Open Access

Recent Progress in Materials

LIDSEN Publishing Inc.

Original Research

Initial Adhesion Behavior of Osteoblast on Titanium with Sub-Micron Scale Roughness

Satoshi Migita *, Tomoya Yamaguchi

Graduate School of Science and Engineering, Yamagata University, 3-4-16 Jonan, Yonezawa, Japan; E-Mails: migita@yz.yamagata-u.ac.jp; cns55@yahoo.co.jp

* **Correspondence:** Satoshi Migita; E-Mail: migita@yz.yamagata-u.ac.jp

Academic Editors: Dorina Lauritano and Hossein Hosseinkhani

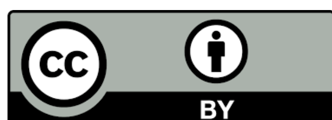
Special Issue: Applications and Development of Biomaterials in Medicine

Recent Progress in Materials
2020, volume 2, issue 1
doi:10.21926/rpm.2001003

Received: September 18, 2019
Accepted: January 10, 2020
Published: January 14, 2020

Abstract

The surface roughness of titanium could regulate various cellular functions such as survival and growth. In the context of cell adhesion to materials, cell spreading and cell shape are also closely linked to various cellular functions. Previously, we found that Ti substrate with a 100-nm scale surface roughness reduced cell survival. However, effects of surface roughness on cell shape were not investigated. Herein, we quantified the initial adhesion behavior of osteoblasts on a Ti substrate with ~100 nm scale surface roughness, which was prepared using silicon carbide (SiC) polishing paper. To evaluate the morphological parameters, such as perimeter, feret's diameter, circularity, and spreading area on initial adhesion, cells were cultured on SiC-polished or mirror-polished Ti for 24 h. These morphological parameters were determined from fluorescence micrographs using image J software. Cells cultured on SiC-polished Ti exhibited poor spreading area. Additionally, pseudopodia formation and actin bundle construction of the cells were also poor on SiC-polished Ti, while the cells cultured on the mirror-polished Ti exhibited clear F-actin and greater overall cytoskeletal activity. On a Ti substrate, ~100 nm scale surface roughness could be expected to inhibit the cell spreading and pseudopodia formation of osteoblasts when compared to those cultured on a smooth



© 2020 by the author. This is an open access article distributed under the conditions of the Creative Commons by Attribution License, which permits unrestricted use, distribution, and reproduction in any medium or format, provided the original work is correctly cited.

substrate. Thus, consistent with our earlier reports, the surface roughness of the substrate is linked to reduced cellular functions such as cell growth and differentiation.

Keywords
Titanium surface roughness; cell spreading; cell shape; morphology

1. Introduction

Cell adhesion to materials is a major concern for biocompatibility. Because the first phase of cell-material interaction is cell adhesion [1], the surface characteristics, such as topography and surface chemistry, of the materials are closely linked to its biocompatibility. Thus, surface treatment is a key to improving the biocompatibility of materials. In the case of metallic biomaterials, surface treatment is carried out to achieve roughened surface [2]. Surface roughness induces changes in cellular responses such as attachment, proliferation, and differentiation. Cells exhibit higher attachment activity on titanium (Ti) with micrometer-scale surface roughness than on Ti with a smooth surface [3]. Moreover, osteoblastic cells cultured on Ti with micrometer-scale roughness show increased differentiation activity [4]. Several studies have shown that cells cultured on Ti with submicrometer-scale surface roughness (surface roughness between 370 nm to 700 nm) exhibit higher cellular function than those cultured on Ti with micrometer-scale roughness [5-10].

Several groups have reported contrary results for cellular function on nanometer-scale surface roughness. Cai et al. reported that there was no difference in cell adhesion, spreading, proliferation and differentiation [11] between cells cultured on Ti with a single nanometer surface roughness and those cultured on tissue culture polystyrene (TCPS). Moreover, Washburn et al. reported that poly-l-lactic acid (PLLA) with a 10 nm root-mean square (RMS) roughness value reduced the proliferation activity of osteoblasts [12]. According to their results, the critical RMS roughness value for reducing proliferation activity was approximately 1.1 nm. Furthermore, we have previously reported that a Ti surface with an arithmetic average of the roughness (Ra) value on the order of 100 nm markedly reduced fibroblast attachment and proliferation [13]. Similar results have been found for osteoblasts [14]. These phenomena are possibly induced by poor cell adhesion on to the material surface because cell adhesion is closely linked with cellular functions such as survival.

In this study, we compared the morphology of osteoblast at initial adhesion on a mirror polished Ti and that on a surface with 100 nm-scale roughness. To adjust the surface roughness without affecting the chemical composition of the surface, we used silicon carbide (SiC) paper and a colloidal silica suspension. Cells from the mouse osteoblast cell line MC3T3-E1 were cultured on each surface, and their attachment activity, spreading area, and cell shapes were analyzed. These cells retained their characteristic morphology on the rough surface. These results provide insight into the effects of surface roughness on initial cell adhesion based on the cell shape for generating cellular functions (i.e. proliferation and differentiation).

2. Materials and Methods

2.1 Preparation of Ti Discs

Commercial pure Ti discs (grade 2, Rare Metallic Co., Tokyo, Japan) were mirror-polished using a silica suspension. To prepare the roughened surface, the Ti discs were re-polished with silicon carbide (SiC) polishing paper, and the discs were rinsed by sonication in acetone three times. The Ti discs were then immersed in a 70% ethanol solution under ultraviolet light for 1 h for sterilization. The surface of each Ti disc was observed under a scanning probe microscope (SPM-9600; Shimadzu, Kyoto, Japan) using a silicon cantilever (NCHR-20; Nano World AG, Neuchâtel, Switzerland). The surface chemical composition of each Ti was determined using SEM-EDS (JSM-7600FA, JEOL). The *Ra* value of each surface was calculated by examining Z-range images. To determine the water contact angle, the 10 µL water drop on the Ti surface was captured by a digital camera (CX4, Ricoh, Tokyo, Japan). These camera images were subsequently used to calculate the contact angle using Image J software (http://imagej.nih.gov/ij/).

2.2 Cell Culture

MC3T3-E1 cells were purchased from Riken Cell Bank (Tsukuba, Japan). The cells were cultured in α-Minimal Essential Medium (α-MEM, Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (Biowest, Nuaillé, France), 100 U/ml penicillin, and 100 µg/mL streptomycin (Nacalai Tesque).

2.3 Initial Cell Adhesion Analysis

To investigate initial cell adhesion, cells were seeded on each Ti surface at a density of 50,000 cells/cm^2 and were incubated in α-MEM for 4 h and 24 h. After incubation, the number of adhered cells was determined using Cell-Counting Kit 8 (Dojindo Laboratories, Kumamoto, Japan). The cells were incubated in test medium containing 10% Cell-Counting Kit solution for 1 h at 37°C in a CO_2 incubator. Next, absorbance of the medium was measured at 450 nm using a spectrophotometer (SmartSpec Plus, BIORAD, Richmond, CA).

2.4 Cell Morphology Analysis

To determine the morphology, the cells were cultured on each Ti surface for an incubation period of 24 h. After incubation, the cytoplasm was stained with 0.5 mg/mL calcein-AM (Dojindo Laboratories) for 1 h at 37 ℃ in a CO_2 incubator. The shape of the cells was observed using fluorescence microscope (IX-71; Olympus, Tokyo, Japan) without fixation, and the images were captured using a CCD camera. The cell spreading area, perimeter, feret's diameter, and circularity, were then calculated from the captured images using Image J.

To observe actin filaments, the cells were fixed with 4% paraformaldehyde for 1 h, washed with PBS, and stained with rhodamine-conjugated phalloidin (Cytoskeleton, Inc., Denver, CO, USA) for 1 h at room temperature. The stained cells were then observed using fluorescence microscope (IX-71; Olympus).

Five samples were used in each analysis. The data represents average ± SD. Differences were examined using Student's *t*-test, with significance defined at $p < 0.05$.

3. Results

3.1 Preparation of the Ti Surface

Figure 1 shows atomic force microscopy (AFM) images of the Ti surfaces. There were no-directional scratches on SiC-polished surface (Figure 1a). The *Ra* values calculated from the AFM images were 108.9 ± 14.3 nm and 3.3 ± 2.3 nm for SiC-polished Ti and mirror-polished Ti, respectively. The *Ra* values and water contact angles of SiC-polished Ti and mirror-polished Ti are listed in Table 1. Although there was a significant difference in the surface roughness, the static water contact angles were similar at 57.5 ± 7.0° and 49.9 ± 1.9° for SiC-polished Ti and mirror-polished Ti, respectively.

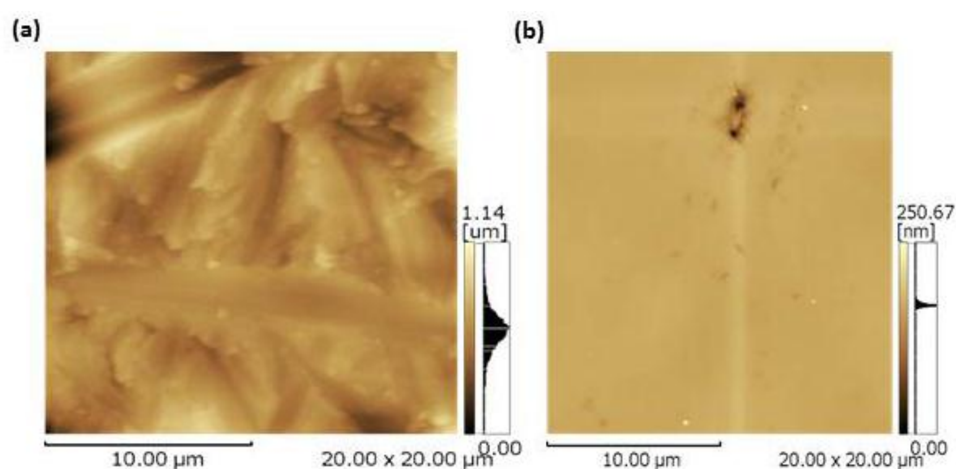


Figure 1 AFM topological images of each surface with an area of 20 µm ×20 µm. (a) SiC-polished Ti and (b) Mirror-polished Ti.

Table 1 *Ra* values, water contact angles of SiC-polished and mirror-polished Ti.

	SiC-polished Ti	Mirror-polished Ti
Surface roughness, *Ra* (nm)	108.9 ± 14.3	3.3 ± 2.3
Water contact angle (deg)	57.5 ± 7.0	49.9 ± 1.9

3.2 Adhesion of Cells

Figure 2(a-d) shows fluorescence microscopic images of the osteoblasts cultured on each Ti surface. After 4 h of cultivation, most of the cells cultured on the SiC-polished Ti substrate exhibited a round shape, and a drastically reduced spreading area compared to those of the cells cultured on mirror-polished Ti. After 24 h of cultivation, cells cultured on both surfaces exhibited a spindle shape. Cells cultured on the SiC-polished Ti substrate displayed a smaller spreading area than those on the mirror-polished substrate. Surface topography of the SiC-polished substrate inhibited cell spreading. Figure 2e shows cell survival on each Ti after 4 h and 24 h cultivation. Cells proliferated on both Ti surfaces, but proliferation on SiC-polished Ti was lower than that on mirror-polished Ti.

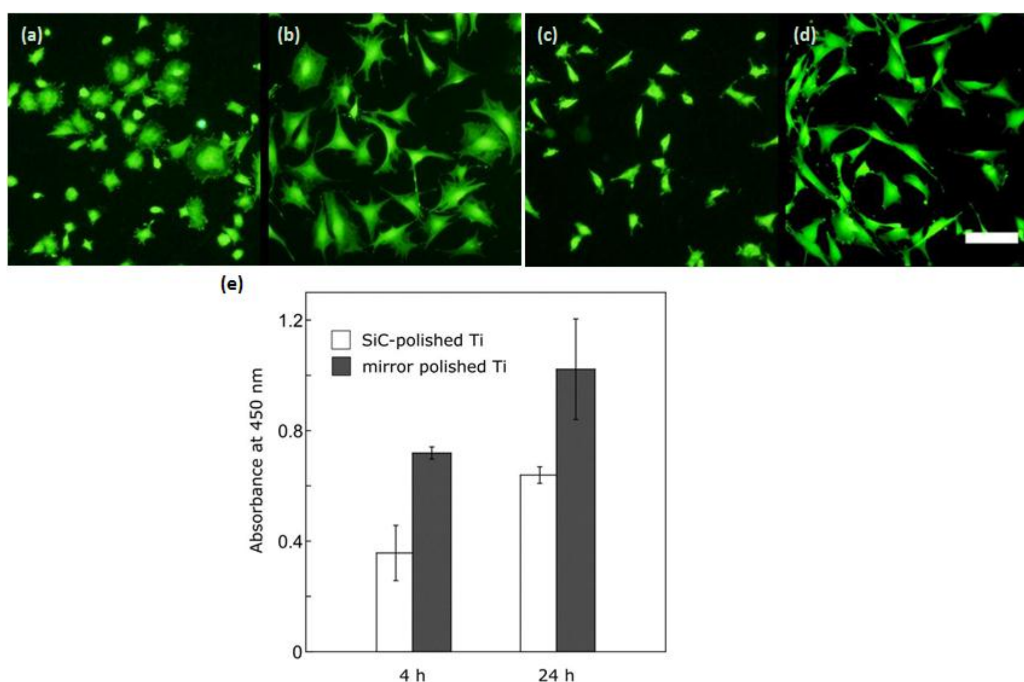


Figure 2 Fluorescence microscopic images for observation of cell morphology. Cells cultured on SiC-polished Ti and mirror-polished Ti after cultivation for 4 h (a, b) and 24 h (c, d). The scale bar represents 100 μm. Cell proliferation on SiC-polished Ti and mirror-polished Ti after cultivation for 4 h, and 24 h (e).

3.3 Analysis of Cell Shape

Cell shape parameters such as, perimeter, feret's diameter, circularity, and spreading area on each substrate after cultivation for 24 h were determined to examine the morphology of adherent cells. Each Ti surface was significantly different with regard to the morphological parameters of the adhered cells. Circularity, which can be used as an indicator of pseudopodium formation, was greater on mirror-polished Ti than that on SiC-polished Ti. These results supported the qualitative observations of cell morphology shown in Figure 3 (a-d).

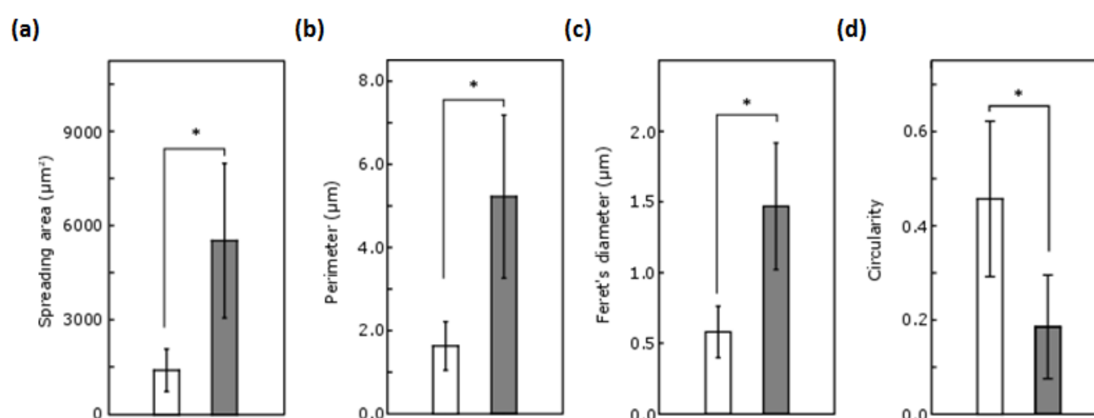


Figure 3 Morphological parameters of cells cultured on SiC-polished Ti (white bar) and mirror polished Ti (gray bar). (a) spreading area, (b) perimeter, (c) feret's diameter, and (d) circularity. The data represents average ±SD (n = 5).

3.4 Observation of Actin

Actin stress fibre alignment has been shown to aid cell migration [24, 25]. Figure 4 shows fluorescence microscopic images of actin distribution in cells cultured on both the SiC-polished Ti and the mirror-polished Ti after 24 h. The cells on the mirror-polished Ti exhibited clear F-actin; however, on the SiC-polished Ti, the cells formed very few discernible actin bundles.

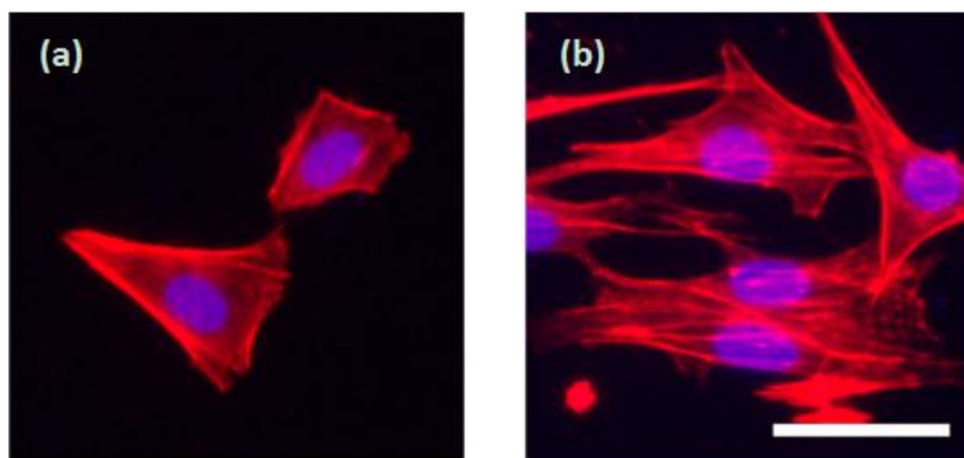


Figure 4 Fluorescence microscopic images of actin fibres in cells cultured on (a) SiC-polished and (b) mirror-polished Ti surfaces. The cells were cultured for 24 h on each surface. The scale bar represents 50 µm.

4. Discussion

Many studies have explored the effect of surface roughness on cell adhesion. In these studies, the roughened surface was created using different processes such as blasting, alkaline or/and acid etching, resulting different surface chemical compositions and topologies. Therefore, it is difficult to directly compare the findings of the different studies. According to EDS analysis, the surface chemical composition of SiC-polished Ti was similar to that of mirror-polished Ti (Figure S1). Furthermore, although the SiC-polished Ti showed higher contact angles than the mirror-polished Ti, the results were not statistically significant. These findings indicate that SiC polishing had a minimal effect on the chemical composition of the Ti surface. Therefore, it is plausible that cells were directly affected by surface roughness and topology. Surface topology is mainly influenced by non-directional scratches on SiC-polished surface and is significantly different from that created by blasting or SLA. Therefore, the effect of surface-to-cell attachment may be different from that observed with surface modified by other methods.

The shape of a cell on the surface of a material is an important parameter for determining the biocompatibility of the material because cell spreading is closely linked to cell survival [15, 16] and differentiation [17, 18]. There was no substantial difference between the morphologies of cells upon initial adhesion on the mirror-polished Ti surface and TCPS. Our results clearly showed that cell spreading is inhibited on SiC-polished Ti. Ridges and pitches of roughened surface produced by sand blasting and chemical etching could provide mechanical stabilization of cell membrane [22, 23]. Therefore, distinctive actin networks were observed on roughened surface [22]. Sub-micron scale surface roughness created by mechanical polishing may have induced the same effects on

the cells. Therefore, cells attached to the roughened surface exhibited indiscernible actin fibers compared to those on smooth surface. Although difficult to compare, data from different research suggest that inhibition of cell spreading and poor actin fiber formation may be closely linked to ridges and pitches of roughened surface. Hence, surface topography information is potentially more important than surface roughness.

The cells exhibited higher circularity on SiC-polished Ti. A previous report suggested that higher cell circularity is observed on surfaces with poor adhesion capacity [21]. Hence, we conclude that cell adhesion on Ti with ~100 nm scale surface roughness may be poor substrate. On such a surface, cells downregulate integrin α_5 subunit expression and exhibit reduced growth activity [14]. Ridges and pitches of the roughened surface could downregulate integrin expression. Arnold et al proposed that distance of cell adhesive points of the surface could be closely linked to integrin clustering and activation for cell spreading and actin fiber formation [24]. The $\alpha_5\beta_1$ integrin helps cell survival [19, 20]. Therefore, ~100 nm scale surface roughness leads to changing cell shape and reducing cell survival via poor cell adhesion. While some reports have shown that sub-micron scale surface roughness produces positive effect on cellular function [5-10], a few contradictory reports suggest negative effect of submicron scale surface roughness on the cellular functions [11-14]. Our results support that sub-micron scale surface roughness induces negative effect on the cells.

5. Conclusions

In this study, we investigated the initial adhesion behavior of MC3T3-E1 mouse osteoblastic cells on sub-micron meter-scale roughened Ti. The spreading of these cells was inhibited, and they exhibited a round shape on the surface. Given that insufficient cell spreading directly relates to poor cell adhesion, it seems likely that the ~100 nm scale surface roughness might be inducing poor cell adhesion and contributing to reduced cell survival.

Author Contributions

Satoshi Migita (SM) reviewed literature and conceived the study. SM and Tomoya Yamaguchi prepared the roughened Ti surface. TY evaluated the cell attachment activity on the roughened surface. SM wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and have approved the final version of the manuscript.

Funding

This work was supported by JSPS KAKENHI Grant Number 19K12798.

Competing Interests

The authors have declared that no competing interests exist.

Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Figure S1: EDS spectra of the SiC-polished Ti (a), and mirror-polished Ti (b).

References

1. Anselme K. Osteoblast adhesion on biomaterials. Biomaterials. 2000; 21: 667-681.
2. Hanawa T. Research and development of metals for medical devices based on clinical needs. Sci Technol Adv Mater. 2012; 13: 64102.
3. Duraccio D, Mussano F, Giulia M. Biomaterials for dental implants : Current and future trends. J Mater Sci. 2015; 50: 4779-4812.
4. Martin J, Schwartz Z, Hummert T, Schraub D, Simpson J, Lankford Jr J, et al. Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). J Biomed Mat Res A. 1995; 29: 389-401.
5. Schwartz Z, Raz P, Zhao G, Barak Y, Tauber M, Yao H, et al. Effect of micrometer-scale roughness of the surface of Ti6Al4V pedicle screws in vitro and in vivo. J Bone Joint Surg Am. 2008; 90: 2485-2498.
6. Gittens RA, Mclachlan T, Cai Y, Berner S, Tannenbaum R, Schwartz Z, et al. The effects of combined micron-/submicron-scale surface roughness and nanoscale features on cell proliferation and differentiation. Biomaterials. 2011; 32: 3395-3403.
7. Guo Y, Hu B, Tang C, Wu Y, Sun P, Zhang X, et al. Increased osteoblast function in vitro and in vivo through surface nanostructuring by ultrasonic shot peening. Int J Nanomedicine. 2015; 10: 4593-4603.
8. Zhao G, Zinger O, Schwartz Z, Wieland M, Landolt D, Boyan BD. Osteoblast-like cells are sensitive to submicron-scale surface structure. Clin Oral Implants Res. 2006; 17: 258-264.
9. Holthaus M, Treccani L, Rezwan K. Osteoblast viability on hydroxyapatite with well-adjusted submicron and micron surface roughness as monitored by the proliferation reagent WST-1. J Biomater Appl. 2013; 27: 791-800.
10. Klein MO, Bijelic A, Ziebart T, Koch F, Kämmerer PW, Wieland M, et al. Submicron scale-structured hydrophilic titanium surfaces promote early osteogenic gene response for cell adhesion and cell differentiation. Clin Implant Dent Relat Res. 2013; 15: 166-175.
11. Cai K, Bossert J, Jandt KD. Does the nanometre scale topography of titanium influence protein adsorption and cell proliferation? Colloids Surfaces B Biointerfaces. 2006; 49: 136-144.
12. Washburn NR, Yamada KM, Simon CG, Kennedy SB, Amis EJ. High-throughput investigation of osteoblast response to polymer crystallinity: Influence of nanometer-scale roughness on proliferation. Biomaterials. 2004; 25: 1215-1224.
13. Migita S, Okuyama S, Araki K. Sub-micrometer scale surface roughness of titanium reduces fibroblasts function. J Appl Biomater Funct Mater. 2016; 14: 65-69.
14. Migita S, Araki K. Effect of nanometer scale surface roughness of titanium for osteoblast function. AIMS Bioengineering. 2017; 4: 162-170.
15. Re F, Zanetti A, Sironi M, Polentarutti N, Lanfrancone L, Dejana E, et al. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J Cell Biol. 1994; 127: 537-546.
16. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science. 1997; 276: 1425-1428.
17. Ross TD, Coon BG, Yun S, Baeyens N, Tanaka K, Ouyang M, et al. Integrins in mechanotransduction. Curr Opin Cell Biol. 2013; 25: 613-618.
18. Mansouri R, Haÿ E, Marie P, Modrowski D. Role of syndecan-2 in osteoblast biology and pathology. Bonekey Rep. 2015; 4: 666.

19. Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999; 285: 1028-1032.
20. Zhang Z, Vuori K, Reed J, Ruoslahti E. The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. P- Natl Acad Sci USA. 1995; 92: 6161-6165.
21. Kuhn S, Kroth J, Ritz U, Hofmann A, Brendel C, Müller LP, et al. Reduced fibroblast adhesion and proliferation on plasma-modified titanium surfaces. J Mater Sci Mater Med. 2014; 25: 2549-2560.
22. Kunzler, TP, Drobek T, Schuler M, Spencer ND. Systematic study of osteoblast and fibroblast response to roughness by means of surface-morphology gradients. Biomaterials. 2007; 28: 2175-2182.
23. Brunette DM, Chehroudi B. The effects of surface topography of micromachined titanium substrata on cell behavior in vitro and in vivo. J Biomech Eng. 1999; 121: 49-57.
24. Arnold M, Cavalvanti-Adam EA, Glass R, Blümmel J, Eck W, Kantlehner M, et al. Activation of integrin function by nanopatterned adhesive interfaces. Chemphyschem. 2004; 19: 383-388.

Enjoy *Recent Progress in Materials* by:

1. Submitting a manuscript
2. Joining in volunteer reviewer bank
3. Joining Editorial Board
4. Guest editing a special issue

For more details, please visit:
http://www.lidsen.com/journals/rpm