REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	5.Jul.06	THESIS

4. TITLE AND SUBTITLE
17B-ESTRADIOL MODULATES THE RESPONSE OF HUMAN OSTEOBLASTS TO TITANIUM SURFACE ROUGHNESS.

5. FUNDING NUMBERS

6. AUTHOR(S)
CAPT TANDY ELIZABETH M

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UNIVERSITY OF TEXAS HSC AT SAN ANTONIO

8. PERFORMING ORGANIZATION REPORT NUMBER
CI04-1822

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
THE DEPARTMENT OF THE AIR FORCE
AFIT/CIA, BLDG 125
2950 P STREET
WPAFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Unlimited distribution
In Accordance With AFI 35-205/AFIT Sup 1

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

17β-Estradiol Modulates the Response of Human Osteoblasts to Titanium Surface Roughness

A

Thesis

Presented to the faculty of the

University of Texas Graduate School of Biomedical Sciences
At San Antonio

in partial fulfillment of

the requirements for the degree of

Master of Science

By

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San Antonio, Texas

March 2002

17-β ESTRADIOL MODULATES THE RESPONSE OF NORMAL HUMAN OSTEOBLASTS TO TITANIUM SURFACE ROUGHNESS.

Publication No. _____

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Estrogen (17β-estradiol) is involved in the regulation of bone formation, and estrogen deficiency results in decreased bone density and bone mass, as well as a decrease in pull-out strengths of implants. In previous studies, implant surface roughness was shown to affect the morphology, proliferation and differentiation of MG63 osteoblast-like cells, by inducing a more mature phenotype and by increasing production of local factors. The purpose of the current study was to investigate whether primary cultures of normal human female osteoblast (NHOst) cells respond in a similar fashion to these same surfaces and how these surfaces modulate cell response to 17β-estradiol. Much of the Brånemark literature supports the use of machined titanium surfaces for cases of suitable bone quality. Patients who present with less than ideal bone quality (i.e. osteoporotic bone) present a unique challenge to clinicians to enhance osseointegration

that will allow masticatory function for many years to come. The possible effects of estrogen or estrogen deficiency on osseointegration are unclear. The *in vitro* model described here was designed to elucidate what effect, if any, did estrogen have on osteoblast response to titanium surface roughness, and conversely, what effect surface microtopography had on osteoblasts response to estrogen.

NHOst cells were obtained from a premenopausal female donor and were cultured on commercially pure titanium (Ti) disks with surfaces of different roughness values: pretreatment (PT) (R_a=0.60±0.02 μm), sandblasted/large-grit/acid-etched (SLA) (R_a=3.97±0.04 μm), and titanium plasma sprayed (TPS) (R_a=5.21±0.24 μm). Upon reaching confluence, cell cultures were treated for 24-hours with 10^{-8}M and 10^{-7}M concentrations of 17β-estradiol. At harvest, cell number, cell and cell layer lysate alkaline phosphatase specific activity, and the levels of osteocalcin, prostaglandin E_2 and transforming growth factor β-1 (TGF-β1) in the conditioned media were determined.

On surfaces with greater surface roughness values, NHOst cells assumed a more cuboidal and elongated shape, with interconnecting extensions on the rougher surfaces. There was an estrogen-independent decrease in cell number with increasing surface roughness. Alkaline phosphatase activity in isolated cells decreased with increasing surface roughness. In comparison, estrogen significantly increased alkaline phosphatase activity in a dose-dependent manner on smooth surfaces; to a slight extent this was also observed at the highest concentration of 17β-estradiol on TPS. Cell layer alkaline phosphatase likewise decreased with increasing surface roughness, and in the presence of estrogen, a dose-dependent increase was only seen on the plastic. For the Ti surfaces, increased alkaline phosphatase activity was only seen at the higher estrogen

concentration. Osteocalcin levels were increased with increasing surface roughness, and were further elevated in the presence of estrogen on SLA and TPS surfaces. PGE_2 and TGF-β1 both were augmented with increasing surface roughness, and this effect was enhanced in a synergistic manner in the presence of estrogen for the cells cultured on the SLA and TPS surfaces.

NHOst cells demonstrate a more mature phenotype on surfaces of greater roughness values. On smooth surfaces, 17β-estradiol affected only alkaline phosphatase, but on rough surfaces, 17β-estradiol increased levels of osteocalcin, TGF-β1 and PGE_2. These results show that NHOst cells are sensitive to surface roughness and that surface roughness alters the cell response to 17β-estradiol. Thus, adequate estrogen levels may be an important consideration in patients who are estrogen deficient and seeking implant therapy.

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I. INTRODUCTION AND LITERATURE REVIEW

A. Implant surface characteristics and osseointegration

The success of endosseous implants has afforded patients options that can improve their quality of life tremendously. From orthopaedics to dentistry, the use of implants is gaining immense popularity.

The bone-implant interface has great influence on the long-term success or failure of titanium implants. Titanium implant surface characteristics play a key role in promoting osseointegration (Schwartz and Boyan 1994). Rough implant surfaces have increased bone-to-implant contact, and require greater forces to remove them from bone (Cochran 1999). On a cellular level, it has been demonstrated that titanium surface roughness characteristics affect osteoblast proliferation, differentiation and matrix synthesis (Martin *et al.* 1995), as well as production of prostaglandin E_2 (PGE_2), and transforming growth factor-β1 (TGF-β1) (Kieswetter *et al.* 1996). Also, different surface materials affect chondrocyte cell growth and metabolism (Hambleton *et al.* 1994) in a manner that is cell-maturation dependent (Schwartz *et al.* 1996). 1_α, 25-$(OH)_2D_3$ treatment of osteoblasts that have been, cultured on surfaces of increasing roughness increases cell layer alkaline phosphatase specific activity and levels of osteocalcin, PGE_2, and TGF-β1 in the conditioned media in a synergistic manner (Boyan *et al.* 1998). That is, the effect of 1,25-$(OH)_2D_3$ on the rougher surfaces was much greater than seen with smoother surfaces. In addition, osteoblast response to 1_α,25-$(OH)_2D_3$ is mediated by prostaglandins, and can inhibited by indomethacin in a time-dependent manner (Batzer *et*

al. 1998, Sisk *et al.* 2001). Furthermore, it has been shown that this response to surface roughness is partially mediated by protein kinase C (PKC), whereas the response to $1,25OH_2D_3$ is mediated exclusively through phospholipase A2 and protein kinase A (Schwartz *et al.* 2001). The effect of surface roughness on PKC can be blocked by the inhibitor cheleythrine. Although parallel comparative studies showed that titanium surface chemistry (machined cp Ti versus machined Ti-alloy) plays a role in cell response, surface roughness is a more important factor in the response of the cell to surface characteristics (Lincks *et al.* 1998, Boyan *et al.* 1999).

Increased bone loss and decreased quality of bone associated with osteoporosis are caused by an imbalance in bone resorption and bone formation. Osteoporosis is a multi-factorial disease that occurs in both males and females, but has a higher frequency in females. Although bone mass is known to decrease with increasing age, decreased estrogen levels after menopause appear to accelerate this process. This is significant because osteoporotic bone may have impaired responses to mechanical stress, and therefore a reduced ability to withstand strain within physiologic limits (Sterck *et al.* 1998). This could be of great clinical significance when taking into consideration the use of implants in these patients.

B. Cell proliferation and differentiation

Cultured osteoblast-like cells and chondrocytes are affected by surface roughness, expressing changes in both proliferation and differentiation (Martin *et al.* 1995, Schwartz *et al.* 1996). The surface of an implant can significantly alter the activity of cells growing on that surface. Depending on a number of factors, there can be marked changes

in proliferation and differentiation. Osteoblasts exhibit greater initial attachment to rough titanium surfaces *in vitro* (Lohmann *et al.* 1999a, Bowers *et al.* 1992, and Michaels *et al.* 1989). Furthermore, better bone fixation to the implant surface occurs with rougher surfaces when compared with smoother surfaces (Buser *et al.* 1991, Wilke *et al.* 1990, Carlsson *et al.* 1988, Thomas and Cook 1985). Markers of cell differentiation are produced at higher levels when osteoblast-like cells are cultured on rougher surfaces (Kieswetter *et al.* 1996).

Cells previously used for studies of this type have included transformed cell lines, which lack the cell cycle regulation that normal diploid cells have, and this can affect not only proliferation, but also whether or not the cells are of a maturation state that is relevant to the questions being investigated in the study. Gender of the derived cells may also play a role in cellular response. Cells from females may behave differently than male-derived cells (Sylvia *et al.* 1998, 2000). The use of immature osteoblasts obtained from a human donor were chosen to attempt to simulate the *in vivo* situation that occurs when an endosseous implant is placed in a patient, and the objective of inducing osseointegration is attempted. The cells chosen for this experimental study were from a pre-menopausal female subject who had not been exposed to an estrogen-deficient environment.

C. Local Factor Production

Bone formation at the implant-bone interface is modulated through surface roughness. For example, osteoblast-like cells demonstrate greater production of two local regulatory factors of bone formation on rough surfaces when compared to smooth surfaces (Kieswetter *et al.* 1995). Surface roughness impacts local regulatory events at

the implant-bone interface and modulates osteoblast activity by both autocrine and paracrine mechanisms. Levels of PGE_2 and TGF-β1 are elevated in osteoblasts cultured on rougher surfaces versus those grown on smoother surfaces. The effects of these two local factors are also biphasic, inducing different effects at differing concentrations. PGE_2 at lower concentrations promotes osteoblastic differentiation, and causes increased alkaline phosphatase activity (Schwartz *et al.* 1992). At higher concentrations, PGE_2 inhibits osteoblasts and stimulates osteoclasts (Raisz *et al.* 1990, Chyun *et al.* 1984). The effects of TGF-β1 are of varying range and are cell type specific. The formation of bone appears to be strongly related to the presence of TGF-β1 in the bone matrix, and osteoblast proliferation, differentiation and matrix production is believed to be modulated by TGF-β1 (Bonewald *et al.*1990, Joyce *et al.* 1990, Dworetzky *et al.* 1990).

D. 17-β estradiol

Estrogen receptors were identified in bone as high affinity nuclear binding sites for 17β-estradiol (E2)in human osteoblast-like cells (Eriksen *et al.* 1988). E2 has direct effects on trabecular bone in ovariectomized rats after localized infusion of E2 (Takano-Yamamoto *et al.* 1990). E2 demonstrated a sex-specific as well as dose-dependent stimulatory effect on bone formation and mineralization, while it was shown to have no effect on bone resorption (Schwartz *et al.* 1991). In chondrocytes, the effect of E2 is dependent also on the stage of maturation and is sex-specific. E2 decreases cell number and tritiated thymidine incorporation in female chondrocytes suggesting that E2 promotes differentiation versus proliferation. E2 also increases collagen synthesis and alkaline phosphatase activity in female cells (Nasatzky *et al.* 1993).

In vivo studies on the osseointegration of implants the pull-out strengths of titanium-alloyed implants was 31% less than in control animals in estrogen-deficient dogs, (Martin *et al.* 1988). Ovariectomized rats revealed a decrease in the amount of bone in contact with the implants, and when titanium screws were placed in tibiae of ovariectomized animals, a decrease in bone mass and implant contact area were observed (Yamazaki *et al.* 1999; Pan *et al.* 2000). Bone volume around the implants was decreased in the cancellous bone area surrounding the implant, which would also suggest that the trabeculae that support the implant may not occur in estrogen deficiency. Thus, estrogen deficiency may be associated with decreased osseointegration.

E. Investigational Objective and Aims

In order for osseointegration to occur successfully, the behavior of the osteoblast at the bone-implant surface is critical. Recent work has shown that osteoblast activities may be influenced by surface roughness, cytokines and hormones. This study was based on the hypothesis that surface roughness modulates the response of osteoblasts to 17β-estradiol. To test this, the following Specific Aims were addressed.

The first specific aim was to examine the combined effects of 10^{-7}M and 10^{-8}M 17β-estradiol and surface roughness on the phenotype of normal female human osteoblasts.

The second specific aim was to examine the effects of 10^{-7}M and 10^{-8}M 17β-estradiol and the effects of surface roughness on female human osteoblasts as expressed

as changes in cell number, alkaline phosphatase specific activity, osteocalcin levels, and local factor production.

II. METHODS AND MATERIALS

A. Preparation and Characterization of the Titanium (Ti) Disks

Cell cultures were performed directly on tissue culture polystyrene (plastic) or on titanium (Ti) disk surfaces. The Ti disks (15 mm in diameter) were punched from 1 mm thick sheets of grade 2 unalloyed Ti (ASTM, no. 67) so as to fit into the well of a 24-well plate and were supplied by Institut Straumann AG (Waldenburg, Switzerland). Disks were processed as described previously (Boyan *et al.*., 2001, Schwartz *et al.*., 2001) and summarized below.

Pretreatment or PT: The disks were degreased and acid-prepickled by washing in acetone, processing through 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55°C for 30 s, and pickling in 2% hydrofluoric acid/10% nitric acid at room temperature for 30 s.

Sandblasted large grit/acid etched or SLA: PT disks were coarse grit-blasted with 0.25-0.50 μm corundum grit at 5 bars until the surface became uniformly gray, followed by acid-etching in hydrochloric acid/sulfuric acid according to a proprietary process of Institut Straumann AG.

Titanium plasma sprayed or TPS: PT disks were processed by coarse grit-blasting as described above for the SLA disks and then plasma spray-coated with titanium hydride powder using a proprietary process of Institut Straumann AG.

After preparation, the disks were rinsed in deionized water, and neutralized by placing in 5% sodium bicarbonate for three 5-min periods in an ultrasonic bath. The surfaces were analyzed by scanning electron microscopy (SEM) using a LEO VP436

scanning electron microscope (LEO Ltd., Cambridge, UK) and profilometry using a Taylor-Hobson Surtronic 3 profilometer (Leicester, UK) at a high sensitivity setting. Three disks from each batch of disks were measured six times to obtain an average roughness value (R_a). R_a values were not determined for plastic surfaces, although the R_a value would be comparatively much less than the test surfaces.

SEM confirmed that the disks in the PT group were smooth, and contained undulating regions with occasional patches of small crystals. The SLA disks contained macro-pits >10μm in diameter and micro-pits approximately 1μm in diameter. Disks in the TPS-treated group had large areas with sheets and globular outcroppings, as well as deep pits and frequent fissures and cracks as previously described (Kieswetter *et al.*. 1996).

The R_a values of the three different surfaces were: PT, 0.60 ± 0.02 μm; SLA 3.97 ± 0.04 μm; and TPS, 5.12 ± 0.24 μm. The SLA and TPS surfaces were significantly rougher than the PT surface (Table 1). In addition, these two surfaces were significantly different from each other and had a distinctly different topography.

B. Experimental Cell Culture

Prior to cell culture, the disks were sterilized by steam autoclaving. For all experiments, cells were cultured on Ti disks placed in 24-well plates (Corning Costar, Cambridge, MA). Control cells were plated directly onto tissue culture plastic (polystyrene).

Normal female human osteoblasts (NHOst) were obtained from Clonetics, San Diego, CA. For all proliferation experiments, the cells were cultured using osteoblast growth media (OGM) purchased from the supplier. This media contains ascorbic acid. The cells form multilayers in culture with focal condensations, but they do not form nodules typical of fetal rat calvarial cultures. These cultures become von Kossa positive,

Table 1: Average Surface Roughness (R_a) Values for Titanium Disks

Surface	**R_a**
PT	0.60 ± 0.02 μm
SLA	3.97 ± 0.04μm
TPS	5.21 ± 0.24μm

suggesting that mineralization has occurred (unpublished data). The cells were received in a T-75 flask at third passage, and were passaged one more time prior to use in the experiments. For each experiment, third passage cells were plated at 5000 cells/cm^2. Because of the opacity of the Ti disks, there was no practical way to assess confluency of the cultures. As a result, when the cells in the control (= plastic) wells reached visual confluence, cultures on all other surfaces were considered confluent as well. Although this approach, leaves the possibility open that all cultures did not achieve the same degree of confluence at the time they were exposed to treatment, we have previously shown by SEM that all surfaces are covered by cells at this time and are at a similar stage of confluency (Kieswetter *et al.* 1996).

To determine whether surface roughness modulates the response of osteoblasts to estrogen, cultures were treated for twenty-four hours with or without 10^{-7}M or 10^{-8}M 17β-estradiol (Sigma Chemical Co., St. Louis, MO) after the cells had reached confluence.

C. Cell Morphology

To determine whether cell morphology varied as a function of surface roughness, the cultures were examined by scanning electron microscopy. At harvest, the culture media were removed and the samples rinsed three times with phosphate-buffered saline (PBS) and fixed with 1% OsO_4 in 0.1 M PBS for 15-30 min. After fixation, the disks were rinsed with PBS, sequentially incubated for 30-45 min each in 50, 75, 90 and 100% *ter*-butyl alcohol, and vacuum-dried. A thin layer of gold-palladium was sputter-coated onto the samples prior to examination using a LEO 435VP scanning electron microscope (LEO Electron Microscopy Ltd., Cambridge, England).

D. Cell Number

At time of harvest, cells were washed twice with Dulbecco's modified Eagle medium (DMEM), and then released from the culture wells by the addition of 0.25% trypsin and in Hank's balanced salt solution (HBSS) containing 1 mM ethylenediamine tetraacetic acid (EDTA) for ten minutes at 37°C, followed by addition of DMEM containing 10% FBS to stop the reaction. Cell suspensions were centrifuged at 500 x g for 10 minutes. Cell pellets were washed with PBS and resuspended in PBS. Cell number was determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Cells harvested in this manner exhibited > 95% viability based on trypan blue by exclusion.

E. Cell Differentiation

Alkaline Phosphatase Specific Activity. At harvest, isolated cells and cell layers were prepared as previously described (Boyan *et al.* 1989) and their protein content determined by use of commercially available kits (Micro/Macro BCA, Pierce Chemical Co., Rockford, IL). Alkaline phosphatase [orthophosphoric monoester phosphohydrolase, alkaline; E.C. 3.1.3.1] activity was assayed as the release of *p*-nitrophenol from *p*-nitrophenylphosphate at pH 10.2 as previously described (Bretaudiere and Spillman 1984), and specific activity determined.

Osteocalcin levels. The levels of osteocalcin in the conditioned media were measured using a commercially available radioimmunoassay kit (Human Osteocalcin RIA Kit, Biomedical Technologies, Stoughton, MA) as described previously (Boyan *et al.* 1998).

F. Local Factor Production

Prostaglandin E_2. The amount of PGE_2 produced by the cells and released into the media was assessed using a commercially available competitive binding radioimmunoassay kit (NEN Research Products, Boston, MA) as described previously (Kieswetter *et al.* 1996).

Transforming Growth Factor-beta-1 (TGF-β1). In order to measure the level of total TGF-β1 in the conditioned media, a commercially available enzyme-linked immunoassay (ELISA) kit (Promega Corp., Madison, WI) specific for human TGF-β1 was used. Immediately prior to assay, conditioned media were acidified to activate latent TGF-β1 (LTGFβ1), and the assay was performed according to the manufacturer's directions as described previously (Kieswetter *et al.* 1996). The amount of TGF-β1 in the cell layer was not examined because of difficulties associated with quantitatively extracting this cytokine from the matrix.

G. Statistical Analysis

Experiments were conducted four times to ensure the validity of the results. The data shown are from one experiment. Both experiments yielded comparable observations. For any given experiment, each data point represents the mean $\pm$ SEM of six individual cultures. Data were first analyzed by analysis of variance; when statistical differences were detected, the Student's t-test for multiple comparisons using Bonferroni's modification was used. Additionally, data were analyzed using two-factor analysis of variance with equal replication using the SPSS program for PCs. P-values ≤ 0.05 were considered to be significant.

III. RESULTS

A. Surface Characteristics

Average Surface Roughness of Titanium Disks Through the use of profilometry, The R_a values of the three different surfaces were: PT, 0.60 ± 0.02 μm; SLA 3.97 ± 0.04 μm; and TPS, 5.12 ± 0.24 μm. The SLA and TPS surfaces were significantly rougher than the PT surface. In addition, these two surfaces were significantly different from each other and had a distinctly different topography. These R_a values were similar to those found for the titanium disks used in other investigations (Boyan *et al.* 1998, Lohmann *et al.* 1999, and Martin *et al.* 1995). The plastic control did not have profilometry performed to determine average surface roughness characteristics.

B. Cell Morphology

Cultured NHOst Cell Morphology: Normal female human osteoblasts (NHOst) were obtained from Clonetics, San Diego, CA. For all experiments, the cells were cultured using osteoblast growth media (OGM) purchased from the supplier. This media contains ascorbic acid. The cells form multilayers in culture with focal condensations, but they do not form nodules typical of fetal rat calvarial cultures. These cultures become von Kossa positive, suggesting that mineralization has occurred (unpublished data). The cells were received in a T-75 flask at third passage, and were passaged one more time prior to use in the experiments. For each experiment, third passage cells were plated at 5000 cells/cm^2. When the cells in the control (= plastic) wells reached visual confluence, cultures on all other surfaces were considered confluent as well. When the NHOst cells

were cultured on the plastic or PT surfaces, they formed a uniform continuous monolayer, and the cells themselves were oval and rather flat in appearance (Figure 1A, 1B). The cells demonstrated a more differentiated phenotype when cultured on SLA surfaces, however, and were more cuboidal and elongated. The cells appeared to grow into the microtopographical cracks and crevices more readily, and demonstrated multiple cellular extensions that allowed them to interconnect with other cells in close proximity (Figure 1C). NHOst cells cultured on the TPS surfaces demonstrated an even more elongated phenotype than observed on the SLA surfaces, and the cellular extensions proved to be more elongated as well, and often bridged the microirregularities of the plasma-sprayed topography.

C. Analysis of Cell Response

NHOst cell number Cell proliferation was affected by surface roughness, but not by treatment with 17β-estradiol (Figure 2). The number of cells on all titanium surfaces was reduced compared with the smooth plastic control surfaces. The PT surfaces exhibited a 45% decrease in cell number, while the SLA demonstrated a 76% decrease and the TPS showed a decrease of 81% versus the plastic.

Alkaline Phosphatase Specific Activity Alkaline phosphatase activity varied with titanium surface roughness, and the effects of 17β-estradiol were surface-dependent. The enzyme specific activity was comparable in isolated cells and cell layer lysates in cultures that were grown on the plastic and on the PT surfaces (Figure 3). Cell culture on the SLA and TPS surfaces resulted in significantly reduced alkaline phosphatase specific activity for both cell and cell layer lysate measurements. The reduction for SLA surfaces was 89% and the reduction for TPS was 91%. When cells were cultured on SLA, there was no effect of 17β-estradiol on isolated cells, yet there was a small, but significant

increase on the TPS surfaces. However, addition of 17β-estradiol to the cultures resulted in a 100% increase in cell layer alkaline phosphatase specific activity on both SLA and TPS surfaces.

Osteocalcin Levels Osteocalcin was also sensitive to surface roughness (Figure 4). Osteocalcin levels were similar for the plastic and PT surfaces, but the SLA and TPS surfaces showed significantly greater levels over both the PT and plastic control. SLA showed a 2-fold increase, and TPS showed a 2.1-fold increase over PT and plastic control surfaces. There was a dose-dependent increase in osteocalcin in the conditioned media of the cells cultured on the SLA and TPS surfaces at the higher concentration of 17β-estradiol. There was no effect on osteocalcin levels for those cultures grown on plastic or PT surfaces.

Prostaglandin E_2 (PGE_2) NHOst cells showed sensitivity to surface roughness, and produced PGE_2 in a surface-dependent manner that was also regulated by 17β- estradiol (Figure 5). Greater production of PGE_2 was observed with increasing surface roughness. Cells cultured on PT surfaces increased 7.4-fold over the plastic control, and there was a 57.2-fold production increase on SLA and a 51.4-fold increase of production of PGE_2 on the TPS surfaces. Production of PGE_2 was not significantly affected by the presence of 17β- estradiol on PT and plastic surfaces, but surface roughness effects were synergistic with the addition of 17β-estradiol to the cultures.

Transforming Growth Factor β1 NHOst cells grown on plastic and PT surfaces demonstrated comparable levels of TGF-β1, and treatment using 17β-estradiol did not affect their levels. Cells cultured on both SLA and TPS surfaces had increased amounts of TGF-β1 in their media, and the effect was surface dependent. A synergistic effect was

observed when NHOst cells were cultured on SLA or TPS surfaces and then treated with 17-β-estradiol (Figure 6).

Figure 1: Cultured NHOst Cell Morphology on Surfaces of Different Roughnesses

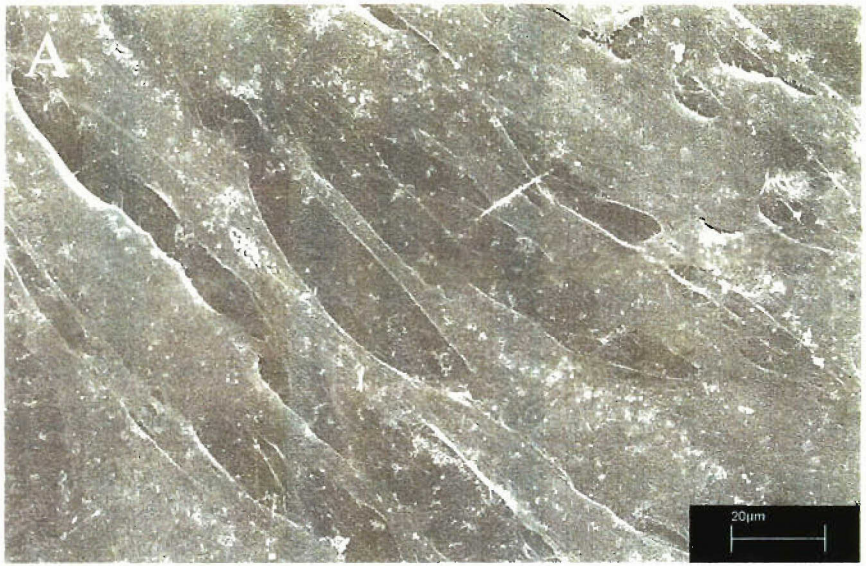
A. Cells cultured on the smooth plastic control. Cells form a continuous monolayer that is flat in appearance

B. NHOst cells cultured on PT surface: Cells also form a uniform monolayer where the cells are flat and oval in appearance

C. Cells cultured on SLA surface: Cells produce multiple extensions to cover the microtopography of the surface. Extensions seem to interconnect between cells in proximity.

D. NHOst cells on TPS surface: More extensions produced, and cells appear to bridge microirregularities of the surface.

Cultures were examined using scanning electron microscopy. At harvest, cell culture media was removed, and cells were rinsed three times with phosphate-buffered saline (PBS), and then fixed with 1% OsO_4 in PBS for 15-30 minutes. Disks were then again rinsed with PBS, and incubated for 30-45 minutes each in 50, 75 and 100% *ter*-butyl alcohol and vacuum dried. A thin layer of gold-palladium was sputter coated onto the samples using a LEO 435VP scanning electron microscope (LEO Electron Microscopy Ltd., Cambridge, England).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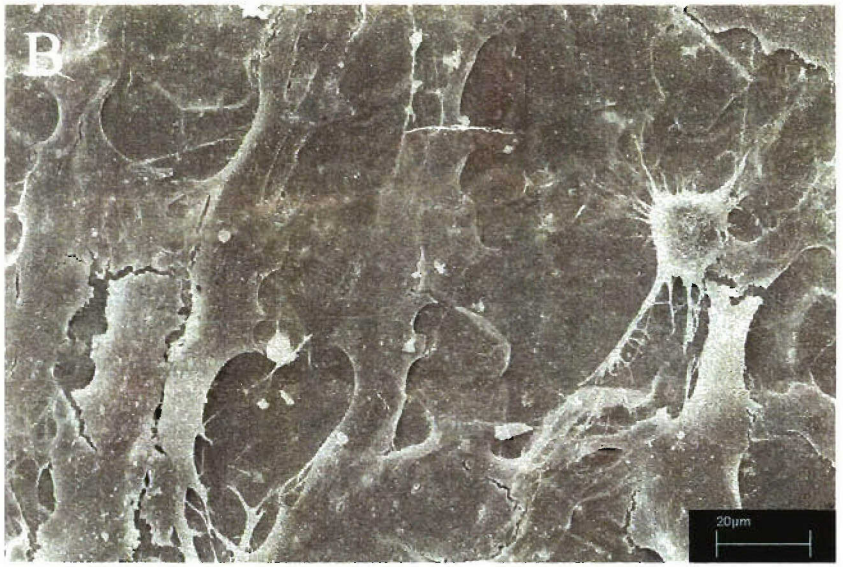
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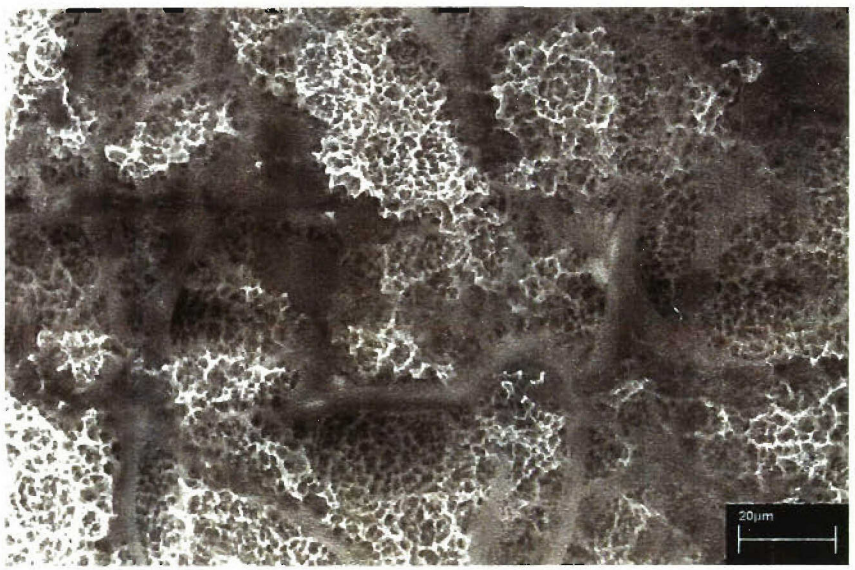
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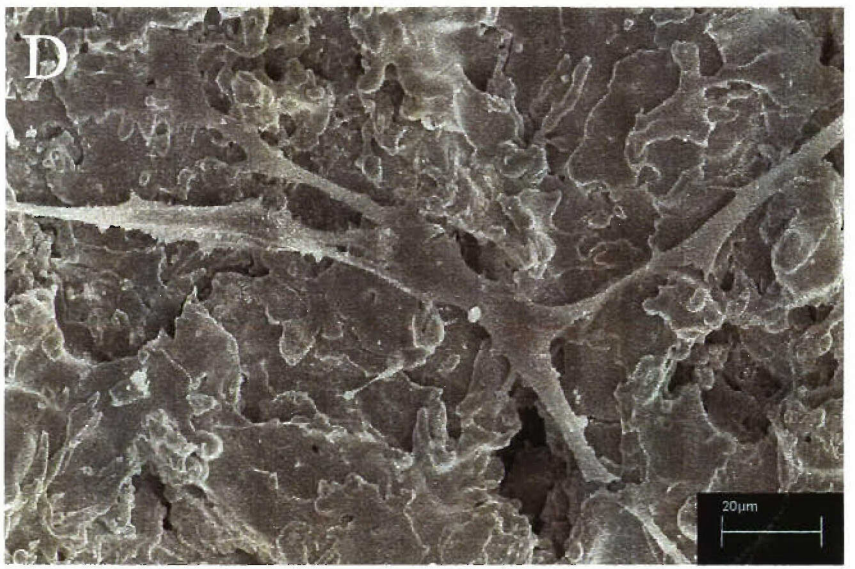
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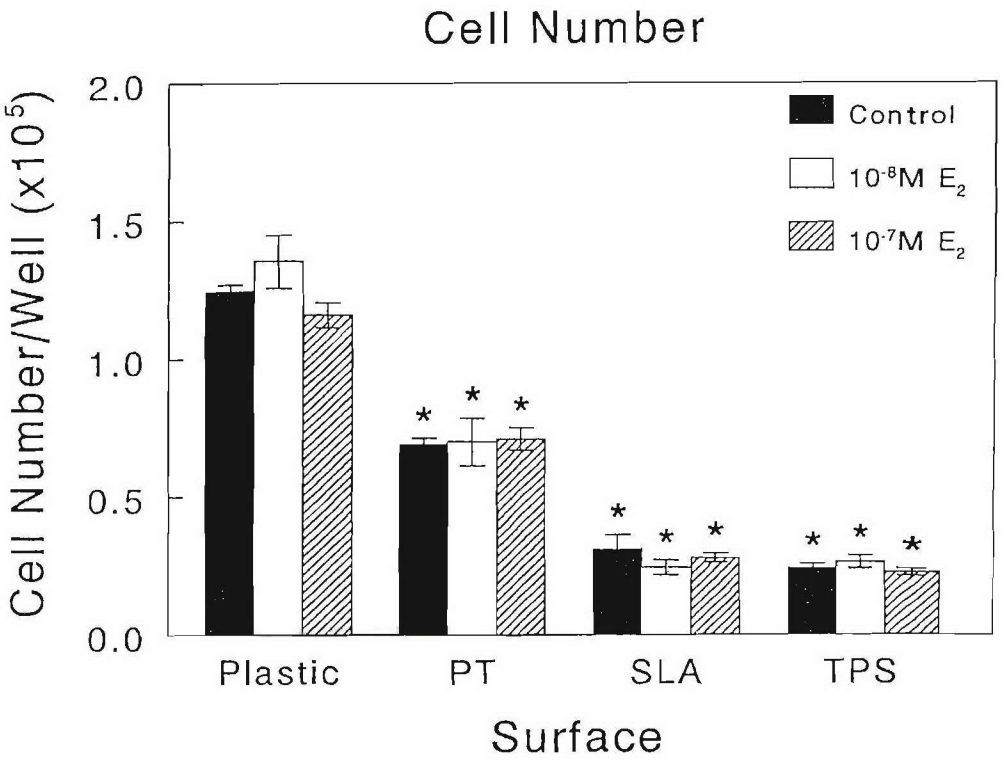
20µm

Figure 2. Number of cells released from culture surfaces.

NHOst cells were cultured to confluence on plastic or Ti disks and then treated for twenty-four hours with 10^{-7}M or 10^{-8}M 17β-estradiol. At harvest, cell layers were trypsinized and cell number determined. Data are from one of four replicate experiments, all yielding nearly identical results. Values are the mean ± SEM of six cultures. *P <0.05, Ti disks vs. plastic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Cell Number/Well (x10^5)
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1.5
1.0
0.5
0.0
Control
10^{-8}M E$_2$
10^{-7}M E$_2$
*
Plastic
PT
SLA
TPS
Surface

Figure 3: Alkaline phosphatase Specific Activity: NHOst cells were cultured to confluence on plastic or Ti disks and then treated for twenty-four hours with 10^{-7}M or 10^{-8}M 17β-estradiol. At harvest, isolated cells (Panel A) and cell layer lysates (Panel B) were prepared and alkaline phosphatase specific activity in each was determined. Data are from one of four replicate experiments, all yielding nearly identical results. Values are the mean ± SEM of six cultures. *$P<0.05$, Ti disks vs. plastic; and #$P<0.05$ treated vs. untreated control for a particular surface.