# INNOVATIVE SUPER-HYDROPHILIC TITANIUM SELECTIVELY PROMOTES FIBRONECTIN ADSORPTION AND OSTEOBLASTS ADHESION AND PROLIFERATION

## *Superfici implantari iperidrofiliche favoriscono l'assorbimento selettivo di fibronectina plasmatica e migliorano la risposta osteoblastica*

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## Abstract

Titanium surface wettability has been shown to affect the adsorption of blood proteins and consequently cell response. The aim of our study was to further investigate the influence of super-hydrophilic surfaces obtained through a proprietary process on the adsorption of specific blood proteins and the corresponding response of osteoblasts.

Fibronectin adsorption on surfaces was monitored over time, and qualitatively assessed by western blot analysis. Subsequently, the influence of super-hydrophilic and fibronectin-enriched surfaces was tested on murine MC3T3-E1 osteoblasts, which were assayed for viability, proliferation, morphology and adhesion.

The super-hydrophilic titanium induced a significant increase of fibronectin adsorption. Osteoblasts showed a faster proliferation, closer adhesion and higher expression of focal adhesions when cultured on super-hydrophilic substrates.

These data showed how the enhanced adsorption of fibronectin influences the response of osteoblasts on titanium surfaces. Moreover, they showed the enhancement of cellular responses that can be achieved by a super-hydrophilic titanium surface modification obtained by a proprietary method

## Riassunto

È stato dimostrato che la bagnabilità della superficie degli impianti in titanio giochi un ruolo fondamentale nell'adsorbimento di proteine presenti nel sangue, influenzando positivamente la risposta cellulare primaria successiva al posizionamento dell'impianto. Pertanto, dopo aver messo a punto un metodo per rendere le superfici implantari iperidrofiliche, abbiamo deciso di studiare

l'influenza della topografia superficiale e della bagnabilità sull'adsorbimento di specifiche proteine plasmatiche e sulla risposta cellulare primaria.

L'adsorbimento di fibronectina sulle superfici è stato monitorato nel tempo e successivamente analizzato qualitativamente attraverso un immunoblotting. Successivamente, l'influenza della super-idrofilicità e del conseguente arricchimento superficiale di fibronectina è stato testato su cellule osteoblastiche murine, di cui sono state valutate vitalità, proliferazione, morfologia e adesione.

I risultati che abbiamo ottenuto hanno mostrato come il nostro metodo permetta un sensibile guadagno di idrofilicità del titanio, inducendo altresì un aumento dell'adsorbimento superficiale di fibronectina. Inoltre, i nostri dati supportano l'idea che l'adsorbimento selettivo di fibronectina possa giocare un ruolo centrale nella risposta osteoblastica. Sulle superfici super-idrofiliche gli osteoblasti hanno infatti mostrato una proliferazione più rapida, un'adesione più stretta al substrato ed una maggiore espressione di adesioni focali.

In conclusione i nostri dati supportano l'efficacia del nostro trattamento nell'ottenimento di superfici implantari super-idrofiliche e nell'aumento della selettività di queste superfici per l'adsorbimento di proteine coinvolte nelle risposte cellulari primarie.

## Introduction

Since their introduction in the clinical scene, dental implants underwent major modifications in terms of material, shape, surface topography and physical properties. In the last years, many authors focused their research on implant surface properties, to enhance their biological and clinical performances [1,2,3]. Many studies demonstrated that surface hydrophilicity can influence protein adsorption and cellular behaviour, with better osseointegration for increased wettability [4,5]. At a microscopic level, high surface hydrophilicity has been shown to preserve protein conformation upon adsorption and consequently promote the development of a favourable micro-environment [6,7]. Hydrophilic titanium surfaces, in fact, allow lower protein denaturation and misfolding during adsorption, preserving their correct molecular function and enhancing surface bioactivity. As a consequence, this leads to a faster and better osseointegration [4].

Fibronectin plays a crucial role in wound healing [8]. Along with fibrin, plasma fibronectin is deposited at injury sites, contributing to form a blood clot that stops bleeding and protects the underlying tissue. Subsequently, it forms a scaffold to which bone cells can bind and proliferate. Considering this, a modified titanium implant surface with a fibronectin coating was found to enhance osteoblasts differentiation, driving to a faster osseointegration [9,10].

We have recently developed a new implant surface treatment to enhance titanium hydrophilicity. The aim of this study is to compare the effects of topographically different implant surfaces with and without super-hydrophilic treatment by measuring the amount and quality of fibronectin adsorbed and bone cell viability, morphology, adhesion and proliferation.

## Materials and Methods

**Titanium discs -** Commercially pure, grade 4 (ISO5832/2) titanium discs (d=8.0mm) with machined or sand-blasted/acid-etched surface were kindly provided by Sweden&Martina S.P.A. (Due Carrare, Padova, Italy). Discs were treated with a proprietary method, which involves a thermal treatment and which cannot be disclosed because under patent, in order to obtain super hydrophilic titanium surfaces.

The four experimental conditions were as follows:

Machined surface= MAC

Super-hydrophilic - Machined surface= SMAC

Sand-blasted/acid-etched surface= SAE

Super-hydrophilic - Sand-blasted/acid-etched surface= SSAE

**Protein adsorption -** To investigate whether hydrophilicity influence the adsorption of proteins involved in primary cells responses, a quantitatively and a qualitatively analysis was performed.

For quantitative analysis titanium discs were soaked in 500µl of a 200µg/ml human fibronectin (Sigma-Aldrich) solution for 2 hours. Protein concentration decrement in the supernatant was measured after 5, 15, 30, 60, 120 and 180 minutes through the Bradford (BIO-RAD) mixing 10µl of sample and 200µl of Bradford Working Solution. Sample absorbances were read at 620nm with a Multiskan FC plate reader (Thermo Fisher Scientific) and the amount of adsorbed fibronectin was calculated subtracting the residual concentration in the supernatants.

For qualitative analysis, discs were incubated in 500µl of 2% human serum (Sigma-Aldrich) solution for 1 hour at room temperature (RT). Discs were subsequently rinsed twice in PBS in order to remove unbound proteins and covered with 80µl of Sample Buffer 1X. Adsorbed proteins were then completely recovered by freezing, thawing, sonicating for 15 minutes and boiling samples for 10 minutes. Equal volumes of samples were then run on a 12% polyacrylamide gel (Acrylamide/Bis-Acrylamide 30%, Sigma-Aldrich) for 1 hour and 180 V. Separated proteins were subsequently blotted on a PVDF membrane (Immobilon-P) at 100V for 1 hour. Non-specific sites were blocked with Tris-buffer saline containing 10% of blocking reagent (Roche SpA) for 1 hour at RT and fibronectin revealed with an anti-Fibronectin (Sigma-Aldrich) primary antibody diluted 1:800 in 0.1 % v/v TBS Tween 20 supplemented with 5% BSA (Sigma-Aldrich) and with a HRP-conjugated secondary antibody (Cell Signaling Technology) diluted 1:10000.

**Cell culture -** *In vitro* assays were performed with murine osteoblasts (MC3T3-E1) obtained from the America Typer Culture Collection (LGC Standards). Cells were cultured in complete Alpha-MEM (Life Technologies) additioned with 10% Fetal Bovine Serum (Life Technologies) and 1% Penicillin and Streptomycin (Life Technologies) at a density of 10000cells/disc.

**Cell viability -** Cell viability was assessed through LIVE/DEAD assay. After 48 hours of culture, culturing medium was removed and cells stained with a 4µM Calcein AM (Calcein AM, Life

Technologies) and 7.5µM PI (PI, Life Technologies) solution for 10 minutes at RT in dark conditions. Samples were then fixed with 4% PFA (Sigma-Aldrich) for 20 minutes in dark conditions and observed with a fluorescence-equipped stereomicroscope (Nikon). Three representative regions of interest (ROIs) were chosen and viable and dead cells counted through the use of the Documentation D3 software (Nikon).

**Cell metabolic activity -** Cell metabolic activity was measured through Resazurin Sodium Salt assay 24, 48 and 72 hours after seeding. At each experimental point, 100µl of Resazurin Sodium Salt stock solution (final concentration 0.15mg/ml) were added to 500µl of serum-free culturing medium and samples incubated at 37°C and 5% $CO_2$ for 4 hours. Fluorescence was finally excited at 560nm and read at 585nm through a Multiskan Ascent microcell plate reader (Thermo Labsystems**).**

**Cell morphology -** Cell morphology and focal adhesion distribution were studied trough cytofluorescence for actin, vinculin and cell nuclei. After 24 hours of culture, culturing medium was removed and cells fixed in a 4% PFA solution for 10 minutes at RT. After twice rinsed in PBS, cells were permeabilized with 0.1% v/v Triton X-100 (Sigma-Aldrich) solution for 5 minutes at RT and washed twice in PBS. One-% BSA solution was then added to the samples for 30 minutes at RT in order to block non-specific sites. Subsequently, cells were stained for focal adhesion with a primary anti-vinculin monoclonal antibody, clone 7F9 (Merck Millipore) for 1 hour at RT and washed twice in PBS. To reveal primary antibody, a secondary anti-rabbit labeled with the AlexaFluor®488 chromophore (Life Technologies) diluted 1:200 in PBS was used and co-incubated with TRITC-conjugated phalloidin (Merck Milliporere) (dilution 1:200) for actin staining. After three rinses in PBS, nuclei counterstaining was performed with a 1:1000 PBS solution of DAPI (Merck Millipore) for 5 minutes at RT.

**Cell adhesion -** To study the interactions of cells with the underlying titanium surface, a scanning electron microscopy (SEM) in combination with the orthogonal sample cutting through a Gallium Focused Ion Beam (FIB) source was performed. SEM-FIB preparation was performed 24 hours after seeding at RT. Culturing medium was removed, cells rinsed in PBS, fixed in a 2.5% gluteraldehyde (Sigma-Aldrich) solution in Na-Cacodylate buffer (Sigma-Aldrich) for 30 minutes, washed in Na-Cacodylate buffer for 5 minutes and dehydrated in ethanol at increasing concentrations (Sigma-Aldrich). Finally, samples were critical point dried with liquid carbon dioxide (Balzer Union) and sputtered with a thin layer of gold through a SCD 040 coating device (Balzer Union). Photographs were taken using a dual beam Zeiss Auriga Compact system equipped with a GEMINI Field-Effect SEM column and a Gallium FIB source (Zeiss). SEM analysis was performed at 5keV, while the cross-sectional analysis at 30kV with a current of 10pA.

**Statistical analysis -** Data were analyzed using Prism 6 (La Jolla). All the values are reported as the mean ± SD of three repeated experiments performed in multiple replicates. Differences between groups were evaluated with the two-way ANOVA statistical test and Tukey's multiple comparison post-test. Differences were considered significant when $p<0.05$.

## Results

**Super-hydrophilicity increases the amount of stable adsorbed fibronectin -** The overall amount of deposited protein onto titanium surfaces was not influenced by hydrophilicity. Bradford assay revealed that after 2 hours a mean of 13.6 ± 0.7µg of fibronectin was present on MAC samples, while 13.9 ± 1.4µg on SMAC discs (fig.1A). Similarly, 12.3 ± 2.5µg of fibronectin were on SAE sample vs the 13.9 ± 1.4 µg on SSAE (fig.1B). Interestingly neither hydrophilicity, nor surface topography seemed to affect this. Surprisingly however, after rinsing with PBS the amount of stable bound proteins was different and heavily influenced by hydrophilicity (fig.1C). As western blot analysis revealed, the amount of adsorbed fibronectin was higher on super-hydrophilic samples than on controls (fig.1D-E).

**Super-hyrophilicity enhances cells proliferation -** The relationship between live and dead cells was not altered through the groups (fig.2A). However, the number of cells was higher on treated samples than on controls both for machined (fig.2B) and for sandblasted/acid-etched surfaces (fig.2C), with statistically significative differences ($p<0.0001$). In spite of this, proliferation was enhanced on super-hydrophilic surfaces and differences were highly different between non-super-hydrophilic and super-hydrophilic discs both for machined and for sanblasted/acid-etched surface ($p<0.0001$).

Moreover, proliferation was significantly higher on SMAC than on SSAE samples ($p=0.0064$), and interestingly these data linearly fitted with the amount of fibronectin adsorbed on samples as reported in figure 1D-E.

On the other hand, hydrophilicity did not seem to influence cell metabolic activity (fig.3), which was influenced by the surface microtopography in a statistically significative way after 48 hours for super hydrophilic surfaces (SMAC-SSAE $p=0.0199$) and after 72 hours for hydrophobic and for hydrophilic surfaces (MAC-SAE $p<0.0001$ ; SMAC-SSAE $p=0.0010$).

**Super-hydrophilicity influenced cell morphology and focal adhesion expression -** Immunostaining revealed the presence of healthy cells on all the samples (fig.4A). In particular, the staining for focal adhesions showed more numerous focal adhesions per cell on treated samples than on controls (fig.4B), with statistically significative differences between the groups (MAC-SMAC $p=0.0100$ ; SAE-SSAE $p=0.0061$ ; SMAC-SSAE $p=0.0140$) and a trend that perfectly fits the amount of stably adsorbed fibronectin on surfaces and analyzed in figure 1D-E.

**Super-hydrophilicity allows a closer adhesion to titanium of cells -** SEM analysis reported in figure 5, revealed a similar cell adhesion to MAC or SMAC samples, where cells displayed a flat and well spread shape. However, a different cell morphology was observed for control and treated sandblasted/acid-etched surfaces: cells adhered more closely to super-hydrophilic surfaces, and showed a flatter shape that allowed to glimpse the underlying micro texture of the underlying titanium profile. FIB analysis confirmed SEM observations, showing how super-hydrophilic surfaces could promote a closer adhesion of cells to the material, and how they appeared thinner, adhering preferentially to surface peaks of SSAE sample.

## Discussion

With this study we intended to assess the efficacy of super-hydrophilicity in improving the adsorption of a target blood protein, fibronectin, which severely affected primary osteoblasts response, on the titanium dental implant surfaces.

The process we designed to obtain super-hydrophilic titanium efficiently led to a gain in surface hydrophilicity both on machined and sandblasted/acid-etched surfaces. Furthermore, the gain of hydrophilicity allowed a stable adsorption of fibronectin on the surfaces and this impacted on the subsequent osteoblasts response (fig.1). The number of cells was higher on treated samples than on the controls regardless of surface topography. Higher cell viability was matched by a higher amount of focal adhesions per cell unit (fig.2-4) and, consistently, a tighter cell adhesion, as visible at SEM and FIB sections (fig.5).

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The authors declare that the treatment method whose effect are described in this work is actually under patent process.

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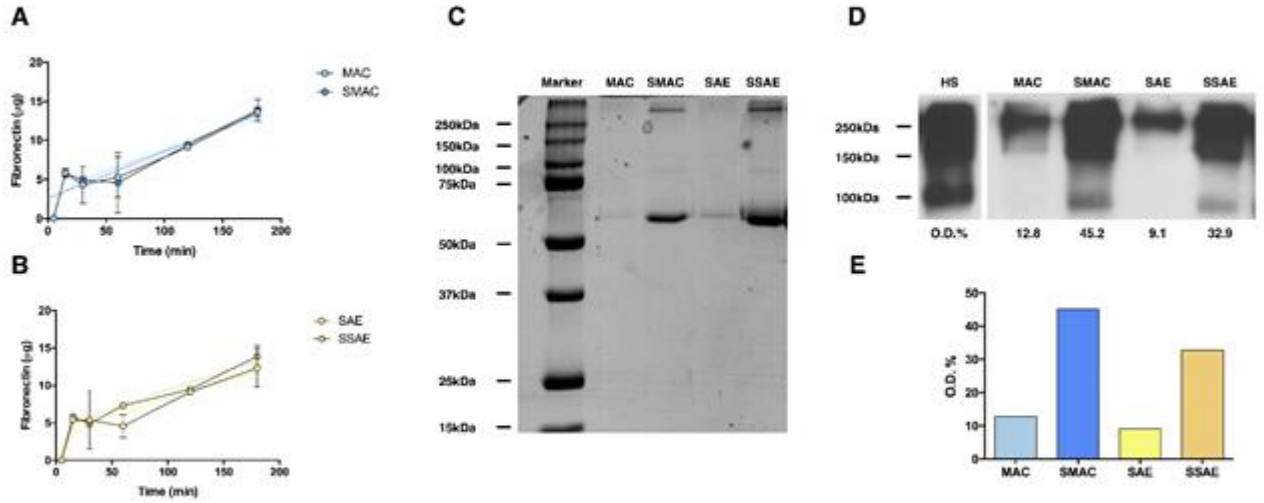


**Figure 1 -** Protein adsorption. (A) Fibronectin adsorption on MAC and SMAC titanium surfaces and quantitated through the Bradford. (B) Fibronectin adsorption on SAE and SSAE titanium surfaces and quantitated through the Bradford. (C) SDS-PAGE separation of human serum proteins after Coomassie Blue staining. (D-E) Immunoblot for fibronectin and optical density percentual plotting.

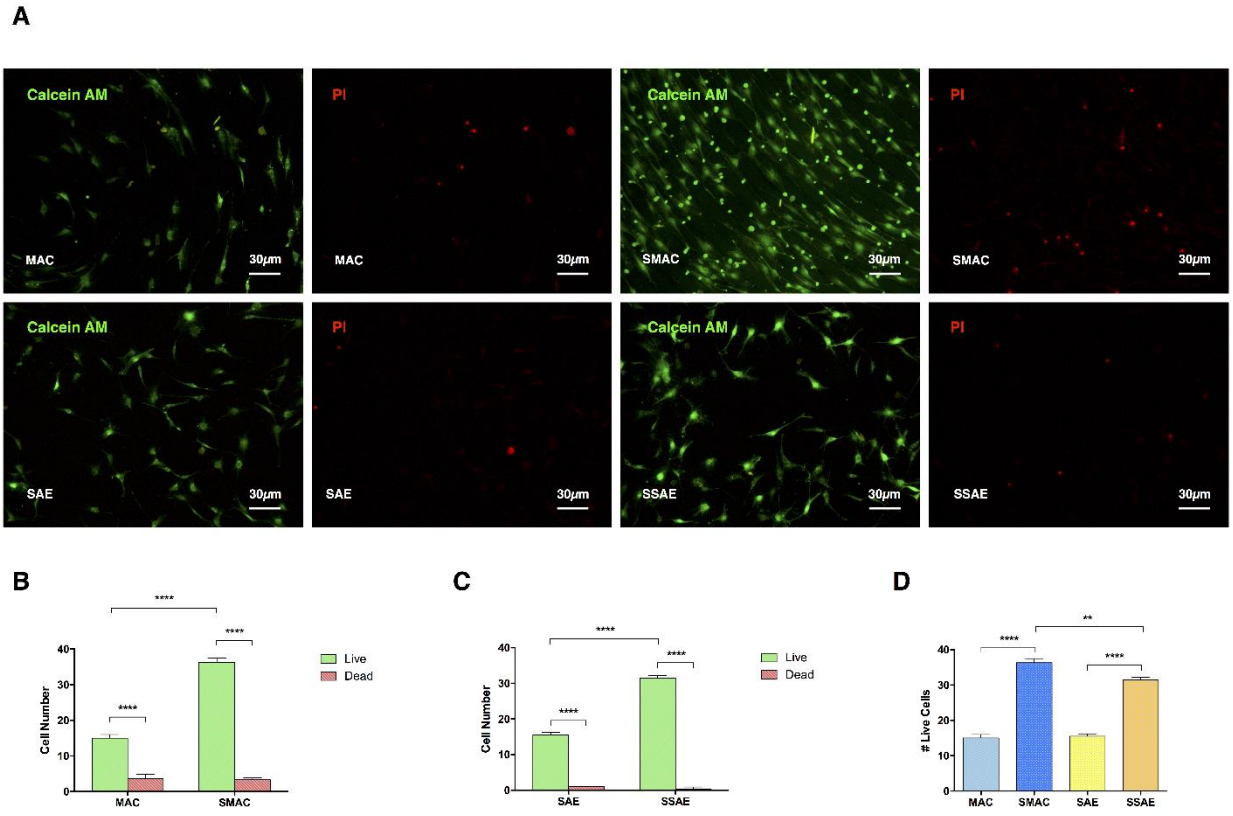


**Figure 2 -** Cell viability. (A) Murine MC3T3-E1 osteoblasts viability on different titanium surfaces. (B) Quantitation of live (green) and dead (red) cells on MAC or SMAC titanium surfaces. (C) Quantitation of live (green) and dead (red) cells on SAE or SSAE titanium surfaces. (D) Quantitation of live (green) cells on different titanium surfaces.

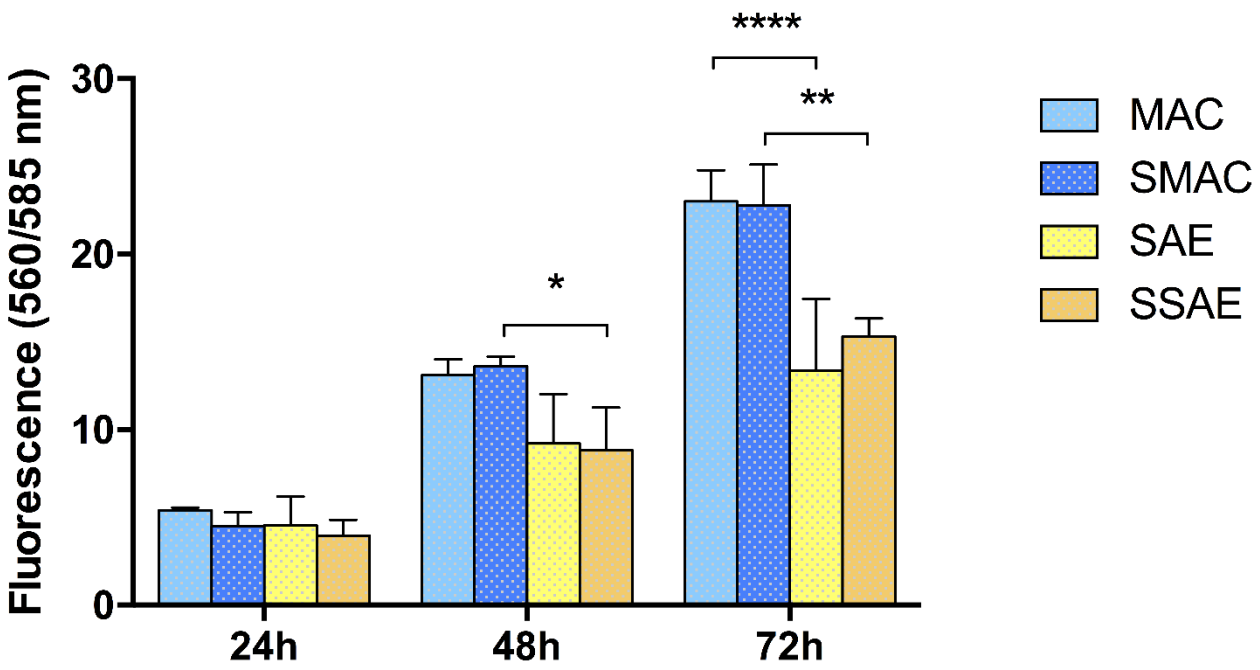


**Figure 3** - Cell metabolic activity on different titanium surfaces, measured through Resazurin sodium salt assay after 24, 48 and 72 hours of culturing.

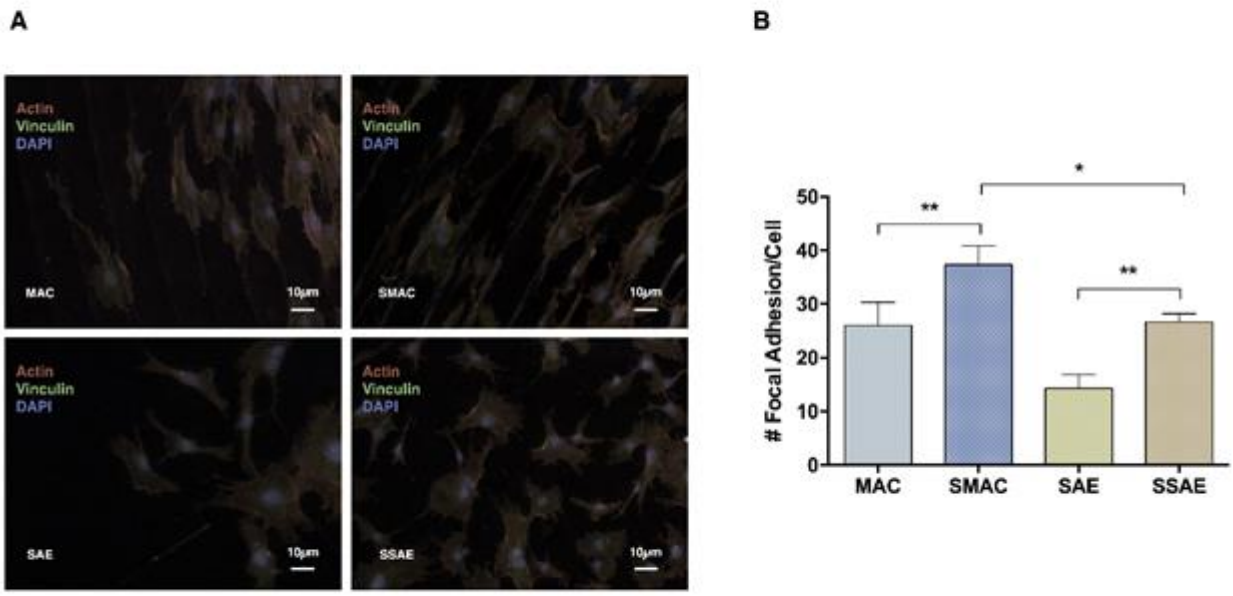


**Figure 4 -** Cell morphology. (A) Murine MC3T3-E1 osteoblasts morphology different titanium surfaces stained for actin (red), focal adhesion (green spots) and cell nuclei (blue). (B) Quantitation of focal adhesion per cell expressed and quantitated through the Documentation D3 software.

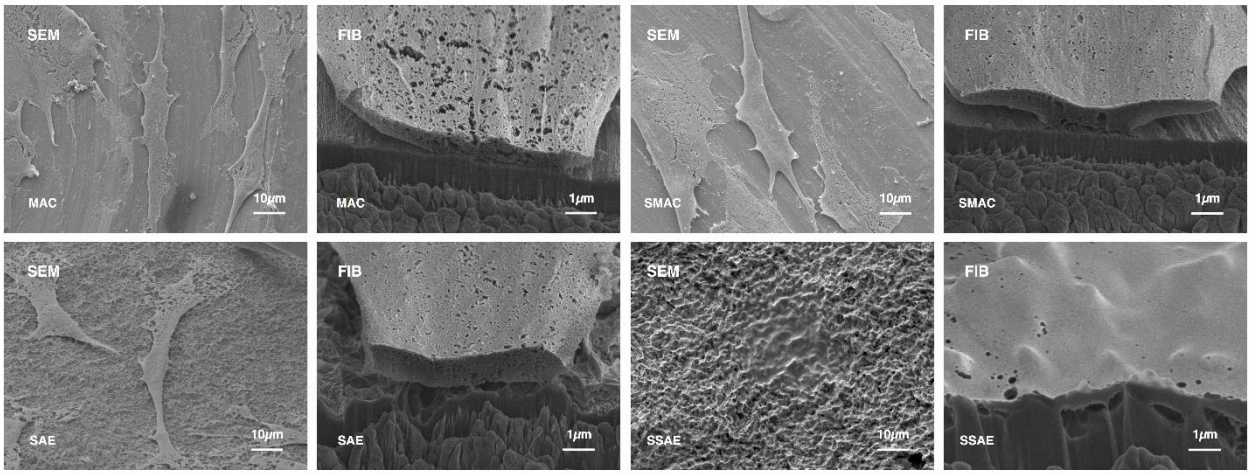


**Figure 5 -** Cell adhesion. Murine MC3T3-E1 osteoblasts morphology and adhesion on different titanium surfaces fixed and observed through SEM-FIB.