A6: c.p. Ti blasted with $Al_2O_3$ particles of 425–600 μm in size.
A9: c.p. Ti blasted with $Al_2O_3$ particles of 1,000–1,400 μm in size.
S3: c.p. Ti blasted with SiC particles of 212–300 μm in size.
S6: c.p. Ti blasted with SiC particles of 425–600 μm in size.
S9: c.p. Ti blasted with SiC particles of 1,000–1,400 μm in size.

For the qRT-PCR experiments, only the smooth, A6 and S6 surface finishes were tested. Tissue culture polystyrene (TCPS) was studied as a positive control for these experiments.

Blasting was carried out with a laboratory blasting machine at 0.25 MPa pressure for the time required for saturation of the roughness of the samples. The particles used for each sample series were as shown above.

After the blasting, the samples were cleaned by sonication in acetone for 15 min, followed by sonication in distilled water for 15 min. Finally, all samples were steam-sterilized at 121°C for 30 min and kept under vacuum.

#### *2.1.2 Fn coating*

Two series of the different surface finishes were performed in parallel for all the studies: (a) fibronectin (Fn) pre-coated surfaces; and (b) uncoated/plain surfaces. The former were coated with human Fn (Sigma-Aldrich) at a concentration of 20 μg/ml for 30 min at 37°C. The latter were not coated prior to the in vitro cell assays.

#### *2.1.3 Cells*

Human osteoblast-like MG63 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% L-glutamine, and 1% pyruvate (Sigma-Aldrich) at 37°C in a 5% $CO_2$/95% air atmosphere and at 100% humidity. The MG63 cells were obtained from American Type Culture Collection (Rockville, MD). The culture medium was changed every 2 days. For the experiments, cells were harvested at 70–90% confluence by trypsin/EDTA, centrifuged and re-suspended in a serum-free medium before being plated at $2 \times 10^4$ MG63 cells per sample.

For the differentiation studies, after 7 days of incubation, the time course of mineralization was accelerated by changing to an osteogenic medium, consisting of a complete medium supplemented with $10^{-8}$ M dexamethasone, 100 μM 2-phosphate ascorbic acid, and 10 mM $\beta$-glycerophosphate.

### 2.2 Methods

#### *2.2.1 Real-time qRT-PCR technique*

Gene expression of integrins $\alpha_5$, $\alpha_3$ and $\alpha_v$ (30 min and 4 h incubation) and of ALP and OC (7 and 14 days of culture) was determined through a real-time reverse transcription-polymerase chain reaction (qRT-PCR) assay. $\beta_2$-μglobulin was used as a housekeeping gene.

The following protocol was applied: (1) mRNA extraction; (2) spectrophotometric quantification of RNA; (3) reverse transcription (RT); and (4) polymerase chain reaction (PCR).

A kit (RNeasy Minikit, QIAGEN, Germany) was used to extract the mRNA from the cell pellets, and quantification was achieved by measuring the absorbance with a NanoDrop® Spectrophotometer ND-1000 (Thermo Scientific, USA) using the ND-1000 software (v 3.5.2) (Thermo Scientific, USA). Reverse transcription is accomplished when the enzyme reverse transcriptase makes a double-stranded cDNA copy of the mRNA molecules (QuantiTect Reverse Transcription Kit, QIAGEN, Germany). The resulting cDNA is then amplified using the SYBR green method (QuantiTect SYBR Green kit, QIAGEN, Germany). This requires a double-stranded DNA dye in the PCR reaction that binds to newly synthesized double-stranded DNA and generates fluorescence.

#### *2.2.2 Cell differentiation markers*

Cell differentiation was also assessed by measuring alkaline phosphatase activity (ALP) and osteocalcin production (OC). Cells were culture for 14 and 21 days in osteogenic media as described above. An additional control group consisted of cells on non-coated surfaces after 3 days in culture was tested. Both ALP and OC activity were

normalised to the total protein concentration quantified with a BCA Protein Assay kit (PIERCE).

ALP activity was measured with a Phosphatase, Alkaline Acid, Prostatic Acid Assay kit (SIGMA Diagnostics). Samples were rinsed twice in phosphate-buffered saline (PBS) and soaked in 500 µl MPER (M-PER Mammalian Protein Extraction Reagent, Thermo Scientific). The MPER was meant to detach the cells from the surfaces and lysate them. Readings were taken at 405 nm on a PowerWaveX Bio-Tek Instruments spectrophotometer.

The osteocalcin (OC) concentration was measured by means of a Metra® Osteocalcin kit (Roche), a competitive immunoassay, and normalized to BCA readings.

Each group was tested in triplicate.

#### 2.2.3 Statistical analysis

ANOVA tables with Fisher's or Tukey's post-hoc multiple-comparison tests were performed using Minitab™ Release 14 software (Minitab Inc., USA) to assess statistically significant differences ($P$ value $\leq 0.05$) between groups. Tukey's test was performed when comparing groups with samples of different size.

## 3 Results

### 3.1 Cell adhesion

#### 3.1.1 $\alpha_5$, $\alpha_v$ and $\alpha_3$ integrin subunit expression

Cells' $\alpha_5$ integrin subunit ($\alpha_5$ mRNA) gene expression increased with time on all surfaces except smooth ones (Fig. 1a). After 30 min in culture, cells on smooth Ti surfaces showed the highest levels of $\alpha_5$ mRNA expression. However, after 4 h of cell culture, cells on rough Ti surfaces expressed higher levels of $\alpha_5$ mRNA than cells on smooth Ti samples. In contrast, gene expression of $\alpha_v$ and $\alpha_3$ integrin subunits increased with time on all surfaces except the alumina-blasted series. The A6 surface finish showed a decrease in $\alpha_3$ mRNA with time (Fig. 1b), while gene expression levels of $\alpha_v$ (Fig. 1c) remained statistically constant with time.

The SiC-blasted series (S6) expressed higher levels of $\alpha_5$ and $\alpha_v$ mRNA than the $Al_2O_3$-blasted ones (A6). This effect was more evident after 4 h of incubation (Fig. 1a, c). The $\alpha_3$ integrin subunit was also expressed more S6 surfaces than on A6, but only in the non Fn-coated series.

Osteoblastic cells on Fn pre-coated surfaces showed a lower level of $\alpha_5$ mRNA expression than cells on uncoated surfaces (Fig. 1a). This effect was most noticeable on the expression of this gene in cells in the TCPS series and least noticeable in cells cultured on smooth surfaces. Fn delayed the expression of $\alpha_5$ mRNA by cells after 4 h in culture on all studied surfaces except for smooth ones. Conversely, Fn coating increased $\alpha_v$ integrin subunit expression after 4 h of

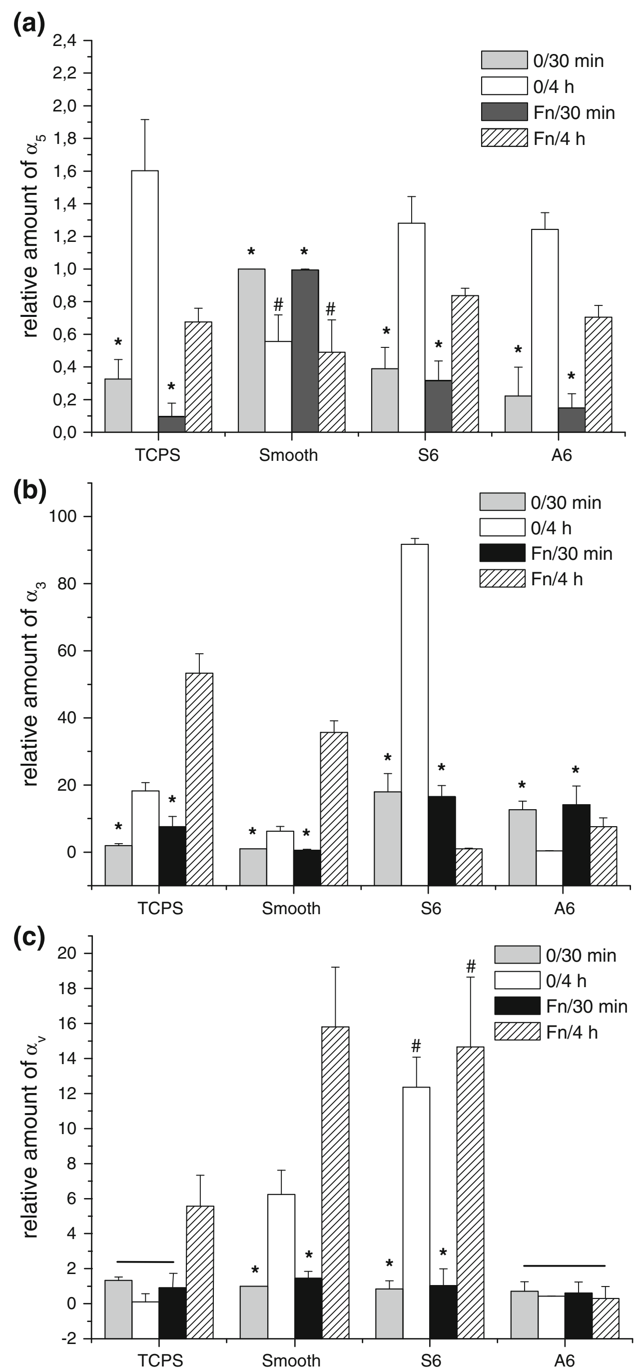


**Fig. 1** Gene expression of **a** $\alpha_5$, **b** $\alpha_3$ and **c** $\alpha_v$ integrin subunits by cells on Ti surfaces with (Fn) or without (0) Fn coating measured by qRT-PCR after 30 min and 4 h of cell culture. Ratios of target genes relative to the housekeeping gene—µglobulin—were expressed as a percentage relative to the uncoated smooth surfaces after 30 min of cell culture. *Bars* with the same symbols (*, #) indicate that the differences between them are not statistically significant when compared in the same surface finish group

incubation time on all surfaces except alumina ones, on which it remained constant (Fig. 1c). Also, Fn-coating surfaces increased gene expression of $\alpha_3$ integrin subunit on all surfaces after 4 h of incubation time, except for S6 on which a notable decrease of the $\alpha_3$ gene expression was detected (Fig. 1c).

## 3.2 Cell differentiation

### *3.2.1 Alkaline phosphatase gene expression*

The ALP mRNA gene expression of MG63 osteoblast-like cells increased with time on all the studied surfaces (Fig. 2). Cells on rough and uncoated surfaces exhibited higher levels of ALP mRNA expression than cells on TCPS surfaces.

After 7 days of cell culture, Fn coatings did not have a prevalent effect on ALP expression. In contrast, after 14 days in culture, cells on Fn-coated surfaces exhibited lower levels of ALP mRNA expression than cells on uncoated surfaces in all cases (Fig. 2).

### *3.2.2 Alkaline phosphatase activity*

The ALP results were normalized to the BCA readings. Figure 3 shows the results of the ALP/total protein ratio for cells on all smooth and rough Ti series. The cells' ALP activity increased with time on all studied surfaces. Differences were statistically significant when the results were compared after 3 days.

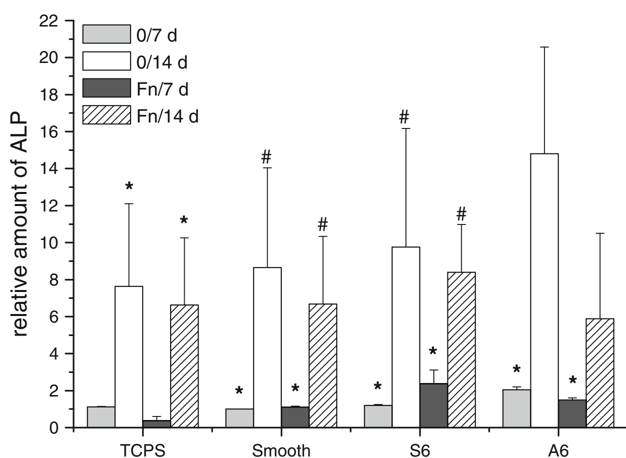


**Fig. 2** ALP gene expression of MG63 osteoblast-like cells on Ti surfaces with (Fn) or without (0) Fn coating measured by qRT-PCR after 7 and 14 days of cell culture. Ratios of target genes relative to the housekeeping gene—μglobulin—were expressed as a percentage relative to the uncoated smooth surfaces after 7 days of cell culture. *Bars* with the same symbols (*, #) indicate that the differences between them are not statistically significant when compared in the same surface finish group

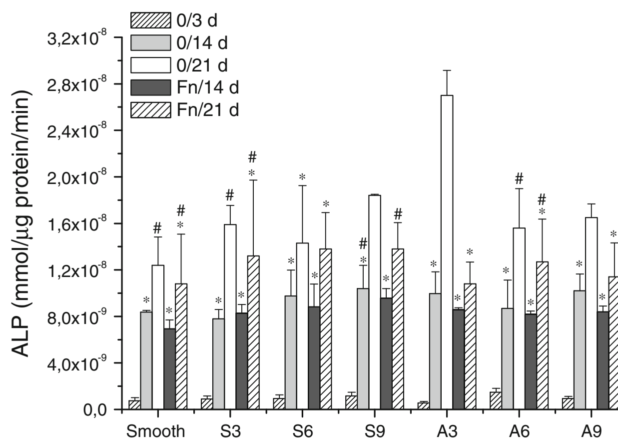


**Fig. 3** ALP activity of MG63 cells after 3, 14 and 21 days in culture on different rough Ti surfaces with (Fn) and without (0) Fn coating. *Bars* with the same symbols (*, #) indicate that the differences between them are not statistically significant when compared in the same surface finish group

Roughness did not influence the cells' ALP activity after 14 days in culture. In contrast, after 21 days of cell culture, roughness was seen to affect ALP activity, which was generally higher on rough surfaces than on smooth ones (Fig. 4). This trend was more noticeable for uncoated surfaces than for Fn-coated ones.

After 14 days of cell culture, Fn did not have a significant impact on ALP activity. Conversely, after 21 days in culture, cells on Fn pre-coated series showed lower levels of ALP activity than cells on uncoated ones (Fig. 3).

### *3.2.3 Osteocalcin gene expression*

OC mRNA expression of MG63 osteoblast-like cells increased with time with all studied surfaces (Fig. 5) except the uncoated alumina-blasted Ti ones.

The effects of roughness and Fn coating on OC mRNA gene expression were unclear. However, blasting particles had a notable effect on it. OC mRNA expression on alumina-blasted surfaces was higher than on SiC-blasted surfaces.

### *3.2.4 Osteocalcin production*

Figure 6 shows the OC production of osteoblast-like cells cultured on all the studied surfaces. OC production decreased with time in cells on uncoated surfaces (Fig. 7, left graph), whereas it showed an increase with time in cells on Fn-coated surfaces (Fig. 7, right graph).

Cells on rough surfaces had higher OC production levels than cells on smooth surfaces. This effect was more pronounced in uncoated samples than in Fn-coated ones.

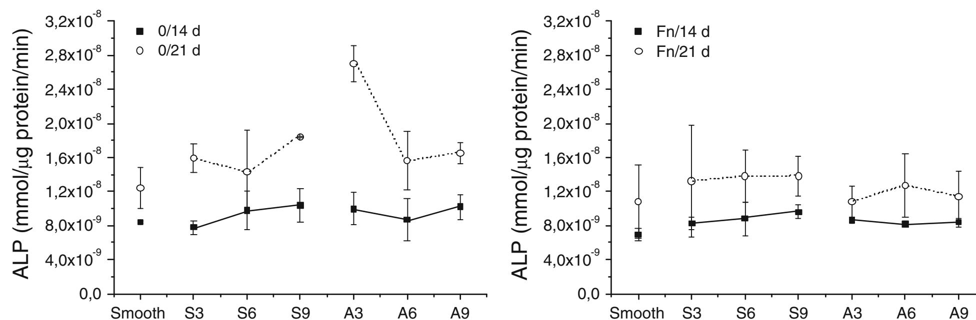


**Fig. 4** ALP activity of MG63 cells after 14 and 21 days in culture on different rough Ti surfaces. The depicted values are the same ones shown in Fig. 3, but the uncoated (*left*) and Fn-coated (*right*) series are separated, and ALP activity after 3 days is not included

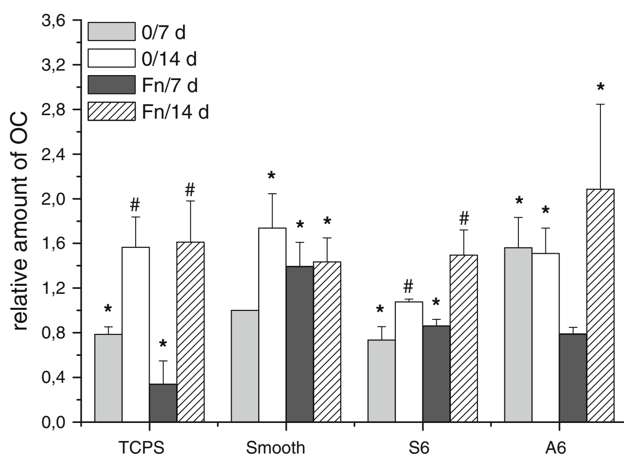


**Fig. 5** Gene expression of OC of MG63 cells after 7 and 14 days in culture on blasted Ti surfaces with (Fn) or without (0) Fn coating measured by qRT-PCR. Ratios of target genes relative to the housekeeping gene—µglobulin—were expressed as a percentage relative to uncoated smooth surfaces after 7 days in culture. *Bars* with the same symbols (*, #) indicate that differences between them are not statistically significant when compared in the same surface finish group

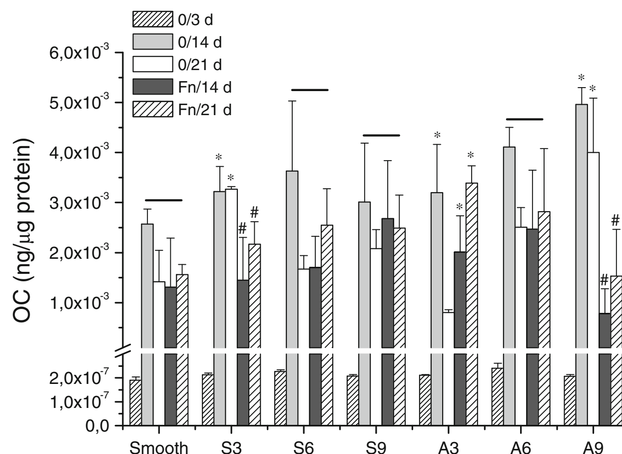


**Fig. 6** Osteocalcin production of MG63 cells after 3, 14 and 21 days in culture on different rough Ti surfaces with (Fn) and without (0) Fn coating. *Bars* with the same symbols (*, #) indicate that differences between them are not statistically significant when compared in the same surface finish group

As for the chemical composition of the blasting particles used to roughen the surfaces, cells on $Al_2O_3$-blasted surfaces showed a greater increase in OC production than cells on SiC-blasted surfaces after the same time in culture on uncoated series (Fig. 7, left graph). No correlation was found between OC production and the nature of the blasting particles for cells on Fn-coated surfaces.

## 4 Discussion

The influence of surface properties and fibronectin coatings on smooth and blasted rough Ti samples on MG63 osteoblast-like cell adhesion and differentiation was studied to learn more about their possible effect on cell osteogenic processes. Expression of $\alpha_5$ integrin subunit mRNA was investigated, since this integrin's specificity for fibronectin makes it a good indicator of the quality of cell adhesion in these experiments. $\alpha_v$ and $\alpha_3$ integrin subunits were also studied, since they compete with $\alpha_5$ to bind to the Fn RDG site; however, these integrins are not specific for this adhesive protein. Cell differentiation was studied not only by analyzing differentiation markers (alkaline phosphatase activity and osteocalcin production) using traditional techniques, but also by investigating alkaline phosphatase and osteocalcin mRNA gene expression using the more reliable qRT-PCR technique.

MG63 osteoblast-like cells, originally isolated from a human osteosarcoma, are widely used and studied for in vitro tests [12, 15, 16, 26]. They are a well-characterized cell model to study the influence of the topography of a surface on the osteoblast adhesion, proliferation and differentiation. Although a tumor cell line, MG63 cells are relatively immature osteoblasts that exhibit bone forming osteoblastic traits. Alkaline phosphatase activity and osteocalcin are examples of phenotypic markers that MG63 cells can exhibit. We focused here on assessing surface

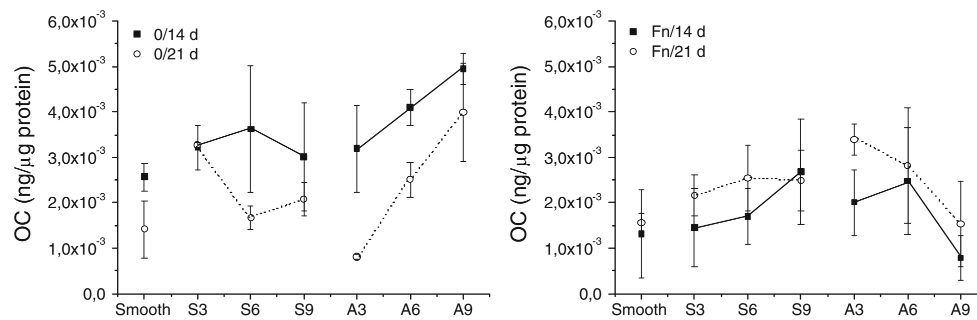


**Fig. 7** OC production of MG 63 cells after 14 and 21 days in culture on different rough Ti surfaces. The depicted values are the same ones shown in Fig. 6, but the uncoated (*left*) and Fn-coated (*right*) series are separated, and OC production after 3 days is not included

effects on osteoblasts response using the model MG63 cell line, but studies using human primary cells will be further evaluated.

### 4.1 Cell adhesion

$\alpha_5$ mRNA expression was considered a good indicator of the adhesion response for osteoblastic cells in view of previous knowledge of the selectivity of the integrin receptor $\alpha_5\beta_1$ for fibronectin. $\alpha_5\beta_1$ binding to fibronectin has been shown to be necessary for bone-like nodule formation in vitro when osteoprogenitor cells are grown on tissue culture plastic and other synthetic biomaterials [27]. However, $\alpha_5\beta_1$ is not the only integrin that binds to fibronectin. Other integrins also compete to bind to the RGD site. This is the case with the $\alpha_3\beta_1$ integrin, which binds to laminin, collagen, and fibronectin [22], and $\alpha_v\beta_3$, which binds to vitronectin, fibrinogen, fibronectin, and osteopontin, among others proteins [21].

The quantitative results for $\alpha_5$ mRNA gene expression indicated that the values of $\alpha_5$ mRNA expression increased with the time of cell culture except in cells cultured on smooth Ti surfaces (Fig. 1a). After 30 min in culture, gene expression in cells on smooth Ti surfaces significantly increased compared to cells in all other series. However, after 4 h in culture, cells on smooth Ti surfaces had the lowest gene expression values, whereas the cells on the rest of the samples maximized the gene expression differences between them. Moreover, the studies performed after 30 min in culture showed nearly no differences between the Fn pre-coated series and the uncoated ones for any of the integrin subunits (i.e. $\alpha_5$, $\alpha_v$ or $\alpha_3$). On the basis of these results, it can be concluded that 30 min of culture is too short a time for cells to develop the appropriate biological mechanisms to adhere to surfaces. Therefore, it is not advisable to differentiate the effects of the different surface treatments on osteoblast adhesion response at 30 min of cell culture.

Osteoblasts interact with the substrate through integrin receptors. The type of substrate determines which integrins and ECM proteins are expressed. After 4 h of cell culture, cells on rough-blasted Ti surfaces expressed higher levels of $\alpha_5$ mRNA than cells on smooth samples (Fig. 1a). Others have reported similar results for MG63 cultured on Ti, in which the expression of integrin subunits $\alpha_2$, $\alpha_3$, $\alpha_5$, $\beta_1$, and $\beta_3$ increased more on rougher surfaces than on polished ones [28].

Our results confirmed the influence of the nature of the blasting particles on $\alpha_3$ and $\alpha_v$ integrin subunit gene expression (Fig. 1). Cells on SiC-blasted Ti surfaces expressed higher amounts of the $\alpha_5$, $\alpha_v$ and $\alpha_3$ mRNA gene than cells on $Al_2O_3$-blasted Ti surfaces after 4 h in culture; the differences were not statistically significant for the $\alpha_5$ integrin subunit. In particular, this behavior was most noticeable with $\alpha_v$ mRNA expression. These differences may be caused by the higher amount of Fn that is absorbed on SiC-blasted surfaces, as determined previously [25]. The higher amount of adsorbed Fn, which is due to the higher surface free energy of SiC-blasted surfaces, may give the surface a higher number of specific cell-binding sites. This, in turn, would result in increased $\alpha_5$, $\alpha_v$ and $\alpha_3$ mRNA gene expression.

We previously determined an increase in number of adhered MG63 cells after 4 h of incubation when surface roughness was increased on uncoated surfaces [25]. Those findings correlate with the increase in $\alpha_5$ mRNA expression found in this work (Fig. 1). Any relation was found between cell adhesion on uncoated surfaces and the rest of the studied integrins. We also demonstrated that Fn coatings on Ti surfaces led to a decrease in number of adhered cells in combination with enhanced cell spreading. Interestingly, those results nicely correlate with decreasing and increasing $\alpha_5$ mRNA and $\alpha_V$ mRNA expression, respectively. Thus, the expression of $\alpha_5\beta_1$ integrin is the main factor influencing cell adhesion in the type of surfaces tested in this work.

Differences in the $\alpha_5$, $\alpha_v$ and $\alpha_3$ mRNA gene expression of osteoblasts detected between the different substrates studied were statistically significant only after 4 h of cell culture. This includes one of the most interesting results obtained, namely, that cells on Fn-coated Ti surfaces showed lower $\alpha_5$ mRNA gene expression than cells on the same type of surface when it was uncoated. This was true of all surface types analyzed. This is an unexpected result. One might speculate that the increased gene expression on Fn-coated surfaces is due to the aforementioned specificity of the $\alpha_5\beta_1$ integrin—and, thus, the associated $\alpha_5$ expression—to bind to fibronectin.

The $\alpha_5$ integrin subunit binds to $\beta_1$ to form the integrin $\alpha_5\beta_1$, and it is via $\alpha_5\beta_1$ that cells initiate the attachment to fibronectin [27, 29]. One reason for cells to express less $\alpha_5$ mRNA on Fn-coated surfaces might be that cells express other integrins instead of $\alpha_5\beta_1$. Moreover, different integrins can bind to Fn through the RDG site, including $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_3\beta_1$ [21]. The fact that Fn coating might provide the surface with a higher number of specific cell-binding sites may be the cause for cells to express other integrins. In this respect, cells tend to provide the necessary integrins to perform their biological activities by up- or down-regulating the required subunits and integrins.

With respect to $\alpha_2\beta_1$ integrin expression, Raz et al. [30] found higher $\beta_1$ integrin subunit expression and lower $\alpha_5$ subunit expression on Ti surfaces with rough microtopographies than on surfaces with smooth ones when MG63 cells were cultured up to confluence. Thus, a preferential $\alpha_2\beta_1$ integrin expression by cells on rough surfaces was assessed. It is worth noting that $\alpha_2\beta_1$ is known to be preferentially expressed when cells reach confluence and undergo osteoblastic differentiation [31], as observed by Raz et al. [30]. However, Anselme et al. [32] did not find any $\alpha_2\beta_1$ expression when human osteoblasts were cultured on Ti-alloy surfaces for 24 h, 7 days or 14 days.

Thus, in our experiments, an up-regulation of $\alpha_2$ integrin subunit is not expected since the shorter adhesion times we tested, i.e., cells are far from being confluent.

However, our results showed that surfaces with Fn coatings induced cells to up-regulate $\alpha_v$ integrin subunit expression on all surface finishes, except for those blasted with $Al_2O_3$ particles. Also, surfaces with Fn coatings induced cells to up-regulate $\alpha_3$ integrin subunit expression on all surface finishes, except for those blasted with SiC particles. Consequently, either the upregulation of $\alpha_v\beta_3$, or $\alpha_3\beta_1$, or both integrin subunits depending on the type of surface finishing can compensate the measured down-regulation of the Fn-specific $\alpha_5\beta_1$ integrin.

Given that both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins are known to bind to the Fn RGD site [20, 21], it is important to note that integrin binding specificity—$\alpha_5\beta_1$ versus $\alpha_v\beta_3$—has been linked in the literature by Garcia and coworkers [33] to the regulation of osteoblastic differentiation and the enhancement of in vivo bone healing and functional implant osseointegration [34]. In this study, cells were grown on rough Ti substrates, which also stimulate osteoblastic differentiation more than smooth surfaces. Improved bone tissue formation and functional osseointegration may be attributed to $\alpha_5\beta_1$-specific Ti implants because it increases recruitment of osteoprogenitor cells and differentiation into osteoblasts at the tissue-implant interface. Moreover $\alpha_5\beta_1$ integrin is the main fibronectin receptor, and its expression has been associated with increased mineralization of osteosarcoma and calvarial osteoblast cells [34]. However, the adsorption of RGD containing proteins such as fibronectin or vitronectin in a non-specific way may support $\alpha_v\beta_3$-mediated adhesion due to the conformation of the adsorbed protein. Our results support this claim, since Fn coating, adsorbed in a non-specific way, increased $\alpha_v$ integrin subunit expression on all studied surfaces except the $Al_2O_3$ ones (Fig. 1c). In fact, Garcia and coworkers [34] grafted a specific sequence of fibronectin (9th type repeat) onto Ti, which in the proper structural context exhibits high selectivity for the integrin $\alpha_5\beta_1$. They also grafted a linear RGD oligopeptide (GRGDSPC) onto Ti, demonstrating that it primarily supports $\alpha_v\beta_3$-mediated adhesion. Keselowsky [33] and Cheng et al. [35] showed that $\alpha_v\beta_3$-mediated surface adhesion suppresses osteoblastic differentiation. This agrees with our results, further explained in the following section, since Fn pre-coated surfaces showed high expression of $\alpha_v$ mRNA integrin and low expression of $\alpha_5$ mRNA integrin, low cell differentiation, and low expression of ALP mRNA and ALP activity compared to uncoated surfaces (Figs. 2, 3).

While these results showed substrate-dependent differences in integrin subunit expression levels, the influence of surface topography on integrin functions, such as binding activity and downstream biological responses, was still unknown. Therefore, further analyses were undertaken to identify the mediation of $\alpha_5$, $\alpha_v$ and $\alpha_3$ mRNA integrin subunit expression in osteoblast differentiation in relation to surface-dependent differences.

### 4.2 Cell differentiation

This study has shown that roughness and Fn coating influences $\alpha_5$, $\alpha_v$ and $\alpha_3$ mRNA gene expression and, thus, cell adhesion. Therefore, it seemed reasonable to assume that Fn coating would alter the time course of differentiation. In all types of surfaces tested, both ALP mRNA expression (Fig. 2) and ALP activity (Fig. 3) were lower (sometimes with statistically significant differences) on Fn pre-coated samples than on uncoated surfaces after 14 and 21 days of cell culture. However, none of these differences between Fn-coated and uncoated series was statistically

significant for ALP activity after 14 days of cell culture. Consequently, the decrease in MG63 $\alpha_5$ as well as increase in MG63 $\alpha_v$ mRNA expression upon cell adhesion is related to the influence of the non-specific Fn adsorption on the osteoblast differentiation pathway.

As expected, increased roughness led to increased ALP activity. This response was more significant for non pre-coated surfaces after 21 days of cell culture (Fig. 2). The same trend was seen in ALP mRNA levels after 14 days of cell culture (Fig. 3). At shorter cell culture times, when cell differentiation was in earlier stages, no statistically significant differences were found for ALP mRNA expression (7 days) or ALP activity (14 days).

Gene expression occurs earlier than the production and activity of the proteins tested as cell differentiation markers. Consequently, the qRT-PCR assays for ALP mRNA and OC mRNA were performed after shorter periods than the tests for the differentiation markers. The time of cell culture at which differentiation markers should be measured is difficult to determine, since many parameters, such as the cell line or the number of cells seeded, can influence the results obtained.

It is generally accepted that smooth surfaces promote early differentiation markers, such as ALP activity [13, 26, 36], whereas rougher surfaces promote later steps in cell maturation, such as mineralization of the ECM [13, 16, 36, 37]. The influence of rough surfaces on ALP activity has been extensively studied. Schwartz et al. [38] concluded that rougher surfaces increased alkaline phosphatase activity and osteocalcin production by cells compared to smooth surfaces. This was also reported by Martin et al. [16], who found that human osteoblast-like MG63 cells and normal human osteoblasts exhibited more differentiated phenotypes when grown on substrates with micro-rough surfaces. Orsini et al. [39] observed that sandblasting and acid-etching can improve cellular adhesion and the proliferation of osteoblast-like MG63 cells. Some of the differences in cellular response to surface roughness may have been due to variations in the degree of cell confluence obtained or the cell line studied.

This study found differences in ALP mRNA expression and ALP activity depending on the nature of the grit-blasting particle. After 14 days, cells cultured on uncoated alumina-blasted surfaces expressed higher levels of the ALP mRNA gene than cells on uncoated SiC-blasted surfaces (Fig. 2). Likewise, after 21 days of cell culture, ALP activity was higher for the A3 and A6 surfaces than for the S3 and S6 surfaces, respectively (Fig. 4, left). The fact that SiC surfaces showed higher Fn adsorption than $Al_2O_3$ surfaces due to their higher SFE [25] led to higher $\alpha_v\beta_3$ and lower $\alpha_5\beta_1$ integrin expression and, thus, less cell differentiation, as explained in the previous section. However, this blasting-particle effect was not found in cells on Fn pre-coated surfaces (Fig. 4, right).

The osteocalcin gene has been shown to be involved in other ways in the process of bone mineralization. OC, which is an osteoblast-specific protein, has been demonstrated to stimulate bone mineral maturation [40]. In this study, OC mRNA gene expression increased with culture time (Fig. 5). It is worth remembering that OC mRNA gene expression usually occurs prior to OC production. Following its occurrence, one might expect OC production to increase with time. However, this sequence of events might not occur, since OC gene expression by cells is not directly related to the final cell protein synthesis. Different factors, such as changes in the dynamic protein layer adsorbed on biomaterials surface, could lead to a change in protein synthesis by cells. No relevant differences in OC production were obtained after 14 and 21 days of cell culture (Fig. 6), but rather the results fluctuated. On uncoated surfaces, OC mRNA gene expression increased with time, while OC production decreased with time. In spite of the fact that OC gene expression increased between 7 and 14 days of incubation, a significant down-regulation of OC mRNA gene expression can occur after that time. This was eventually detected as a decrease in OC production following the longest cell culture periods.

One possible explanation for these results might be the differences in the protein layer adsorbed on Ti surfaces, which would affect integrin expression. With regard to the results for ALP activity, the up-regulation of $\alpha_v\beta_3$ expression could also decrease cell differentiation, i.e., decrease OC activity with time.

Cell maturation stage may also explain the decrease in OC production after 21 days of cell culture. Depending on the state of cell confluence and the cell line, the detection periods for cell differentiation markers can vary [41]. Furthermore, the use of total protein concentration to normalize the values of cell differentiation markers may not be the most appropriate choice. Total protein concentration depends on the total number of lysed cells. These cells may be shielded by the extracellular matrix. Zinger et al. [42] suggest using the number of cells to normalize ALP and OC results. The cell number is obtained by trypsinizing the culture cells and then counting them with a cytometer. Other methods, such as lactate dehydrogenase (LDH), are not recommended since they also rely on cell lysis.

Roughness has been shown to influence cell adhesion and differentiation. In general, cells cultured on rougher surfaces tend to exhibit attributes of more highly differentiated osteoblasts and higher OC production than cells cultured on smoother surfaces for comparable periods. However, Fn coating affected cells' OC production and OC

mRNA expression. With Fn-coated surfaces, both OC gene expression and OC production by cells increased with time throughout the experiment. This indicates that the cells on those surfaces remained in an earlier stage of maturation than the cells on the non-Fn-coated surfaces, i.e., they had not yet reached the mineralization stage.

General relationships between ALP and OC were described by Owen et al. [43] in a study of differentiation-associated genes (ALP and OC) based on the mRNA levels expressed by calvarial-derived osteoblasts. A model of the relationship between proliferation and differentiation was established. One of the main conclusions was that, as the cultures progressed into the mineralization stage, cellular levels of alkaline phosphatase declined. Osteocalcin exhibited the opposite pattern of expression. In general, there is an initial period of active proliferation in which cells grow and related genes are actively expressed. Then, the down-regulation of proliferation leads to a period of matrix maturation. Finally, when the alkaline phosphatase gene is maximally expressed, the extracellular matrix is competent for mineralization. The results of our study show that cells' ALP activity is either still under or near the maximum values at 21 days of culture. Consequently, OC mRNa expression and the associated OC production were measured when cells were still in an immature stage, at which point their values may fluctuate.

## 5 Conclusions

Cells on SiC-blasted Ti surfaces expressed higher amounts of the $\alpha_5$, $\alpha_3$, and $\alpha_v$ mRNA gene than cells on $Al_2O_3$-blasted Ti surfaces. This may be related to the fact that SiC-blasted surfaces adsorb higher amounts of fibronectin due to their higher surface free energy and, thus, offer a higher number of specific cell-binding sites.

Ti surfaces coated with fibronectin decreased the $\alpha_5$ mRNA gene expression by cells. Either the upregulation of $\alpha_v\beta_3$, or $\alpha_3\beta_1$, or both integrin subunits depending on the type of surface finishing compensate the measured down-regulation of the Fn-specific $\alpha_5\beta_1$ integrin.

The changes in $\alpha_5$ and $\alpha_v$ mRNA expression induced by the presence of fibronectin coatings can also influence the osteoblast differentiation pathway, as fibronectin coatings on Ti surfaces decreased both cell ALP mRNA expression and ALP activity after 14 and 21 days of cell culture.

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