DNA microarray analysis of gene expression of MC3T3-E1 osteoblast cell cultured on anodized- or machined titanium surface

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ABSTRACT

Purpose: The aim of this study was to evaluate adhesion and gene expression of the MC3T3-E1 cells cultured on machined titanium surface (MS) and anodized titanium surface (AS) using MTT test, Scanning electron micrograph and cDNA microarray.

Materials and Methods: The MTT test assay was used for examining the proliferation of MC3T3-E1 cells, osteoblast like cells from Rat calvaria, on MS and AS for 24 hours and 48 hours. Cell cultures were incubated for 24 hours to evaluate the influence of the substrate geometry on both surfaces using a Scanning Electron Micrograph (SEM). The cDNA microarray Agilent Rat 22K chip was used to monitor expressions of genes.

Results: After 24 hours of adhesion, the cell density on AS was higher than MS ($p < 0.05$). After 48 hours the cell density on both titanium surfaces were similar ($p > 0.05$). AS had the irregular, rough and porous surface texture. After 48 hours incubation of the MC3T3-E1 cells, connective tissue growth factor (CTGF) was up-regulated on AS than MS (more than 2 fold) and the insulin-like growth factor 1 receptor was down-regulated (more than 2 fold) on AS than MS.

Conclusion: Microarray assay at 48 hours after culturing the cells on both surfaces revealed that osteoinductive molecules appeared more prominent on AS, whereas the adhesion molecules on the biomaterial were higher on MS than AS, which will affect the phenotype of the plated cells depending on the surface morphology. ***(J Korean Acad Periodontol 2008;38:299-308)***

KEY WORDS: cDNA microarray; MTT assay; SEM; anodized titanium surface.

Introduction

The pure machined titanium implants are widely used in orthopedic and dental surgeries due to their excellent biocompatibility and mechanical properties which lead to successful clinical performance in implant dentistry. Despite the favorable biological performance of a thin native oxide film, formed on titanium surface spontaneously, titanium oxide seldom forms a direct chemical bond to bone tissue. In bone of poor quality and quantity, the results have not always been so good, motivating the development of new surface processing technologies. Albrektsson and Wennerberg reviewed the clinical knowledge of 5 selected oral implant surfaces and clinical studies with different surfaces were analyzed separately in conjuction with bone grafts[1]. Recently, various attempts to modify the surface of implants to coat materials or anodize electrochemically the pure titanium surface have been developed based on the methods of the surface modification of Ti implants[2,3,4,5]. Implant success has been reported to be largely dependent on the surface composition and structure, surface energy, and morphology of implants. Understanding of Bone-biomaterial interface and methods to control was reviewed by Puleo and Nanci[6].

The surface roughness of implant can influence adherence, attachment, spreading of osteoblast and metabolism, modifying and controlling the osseointegration process. MG63 cells, originally derived from a

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male human osteosarcoma, cultured on rough Ti surfaces exhibit decreased proliferation and increased adhesion and phenotypic differentiation compared with cultures on plastic and smooth Ti surfaces[7]. Surface microtopography also affects cell response to the systemic hormones 17β-estradiol and 1a,25(OH)2D3, mechanical stimuli such as shear force, and local regulatory like bone morphogenetic protein-2[7].

The chemical property in implant surface is one of the most important key factor in the reaction of osteoblast to the implant, since it influences charge and wettability. Surface wettability is highly affected by surface energy and influences the degree of contact with the physiologic environment. Increased wettability enhances interaction between the implant surface and surrounded tissues. Fluoride-modified titanium surfaces, in synergy with surface roughness, had a greater expression level of cbfa1 (a key regulator for osteogenesis) than the unmodified titanium surfaces[8]. Improved bone bonding and accelerated bone formation seems possible with roughened surface further modified with certain acid treatment.

One of the techniques for modifying Ti implant surface is an anodization. This method is an electrochemical one that forms rough, thick, and porous oxide films. The chemical composition, crystallization, roughness, and topography of the implant surface are changed. Recently, published in vivo investigations have shown significantly improved bone tissue reactions by modification of the surface oxide properties of titanium[8]. Findings in animal studies have indicated that bone tissue reactions were strongly reinforced than 600nm, a porous surface structure and an anatase type of titanium with oxidized Ti implants characterized by a titanium oxide layer thicker oxide with largely unaltered surface roughness compared with machined implants. In vitro studies have shown that differences in the microtopography of an implant surface can affect the expression of key bone matrix-related proteins and osteogenic transcription factors that will enhance osteogenesis on implant surfaces[9].

DNA microarray is a new molecular technology that enables the analysis of gene expression in parallel on a very large number of genes, spanning a significant fraction of the genome. It is a qualitative analytical technique (e.g., it can differentiate each single gene) and is also quantitative, because it has the sensitivity to detect a change of expression in the investigated cells when compared to normal samples. Recently, to compare the gene expression from the cells cultured on the different microtopography, chemicals, component of the implant surface, various kinds of studies using microarray assay have been performed[10-15].

The purpose of this study was to evaluate adhesion and gene expression of the MC3T3-E1 cells cultured on machined titanium surface and anodized titanium surface using MTT test, Scanning electron micrograph and cDNA microarray.

Materials and Methods

1. Materials

1) Titanium disk preparation

The Ti disks used for the cell culture were fabricated from grade 4 commercially pure Ti (Dynamet, A Carpenter Co., Washington, USA). The pure Ti disks were used as control, and the disks were prepared to be 12 mm diameter and 1 mm thick. The disks Osstem (Pusan, Korea) for the anodization were washed and sonicated in acetone, then pretreated with a solution of 1HF and $3HNO_3$. The electrolyte solution contained 0.25 M sulfiric acid and 0.25 M phosphoric acid. The electrolyte solution were mixed with dH_2O and stirred more than 1 hour. The pretreated disks were anodized using 380 A/m^2, 300 V at electrode. The positive electrode was connected to the disks, and the negative electrode was to the platinum plate, and the temperature of the electrolyte solution was maintained and circulated at 0-20°C. The anodized disks were wash-

ed, dried, and sterilized with γ-ray and stored.

The disk samples were classified as described below.

MS Group: Machined surface, used as control.
AS Group: Anodized surface using electronic current and voltage.

The surface characteristics of the machined and anodized titanium disks were analyzed by using SV-3000S4 (Mitutoyo, Kawasaki, Japan). The surface roughness of anodized surface was 0.8-1.2μm, whereas machined surface was 0.1-0.2μm.

2. Methods

1) Cell culture and RNA extraction

MC3T3-E1 cells, osteoblast like cells from Rat calvaria, were obtained from the Korean Cell Line Bank and cultured in sterile cell culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) containing alpha minimal essential medium. For all experiments, cells were cultured on disks placed in 10 cm-diameter dishes. The control groups consisted of cells cultured on the machined surfaces (MS). The experimental groups were composed of cells cultured on the anodized titanium surfaces (AS). Cells were collected and seeded at a density of 1×105 cells /ml by using 0.1% trypsin, 0.02% EDTA of pH 7.2 for cell release. One set of wells contained sterile 20 disks of MS (grade 4 titanium) whereas another contained 20 disks of AS at 37°C in 5% CO_2 for 24 hours and the samples were moved to new dishes and media were added and the plated disks were cultured at 37°C in 5% CO_2 for 24 hours. All Cell culture media were supplemented with 100 units/ml penicillin-G, 100g/ml streptomycin, and 0.25g/ml fungizone (Gemini Bio-Products, Inc, Woodland, CA, USA). Total RNA extractions were performed with Qiagen mini kit (Qiagen, Chatsworth, CA, USA) for microarray assay.

2) MTT &Assay

The MTT test assay (Sigma, St. Louis, Mo, USA) were used for examining the proliferation of cells after culturing the cells on the machined surface and anodized surface for 24 hours and 48 hours. The substance used for MTT test was a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium salt, which turns into a blue formazan product due to the viable mitochondria in active cells. The cells were cultured on the 10 disk placed in 100ml dishes and incubated at 37°C in 5% CO_2 for 24 hours and 48 hours. The disks were move to 24 well plate after 24 hours and 48 hours incubation and new media were added, after which, the media were removed and replaced by diluted MTT solution (5mg/ml) and incubated at 37°C in 5% CO_2 for 4 hours. The media was discarded and 400 μl of isopropanol with 0.04 N HCl was added in each well. The product solution was moved into 96 well plate. The obsorbance at 570 nm was measured using microplate reader (Bio-Rad, Hercules, CA, USA).

3) Scanning electron micrograph

Cell suspension (1×10^5 cell/ml) was seeded to MS and AS. The cultures were incubated for 24 hours to evaluate the influence of the substrate geometry on machined titanium surface and anodized titanium surface using a Scanning Electron Micrograph (SEM). At the end of the various incubation times, the non-attached cells on the different substrates were removed by rinsing twice with 0.1 M sodium cacodylate buffered solution, dehydrated in a series of ethanol, dried by tetramethylsilane (Merck, Darmstadt, Germany) and sputter-coated with Au-Pd (Bio Rad, SC-500, UK). Finally, they were observed in a SEM (JSM-6430F, JEOL, Tokyo, Japan) at an accelerating voltage of 15kV. Three runs of experiments were carried out, which included all different samples in threefold.

4) Microarray assay

The cDNA microarray Agilent Rat 22K chip (Digital Genomics, Seoul, Korea) was used and monitored the

expression of 21575 genes. The samples (10μg total RNA per condition) were processed according to the manufacturer's recommendation (Fig. 1). Fluorescent-labeled cDNA for Oligo micorarray analysis was prepared by amplification of total RNA in the presence of aminoallyl-UTP followed by the coupling of Cy3 or Cy5 dyes (Amersham Pharmacia, Uppsala, Sweden). RNA extracted from cells grown on machined titanium disks was labeled with Cy3 and used as control against the Cy5-labeled, treated (AS) cDNA in the first experiment and then switched. Oligo Microarray kit was hybridized with the fluorescently labeled cRNA at 60°C for 16 hours and then washed. This micro-array contains 21575 distinct sequences (1075 control spot and 20500 gene spot). DNA chips were scanned using GenePix 4000B (Axon Instruments, Union City, CA, USA). Scanned images were analyzed with GenePix Pro 3.0 software (Axon Instruments, Union City, CA, USA) to obtain gene expression ratios. Logged gene expression ratios were normalized by LOWESS regression.

5) Statistical analysis

Test mean values and standard deviations were computed for MTT test and the Student's *t*-test were used to assess the significance level of the differences between the experimental group (N=10). All statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). P-values of <0.05 were considered statistically significant.

Results

1. MTT Assay

The density of MC3T3-E1 cells was measured over 2 different time-periods (24 hours and 48 hours) by using the MTT assay, as shown in Fig. 2. After 24 hours of adhesion, the cell density on AS was higher than MS ($p<0.05$). After 48 hours of adhesion the cell density on both titanium surfaces were similar ($p>0.05$).

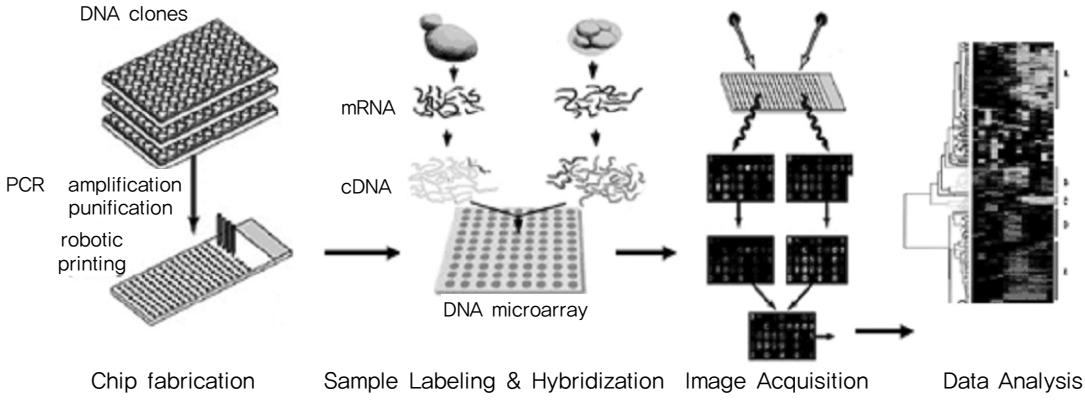

Figure 1. *Overview of DNA microarray methodology.*

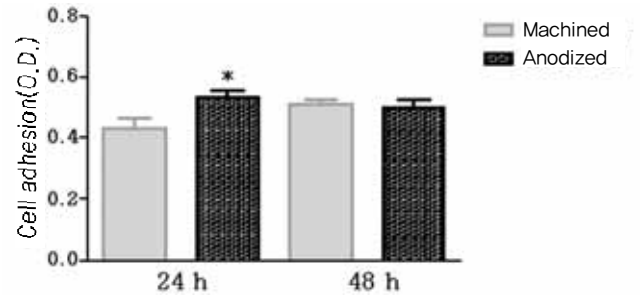

Figure 2. *Evaluation of cellular viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on MC3T3-E1 cells during 24 hours and 48 hours of cell culture on MS and AS.*

2. Scanning electron micrograph (SEM)

The surface oxide layers on AS were obtained by anodic oxidation compared with MS. Fig. 3 shows representative scanning electron micrographs of the machined surface and anodized surface. The anodic the anodized oxide layer were made from micro arcing which occurs on the surface of titanium anode which suggest that the exposure of the titanium surface to anodic oxidation result in the formation of a rough, thick and porous crystalline oxide layer.

3. Microarray assay

After 48 hours incubation of the MC3T3-E1 cells, we were evaluated the different gene expression according to the surface treatment. As shown in Fig. 1 after scanning of the two slides containing the 21,575 rat genes in duplicate, local background was calculated for each target location. A normalization factor was estimated from ratios of median. Normalization was performed, by adding the $\log_2$ of the normalization factor to the $\log_2$ of the ratio of array were then calibrated using the normalization factor. If $\log_2$ Cy5/Cy3 (Cy5: MS, Cy3: AS) is lower than -1, there is a two-fold expression in AS than MS, if $\log_2$ Cy5/Cy3 (Cy5: MS, Cy3: AS) is higher than 1, there is a two-fold expression in MS than AS.

The genes differently expressed in machined surface and anodized surface were reported in Table 1 and Table 2, whereas Fig. 4 showed the general view of the genetic profiling (M-A plot).

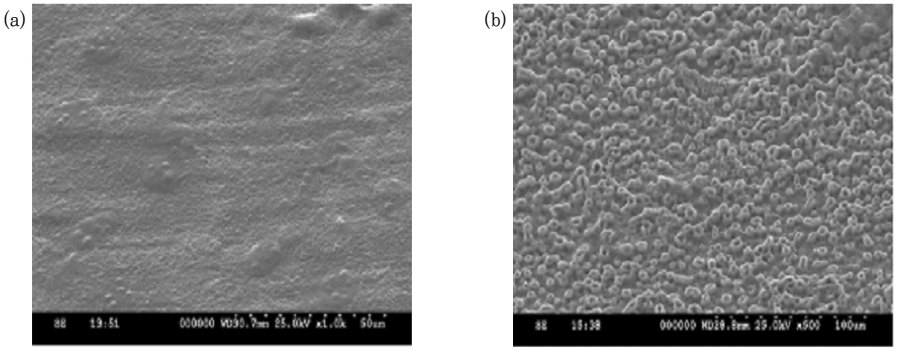

Figure 3. Scanning electron micrograph of MS (a) and AS (b) before seeding the cells.

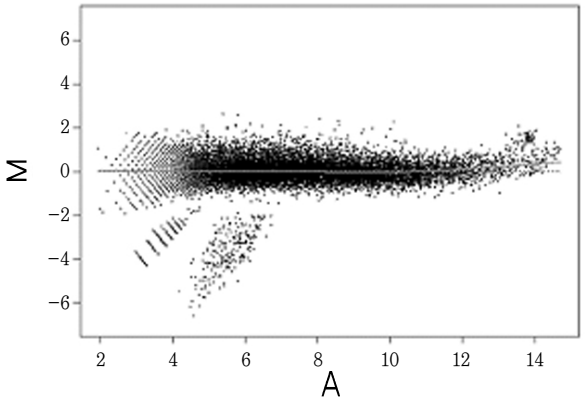

Figure 4. M-A plot.

$M=\log_2(R/G)$
$A=\{\log_2(RXG)\}/2$
R = Cy5 signal - background
G = Cy3 signal - background

Table 1. The down-regulated genes expressed lower on AS than MS

ID	Cytoband	Title	Score
14886	2q34	Extracellular matrix protein 1	0.3323292
5582	9q33	Fibronectin 1	0.3386347
3563	10q25	Vitronectin	0.3902806
3818	7q36	Insulin-like growth factor binding protein 6	0.3220125
5113	1q22	Insulin-like growth factor 1 receptor	1.199063
889	14p22	Fibroblast growth factor 5	0.8153113
2054	14q21	Fibroblast growth factor receptor 3	0.5838624
4106	18p11	Fibroblast growth factor 1	0.32209
4423	3q35	Fibroblast growth factor 7	0.8470103
7087	14q21	Fibroblast growth factor binding protein 1	0.8986635
10843	1q22	Fibroblast growth factor 21	0.4266481
17346	7q11	Fibroblast growth factor 22	0.389941
19384	1q42	Fibroblast growth factor 4	0.3734014
17327	2q33	Platelet-derived growth factor, C polypeptide	0.832271
6233	15p14	Bone morphogenetic protein 4	0.3101672
9001	15p11	Bone morphogenetic protein 1 (procollagen C-proetinase)	0.7528597
18436	15p11	Bone morphogenetic protein 1 (procollagen C-proetinase)	0.6310773
21133	--	Transforming growth factor, beta 1	0.4189094

Ratio = $\log_2$ Cy5/Cy3 (Cy5: MS, Cy3: AS).

Table 2. The up-regulated genes expressed higher on AS than MS

ID	Cytoband	Title	Score
1520	1q11	Insulin-like growth factor 2 receptor	-0.5445057
5375	15p11	Fibroblast growth factor 17	-0.3036314
10373	1q37	Fibroblast growth factor receptor 2	-0.3071231
13715	2q15	Fibroblast growth factor 10	-0.3514244
14870	Xq31	Fibroblast growth factor 16	-0.4562805
15980	4q42	Fibroblast growth factor 23	-0.836829
17241	--	Fibroblast growth factor 18	-0.3649532
7120	12q11	Platelet derived growth factor, alpha	-0.363867
16844	3q42	Bone morphogenetic protein 7	-0.3730925
20832	Xq13	Bone morphogenetic protein 15	-0.845949
508	1p12	Connective tissue growth factor	-1.019226
9987	13q26	Transforming growth factor, beta 2	-0.3889263

Ratio = $\log_2$ Cy5/Cy3 (Cy5: MS, Cy3: AS).

Discussion

In this study, we examined whether the anodization of the titanium surface affects proliferation and phenotype of the rat calvarial osteoblast cultured on the implant disk surface.

The success of implant in dentistry depends on "osseointegration" at the bone-implant interface. The "osseointegration" of biomaterials to bone comprises two components, the response of the host to the implant and the behavior of the material in the host. The host response to implant placed in bone involves a series of cell and matrix events, ideally culminating in intimate apposition of bone to biomaterial. The almost immediate event occurring upon implant in the host is adsorption of proteins which first come from blood and tissue fluids at the wound site and later from cellular activity in the periprosthetic region.

Different approaches are being used in an effort to obtain the desired bone-implant interface. The ideal implant should present a surface conducive that will induce osseointegration, regardless of implantation site, bone quantity, etc. The biological tissues interact with mainly the outermost atomic layers of an implant and the "primary interaction zone" is generally about 0.1-1nm. Consequently, much effort is being devoted to methods of modifying surfaces of existing biomaterials to achieve desired biological responses and the process of osseointegration in the bone-implant interface was reviewed[6].

The dioxide layer (TiO_2) spontaneously forming at the surface of the implant were very stable, it slow down the corrosion, maintaining mechanical properties and lowering material dissolution out to the tissues. Even when attacked, it only releases non-toxic and non-reactive products. The ability of titanium to replace mechanical functions of the body is broadly due to its strength. Its low weight increases the life quality of the patient and its ductility facilitates the adaptation work of the surgeon[16].

In this study we compared the cell viability cultured on the machined surface and anodized titanium surface. We used the rat calvarial osteoblast-like cells (MC3T3-E1 cells) to examine the viability of cells plated on the machined surface and anodized surface. During 24 hours of cell culture on AS showed higher viability than MS ($p<0.05$). After 48 hours of cell culture both titanium surfaces showed similar viability ($p>0.05$).

Comparing the surface roughness the machined surface and anodized surface, the machined surface was smooth, whereas the anodized surface was rough and porous which will affect the phenotype of the attached cell and adhesion between biomaterials and the cells[2,12,17]. To evaluate the phenotype of attached osteoblast-like cells, cells were plated on the machined surface and anodized surface and observed in a Scanning Electron Micrograph (SEM). These results will affect the initial stability and removal torques and histological osseous healing of the implant[3,5,18,19]. These kinds of alterations in surface morphology and roughness have been used to influence cell and tissue responses to implants. Porous coating was developed with the rationale that, because of mechanical interlocking, bone ingrowth would increase fixation and stability of implant. Son et al. revealed that the removal torque strength was significantly higher for anodized implant than the untreated implant at 6 weeks after implantation[5].

The goal of modification implant surface is to immobilize proteins, enzymes, or peptides on biomaterials for the purpose of inducing specific cell and tissue responses or, in other words, to control the tissue-implant interface with molecules delivered directly to the interface. One approach to control cell-biomaterial interactions was to utilize cell adhesion molecules (fibronection, vitronectin, type I collagen, osteopontin, and bone sialoprotein, etc). A second approach to biochemical surface modification uses biomolecules involved in bone development and fracture healing which effects for mitogenecity (e.g., IGF-I, FGF-2, and PDGF-BB) to increasing activity of bone cells (e.g. TGF-β1) to osteoinduction (e.g. BMPs). Hybridization of mRNA-derived probes to cDNA microarrays allows us to perform systemic analysis of expression profiles for thousands of genes simultaneously and provide primary information on transcriptional changes related to different implant surfaces. We identified several genes whose expression was importantly related with cell-biomaterial interaction and mitogenecity.

The insulin-like growth factor 1 receptor binds insulin-like growth factor with a high affinity. It has tyrison kinase activity and plays a critical role in transformation events. Cleavage of the precursor generates a and β subunits. It is highly overexpressed in most malignant tissues, where it functions as an antiapoptotic agent by enhancing cell survival. It was down-regulated (more than 2 fold) on AS than MS. As shown in Table 1, other cell adhesion molecules

(Extracellular matrix protein 1, Fibronection 1, Vitronectin, Insulin-like growth factor binding protein 6) were more expressed in MS than AS.

The connective tissue growth factor (CTGF) is up-regulated on AS than MS (more than 2 fold). CTGF binds IGF and may have a role in regulating cell growth.

Among the molecules affecting the cell mitogenecity, fibroblast growth factor-7, 5, Platelet derived growth factor C peptide, Bone morphogenic protein 1, Transforming growth factor ß1 were definitely down regulated on AS compared with MS. On the other hand, Insulin-like growth factor 2 receptor out of the adhesion molecules was more expressed on the anodized surface than machined surface. Fibroblast growth factor 23 and bone morphogenic protein 15 out of the mitogenetic molecules were definitely up-regulated on the anodized surface compared with machined surface, but rest of the molecules up-regulated from the mitogenitic molecules on the anodized surface were not remarkable.

The genes discussed are only a limited number among those differentially expressed and the down-regulated on the anodized surface was reported in Table 1, and the up-regulated on the anodized surface was in Table 2. We briefly analyzed some of those better-known function.

Recent studies on animal models have demonstrated that titanium surfaces are important to influence the timing of bone healing and rougher surfaces induced a higher quantity of bone-implant contact percentage and higher removal torque values.

The anodized titanium surface had the irregular, rough, porous surface texture, which plays an important role in the regulation of the cells (MC 3T3-E1, rat calvarial osetoblast-like cells) to higher viability of the cells on the implant disk surface after 24 hours compared with machined surface but similar viability of the cells plated on the anodized surface compared with machined surface was observed 48 hours after plating the cells.

Microarray assay at 48 hours after culturing the cells on the machined surface and anodized surface revealed that osteoinductive molecules appeared more prominent on the anodized surface, whereas the adhesion molecules on the biomaterial were higher on the machined surface than anodized surface, which will affect the phenotype of the plated cells depending on the surface morphology.

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