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Research Article

A Comparative Study on the Effect of Integrin Subunits Beta One and Beta Three on Osteoblast Implant Interactions

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Abstract

Background: Implant surface features affect bone formation and adherent cellular activities. Integrin transmembrane receptors has emerged as central regulators of cell biomaterial interactions.

Aim: To compare and contrast the effect of integrin beta 1 and beta 3 subunits on initial interaction of osteoblast like cells with titanium surfaces of different surface topographies.

Materials and Methods: CpTi disks were prepared to produce two different surface topographies, smooth (turned) and micro-rough (sand blasted and acid etched). MC3T3-E1 cells were used and they were pretreated with either anti- beta 1/anti- beta 3 monoclonal antibodies or IgG control. Cells were plated on the disks for 24 hours then RT^2 Profiler™ PCR Arrays were used to quantify the expression of a panel of osteogenesis related genes. Moreover, adherent cells were fixed at 2, 4, and 24 hours on the disks for SEM evaluation of the different experimental groups. Number of total, spread, and round cells were measured on three random areas per each disk and mean number of cells were calculated for statistical comparisons. Furthermore, SEM images were made at higher magnification for subjective evaluation.

Results: 1. Mean numbers of total and spread cells were significantly higher on the control group in comparison to the anti- beta 1 or anti- beta 3 groups which were not significantly different.

2. Large number of genes related to osteogenesis showed significant difference in mRNA fold induction. Differences were both surface and treatment dependant.

Conclusions: 1. Blocking integrin-beta 1 and integrin- beta 3 subunits with antibodies has an inhibitory effect on osteoblast like cell binding, and spreading to CP titanium surfaces *in vitro.*

2. Both beta 1 and beta 3 integrins are involved in mediating surface specific changes.

Keywords: *Intergrin B1; Integrin B3; Implant surface; Implant surface topography; Osteoblast; Osseointegration; Cell spreading; Cell attachment; Osteogenesis*

Introduction

The introduction of new medical techniques intended to help patients in overcoming their disabilities and improve their quality of life has always been a priority. However, any novel concept should be based on solid scientific evidence to ensure long term success with minimal side effects. Osseointegration is a concept that was introduced by Branemark and his colleagues in the mid 1960's, and since then it has been a leading topic of interest. Based on this concept many new techniques and materials have evolved and are available to facilitate treatment and rehabilitation of patients.

Branemark placed his first intraoral implant in 1965. Despite having poor clinical results in the following 5 years with a success rate of about 50%, Osseointegration research continue to expand with the introduction of new surgical protocols and implant designs. Indisputable progress was made during the 1970's. This resulted in significant improvement and scrupulous documentation in the field of implantology leading to its general acceptance in Europe. Another important historical event in endosseous implant evolution was their introduction in North America in 1982. The last three decades were marked by expanded Osseointegration research, and tremendous increase in endosseous implants usage [1].

Orthopedics, bone-anchored hearing aids, craniofacial prosthetics, and Prosthodontics are some of the fields that were positively impacted and rejuvenated by the use of endosseous implants. Tissue integrated prostheses are currently a predictable and highly reliable technique. Endosseous implant markets are growing very rapidly world-wide [2-4]. Endosseous implants are used to support and retain all kinds of prostheses in Dentistry and Maxillo-facial prosthetics from single tooth to prosthetic ears and noses. Moreover, they have a wide variety of uses in medicine particularly in the joint replacement field. The impact of those new treatment modalities on patient's life as well as on treatment planning procedures has been tremendous. Implant supported prostheses are the standard of care nowadays to replace missing teeth.

Despite the success, many unanswered questions remain unanswered. Studying the molecular aspects of Osseointegration is an important mode of its research. The tissues that oppose endosseous implants are multidimensional and represent diverse and dynamic living entities. Interfacial tissues include epithelium, soft fibrous connective tissues, and calcified bone [5]. These living tissues are regulated at the molecular level. It is important to consider that the clinical success of endosseous implants is associated with the formation and maintenance of bone at implant surfaces [6]. This research project aimed to understand some of the fundamentals of Osseointegration, and to reveal the importance of the integrin receptors in the early stages of this process. Integrins are a group of transmembrane proteins that mediate the interaction and cross-talking of the cells with extracellular matrix components and other cells (Figure 1). These receptors particularly integrin beta1 (Itgβ1) and integrin beta3 (Itgβ3) are thought to play a critical role in Osteoblast interaction with implant surface. Nevertheless, their exact role in this process is yet to be explicitly elucidated.

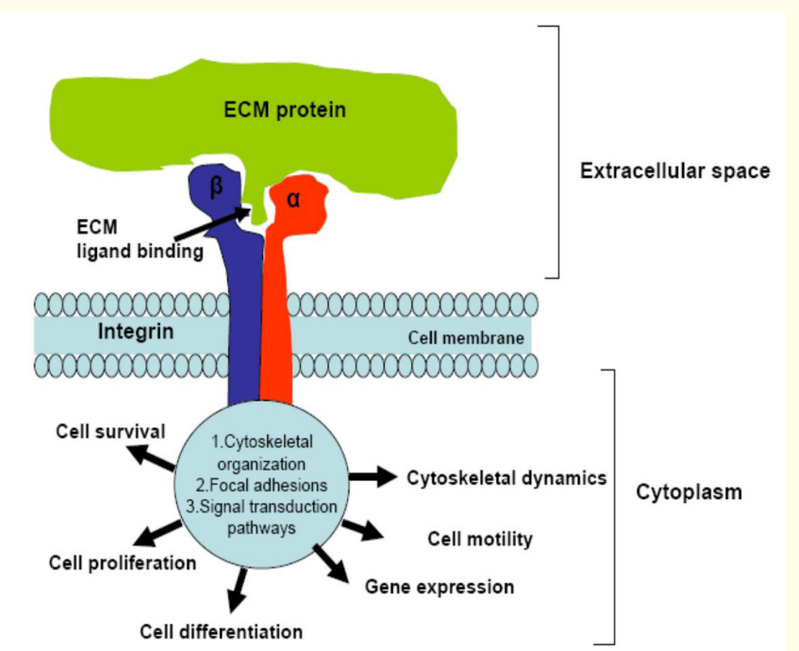

Figure 1: *Schematic representation of the integrin receptor and its role in signal transduction.*

Thus, this project was conducted to compare and contrast the effects of β1 and β3 integrins on early osteoblast implant interactions and their ability to mediate surface specific changes.

Materials and Methods

Commercially pure titanium surface preparation

Commercially pure grade IV titanium (cpTi) disks were provided by the University of North Carolina (UNC) Dental Research Center, bone biology and implant therapy laboratory. The disks were 13 mm in diameter and 2 mm thick. Disks were randomly selected to prepare two different surface roughnesses, smooth (turned) and rough (sandblasted and hydrochloric acid etched). All disks were initially prepared by polishing using silicone carbide abrasive paper (3M, Saint Paul, MN). Disks were gradually polished by 200,400 and 600 grit carbide abrasive papers in a consecutive manner. While the disks were polished they were washed consequently with 70% ethanol to clean the debris and to minimize heat production. Afterwards, the disks were washed thoroughly using distilled water. The disks that were randomly selected to have smooth surface topography were ultrasonically cleaned using deionized distilled water (ddH_2O). The disks went through 5 phases of ultrasonic cleaning for 5 minutes duration for each phase. The ddH2O was changed each time and the disks were further washed and rinsed thoroughly in ddH_2O between phases.

The rough surface were prepared and passivated according to the preparation method proposed by Keller., *et al* [7]. Disks were further grit-blasted with 100 μm aluminum oxide Al_2O_3 particles using a sandblasting machine (MicroBlaster, Comco Inc, Burbank, CA). Afterwards, sandblasted surfaces were washed with ddH_2O, ultrasonically cleaned five times, 5 minutes each time with ddH_2O in a similar fashion to the smooth surfaces. Then the disks were acid etched with 5 mol/L hydrochloric acid (5N HCL) overnight. This procedure will result in grit blasted acid etched surface of the micron-scale level of roughness [8].

Finally, both smooth and rough surface disks were rinsed and washed thoroughly using ddH_2O and passivated by soaking the disks 40% nitric acid for 5 minutes. After the passivation process, the disks were further washed by ddH_2O and then were soaked in 70% alcohol for at least 24 hours. Prior to cell plating, the disks were exposed to ultraviolet light in a sterile tissue culture hood for 24 hours to dry and sterilize the disks.

Surface analysis

To gain more detailed information regarding the prepared surfaces topography, a surface analysis was conducted using atomic force microscopy (AFM). Disks were subjected to AFM analysis (AFM; Auto Probe CP, Park Scientific Instruments, Sunnyvale, CA). The atomic force microscope uses a non-contacting stylus to image the surface of the disks and create a digitized image from which numerous surface parameters can be calculated. Scans (50 μm X 50 μm) were made for each surface, and three disks were analyzed for each individual surface. Packaged algorithms provided calculations for area statistics, which included average roughness (Ra) and root mean square roughness (RMS). Average roughness (Ra) is the most commonly reported surface parameter and it represents the arithmetic mean of deviations in the roughness profile from the mean line [9]. On the other hand, RMS represents the standard deviation of the distribution of surface heights. It is important parameter to describe surface roughness by statistical methods.

Cells and cell culture

The MC3T3-E1 osteogenic cell line (American type culture collection, Manassas, VA) was used in this project. The cells were maintained in Gibco Minimal Essential Medium Eagle, Alpha modification (α-MEM) (Invitrogen Corporation, Carlsbad, CA). The medium was supplemented with 10% fetal bovine heat inactivated serum (FBS) (Invitrogen Corporation, Carlsbad, CA) and 1% antibiotics (penicillin/streptomycin) and antimicotic agents (Sigma, Saint Luis, Missouri). The cells were cultured in a fully humidified atmosphere consisting of 95% air, 5% CO_2 at 37°C. The cells were passaged every third day. Cells at 90% confluent were removed by using Trypsin/EDTA reagent (Sigma, Saint Louis, Missouri). At the time of the experiments the cells were centrifuged at 1200 rpm for 4 minutes, suspended in complete medium counted and prepared for each experiment. Each experimental condition was performed in triplicate for statistical confidence.

Cell treatment with monoclonal antibodies and IgG control

Before plating the cells on the disks for both the molecular and morphological studies, the cells were treated with either specific monoclonal antibodies for β1 and β 3 integrin subunits or with control immunoglobulin G antibody (IgG). After the cells were trypsnized, counted, and centrifuged, they were resuspended in medium to provide the concentration of cells needed for the particular experiment. Afterwards, the cells were centrifuged again at 1200 rpm for 4 minutes and the medium was removed. The cell pellet was resuspended in media containing the specific monoclonal antibody or the IgG control at the defined concentration.

Monoclonal anti-mouse integrin β1/ CD29 antibody (R and D systems, Minneapolis, MN) was used to block integrin β1 function. The stock solution was diluted with 0.2 ml of ice cold 1X Phosphate buffered saline (PBS). This resulted in an antibody concentration of 500 μg/ml. Moreover, monoclonal anti- β3 CD61 mouse antibody (Fitzgerald industries international incorporation, Concord, MA) was used to block β3 integrin function. The stock solution was reconstituted with 1 ml of ice cold 1X PBS that resulted in a concentration of 500 μg/ml. Finally, for the control groups a Rat IgG isotype control antibody (R and D systems, Minneapolis, MN) was used. The stock solution was reconstituted with 1ml of ice cold PBS to produce a concentration of 500 μg/ml. For each of the three reagents, 60 μg was needed to treat one million cells. The cell pellet after centrifuging was washed twice with ice cold 1X PBS and was resuspended in the solution containing either the anti- β1 monoclonal antibody, anti- β3 monoclonal antibody, or the IgG isotype control. The tubes were kept in a cell culture incubator at 37°C with intermittent agitation. After one hour α-MEM was added to produce a concentration of 20 μg/ml for the three reagents in the RT^2 Profiler™ PCR Arrays experiment, and 8 μg/ml for the SEM experiment. For both the molecular and the morphological studies cells were resuspended in 300 μl media for plating on each disk. This resulted in final concentration of 6 μg/ 100,000 cells for the molecular study and 2.4 μg/40,000 cells for the morphological study.

Osteogenesis gene expression profiling with RT^2 Profiler™ PCR Arrays experiment

After the completion of the cell treatment with anti- β1 monoclonal antibody, anti- β3 monoclonal antibody, or IgG isotype control and the addition of the required amount of α-MEM medium, the cells were mixed thoroughly and were ready to be plated. This experiment was performed twice; once the cells were treated with anti- β1 monoclonal antibody in the test group and the second time cells were treated with anti-β3 monoclonal antibody in the test group. In both experimental conditions, cells in the control groups were treated with IgG isotype control. Having two different surface preparations (rough, smooth) and two different cell treatment protocols (anti β1/ β3, Control) resulted in four different groups in each experimental condition (Figure 2.1).

The cells were seeded on the disks with a density of 10^5 cells/ disk in 300 μl volume of medium. Seven disks were used for each group the disks were placed in cell culture plates and incubated in fully humidified atmosphere consisting of 95% air, 5% CO_2 at 37°C for 24 hours. The cells were allowed to attach initially to the surface for 3 hours and after 3 hours α-MEM medium was added to cell culture plates until the disks were completely covered and were placed back in the incubator for the remainder of the 24 hour culture period. After 24 hours, the cells were harvested using TRI REAGENT™ (Sigma, Saint Louis, Missouri). This reagent is a mixture of guanidine, thiocyanate, and phenol in a mono-phase solution. After the removal of the medium the disks were washed twice with ice cold 1X PBS, then the cells were harvested carefully using the TRI REAGENT™ and were ready for ribonucleic acid (RNA) isolation. After the cells were homogenized in TRI REAGENT, samples were stored at -70°C till the time of RNA isolation less than a week after the completion of the experiment.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from cell layers using TRI REAGENT™ (Sigma, Saint Louis, Missouri), based on the single-step method described by Chomczynski and Sacchi [10].

1. The homogeneous mix sample was allowed to stand for 5 minutes at room temperature. Afterwards, 0.2 ml of chloroform was added per ml of TRI REAGENT used. The resulting mix was centrifuged at 12,000 x g for 10 minutes at 4°C mix. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interface (containing DNA), and a colorless upper aqueous phase (containing RNA).

2. The aqueous phase was transferred to fresh tube and 0.5 ml of isopropanol per ml of TRI REAGENT used in sample preparation. The resulting mixture was allowed to stand for 5 - 10 minutes at room temperature, and then it was centrifuged at 12,000g for 10 minutes at 4°C. This step precipitated the RNA which formed a pellet on the side and bottom of the tube.

3. The supernatant was removed and the RNA pellet was washed with 75% ethanol per 1 ml of TRI REAGENT used in sample preparation. The mix was then shaked vigorously using Vortex and then it was centrifuged at 7,500 x g for 5 minutes at 4°C.

4. The RNA was air dried for 5 - 10 minutes without completely drying the pellet. Afterwards, the RNA was redissolved using 10 – 20 µl of RNase free diethylpyrocarbonate (DEPC) water. To facilitate the dissolution, the liquid was mixed by repeated pipetting at 55 - 60°C for 10 - 15 minutes.

5. The RNA was quantified by UV spectrophotometry (Beckmann DU-600). One µg of total RNA was needed to make cDNA using RT^2 first stand kit (SuperArray, Bioscience Corporation, Fredrick, MD) for each 96-well plate formats of RT^2 Profiler™ PCR Arrays.

6. For cDNA synthesis, a genomic DNA elimination mixture was prepared by mixing total RNA with GE reagent (5x gDNA elimination buffer) and DPEC water. The mixture was incubated at 42°C for 5 minutes and chilled immediately on ice. Afterwards 10 µl of the Reverse transcriptase (RT) cocktail was added to 10 µl of genomic DNA elimination mixture and they were mixed very well using a pipettor and were incubated at 42°C for 15 minutes. Afterwards, they were heated to 95 °C for 5 minutes to degrade the RNA and to inactivate the reverse transcriptase. Finally, 91 µl of ddH_2O was added to each 20 µl of cDNA synthesis reaction. They were mixed well and the finished first strand cDNA was stored at -20°C. The cDNA for each experimental group was equally divided into 3 samples to perform the PCR array in triplicate format.

Performing Real-Time PCR using RT^2 Profiler™ PCR Arrays

To determine the relative differences in gene expression of osteogenesis specific genes the mouse osteogenesis RT^2 Profiler™ PCR Arrays system was used. This system brings together the quantitative performance of real-time PCR and the multiple gene profiling capability of microarrays. This PCR array profiles the expression of a panel of 84 genes related to osteogenic differentiation, skeletal development, bone and mineral metabolism, growth factors, cell adhesion and extracellular matrix molecules related to bone development, and genes mediating osteogenesis, cell proliferation, growth, and differentiation. The whole list of genes included in this array is represented in appendix A. The protocol for performing the PCR array took about two hours for each sample. The PCR was done in triplicate for each experimental or control group for statistical confidence. This resulted in 12 samples for the anti- β1 experiment and another 12 samples for the anti- β3 experiment.

For each sample (PCR plate) 102 µl of diluted first strand cDNA was mixed with 1275 µl of 2X super Array RT^2 qPCR master mix and 1173 µl of ddH_2O, this resulted in 2550 µl of total volume. Equal aliquots of 25 µl were added to the 96 wells containing the pre-dispensed gene-specific primer sets using a multi-channel pipette and the wells were covered tightly with a plastic led. PCR was performed using

ABI prism 7000 real-time PCR thermocycler. The instrument's software was used to calculate the threshold cycle (Ct) values for all genes on each PCR array. Five internal control genes presented in PCR array were used for normalization. A simple examination of Ct value consistency of these internal control genes quickly indicated the proper normalization method. Fold changes in gene expression for pair-wise comparison was calculated using comparative Ct method (ΔΔ Ct method). This method was used to calculate the relative amount of the transcripts in the experimental sample and the control sample, both of which were normalized to the internal controls. Δ Ct is the $\log_2$ difference in Ct between the gene and internal controls, ΔΔ Ct= ΔCt (experimental)-ΔCt (control) for biological RNA samples [11].

Cell binding experiment using scanning electron microscopy

Evaluation of the effect of anti-β1 and anti- β3 monoclonal antibodies, time, and surface topography on MC3T3-E1 initial cell binding to Cp titanium surfaces as well as cell spreading were examined both quantitatively and qualitatively using SEM. The experimental design was similar to the PCR array experiment (Figure 2.1). However, the effect of time on the initial cell binding and spreading was evaluated at 3 different time points for all different experimental groups (2 hours, 4 hours, and 24 hours). Unlike the PCR array experiment, anti- β1 and anti- β3 and control (IgG) were evaluated on both smooth and rough Cp titanium surfaces in the same experiment. This resulted in six different experimental groups that were evaluated at three different time points (Figure 2.1). Two disks were used for each group at any single time point. The cells were treated with either anti- β1 or anti- β3 and IgG isotype control in a similar fashion to the PCR array experiment. However, only 40,000 cells in 300 µl of medium were plated on each disk to decrease cell density and be able to identify individual cells when performing the SEM.

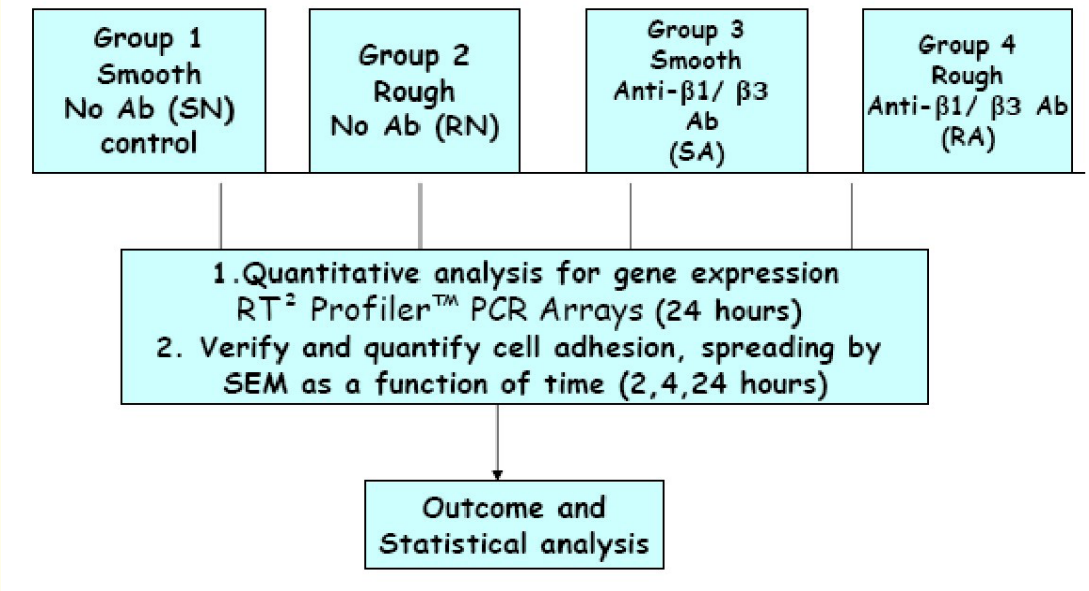

Figure 2.1: *A flow chart representing the experimental design for The SEM and the RT² Profiler™ PCR Arrays experiments.*

SEM preparation

After plating the cells on the disk, adherent cells and disks at the particular time points were rinsed three times with ice cold 1X PBS and fixed for 60 minutes with 4 % Para- formaldehyde and left at room temperature for one hour, then they were refrigerated a -4°C for

at least 24 hours. For the 4 and 24 hour groups α-MEM medium was added to cover the disks after 3 hours, then it was removed before washing the adherent cells and disks with 1X PBS and fixation with paraformaldehyde. After fixation for 24 hours the paraformaldehyde was removed and the disks were further washed three times with ice cold 1X for 15 minutes each time. Afterwards, the disks and adherent cells were dehydrated using graded ethanol solutions from 50% to 100% for 15 minutes each time in a 12 well-plate. The 100% ethanol step was repeated for 3 times. After the last 100% ethanol drying step, 2 ml of hexamethyldisilazane (HDMS) (Electron microscopy sciences, Fort Washington, PA) was added for each well and let to evaporate overnight under a fume hood for complete dehydration. Before performing the SEM imaging the samples were coated with conductive material according to SEM manufacturer recommendation. Hitachi S-4700 Scanning electron microscope (Hitachi High Technologies America, inc., Pleasanton, CA) was used to obtain SEM images for the different groups.

Quantitative and qualitative evaluation of osteoblast like cell adhesion, spreading and morphology on Cp titanium surfaces

Cell adhesion was determined by averaging the number of the cells counted at low magnification (X 300) from three random areas per disk. Two disks were used for each group at any particular time point. The number of cells was counted three times by three different investigators working independently and who were blinded to the experimental group for each sample. The investigators were calibrated before performing the cell counting process and inter-examiner reliability test showed a very good agreement among the three investigators (Kappa value 0.78). This protocol resulted in 18 readings for any experimental group at any particular time point. Descriptive statistics were calculated for each group using SPSS software (SPSS inc. Chicago, Illinois) and comparisons among groups and between particular groups were performed. Furthermore, the spreading of the cells was evaluated by using the presence of cell processes, elongation of the central cytoplasm region, cell diameter at its longest axis as criteria for spread cell to score the cell morphology manually. Copies of all the SEM images at X300 magnification were printed out for each investigator on an A2 size (8.5X11) paper sheet. Cells that scored 10 mm or more in its longest dimension were considered as spread cells and the ones that scored less than 10 mm were considered as round cells. Data acquisition and analysis for round and spread cells were performed in identical fashion to total number of cells with three blind investigators counting cells independently.

SEM images at higher magnification (X 2000) for all experimental groups were made to evaluate cell shape, spreading and attachment for subjective comparison among different surfaces and treatment protocols.

Statistical analysis

For the RT^2 Profiler™ PCR Arrays experiment, a specific data analysis web portal provided by the PCR arrays system was used to perform the data analysis. This web portal automatically performs calculations and interpretations of the control wells upon including threshold cycle data from the real-time PCR instrument. Statistical comparisons between fold changes among any two groups were performed using T-test and any p-value less than 0.05 was considered statistically significant.

As for the quantitative analysis of cell adhesion and spreading using SEM, SPSS software was used to analyze the raw data. Descriptive statistics comparisons were performed for the different experimental groups. Moreover, factorial ANOVA was used to compare the mean number of total, spread, and round cells among the different experimental groups and at different time points and any P value less that 0.05 was considered statistically significant. For pair wise comparisons between individual groups post-hoc Tuckey test was used and any p value less than 0.05 was considered statistically significant.

Results

Atomic force microscopy surface analysis

AFM analysis of the disks provided measures of average surface roughness (Ra) and root mean square roughness, for comparisons among individual surfaces (Table 1). Each surface displayed a unique topography (Figure 3.1). Smooth surfaces had relatively low peak-to-valley measurements compared to rough surfaces. Moreover, the Ra and RMS values were considerably greater for rough surfaces compared to smooth surfaces.

Surface	Ra	RMS
Rough	0.39 (± 0.019) µm	0.46 (±0.027) µm
Smooth	0.036 (± 0.006) µm	0.047 (± 0.007) µm

Table 1: *Surface parameter measurements as calculated by atomic force microscopy for smooth and rough surfaces.*

Ra: Average Surface Roughness; RMS: Root Mean Square Roughness

Data presented as the mean (± Standard error of the mean)

Atomic force microscopy representative images of 50 µm X 50 µm of the test surfaces for surface parameters analysis are presented in figure 3.1.

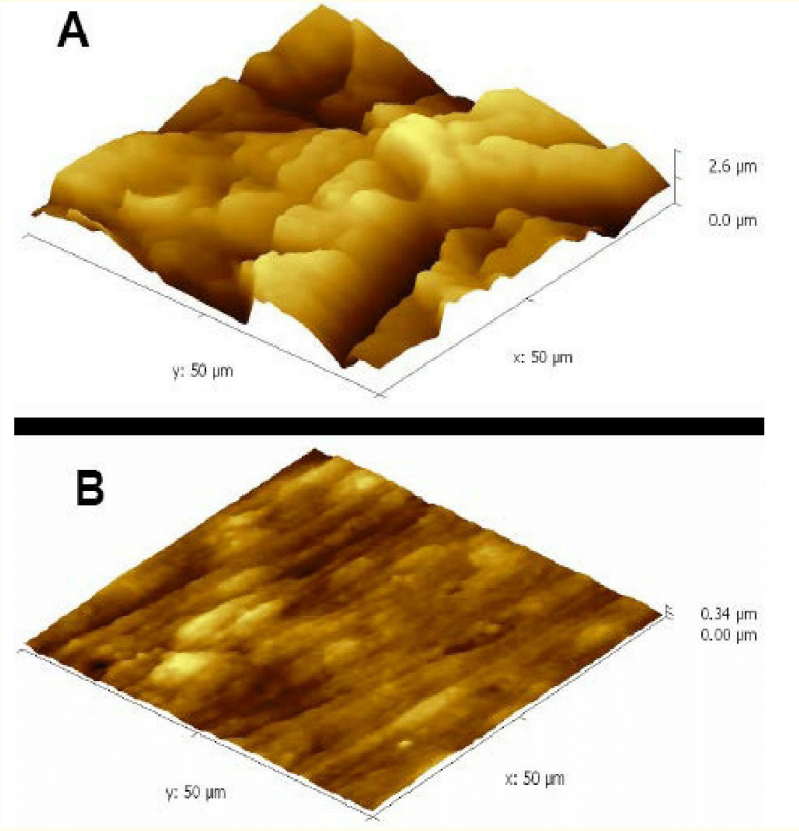

Figure 3.1: *Atomic force microscopy surface topography analyses representative of 50 µm X 50 µm areas of test surfaces (A: Rough surface, B: Smooth surface).*

Initial cell attachment and total cell count

The number of total cells per surface area was measured as a function of cell attachment to the cpTi surfaces. The input number of cells was held constant in this experiment. Mean number of adherent cells in the different experimental groups at three different time points on both smooth and rough surfaces are shown in figure 3.2.

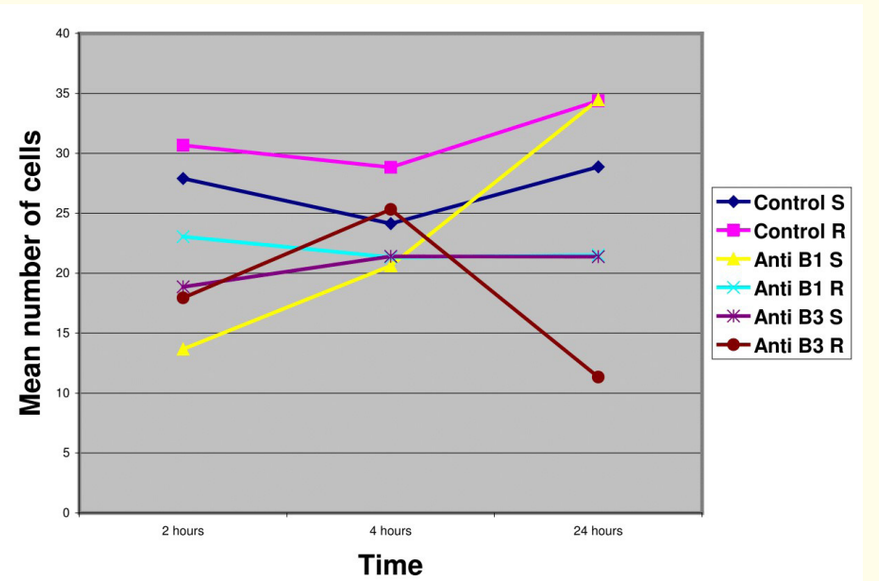

Figure 3.2: *Mean number of cells at different time points among the different experimental groups on both smooth and rough surfaces.*

S: Smooth surface; R: Rough surface

The number of cells in the control group is slightly higher on rough surface than smooth surface and they increase slightly at 24 hours in comparison to 2, 4 hours. Nevertheless, in the anti-β1 and anti-β3 groups the cell numbers initially at the 2 hours time point are less than the control group and they increase with time on the smooth surface particularly in the anti- β1 group where cell numbers return to the level of the control group at 24 hours. On the other hand, cells decrease in number on rough surface between 4 and 24 hours in the anti- β3 group and did not change in the anti- β1 group. Nonetheless, when performing factorial ANOVA statistical test, the only factor which had a significant influence on number of cells was treatment (anti- β1 Ab, anti- β3 Ab, or IgG control). Figure 3.3 shows the mean number of cells at different time points among the different experimental groups on both surfaces.

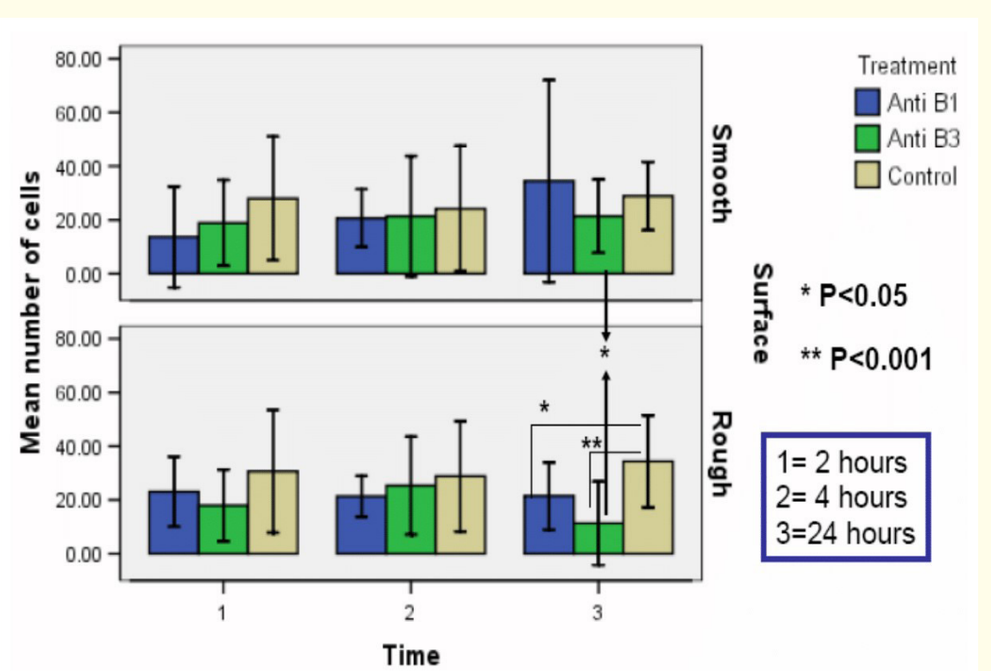

Figure 3.3: *Clustered bar chart showing the mean number of cells among the different experimental groups on both smooth and rough surfaces.*

The control group had higher number of cells than the anti-β1 or the anti- β3 groups on both surfaces and at all time points except for the anti- β1 group at 24 hours on smooth surface. However, the difference was only statistically significant at 24 hours on rough surface as determined by post-hoc Tuckey test. Moreover, smooth surface has significantly higher number of adherent cells at 24 hours than rough surface in the anti-β3 group as determined by one way ANOVA statistical test.

Initial cell spreading and spread and round cell counts

Cell spreading was evaluated by measuring the number of spread cells versus round or spherical cells. The presence of cell processes the elongation of the cytoplasm and the ability of the cell to spread for more than 10 mm as measured by the cell longest dimension were considered as surrogates for cell spreading. Figure 3.4 shows the mean number of spread cells at different time points among the different experimental groups on both smooth and rough surfaces.

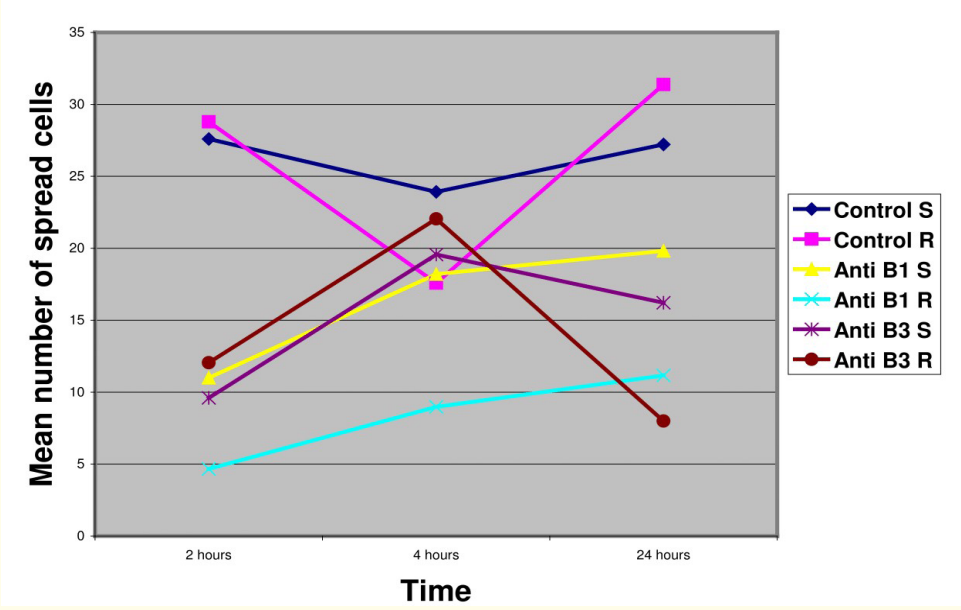

Figure 3.4: *Mean number of spread cells at different time points among the different experimental groups on both smooth and rough surfaces.*
S: Smooth surface; R: Rough surface

In the control group numbers of spread cells on both surfaces were higher than for the anti-β1 and the anti- β3 groups. However, cells in the control group adherent to the rough surface decreased from 2 to 4 hours then increased at the 24-hour mark. Notably, spread cells on smooth surface where higher than on rough surface. Similar finding was noted in the anti- β1 and anti- β3 groups with more spread cells in the anti- β3 group than anti- β1 group and more spread cells on smooth versus rough surface. Another finding was that cells slightly increased between the 4 and 24-hour time points in the anti- β1 group. On the other hand, they decreased in the anti- β3 group particularly on rough surface. Nonetheless, when factorial ANOVA statistical test was performed, the only factor that had a significant influence on number of spread cells was treatment (anti- β1 Ab, anti- β3 Ab, or IgG control). Figure 3.5 shows the mean number of spread cells on both surfaces at the different time points among the different experimental groups.

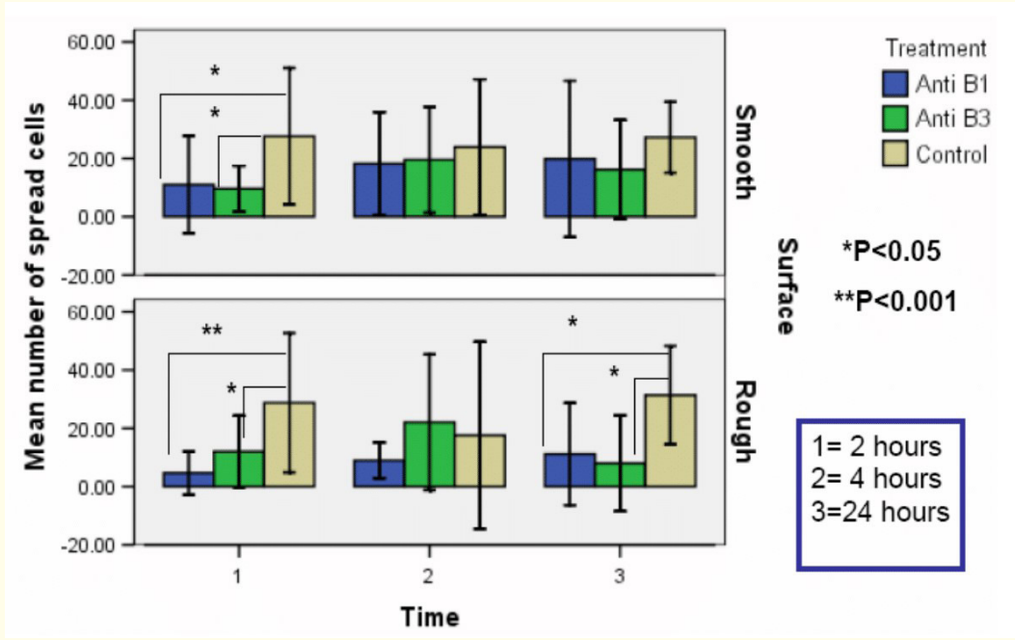

Figure 3.5: *Clustered bar chart showing the mean number of spread cells among the different experimental groups on both smooth and rough surfaces.*

The control group had higher number of spread cells than anti- β1 and anti- β3 groups on both surfaces at all time points. Nevertheless, the difference was only significant at 2 hours on both surfaces and at 24 hours on rough surface only, as determined by post-hoc Tuckey test. The mean number of round or spherical cells at different time points among the different experimental groups are shown in figure 3.6.

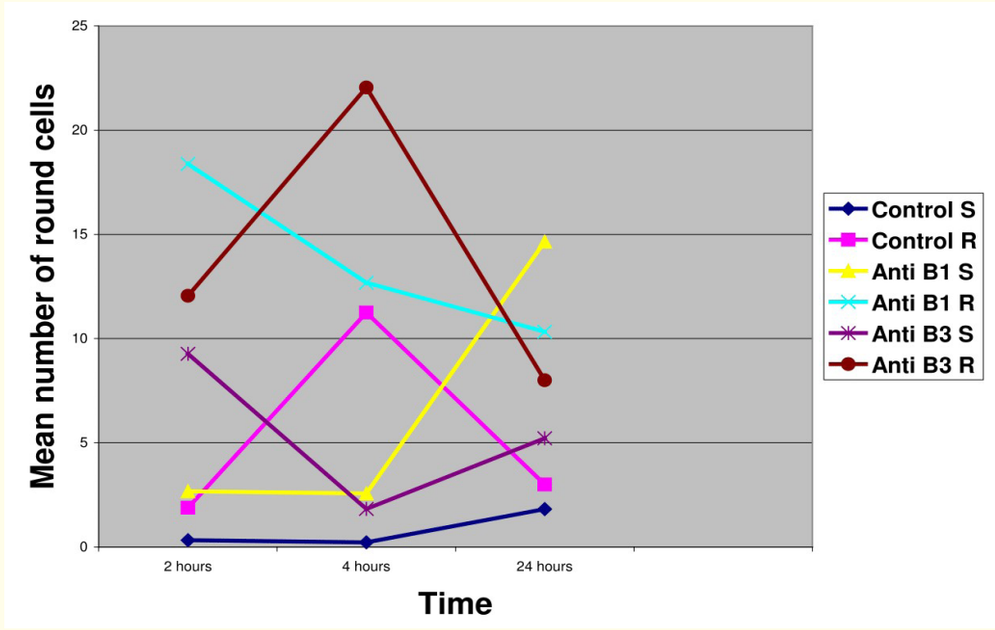

Figure 3.6: *Mean number of round cells at different time points among the different experimental groups on both smooth and rough surfaces.*
S: Smooth surface, R: Rough surface

This figure shows that the control group on both smooth and rough surfaces has less number of round cells in comparison to the anti-β1 and the anti- β3 groups although round cell number on rough surface increased at the 4 hours mark on rough surface but they decreased on the 24 hours mark. Moreover, round cells were higher on rough surface in comparison to smooth surface. This observation was also noticed in the anti- β1 and the anti- β3 groups although at the 24 hour mark the highest number of round cells was measured at the smooth surface in the anti- β1 group. Another observation was that in both anti- β1 and anti- β3 groups round cell number decreased between the 4 and 24 hour time points on rough surface and increased on smooth surface.

When factorial ANOVA statistical test was performed the factors that had significant influence on number of round cells where treatment (P value < 0.0001) and surface (p value < 0.05). When post-hoc Tuckey test was performed for pair wise comparisons, anti-β1 group had a significant higher number of round cells on rough surface than both anti-β3 group and the control group at the 2 and 24 hours time points. Nevertheless, anti-β3 had a significantly higher number of adherent round cells on smooth surface at the 2 hour time point than the anti-β1 and the control groups. Furthermore, one way ANOVA statistical test was performed for pair wise comparisons of the different experimental groups on both surfaces. These comparisons showed that the anti-β1 and the control groups had significantly higher number of round cells on rough surface than on smooth surface at the 2 and 4 hours time points (Figure 3.7).

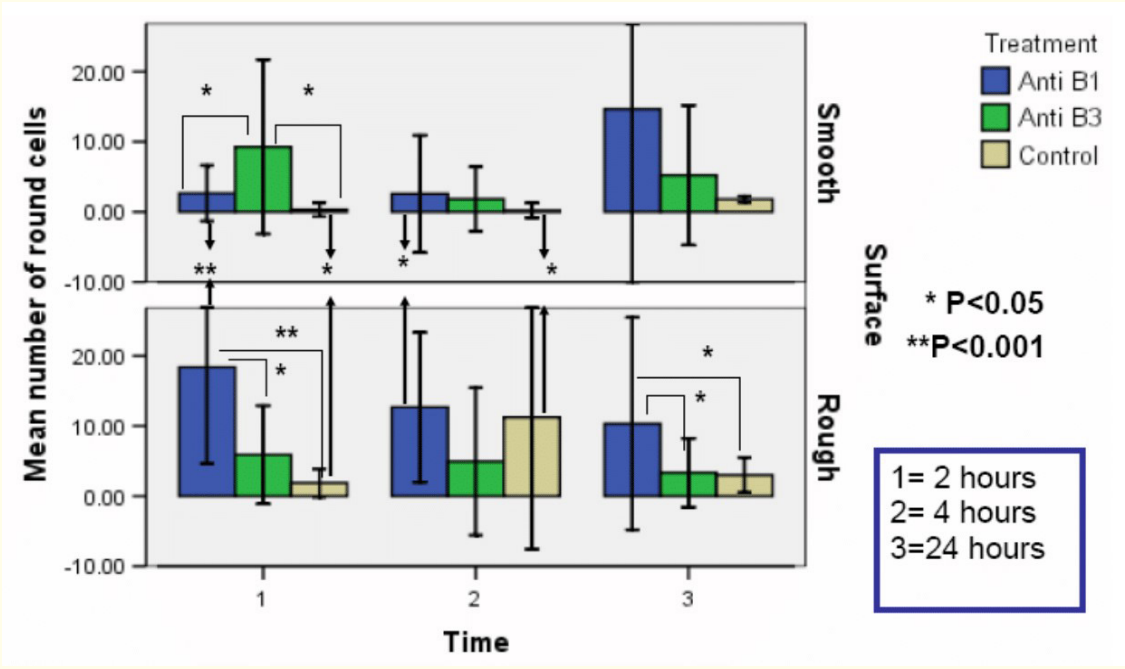

Figure 3.7: *: Box plot graph showing the mean number of round cells among the different experimental groups on both smooth and rough surfaces.*

Different examples of SEM images that were used for counting the number of total, spread, and round cells are shown in figure 3.8. These sections show that the experimental groups anti-β1 and anti- β3 had less number of cells and higher number of round cells or cells that couldn't spread enough on the surface.

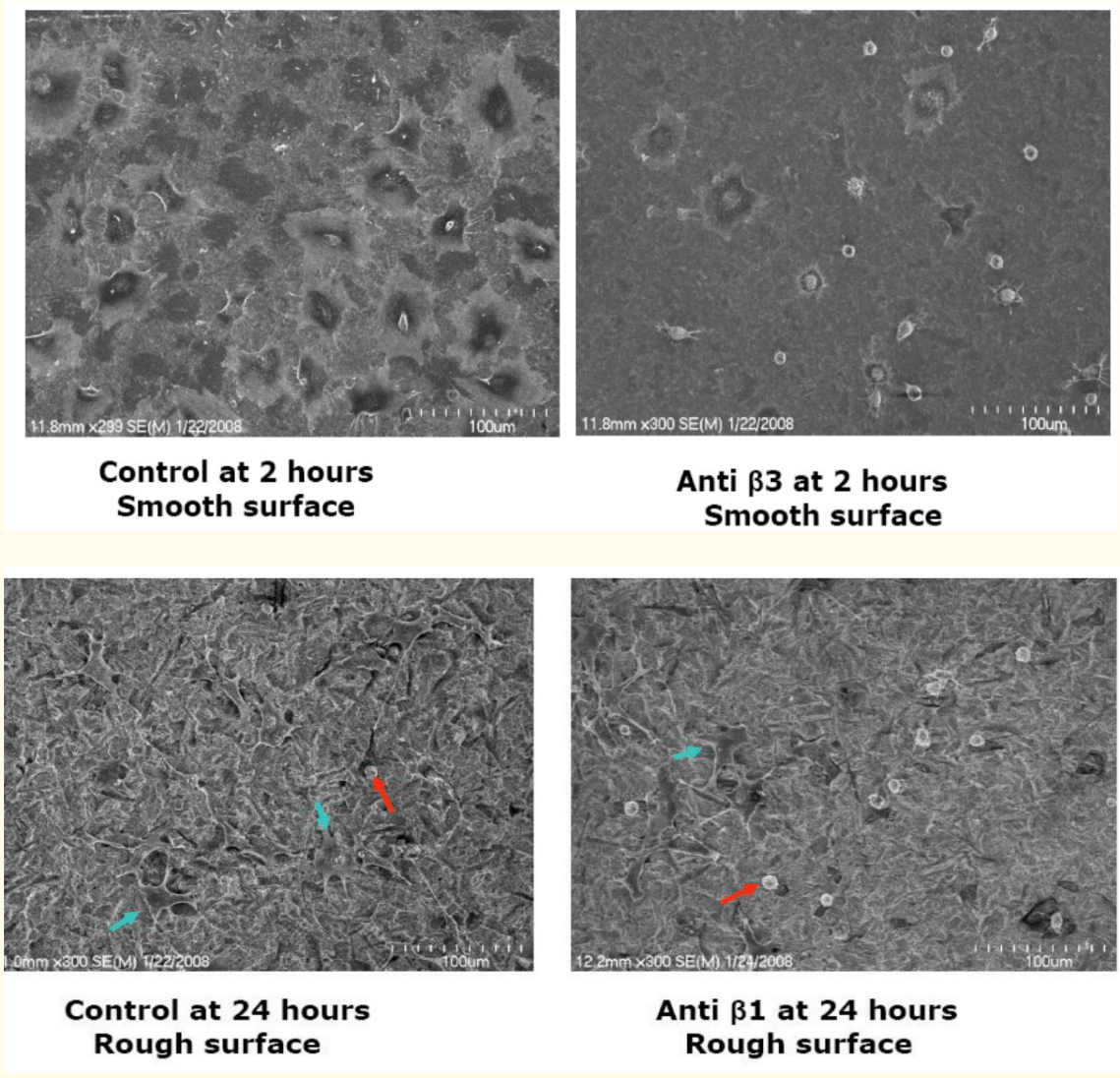

Figure 3.8: *Examples of SEM images from different experimental groups and different time points that were used for data acquisition.*

Blue arrows: Spread cells; Red arrows: round cells

Subjective evaluation of SEM images at higher magnification

SEM images were made at higher magnification (X 2000) for subjective evaluation of the cell shape, spread, and interaction of the surface. Some examples of the SEM images are shown in figure 3.9. The highly magnified SEM images show that cells adhere differently on smooth and rough surfaces. By comparing images A and B the cells on both surfaces did spread over the surface. However, the cell on the smooth surface had still a round cytoplasm and less number of cell processes attaching the cell to the surface and it seems as if it is raised over the surface. On the other hand, cells on rough surface at the same time point had a more elongated cytoplasm and processes and they looked as if they were closely adapted to the configurations of the surface.

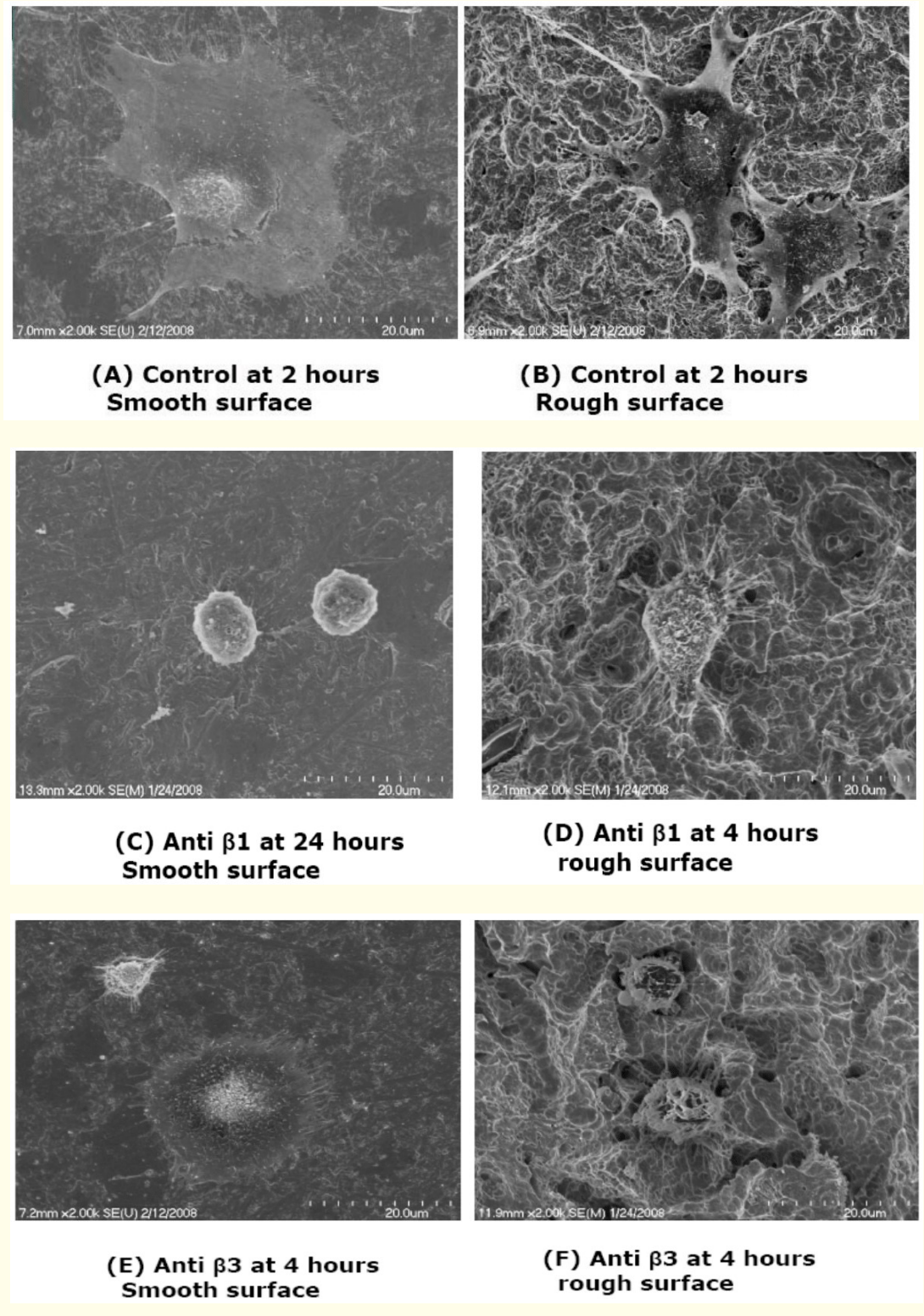

Figure 3.9: *SEM images at higher magnification (X 2000).*

Similar findings can be observed when comparing images C and D. Cells on images C and D are round and couldn't spread over the surfaces Nonetheless, these cells still could attach to the surface. Nevertheless, cells on the rough surface seem to have better ability to attach to the surface and to develop more cellular processes even earlier in the process. Image F shows cells that were treated with anti-β3 antibody; both cells were round and didn't spread over the surface. However, one of the cells attached better to the surface (rough) with multiple processes and better adaptation to surface configurations. On the other hand, the other cell was probably only mechanically retained by the pits and irregularities produced by surface treatment. Finally, image E shows that cells were differentially affected by the anti-β3 treatment. One cell could spread over the surface and the other did not. However, both of them developed some processes to attach them to the surface although the images show that they were less intimately attached to the smooth surface in comparison to the rough.

RT^2 Profiler™ PCR Arrays results

Mouse RT^2 Profiler™ PCR Arrays system was used to determine the relative differences in gene expression of a panel of 84 genes associated with the process of osteogenesis. This system profiles the expression of genes from different functional groups related to many cellular activities such as skeletal development, bone mineral metabolism, cell growth and differentiation, ECM proteins, cell adhesion molecules, collagen proteins, and transcriptional factors and regulators. A complete list of all genes available in this system is presented in appendix A. Nevertheless, for easier presentation of data, selective classes of functional genes will be considered in the data analysis. Table 2, presents the functional group of genes and the genes that showed significant changes among different experimental groups.

Functional group of genes	Genes presented significant changes (anti-β1)	Genes presented significant changes (anti-β3)
Bone morphogenic protein superfamily	Tgfβ1, Bmp2, Bmp6	Gdf10, Bmp2, Bmp3, Bmp5, Bmp6
Bone matrix proteins	Ambn, Sost, Ahsg, ALP	Ambn, Sost, Ahsg, ALP
Integrin receptors	Itgα2, Itgβ1, Itgαv	
Growth factors	Vegfa, Vegfb, pdgfα, fgf3, Csf2, Csf3	Vegfa, Fgf2, Fgf3, Egf, Csf2,Csf3
Transcriptional factors	Smad4, Runx2, Msx1, Twist1	Msx1

Table 2: *Functional group of genes and genes that presented significant changes among different experimental groups (significant: either statistically significant or has more that 2 fold difference in mRNA expression).*

In the following sections the fold difference in mRNA expression between the anti-β1/anti-β3 and the control group on both smooth and rough surfaces will be presented for selective functional gene groups. Furthermore, the group of genes which are not presented in the following sections showed similar trends.

Bone morphogenic protein superfamily

The bone morphogenetic proteins (Bmps) are a family of secreted signaling molecules that can induce ectopic bone growth. Many Bmps are part of the transforming growth factor-beta (Tgfβ) superfamily. Bmps were originally identified by an ability of demineralized bone extract to induce endochondral osteogenesis in vivo in an extraskeletal site. Figures 3.10 and 3.11 represent bar graph charts of fold difference in mRNA expression of Bmp superfamily genes between the anti-β1/anti-β3 and the control group on both smooth and rough surfaces.

The results show that there is a different trend in Bmp superfamily gene expression when β1 and β3 integrins were blocked with specific antibodies particularly on smooth surface (Figure 3.10, 3.11). Pre-treatment of MC3T3-E1 cells with anti- β1 antibody on smooth surface resulted in down regulation of the Bmp superfamily gene expression of about 2 folds, with the exception of Bmp2, and Gdf10 which were up-regulated. On the contrary, when cells were pretreated with anti- β3 antibody, all Bmp superfamily genes were up- regulated on smooth surface and Bmp5 and Bmp6 were significantly up- regulated. Nevertheless, on rough surface, Bmp superfamily gene expression

show a similar trend, when cells were pretreated with anti-β1 and anti- β3 antibodies. All Bmp superfamily genes in the anti- β1 group were up-regulated or rough surface with Bmp2 and Bmp6 significantly up regulated (more than 8 folds) and Bmp3 and Bmp5 with more that 4 folds of up-regulation. In the anti- β3 group all Bmp superfamily genes were significantly up-regulated (over 4 folds) with the exception of Tgfβ.

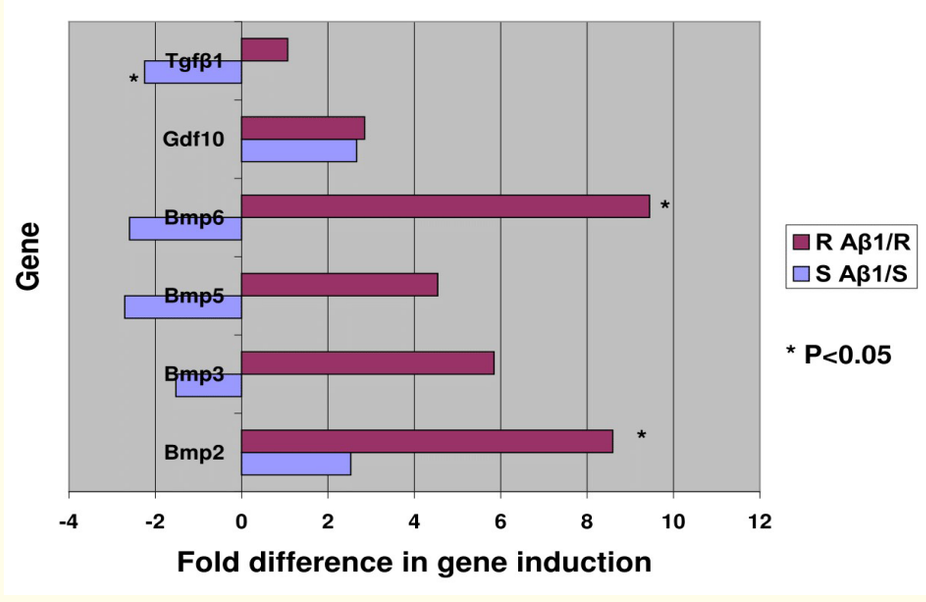

Figure 3.10: *Bar graph showing the difference in mRNA expression of Bmp superfamily genes between anti-β1 and control groups on both smooth and rough surfaces.*

R: rough surface, S: smooth surface.

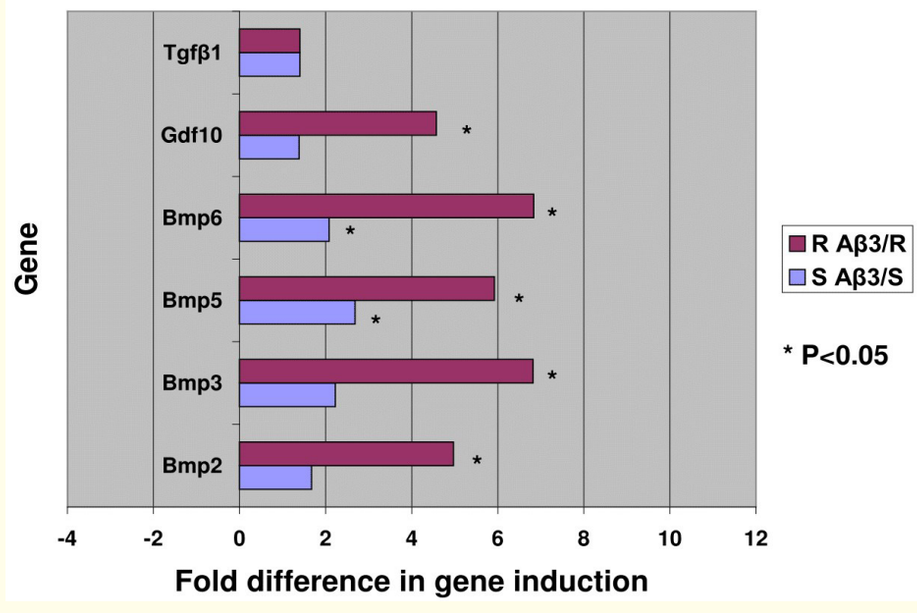

Figure 3.11: *Bar graph showing the difference in mRNA expression of Bmp superfamily genes between anti-β3 and control groups on both smooth and rough surfaces.*

R: rough surface, S: smooth surface.

Bone matrix proteins

This group of genes contains some of the matrix proteins that are associated with osteogenesis and tissue development. Alkaline phosphatase (ALP) is a tetrameric glycoprotein found on the surface of osteoblast and is responsible for laying down matrix for bone. It is considered as a marker for early bone formation. Sclerostin (Sost) is a secreted glycoprotein that works as a Bmp antagonist. A mutation in the Sost gene is associated with an autosomal recessive disorder called sclerosteosis which causes progressive bone overgrowth. Alpha-2-HS-glycoprotein (Ahsg) is a glycoprotein that is present in serum and is involved in bone development and formation as well as development of other tissues. Finally, Biglycan (Bgn) is a cellular or peri-cellular proteoglycan, it is thought to function in connective tissue metabolism by binding to collagen fibrils and Tgfβ. Figures 3.12 and 3.13 represent bar graph charts of fold difference in mRNA expression of these bone matrix proteins between the anti-β1/anti-β3 and the control group on both surfaces.

Figures 3.12 and 3.13 show similar trends in bone matrix proteins gene expression in both anti- β1 and anti- β3 experiments. When MC3T3-E1 were pre-treated with anti- β1 antibody bone matrix proteins gene expression were slightly down regulated on smooth surface with the exception of Sost and Bgn that were slightly up-regulated. Nevertheless, these differences were not statistically significant. On the contrary, on rough surfaces all bone matrix proteins showed up regulation in gene expression specifically Sost, and ALP that had at least 4 or more fold increase in gene expression (Figure 3.12). Likewise, when MC3T3-E1 cells were pre-treated with anti- β3 antibody similar findings were noted. On smooth surface, bone matrix proteins were not regulated without any significant changes in gene expression except for Sost that had 2 fold increase in gene expression. Yet, on rough surface the difference was more pronounced and bone matrix proteins gene expression show a highly significant up regulation with 4 fold or more with the exception of Bgn which didn't show a marked difference in gene expression (Figure 3.13).

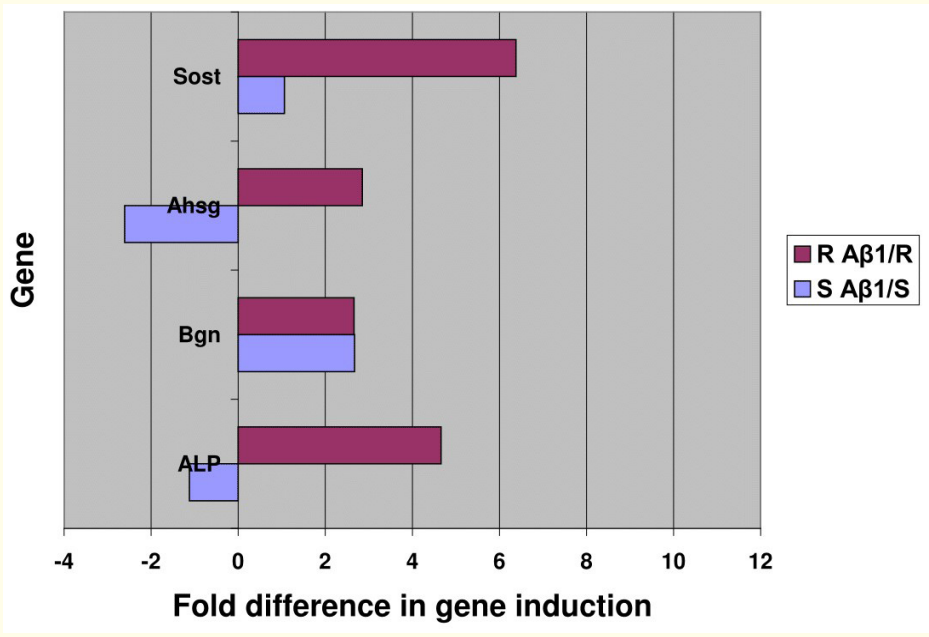

Figure 3.12: *Bar graph showing the difference in mRNA expression of bone matrix proteins between anti-β1 and control groups on both smooth and rough surfaces.*

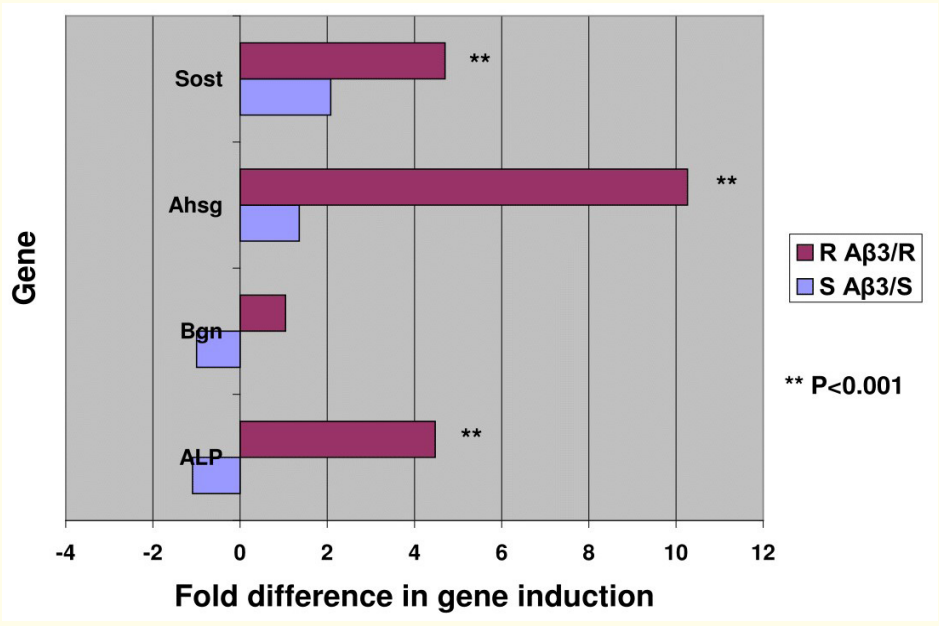

Figure 3.13: *Bar graph showing the difference in mRNA expression of bone matrix proteins between anti-β3 and control groups on both smooth and rough surfaces.*

Growth factors

This group of genes includes multiple growth factors that are associated with growth and development of many tissues and cells. Vascular endothelial growth factors (Vegfa, Vegfab) are important factors in increasing vascular permeability and promoting angiogenesis and cell migration. The colony stimulating factors (Csf2, Csf3) are cytokines that controls the function and differentiation of macrophages and granulocytes. Platelet derived growth factor alpha (Pdfgα) is an important mitogenic factor for cells from mesenchymal origin. Epidermal growth factor (Egf) is another mitogenic factor that has a potent effect on the differentiation of variety of cells from ectodermal and mesodermal origin. Finally, the fibroblast growth factor family (Fgf) is a family of growth factors that have broad mitogenic and angiogenic activities which play an important role in tissue repair, cell growth, and morphogenesis. Figures 3.14 and 3.15 represent bar graph charts of fold difference in mRNA expression of these growth factors between the anti-β1/anti-β3 and the control group on both surfaces.

Figure 3.14 shows that when MC3T3-E1 cells were pre-treated with anti-β1 antibody prior to plating on smooth surfaces, vegfa, pdgfα, Fgf3, Egf, Csf3 were not regulated with less than 2 folds increase or decrease in gene expression. Nevertheless, Vegfb was down regulated with over two folds and csf2 on the contrary had over five fold increase in gene expression which was statistically significant. However, on rough surface all growth factors showed up regulation of gene expression when cells were pre-treated with anti- β1 antibody with the exception of pdgfα which was not regulated. The fold increase in gene induction of Csf2, and Csf3 was pronounced with over 10 folds increase which was statistically significant. Moreover, Fgf3 showed over 4 fold increase in gene induction which was statistically significant as well.

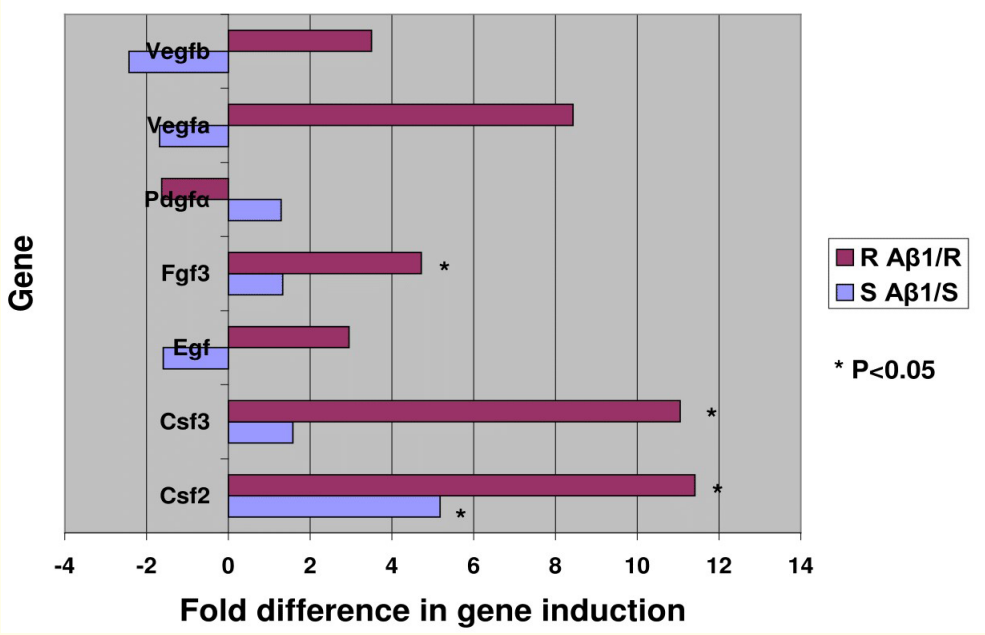

Figure 3.14: *Bar graph showing the difference in mRNA expression of selective growth factors between anti-β1 and control groups on both smooth and rough surfaces.*

Figure 3.15 shows the relative difference in gene induction of the growth factors family when MC3T3-E1 cells were pretreated with anti-β3 antibody before plating on both experimental surfaces. The key difference in comparison to the anti- β1 group was the relative expression of the Vegf family. Vegfa did not show much change on both surfaces while Vegfa was down regulated for about two folds on

rough surfaces although it was up regulated for more that 8 folds in the anti- β1 experiment. Other growth factors showed similar trends in gene induction to the anti- β1 experiment, with Fgf3, Egf, Csf2, Csf3 being significantly up regulated on rough surface when cells were pretreated with anti- β3 antibody before plating.

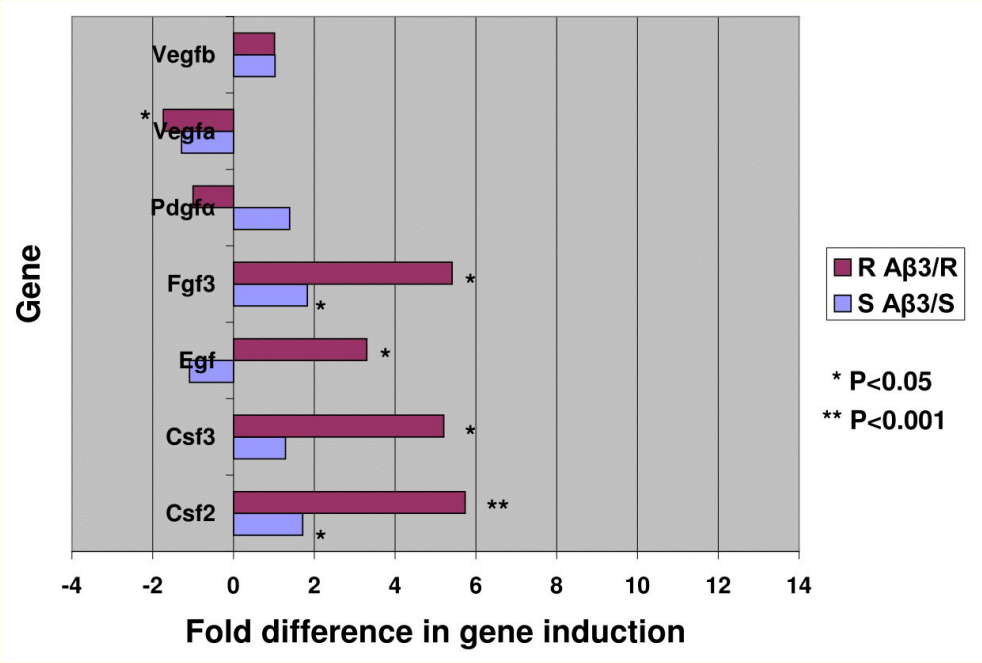

Figure 3.15: *Bar graph showing the difference in mRNA expression of selective growth factors between anti-β3 and control groups on both smooth and rough surfaces.*

Transcriptional factors

This group of genes includes some transcription factors that are important for osteoblastic differentiation, cell lineage determination, and signal transduction. Twist homolog 1 (Twist1) is a transcriptional factor that has been implicated in cell lineage determination and differentiation. Smad proteins are signal transducers and transcription modulators that mediate multiple signaling pathways. Smad 2 mediates Tgfβ signal and it is associated with Smad4 protein which plays an important role in the translocation of Smad2 into the nucleus, where it binds to target promoters and forms a transcription repressor complex. Runt related transcription factor 2 (Runx2) is essential for osteoblastic differentiation and skeletal morphogenesis. It acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression. Mutations in this gene are associated with cleidocranial dysplasia 12. Finally, Msh homebox1 (Msx1) plays an important role in limb-pattern formation and craniofacial development particularly odontogenesis besides it role in embryogenesis. Figures 3.16 and 3.17 represent bar graph charts of fold difference in mRNA expression of these transcriptional factors between the anti-β1/anti-β3 and the control group on both surfaces. Figure 3.16 and figure 3.17 show that most of the transcriptional factors didn't show dramatic changes in relative gene expression in both experimental conditions. Nevertheless, Msx1 levels show statistically significant up regulation of gene expression in the anti- β1 experiment on both smooth and rough surface. However, the expression was more pronounced on the smooth surface. Moreover, Smad4 gene showed more that two folds down regulation on smooth surface in the anti- β1 experiment and Runx2 showed more that two folds up regulation on the smooth surface in anti- β1 experiment. All the other transcriptional factors in both experiments were not regulated with relative gene induction of less than 2 folds.

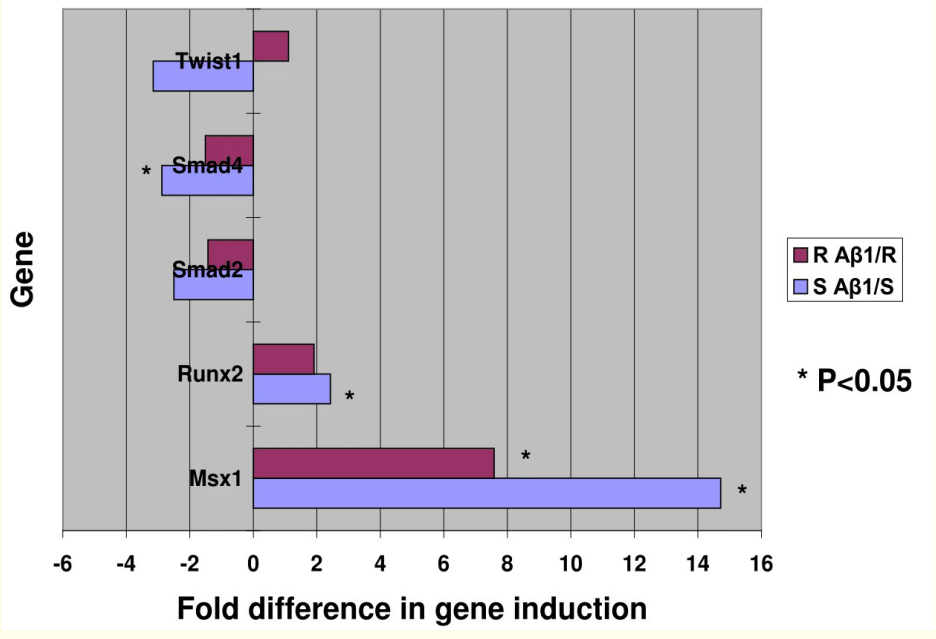

Figure 3.16: *Bar graph showing the difference in mRNA expression of selective transcriptional factors between anti-β1 and control groups on both smooth and rough surfaces.*

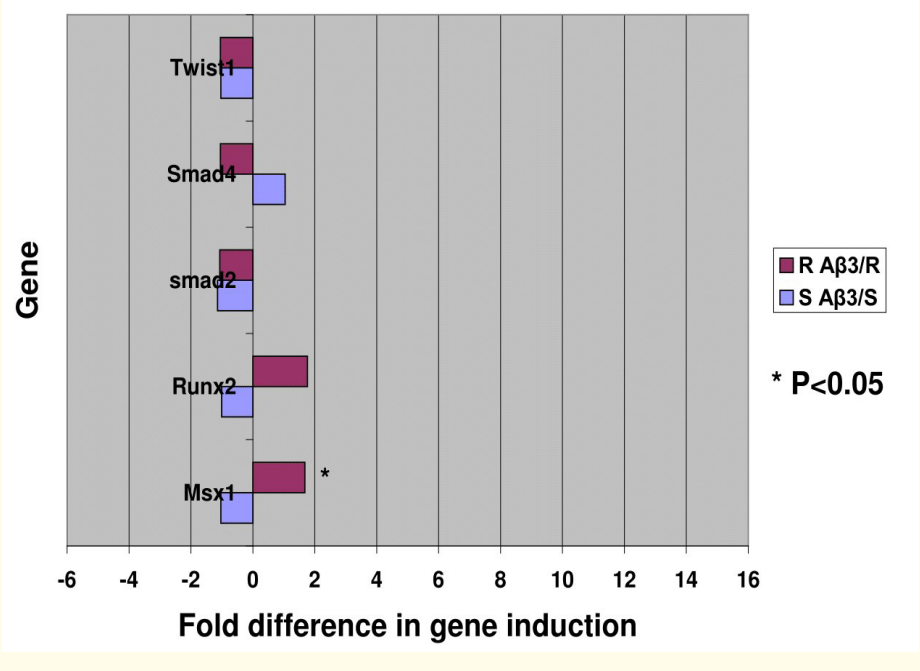

Figure 3.17: *Bar graph showing the difference in mRNA expression of selective transcriptional factors between anti-β3 and control groups on both smooth and rough surfaces.*

Discussion

The success of endosseous implants is determined by the integration of the biomaterial substances with the surrounding tissues and the formation of direct bone to implant contact [13-15]. The peri-implant bone healing is a complex and synchronized process that involves multiple cellular and molecular mechanisms which ends in bone formation and wound healing [16]. The initial adhesion and spreading of osteoblast-like cells on the implant surface is crucial in implementing an appropriate cell response to the surface and is related to the skeletal development, homeostasis and maturation of osteoblastic phenotype [17-20].

There is growing evidence suggesting that implant surface features affect bone formation and related adherent cellular activities [21-23]. Integrin transmembrane receptor has emerged as a central regulator of cell biomaterial interactions. Moreover, the ability of the osteoblast-like cells to sense and react to different surface characteristics has been attributed to the integrin receptors particularly specific heterodimers containing β1 and β 3 integrin subunits [24-27]. Nonetheless, the exact role that integrins play in mediating osteoblast adhesion to the implant surface and its effect on subsequent cell spreading, motility, proliferation, differentiation, and matrix mineralization is not completely understood. Thus, this project was conducted to compare and contrast the effects of β1 and β3 integrins on early osteoblast implant interactions and their ability to mediate surface specific changes.

Experimental model

A cell culture model utilizing the mouse osteoblast like MC3T3-E1 cells was used in this project. Cell culture models have proved to be a very successful and valuable in investigating the aspects of bone formation and osteoblast implant interactions [28-30]. In addition, MC3T3-E1 cells are a good model for this project taking into consideration their ability of showing different stages of growth and development under cell culture conditions [20]. Commercially pure grade IV titanium disks were prepared using two different protocols to produce surfaces with different roughness topographies (turned or machined versus micro-rough surface) to investigate the cell behavior on different surface topographies and the ability of β1 and β3 integrins to mediate surface specific changes. The AFM surface analysis showed that the rough surface has an average surface roughness values that are compatible with minimally rough surfaces, which reflects the spectrum of micro-rough implant surfaces. Moreover, the Ra values for rough surface were considerably greater than the smooth surface. Similar results were documented with Abron and collaborates who used similar protocol for surface preparation [8].

Functional perturbation of β1 and β3 integrin subunits using integrin-specific monoclonal antibodies was used to evaluate the role of these transmembrane receptors in mediating osteoblast implant interactions. This procedure has been utilized to identify specific integrin subunits and integrin-ligand pairs that mediate osteoblast adhesion to biomaterials and their influence on mediating vital cellular mechanisms [18,24,31-33]. Furthermore, in order to offset any IgG non-specific effects an isotype IgG control was used in the control groups in the same concentration as the monoclonal antibodies. In a similar experimental protocol, Siebers and collaborates examined the influence of integrin subunit-β1 and subunit- β3 on the behavior of primary osteoblast-like cells (rat bone marrow cells), cultured on calcium phosphate coated and non-coated titanium. They treated the cells with specific monoclonal antibodies in a similar fashion to this project. Nevertheless, they didn't use isotype IgG control to match nonspecific IgG effects. Besides, in their experiment they treated the cells with antibody concentration of 50 μg/ml which is very likely a saturating concentration that could possibly have had some inhibitory effect on cell adhesion. Nevertheless, their results showed that cell adhesion was only slightly affected by pre-treatment with anti-β3 antibody. However, their molecular data showed that pretreatment with either anti-β1 or anti- β3 resulted in decrease of ALP expression [24].

Another important factor in blocking the integrin subunits with specific monoclonal antibodies is the timing of the antibody treatment. In our protocol, cells were treated with the antibody one hour before the experiment and no serum was added to the cells. Cells, PBS, antibody or IgG mixture was incubated in cell culture incubator at 37°C for one hour before the cells were plated on the titanium disks. This protocol, gives the antibody the chance to block integrins on all surfaces of the cells. Furthermore, no serum was added to avoid competitive binding of the antibodies with serum proteins. Other investigators performed the blocking protocol by adding the antibodies

to the cell culture medium after the cells were initially attached [26,33,34]. The problem with such protocol is that integrins are also expressed on non-binding surfaces of the cells, thus the effect of the antibody will be through integrin not involved in cell substrate interaction and this will influence only the cell signaling without affecting the cell adhesion as cells had already adhered. Moreover, the initial cell adhesion before the antibody blocking changes integrin expression. Other inherent problems with antibody blocking experiments include binding affinities, as well as the variable expression levels between the two integrins. More conclusive studies could be performed to compare the functions of these two integrins. Examples include, using knock-out animals or silencing of the integrin subunits. Such studies not only encompass transcription, but also, can be complemented by experiments verifying the integrin protein expression changes.

Three variables than influence initial cell adhesion and spreading (the effect of anti-body treatment (β1, β3), surface, and time), were explored using SEM. The numbers of total, spread, and round cells were counted in three random areas on each disk. Two disks were used for each group at each particular time point. Three calibrated and blinded investigators counted the number of cells separately to decrease any risk of bias in the results. SEM images at higher magnification were done for subjective evaluation of cell shape, spread and interaction with the respective surface. A specific criteria regarding the elongation of the cell cytoplasm, the presence of cell processes, as well the diameter of the cell at its longest dimension were utilized as guide lines for cell spreading. This method provides the ability to evaluate initial cell adhesion as well as spreading both quantitatively and qualitatively in the same experimental setting. Nevertheless, there are some inherent disadvantages with this method including:

1. Cells with different stages of maturation might present with different sizes and shapes.
2. The differential cell density on different sites of the disks depending on cell plating uniformity and accuracy.

To overcome these potential problems, semi confluent cells of passage 6 or less were used in the experiments and three random areas were selected for each disk. These random areas were replicated in each disk to account for plating imperfections. Other investigators measured cell number on various substrates when cells were treated with specific monoclonal antibodies, or by silencing integrin β1. The cell count was performed by detaching the cells using trypsin and counting the cells with a cell counter [24,26]. This method doesn't provide the ability to evaluate cell shape and spread in the same experimental setting. Additionally, other methods for evaluation of cell adhesion and spreading had been reported in the literature such as; using cell adhesion assays, cell spreading assays, confocal microscopic analysis, immunofluorescence assays, and cell staining. Some of these methods were used to evaluate cell adhesion as a function of focal contacts distribution, cytoskeletal proteins organization, and staged cell spreading [27,35]. These methods can be very valuable in future studies to verify cell adhesion and spreading in a more characteristic fashion regarding the organization, distribution of integrin receptors, focal adhesion contacts, and cytoskeletal proteins. Furthermore, developing a cell adhesion assay model where mean cell surface area can be calculated as a guide line for cell spreading is recommended. This method would provide a more objective mechanism for evaluating cell spreading.

In order to further investigate the effect β1 and β3 integrin subunits and surface roughness on osteogenesis, the relative differences in gene expression of osteogenesis specific genes were quantified using the mouse osteogenesis RT^2 Profiler™ PCR Arrays system. This system brings together the quantitative performance of real-time PCR and the multiple gene profiling capability of microarrays. This PCR array profiles the expression of a panel of 84 genes related to osteogenesis. Thus, it provides a unique opportunity to survey these multifunctional genes and investigate the effect of integrin-ligand blocking on multiple cellular mechanisms as well as the effect of increased surface roughness on cellular adhesion, and osteoblastic differentiation. RT^2 Profiler™ PCR Arrays system was utilized for a variety of molecular biology applications such as; toxicology, oncology, and immunology research and proved to be a reliable and accurate tool of analyzing the expression of a focused panel of genes [36,37].

Cell adhesion

The initial cellular attachment as measured by cell count showed similar number of cells on both surfaces in the control group. Nevertheless, numbers of cells were slightly higher on rough surface in comparison to the smooth surface and they slightly increase from 4

to 24 hours as expected. Although, these differences were not statistically significant, they still support the hypothesis that micro- rough surfaces support early cellular adhesion. Moreover, by careful observation of SEM images with higher magnification we can clearly see that cells on rough surface developed more cellular processes and adopted more irregular elongated shape and spanned across the pits on the surface. On the contrary, cells on smooth surface were more spherical, flattened, had fewer cellular processes, and seem to be differently adhered to the surface (figure 3.9 (A, B)). These observations are consistent with the findings of other investigators who found direct correlation between cell attachment and increased roughness on the micron-scale level [21,38]. Nevertheless, the literature shows that greater cellular adhesion is not necessarily associated with more adherent cell numbers. Keselowsky and collaborates found out that α5β1 integrin binding and FAK phosphorylation was directly related to surface roughness. Nonetheless, their results also showed that surface roughness was inversely related to adherent cell numbers [34]. Similarly, other investigators documented the presence of fewer numbers of cells on rough surfaces in comparison to smooth titanium surfaces. Kim and collaborates using titanium alloy and MG63 cells, found that after 3 days of cell culture proliferation was inhibited by 17% on sandblasted and acid etched surface in comparison to smooth surface39. Wang and collaborates, using MG63 cells and grade 2 unalloyed titanium, found after 24 hours in culture, that cell numbers on sandblasted and acid etched surfaces and titanium plasma- sprayed (TPS) surfaces were less than cell numbers on smooth titanium surfaces and plastic surfaces, which had similar number of cells [26]. However, in a similar study design Martin and collaborates, found that micro- rough surfaces had higher number of cells in comparison to TPS surfaces and similar number to smooth surfaces [40].

When MC3T3-E1 cells were pre-treated with anti-β1 or anti- β3 monoclonal antibodies, the numbers of adherent cells on both smooth and rough surfaces were reduced. Nevertheless, the difference was only statistically significant on the rough surface at the 24 hour time point among the experimental and the control groups. An interesting finding was that at the 24- hour time point the difference in cell numbers among the experimental groups was higher on smooth surfaces versus rough surfaces with a significant difference in the anti-β3 group. Moreover, cell numbers in the anti- β3 group were less than the anti- β1 group, where cell numbers returned to the control levels on smooth surfaces. These findings may suggest that either the cells plated on smooth surfaces had better ability to overcome the inhibitory effects of the function-blocking antibodies over time, or that function-blocking antibodies had a more pronounced effect on cells plated on rough surfaces. In addition, these findings may also suggest that β3 integrins are more active later in the process (24 hours) of cell binding, in comparison to β1 integrin, which might be more active at earlier stages. Our results agree with results from other studies. Wang collaborates using MG63 cells found out that integrin β1 silencing resulted in 40% decrease in cell numbers after 24 hours on 5 different surfaces including plastic, polished smooth titanium surface, sandblasted/ acid etched titanium surface, and TPS surface. Moreover, their results showed the presence of more adherent cells at 24 hours on smooth surfaces versus rough surfaces [26]. Keselowsky and collaborates reported that cell number on smooth and TPS surfaces were reduced at 3 days when MG63 cells were pre-treated with anti-α5 antibody to block α5β1 integrin function [34]. On the otherhand, Siebers and collaborates using rat bone marrow cells found out that cell binding was affected differently on calcium phosphate (CaP) coated surfaces versus bare titanium surfaces, when cells were pretreated with anti- β1 and anti- β3 antibodies. On CaP-coated surfaces, they found that cell numbers decreased around 20 - 30% after pre-treatment with anti- β1. This decrease in cell numbers was significant from 30 minutes up to 1 day after plating. On the other hand, cell numbers decreased on CaP- coated surfaces around 40-50% when cells were pre-treated with anti- β3 antibody. This decrease was significant from 30 minutes up to 3 days after plating. Nonetheless, their results on bare titanium surfaces did not show significant decrease in cell numbers in the anti- β1 group. But, pre-treatment with anti- β3 showed 30% decrease in cell number after 30 and 60 minutes of plating on the bare titanium surfaces [24]. These results show clearly that cell binding is not only integrin dependent but also, surface dependent. Moreover, their results on CaP-coated surfaces might suggest that β3 integrin is more important later in the process of cell binding (1 - 3 days) in comparison to β1 integrin.

Cell spreading

One of the aims of this investigation was to evaluate the importance of β1 and β3 integrins as well as, surface topography in mediating cell spreading. Cell spreading on substrate surface, is one of the parameters frequently reported in cell biomaterial interaction research,

due to its relation to cell migration, growth, and differentiation [41,42]. Nevertheless, cell spreading evaluation methods are not standardized. Some investigators used a special soft-ware to calculate mean cell surface area as an indication of cell spreading [27,38]. On the other hand, Lumbikanonda and Sammons developed a model to classify cell attachment/ spreading into four stages depending on morphological criteria [35,43]. In the current investigation cell spreading was evaluated as a dichotomous variable where cells were classified as spread or round depending on specific criteria related to cell morphology and dimensions [44].

Our results showed that pre-treatment with anti-β1 and anti- β3 antibodies, reduced the number of spread cells on both surfaces. This reduction was statistically significant on both surfaces at the 2 hour time point and on rough surface at the 24 hour time point. Moreover, the results showed that, at the 24 hour time point there were more spread cells on smooth surfaces in comparison to rough surfaces in both anti- β1 and anti- β3 groups. This observation might indicate that either the cells had better ability to overcome the inhibitory effects of function-blocking antibodies on smooth surface, or that surface roughness modulates antibody function. Although, our results didn't show significant difference between number of spread cells on rough and smooth surfaces in the control groups, yet they are consistent with observations made by other investigators. Several studies reported an inverse relationship between cell spreading as measured by mean surface area, and surface roughness as well as cellular adhesion [27,34,38]. Woodruff and collaborates, stated that cell adhesion is not indicative of how supportive a substrate is to cell spreading which doesn't correlate with focal contact formation [45]. On the contrary, Sammons and collaborates, reported that rough surfaces of porous microstructure may enhance the rate of cell spreading. Nevertheless, their observations were based on morphological criteria rather than the mean cell surface area [35]. These observations agree with our subjective results using highly magnified SEM that showed more morphological variation in cell shape on rough surfaces in comparison to smooth surfaces.

The mean numbers of round cells in the different experimental groups confirm our findings that both integrin β1 and β3 are involved in cell spreading. Furthermore, these results show that the anti- β1 group had significantly higher number of round cells in comparison to the anti- β3 group on rough surface. These results are consistent with observations made by Luthen and collaborates, who reported that β1 integrins are more involved in the formation of fibrillar adhesion than β3 integrins, which is affected by the surface roughness of titanium [27].

Relative expression of osteogenesis genes

The effect of β1 and β3 integrin on the relative expression of a panel of osteogenesis related genes, was evaluated using RT^2 Profiler™ PCR Arrays. Several functional group of genes related to osteogenesis were examined using this method (appendix A). Nevertheless, for simplicity purposes only the results of specific functional groups of genes were presented in this thesis and will be discussed accordingly.

Bone morphogenic protein superfamily

Our results show differential relative expression of Bmp superfamily proteins when cells were pre-treated with anti-β1 antibody versus anti-β3 antibody. Pre-treatment of MC3T3-E1 cells with anti-β1 antibody before cell plating, resulted generally in slight down regulation of Bmp superfamily genes on smooth surface. On the contrary when MC3T3-E1 cells were pre-treated with anti-β3 antibody before cell plating, the Bmp superfamily gene expression were slightly up-regulated on smooth surface. Nevertheless, the expressions of Bmp superfamily genes on rough surfaces in both experimental groups were similar. They were pronouncedly up-regulated particularly Bmp2 and Bmp6. This differential expression might suggest that the effect of the surface on Bmp superfamily gene expression is beyond the effect of β1 and β3 integrins. Moreover, it might suggest that the cells respond to integrin blocking by compensatory effect through other integrin and non-integrin signals. Another explanation of this finding could be that the antibody it self creates signals that is reflected in up regulation of Bmp superfamily gene expression. Nonetheless, these results still show that both β1 and β3 integrins might be involved in mediating surface specific changes, which is evident in the differential expression of Bmp superfamily proteins on rough versus smooth surfaces.

These results agree with observations from previous studies which show that Tgfβ superfamily and Bmps particularly Bmp2 are correlated and work closely in mediating cell adhesion. Nissinen and collaborates showed that human recombinant Bmp2, regulate cell matrix interactions by modifying the expression of integrin α3β1 that mediates cell adhesion to laminin-546. On the other hand, Shah and collaborates reported that pre-treatment of primary human osteoblastic cells with Bmp-2 for 12 hours before plating on titanium alloy, resulted in increased expression of α5 and β1 integrin subunits, fibronectin, and focal adhesion kinase expressions. In addition, they showed that this increased expression was associated with stimulated cell adhesion and proliferation of osteoblastic cells, which was reflected on long term mineralization [47]. Similarly, Lai and Su documented that Bmp2 up-regulates the expression of αvβ integrins which in turn, play a critical role in Bmp2 osteoblastic function [48].

Bone matrix proteins

For this group of genes, the results were similar when the cells were pre-treated with anti- β1 or anti- β3 antibodies particularly for ALP and Sost genes. On smooth surface, there was slight down-regulation of ALP and slight up-regulation of Sost. However, there was a significant up-regulation of both genes expression on rough surface. An interesting observation was the up-regulation of Sost gene expression, when cells were pre-treated with either anti- β1 or anti- β3 antibodies. This gene is a Bmp antagonist, and it is associated with reduction of the expression of proteins associated with osteoblastic differentiation, proliferation, and matrix mineralization [49]. These results may indicate that β1 and β3 integrins are involved in mediating osteoblastic differentiation in a surface dependent manner.

The ALP relative gene expression was surface dependent with slight down- regulation on smooth surfaces and significant up-regulation on rough surfaces. These observations agree and disagree with observations made by other investigators in similar study designs. Wang and collaborates reported that blocking β1 integrin function with specific antibody, resulted in reduction of ALP expression in Mg63 cells. Moreover, they observed that this decrease in gene expression was dependent on time of antibody treatment, dose of antibody, and substrate on which the cells were plated [26]. Similarly, Siebers and collaborates reported that pre-treatment of rat bone marrow cells with either anti- β1 or anti- β3 antibodies before plating, resulted in surface dependent decrease in ALP gene expression [24]. Although, it is difficult to compare these contradictory results, due to differences in cell types, substrates, and experimental conditions, yet, they all agree that blocking β1 and β3 integrin function resulted in substrate dependent change in ALP gene expression.

Growth factors

The relative gene expressions of several growth factors were evaluated. The results were similar in both the anti- β1 and anti- β3 groups with the exception of the vascular endothelial growth factors. These results further confirm the observation that blocking β1 and β3 function with specific monoclonal antibodies resulted in surface dependent change in growth factors gene expression. These results are similar to observations made with other functional group of genes and the same explanations may be applied. Nevertheless, the differential expression of the Vegf genes, particularly Vegfa among the anti- β1 and anti- β3 experimental groups was of interest. In the anti- β1 group, Vegfa was slightly down regulated on smooth surface and up regulated on rough surface. Nevertheless, in the anti- β3 group it was down regulated on both surfaces with more down regulation on the rough surface. It is well documented that integrins β1, β3, and β5 are expressed in endothelial cells and are involved in the process of angiogenesis through their effect on Vegf [50,51]. Nevertheless, integrin β3 particularly αvβ3 has the most potent effect on angiogenesis. Mahabeleshwar and collaborates showed that inhibition of β1, β3, and β5 integrin expression in endothelial cells resulted in down regulation of endothelial cell adhesion and migration. Moreover, they reported that inhibition of β3 integrin resulted in the most potent reduction in capillary growth stimulated by Vegf. Our results agree with these observations reported in relation to endothelial cells. Further investigation of the correlated effect of integrins particularly β3 and Vegf in osteoblasts is of interest, since angiogenesis is a crucial process in peri-implant bone healing.

Transcriptional factors

The results in this group of genes surprisingly did not show much difference in the relative expression among the different experimental groups on both surfaces, with the exception of Msx1 gene in the anti- β1 group. Nevertheless, an interesting finding was that the Smad

proteins were down regulated in the anti- β1 group for more that two folds on smooth surface. This correlates with the fact that these proteins works as signaling molecules, and are part of the down stream signaling mechanism of Tgfβ and Bmps52. This might further indicate the involvement of β1 integrin in modulating osteoblastic differentiation. The other interesting observation was the significant up-regulation of Msx1 gene expression when cell were pre-treated with anti-β1 antibodies particularly on smooth surface. There is no known connection between this gene and integrins. This observation might suggest that the effect of the surface is complex and goes beyond the integrin transmembrane receptor. Moreover, there is a possibility that the blocking antibody, might un-mask key non-integrin signals. Nevertheless, it is interesting to further explore the possibility of having a correlation between β1 integrin function and Msx1 gene expression in osteoblasts.

Summary of findings and future recommendations

The findings of this *in-vitro* study can be summarized as following:

1. Both β1 and β3 integrins are involved in mediating osteoblast implant surface interactions. They have a direct effect on initial cell adhesion and spreading in a surface and time dependant manner.
2. Integrin β1 and β3 seem to be active at different stages of cell adhesion, with β1 being more active early in the process in comparison to β3 which has more potent effect later in the process.
3. Function blocking of both integrin subunits resulted in variable and surface dependant differences in gene expression of multiple genes related to osteogenesis. Nevertheless, function blocking antibodies seem to initiate signals that translate in up-regulation of multiple genes.
4. The molecular results might suggest that, cell biomaterial interaction is a complex process, which can be further mediated by other integrin and non-integrin molecules.
5. It seems like osteoblast like cells have the ability to compensate to a great extent for the blocking strategy applied in this investigation.
6. More conclusive comparative studies are recommended. These studies should be performed at different time points, and involve innovative techniques to knock out the integrin subunits such as; knock- out animals or RNA silencing (siRNA). Such designs can result in more precise evaluation of the effect of integrin receptors on cell biomaterial interaction and provide better insight on the long term effect of these interactions.

Conclusions

Within the limitations of this *in-vitro* investigation we can make the following conclusions:

1. Blocking integrin-β1 and integrin- β3 subunits with antibodies has an inhibitory effect on osteoblast like cell binding, and spreading to commercially pure titanium surfaces in vitro.
2. Beta one and beta three integrin mediation of initial cell adhesion and spreading is both time and surface dependent.
3. Both beta one and beta three integrins are involved in mediating surface specific changes, that modulate osteogenesis related gene expression. However, other integrin and non-integrin molecules might be involved in this process.
4. Function-blocking antibodies (that block cell binding) may activate signaling which result in a substrate dependent temporal expression of osteogenesis related genes.

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