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Osteoblasts preferentially adhere to peaks on micro-structured titanium

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The aim of the study was to investigate cell adhesion to micro-structured titanium. Osteoblastic MC3T3 cells were cultured on smooth (P) or sand-blasted/acid-etched (SLA) titanium discs and were observed at scanning electron microscope/focused ion beam (SEM/FIB). Myosin II and actin microfilaments were labelled for epifluorescence microscopy. FIB revealed that cell adhesion initiated centrally and expanded to the cell periphery and that cells attached on the substrate by bridging over the titanium irregularities and adhering mostly on surface peaks. Gaps were visible between concave areas and cytoplasm and areas around ridges represented preferred attachment points for cells. A different myosin distribution was observed between samples and myosin inhibition affected cell responses. Taken together our data indicate that cells attach on micro-rough titanium by bridging over its irregularities. This is likely mediated by myosin II, whose distribution is altered in cells on SLA discs.

Keywords: Osteoblast, Implant, Topography, Titanium, Surface

INTRODUCTION

Micro-structured surfaces have long been used to promote the integration of endosseous implants in bone, as rough surfaces have been extensively shown to improve the differentiation of mesenchymal and bone cells, bolstering the expression of osteoblast-specific genes[1-4]. Adhesion is a key factor in determining the ability of a cell to effectively colonise a biomaterial, and thus to deposit a matrix that can serve as basis for newly formed tissue. Cells adhering to biomaterials with complex topographies have been shown to acquire a shape that adapts to the underlying substrate[5-8], and convincing evidence has been presented to support the hypothesis that cell shape actually affects cell activity and differentiation fate[9-11], although how cells can sense the geometrical features of endosseous implant surfaces and transduce them into pro-differentiation stimuli is still being actively investigated. Biomaterials that can control cell shape have thus the potential to provide cells with potent stimuli that can affect cell commitment toward a phenotype lineage, and investigating the 3D morphological conformation of a cell on a biomaterial surface is therefore of pivot importance to predict its behavior.

Cell shape is determined by the complex interaction between cytoskeletal components that regulate the cellular internal structure and the mechanical integration of the cell with the extracellular matrix[12-14]. According to the tensional integrity theory, cell shape is maintained through a balance of forces generated by actomyosin filaments and withstood by microtubules, which act like rigid support struts for cells[15]. This cell balance dictates how cells interact with the culture substrate, including titanium micro-topography. Availability of improved investigation tools such as focused ion beam microscopy (FIB)[16] allow for detailed analysis of how cells contact micro-rough surfaces and the role of cell contractility in cell adhesion. FIB is a similar instrument to conventional scanning electron microscopes (SEM), but relies on an ion source, mostly Gallium, as with the instrument used in the present study, instead of electrons. Ions can mill or cut samples, and thanks to beam control, it is possible to create very precise sample sections, even with metals such as titanium (Fig. 1). This allows for unprecedented possibilities to investigate the spatial and geometric relationship between cells and biomaterials, because it is possible to get a glimpse at the interface between cell cytoplasm and substrate, right underneath the cellular body.

The present study focuses on the use of FIB as a tool to investigate cell adhesion on micro-topographyed titanium at different time points to get a better glimpse at how cells attach to their substrates.

MATERIALS AND METHODS

Titanium surfaces

Pickled and acid-etched, sand-blasted (SLA) commercially pure titanium samples were kindly provided by Straumann Institut (Basel, Switzerland) These surfaces have been extensively described and characterized in the literature[17,18]. The samples were provided as sterile discs of 1 mm thickness, 16 mm diameter, and were used in 24 well plates (Euroclone, Milano, Italy) for the biological assays.

Color figures can be viewed in the online issue, which is available at J-STAGE.

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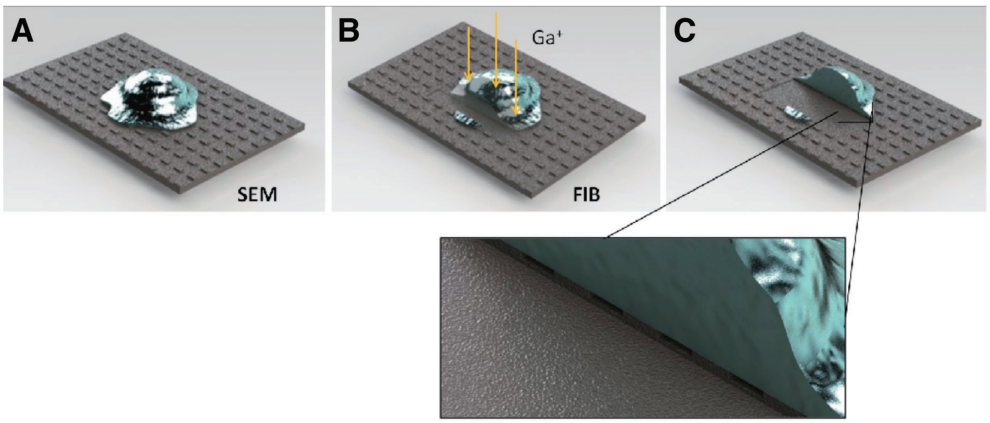

Fig. 1 Diagram showing the difference between SEM and FIB microscopes.
SEM instruments (A) rely on an electron beam to visualise the surface of the sample. The ion beam used with FIB instruments however (B) is able to cut the sample along a finely tunable plane. Conventional SEM techniques, usually integrated in the same microscope (C) allow for section analysis (C), with high magnification details (inset).

Cell cultures

The MC3T3-E1 cell line was obtained from the American Type Culture Collection (LGC Standards, Milano, Italy) and cultured in DMEM medium as described above. MC3T3 are osteoblastic cells from mouse calvaria. They typically retain strong similarities with primary cells, such as contact inhibition, and are thus an established *in vitro* model of osteoblasts[19-21]. To perform the morphological assays 1×10^4 MC3T3 cells were plated on Pickled or SLA discs in 1 mL of complete medium in 24 well plates (Corning, Tewksbury, MA, USA), in triplicate and assayed 1, 3, 6 or 24 h after plating. For viability assays 2×10^4 MC3T3 cells were plated on titanium discs as described above and assayed after 24 h. For transfection assays, MC3T3 cells were plated on titanium surfaces in 1mL/well OptiMEM (Life Technologies Italy), 5% FBS, 1% Penstrep at the density of 1×10^5 cells/well and the cells were assayed after 24 h.

SEM and FIB

Ten thousand cells/mL were seeded in 24 multiwell plates in DMEM on Pickled titanium sample and SLA titanium sample. The plates were maintained at 37°C and 5% CO_2 in a humidified atmosphere for 1, 3, 6 and 24 h. For SEM observation, the samples were first washed with PBS at 37°C and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30' at RT. After that, samples were dehydrated in ethanol at increasing concentrations. Finally, the samples were critical point dried with liquid carbon dioxide (CPD 030 Baltec, BALTEC, Wallruf, Germany) and they were covered by a nm thick gold layer (PLANO, Wetzlar, Germany) deposited by sputtering using a SCD 040 coating device (Balzer Union, Wallruf, Germany). The images of cells distribution and morphology over Titanium sample were characterized by SEM, using a dual beam Zeiss Auriga Compact system equipped with a GEMINI Field-Effect SEM column and a Gallium FIB source. The SEM analysis was performed at 5 keV. This instrument allows, also, to cut cells on the samples. Dual beam FESEM-FIB apparatus was used to section the samples by using a Gallium ion beam accelerated at 30 kV with 500 pA current; this allowed us to perform *in situ* cross-sectional analysis of the cells and their interactions with the underlying surface.

Immunofluorescence

Cells were seeded at the concentration of 2×10^4 cells/well in complete medium and after 24 h they were fixed with 4% paraformaldehyde (Sigma-Aldrich, St.Louis, MO, USA) for 10 min followed by three rinses with PBS. They were then permeabilized with 0.1% Triton-X100 (Sigma-Aldrich) for 5 min followed by three rinses with PBS. Non-specific binding sites were blocked by incubating the samples in 1% bovine serum albumin in PBS for 20 min. Cells were first stained with a mouse monoclonal anti-myosin II antibody (Ab684, Abcam, Cambridge, UK) for 1 h followed by three rinses with PBS. The cells were labelled with FITC-anti-mouse IgG antibody (AP124F, Chemicon) in combination with TRITC-conjugated phalloidin (FAK100, Merck Millipor, Burlington, MA, USA) for 1 h followed by three rinses with PBS. Nuclear counterstaining was performed by incubation with DAPI (D1306, Molecular Probes, Life Technologies) for 5 min followed by three rinses with PBS. All the steps were carried out inside the culture well at room temperature. The treated discs were then transferred to microscope slides and were mounted under glass cover slips using an antifade-mounting medium (P7481, Molecular Probes, Life Technologies) for photo bleaching reduction. Samples were examined using a Nikon Eclipse 90i (Nikon, Tokyo, Japan) microscope equipped for fluorescence analysis.

Inhibitors
For Blebbistatin experiments, cells were plated as described above and stimulated with 1 μM Blebbistatin (Inalco, Milano, Italy) 4 h after plating for the whole duration of the experiments. An equal amount of PBS was used as a control. Pilot experiments were performed to choose the reagent concentrations, which were chosen based on the literature as the minimal dose to induce consistent and predictable morphological changes at immunofluorescence[22].

Cell viability
A bioluminescence assay for cell viability (CellTiter-Glo®, Promega, Madison, WI, USA) was also used, according to the recommendations of the manufacturer. Briefly, a lysis reagent was added to the culture wells, to release intracellular ATP into the culture medium. A reagent containing the luciferase enzyme was then added, generating a bioluminescent signal proportional to the amount of ATP present. This signal correlates with the number of viable cells in the well. The samples were read with a Glomax 20/20 Luminometer (Promega) with double injectors.

Statistical analysis
Data were analyzed using Prism 6 (GraphPad, La Jolla, CA, USA). All values are reported as the mean±standard deviation of three repeated experiments. Differences between group means were evaluated with two-way ANOVA statistical test and Bonferroni post-test and differences were considered significant when $p<0.05$.

RESULTS

Early attachment
MC3T3 cells were first observed 1 h after plating them on Pickled (P) or sand-blasted/acid-etched discs (SLA). At this time point, cells on both substrates appeared round, with visible villi on their surfaces (Fig. 2). Cells already established early but broad contact areas with the underlying surface on P discs (Fig. 2C) and smaller contacts with the protruding elements of SLA sample surfaces (Fig. 2F).

Osteoblastic cells display early preferential attachment to peaks
Three hours after seeding cell appearance turned from globular to flat and elongated (Fig. 3). Cells on P discs appeared mostly round with short and broad podosomes (Fig. 3A). Cell edges were often raised, an artefact indicative of weaker adhesion along the cell edge and confirmed by our previous studies showing early concentration of focal adhesions around the perinuclear area 3 h after seeding[23]. Higher magnification showed that adhesion was limited to small and frequent areas often around minor peaks, with cells bridging small concavities (Fig. 3C arrowhead, Fig. 4). Cell shape in cells on SLA discs already reflected the topography of the substrate, and was mostly elongated with cytoplasmic projections that followed the main valleys of the surface (Fig. 3D). Interestingly, FIB sections revealed that cells were similar to a tent or canopy tensed over spikes, or peaks of titanium, with small contact areas (Fig. 3F).

As adhesion progresses cells are tensed over peaks
Cell morphology became even more deeply affected by surface topography 6 h after seeding (Fig. 5). Pickled surfaces were not completely flat, although their profile was smoother than SLA discs. This implied the presence of preferential adhesion routes that modelled cell morphology into a broad but elongated star-like shape (Fig. 5A). Cells appeared as lying on top of the surface, resting on peaks and skipping the underlying valleys (Fig. 5C, Fig. 6). MC3T3 cells behaved similarly on SLA surfaces, albeit in an even more dramatic fashion. Cells were narrower (although often thicker) than on P

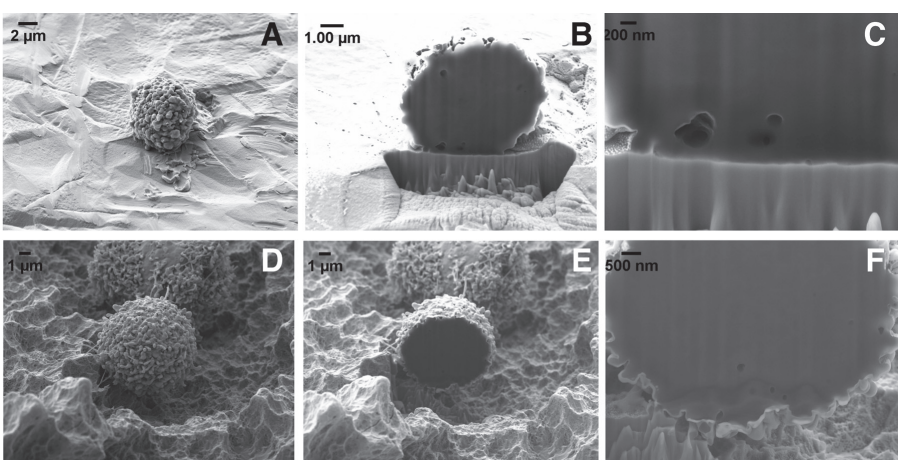

Fig. 2 SEM microphotograph of MC3T3 cells on Pickled (A–C) or SLA (D–F) titanium surfaces 1 h after seeding, before and after sectioning with FIB. Scale bar is indicated on every microphotograph.

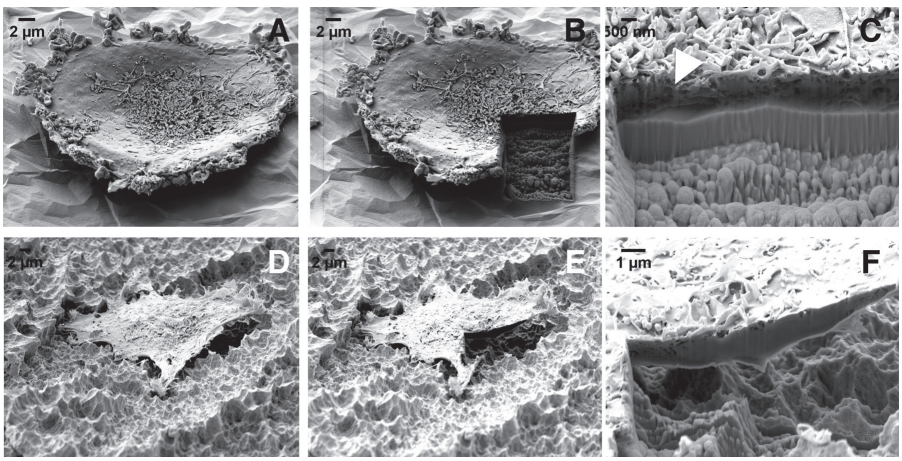

Fig. 3 SEM microphotograph of MC3T3 cells on Pickled (A–C) or SLA (D–F) titanium surfaces 3 h after seeding, before and after sectioning with FIB. Scale bar is indicated on every microphotograph.

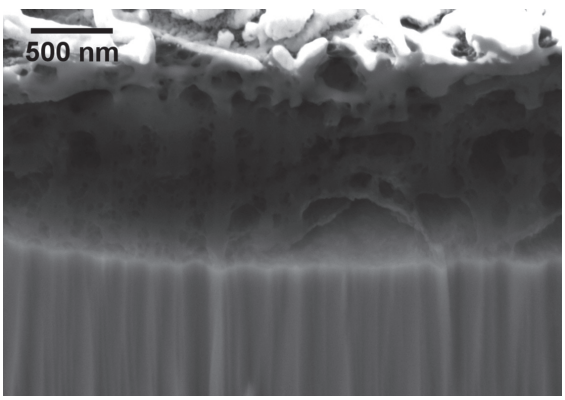

Fig. 4 SEM microphotograph of a MC3T3 cell on Pickled titanium surface 3 h after seeding, after sectioning with FIB.

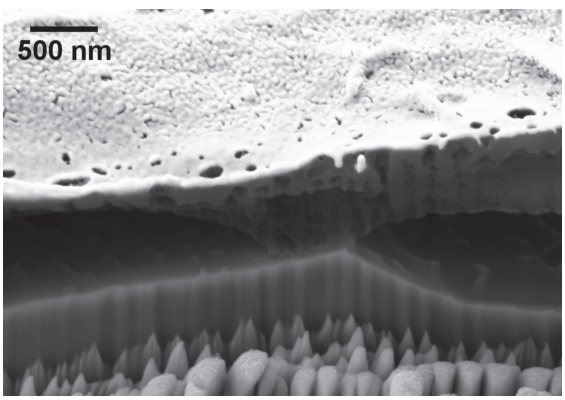

Fig. 6 SEM microphotograph of a MC3T3 cell on Pickled titanium surface 6 h after seeding, after sectioning with FIB.

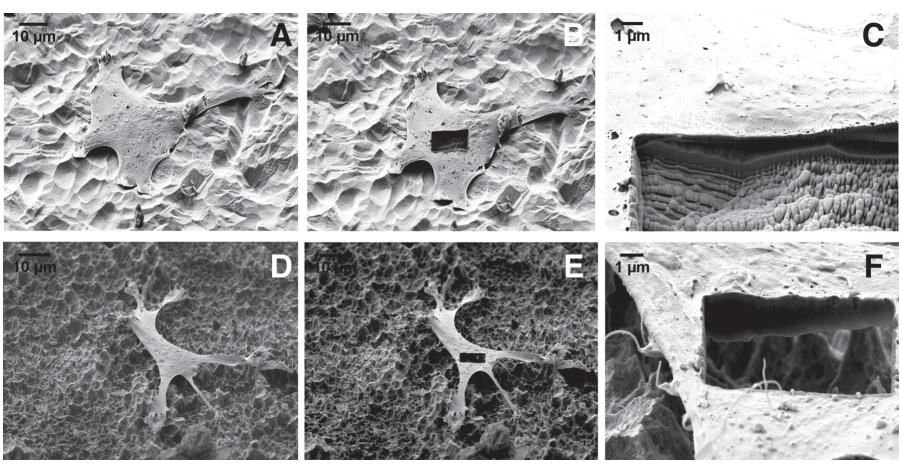

Fig. 5 SEM microphotograph of a MC3T3 cell on Pickled (A–C) or SLA (D–F) titanium surfaces 6 h after seeding, before and after sectioning with FIB. Scale bar is indicated on every microphotograph.

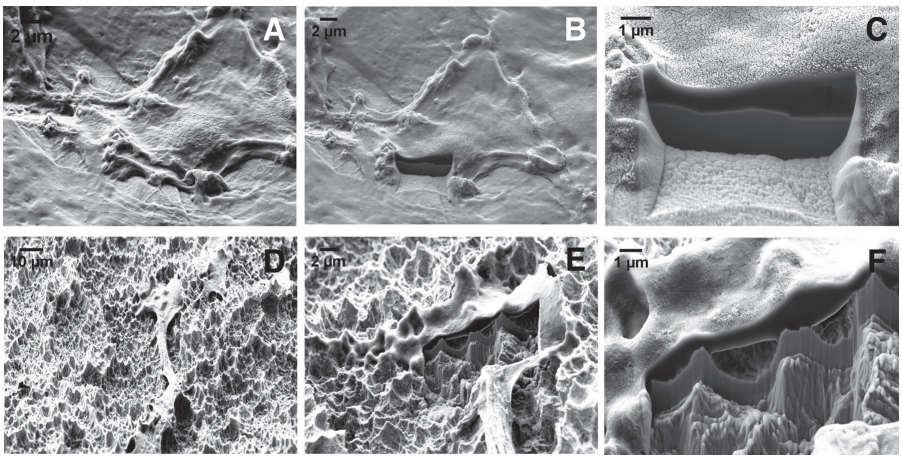

Fig. 7 SEM microphotograph of a MC3T3 cell on Pickled (A–C) or SLA (D–F) titanium surfaces 24 h after seeding, before and after sectioning with FIB. Scale bar is indicated on every microphotograph.

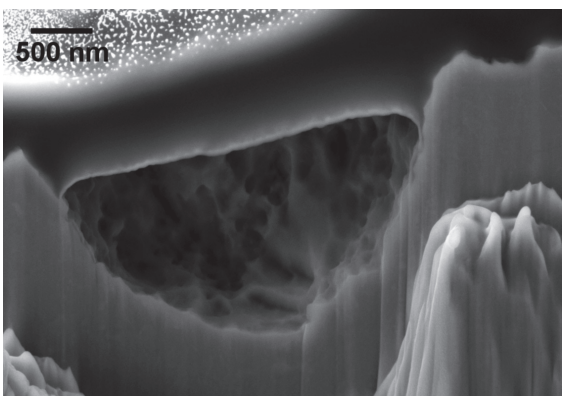

Fig. 8 SEM microphotograph of a MC3T3 cell on SLA titanium surface 24 h after seeding, after sectioning with FIB.

surfaces with long and thin philopodes anchoring them on titanium (Figs. 5D–F). Morphology was stable after 24 h of culture (Fig. 7). Cells on Pickled surfaces were firmly adherent on the substrate, with broad adhesion surfaces whenever flat portions of titanium were available (Fig. 7C). Cells were also firmly gripping to titanium peaks on SLA surfaces, while bridging over the deep valleys and creating small secluded spaces, where only thin cytoplasmic projects appeared to crawl in (Fig. 8).

Cellular cytoskeletal apparatus is modulated by micro texture

We then stained cells on Pickled or SLA surfaces for myosin II and actin (Fig. 9). Cells on all surfaces surfaces displayed visible myosin labelling across the cell body, co-localized with actin microfilaments. These fibres crossed the whole cytoplasm and outlined preferential force vectors, which could not be observed in cells on SLA (Figs. 9B, D, F). These presented with

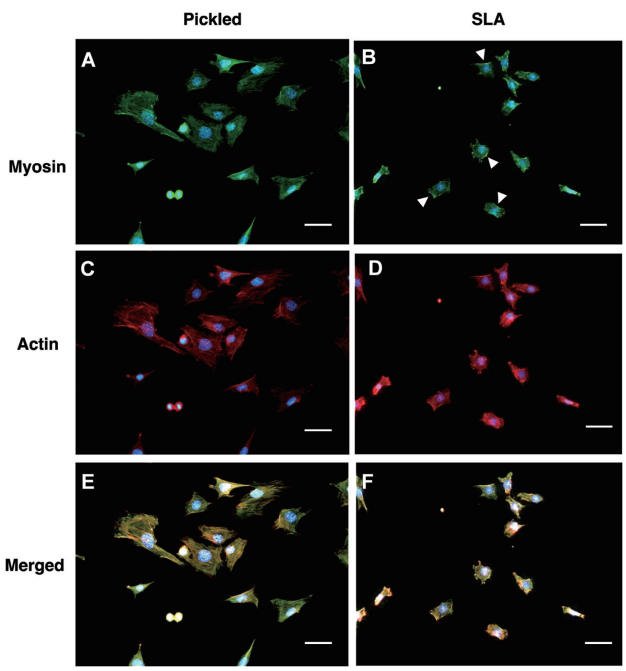

Fig. 9 Microphotographs of MC3T3 cells on Pickled (A,C,E) or SLA (B,D,F) titanium surfaces 48 h after seeding, labelled with FITC anti myosin II antibody (green) or TRITC-phalloidin for actin microfilaments (red).
Magnification 400×. Bar=10 µM.

a more intense fluorescence along the cell edges and cytoplasm protrusions (Figs. 9B, D, F). Myosin was visible especially along straight cell edges, suggestive of cytoplasm tension (Fig. 9B, arrowheads).

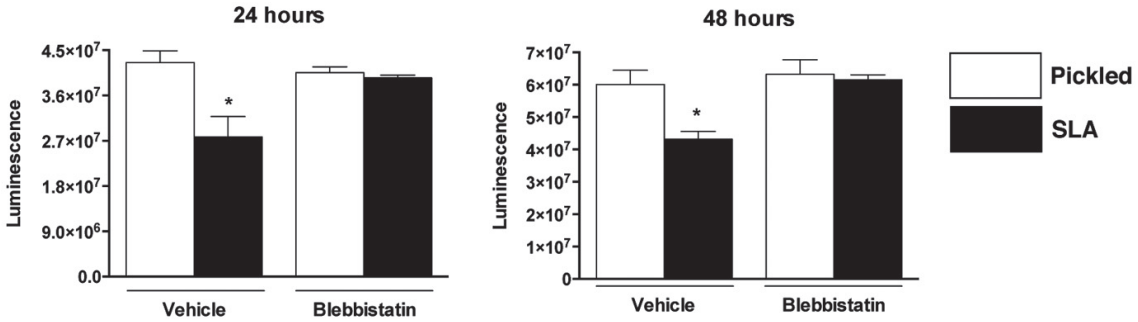

Fig. 10 Cell viability of MC3T3 cells after 24 or 48 h on Pickled or SLA surfaces measured by chemiluminescence in the presence or in the absence of 1 μM Blebbistatin.

Cytoskeletal contractility affects cell growth on microtextured surfaces

The realisation that MC3T3 cells were tensed over the titanium surfaces, and that adhesion areas were mostly limited to surface peaks lead us to investigate the effects of the modulation of cell contractility on cell growth. When MC3T3 cells were plated on Pickled or SLA surfaces, cell viability appeared to increase on both surfaces over time but it remained significantly higher on smooth surfaces both 24 and 48 h after seeding (Fig. 10). However when cell contractility was inhibited by addition of Blebbistatin, an inhibitor of the myosin light chain kinase enzyme, no difference was observed between the two surfaces at both time points (Fig. 10), and cell viability on SLA surfaces reached similar levels to cells on pickled discs.

DISCUSSION

It is well known that cells can sense the three dimensional profile of the substrate on which they grow and conform to its topography thanks to an adaptable cytoskeleton[5,8,24]. Ever since implant surfaces with different degrees of roughness were introduced into the clinical use, investigators realised that the way cells and tissue interacted with smooth or rough surfaces was profoundly different[2,17,25]. Tailored experiments have since proved that cells are indeed capable to translate geometric cues into intracellular signals that then can affect cell fate and activity[7,9,26,27]. More specifically micro-rough surfaces were demonstrated to enhance the expression of osteoblast-specific genes such as alkaline phosphatase, osteoprotegerin or osteocalcin[2,25,28,29], thus attracting the attention of the scientific world as good candidates for endosseous devices because of their improved integration in bone[30]. The use of recent tools such as FIB has allowed for unprecedented insights into how cells adhere to their substrate, providing a way to better understand the role of cell forces in adhesion to microtextured surfaces[16]. More specifically, FIB allows to take a glimpse at the relation between a cell and its underlying surface and we employed this feature to investigate whether cells filled all the surface irregularities with their cytoplasm while growing on a rough substrate. According to Ingber's tensegrity theory, both adhering and non-adhering cells are in a state of mechanical balance between opposing forces that determine their shape[15,31,32]. The whole cell is under mechanical tension, also known as pre-stress, which is generated by myosin motor proteins that pull on actin microfilaments. Thus, every cell possesses a certain degree of pre-stress, which explains the round shape of non-adhering cells. Our data are consistent with previous reports indicating that cells can adhere to flat surfaces thanks to broad adhesion areas, as suggested by big FA areas in immunofluorescence, and we demonstrated that when cells grow on micro-textured substrates their adhesion areas are limited to the peaks of such surfaces[23]. Gaps remain between the cellular body and the substrate and these are not filled by cell cytoplasm. This behavior started to manifest itself from early time-points, up to 3 h after seeding, and became more evident by 24 h of culture, a time-point by which adhesion is considered mostly stable. Even on substrates with low roughness such as Pickled surfaces, where cells exhibit differentiation behaviors quite distinctively different from SLA discs[1], cells preferentially adhered to titanium peaks, and areas of the surface near to peaks (Fig. 6). It can be hypothesized that differences in the physical properties of the surface, such as hydrophilicity, may contribute to such confinement of focal adhsions to titanium peaks. This was dramatically more visible on SLA samples, where MC3T3 cells grasped on titanium ridges and bridged over the substrate valleys (Fig. 8). Cells were quite similar to canopies, to tents, which rested on the protrusions on the surface and did not fill the cavities delimited by the ridges, and FIB allowed to incontrovertibly demonstrate it for the first time, to the best of our knowledge. Cells were so tensed that the underlying surface features could not be seen through the cell body, which appeared flat, even on SLA samples, where surface features are quite visible on a cellular scale. As time progressed, cells on P surfaces tended to occupy all the available space and rested on the titanium uniformly, whereas cells on SLA samples limited their attachments to extremely discrete areas, although their contact points became wider. By 24 h cells on SLA reached the familiar star-like shape with protrusions

grasping surrounding peaks, whilst cells on P surfaces remained rather square-shaped. In order to maintain such a morphology cells must exert quite a considerable tension within the cell bodies or their cytoplasm would just collapse in the valleys of the substrate. Moreover, we have shown that although MC3T3 cells grew on either smooth or rougher substrates, significantly fewer cells were detectable on SLA surfaces after both 24 and 48 h of culture. Inhibition of cell contractility by addition of MLCK inhibitor blebbistatin did not affect cell viability on Pickled discs, but increased cell viability on SLA discs. This maneuver resulted in a decrease in cell tension and caused cells on SLA surfaces to behaved similarly to those on P surfaces, consistently with viability data on micro-structured surfaces after inhibition of ROCK, a kinase that operates upstream of MLCK[22]. This would also imply that the difference in cell proliferation observed on smooth and rough surfaces is not due to mechanical hindrance of titanium ridges to cell motility but is rather consequence of the activation of intracellular pathways downstream of MLCK. Future studies will have to further investigate the physicochemical basis for this cell behavior, particularly which surface characteristics promote cell adhesion mostly around surface peaks, and the molecular effectors that mediate the effects of cell tension on cell behavior. Taken together, our observations quite clearly show that cell adhesion on micro-structured titanium surfaces is limited to localised areas on peaks and that cell bodies bridge over the valleys of titanium discs.

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