Genome Wide assessment of Early Osseointegration in Implant-Adherent Cells

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ABSTRACT

GHADEER N. THALJI: Genome wide assessment of early osseointegration in implant-adherent cells
(Under the direction of Professor Lyndon F. Cooper)

Objectives: To determine the molecular processes involved in osseointegration.

Materials and methods: A structured literature review concerning in vitro and in vivo molecular assessment of osseointegration was performed. A rat and a human model were then used to identify the early molecular processes involved in osseointegration associated with a micro roughened and nanosurface superimposed featured implants. In the rat model, 32 titanium implants with surface topographies exhibiting a micro roughened (AT-II) and nanosurface superimposed featured implants (AT-I) were placed in the tibiae of 8 rats and subsequently harvested at 2 and 4 days after placement. Whereas in the human model, four titanium mini-implants with either a moderately roughened surface (TiOblast) or super-imposed nanoscale topography (Osseospeed) were placed in edentulous sites of eleven systemically healthy subjects and subsequently removed after 3 and 7 days. Total RNA was isolated from cells adherent to retrieved implants. A whole genome microarray using the Affymetrix 1.1 ST Array platform was used to describe the gene expression profiles that were differentially regulated by the implant surfaces.

Results: The literature review provided evidence that particular topographic cues can be specifically integrated among the many extracellular signals received by the cell in its signal transduction network. In the rat model, functionally relevant categories related to ossification, skeletal system development, osteoblast differentiation, bone development and biomineral tissue development were upregulated and more prominent at AT-I compared to AT-II. In the human model, there were no

significant differences when comparing the two-implant surfaces at each time point. However, the microarray identified several genes that were differentially regulated at day 7 vs. day 3 for both implant surfaces. Functionally relevant categories related to the extracellular matrix, collagen fibril organization and angiogenesis were upregulated at both surfaces. Abundant upregulation of several differential markers of alternative activated macrophages was also observed. The biological processes involved with the inflammatory/immune response gene expression were concomitantly downregulated.

Conclusions: The presence of micro-roughened and nanosurface features modulated in vivo bone response. This work confirms previous evaluations and further implicates modulation of the inflammatory/immune responses as a factor affecting the accrual of bone mass shortly after implant placement.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	analysis of variance
BMP	bone morphogenic proteins
Ca	calcium
Cr	chromium
Co	cobalt
cDNA	complimentary deoxyribonucleic acid
COX	cyclooxygenase
Cp Ti	commercially pure
ELISA	enzyme-linked immunosorbent assay
ECM	extracellular matrix
FC	fold change
GO	gene ontology
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAG	glycosaminoglycans
hMSC	human mesenchymal cells
HUVEC	human umbilical vein endothelial cells
HCL	hydrocluoric acid
HF	hydrofluoric acid
H2O2	hydrogen peroxide
HA	hydroxyapatite
ID	identification
ISQ	implant stability quotient
IL	interleukin
Fe	iron
Mg	magnesium
Mn	manganese
mRNA	messenger ribonucleic acid

Mod	modified
Mo	Molybdenum
MAO	monoamine oxidation
Ni	nickel
Nb	niobium
NS	not significant
OS	osseospeed
P	phosphate
PMMA	polymethylmethacrylate
qRT-PCR	quantitative real-time polymerase chain reaction
RhoA	Ras homolog gene family, member A
RBM	rat bone marrow cells
RT-PCR	real-time polymerase chain reaction
RANKL	receptor activator of nuclear factor kappa-B ligand
SEM	scanning electron microscopy
SBF	serum blood fluid
Si	silicon
Ag	silver
SMO	smooth
Sst	stainless steel
Sa	surface roughness
TRAP	tartrate resistant acid phosphatse
Sn	tin
Ti	titanium
TiO	TiOBlast
TiO_2	titanium oxide
TNF	tumor necrosis factor
UNC	University of North Carolina at Chapel Hill

UV	ultraviolet
Wt	weight
XPS	X-ray photoelectron spectroscopy
Zn	zinc
Zr	zirconium
α	alpha
β	beta
γ	gamma
μ	micro

Introduction

Current tooth replacement strategies typically consider the alloplastic integrated replacement of missing teeth using endosseous dental implants as a primary choice among available modes of therapy. The success of contemporary dental implants is largely attributed to the process of osseointegration. Osseointegration is defined as a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant [1]. Despite the high success rates achieved, implant failures that mandate implant removal do occur. Factors attributing to implant failures include local and systemic conditions such as reduced bone volume, reduced bone density, periodontitis, and impaired wound healing (e.g., diabetes, smoking, osteoporosis, radiation therapy, chemotherapy) [2-6]. Efforts to enhance osseointegration of dental implants allowing for faster prosthetic rehabilitation and improved success rates in clinically challenging situations, included modifications to the physical and chemical properties of the implants surfaces.

It is well demonstrated that implants with moderately rough surfaces (average height deviation of 1-2μm) enhance the rate and quality of osseointegration with greater bone-to-implant contact and higher resistance to torque removal [7-11]. Human clinical investigations have provided histomorphometric data indicating the importance of surface topography for improvement of osseointegration. At the histological level, pair-wise comparisons of machined implant surfaces with etched or grit-blasted surfaces revealed marked increases in bone-to-implant contact for the rough surface implants. The majority of the studies focusing on this comparison involved implants placed in the posterior regions of the maxilla and mandible (presumed type III or IV bone) and represented healing periods of greater than 3 months duration [12-15].

In contrast to micron-features of alloplastic materials, bone is composed of constituent nanofeatures [16]. Nanostructured materials are those with features less than 100nm in at least one dimension [17]. In the dynamic research field of implants, a multitude of biomaterial modification approaches with nanoscale features have been pursued. Typically applied techniques include lithography, ionic implantation, anodization, acid etching, alkali treatment, peroxidation and sol-gel deposition [18]. Various reports support that embellishment of the micron-rough surface with nanoscale features further enhances osteoblast differentiation [19-21], which could also promote stability and increase interfacial biomechanical locking with bone [22-24].

Precisely how this is achieved has not been clearly delineated. Only recently has the molecular basis of osseointegration been considered. A comprehensive understanding of the molecular and cellular processes relevant to peri-implant healing is critical for achieving therapeutically relevant targets to positively influence implant osseointegration.

The scope of work presented in this dissertation included a comprehensive structured literature review of current molecular data in vitro and in vivo and genome-wide evaluation of nanoscale topography influences on osseointegration in a rodent model and in man.

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Chapter 1

Molecular Assessment of Osseointegration *In Vitro*; A review of current literature

Abstract:

This paper represents the results of a structured review of the literature concerning in vitro molecular assessment of osseointegration at the level of cell – surface topography interactions. A search of the electronic databases was performed up to and including November, 2010. 320 articles met the inclusion criteria. Characteristics of the included in-vitro reports were model systems used, genes examined, techniques used for molecular assessment of the osseointegration process, and wide gene expression profiling studies. There exists a growing body of in vitro evidence to support a role for surface topography in the direct influence of cellular phenotypes as related to the process of osseointegration. Most recently, functional or mechanistic studies have provided evidence that particular topographic cues can be specifically integrated among the many extracellular signals received by the cell in its signal transduction network. Such investigations begin to define linkages between the character of the implant surface and adherent cellular responses, including cells from extravasated blood (e.g., platelets) and of the immune system (e.g., monocytes). In vitro studies involving cell culture on endosseous implant related biomaterials offer important and beneficial insight into the clinical control of the implant/bone interface.

Introduction:

The dental implant surface represents one of the key factors affecting osseointegration success. Earliest studies indicated that implant surfaces could dramatically influence the possible formation of a direct bone to implant contact [1], despite the recognized influence of surgical technique and loading conditions [2]. In the influential report of Buser et al (1991), comparison of experimental implant surfaces bearing different surface topography ranging from electropolished to electropolished and plasma sprayed hydroxyapatite-coated surfaces revealed that increasing surface roughness resulted in increased bone-to implant contact [3]. The efforts of Wennerberg and co-workers also systematically revealed that compared to machined cpTitanium surfaces, increasing surface roughness improved both bone-to-implant contact and physical interaction with bone (removal torque) [4-8]. In summary reviews, it was concluded that numerous investigations demonstrated that smooth (S(a) <0.5 micron) and minimally rough (S(a) 0.5 – 1.0 micron)surfaces showed less strong bone responses than rougher surfaces [7,9].

The current literature involving *in vivo* studies of surface topography effects on osseointegration at the molecular level further supports both histomorphometric and biomechanical data of improved osseointegration [8,10]. These studies demonstrate that surface topography is, in part, responsible for changes in the interfacial tissue responses to the endosseous implant that lead to greater bone to implant contact. These changes are manifest by a) greater bone-to-implant contact and b) increased biomechanical interlocking with bone in animal and human studies.

Human clinical investigations have provided histomorphometric data indicating the importance of surface topography for improvement of osseointegration. At the histological level, pair-wise comparisons of machined implant surfaces with etched or grit-blasted surfaces revealed marked increases in bone-to-implant contact for the rough surface implants. The majority of the studies focusing on this comparison involved implants placed in the posterior regions of the maxilla and mandible (presumed type III or IV bone) and represented healing periods of greater than 3 months duration [11-14]. Implants with altered topography, indicated partly by increased S(a) values, supported greater bone accumulation at the interface. It is assumed that the surface topographic changes of the surface represent alloplastic cues that promote tissue changes leading to increased bone accrual at the implant surface.

Several *in vivo studies* at the molecular level indicate that surface topography influences adjacent cellular functions including the process of bone matrix biosynthesis and mineralization [15]. Most recent investigations focusing on mRNA expression profiles within cells adjacent to tissue at implants or cells adherent on implant surfaces. The findings include increased expression of mRNAs involved in the regulation of cell proliferation, angiogenesis, osteoinduction and osteogenesis [16].

The complexity of wound healing is self evident in the many changes in gene expression observed in gene profiling studies such as those recently published regarding osseointegration. For example, examination of gene expression events at 4, 7 and 14 days in tissues surrounding SLActive surface implants in the retromolar area of 9 human volunteers revealed thousands of differentially significant genes that increased and decreased with time [17]. The biological processes identified at day 4 prominently involved the cell cycle, the immune response and inflammatory gene regulation, phagocytosis and macrophage activation. These prominent changes suggested the induction of fundamental wound healing events. At day 14, gene expression events representing skeletal development, ossification and cellular differentiation were prominently revealed. The complexity of these processes requires detailed analysis and model systems that permit the testing of hypotheses to confirm what descriptive analyses have suggested.

In vitro studies that pursue aspects of osseointegration, particularly cellular responses to implant surface topography, provide insight into the role that the implant surface plays in modulating cellular responses during the complex process of osseointegration. It is the aim of this review to assess the knowledge that has been gained to date from molecular biological evaluations of cellular responses to endosseous dental implant surface topography.

Materials and methods:

For the literature to be included in this review, the following eligibility criteria were used: in vitro reports assessing the molecular process of osseointegration, and articles published only in English. Studies related to wear particles, letters to the editor and reviews were excluded. Articles limited to assessment of Alkaline phosphatase activity were excluded. Articles limited to biological coatings were excluded in this review.

Search Strategy: A thorough search was performed up to and including November, 2010 through the following databases: PubMed (1948 to November, 2010), using the following terms: (titanium[tw] OR zirconium[tw] OR dental implant*[tw]) AND (gene expression[MeSH Terms] OR gene expression[tw] OR differentiation[tw] OR rna[MeSH Terms] OR rna[tw] OR messenger rna[tw] OR mrna[tw]), EMBASE via OVID (1947 to November, 2010) using: (Titanium.mp. or zirconium.mp. or tooth implantation/ or dental implant*.mp. and gene expression.mp. or exp gene expression/ or exp DIFFERENTIATION/ or differentiation.mp. or ena.mp. or exp RNA/ or messenger rna.mp.[mp=title, abstract, subject headings, heading words, drug trade name, original title, device manufacturer, drug manufacturer] or mrna.mp.[mp=title, abstract, subject headings, heading words, drug trade name, original title, device manufacturer, drug manufacturer], and BIOSIS Previews via ISI Web of Science (1969 to November, 2010), ISI Citation via ISI Web of Science (1955 to November, 2010) using the following words: Topic=((titanium OR zirconium OR "dental implant*") AND ("gene expression" OR differentiation OR rna OR rna OR "messenger rna" OR mrna)). Titles and abstracts were screened for possible inclusion in the review. The full text of the articles judged to be relevant by the title and abstract was read and independently evaluated against the eligibility criteria. In addition, a hand search of the reference lists of original studies that were found to be relevant was conducted.

Results:

320 articles met the inclusion criteria. Characteristics of the included in-vitro reports including surfaces examined, model systems used, genes examined, techniques used for molecular assessment of the osseointegration process, and wide gene expression profiling studies are presented in Tables 1, 2, 3, 4 and 5 respectively.

The spectrum of surfaces investigated in *in vitro* studies of cellular aspects of the osseointegration process is widely represented by metallic and ceramic materials (Table 1). It is apparent that, when limited to critical appraisal of studies involving osteoblast-like or osteoblastic cell performance in relationship to endosseous implant material influences, studies performed in cell culture are possible and provide information regarding cytotoxicity, cell adhesion, proliferation and differentiation. The vast majority of these studies have been conducted using typical cell culture conditions that include the use of 5 – 15% serum, typically bovine derived. It is acknowledged that adhesion – related phenomena involved the interaction of cellular receptors with serum

derived proteins such as Fibronectin. The study of direct cell (or cell-derived protein) - surface interaction has not been widely pursued.

Interestingly, despite many preliminary studies of cell function with regard to bulk endosseous implant material, the majority have focused on osteoblastic or osteoblast-like cell behavior without regard to the key cellular components of the immune system. More recent interest in the role of immunomodulation of osteogenesis in general [18] suggests the importance of other cell types in the process of osseointegration. The more recent investigations of monocyte/macrophage function, platelet function and inflammatory gene regulation in vitro demonstrate that surface topography influences cellular processes beyond osteoblast functions and these processes may be evaluated in cell culture [19,20].

While the obvious variable in studies of topography-dependent modulation of osseointegration is the implant surface, the previous statement indicates that in vitro studies are highly dependent on the model system and particularly upon the cell line selected for investigation. Comprehensive review revealed approximately 50 different types of cells, the majority representing the osteoblastic lineage, has been used to study implant surface-cell interactions in culture (Table 3). The majority of studies employed one or another osteoblastic cell model able to differentiated under culture stimulation. Such models have included primary cultured bone marrow-derived stem cells (e.g., hMSCs), stem cell-like cell lines (e.g., $C_3H_{10}T_{1/2}$) or C_2C_{12}), bone-derived osteoprogenitors (e.g., RBM cells), transformed osteoblastic cells (e.g. FOB), osteoblastic cell lines (e.g., MC3T3-E1), and osteosarcoma derived cell lines (e.g., MG63). Cells of human, mouse and rat origin dominate these models, however, canine, chick and bovine cell models have also been utilized. Each model system offers select advantages and disadvantages [21], despite the generalized ability to adhere to and proliferate on the alloplastic substrates. Differences in phenotypes such as the relative expression of integrin receptors, matrix proteins or signal transduction molecules must be understood for proper deployment and interpretation of results. Irrespective of important distinctions that must be made for the different models deployed to investigate “osteoblast” – implant surface interactions, it may be generally concluded that osteoblastic cell adhesion and culture on titanium (or Zirconia) surfaces with enhanced surface topography in the micron-scale range results in increased osteoblastic differentiation as evidenced by a) increased osteoinductive transcription factor mRNA expression (RUNX-2 and Osterix), b) elevated type 1 collagen expression, c) increased bone

specific matrix protein expression including alkaline phosphatase, osteopontin, osteocalcin and bone sialoprotein, and d) cell matrix layer mineralization.

The array of surface topographies investigated to date spans the range of applicable technologies represented by clinical dentistry and orthopedics. They include grit blasting, acid etching, anodizing, laser ablating, micromachining, and photolithography methods among them. While most investigations have examined one or two surfaces representing the result of a modification process, a select few investigations have attempted to systematically interrogate the role of a specific surface modification parameter on adherent cell behavior. For example, Sjotstrom et al. (2009) [22] utilized titania nanopillar structures of 15 – 100 nm to examine hMSC osteoblastic differentiation as a function of nanoscale topography. In this instance, diameter and spacing parameters were not controlled. In this particular review, the biological activation of metallic and ceramic implant surfaces by linking of receptor targets (e.g., matrix proteins, growth factors, or cytokines) was not considered. [23]

Cell Adhesion – A general appreciation for cell adhesion phenomena at endosseous implant surfaces includes specific roles for adherent extracellular matrix proteins (ECM, e.g., fibronectin, osteopontin) and related transmembrane receptors (e.g., integrins) present on the adherent cells [24]. Adherent osteoprogenitors produce ECM proteins in a manner that is influenced by the nature of the titanium surface, suggesting that surface topography provides environmental cues that change cell signaling at the most fundamental levels [25]. Surface topography does influence the expression of integrins important in osteoblastic cell adhesion and differentiation [26]. The systematic knockdown of alpha 2 integrins in MG63 osteosarcoma cells revealed the requirement of alpha 2-beta1 integrin signaling of titanium topographic cues affecting osteoblastic differentiation [27]. The role of integrins in cell adhesion involves many different integrins and common integrin subunits (e.g., b1) appear to mediate binding to many different surfaces in cell culture [28], leaving open the question of precisely how specificity of adhesion is controlled at the cellular level. One other mechanistic evaluation of topographic cue-based signaling has focused on b-Catenin activation involving the cytoskeleton, RhoA, as well as WNT pathway mediators. Using reporter gene tools to study the activity of b-Catenin (a known mediator of osteoinduction), Macaluso and colleagues have provided among the few investigations probing the mechanisms by which surface topographic cues influence adherent cell function [29].

The surface cues imposed through adhesion –based signaling leads to functionally and structurally significant changes in the cultured cell layer, suggesting that similar influences are imposed at the implant bone interface *in vivo*. Ogawa and colleagues' comparison of rat BMC cultured on titanium and tissue culture polystyrene (TCPS) revealed a collagen rich, calcium phosphate layer at the titanium but not TCPS interface. The titanium interfacial tissue demonstrated greater hardness, elastic modulus and interfacial strength [30]. Changes of the titanium surface in fact alter abundance of key signaling proteins such as the small GTPase RAS and the MAP kinase pathway within adherent osteoblastic cells [31]. When considering the influence of topography, the stimulation of matrix formation can influence a range of cellular processes including migration, matrix organization, cytoskeletal organization and intracellular signaling [32]. An elegant investigation of hMSC adhesion to nanoporous TiO_2 surfaces measured displacement of adherent cells using atomic force microscopy methods which led to hypothesize that initial adsorption is a function of proteins adsorbed from serum (e.g., fibronectin; [33]). Additional studies have revealed that particular signals area mediated from topographically modified titanium substrates to cells via integrins including the a2b1 integrin [27] . Most recently, MC3T3-E1 osteoblastic cell culture on smooth versus SLA surfaces was examined at the level of ERK signaling. Greater Osterix (Sp7), osteopontin and osteocalcin expression were observed in cells cultured on SLA surfaces. Associated with this enhanced Osteoinduction was reduced ERK1/2 phosphorylation, suggesting that topography-mediated signals involve the downregulation of the ERK pathway [34]. Continued mechanistic investigations are needed to define precisely how cells perceive, signal and integrate topographic cues from alloplastic materials to physiologic changes that result in tissue integration.

Osteoblastic differentiation - Runx2 is an essential transcription factor for osteogenesis; its removal results in no bone formation or osteoblastogenesis. It binds to target gene promoters to affect their expression during osteoblastic differentiation and bone formation [35]. If surface topography alters adherent cellular differentiation, then one mechanism could involve topography-related changes in Runx2 expression. Earliest reports of surface topography influences on RUNX-2 included studies of Masaki et al (2005) [36] and Guo et al (2007) [37] that evaluated the influence of HF treatment of Ti02 grit blasted implant surfaces on adherent MSC function. Recently, elevated RUNX-2 expression was reported for human periodontal ligament cells cultured on the SLactive surface [38].

Osterix (Sp7) represents the second key transcriptional regulator of Osteoinduction. Sp7 is required downstream of Runx2. Importantly, Sp7 is necessary for postnatal osteoblast and osteocyte function [39]. Masaki et al[36] first indicated that enhancing titanium substrate microtopography led to elevated Sp7 expression in adherent osteoprogenitor cells. Others including Perrotti et al (2012) [40] have demonstrated that compared to machined surfaces, adherent cell Sp7 expression on micron rough surfaces is elevated. hMSC Sp7 expression is positively influenced by culture on titanium substrated bearing nanoscale topographic enhancement [37,41-43] In confirmation of the significance of Osterix's role in promotion of osseointegration, in vivo studies reveal that Osterix overexpression in labeled mouse bone marrow stem cells led to accelerated osseointegration[44]. Thus, *in vitro* studies of surface topography effects on Osteoinduction suggest that greater bone formation in vivo is due, in part, to surface dependent modulation of the osteoinductive transcription factors Runx2 and Osterix (Sp7).

More recently, investigations have pursued descriptive studies of cell – implant surface interactions in vitro using genome wide expression profiling techniques. At least 13 publications report use of different arrayed gene assays for the purpose of characterizing the changes in gene expression that occur over time or as a function of surface modification. For example, Vlacic-Zischke et al (2011) compared human mandible derived osteoblast gene expression following growth on SLA vs. SLactive surfaces [45]. When subjected to pathway analysis, gene expression data revealed the up-regulation of BMP pathway signaling as well as molecules involved in Wnt signaling. Zhao et al (2010) examined rat osteoblastic cell culture on a unique porous Ti alloys surface [46]. When comparing data from studies using different cell models, markedly different results are observed (e.g. MG63 cells [47] vs. hMSC [48]). Culture of hMSCs on topographically modified titanium substrates led to increased expression of both Runx2 and BSP where as MG63 cell culture at 24 hours did not reveal elevations in these key markers of Osteoinduction.

The aggregate data from focused evaluations of directed studies of one or another osteoinductive regulatory gene or from gene profiling studies regarding cells adherent to different implant surfaces indicates that surface topography can influence Osteoinduction. With increasing surface roughness, elevated and / or earlier expression of Runx2, Osterix (Sp7), BMPs (notably BMP2 and BMP6), and Wnt pathway molecules including b catenin, has been reported. In vitro, cells adherent to modeled dental implant surfaces demonstrate

topography-dependent changes in the expression and function of several key regulators of Osteoinduction (Figure 1).

Extracellular matrix formation - Among the first observations of surface topography effects on adherent cell expression of ECM protein expression was the study of fibronectin mRNA levels that concluded that topography-induced changes of the cell led to increased fibronectin expression [49]. With specific emphasis on interfacial bone formation, attention has focused on bone matrix proteins known to be the product of osteoblastic cells [50].

Alkaline phosphatase is a commonly utilized marker of early osteoblastic differentiation that is repeatedly observed to be elevated in cells adherent to surfaces of increasing surface topography and Osteocalcin has been utilized as a marker of terminal osteoblastic differentiation [51,52]. Ogawa and colleagues have utilized both osteopontin and osteocalin expression levels to indicate the extent of osteoblastic differentiation [53,54]. These and many other investigations have revealed that culture-induced differentiation of osteoblast-like cell lines and mesenchymal stem cells is surface topography dependent. This process is modeled by the sequential expression of alkaline phosphatase, osteonectin, osteopontin, and the penultimate emergence of bone sialoprotein and osteocalcin expression. With increasing topographic complexity and / or increasing R(a) or S(a) values, cultured cells produce greater abundance of mRNAs encoding these matrix proteins at earlier time points. Suggested is earlier and greater bone formation *in vivo* at implants with enhanced surface topography.

Bone matrix is largely composed of type I collagen. Cooper and co workers described the differential regulation of collagen biosynthesis in cells adherent to smooth and rough titanium surfaces [55]. This revealed greater collagen biosynthesis and higher levels of cross-linking gene expression within cells adherent to the topographically enhanced titanium substrates. Similar investigations have revealed the positive influence of surface roughness on type I collagen mRNA expression, as well as other bone matrix components including osteopontin, osteocalcin and alkaline phosphatase [26].

These different molecules and the molecular knowledge of their regulation enables investigators to pursue studies that may indicate the mechanisms by which surface topographic cues enable cellular differentiation along the osteoblast lineage. In vitro studies to date have **a**) confirmed that adherent cell expression of ECM protein and encoding mRNAs is affected by topography, **b**) demonstrated that changes in ECM expression in

turn affect adherent cell behavior, and **c**) shown how alterations in ECM protein biosynthesis of adherent cells is associated with changes in the physical properties of the protein / cell layer and its attachment to the modeled implant surface.

Osteoclastogenesis: The accrual of bone mass at the implant surface requires the formation of new bone matrix and its mineralization. The vast majority of cell culture studies addressing osseointegration have focused on osteoblastic production of bone matrix and mineralization. However, bone accrual is balanced by osteoclastogenesis. Few clinical, pre-clinical or in vitro studies of osseointegration have investigated osteoclastogenesis at the implant-bone interface or as a function of the implant surface per se.

Osteoclastogenesis is controlled by soluble inducing factors as well as insoluble membrane-bound ligands present on osteoprogenitor cells. These factors and ligands represent both positive and negative mediators of osteoclast production. The MSC expresses the central insoluble ligand that triggers monocyte differentiation to osteoclasts, namely RANKL. The MCS and osteoblastic cells produce and secrete the key inhibitor of osteoclastogenesis, termed osteoprotegrin (OPG). Additionally, osteoclastogensis is modulated by immunoregulatory cytokines including TGFb and IL6. Other mediators include mechanostimulatory events and particulate debris that are beyond the scope of this review. Three similar investigations using MG63 osteosarcoma cells [56] , human periodontal ligament cells [38] ,and primary human osteoblasts cultured on smooth versus SLA and / or SLactive substrates [57,58]. RANLK expression was lower, while OPG expression was increased in cells adherent to the rougher surfaces. Increased expression of OPG on a rough versus smooth surface was observed for both MG63 cells and alveolar primary osteoblasts [59]. In the MG63 model, integrin mediate signaling and protein kinase C activity were implicated in the increased expression of OPG [60], further demonstrating that surface cues directly impact adherent cell physiology.

Other cellular aspects of osseointegration modeled in vitro: While the majority of studies of osseointegration in cell culture have centered about the osteoblastic cell lineage, the process of osseointegration in vivo is not fully represented by surface – osteoblast interactions alone. Other cell types are clearly observed to adhere before the attachment of osteoprogenitor cells or mesenchymal stem cells [61]. Primary among these other cells are the components of extravasated blood, notably the platelet. Additionally, monocytes from the

circulation and cells of the innate immune system are eventually recruited to this site of surgical tissue damage. The direct interaction of these cells with the implant surface has received limited attention.

Davies and co-workers introduced the general concept that the topographic enhancement of blood platelet – implant surface interaction was central to the process of osteoconduction [62]. Subsequently, it was demonstrated that adhesion of platelets to microrough surfaces of increasing topographic complexity and dimension enhanced platelet activation [63,64]. More recently, assessment of the thrombogenic response with regard to surface topography was performed. Thor et al (2007) demonstrated that 15 fold greater activation (transglutaminase release) for whole blood plated on grit blasted vs machined or HA surfaces [65]. Cell culture investigation of thrombogenesis was further able to distinguish greater transglutaminase release from blood platelets on SLactive vs. SLA surfaces [66]. In the context of this review, it is important to recognize the lack of transcription and mRNA expression occurring in platelets. However, as remarkably rich sources of growth factors and cytokines, their regulated presence and activity may be important, indirect significance to the surface-mediated changes in osteoprogenitor cell phenotype that is of direct concern herein.

Monocytes or macrophages represent important cellular mediators of tissue responses to implanted biomaterials and have been known to be topography dependent in this function [67]. Monocytes display marked haplotaxis and monocyte adherence increases with surface topography [68]. Takebe et al (2003) [69] utilized anti beta3 integrin antibody treatment to demonstrate that J774A.1 cell adhesion and spreading as a function of surface topography was integrin- dependent and involved alterations in the cytoskeleton. When murine macrophage like cells RAW 264.7 cells were cultured on surfaces of increasing roughness, unstimulated macrophages increased TNF-α secretion . Upon LPS stimulation, cells cultured on roughest surfaces produced the highest levels of IL-1b, IL-6 and TNF-α [70]. Using the J744A.1 cell line, in the absence of inflammatory LPS stimulation, only IL-1 levels were increased on the rougher surface [71] , once again indicating the important role cell models play in modeling biologic events surrounding the impact of surface topography on adherent cell phenotypes *in vitro*. Further study of the role surface topography place in the initial interactions of an implant surface with blood and tissues is requires and can be facilitated using cell culture models and in vitro systems.

Conclusions:

The present literature concerning the molecular phenotype of cells adherent to and cultured on endosseous implant materials subjected to topographic and biological modification confirms *in vivo* and clinical data suggesting that topographic enhancement of the implant surface influences cell function directly. Depending upon the adherent cell type, different biological processes may be modeled in cell culture. With regard to osteoinduction and osteogenesis, cell culture studies clearly indicate that adhesion to surfaces of increasing topographic magnitude and complexity results in increased osteoinductive and bone matrix-specific gene expression. These changes are associated with increases in BMP expression and Wnt pathway gene expression, suggesting that these pathways are influential in promoting osteogenesis of adherent osteoprogenitors. The role of other adherent cell types, including platelets, monocytes, and lymphocytes has been recognized, but comprehensive evaluation of the impact of these cell types as well as the influence of surface topography upon their function adherent to the implant surface remains to be defined. Studies of cell – substrate interactions involving molecular assessment have largely provided descriptions of adherent cell phenotypes. Future studies can utilize the cell culture environment to investigate the mechanisms by which topographic cues influence adherent cell behavior related to the process of osseointegration.

Table 1: Surface modification and coatings examined in the in-vitro studies.

Material (Bulk) Titanium	Surface modification (With nanotopographic features*)	Coatings after surface modification	Coatings only (With nanotopographic features*)
	Untreated/mirror polished/machined/electromachined [52,72-93]		Hydroxyapatite coating-Calcium phosphate [94-111, 112*, 113-116, 117*, 118*, 119,120*, 121]
	Wet ground-grit [77,122-124]		Carbonated hydroxylapatite [125]
	Grooved [126-128]		Hydroxyapatite outer layer and Fluorhydroxyapatite inner layer [97]
	Drill channels [129]		Fluorhydroxyapatite [97]
	Porous-powder metallurgy [130]		Calcium metaphosphate Anodized[108]
	Acid etched [42,46,53,54,59,90,111,117,120,127,131-152]	Sputter deposited TiO2 [149,151*, 153*]	Octacalcium phosphate (OCP) coated Ti [154]
	Acid etching with chemical modification by exposing to NaOH (Alkali acid etched) [155]		Titania hydroxyapatite coatings (composite coating of HA+TiO(2)) [116] [156,157]
	Mod A [59,141-144]		Zinc Hydroxyapatite coating [115]
	Acid etched with Photofunctionalization of titanium oxide: ultraviolet light mediated changes [158-160]		dicalcium pyrophosphate ($Ca_2P_2O_7$, DCPP) [110]
	Acid etched aged [160]		Mn(2+)-doped carbonate hydroxyapatite (Mn-CHA) [154]
	Acid etching/Anodization [46]*		Porous surface-Laser Engineered Net Shaping (LENS) method- ti powder[161]
	Blasted [43,69,118,120,134,135,137,145,162-180]	Hydoxyapatite[182] Calcium phosphate coating [172-174] Calcium phosphate coating with heat treatment [174]	Ag particles electrodeposited onto the TiO_2 nanotubular surface [182*]
	Sandblasted with Photofunctionalization of titanium oxide: ultraviolet light mediated changes [160]		Hydroxyfluorine coating [95]
	Sandblasted aged [160]		Titanium nitrate coating[95]
	Blasted Acid etched [27,36*, 41,43*, 48,51,52,55,57,58-60,71,94,95,106,107,111,118,120,126, 134,139-145,164*, 166*, 167*, 168*, 183,184,185*, 186-228,236*]	Hydroxyapatite coating [210] Anatase coating [228]	Calcium titanate-amorphous carbon (CaTiO(3)-aC) coating [105]
	Blasted acid etched with heat treatment [226,227]		Ti-Plasma sprayed [51,57,60,94,107,114,134, 135,140,162,163,190,191,193

			-199,201,207-209,229] ANODIZED [114,179,229-231]
	Mod SLA [27, 36,48,58,59,141-144, 202,211-221]		Diamond-like carbon coating [95]
	Blasted etched with modification using hydrogen peroxide [199]		Anatase titania coating [41*,47*,233*,234*]
	Blasted Amino group ion implanted [179]		Rutile titania coating [41*]
	Alkali heat treated [234-237]		Ti carbide coatings [238]
	Anodized Alkali treatment [239]		Plasma sprayed and Amino group ion implanted [179]
	Alkali heat treated with SBF [118,234,236]		Carbon-coated TiO(2) nanotubes [240*]
	Oxidation: immersion in a solution of 8.8 M H_2O_2/0.1 M HCl at 80°C for 30 min [241]		
	Oxidation of either PT or SLA [242*]		Coating with titanium nitride oxide [243]
	Anodization[22*,136,165,182*,240*, 244*,245,246*,247,248,249*,250,251* ,252*,253*,254]	Hydroxyapatite [99*,255]	Titania coating non-specified [116,256,257]
	Anodization Mg ion incorporated [165]		Phosphonic acid coated [258]
	Anodization Mg ion incorporated with SBF [118]		Plasma-immersion implanting Si ion [259*]
	Electrochemical treatment either with Ca or Ca and P [260]		Calcium ion implanted [261]
	Heat treatment [73]		Hydrothermal treatment: Strontium ions and/or phoshate [262] phosphate [263] magnesium- [264*]; Calcium [265]
	Glow discharge plasma (GDP) pretreatment[266]		A copolymeric hydrogel coating, poly-2-hydroxyethyl methacrylate-2-methacryloyloxyethyl phosphate (P(HEMA-MOEP)) [268]
	Unspecified smooth/rough [268]		Zirconium coating [41*,99]
			Alumina coating [41*,42*]
			Anodized alumina coating [269*]
Titanium alloys Ti6Al4V alloy			
	Grade polished/ grooved/ untreated [77,86,135,137,270-283]		Hydroxyapatite- calcium phospate [284-288]
	Wet ground [77,289-291]		Coating with porous alumina ceramics [292,293]
	Acid etched [137,294,295]	Hydroxyapatite [295]	Titanium plasma spraying [297]
	Blasted[180,270-273,277,293,298-309]	Hydroxyapatite [181] Titanium nitride coating [307]	Titanium plasma spraying in addition to titanium nitride coating [297]

	Blasted acid etched [311]		CaO-ZrO2-SiO2 (CZS) coating[311]
	Shot peened [181]	Hydroxyapatite [181]	blends of hydroxyapatite and fluorapatite, with concentrations ranging from 0 to 100% fluorapatite[286]
	Selective Laser Sintering (SLS) [294]		Electrochemically deposited dicalcium phosphate dehydrate, so-called brushite [312]
	MAO [306*]		Silicon-substituted hydroxyapatite (Si-HA) coatings [286]
	Severe plastic deformation [313*, 314*]		Fluorohydroxyapatite [315]
	Nanopore [308*,316*]		Titanium plasma spraying in addition to HA[231]
	Electron beam melting process [317]		silicon (Si) nanofibers coating [318*]
	Oxidation [93,319]		silicon oxide (SiO(2)) nanofibers coating [318*]
	Aged treatment (A:Passivated tt followed by aging in boiling deionized water for 10h)[320,321]		titanium oxide (TiO(2) nanofibers surface coating [318*]
	Unspecified smooth/rough [268]		Bioactive glass coating [322]
			Ion implantation with zinc (Zn–Ti–6Al–4V) [31]
			Ion implantation magnesium (Mg–Ti–6Al–4V) [31]
			Coating alkoxide-derived hydroxy carbonate apatite (CHAP–Ti–6Al–4V) [31]
TiMo12Zr6Fe2 (TMZF) discs [323]			
NiTi			
	Mirror polished [75,93]		Hydroxyapatite[181]
	Oxidized [93]		
Ti-6Al-7Nb			
	Untreated [80]		
TiZr (Ti-50 wt %Zr)			
	Grit ground [124]		
TiNb (Ti-50 wt %Nb) alloy discs			
	Grit ground [124]		
	fine-ground[135]		
Ti-5Zr-3Sn-5Mo-15Nb alloy			
	MAO[324]		
Ti13Nb13Zr alloy			
			Hydroxyapatite coating [288*]
CrCoMo alloy Zimaloy			
	Untreated/polished [73,85,135,273,274,277]		Porocoated [277]
	Etched [147]		
	Heat treatment [73]		
	Blasted [273,305]		
Stainless steel			
	[75,76,80,135,277,325,326]an		

	austenitic Ni-reduced SSt [276,281]	
	Etched [147]	
Tantalum	Untreated [73,85]	chemical vapour deposition (CVD) process [73]
Nickel	[75]	
ZrO	[326]	
ZrO2 ceramics		
	Untreated [327]	
Zirconia (yttria-stabilized tetragonal zirconia: ZrO_2 93.5%, Y_2O_3 5.1%)		
	Untreated [78,328,329]	Zi-Unite [250]
	Grooved [328]	
	UV light [330]	
Yttrium-stabilized tetragonal zirconia polycrystals zirconia discs reinforced with 25% alumina		
	Machined [225,250]	ATZ discs with a modified surface (ATZ-mod)[250]
	Abraded[225]	
	Abraded acid etched [225]	
Novel zirconia alloy ((Y,Nb)-TZP/alumina- zirconia/alumina composite		
	Smooth [331]	
Alumina (polycrystalline alumina ceramic Al_2O_3 99.5% purity) ceramics		
	Untreated [78,332]	Magnesium ion implanted [332]
		Polymeric powder coating with TiO2 or silica nanoparticles [333*]
Glass ceramics[84]		
CaSiO(3) ceramic disks [334,335]		
calcium silicate ceramics, sphene ($CaTiSiO_5$) coating [335]		
Hardystonite (Ca(2)ZnSi(2)O(7)) ceramics [334]		
Hydroxyapatite		
	As received [112,229,291,325,336-339]	Plasma sprayed with HA[229]
	Calcined [338]	
	Sintered [84,113,282,338,340]	
	Sintered with microchannels[342]	
calcium titanium phosphates CaTi(4)(PO(4))(6) [106]		
calcium titanium zirconium phosphates CaZr(4)(PO(4))(6) [106]		
Silicon wafers		
	Etched [49,342]	Coated with cpTi[49,342,343]
	Untreated smooth [49,342]	Coated with cpTi[49,342]
Polysterene culture plates		
		Sputter coated with titanium, titanium oxide, Alumina oxide,zirconia oxide and calcium phosphate [344] ; Titanium [30,72]
Polycarbonate membranes		
		Titanium [345*]
Fiber reinforced composites		
	Blasted [346]	Bioactive Glass [346]
Bioactive glass 45S5, S53P4 [256,325]		
Glass slides		
		Titanium- Tobacco Mosaic Virus and TMV

	-Phos [345,347*]
	Polyelectrolyte multilayer film self assembly[348]
	Nanocrystalline deposition of Cp Ti, Ti6Al4V, TiNb30 and TiNb13Zr13) [349]
PMMA [325]	
Ultra-high-molecular-weight polyethylene (UHMWPE) [339]	
Polyethylene [134]	
Carbon/carbon composite [134]	

Table2: Cell culture models used at different implant surfaces.

Cell models	References
SaOS-2	[106,107,120,124,162,163,186,187,189,190,260,274,275,279,281,305 ,321,340,350,351]
MC3T3-E1	[37,72,74,81,82,87,92,98,105,114,115,152,177-180,185,186,226-228, 262-265,318,322,348,352-356]
MG-63	[27,47,51,52,58-60,79,93,95,99,102,108,134,136,137,140,143-145, 157,169,170, 188,189,192,195-197,201-203,206-209, 215, 216, 218, 221-224,231-233, 235, 242, 247, 255, 261, 267, 281, 285, 292, 293, 298,300,304,308,316,326,327,329,357-359]
C3H10T1/2	[74]
C2C12	[354]
Osteosarcoma cell line U2OS	[113]
ROS-17/2.8 osteosarcoma cell line	[75]
Human osteosarcoma HOS TE85 cells	[96,97,331]
HEPM (Human Embryonic Palatal Mesenchymal Cells	[36,121,155,168,171,272,338]
Primary human osteoblast cells	[31, 57-59, 73, 76, 80, 83, 86, 106, 107, 116, 118, 125, 129, 130, 135, 176,181,184,198,199,211,212,214,215,218,220,225,229,238, 239, 244,273,279,283,294,301,302,309,319,320,323,332,334,335,341,360, 361]
Human bone marrow derived osteoblast cells	[89,154,282,287,289,290,325]
Human mesenchymal stem cells(hMSCs) and human bone marrow cells	[22,41,43,48,58,85,111,125,146,150,158,160,164,166,167,183,216, 217,240,241,252,254,269,277,280,284,288,295,297,307,311,315, 339,345,362]
hFOB 1.19	[147,238,250,312,317,362]
Human fetal osteoblast cells (hFOB)	[119]
osteoblastic precursor cell line (OPC1) established from fetal bone tissue	[161,244,245]
Rat bone marrow (RBM) cells	[42,84,101,103,104,110,172-174, 236, 237, 251, 256, 259, 330, 347, 363]
Primary rat osteoblasts	[46,90,91,100,156,200,205,219,248,249,253,276,291,299,313,314, 324,349,364]
Rat bone marrow derived osteoblasts	[30,53,54,132,137,151,153,159,257,346,365]
Rat periosteum derived cells	[131,149]
Rat osteoblastic cells-CRP10/30 cells	[258]
UMR-106-01-BSP /non-mineralizing subclone of UMR-BSP; UMR-U1 cells (negative mineralization control)	[271]
Rat osteoblastic cells (ROS.MER#14), an osteosarcoma line of immortalized cells	[238]
Calvarial chick osteoblasts	[336]
Bone formative group cells	[138]

(BFGCs) derived from bone marrow of beagle's femur : consisted of hematopoietic stem cells (HSC) and osteogenetic stem cells (OSC)	
2T9 mouse osteoblast progenitor cell line	[122]
Primary mouse osteoblasts	[74,112,234]
Mouse bone marrow derived mesenchymal stem cells	[296]
Rabbit bone marrow derived mesenchymal stem cells	[210]
Primary chondrocytes from Rats- resting zone and growth plate zone	[268,344]
J774A.1	[69,71,109,278]
RAW264.7	[117,122]
Mouse bone marrow cell culture along with osteoclast differentiation media M-CSF and RANKL	[266]
Human monocytes	[166,246,254,335]
Dendritic cells differentiated from human mononuclear cells	[213]
Human aortic endothelial cells (HAEC)	[218]
HUVECs	[88,141,144]
Purified and immortalized human dermal microvascular endothelial cells (HMEC-1)	[335]
Human gingival fibroblasts (HGF)	[49,127,128,182,204,328,342,343]
Rat oral fibroblasts	[133]
NIH3T3 fibroblasts	[152,366]
Adipose derived MSCs	[280]
Platelet concentrate- human	[142]
Schwann cells derived from neonatal rats	[94]

Table 3: Genes examined in in-vitro culture models with different implant surfaces along with their functional gene ontology annotation.

Genes examined	Biological process GO annotation	References
miRNA		[79,232,233,326,327]
Hsp47 (heat shock protein 47)	response to unfolded protein; negative regulation of endopeptidase activity; regulation of proteolysis	[55]
HSP70 (heat shock 70kDa protein)	protein folding; post-Golgi vesicle-mediated transport; cellular membrane organization	[275]
cyclin-dependent kinase 2; cyclin-dependent kinase 4: cyclin-dependent kinase 6	cell division	[127,128]
cyclin A1	Cell division	[250]
cyclin A2	Cell division	[128]
cyclin D1	Cell division	[112,127,128,331]
cyclin E	Cell division	[127,128]
cyclin-dependent kinase inhibitor 1A (p21, Cip1); cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Cell cycle checkpoint	[127,128]
cyclin-dependent kinase inhibitor 2A	Cell cycle checkpoint	[250]
PCNA (proliferating cell nuclear antigen)	cell proliferation	[250]
antigen identified by monoclonal antibody Ki-67	cell cycle; meiosis; cell proliferation	[239]
c-myc	cell proliferation	[127]
Ubiquitin C	cell cycle checkpoint;DNA damage response	[85]
RP59	multicellular organismal development; cell differentiation; regulation of osteoblast differentiation	[234]
RUNX3 (runt-related transcription factor 3)	regulation of transcription, DNA-dependent; transcription from RNA polymerase II promoter; protein phosphorylation; induction of apoptosis; cell proliferation; negative regulation of cell cycle; negative regulation of epithelial cell proliferation	[250]
SOX9	cartilage condensation; regulation of	[149,296]

(SRY (sex determining region Y)-box 9)	transcription from RNA polymerase II promoter; transcription from RNA polymerase II promoter	
MSX-2 (msh homeobox 2)	Sequence-specific DNA binding transcription factor activity;osteoblast differentiation; multicellular organismal development; negative regulation of cell proliferation;	[234]
DLX5 (distal-less homeobox 5)	Skeletal system development; osteoblast differentiation; endochondral ossification; multicellular organismal development	[234,264]
RUNX2 (Runt-related transcription factor 2)	Ossification; osteoblast differentiation; transcription	[36,37,41-43, 46, 48, 55, 73,85, 90, 112, 114, 130,146,166,168,170,171 ,200,212,228,234,238, 243,248,249,260-262, 269-271,280, 283, 286, 289,290,294-296, 322, 324,333,345,347,362]
Sp7 transcription factor	Regulation of transcription; osteoblast differentiation	[36,37,41,43,55,90,112,166, 168,228,295]
ALP (alkaline phosphatase)	Skeletal system development; biomineral tissue development	[36,41,43,46,53,55,84-86, 90, 92,100,101,104-107, 112, 114,119,121,130,135,143, 144,146,152,161,168,180, 185,211,212,226,227,238, 239,241,243-245, 248-250, 260,262-265, 274, 279, 280, 284, 285, 287, 295, 301, 302, 311,312,317,333-335, 340, 345,354, 355,360,362]
BSP (integrin-binding sialoprotein)	Ossification; cell adhesion; extracellular matrix organization; biomineral tissue development	[36,37,41,43,48,73,84,86,106 ,107,112,116,125,162,164, 168,178,183,201,220,228, 234, 250, 253, 260, 262, 264, 271,274,277,286,294,295, 301,302,311,317,333,335, 345,346,348,362]
OC (Osteocalcin)	Skeletal system development; osteoblast differentiation; osteoblast development; bone mineralization; regulation of osteoclast differentiation	[22,30,36,41-43,46,52-55,57, 59, 73, 76, 80, 82, 83, 85, 86, 90-93,96-98, 100, 101, 103, 106, 107, 110, 112, 114, 115, 118,120,124,129,130,131, 134,136,139,143-147,150-153, 155-160, 164, 167, 168, 170, 171, 174, 176, 180, 185,188-190,195,196,199-202, 206, 207,209-212, 218, 220-229, 234,237,238,240,242,243, 246,248-253, 256, 257, 262-265,267,269,272,274,276, 277,280-282, 285, 286, 289, 290,292,295,297,300-302, 307,309, 311, 314,317-319,

		321,324,330,334,335,345-348, 351, 352,355,357,359-361,364,365]
OPN (Osteopontin)	Ossification; cell adhesion; positive regulation of cell-substrate adhesion; biomineral tissue development	[22,30,42,43,46,48,53-55, 72, 84-86,91,98,105-107, 112, 114,116,131,132,146,149-151,153,155,158-160, 162, 164,177,180,210,211,234, 239-241,248-251, 257, 260, 267,262,263,265,277,286, 288,289,291,294,295, 297, 309,311,314, 321, 324, 330, 334,347-349,353,355]
ON (secreted protein, acidic, cysteine-rich) (osteonectin)	Ossification; regulation of cell proliferation; cellular response to growth factor stimulus	[81,86,106,107,116,125,126, 250,288,291,294,296, 309, 321,331]
DMP1 (dentin matrix acidic phosphoprotein 1)	ossification; positive regulation of cell-substrate adhesion; extracellular matrix organization; biomineral tissue development	[234]
OMD (osteomodulin)	cell adhesion	[178]
Wnt 5a	multicellular organismal development; positive regulation of gene expression;	[48]
CTNNB (catenin (cadherin-associated protein), beta 1)	canonical Wnt receptor signaling pathway;	[283,354]
Axin2	intramembranous ossification; chondrocyte differentiation involved in endochondral bone morphogenesis; dorsal/ventral axis specification; regulation of Wnt receptor signaling pathway; bone mineralization;	[354]
Snail	osteoblast differentiation; mesoderm formation; multicellular organismal development; positive regulation of transcription, DNA-dependent; regulated by Wnt signaling pathway	[354]
Wisp2 (WNT1 inducible signaling pathway protein 2)	regulation of cell growth; cell adhesion; signal transduction; cell-cell signaling; negative regulation of cell proliferation	[354]
Connexin43	transport; apoptosis; muscle contraction; signal transduction; cell-cell signaling; gap junction assembly	[354]
BMP1 (bone morphogenetic protein 1)	skeletal system development; cartilage condensation; ossification; multicellular organismal development; cell differentiation	[55]
BMP2 (bone morphogenetic protein 2)	skeletal system development; osteoblast differentiation;positive regulation of bone mineralization	[46,69,71,109,114,164,171, 220,243,248,249,287,295, 324,362]
BMP4 (bone morphogenetic protein 4)	osteoblast differentiation; positive regulation of pathway-restricted SMAD protein phosphorylation; positive regulation of bone mineralization;	[324]
BMP6 (bone morphogenetic protein 6)	skeletal system development;positive regulation of pathway-restricted SMAD protein phosphorylation; positive regulation of bone mineralization	[220]
BMP7	skeletal system development; ossification;	[130,250]

(bone morphogenetic protein 7)	positive regulation of bone mineralization; SMAD protein signal transduction	
SMAD1 (SMAD family member 1)	osteoblast fate commitment; regulation of transcription, DNA-dependent; transforming growth factor beta receptor signaling pathway	[280]
TGFB1 (transforming growth factor, beta 1)	chondrocyte differentiation; SMAD protein complex assembly; regulation of cell proliferation; response to wounding; regulation of collagen biosynthetic process	[52,55,59,60,69,71,75,83,118 ,134-136, 140, 145, 154, 175, 195,196,201,207-209,221-224, 238, 243, 250, 268, 276, 278,281,300,314,356,357, 359]
TGFBR1(transforming growth factor, beta receptor 1); TGFBR2(transforming growth factor, beta receptor 2)	positive regulation of mesenchymal cell proliferation; positive regulation of SMAD protein import into nucleus	[127,128]
EGFR (Epidermal growth factor receptor)	cell morphogenesis; ossification; cell proliferation; positive regulation of cell proliferation; cell-cell adhesion	[127]
FGFR1 (fibroblast growth factor receptor 1)	skeletal system development; positive regulation of cell proliferation	[127]
FGF-2 (fibroblast growth factor 2 (basic))	regulation of cell proliferation; wound healing; positive regulation of blood vessel;regulation of cell cycle	[218]
EGF (epidermal growth factor)	angiogenesis; platelet degranulation; DNA replication; positive regulation of cell proliferation	[218]
IGF1 (insulin-like growth factor 1)	skeletal system development; blood coagulation; positive regulation of cell proliferation; bone mineralization involved in bone maturation; positive regulation of osteoblast differentiation	[314]
Collagen production	Skeletal system development; blood vessel development; osteoblast differentiation; intramembranous ossification; endochondral ossification	[30,46,51,55,76,82,89,98,102 ,129,133,147,151,154,157, 170,181,186,197,219,231, 248,258,268,276,292,295, 305,315,323,336,341,344, 350,357]
COL1 (collagen, type I, alpha 1)	Skeletal system development; blood vessel development; osteoblast differentiation; intramembranous ossification; endochondral ossification	[30,36,53,55,72,73,84-87, 90, 91,103,105-107, 112, 114, 116,121,125,130-133, 143, 146,149,152,153,158-160, 162-164, 167, 168, 177, 179, 182,185,187,211,212,226, 228,234,238,250,257,260, 265,274,277,279,280,285-288,296,298,302,309,311, 312,314,317,319,322,324, 328,333-335, 345, 347, 348, 353,355,360,361,365]

COL II	Skeletal system development	[133,149,250,277,296,339]
COL III (collagen, type III, alpha 1)	skeletal system development; blood vessel development; cell-matrix adhesion	[30,53,182,250,328,339]
COLIX (collagen, type IX, alpha 1)	chondrocyte differentiation; cell adhesion; organ morphogenesis	[149]
COL10a1 (collagen, type X, alpha 1)	skeletal system development	[296]
lysyl hydroxylases (LHs);LH1,LH2,LH3	protein modification process; hydroxylysine biosynthetic process; collagen fibril organization	[55,87]
Lysyl oxidases (LOX, LOXL1–4]	protein modification process; collagen fibril organization	[55]
Prolyl hydroxylases (LEPRE1, P4HA1)	collagen fibril organization	[53,55,133]
Sulfatase 1	Heparan sulfate proteoglycan metabolic process; negative regulation of fibroblast growth factor receptor signaling pathway; bone development	[178]
Decorin	organ morphogenesis; wound healing; extracellular matrix binding ;peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan;	[55,87,250]
Biglycan	Blood vessel remodeling; peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan; extracellular matrix binding	[55,87]
Fibromodulin	Transforming growth factor beta receptor complex assembly; wound healing	[87]
Lumican	collagen fibril organization; cartilage development; collagen binding	[87]
Laminin	regulation of cell adhesion; regulation of cell migration; extracellular matrix structural constituent;	[309,328,339,345]
Vitronectin	cell adhesion; cell-matrix adhesion; extracellular matrix organization	[88,119,161-163,280,309]
Vitronectin receptor	----	[88]
TN (tenascin)	cell adhesion; signal transduction; response to wounding	[162,163,187,188]
fibronectin	extracellular matrix structural constituent; angiogenesis; cell adhesion; response to wounding; cell migration; peptide cross-linking; platelet activation; substrate adhesion-dependent cell spreading; leukocyte migration; regulation of cell shape	[46,49,88,126-128, 133, 162, 163, 182, 186, 187, 248, 280, 292,293,309,321,324,328, 339,343,347]
CD44 molecule	cell-matrix adhesion; cell-cell adhesion; cytokine-mediated signaling pathway	[259,349]
ICAM-1 (intercellular adhesion molecule 1); selectin E	Leukocyte migration; cell-cell adhesion; leukocyte cell-cell adhesion	[141,182]
VCAM-1(vascular cell adhesion molecule 1)	Inflammatory response; cell adhesion; leukocyte cell-cell adhesion regulation of immune response; leukocyte tethering or	[135]

	rolling	
EPCR (Endothelial cell protein C-receptor)	Immune response; blood coagulation; antigen processing and presentation	[141]
CADHERIN E	Cell-cell adhesion	[162,292]
VE-cadherin	Cell-cell adhesion	[88,335]
N-cadherin	Cell-cell adhesion	[292,293]
MOUSE Emr(F4/80)	cell adhesion; G-protein coupled receptor protein signaling pathway; neuropeptide signaling pathway	[266]
Vinculin	blood vessel development; regulation of Wnt receptor signaling pathway; canonical Wnt receptor signaling pathway involved in positive regulation of wound healing	[244,245,251,283,293]
vWF (von Willebrand factor)	Cell adhesion; blood coagulation; response to wounding; cell-substrate adhesion	[88,141]
PECAM-1 (platelet/endothelial cell adhesion molecule)	platelet degranulation; cell adhesion; signal transduction; blood coagulation; cell recognition; platelet activation; leukocyte migration	[88]
vascular endothelial growth factor A	angiogenesis; patterning of blood vessels; vasculogenesis; response to hypoxia	[59,142,182,218,242]
iNOS	response to hypoxia;nitric oxide biosynthetic process;blood coagulation; positive regulation of vasodilation	[81]
PDGF (platelet-derived growth factor alpha polypeptide)	Angiogenesis; response to hypoxia; positive regulation of mesenchymal cell proliferation; platelet degranulation; cell-cell signaling	[142]
Ang-1 (angiopoietin 1)	regulation of endothelial cell proliferation; sprouting angiogenesis; leukocyte migration; positive chemotaxis	[218]
TM (Thromobomodulin)	Negative regulation of blood coagulation; leukocyte migration	[141]
integrin, alpha 1	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; cell chemotaxis	[128,172,173,187,264,273, 305]
integrin, alpha 2	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; organ morphogenesis	[163,172,173,187,264,273, 305]
integrin, alpha 3	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway	[113,172,173,273,345]
integrin, alpha 4	cell adhesion; heterophilic cell-cell adhesion; leukocyte cell-cell adhesion;	[113,172,173,273,345]

	integrin-mediated signaling pathway; regulation of immune response; leukocyte migration	
integrin, alpha 5	angiogenesis; cell-substrate junction assembly; cell adhesion; heterophilic cell-cell adhesion; leukocyte cell-cell adhesion; integrin-mediated signaling pathway; leukocyte migration	[113,127,128,163,172,173, 188,264,273,305,309]
integrin, alpha 6	cell-substrate junction assembly; cell adhesion; integrin-mediated signaling pathway; regulation of apoptosis;leukocyte migration	[113,172,173,187,273]
integrin, alpha V	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway	[113,188,212,273,305,340, 344]
integrin, beta 1	cell migration involved in sprouting angiogenesis; cellular defense response; cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; multicellular organismal development; cellular response to mechanical stimulus	[31,46,104,113,163,172,173, 182,187,188,203,212, 248-250, 259, 264, 273, 305, 309, 313, 324, 328, 331, 332,340]
integrin, beta 3	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; regulation of bone resorption; leukocyte migration; angiogenesis involved in wound healing	[104,172,173,188,250,305]
integrin, beta 5	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; multicellular organismal development	[305,345,250]
integrin alpha2 beta1	platelet degranulation; cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; blood coagulation; platelet activation	[292,332]
integrin alpha3 beta1	cell-matrix adhesion; integrin-mediated signaling pathway	[332]
Integrin alpha 5 beta 1	cell-matrix adhesion; integrin-mediated signaling pathway	[88,292,332]
Integrin, alpha V beta3	----	[88,163,246,332]
CSF (colony stimulating factor 1 (macrophage))	macrophage differentiation; regulation of ossification; osteoclast differentiation; positive regulation of cell migration; positive regulation of mononuclear cell proliferation; monocyte activation	[100,238,278]
OPG (tumor necrosis factor receptor superfamily, member 11b)	Skeletal system development; apoptosis; extracellular matrix organization; negative regulation of bone resorption	[41,57,59,60,73,100,143,144, 166,167,190,196,199, 202, 206,242,243,278,286,300, 319,334,335,354,358, 361]
RANK (tumor necrosis factor receptor superfamily, member 11a, NFKB activator)	cell-cell signaling; positive regulation of cell proliferation; osteoclast differentiation	[122]
RANKL (tumor necrosis factor (ligand) superfamily,	Positive regulation of osteoclast differentiation; positive regulation of bone	[73,81,100,167,196,206,242, 243,286,319,334,335, 358,

member 11)	resorption; cytokine-mediated signaling pathway	361]
TRAF6 (TNF receptor-associated factor 6)	signal transduction; regulation of apoptosis; positive regulation of osteoclast differentiation; negative regulation of transcription, DNA-dependent; positive regulation of NF-kappaB transcription factor activity	[122]
catK (Cathepsin K)	Proteolysis; bone resorption	[122,336]
calcitonin receptor	Activation of adenylate cyclase activity by G-protein signaling pathway	[267,336]
carbonic anhydrase II	Positive regulation of osteoclast differentiation; positive regulation of bone resorption; secretion	[335]
TRAP (acid phosphatase 5, tartrate resistant)	response to cytokine stimulus; bone resorption; bone morphogenesis	[122,246,266,277]
MMP1 (matrix metallopeptidase 1)	Proteolysis; blood coagulation; metabolic process; collagen catabolic process; leukocyte migration	[135]
MMP2 (matrix metallopeptidase 2)	Angiogenesis; proteolysis; metabolic process; collagen catabolic process	[55,342]
MMP9 (matrix metallopeptidase 9)	Skeletal system development; proteolysis; extracellular matrix organization; collagen catabolic process	[55, 335]
TIMP2 (TIMP metallopeptidase inhibitor 2)	Negative regulation of cell proliferation; regulation of cAMP metabolic process; regulation of MAPKKK cascade	[335]
TIMP1–4 (TIMP metallopeptidase inhibitor1-4)	----	[55]
TNF-α (tumor necrosis factor)	Inflammatory response; immune response	[73,116,134,165,213,276, 278]
Interleukin 1, alpha- pro-inflammatory cytokine	Inflammatory response; immune response; cell proliferation; wound healing	[278]
Interleukin 1, beta	Inflammatory response; immune response; signal transduction; cell-cell signaling;	[71,117,213,278]
Interleukin 6	Inflammatory response; immune response	[71,75,117,187,238,276,281, 357]
Interleukin 8	Inflammatory response; immune response	[135,213]
Interleukin 10	Inflammatory response; cell-cell signaling; regulation of gene expression; leukocyte chemotaxis	[71,165,213,278]
Interleukin 11	Inflammatory response; immune response	[73]
interleukin 12, interleukin 17A	immune response	[117]
IL-1 R alpha , IL-12p70, IL-15, IL-18 ,IL-16	immune response	[213]

MCP-1 (CCL2)	angiogenesis; response to hypoxia; chemotaxis; inflammatory response; cytokine-mediated - signaling pathway	[117,213,278]
MIP-1α (CCL3) ; MIP-1 β (CCL4) ; RANTES (CCL5); Eotaxin (CCL11);MDC (CCL22)	chemotaxis; inflammatory response; immune response	[117]; MIP-1 [213]
SDF-1 (CxCL12) (chemokine (C-X-C motif) ligand 12)	chemotaxis; immune response; cell adhesion; signal transduction	[117]
Cox-2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	regulation of inflammatory response; response to glucocorticoid stimulus	[354]
PGE2 (prostaglandin E synthase 2)	prostaglandin biosynthetic process; fatty acid biosynthetic process; cell redox homeostasis; secretion	[52,134,136,140,145,174,175 ,195,196,201,207,208, 221-224,268,300,352,359]
NPAS2(neuronal PAS domain protein 2)	Transcription, DNA-dependent; central nervous system development	[296]
BDNF (brain-derived neurotrophic factor)	Nervous system development; axon guidance	[94]
NGF (Nerve growth factor)	Peripheral nervous system development; regulation of neuron differentiation; regulation of axonogenesis	[94]
FAK		[171,214,299,332,340]
p-ERK; p-JNK ;p-FAK	-----	[81,192,309,340]
Erk; p-eErk1	------	[31,74]
Akt-1; MEK1 ;ERK2	------	[127]
c-fos	transcription, DNA-dependent	[31,299,340]
c-jun	transcription, DNA-dependent	[29,340]
RhoA; Rac1 ;cdc42 ;Rho Kinase		[127]
Shc (SHC (Src homology 2 domain containing) transforming protein 1)	angiogenesis; response to hypoxia; epidermal growth factor receptor signaling pathway; Ras protein signal transduction; positive regulation of cell proliferation; cell-cell adhesion; neuron differentiation; organ regeneration; actin cytoskeleton reorganization; intracellular signal transduction	[31,332]
PLD1a,1b,2a&2b (phospholipase D1, phosphatidylcholine-specific)	phosphatidic acid biosynthetic process; chemotaxis; cell communication; small GTPase mediated signal transduction; Ras protein signal transduction	[202]

phospholipase C-gamma2 (Plc-gamma2)	intracellular signal transduction	[298]
protein kinase C, alpha	intracellular signal transduction	[127,202]

Table 4: Technique used in in-vitro culture models for molecular assessment of osseointegration with different implant surfaces

Method of testing	References
Northern blotting	[84,236,274,279,291,301,302,309,342]
RT-PCR	[30,53,54,69,71,73,98,100,103,109,116,122 ,127-129,131-133, 137, 138, 143, 147, 149, 150, 151, 153,158-160, 164, 170, 171, 178, 198,202-204, 214, 234, 238, 253, 257, 260, 261,266,277-279,285-290, 294-296, 298, 301,302,312-314, 324, 328, 330, 331, 333, 364-366]
Quantitative real time RT-PCR	[27,36,37,41-43, 46, 48, 71, 72, 85, 87, 90, 91, 94, 101, 104, 105, 112, 114, 121, 122, 130,141,144,146,152,168,177,180,182,183, 185,187,188,200, 206,210,212,215-217, 220,226-228, 235, 238, 240, 241,243,248-250,254,259,262-265, 267, 271, 272, 280, 284,293,311,317,321,322,333-335, 340, 345-348,353-355,358,360,362]
In situ hybridization	[86,106,107]
Immunoassay-ELISA (OC, PGE2, TGF-B1, IL-1 alpha, IL-1B, MCP-1, IL-10, IL-12, IL-17A, SDF-1 (CxCL12), RANTES (CCL5), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1 β (CCL4), MDC (CCL22) and Eotaxin (CCL11), FGF2, EGF, M-CSF, BDNF, NGF, IL-6, OPG, VEGFA, DKK1, DKK2, BMP2-BMP4, BMP6, OPN, RANKL, COLI, IGF-1, PDGF, TNF-alpha, MMP1, VCAM1)	[27,52,57-60, 73, 75, 76, 80, 82, 83, 93, 94, 96, 101,103,109,110,115,117,118,129,134-137, 139, 140,142,144,145,147,154-157, 165, 167, 175, 176, 179, 181, 187, 189, 190, 195,196,199,202, 205-209,216-218,220-227, 231, 234, 237, 242, 243, 252, 254, 256, 268, 276, 278, 281, 292, 297,300,307, 314, 315, 318, 319, 321, 348, 351, 352,254,357,359, 361]
Immunochemistry (Vinculin, ALP, PECAM-1, Vitronectin, Fibronectin receptor, VN receptor, VE-cadherin, von Willebrand factor, Fibronectin, α5β1 and α (v) β3), OC, BSP, OPN, TN, Runx2, collagen)	[86,88,92,106,107,119,124,161,162,163, 181, 200,211,244-246,273,283,309]
Radioimmunoassay	[27,52,97,123,134,136,140,145,147,169, 175, 195,196,201,202,205,207,208-210,215-218,221-224, 229-242, 268, 282, 300, 338, 359]
Gelatin Zymography and reverse zymography/ MMP-TIMP2	MMP2 [342]
Immunoblotting	[31,74,81,98,127,192,235,275,292,293,305, 309, 332,340,356]
Immunofluorescence	[22,125,203,239,251,269,277,293,332,341]
Collagen synthesis: 3H-proline incorporation into collagen fibers; Sirius red based stain assay	[30,51,53,102,133,151,170,197,219,295, 305, 336,365]
Immunogold labeling & SEM	[126]
Cytochemical stain TRAP	[246,266]
Flow cytometry	[113,163,172-174, 187, 204, 261, 292, 332, 339, 349]
[35S] Methionine-labeled fibronectin	[49,343]

[35S] Sulfate incorporation	[51,197,268]
miRNA microarrays	
miRNA microarrays containing 329 probes designed from the human miRNA sequence	[79,232,233,326,327]
Wider genome profiling	
Human 19.2 K DNA microarray	[304,308,316,329]
Human 20K DNA microarray (MWG Biotech AG, Ebersberg, Germany)	[47]
cDNA microarray-18,401 genes	[120]
GeneChip Mouse Genome 430 2.0 Array (34,000 genes)-affymetrix	[178]
Whole genome-Illumina Sentrix® Human-6 v2 expression BeadChips-47,000 probe sets	[48,220]
Genefilters GF211: 12,626 genes	[320]
cDNA microarray GCK 5.0 K human chip (Genocheck, Korea) - 5049 genes	[247]
Rat gene microarray: Research Genetics GF300 nylon microarray (Invitrogen Corporation, Carlsbad, CA). These arrays have 1633 named rat gene sequences.relative level of specific groups of genes related to bone and cartilage development, cell adhesion and extracellular matrix proteins, transcription factors, bone morphogenetic proteins, phospholipases, and protein kinases	[363]
Atlas™ Human Cancer 1.2 gene array containing 1176 genes	[184,198]
Human cDNA microarray (1152 elements)	[95,99,108,255]
A microarray containing a total of 687 cDNA sequences	[325]
cDNA GEArrayTM kit for Human Osteogenesis, HS-026-4 www.superarray.com)	[110]
An array of osteogenesis-related genes (human osteogenesis RT^2 Profiler PCR array, PAHS-0026A – SuperArray Bioscience, Frederick, MD)) 76 related genes	[41,55]
RT profiler PCR array for ECM and adhesion molecules	[345]
Murine PCR Array (PAMM-011, SABiosciences, Frederick, MD, USA). This array profiles 84 key genes involved in mediating immune cascade reactions during inflammation and includes chemokines, cytokines, interleukins and their receptors..	[116]
GE-Superarray system,>80 genes	[166]

Table 5: Genome wide expression profiling in in-vitro culture models for molecular assessment of osseointegration.

Model	Surface examined	Technique used	Days of analysis	Reference
Primary human osteoblasts - 3rd molar extraction sites	SLA, mod SLA vs. Glass cover slips	Whole genome-Illumina Sentrix® Human-6 v2 expression BeadChips-47,000 probe sets	72 hrs	[220]
hMSCs	SLA, SLActive, SMO (polished)	Whole genome-Illumina Sentrix® Human-6 v2 expression BeadChips-47,000 probe sets	3, 24, 72 or 120 h	[48]
MC3T3-E1 pre-osteoblastic cells	FN-immobilized titanium (Fibronectin coating using the tresyl chloride activation technique) vs untreated titanium	GeneChip Mouse Genome 430 2.0 Array (34,000 genes)-affymetrix	14 days	[178]
MG-63	Anatase coating of titanium	Human 20K DNA microarray (MWG Biotech AG, Ebersberg, Germany)	24 hrs	[47]
MG-63	Ti6Al4V disks machined (SR=0.77+_0.28) vs. Biolok, Blasted with tricalcium phosphate and light nitric acid tt (SR=1.93+_0.37) Biolok, Nanopore SR=1.05+_0.34 vs. TPSS surface 2.74, or only medium and cells	Human 19.2 K DNA microarray	24 hrs	[308]
MG-63	zirconium oxide discs (Cercon, Degussa Dental, Hanau, Germany)	Human 19.2 K DNA microarray	24,48 hrs	[329]
MG-63	Disks of machined titanium , vs. nanoPORE (Out-Link, Sweden and Martina, Due Carrare, Padova, Italy).The surface roughness (Sa) was 0.77±0.28 for the machined disks and 1.05±0.34 for the nanoPORE disks	Human 19.2 K DNA microarray	24,48 hrs	[316]
MG-63	Machined grade 3 titanium vs. titanium pull spray superficial [TPSS]:produced through micromechanical removal of parts of the superficial oxide layer with the use of aluminum oxide 0.5 mm/micropoints.The Ra value was 0.30 for the machined surface and 2.74 for the TPSS surface.	Human 19.2 K DNA microarray	24,48 hrs	[304]

MG-63	Machined vs anodized Ti surface:The roughness of machined Ti surface was 0.54 °+_ 0.16 μm and the roughness of anodized one was 0.88 °+_ 0.13 μm	cDNA microarray GCK 5.0 K human chip (Genocheck, Korea) - 5049 genes	24 hrs	[247]
Saos-2	1)Tapered Internal (BioHorizons Implant Systems, Inc., Birmingham, AL) and Resorbable Blast Texturing with Laser-Lok collar; (Ra, not available).2)Nanotite (3i Implant Innovations, Inc., Palm Beach Gardens, FL), osseotite surface combined with a discrete crystalline deposition of nanometer scale calcium phosphate; (Ra, 0.28 ± 0.06 μm). 3) Full Osseotite (FOSS; 3i Implant Innovations, Inc.), osseotite surface: dual acid-etched with HF and then HCl/H_2SO_4; (Ra, 0.86 ± 0.14 μm).4)Straumann SLActive Standard Implant (Institut Straumann AG, Basel, Switzerland), sandblasted with large grits (0.25–0.50 mm), acid-etched with HCl/H_2SO_4, rinsed under N_2 protection, and preserved in isotonic NaCl solution; (Ra, 2.93 ± 0.46 μm).5)SwissPlus (Zimmer Dental, Inc., Carlsbad, CA), grit-blasted with hydroxylapatite (Microtextured Titanium, MTX). As referring control, cells grown in absence of dental implant were used (1–2 μm, as provided by the manufacturer). TCP was used as control	DNA microarray-18,401 genes	72 hrs	[120]
primary human osteoblasts (hOB)	Ti6Al4V polished (control) vs. passivated samples (P: nitric acid treatment-30%-1 hr) vs. Aged treatment (A:Passivated treatment followed by aging in boiling deionized water for 10h)	Genefilters GF211: 12,626 genes	4,24,48 and 120 hrs	[320]

Figure 1:

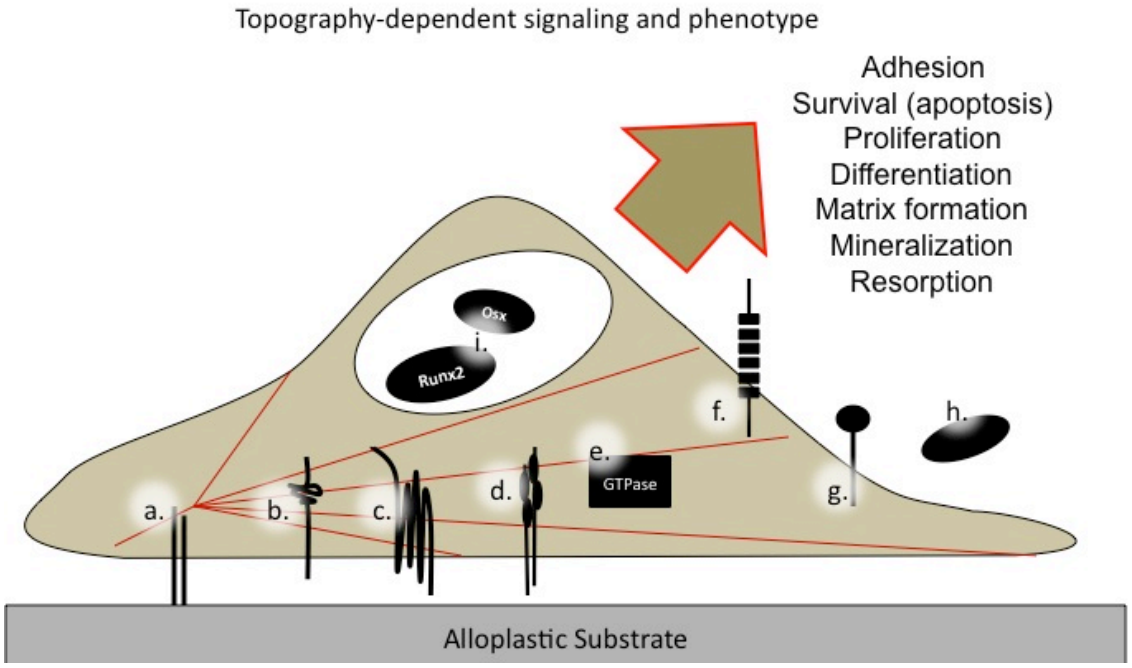

Figure 1: **Topography-dependent signaling and phenotype**. Alloplastic substrates vary widely in bulk composition and topography (Table 1). Multiple signaling pathways directly or indirectly mediate topography dependent changes in the adherent cell phenotype. Numerous primary, immortalized, and tumor-derived cell models have been involved (Table 2). These pathways represent those involved in cell-adhesion and bone development and repair. Included are *(a)* integrins [46,248,324] that interact directly with the surface, *(b)* serine/threonine kinase receptors (eg, TGFβ and BMP signaling [69,196,224], *(c)* G-protein coupled receptors (eg, PTH, Wnt), *(d)*tyrosine kinase receptors (eg, PDGF, IFG-1 [142,314]) *(e)* GTPases (eg, Rho1, RAS), *(f)* b-cadherin, *(g)* RANKL [100], (OPG) [243], *(i)* osteoblast differentiation transcription factors (Runx2, Osx). Cellular responses examined focus on osteoblast-specific phenotype but include a wider range of physiologic responses (Table 3)

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Chapter 2

Molecular Assessment of Osseointegration *In Vivo*; A review of current literature

Abstract:

This paper reports on the results of a structured review of the literature concerning in vivo molecular assessment of osseointegrated endosseous dental implants. A search of electronic databases was performed up to and including August 2011. Thirty articles met the inclusion criteria. A descriptive evaluation and analysis of the gene expression date concerning the process of osseointegration were performed. Broad consensus was observed among the study results, perhaps as a result of the similar targeted gene expression events. More recent investigations using gene arrays or gene profiling techniques offer new insights into the fundamental molecular events that support the osseointegration process. Evidence for the influence of surface topography on osteogenesis and osteoinduction has been reported. Additional investigations are required to further solidify the functional associations between individual or orchestrated gene expression events and the clinical result of osseointegration.

Introduction:

Current tooth replacement strategies typically consider the alloplastic-integrated replacement of missing teeth using endosseous dental implants as a primary choice among available modes of therapy. Among the many reasons for selection of this mode of therapy include the often-cited reproducibility of the biologic integration of the endosseous implant. Such reproducibility in the clinical management of a biological response is accepted without question. Yet, important questions remain. Do current implant technologies address all current needs? Can clinicians explain experienced failures? Answers to such questions require careful understanding of the biologic process of osseointegration.

Osseointegration is defined as a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant [1]. Essential elements of this definition include the implicit understanding that vital bone remains in apposition to the endosseous implant surface throughout its functional (loaded) lifetime. Implant success is often discussed in qualitative and quantitative terms of the amount of bone formed at the endosseous implant interface. The earliest identifiable influences on this feature of osseointegration were the qualitative and quantitative clinical perceptions of local bone prior to implant placement. [2] Early failures were attributed to clinical error leading to lack of primary stability or infection. Delayed failures were attributed to reduced bone volume (low bone to implant contact) attributable to diminished bone volume and quality. The role of systemic diseases or conditions on osseointegration further suggested that the healing potential of the local bone tissues affected bone formation at the endosseous implant.

The past decade has revealed an increasing interest in the improvement of osseointegration through modification of the endosseous dental implant surface. Implant surface topography, as recently reviewed by Wennerberg and Albrektsson [3] can be modified to alter the interfacial bone response. With the caveat that our definition of a 'rough surface' is limited, moderately rough (S(a)> 1 -2 micron) surfaces show stronger bone responses (increased BIC, increased torque removal) than other surfaces. Another recent review of experimental surface alterations ranging from the application of structural peptides and proteins (e.g., collagen) to diverse growth factors and morphogens (e.g., BMPs) revealed more diverse positive and negative outcomes for these different approaches. New techniques to apply ultra thin CaP coatings (vapor deposition, sputter coating, ESD, biomimetic deposition) were observed to improve bone integration as compared to non-coated

titanium implants [4]. These recent reviews display a wide range of current activities that seek to improve the osseointegration result in attempts to address the increasing challenges clinicians address by placing implants in individuals with reduced bone volume, reduced bone density, and impaired wound healing (e.g., diabetes, osteoporosis, radiation therapy, chemotherapy).

Evaluation of the result of osseointegration is possible by simple clinical methods. Tactile approaches that include tapping and reverse torque estimations serve the majority of clinicians well in discerning successful versus failed osseointegration. While more controversial in its physical interpretation, quantifying stability using resonance frequency (ISQ) can reveal changes in implant integration over time that reflect the gain or loss of interfacial bone supporting the osseointegration result [5]. When the body of literature concerning bone-to-implant contact was reviewed at the histological level, a clear advantage for increased surface topography was revealed [6]. Human clinical investigations comparing different implant surfaces also suggest that modification of surface topography leads to alteration in the local bone responses leading to increased bone-to-implant contact [7-10]. There is sufficient data at multiple levels ranging from animal studies to direct human histology indicating that the process of osseointegration can be modulated to increase bone to implant contact. Precisely how this is achieved has not been clearly delineated. Without a complete understanding of the molecular and cellular process of osseointegration, therapeutically relevant targets for improving the result may be poorly defined.

The molecular basis of biology and of disease has been established as a principle determinant in managing human health. Only recently has the molecular basis of osseointegration been considered (Figure 1). The nature of tissue biology at, on or adjacent to the implant surface has not been carefully elucidated. However, recent activities have begun to highlight both the fundamental processes that contribute to interfacial bone formation and how different implant-surface parameters may influence these processes that eventually lead to bone formation at the alloplastic / tissue interface. Several laboratories demonstrated that it is possible to interrogate these molecular processes by retrieval of tissues and implanted endosseous devices from animal models (Figure 2). Early reports focused on demonstrating that gene expression events known to be evident in the process of bone formation were recapitulated at the forming bone to implant interface [11] and others demonstrated that the osteoinductive genes that promote stem cell differentiation to osteoprogenitor cells (namely RUNX-2 and Osterix) were expressed by implant-adherent cells in vivo and were elevated on surfaces

with topographies that enhanced bone to implant contact [12]. Most recently, wider evaluations of gene expression in tissues surrounding dental implants have indicated that other significant biologic processes may be involved in healing of the endosseous implant [13,14]. The aim of this review is to identify the model systems, molecular platforms and targeted gene expression events involved in existing phenotype at the level of protein-encoding mRNA expression. Anticipated (and repeatedly demonstrated in the literature) is the surface-dependent modulation of mRNA expression within the implant-surface adherent cells.

Materials and methods:

For the literature to be included in this review, the following eligibility criteria were used: in vivo reports assessing the molecular process of osseointegration, and articles published only in English. Studies related to wear particles , letters to the editor and reviews were excluded.

Search Strategy: A thorough search was performed up to and including August, 2011 through the following databases: PubMed (1948 to August, 2011), using the following terms: (titanium[tw] OR zirconium[tw] OR dental implant*[tw]) AND (gene expression[MeSH Terms] OR gene expression[tw] OR differentiation[tw] OR rna[MeSH Terms] OR rna[tw] OR messenger rna[tw] OR mrna[tw]), EMBASE via OVID (1947 to August,2011) using: (Titanium.mp. or zirconium.mp. or tooth implantation/ or dental implant*.mp. and gene expression.mp. or exp gene expression/ or exp DIFFERENTIATION/ or differentiation.mp. or ena.mp. or exp RNA/ or messenger rna.mp.[mp=title,abstract,subject headings,heading words, drug trade name, original title,device manufacturer, drug manufacturer] or mrna.mp.[mp=title,abstract,subject headings,heading words, drug trade name, original title,device manufacturer, drug manufacturer], and BIOSIS Previews via ISI Web of Science (1969 to August,2011), ISI Citation via ISI Web of Science (1955 to date) using the following words: Topic=((titanium OR zirconium OR "dental implant*") AND ("gene expression" OR differentiation OR rna OR rna OR "messenger rna" OR mrna)). Titles and abstracts were screened for possible inclusion in the review. The full text of the articles judged to be relevant by the title and abstract was read and independently evaluated against the eligibility criteria. In addition, a hand search of the reference lists of original studies that were found to be relevant was conducted.

Results:

Thirty articles met the inclusion criteria. Characteristics of the included in-vivo reports including model systems used, techniques used for molecular assessment of the osseointegration process, genes examined and wide gene expression profiling studies are presented in Table 1,2,3 and 4 respectively.

Different in vivo models are represented and include mouse, rat, mini-pig and rabbit animal models. Human studies have recently provided additional molecular data concerning the molecular processes surrounding osseointegration. These various studies all share similar major observations. The molecular processes of bone formation suggested by previous histological assessments and in vitro examinations of implant surface effects on osteoblast function are supported by these targeted assessments of osseointegration. Broad consensus is observed among these study results. One possible explanation for this consensus is that the early questions asked regarding the molecular process underscoring osseointegration have been directed about known aspects of bone formation and repair in general.

The spectrum of molecular events targeted in these studies in narrow. Osteoinduction represented by the expression or abundance of Runx-2 and Osterix, key transcriptional regulators of stem cell commitment to osteoblastogenesis, has been explored in many of these investigations. Osteogenesis reflected by the expression of collagen type I and bone-specific or enriched protein encoding mRNAs has also been observed. The increased expression of mRNAs including Collagen type I, osteopontin, osteonectin, bone sialoprotein, and decorin has been observed as a function of time as well as 'enhanced' surface topography in all of the reported studies. One general conclusion is that, irrespective of possible modulation of mRNA abundance temporally or quantitatively, osseointegration involves osteoinduction from progenitor cells and the subsequent elaboration of a bone matrix comprised of the basic components involved in bone repair.

Genome wide assessment of bone formation and repair conducted in vivo reveal a standard progression of cellular events indicated by gene expression [15]. Most investigations have focused on the impact of implant surface on the molecular process of osseointegration. Seventeen studies have specifically explored bone-specific gene expression as a function of implant surface and time. The general observation is that surface topography influences the pattern of gene expression for bone-related proteins (OPN, ON, BSP, COLI, ALP).

Perhaps more significantly, the impact of surface topography (and related chemistry) on osteoinduction has also been specifically addressed by measuring expression of Osx and Runx2.

Gene profiling studies ask a much broader question and can explore a wide range of possible biological processes including osteoinduction and osteogenesis. Only several gene profiling studies involving osseointegration have yet to be reported. For example, results from a human model reported on biological events surrounding osteogenesis, related angiogenesis and some interest on osteoclast recruitment and activity [13]. In a most recent report using targeted gene arrays, Bryington et al [14] explored gene expression events surrounding inflammation as related to early wound healing. Most interestingly, these reporting of these studies have focused on osteogenesis and related angiogenesis. The full value of >20,000 genes representing multiple processes remain underexplored.

Discussion:

Model systems: The selection of models for in vivo investigations involves understanding the value of desired endpoints and the applicability and translation to the human clinical situation. Much of the early research involving osseointegration was performed in extraoral sites that proved valuable in assessment of the extent of bone formation along the implant surface [16]. Mechanical testing of the implant / bone interface required the use of surrogate tests of function that include push out or reverse torque testing that do not fully replicate the biomechanics of dental implant function [17]. Typically, these models involved implants that approximated the dimension and form of clinical implants, but often were designed to best meet the demands of the animal model. (e.g., [18]). Despite these limitations, use of in vivo models to study osseointegration in extraoral sites has contributed to our knowledge of surface topography and mechanical loading on the histological result of osseointegration [3,6].

Studies on dental implant osseointegration have focused broadly on the effects of implant design, local bone physiology effects, masticatory (dys)function effects, or oral environmental effects (biofilm-mediated processes of peri-mucositis and peri-implantitis) largely through the histological evaluations of osseointegration. The majority of studies published represent single time-point studies that infer one or several biological processes were responsible for the results observed through histology (and radiography). A molecular and cellular interrogation of the biologic processes that contribute to osseointegration of endosseous implants requires

careful model selection. In addition to anatomic, dimensional and biomechanical concerns, an *in vivo* examination of the molecular and / or cellular processes contributing to osseointegration requires selection of model systems for which sufficient molecular and cellular tools of assessment are readily available. Unfortunately, the commonly utilized models for histology (rabbits, dogs, and to a lesser extent mini-pigs and sheep) provide fewer advantages for molecular assessment than do small animal models including rats and mice. The main advantages of using rats and mice for studies of biologic processes of physiology and pathology include 1) known genomes [19,20], 2) ready access to tools for molecular assessment, 3) relative low cost, 4) a wealth of knowledge regarding the molecular basis of biologic processes (including osteoinduction, osteogenesis, and osteoclastogenesis) in these specific models, and 5) the ability to specifically alter the genomes of mice and rats to create models that test individual genes encoding proteins that may directly affect osseointegration. Recent review of this field suggests that mice have become a preferred model system for bone research because of their genetic and pathophysiological similarities to humans, relatively low costs and the availability of the genome sequence information [21]. The value of genetic mouse models of bone physiology was recently underscored in a review of osteoclastogenesis where the authors stated that this approach has identified novel molecules and highlighted their involvement in vivo, confirmed human mutations and expanded the knowledge of mechanisms [22].

The rat model for study of bone physiology and repair is advantageous because of its relatively large size that facilitates surgical intervention. Several mutations or conditions of the rat have permitted forward genetic studies. For example, diabetic or osteopetroic conditions have been evaluated in terms of osseointegration using mutant rat strains [22,23]. Further advances are now promised with using reverse genetic approaches that can systematically and controllably alter the genome to affect phenotypic changes. Only recent technical advances in manipulating the rat genome have made this a realistic possibility [24]. Anticipated is the use of this technology to demonstrate the role of specific genes in the process of osseointegration.

Methods to assess gene expression: A major advantage of studying the process of osseointegration at the molecular level is the potential to identify key steps in the process that may be clinically influenced. These 'targets' are identifiable, but are dependent on the methods used to assess gene expression. Gene expression data is readily available at the level of mRNA that encodes proteins, which define the phenotype of interest (here interfacial bone formation). At the level of mRNA, assessments can be made using exploratory methods

(identification of novel or relatively high or low expressed molecules), qualitative methods (present or not), semi-quantitative methods, and quantitative methods. Most techniques all depend upon nucleotide complimentarily and RNA hybridization methods that require knowledge of all mRNA sequences now available through the sequencing of the rat genome[25].

Building on histological methods, sections of tissues representing the implant/bone interface can be treated with antibodies for specific proteins and these proteins can be identified using immunohistochemistry. This technique, widely used in pathology, is not readily applied to ground sections embedded in acrylic resins that are frequently used for endosseous implants. When applied to fractured sections with the implant removed, the risk of interface damage exists. If successful, the result remains semi-quantitative. Another means of phenotypic characterization involves identification of specific mRNAs within tissues on histologic sections. In situ hybridization is the process of RNA hybridization of known RNA sequences within tissues (i.e. express by a particular cell). The methodology is robust, yet technically challenging. When accomplished it too offers a semi-quantitative assessment of the expression of an individual RNA encoding a protein within cells and tissue.

Ogawa and colleagues [26] used a exploratory method termed Differential display to identify differences among mRNAs expressed on two different implant surfaces in vivo. This approach identified at least three mRNAs (TO1, TO2, TO3) with elevated expression in tissues adjacent to implant surfaces following placement in the rat tibia. This and other subtractive gene cloning methods reveal differences by dismissing what appear to be the more common and similar mRNAs and is a powerful exploratory tool in biology. The ultimate role or impact of these identified genes on the process of osseointegration remains to be fully elucidated.

mRNA quantification has been enabled by a process termed quantitative-Reverse Transcription Polymerase chain reaction (qRT-PCR). This method has been largely automated and today it is possible to perform this process on sets of targeted mRNAs or on all mRNAs represented in a tissue (gene profiling) simultaneously. These approaches offer opportunities to explore many aspects of physiology through interpretation of large datasets using complex computer based algorithms. One such data base is ‘the Gene Ontology”, a project that collaboratively assists in structuring vocabularies that describe gene products in terms of a) biologic processes, c) cellular components and c) molecular functions. Genes observed by a process of gene profiling (looking a the relative abundance of all genes expressed) can be organized according to specific functions or

processes[27]. With respect to osseointegration, some of these processes might include ”differentiation” or “ossification” or “skeletal development” and they are identified by the GO program based upon the expression of mRNA molecules. Where one or another single mRNA expression event might not provide insight to an important process, the organized expression of multiple mRNAs can often point toward a specific cellular components, molecular function or biologic process. These powerful tools can be applied to defining in more complete terms the process of osseointegration. This elucidation can provide new clues to the questions all clinicians have regarding the underlying causes of implant failure.

Genes examined: Distinct yet overlapping phases of healing are associated with osseointegration including clot formation, inflammation, bone repair and remodeling. The sequential changes in cell populations are orchestrated in a manner to weigh the balance toward bone formation. This involves the migration of mesenchymal stem cells, proliferation and promotion of osteoblastic differentiation.

Cell proliferation around implants in-vivo was modestly investigated (3,11). Proliferating cell nuclear antigen (PCNA) ; a protein that acts as a processivity factor for DNA polymerase delta in eukaryotic cells [28]was used to indicate cell proliferation(3). This protein is also involved during DNA repair (Bravo R, Celis JE, 1980). Period Homolog 2 (Per2); involved in the circadian rhythm also plays a role in the regulation of the cell proliferation [29]Expression of *Per* genes in osteoblasts negatively regulates osteoblast proliferation. In the context of osseointegration, per2 expression period homolog 2 (Per2), were upregulated around implants vs. osteotomy sites at 2 weeks and diminished by vitamin D deficiency [30]. This may be expected as proliferation and differentiation of osteoblastic cells are inversely related.

Osseointegration at endosseous implants requires activation of processes relevant to osteoinduction, osteoconduction and osteogenesis [31]. Osteoinduction is the process by which primitive, undifferentiated cells are stimulated to develop into osteoprogonitor cells [32]. Key effectors of osteoinduction include Runx2 and Osx. Both are transcription factors necessary for osteoblast differentiation by controlling osteoblast-specific gene expression at target genes such as ALP, COLI, OPN and OC [33-38]. Runx2 or Osx gene ablation in mice results in complete absence of bone development [39,40]. Modulation of Runx2 and Osx expression by the implant surface topography has been observed by several investigations. In-vivo reports modestly investigated the role of Osx (1 study). Runx2 and Osx expression are positively influenced by increased surface roughness

[12,41-43]. The elevated expression of Runx2 or Osx are paralleled by upregulation of bone-specific proteins (ALP, OC ,BSP expression [12,42,43] . PPAR-y ; a key transcription factor for adipocyte differentiation, is also implicated in the proliferation and differentiation of several cell types including osteogenic cells [44] and macrophages [45]. The PPAR-y directs MSC differentiation toward the adipocyte lineage with a negative dominant regulatory effect on osteoblast differentiation [46]. Omar et al [43], investigated the expression of PPAR-γ as a function of time and surface treatment. PPAR y was significantly higher at the machined surface (0.3um) compared to oxidized (Ra=1.2um) surface 1 day after implantation. Nevertheless, at day 3, the expression of PPAR-γ was higher at the oxidized surface. The authors proposed further studies are needed to establish the role of this transcription factor in implant adherent cells.

Osteogenesis was monitored by measurement of bone-specific proteins (ALP, BSP, OPN, ON, OC and COL1). Greater expression of bone-specific proteins is suggestive of greater osteogenesis and more bone formation. Elevations in expression of specific-bone proteins were reflected by higher torque removal values [41,47] at rougher implant surfaces at the micron and nanoscale levels. Collagen type I is the most abundant protein in bone [48] and is secreted at the early stages of osteoblast differentiation. The mechanical strength of bone reflects the inherent properties of its constituents. Collagen biosynthesis at the implant surfaces has been investigated in-vivo widely by means of type I COL expression. On the other hand, stability of collagen is largely dependent on several posttranslational modifications catalyzed by prolyl 4-hydroxylase, prolyl 3-hydroxylase and the family of Lysyl hydroxylases. Additional modifications by Lysyl oxidase (LOX) allow intermolecular and interfibrillar crosslinks between collagen fibrils [49,50]. An in-vitro investigation [51] demonstrated a positive influence of surface roughness on expression of the aforementioned collagen modifying genes. Current in-vivo reports have not examined the potential role of collagen modifying genes in the process of osseointegration. Other ECM components investigated in the context of osseointegration include vinculin [52], fibronectin [53] and various proteoglycans [54]; decorin and biglycan. Proteoglycans role in establishing bone-titanium interfacial adhesion has been established in-vitro by demonstrating that administration of GAG degradation enzyme reduced the bond strength of titanium and cultured rat bone marrow-derived osteoblastic cells [55].

The process of osteogenesis is affected by several growth and differentiation factors. BMPs (Bone morphogenic proteins), members of transforming growth factor family, are one group of stimuli known to induce MSCs or osteoblast progenitors to undergo osteogenic differentiation. The role of BMP signaling pathway in in-vivo osseointegration was mostly investigated through monitoring expression levels of BMP2 (7 studies). However, BMP signal transduction is regulated at different levels in the cell. It can be influenced by extracellular antagonists such as noggin, chordin, follistatin, etc which bind to BMPs and prevent their interaction with receptors [56]. The only extracellular BMP antagonist that was examined in vivo was Noggin. The reported study was examining the effects of local application rh-TGF-b2 in an implant model on modulating gene expression [57] . Elucidation of the role of the various regulatory mechanisms in BMP signaling in osseointegration requires further elucidation. Reports on factors that may play a role in osseointegration also included studies examining TGF-beta and its receptors [43,54,57] . TGFB acts as an important autocrine and paracrine factor in the regulation of bone formation and resorption. It has been implicated to stimulate the replication of precursor cells of the osteoblastic lineage, in addition to its stimulatory effect on bone collagen synthesis [58]. Yet, it also plays a role in stimulating bone resorption by differentiated osteoclasts [59]. TGF-b exerts its function by interacting with a heterotetrameric complex of type I and type II TGF-b receptors. The expression of dominant-negative TGFb receptors in osteoblast cells in vivo causes an age-dependent increase in trabecular bone mass, due to decreased bone resorption by osteoclasts [60].

Angiogenesis is of pivotal importance during the initial healing process of osseointegration. Factors that play a role in angiogenesis include but not limited to VEGF, bFGF, Ang-1 [61], PDGF-B, Insulin-like growth factor and HIF (Hypoxia-inducible transcription factor). VEGF, a molecule known to induce neovascularization, has also been shown to stimulate bone healing and skeletal growth [62,63]. VEGFA acts through its receptor Flk-1/KDR. VEGF leads to an upregulation of BMP2 in endothelial cells that can act reciprocally on the osteoblast lineage inducing osteogenesis [64]. Few studies investigated angiogenesis in the context of osseointegration.

Modulation of the inflammatory process in the early phases of healing following implant placement provides a key element in implant fixation. Inflammatory related processes in implant adherent cells may pioneer biological mechanisms for driving differentiation of cells, including osteoprogenitors, osteoclast precursors, etc that affect osseointegration. Furthermore, differential expression of chemokines and their

receptors affects recruitment of several cells including inflammatory cells, osteogenic cells and their progenitors [65,66]. Inflammatory response to different implant surfaces was investigated by the quantification of the relevant gene expression of TNF-alpa (7 studies), IL-1 beta (3 studies), IL-6 (2 studies), IL-8R (1 study), IL-10 (1 study), IL-11 (1 study), CCL2 (2studies) and CXCR4 (1 study).

Bone accrual at the implant surface also involves resorption and remodeling. Osteoclast differentiation is a tightly regulated process dependent on an osteoblast-derived member of the tumor necrosis factor (TNF) superfamily; RANKL (receptor activator of the NFkB ligand), which binds to its receptor RANK on monocytes [67]. Osteoprotegrin (OPG) plays an essential role in the control of bone resorption by acting as soluble decoy receptor for RANKL. Thus, OPG functions to decrease osteoclast formation and activity [68,69] .Efficient degradation of bone is dependent on the production and activation of various matrix-degrading enzymes; cathepsin K [70,71] , several MMPs [72,73] and TRAP [74]. Activation of the matrix-degrading enzymes is dependent on the acidification of the lacunar space [75] achieved by the action of an essential enzyme carbonic anhydraseII, in addition to a vacuolar ATPase electrogenic proton pump [76,77]. Another phenotypic marker for osteoclast is Calcitonin receptor, which expression correlates well with bone resorption [78,79] . Quantification of osteoclast-related genes was investigated briefly in vivo. Implant surface features modulated expression of bone resorption transcripts [47,80,81].

In an extraoral model of bone healing against endosseous implant surfaces; Donos et al (2011) [82] extended previous investigations of guided bone regeneration that indicated more broad gene regulation involving increased skeletogenesis and resuced inflammatory gene regulation over a 7 – 14 day period. In this study, they found that SLA surfaces displayed relevant gen ontology changes for many cellular processes, particularly at 14 days. Included were skeletal system development and signaling changes associated with the Wnt pathway, and Ras and Rho pathways. The authors highlighted 41 genes upregulated in the mesenchymal stem cell differentiation, angiogenesis ontologies as well. They concluded that regeneration associated ith microrough and polished implant surfaces is associated with unique gene expression profiles. Another gene profiling study focused on early healing (days 4, 7 and 14) of SLActive implants inserted in the retromolar pad area of human volunteers [83]. Large numbers of differentially regulated genes (increasing and decreasing) were observed at each time point. At 4 days, several central inflammation-related biological processes were represented (NFkB cascade, lymphocyte proliferation, macrophage activation). Several osteoinductive genes

including SATB2 and Sp7 (Osterix) were elevated between 4 and 14 days. This investigations highlights important molecular differences between early healing events that include inflammation and Wnt signaling and late healing events that included VEGF signaling, skeletal system development, and continued Wnt signaling. Further investigations have included use of gene profiling to compare the molecular events that occur at SLA vs. SLActive implant surfaces. Clear differences were observed in gene ontologies. For example, at day 7, the MAPK singling pathway was significantly elevated ($P< 0.0004$) in tissues associated with the SLActive implant. At day 14, for example, BMP signaling pathway was also increased ($P< 0.03$). In general, these early gene profiling studies of the osseointegration process have confirmed that surface topography or hydrophobicity influences the behavior of tissues in ways that include fundamental processes of inflammation and wound healing, as well as many aspects of osteogenesis and biomineralization.

Conclusions:

In vivo molecular assessment of osseointegration can be achieved at multiple levels involving protein and mRNA. The quantitative assessment of bone-related mRNA expression is commonly reported with data supporting the concept that implant surface features that are associated with increased bone to implant contact are able to positively influence the process of bone formation. Several reports demonstrate that surface features also promote osteoinduction by the elevated expression of key transcriptional regulators of this process. Recent studies using gene profiling successfully characterized gene expression events in the tissues surrounding implants and within implant adherent cells. Processes preceding osteoinduction and events that follow the elaboration of a mineralized matrix have been highlighted by gene profiling studies. These molecular details may represent additional targets for therapeutic improvement. Further investigations using in vivo molecular assessment, combined with reverse genetic approaches will enable identification of biological factors that both promote osseointegration and underscore its failure.

Figure1:

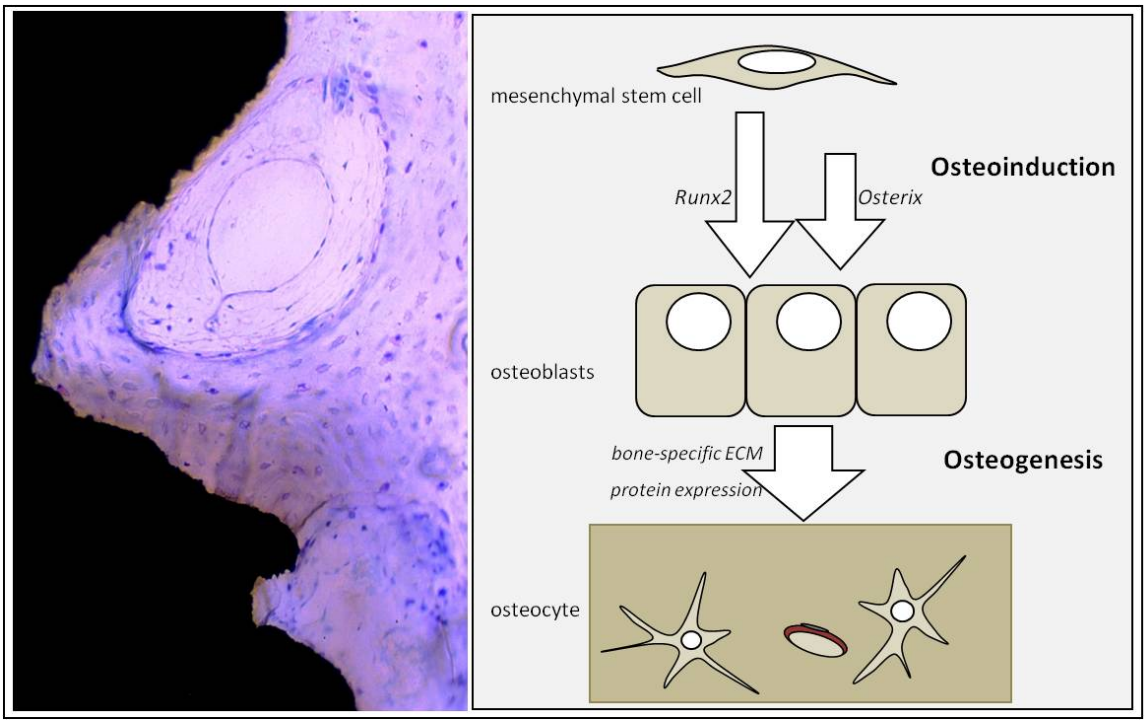

Figure 1. The result of osseointegration can be appreciated histologically (left). Note the presence of cells within the bone and adjacent to the implant surface as bone has formed over a four week period in a canine model. The process(es) that lead to this result are dependent on the differentiation of mesenchymal stem cells to osteoblasts by the process of osteoinduction(right). The key transcriptional regulators that control osteoblastogenesis are Runx2 and Osterix. One important question regarding implant surface influence on osseointegration is "what is the effect of surface on the expression of these transcriptional regulators in implant adherent or adjacent cells?" Committed osteoblasts elaborate a collagen-rich matrix that is embellished with bone-specific extracellular matrix proteins that control tissue formation and mineralization. Many studies have revealed that the nature of the implant surface alters the expression of bone-specific extracellular matrix proteins. These fundamental relationships between the implant and cells that produce bone matrix are accompanied by many other cell type-implant relationships that have not yet been elucidated.

Figure2:

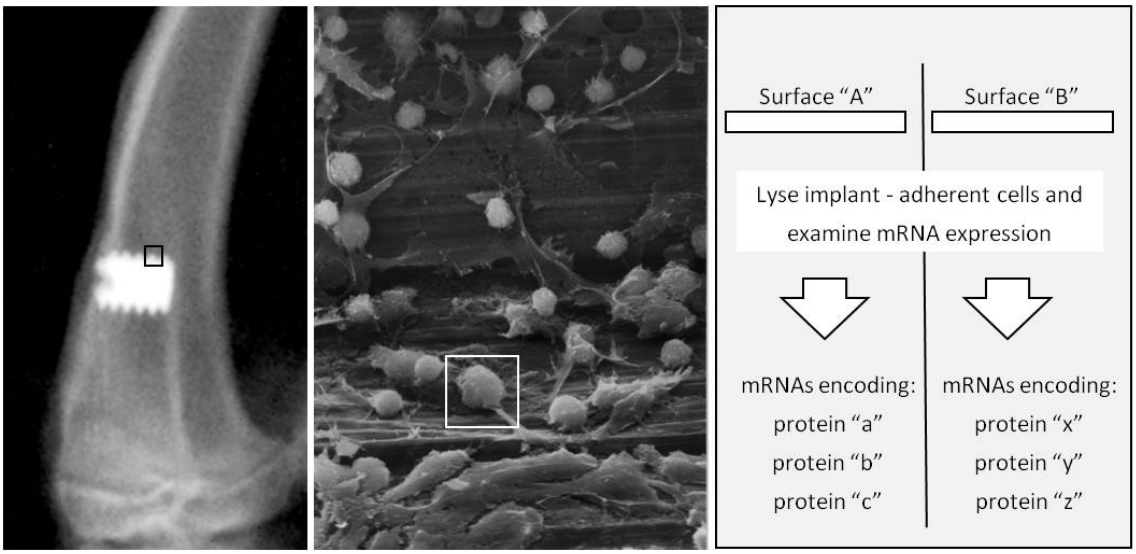

Figure 2: Obtaining molecular information from endosseous implants begins by placement of an implant with defined surface character into the bone marrow (left). Intentional explantation of the implant at early time points relative to mineralization leads to implant retrieval with adherent cells (center). When different implant surfaces are implanted, retrieval of implants with adherent cells permits the evaluation of adherent cell phenotype at the level of protein-encoding mRNA expression. Anticipated (and repeatedly demonstrated in the literature) is the surface-dependent modulation of mRNA expression within the implant-surface adherent cells.

Table1: In vivo models used for molecular assessment of osseointegration

Model Rats – Sprague Dawley;Wistar	Site –intervention if present	References
	Tibiae	[84] [85] [12] [86] [41] [52] [43] [47]
	Femurs	[87] [30] [26] [11] [54][53]
	Femurs- Intramedullary implants	[57] [88]
	Femurs - nicotine exposure	[89]
	Ovariectomzed female rats	[11] [90]
	Tibiae-unloaded by tail suspension-osteopenic model	[91]
	Maxilla	[92]
	Calvarial defects covered by teflon membrane and Ti disks	[82]
New Zealand white rabbits		[81] [93] [94] [95]
Minipigs	Frontal skull	[42]
Mice		
	Mouse Tibiae	[96]
	Femurs ; COX-2−/− and COX-2+/+ mice	[97]
Human volunteers	The retromolar area-trephine	[13] [83]

Table2: Methods used to assess gene expression adjacent to different implant surfaces in in-vivo models.

Method used	**References**	**Role**
Differential display-polymerase chain-reaction	[11]	Exploratory
Immunohistology	[95] [88] [30] [84] [86] [52][43]	Qualitative
Immunofluorescence	[85]	Qualitative
In situ hybridization	[87] [96] [92]	Qualitative and semi-quantitative
Histochemistry; TRAP and ALP	[96] [84]	Qualitative and semi-quantitative
RT-PCR	[57] [30] [26] [54] [90]	Semi-quantitative
qReal-Time-PCR	[97] [12] [53] [41] [81] [52] [43] [47] [93] [94] [91] [42] [89]	Quantitative
Wide genome expression profiling	[13] [83] [82] [30]	Quantitative

Table 3: Molecular assessment of osseointegration in in-vivo models along with Gene Ontology classification

Genes examined	Gene Ontology Classification	References
PCNA (proliferating cell nuclear antigen)	Cell proliferation	[85]
Per2 (period homolog 2)	regulation of transcription, DNA-dependent; circadian rhythm	[30]
PPARy (peroxisome proliferator-activated receptor gamma)	Transcription, DNA-dependent; positive regulation of fat cell differentiation	[43]
RUNX2 (runt-related transcription factor 2)	Ossification; osteoblast differentiation; transcription	[96] [57] [12] [41] [81] [43] [47] [93] [94] [89]
Sp7 transcription factor	Regulation of transcription; osteoblast differentiation	[42]
ALP (alkaline phosphatase)	Skeletal system development; biomineral tissue development	[96] [57] [84] [12] [41] [43] [47] [42]
BSP (integrin-binding sialoprotein)	Ossification; cell adhesion; extracellular matrix organization; biomineral tissue development	[12] [41] [26] [90] [95] [89]
OC (Osteocalcin)	Skeletal system development; osteoblast differentiation; osteoblast development; bone mineralization; regulation of osteoclast differentiation	[97] [96] [57] [84] [85] [87] [41] [81] [26] [54] [43] [47] [90] [93] [94]
OPN (Osteopontin)	Ossification; cell adhesion; positive regulation of cell-substrate adhesion; biomineral tissue development	[96] [57] [53] [41] [30] [26] [54] [90] [95] [89] [57]
ON (secreted protein, acidic, cysteine-rich) (osteonectin)	Ossification; regulation of cell proliferation; cellular response to growth factor stimulus	[53] [87] [30] [26] [90] [95]
COL1A (collagen, type I, alpha 1)	Skeletal system development; blood vessel development; osteoblast differentiation; intramembranous ossification; endochondral ossification	[96] [57] [87] [30] [81] [26] [54] [90] [93] [95] [94] [42]
COL IIA (collagen, type II, alpha 1)	Skeletal system development	[96] [30] [26] [54] [95] [89]
COL IIIA (collagen, type III, alpha 1)	skeletal system development; blood vessel development; cell-matrix adhesion	[90]
COLIXA (collagen, type IX, alpha 1)	chondrocyte differentiation; cell adhesion; organ morphogenesis	[54]
COLXA1 (collagen, type X, alpha 1)	skeletal system development	[30]
collagen XIIA1 (collagen, type XII, alpha 1)	skeletal system development; cell adhesion; collagen fibril organization	[92]
Vinculin	Blood vessel development; regulation of Wnt receptor signaling pathway; canonical Wnt receptor signaling pathway involved in positive regulation of	[52]

	wound healing	
Fibronectin	Extracellular matrix structural constituent; angiogenesis; cell adhesion; response to wounding; cell migration; peptide cross-linking; platelet activation; substrate adhesion-dependent cell spreading; leukocyte migration; regulation of cell shape	[53]
integrin, beta 1	cell migration involved in sprouting angiogenesis; cellular defense response; cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; multicellular organismal development; cellular response to mechanical stimulus	[26] [52] [90]
integrin, beta 2	cell-matrix adhesion; integrin-mediated signaling pathway; cell-cell signaling; multicellular organismal development	[52]
integrin, beta 3	cell adhesion; cell-matrix adhesion; integrin-mediated signaling pathway; regulation of bone resorption; leukocyte migration; angiogenesis involved in wound healing	[26] [52] [90]
Decorin & Biglycan	organ morphogenesis; wound healing; extracellular matrix binding ;peptide cross-linking via chondroitin 4-sulfate glycosaminoglycan;	[54]
BMP2 (bone morphogenetic protein 2)	skeletal system development; osteoblast differentiation;positive regulation of bone mineralization	[57] [84] [85] [54] [43] [89]
BMP4 (bone morphogenetic protein 4); BMP7 (bone morphogenetic protein 7);	osteoblast differentiation; positive regulation of pathway-restricted SMAD protein phosphorylation; positive regulation of bone mineralization;	[57]
The BMP antagonist Noggin	skeletal system development; osteoblast differentiation; negative regulation of BMP signaling pathway	[57]
TGF-β 1 (transforming growth factor, beta 1)	chondrocyte differentiation; SMAD protein complex assembly; regulation of cell proliferation; response to wounding; regulation of collagen biosynthetic process	[57] [54] [43]
TGF-β2 (transforming growth factor, beta 2); TGF-β3 (transforming growth factor, beta 3)	SMAD protein import into nucleus; cell proliferation	[57]
TGF-BR1(transforming growth factor, beta receptor 1); TGF-	positive regulation of mesenchymal cell proliferation;	[57]

BR2(transforming growth factor, beta receptor 2)	positive regulation of SMAD protein import into nucleus	
IGF1 (insulin-like growth factor 1)	skeletal system development; blood coagulation; positive regulation of cell proliferation; bone mineralization involved in bone maturation; positive regulation of osteoblast differentiation	[57] [81]
IGF-1 Receptor [IGF-1R]	signal transduction; positive regulation of cell proliferation; positive regulation of cell migration	[57]
FGF-2 (fibroblast growth factor 2 (basic))	regulation of cell proliferation; wound healing; positive regulation of blood vessel;regulation of cell cycle	[91]
PDGF-B (platelet-derived growth factor beta polypeptide)	positive regulation of endothelial cell proliferation; monocyte chemotaxis; platelet degranulation; response to wounding	[43]
Hif-1 α (hypoxia inducible factor 1, alpha subunit)	response to hypoxia; positive regulation of endothelial cell proliferation;transcription, DNA-dependent; positive regulation vascular endothelial growth factor production; positive regulation of chemokine production; regulation of transforming growth factor-beta2 production	[91]
vascular endothelial growth factor A	Angiogenesis; patterning of blood vessels; vasculogenesis; response to hypoxia	[57] [84] [91]
Fms-like tyrosine kinase 1 [Flt-1])	patterning of blood vessels; response to hypoxia; sprouting angiogenesis; vascular endothelial growth factor receptor signaling pathway	[57]
Epas1 (endothelial PAS domain protein 1)	angiogenesis; response to hypoxia; multicellular organismal development; cell differentiation	[91]
Ang-1 (angiopoietin 1)	regulation of endothelial cell proliferation; sprouting angiogenesis; leukocyte migration; positive chemotaxis	[91]
OPG (tumor necrosis factor receptor superfamily, member 11b)	Skeletal system development; apoptosis; extracellular matrix organization; negative regulation of bone resorption	[86]
RANK (tumor necrosis factor receptor superfamily, member 11a, NFKB activator)	Cell-cell signaling; positive regulation of cell proliferation; osteoclast differentiation	[86]
RANKL (tumor necrosis factor (ligand) superfamily, member	Positive regulation of osteoclast differentiation; positive regulation	[86] [91]

11)	of bone resorption; cytokine-mediated signaling pathway	
TRAP (acid phosphatase 5, tartrate resistant)	response to cytokine stimulus; bone resorption; bone morphogenesis	[96] [81] [47] [93] [94] [42] [43] [91]
catK (Cathepsin K)	Proteolysis; bone resorption	[53] [43] [47]
calcitonin receptor	Activation of adenylate cyclase activity by G-protein signaling pathway	[81]
H+-ATPase	ATP hydrolysis coupled proton transport; proton transport	[81]
TNF-α (tumor necrosis factor)	Inflammatory response; immune response	[81] [52] [43] [47] [91] [57] [94]
Interleukin 1, beta	Inflammatory response; immune response; signal transduction; cell-cell signaling;	[52] [43] [47]
Interleukin 6	Inflammatory response; immune response	[81] [94]
Interleukin 8 receptor	Inflammatory response; immune response	[52]
Interleukin 10	Inflammatory response; cell-cell signaling; regulation of gene expression; leukocyte chemotaxis	[81] [94]
Interleukin 11	Inflammatory response; immune response	[91]
MCP-1 (CCL2)	Angiogenesis; response to hypoxia; chemotaxis; inflammatory response; cytokine-mediated - signaling pathway	[52] [53]
CXCR4 (chemokine (C-X-C motif) receptor 4)	Response to hypoxia; inflammatory response; regulation of chemotaxis	[52]
Cox-1 (prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Prostaglandin biosynthetic process; regulation of cell proliferation	[97]
Cox-2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Regulation of inflammatory response; response to glucocorticoid stimulus	[97] [57]
ED1 (CD68 molecule)	Marker of macrophages	[88]
CD 163	A marker for tissue macrophage	[43]
Periostin	A marker for osteogenic cells	[43]
PGP 9.5 (marker of axons), Calcitonin Gene-Related peptide (constituent of sensory axons and neuropeptide involved in bone remodeling), GAP-43 (marker of sprouting axons)		[88]

Table 4: Wide genome expression profiling studies in vivo.

Model	Surface examined	Technique used	Day of analysis	Reference
Retromolar area - human volunteers-trephine	SLA surface vs. SLActive	Human WG-6 V3 array (Illumina)	4,7 and 14 days	Donos et al, 2011[13]
Retromolar area - human volunteers-trephine	SLActive	Human WG-6 V3 array (Illumina)	4,7 and 14 days	Ivanovski,S. et al,2011[83]
Calvarial defects - Wistar rats total covered by teflon membrane and Ti disks	Polished Ti- SMO vs. microrough surface SLA	Affymetrix Gene Chip Rat Expression Set 230 v 2.0	7 and 14 days	Donos et al, 2011[82]
Rat femur	T-shaped Ti4Al6V implant-surface treated by dual acid-etching followed by cystalline deposition of HA particles –(tissue collected in the hollow chamber vs. osteotomy sites without implants were analyzed) – control vs. vitamin D deficient diet	Agilent Rat Whole Genome- two color channel microarray-41,000 genes	2 weeks	Mengatto,C.M et al,2011[30]
Rat femur	cp Titanium implants vs osteotomy healing sites	DNA microarray where 20,000 rat genes (Agilent Rat Oligo array, Agilent Technologies)	1 wk,2 wks and 4 wks	Kojima et al, 2008 [53]
Rat femur	Experimental cp Ti T-shaped implants .The implant surface was either machined (turned by a lathe) or treated by acid-etching (Osseotite)-(1) the osteotomy control group, (2) the machined titanium implant group, and (3) the acid-etched titanium implant group (4) untreated control group	Differential display-polymerase chain-reaction	3 days, 1,2 and 4 weeks post-surgery	Ogawa,T.et al, 2006[11]

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Chapter 3

Comparative Molecular Assessment of Early Osseointegration in Implant-Adherent Cells: Rat model

Abstract:

Objective: to identify the early molecular processes involved in osseointegration associated with a micro roughened and nanosurface superimposed featured implants.

Materials and methods: Thirty-two titanium implants with surface topographies exhibiting a micro roughened (AT-II) and nanosurface superimposed featured implants (AT-I) were placed in the tibiae of 8 rats and subsequently harvested at 2 and 4 days after placement. Total RNA was isolated from cells adherent to retrieved implants. A whole genome microarray using the Affymetrix Rat gene 1.1 ST Array followed by validation of select genes through qRT-PCR was used to describe the gene expression profiles that were differentially regulated by the implant surfaces.

Results: Whilst significant differences at the gene level were not noted when comparing the two-implant surfaces at each time point, the microarray identified several genes that were differentially regulated at day 4 vs. day 2 for both implant surfaces. A total of 649 genes were differentially regulated at day4 vs. day2 in AT-I and 392 genes in AT-II implants. Functionally relevant categories related to ossification, skeletal system development, osteoblast differentiation, bone development, bone mineralization and biomineral tissue development were upregulated and more prominent at AT-I (day 4 vs. day2) compared to AT-II. Analysis of the downregulated gene lists (day 4 vs. day 2) with average fold change >2 (were not stastically significant) revealed the biological processes involved with the inflammatory/immune response gene expression. The

number of genes that were associated with the inflammatory/immune response category was greater for AT-I than AT-II.

Conclusions: The presence of nanosurface features modulated in vivo bone response. This work confirms previous evaluations and further implicates modulation of the inflammatory/immune responses as a factor affecting the accrual of bone mass shortly after implant placement.

Introduction:

The placement of endosseous implants is a common treatment option to treat edentulism. The success of dental implants is based on the concept of osseointegration introduced by Branemark[1]. Despite the high success rates achieved, implant failures that mandate implant removal do occur. Factors attributing to implant failures include local and systemic conditions such as reduced bone volume, reduced bone density, periodontitis, and impaired wound healing (e.g., diabetes, smoking, osteoporosis, radiation therapy, chemotherapy) [2-6]. Efforts to enhance osseointegration of dental implants allowing for faster prosthetic rehabilitation and improved success rates in clinically challenging situations, included modifications to the physical and chemical properties of the implants surfaces. It is well demonstrated that implants with moderately rough surfaces (average height deviation of 1-2μm) [7] enhance the rate and quality of osseointegration with greater bone-to-implant contact and higher resistance to torque removal[8-12].

In contrast to micron-features of alloplastic materials, bone is composed of constituent nanofeatures[13]. Nanostructured materials are those with features less than 100nm in at least one dimension[14]. Simulation of nanofeatures at implant surfaces has shown favorable bioactivities with titanium surfaces[15]. Enhanced in vivo bone responses to implants with nanofeatures compared to machined or micro roughened surfaces measured by histological and biomechanical means have been shown in several animal models (eg, rabbits, dogs, rats) [16-23]. The topography of titanium surface at the nanolevel has been reported to modulate differentiation, proliferation, and increase expression of osteogenic markers[17,24,25]. Yet, the exact role of nanosurface topography on the molecular events occurring early in the process of osseointegration remains poorly understood. Prior studies mainly focused on select target genes; typically markers for osteoblasts including *Cbfa1/Runx2* [26,27] , *Osterix (Osx)* [26-29] , *Osteocalcin (Ocn)* [30-34] , *Osteopontin (Opn)* [35-38] , *Collagen I* [39-42] and *Alkaline phosphatase (Alp)* [27,29,43,44] .

It is of interest to further investigate the overall gene expression profiles by implant adherent cells during the early phase of osseointegration. The advent of gene expression microarrays allows the rapid and high-throughput quantification of thousands of genes simultaneously [45]. Microarray analysis may unveil the regulation of individual genes that might not be identified otherwise. These molecular details may represent targets for future therapeutic improvement. Most recently, this approach has been applied in vitro in the analysis of MG63 osteoblastic- cell response to a nanoporous Ti6AL4V surface (produced by blasting with tricalcium

phosphate and light nitric acid treatment; nanoPORE, Out-Link, Sweden and Martina, Due Carrare, Padova, Italy) [46]. However, we recognize that the biological environment in vivo is very different from the in vitro conditions and contains a variety of cells that each can respond to the implant surface and produce several cytokines and growth factors influencing each other's behavior. Few whole genome-wide profiling studies have been reported using in-vivo models [47-49] . Profiles of gene expression of in vivo bone healing with or without titanium implants (osteotomy sites) were described in a rat model [48,50] at different time points with 1 week time point representing the earliest analysis[50]. Moreover, Donos et al [47] reported on the gene expression profiles associated with a moderately rough surface (SLA) compared to a chemically modified moderately rough surface (SLActive) in a human model. The microarray analysis was carried out at 4, 7 and 14 days post surgery.

Various methods have been developed in order to create a nanosurface. Typically applied techniques include lithography, ionic implantation, anodization, acid etching, alkali treatment, peroxidation and sol-gel deposition[15,51]. Recently, Johansson et al. [52], reported on a newly developed nanosurface (AT-I) produced by sequential chemical treatment with oxalic acid and hydrofluoric acid preceded by blasting with titanium oxide particles. These implants were tested in a rabbit model and compared with implants with micro-roughened surface (AT-II). The results demonstrated greater 2D bone-to-implant contact and 3D removal torque values for the nanosurface implants. The objective of the present study was to further investigate the effect of this newly developed nanosurface (AT-I) topography imposition on the whole genome expression profiles at early time points in the process of osseointegration using an in-vivo rat model and compare it to those of the micro-roughened surface (AT-II).

Materials and methods:

Implants:

Newly developed implant surfaces of commercially pure titanium grade IV screws (2.0mmx3.0mm) were used in this study. The test samples were manufactured per protocol described previously by Johansson et al, 2011[52]. Briefly, the samples were degreased, blasted with titanium oxide particles and rinsed in sterile water. These samples were then treated in a different sequential process resulting in two different surface structures. One sample group was treated in oxalic acid, named AT-II, while the other group was treated in oxalic acid and hydrofluoric acid sequentially (AT-I). All implants are washed by sterile water and beta-sterilized.

Surface characterization:

The implant surfaces were examined by a high-resolution scanning electron microscope [ESEM XL30, FEI Company,5651 GG Eindhoven, the Netherlands] . The three-dimensional surface parameters were determined using optic interferometry (MicroXam, Phase-Shift, Tucson,AZ). Three specimens of each surface type was analyzed , and each specimens was analyzed in three areas. Errors of form were removed using a Gausian filter size of 50x50mm. Surface roughness values were reported by the S_a and S_{dr}% values per the suggested guidelines by Wennerberg A & Albrektsson T for the topographic evaluation of implant surfaces. [53]

The Sa represents the average height of the analyzed area

The Sdr represents the developed interfacial area ratio %

The chemical nature of the surfaces was examined using X-ray photoelectron spectroscopy (XPS). A Quantum 2000 ESCA Scanning Microscope A Quantum 2000ESCA Scanning Microscope (Physical Electronics, Chanhassen,MN, USA) with an X-ray source of monochromatic A1*K*a was used to obtain a spectrum for each surface. The mean numbers and standard deviation (SD) were deduced from measurements of three implants per group with two regions per implants resulting in n=6 per implant type. The wettability was measured by using the sessile drop technique on titanium coins with the AT-I and AT-II surfaces.

Model:

A rat tibia model of osseointegration was used [18,54]. All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina, Chapel Hill (IACUC ID: 10-127.0). Eight male Sprague Dawley rats (326-350 g) were purchased from Harlan laboratories and acclimated for 7 days prior to initiation of studies. Anesthesia was achieved using Ketamine/Xylazine (80-100mg/kg and 10 mg/kg respectively) along with supplemental local anesthesia (Lidocaine 2% with epinephrine (1:100,000) .The dorsal/medial aspect of the tibiae was identified, shaved, and disinfected using betadine and 70% ethanol scrub. Using aseptic technique, a full thickness myocutaneous flap was made and carefully retracted to reveal the medial aspect of the tibia bone. With sterilized stainless steel burs, two drill holes were created with copious irrigation. The drill holes were made approximately 5 mm apart .Two implants (cp titanium) were placed in each tibia to provide sufficient RNA for each experimental sample. For every time point (day2 and 4), 4 rats were used. Each animal received two AT-I implants in one tibia and two AT-II implants in the contralateral

tibia (randomly distributed). The periosteum was adapted over the site using interrupted 4-0 chromic gut sutures for subcuticular closure. The skin was then closed using vicryl sutures. Animals were monitored continuously following surgery. Ambulation was the defined criteria for immediate recovery. A postoperative analgesic was provided for 48 hours after surgery, by means of subcutaneous ketoprofen injections (5mg/kg) once daily. At the time of sacrifice, each animal was placed in a CO_2—saturated chamber. Death was assured by thoracotomy and severance of the inferior vena cava for exsanguination.

RNA isolation:

At 2 and 4 days following surgery, animals were euthanized. Immediately thereafter, the tibia sites were isolated and the implant site was exposed using sterile technique and the entire tibias were harvested and the implants were explanted by fracture of the tibia. For evaluation of mRNA expression in cells adherent to explanted endosseous implant surfaces, the implants were rinsed in cold PBS immediately following retrieval and then placed into 1000 µl of Tri-reagent. Total RNA was isolated from the lysates using the standard Tri-reagent protocol and collected by ethanol precipitation. This was followed by purification using RNeasy MinElute Clean up kit (Qiagen, Valencia, CA, USA). RNA was assessed for quality and quantity using a bioanalyzer (Agilent, Santa Clara, CA, USA) and nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) respectively. Samples were processed and hybridized to the Affymetrix Rat gene 1.1 ST Array at the UNC core facility following the manufacturer's recommended protocols and reagents (Affymetrix, San Clara,CA). The Rat Gene 1.1 ST Array Plate interrogates more than 27,000 well-annotated genes with more than 720,000 distinct probes. The raw microarray data is available online at the NCBI-GEO (http://www.ncbi.nlm.nih.gov/geo/) database, accession number GSE35976.

Microarray data analysis:

Data analysis was completed using GeneSpring software v.11.5.1 (Agilent Technologies, Santa Clara, CA). 2-Way ANOVAs statistical analysis was applied to determine differentially expressed genes among the various parameters (implant surface type and time points). Further analysis included pairwise comparisons of each implant surface independently at the different time points (day4 vs. day 2). A p-value of 0.05 was used as the threshold for statistical significance. Exported lists included significant genes, fold changes and p-values for comparisons. These lists of genes were then condensed into organized classes of related biology using The GO Ontology Browser function in the GeneSpring. Significant GO $P<0.05$ was corrected for multiple sampling

using the method of Benjamini and the Hochberg false discovery rate method[55]. In addition, to focus on the major gene expression changes, we analyzed genes with over twofold-changed expression compared to control.

Real-time quantitative PCR:

Following microarray analysis, quantitative real-time PCR (qRT-PCR) was performed using the same RNA samples in order to validate expression patterns for select genes. We have selected the following genes for this RT-qPCR validation: highly upregulated genes (*Phex, Aspn, Satb2*), known osteoblast marker (*Bsp*), highly downregulated genes with greater than 2 fold change (*Il-1ß, Cxcl2, Ccl3* (all belonged to the inflammatory response). First strand cDNA was synthesized (Superscript III, Invitrogen) from total RNA (500 ng) in a standard 20-µl reaction. Subsequently, equal volumes of cDNA were used to run real-time PCR reactions specific for mRNAs encoding the following markers: phosphate regulating endopeptidase homolog, X-linked (Assay ID: Rn00448130_m1), pannexin 3 (Assay ID: Rn01640170_m1), SATB homeobox 2 (Assay ID: Rn01438160_m1), Ibsp (Assay ID: Rn00561414_m1), interleukin 1 beta (Assay ID: Rn00580432_m1), chemokine (C-C motif) ligand 3 (Assay ID: Rn01464736_g1), chemokine (C-X-C motif) ligand 2(Assay ID: Rn00586403_m1). Reactions were performed using TaqMan Universal PCR Master Mix and thermocycling in an ABI 7200 real-time thermocyler (Applied Biosystems, Foster City, CA). Relative mRNA abundance was determined by the $2^{-\Delta\Delta Ct}$ method and reported as -fold induction. GAPDH mRNA abundance was used for normalization[56]. Samples were run in triplicate and the average was used for further analysis. The data points analyzed were 2 and 4 days. The surface-matched Day2 was used as a calibrator.

Results:

Surface analysis:

SEM images (Figure1) revealed a micro-roughened surface for AT-II, whereas AT-I produced by the additional treatment of hydrofluoric acid is comprised of nanostructures superimposed on a moderately roughened surface. Measurements of surface parameters (Table 1) showed that the two surfaces examined have similar S_a values. XPS analysis (Table 2) showed a significantly higher carbon signal for the AT-II sample and presence of oxalate in the surface oxide for this sample. Both surfaces were hydrophobic (AT; a contact angle of 90-110°, AT-II; a contact angle of ~ 90°).

Microarray data:

To gain insight into the mechanisms by which surface topography influences the process of

osseointegration, quadriplicate genome wide expression studies of implant adherent cells were performed as a function of time (t=2,4 days) with either micro (AT-II) or nanosurface topology implants (AT-I). 2-Way ANOVA was applied to determine differentially expressed genes among the various parameters (implant surface type and time points). No genes were identified (p-value<0.05) to be different when comparing the 2 implants surfaces at each time point. Further analysis included pairwise comparisons of each implant surface independently at the different time points (day 4 vs. day2) to allow understanding of the early molecular events occurring at either surface (AT-I, AT-II). 649 genes were found to be differentially expressed (p-value ≤0.05) at AT-I; of these, 601 were upregulated, and 48 were downregulated. On the other hand, 392 genes were found to be differentially expressed (p≤0.05) at AT-II; of these, 294 were upregulated and 98 were downregulated (Table 3). The identity of the top 35 genes differentially expressed at the two implants surfaces (day 4 vs. day 2; p-value<0.05) is shown in tables 4 and 5. Another table (table 6) was created to include list of top 25 differentially expressed genes (p-value≤0.05) at AT-I (Day 4 vs. day2) with their fold regulation in AT-I, AT-II. Nevertheless, the downregulated subset of genes that reached statistical significance (p-value <0.05) did not include any with fold regulation more than 2. The top 25-downregulated genes at AT-I and AT-II are listed in tables 7 and 8.

Validation of Affymetrix microarray data by qRT-PCR

The abundance of selected gene transcripts was analyzed on each implant surface at the different time points using RT-qPCR. Table 9 summarizes results obtained with the two techniques for the selected genes (*Aspn, Phex, Satb2, Bsp, Cxcl2, Ccl3, Il-1ß*). The results were expressed as mean fold change. Results obtained from RT-qPCR were in full concordance with those from microarray, validating our microarray results.

Identification of significant gene ontologies associated with the different implant surfaces

GO analysis was carried out on the differentially regulated genes between day 4 and day 2 (fold change ≥2; p ≤0.05) at both implants surfaces. These sets of genes comprised only upregulated genes and did not include downregulated genes. As mentioned earlier, the downregulated subset of genes that reached statistical significance included only genes with less than 2 fold downregulation. To focus on the major changes that might have been caused by reduced expression, another analysis was carried out on the downregulated genes

with more than 2 fold (p-value>0.05).

GO terms upregulated at AT-I, AT-II (day 4 compared with day 2)

The gene functional annotation identified at AT-I consisted of a large variety of processes. 80 terms satisfied p≤0.05. Functionally relevant categories relevant to ossification, skeletal system development, skeletal system morphogenesis, osteoblast differentiation, bone development, bone mineralization and biomineral tissue development were clearly demonstrated at AT-I. Fourteen genes associated with ossification were differentially upregulated (P≤0.05) at AT-I. The increased expression of ossification genes at the AT-I surface is consistent with the notion that there is an active and accelerated bone formation at this time point (Day4). These genes included *Fgfr2* (4.55 fold), *Ostn* (2.23 fold), *Dmp1* (9.67 fold), *Bmp3* (8.25 fold), *Aspn* (14.61 fold), *Col11a1* (5.91 fold), *Mef2c* (2.03 fold), *Col11a2* (2.3 fold), *Dlx5* (3.86 fold), *Ptn* (2.63 fold), *Sp7* (3.55 fold), *Pth1r* (5.88 fold), *Runx2* (2.33 fold) and *Satb2* (9.15 fold). This list also demonstrates the representation associated to extracellular matrix, developmental processes, cell adhesion, collagen fibril organization and regulation of cell proliferation. Interestingly, there was a representation of neurogenesis and axonogenesis.

Nevertheless, 70 GO terms satisfied the p-value cut off 0.05 in AT-II. Several functionally GO categories were over-represented in the list of genes that were up regulated at AT-II, including the category of extracellular matrix, cell adhesion and collagen fibril organization. Likewise to AT-I, neurogenesis was also prominent at AT-II. Significantly, the category related to bone was limited to ossification and skeletal system development. Bone biomineralization and biomineral tissue development were not identified by this GO analysis at AT-II surface. The counts of genes involved in both categories in AT-II were less than those in AT-I; with skeletal development count limited to 7 genes in AT-II opposed to 15 genes in AT-II and ossification count limited to 3 in AT-II opposed to 14 in AT-I. Furthermore, our results identify higher p-values for both ossification (p=0.0025) and skeletal system development (p=0.00122) in AT-II in comparison with the p-values for these categories in AT-I (ossification; p-value=8.66E-09, skeletal system development; p-value=1.01E-07). The p-value for each GO term reflects the enrichment in frequency of that GO term in the entity list, which further emphasizes the presence of enhanced bone response in AT-I at this early stage of osseointegration (Day4).

GO terms downregulated at AT-I, AT-II (day 4 compared with day 2)

Further functional annotation analysis of the downregulated lists of AT-I and AT-II highlighted several categories in this domain. 205 terms satisfied $p \leq 0.05$ in AT-I, with only 21 terms in AT-II. Table 12 outlines the top 25 GO terms in AT-I and table 13 outlines the 21 terms that met the eligibility criteria in AT-II.

An overrepresentation of functional annotations associated with inflammatory response, immune response and chemotaxis were essentially demonstrated by both surfaces. However, examination of the number of genes associated with each GO term in both lists show that AT-I is by far associated with a higher number of genes in each functional GO term. This implies that AT-I surface might have a role in rapidly downregulating a wide range of genes associated with the inflammatory and immune response processes in the early days following implant placement. This is also highlighted when we examine the list of genes downregulated at both surfaces (Tables 7 and 8) with further more downregulation of several inflammatory genes at AT-I. We recognize that these lists of genes did not reach statistical significance. However, this could be due to the proximity of the two data points analyzed.

Discussion:

This study describes the detailed genetic responses to a micro-roughened (AT-II) and nanostructured (AT-I) implant surfaces in a rat tibia model. With the advent of o molecular biology techniques, comparative analysis of gene expression of a large number of genes spanning a significant fraction of the genome using microarrays and not only a few selected activities is possible. This also allows interrogation of broad biological processes as well as individual genes. This analysis effectively reveals the early cellular and molecular mechanisms that drive the process of osseointegration at both surfaces using the Affymetrix Rat gene 1.1 ST Array. The transcriptional analysis described in this study reports the gene expression profiles associated with osseointegration at days 2 and 4 following implant placement. The choice of time points was taken to detect very early molecular events.

Whilst significant differences at the gene level were not noted when comparing the two-implant surfaces at each time point, the general trend was striking. Pairwise comparisons of each implant surface at the two time points (day 4 vs. day 2) allowed us to further comprehend the initial wound healing process associated with

each implant surface. 649 genes were found to be differentially expressed at AT-I and 392 genes were found to be differentially expressed at AT-II. The gene expression profile in AT-I at day 4 is characteristic of induced bone formation with the over-representation of the functional biological processes related to ossification, skeletal system development, osteoblast differentiation and biomineral formation with greater number of genes that were up-regulated in each category in response to the AT-I surface compared with the AT-II surface. Hence, more rapid bone formation is expected with AT-I surface. This is in agreement with published histological and biomechanical data comparing these two implant surfaces at 6 weeks in a rabbit model showing greater implant-bone contact and higher torque removal values for AT-I surface compared with AT-II surface [52]. The genes associated with skeletogenesis at AT-I included *Fgfr2, Ostn, Dmp1, Bmp3, Aspn, Col11a1, Mef2c, Col11a2, Dlx5, Ptn, Sp7, Pth1r, Runx2, Satb2, Shox2, Papss2, Pdgfra, Gli3, Mmp2, Tbx15, Ryk, Vdr and Phex*. Implants placed in the jaws often penetrate the bone marrow cavity coming in direct contact with several different cell types including hematopoietic cells, fibroblasts, vascular pericytes and those with an osteogenic capacity (including osteoblasts and MSCs), which was definitely the case in our rat tibia model. The potential of a surface to elicit osseointegration is dependent on its ability to induce the differentiation of pluripotent mesenchymal stem cells along the osteoblast cell lineage and stimulate the secretion of a calcified matrix by these osteoblasts [57]. This is orchestrated by several transcriptional factors, among which is RUNX2, OSX and SATB2 which regulate the expression of several bone matrix extracellular protein genes that encode for bone sialoprotein, osteocalcin and collagen type I [58-61]. Modulation of *Runx2* and *Osx* expression by the presence of nanosurface features on implant surfaces topography has been observed by several investigations [18,24,26]. Recent evidence showed that SATB2 is a downstream of OSX. *Satb2* knock out mice exhibit multiple craniofacial defects including a significant truncation of the mandible, a shortening of the oral maxillofacial bones, and defects in osteoblast differentiation [61] . The potential role of *SATB2* in osseointegration has been observed in a recent microarray data demonstrating its differential upregulation (2.5 fold) on a chemically modified surface (SLActive) between day 4 and day 14 [49] in a human model. Other genes in our data lists associated with skeletogenesis that were also upregulated in the aforementioned microarray data included COL11A1, COL11A2, PHEX, DLX5, SP7, PTHR1, PTN and PDGFRA. The precise role of each of these genes in the modulation of osseointegration requires further investigation.

Furthermore, day 4 demonstrated the representation associated to extracellular matrix, cell adhesion,

collagen fibril organization and regulation of cell proliferation in both surfaces.

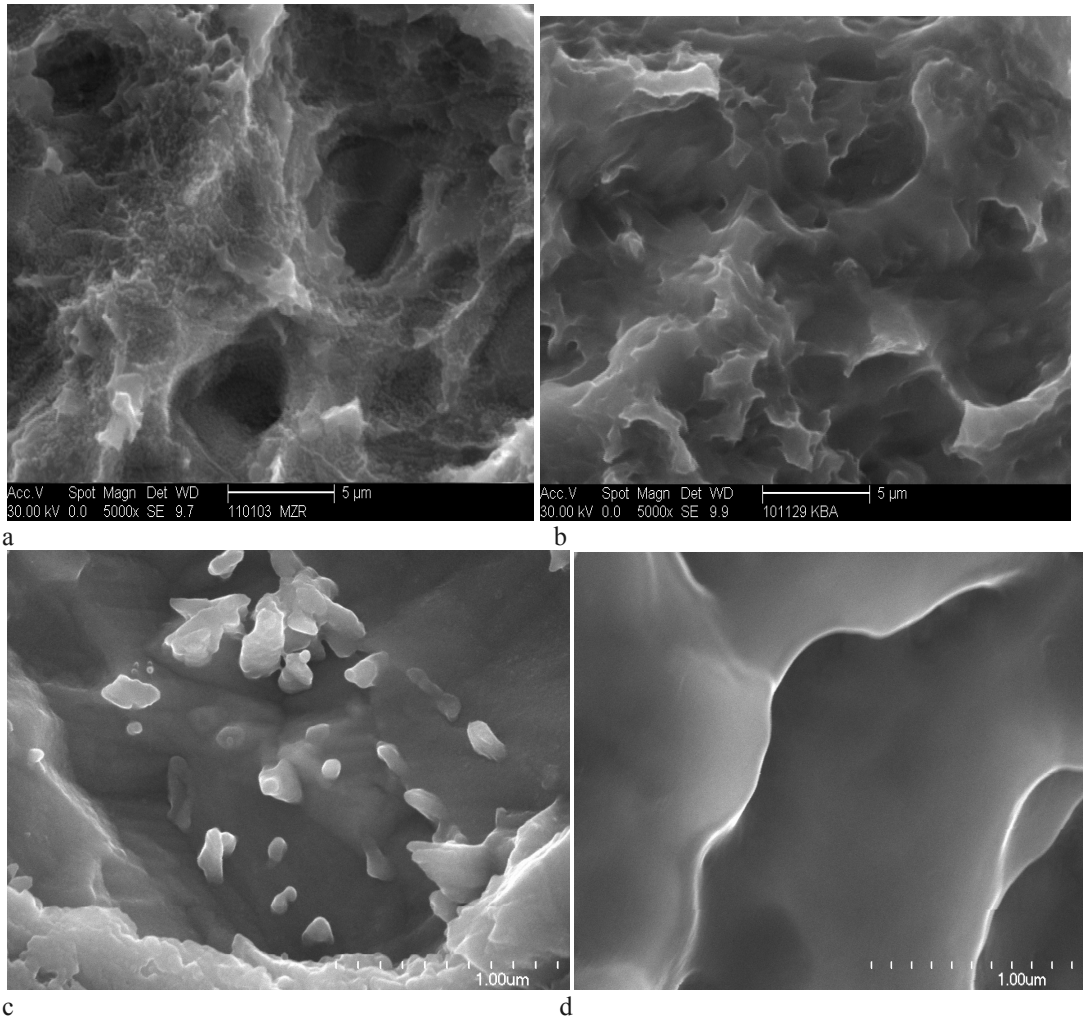
We realize that the process of osseointegration maybe affected by regulation of other cells resident in the blood clot and bone marrow. Analysis of implants adherent gene expression profiles that were downregulated at day 4 suggested a decline in the inflammatory/immune responses. Even though this subset of genes did not reach statistical significance; which could be due to the proximity of the two data points analyzed, its relevance is very critical. Indeed, AT-I was by far associated with greater number of genes associated with inflammatory/immune responses that were downregulated. Furthermore, this is highlighted with further more downregulation of several inflammatory associated genes at AT-I (Tables 7 and 8). The role of implant surface topography and/or chemistry on modulating the inflammatory/immune response has been suggested by several studies. An in vivo study using the rat tibia model demonstrated a higher expression of *Tnf-α* (after 6 and 28 days) and *Il-1β* (6, 14 and 28 days) on machined surfaces compared to the oxidized implants[62]. Furthermore, an in vitro investigation using RAW264.7 cells grown on a nanoscale calcium phosphate coated titanium implants elicited a gene expression profile with marked downregulation of several pro-inflammatory cytokines (*Tnf-α, Il-1β, Il-1f8*), chemokines (*Ccl2, Ccl3, Ccl5, Ccl8, Ccl11, Ccl12*) and the chemokine receptors *Ccr1* and *Xcr1*[63]. On the other hand, the effect of chemical modification of titanium implants on macrophage cytokine gene expression has been reported; hydrophilic implant surfaces (SLActive) resulted in a significant downregulation of key pro-inflammatory cytokines *Tnf-α, Il-1α, Il-1β* and the chemokine *Ccl-2* in RAW 264.7 cells 24 hours post seeding compared with either smooth polished or sandblasted acid-etched (SLA) surfaces [64].

These surface specific responses suggest that the presence of nanotopographic cues on the titanium surface topography may modulate the various implant adherent cells resulting in unique differential phenotypes affecting inflammatory, mesenchymal stem cells, and osteoblastic cells. These biological advantages acquired by the imposition of nano-features through triggering of gene expression crucial for osteoblast differentiation, skeletal development and the rapid decline in inflammatory related processes in nanosurface adherent cells may provide a cardinal role into how we can make more bone faster. However, the present study did not segregate potential effects of surface chemistry.

Conclusion:

The present study investigated experimental surfaces with either nanosurface vs. a moderately rough surface in a rat tibia model at early implantation times. Here, initial efforts were deployed to unravel the molecular mechanism early in the process of osseointegration associated with micro-roughened and nanosurface dental implants. The nanosurface modulates earlier and higher bone repair response as revealed by the recruited expression of gene involvements in the processes related to skeletal development, ossification and osteoblast differentiation. A potential role of the nanosurface in downregulating the inflammatory/immune responses at a faster rate than a micro-roughened surface is implicated. Comprehensive understanding of the complex biological events occurring the bone implant interface will ultimately lead to improve biologically driven strategies for improved osseointegration. In this study distinct biochemical responses that underlie the process of osseointegration to the nanosurface were revealed, which could not have been observed through the use of selected gene studies or through in vitro exams. Further detailed studies using additional points may verify the significance of the results obtained. In addition, complementary in vitro and in vivo studies (using genetically modified animals) are required to identify the biological roles of the isolated gene transcripts.

Figure 1. Scanning electron microscopic (SEM) evaluation of tested implant surfaces (a) AT-I surface blasted with TiO2 particles and sequentially treated in oxalic and hydrofluoric acid (b) AT-II surface blasted with TiO2 particles and treated in oxalic acid at 5000X magnification (c) AT-I and (d) AT-II at 50000X.Note that AT-I surface is randomly covered with topography features of approximately 100nm.



a b

c d

Table 1:
Surface roughness values as determined by optical interferometry ((MicroXam, PhaseShift).

Sample	S_a (µm)	S_{dr} (%)
AT-I	1.50 (0.15)	45.52 (8.98)
AT-II	1.58 (0.16)	66.01 (10.43)

The mean numbers and standard deviation (SD) are deduced from measurements of three implants per group with three regions per implant type (n=27).

Table 2:Quantitative chemical surface composition (atomic percentage) as determined by X-ray photoelectron spectroscopy (Quantum 2000ESCA Scanning Microscope, Physical Electronics)

Surface type	C1s	N1s	O1s	Ti2p	Si2p	F1s
AT-I	24.48 (5.36)	1.06 (0.59)	52.31 (3.72)	20.88 (3.47)	0.50 (1.11)	0.31(0.79)
AT-II	30.41 (7.66)	1.40 (0.62)	48.62 (5.02)	19.36 (3.56)	0.13 (0.2)	-----

The mean numbers and standard deviation (SD) are deduced from measurements of three implants per group with two regions per implants resulting in n=6 per implant type.

Table 3. Number of genes differentially expressed (Day 4 vs. Day 2)

Comparison	Differentially expressed genes *	Downregulated genes*	Upregulated genes*	Upregulated genes with FC >2	Downregulated genes with FC >2
AT-I	649	48	601	419	93
AT-II	392	98	294	314	19

* Upregulated (>1.1) and downregulated (<1.1) genes with p-value <0.05. FC; fold change

Table 4.: List of the top 35 differentially regulated genes at AT-I (Day4 vs. Day2). P-value <0.05.

Transcripts Cluster Id	Gene symbol	Gene description	Fold change	P-value
10916247	Panx3	pannexin 3	14.85	0.016
10797660	Aspn	asporin	14.61	0.018
10933664	Phex	phosphate regulating endopeptidase homolog, X-linked	13.14	0.015
10797657	Omd	osteomodulin	10.70	0.020
10744687	Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	10.15	0.018
10810817	Smpd3	sphingomyelin phosphodiesterase 3, neutral	9.89	0.022
10775375	Dmp1	dentin matrix acidic phosphoprotein 1	9.67	0.016
10928191	Satb2	SATB homeobox 2	9.15	0.023
10775573	Bmp3	bone morphogenetic protein 3	8.25	0.043
10867761	Mmp16	matrix metallopeptidase 16	7.00	0.022
10726676	Ifitm5	interferon induced transmembrane protein 5	6.65	0.016
10853963	Ptprz1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	6.60	0.020
10729377	Mamdc2	MAM domain containing 2	6.36	0.015
10895075	Dcn	decorin	6.26	0.044
10809540	Mmp2	matrix metallopeptidase 2	6.18	0.036
10869616	Ptprd	protein tyrosine phosphatase, receptor type, D	6.14	0.015
10818502	Col11a1	collagen, type XI, alpha 1	5.91	0.018
10883212	Cgref1	cell growth regulator with EF hand domain 1	5.90	0.034
10920524	Pth1r	parathyroid hormone 1 receptor	5.88	0.039
10811248	Adamts18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	5.52	0.029
10896263	Cthrc1	collagen triple helix repeat containing 1	5.43	0.048
10834982	Cercam	cerebral endothelial cell adhesion molecule	5.23	0.015
10738177	Fkbp10	FK506 binding protein 10	5.22	0.023
10803323	Cdh2	cadherin 2	5.22	0.016
10890951	Slc8a3	solute carrier family 8 (sodium/calcium exchanger), member 3	5.02	0.015
10744939	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	5.00	0.038
10759177	Tmem119	transmembrane protein 119	4.96	0.023
10815679	Mme	membrane metallo endopeptidase	4.85	0.023
10733258	Adamts2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	4.80	0.022
10847474	Creb3l1	cAMP responsive element binding protein 3-like 1	4.76	0.023
10764050	Fmod	fibromodulin	4.76	0.022
10846340	Fkbp7	FK506 binding protein 7	4.70	0.034
10729667	Dkk1	dickkopf homolog 1 (Xenopus laevis)	4.69	0.039
10842500	RGD1562846	similar to Docking protein 5 (Downstream of tyrosine kinase 5) (Protein dok-5)	4.63	0.050

Table 5. List of the top 35 differentially regulated genes at AT-II (day4 vs. day2). P-value ≤0.05.

Transcripts Cluster Id	Gene symbol	Gene description	Fold change	P-value
10933664	Phex	phosphate regulating endopeptidase homolog, X-linked	10.90	0.037
10775375	Dmp1	dentin matrix acidic phosphoprotein 1	9.74	0.036
10744687	Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	7.87	0.046
10928191	Satb2	SATB homeobox 2	7.22	0.045
10761446	LOC687426	similar to G protein-coupled receptor 133	6.65	0.031
10853963	Ptprz1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	6.02	0.037
10726676	Ifitm5	interferon induced transmembrane protein 5	5.84	0.032
10818502	Col11a1	collagen, type XI, alpha 1	5.64	0.047
10817583	Itga10	integrin, alpha 10	5.60	0.031
10764050	Fmod	fibromodulin	5.38	0.032
10871775	Rhbdl2	rhomboid, veinlet-like 2 (Drosophila)	5.30	0.031
10809540	Mmp2	matrix metallopeptidase 2	5.24	0.049
10729667	Dkk1	dickkopf homolog 1 (Xenopus laevis)	5.23	0.031
10883212	Cgref1	cell growth regulator with EF hand domain 1	4.98	0.049
10739455	Gprc5c	G protein-coupled receptor, family C, group 5, member C	4.76	0.031
10890951	Slc8a3	solute carrier family 8 (sodium/calcium exchanger), member 3	4.71	0.031
10869616	Ptprd	protein tyrosine phosphatase, receptor type, D	4.67	0.037
10715416	Loxl4	lysyl oxidase-like 4	4.50	0.032
10915131	Fat3	FAT tumor suppressor homolog 3 (Drosophila)	4.39	0.041
10847474	Creb3l1	cAMP responsive element binding protein 3-like 1	4.38	0.032
10759177	Tmem119	transmembrane protein 119	4.37	0.039
10733258	Adamts2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	4.33	0.034
10773435	Cpz	carboxypeptidase Z	4.30	0.034
10744939	Serpinf1	serine (or cysteine) peptidase inhibitor, clade F, member 1	4.23	0.046
10742766	Slc36a2	solute carrier family 36 (proton/amino acid symporter), member 2	4.20	0.032
10726620	Sprn	shadow of prion protein homolog (zebrafish)	4.16	0.041
10895075	Dcn	decorin	4.06	0.044
10855348	Zfp862	zinc finger protein 862	3.91	0.034
10752695	Robo2	roundabout, axon guidance receptor, homolog 2 (Drosophila)	3.89	0.037
10846340	Fkbp7	FK506 binding protein 7	3.88	0.046
10739449	Gprc5c	G protein-coupled receptor, family C, group 5, member C	3.85	0.039
10860499	Sema3d	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	3.70	0.031
10715667	Kazald1	Kazal-type serine peptidase inhibitor domain 1	3.67	0.034
10866535	Rerg	RAS-like, estrogen-regulated, growth-inhibitor	3.65	0.031

Table 6. List of top 25 differentially expressed genes at AT-I (Day 4 vs. day2) with their fold regulation in AT-I, AT-II.

Gene symbol	Gene description	Fold change ([ATI-Day4] vs [ATI-Day2])	Fold change ([ATII-Day4] vs [ATII-Day2])
Panx3	pannexin 3	14.85*	10.79
Aspn	asporin	14.61*	6.66
Phex	phosphate regulating endopeptidase homolog, X-linked	13.14*	10.90*
Bglap	bone gamma-carboxyglutamate (gla) protein	12.66	10.05
Omd	osteomodulin	10.70*	7.11
Slc13a5	solute carrier family 13 (sodium-dependent citrate transporter), member 5	10.15*	7.87*
Smpd3	sphingomyelin phosphodiesterase 3, neutral	9.89*	6.85
Dmp1	dentin matrix acidic phosphoprotein 1	9.67*	9.74*
Satb2	SATB homeobox 2	9.15*	7.22*
Bmp3	bone morphogenetic protein 3	8.25*	5.93
Mmp16	matrix metallopeptidase 16	7.00*	5.42
Ibsp	integrin-binding sialoprotein	6.94	4.91
Ifitm5	interferon induced transmembrane protein 5	6.65*	5.84*
Ptprz1	protein tyrosine phosphatase, receptor-type, Z polypeptide 1	6.60*	6.02*
Mamdc2	MAM domain containing 2	6.36*	5.36
Dcn	decorin	6.26*	4.06*
Mmp2	matrix metallopeptidase 2	6.18*	5.24*
Ptprd	protein tyrosine phosphatase, receptor type, D	6.14*	4.67*
Col11a1	collagen, type XI, alpha 1	5.91*	5.64*
Cgref1	cell growth regulator with EF hand domain 1	5.90*	4.98*
Pth1r	parathyroid hormone 1 receptor	5.88*	4.46
Adamts18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	5.52*	5.03
Cthrc1	collagen triple helix repeat containing 1	5.43*	4.09
Cercam	cerebral endothelial cell adhesion molecule	5.23*	4.49

*P-value <0.05.

Table 7. Top 25 downregulated genes at AT-I.

Gene symbol	Gene description	Fold change ([AT-I-Day4] vs. [AT-I-Day2])
Cxcl2	chemokine (C-X-C motif) ligand 2	-9.74
Niacr1	niacin receptor 1	-5.88
Irg1	immunoresponsive 1 homolog (mouse)	-5.33
Car4	carbonic anhydrase 4	-5.06
Nos2	nitric oxide synthase 2, inducible	-4.90
Ccl3	chemokine (C-C motif) ligand 3	-4.72
Il1b	interleukin 1 beta	-4.56
Cxcl1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-4.29
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	-4.29
Il1a	interleukin 1 alpha	-3.73
G0s2	G0/G1switch 2	-3.72
Isg15	ISG15 ubiquitin-like modifier	-3.64
Ptgs2	prostaglandin-endoperoxide synthase 2	-3.46
Il6	interleukin 6	-3.21
Il23a	Interleukin 23, alpha subunit p19	-3.17
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	-3.14
Upp1	uridine phosphorylase 1	-3.13
Cxcl3	chemokine (C-X-C motif) ligand 3	-3.10
Clec4e	C-type lectin domain family 4, member e	-3.09
Rab20	RAB20, member RAS oncogene family	-3.07
Ier3	immediate early response 3	-2.96
Cd274	CD274 molecule	-2.81
Osm	oncostatin M	-2.74
Slpi	secretory leukocyte peptidase inhibitor	-2.73
Il1rn	interleukin 1 receptor antagonist	-2.64

Table 8. Top 25 downregulated genes at AT-II.

Gene symbol	Gene description	Fold change ([ATII-Day4] vs. [ATII-Day2])
Cxcl2	chemokine (C-X-C motif) ligand 2	-3.22
Niacr1	niacin receptor 1	-3.08
Il6	interleukin 6	-2.87
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	-2.47
Upp1	uridine phosphorylase 1	-2.24
Ass1	argininosuccinate synthetase 1	-2.20
Rab20	RAB20, member RAS oncogene family	-2.20
Irg1	immunoresponsive 1 homolog (mouse)	-2.15
Il1b	interleukin 1 beta	-2.13
Fam111a	family with sequence similarity 111, member A	-2.12
G0s2	G0/G1switch 2	-2.10
Far2	fatty acyl CoA reductase 2	-2.09
Clec4e	C-type lectin domain family 4, member e	-2.07
Prtn3	proteinase 3	-2.06
Plekhb1	pleckstrin homology domain containing, family B (evectins) member 1	-2.05
Ms4a3	membrane-spanning 4-domains, subfamily A, member 3	-2.03
Fetub	fetuin B	-1.97
RGD1565374	similar to hypothetical protein LOC199675	-1.96
Slpi	secretory leukocyte peptidase inhibitor	-1.94
Car4	carbonic anhydrase 4	-1.93
Cxcl3	chemokine (C-X-C motif) ligand 3	-1.87
Mx2	myxovirus (influenza virus) resistance 2	-1.86
Tspo	translocator protein	-1.82
Nos2	nitric oxide synthase 2, inducible	-1.82
Il1r2	interleukin 1 receptor, type II	-1.81

Table 9. The validation of the array data was carried out by qRT-PCR of select number of transcripts for equal number of AT-I and AT-II samples (n=3).

Gene symbol	Gene description	Affymetrix fold change AT-I (day 4 vs. day 2)	qRT-PCR fold change AT-I (day 4 vs. day 2)	Affymetrix fold change AT-II (day 4 vs. day 2)	qRT-PCR fold change AT-II(day 4 vs. day 2)
Panx3	pannexin 3	14.85*	16.95	10.79	10.18
Phex	phosphate regulating endopeptidase homolog, X-linked	13.14*	14.31	10.90*	10.74
Satb2	SATB homeobox 2	9.15*	10.05	7.22*	7.25
Ibsp	integrin-binding sialoprotein	6.94	7.99	4.91	5.73
Cxcl2	chemokine (C-X-C motif) ligand 2	-9.74	-10.4	-3.22	- 3.7
Ccl3	chemokine (C-C motif) ligand 3	-4.72	-4.93	-1.71	- 2.02
Il1b	interleukin 1 beta	-4.56	-4.17	-2.13	-2.17

Table 10. Top 60 GO terms for differentially regulated genes at AT-I (Day 4 vs Day2). 80 terms satisfied $p < 0.05$

GO ACCESSION	GO Term	Corrected p-value	Count in Selection
GO:0031012	extracellular matrix	5.39E-12	24
GO:0007275	multicellular organismal development	4.90E-10	46
GO:0032502	developmental process	1.35E-09	50
GO:0044421	extracellular region part	8.66E-09	24
GO:0001503	ossification	8.66E-09	14
GO:0048856	anatomical structure development	1.73E-08	41
GO:0007155	cell adhesion	1.73E-08	25
GO:0022610	biological adhesion	1.73E-08	25
GO:0048731	system development	2.15E-08	38
GO:0005576	extracellular region	9.42E-08	42
GO:0001501	skeletal system development	1.01E-07	15
GO:0005578	proteinaceous extracellular matrix	1.18E-07	14
GO:0048513	organ development	5.24E-07	28
GO:0009653	anatomical structure morphogenesis	1.50E-06	23
GO:0048705	skeletal system morphogenesis	8.29E-06	6
GO:0002062	chondrocyte differentiation	2.37E-05	7
GO:0009887	organ morphogenesis	3.24E-05	10
GO:0031175	neuron projection development	7.56E-05	14
GO:0048812	neuron projection morphogenesis	7.56E-05	12
GO:0007409| GO:0007410	axonogenesis	8.38E-05	11
GO:0000904	cell morphogenesis involved in differentiation	8.56E-05	12
GO:0048666	neuron development	9.23E-05	14
GO:0048468	cell development	1.02E-04	19
GO:0030030	cell projection organization	1.53E-04	14
GO:0048667	cell morphogenesis involved in neuron differentiation	2.46E-04	11
GO:0048858	cell projection morphogenesis	2.54E-04	12
GO:0007399	nervous system development	2.71E-04	19
GO:0048699	generation of neurons	2.71E-04	14
GO:0032990	cell part morphogenesis	2.71E-04	12
GO:0007156	homophilic cell adhesion	2.71E-04	10
GO:0009888	tissue development	2.88E-04	13
GO:0022008	neurogenesis	3.56E-04	14
GO:0004222	metalloendopeptidase activity	4.03E-04	10
GO:0030154	cell differentiation	4.43E-04	27
GO:0051216	cartilage development	5.73E-04	9
GO:0005886| GO:0005904	plasma membrane	6.57E-04	50
GO:0000902| GO:0007148| GO:0045790| GO:0045791	cell morphogenesis	6.57E-04	13
GO:0048869	cellular developmental process	8.35E-04	27
GO:0030182	neuron differentiation	8.75E-04	14
GO:0001649	osteoblast differentiation	9.74E-04	5
GO:0071944	cell periphery	0.001	50
GO:0032989	cellular component morphogenesis	0.002	13
GO:0016337	cell-cell adhesion	0.002	11
GO:0005509	calcium ion binding	0.003	20

GO:0035107	appendage morphogenesis	0.003	7
GO:0035108	limb morphogenesis	0.003	7
GO:0030199	collagen fibril organization	0.003	5
GO:0048736	appendage development	0.004	7
GO:0060173	limb development	0.004	7
GO:0008237	metallopeptidase activity	0.004	11
GO:0043062	extracellular structure organization	0.005	8
GO:0005913	cell-cell adherens junction	0.005	6
GO:0060348	bone development	0.006	2
GO:0030198	extracellular matrix organization	0.007	8
GO:0030326	embryonic limb morphogenesis	0.007	7
GO:0035113	embryonic appendage morphogenesis	0.007	7
GO:0030282	bone mineralization	0.009	4
GO:0031214	biomineral tissue development	0.009	5
GO:0042127	regulation of cell proliferation	0.011	13
GO:0004714	transmembrane receptor protein tyrosine kinase activity	0.014	2

Table 11. Top 60 GO terms for the differentially regulated genes at AT-II satisfying the criteria of fold upregulation >2 and p-value <0.05. 70 terms satisfying the p-value cut off of 0.05

GO ACCESSION	GO Term	Corrected p-value	Count in Selection
GO:0031012	extracellular matrix	3.92E-10	18
GO:0005576	extracellular region	3.78E-07	32
GO:0005578	proteinaceous extracellular matrix	3.78E-07	11
GO:0044421	extracellular region part	6.84E-07	18
GO:0007155	cell adhesion	7.27E-07	19
GO:0022610	biological adhesion	7.27E-07	19
GO:0032502	developmental process	2.98E-05	25
GO:0048856	anatomical structure development	5.09E-05	21
GO:0007275	multicellular organismal development	6.86E-05	25
GO:0030030	cell projection organization	6.86E-05	11
GO:0048731	system development	1.69E-04	21
GO:0048699	generation of neurons	2.17E-04	12
GO:0031175	neuron projection development	4.09E-04	11
GO:0048468	cell development	5.76E-04	12
GO:0022008	neurogenesis	6.87E-04	12
GO:0000904	cell morphogenesis involved in differentiation	7.09E-04	9
GO:0030198	extracellular matrix organization	7.14E-04	8
GO:0022603	regulation of anatomical structure morphogenesis	7.75E-04	2
GO:0048666	neuron development	7.82E-04	11
GO:0007156	homophilic cell adhesion	8.56E-04	8
GO:0048812	neuron projection morphogenesis	8.56E-04	9
GO:0008237	metallopeptidase activity	8.84E-04	10
GO:0004222	metalloendopeptidase activity	0.001	8
GO:0001501	skeletal system development	0.001	7
GO:0043062	extracellular structure organization	0.001	8
GO:0007409| GO:0007410	axonogenesis	0.001	8
GO:0009653	anatomical structure morphogenesis	0.001	11
GO:0000902| GO:0007148| GO:0045790| GO:0045791	cell morphogenesis	0.002	10
GO:0048858	cell projection morphogenesis	0.002	9
GO:0032990	cell part morphogenesis	0.002	9
GO:0001503	ossification	0.003	3
GO:0030182	neuron differentiation	0.003	11
GO:0005886| GO:0005904	plasma membrane	0.003	35
GO:0048667	cell morphogenesis involved in neuron differentiation	0.003	8
GO:0032989	cellular component morphogenesis	0.004	10
GO:0007399	nervous system development	0.005	15
GO:0071944	cell periphery	0.005	35
GO:0045595	regulation of cell differentiation	0.005	3
GO:0016043	cellular component organization	0.006	20
GO:0008046	axon guidance receptor activity	0.006	3
GO:0060284	regulation of cell development	0.009	2
GO:0021891	olfactory bulb interneuron development	0.009	2
GO:0030154	cell differentiation	0.009	15
GO:0030199	collagen fibril organization	0.010	4

GO:0021889	olfactory bulb interneuron differentiation	0.013	2
GO:0048513	organ development	0.013	14
GO:0048869	cellular developmental process	0.013	15
GO:0071840	cellular component organization or biogenesis	0.013	20
GO:0002062	chondrocyte differentiation	0.014	3
GO:2000026	regulation of multicellular organismal development	0.015	2
GO:0005592	collagen type XI	0.017	2
GO:0050925	negative regulation of negative chemotaxis	0.017	2
GO:0009887	organ morphogenesis	0.018	4
GO:0016337	cell-cell adhesion	0.021	8
GO:0051128	regulation of cellular component organization	0.023	2
GO:0019838	growth factor binding	0.030	1
GO:0050793	regulation of developmental process	0.030	3
GO:0009888	tissue development	0.032	5
GO:0009880	embryonic pattern specification	0.038	2
GO:0010453	regulation of cell fate commitment	0.038	1

Table 12. Top 25 GO terms for downregulated genes at AT-I (Day 4 vs Day 2). 205 terms satisfied p <0.05 and fold dowregulation >2.

GO ACCESSION	GO Term	Corrected p-value	Count in Selection
GO:0006954	inflammatory response	1.79E-14	17
GO:0005126	cytokine receptor binding	1.81E-12	10
GO:0006952| GO:0002217| GO:0042829	defense response	1.81E-12	19
GO:0005125	cytokine activity	3.36E-12	13
GO:0009611| GO:0002245	response to wounding	4.65E-12	18
GO:0009607	response to biotic stimulus	8.17E-12	15
GO:0006955	immune response	8.61E-11	16
GO:0006950	response to stress	9.78E-11	23
GO:0002376	immune system process	2.06E-10	18
GO:0051707| GO:0009613| GO:0042828	response to other organism	1.56E-09	14
GO:0051704| GO:0051706	multi-organism process	3.37E-09	14
GO:0009617| GO:0009618| GO:0009680	response to bacterium	1.50E-08	12
GO:0032496	response to lipopolysaccharide	2.85E-08	10
GO:0002237	response to molecule of bacterial origin	4.57E-08	10
GO:0008009	chemokine activity	4.57E-08	7
GO:0042379	chemokine receptor binding	5.14E-08	7
GO:0042493| GO:0017035	response to drug	8.89E-08	14
GO:0009605	response to external stimulus	1.26E-07	13
GO:0030593	neutrophil chemotaxis	1.33E-07	6
GO:0050896| GO:0051869	response to stimulus	1.58E-07	35
GO:0051384	response to glucocorticoid stimulus	2.02E-07	9
GO:0031960	response to corticosteroid stimulus	3.71E-07	9
GO:0050900	leukocyte migration	8.66E-07	7
GO:0030595	leukocyte chemotaxis	2.32E-06	6
GO:0060326	cell chemotaxis	4.96E-06	6

Table 13. GO terms related to downregulated genes at AT-II. 21 terms satisfied p <0.05 and fold dowregulation >2.

GO ACCESSION	GO Term	corrected p-value	Count in Selection
GO:0001780	neutrophil homeostasis	0.008	2
GO:0001781	neutrophil apoptosis	0.004	2
GO:0002237	response to molecule of bacterial origin	0.013	4
GO:0002262	myeloid cell homeostasis	0.016	2
GO:0002376	immune system process	0.016	6
GO:0002526	acute inflammatory response	0.016	2
GO:0005125	cytokine activity	0.047	2
GO:0006952| GO:0002217| GO:0042829	defense response	0.014	5
GO:0006953	acute-phase response	0.004	2
GO:0006954	inflammatory response	0.003	5
GO:0006955	immune response	0.013	5
GO:0009611| GO:0002245	response to wounding	0.016	5
GO:0009617| GO:0009618| GO:0009680	response to bacterium	0.024	4
GO:0010574	regulation of vascular endothelial growth factor production	0.016	1
GO:0030593	neutrophil chemotaxis	0.047	2
GO:0032496	response to lipopolysaccharide	0.012	4
GO:0032755	positive regulation of interleukin-6 production	0.047	2
GO:0033028	myeloid cell apoptosis	0.005	2
GO:0045429	positive regulation of nitric oxide biosynthetic process	0.047	2
GO:0050995	negative regulation of lipid catabolic process	0.016	2
GO:0051707| GO:0009613| GO:0042828	response to other organism	0.047	4

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Chapter 4

Early molecular assessment of osseointegration in humans

Abstract:

Objective: To determine the early temporal wide genome transcription regulation by the surface topography at the bone-implant interface of implants bearing micro-roughened or superimposed nanosurface topology.

Materials and methods: Four commercially pure titanium implants (2.2 x5.0mm) with either a moderately roughened surface (TiOblast) or super-imposed nanoscale topography (Osseospeed) were placed (n=2/surface) in edentulous sites of eleven systemically healthy subjects and subsequently removed after 3 and 7 days. Total RNA was isolated from cells adherent to retrieved implants. A whole genome microarray using the Affymetrix Human gene 1.1 ST Array was used to describe the gene expression profiles that were differentially regulated by the implant surfaces.

Results: There were no significant differences when comparing the two-implant surfaces at each time point. However, the microarray identified several genes that were differentially regulated at day 7 vs. day 3 for both implant surfaces. Functionally relevant categories related to the extracellular matrix, collagen fibril organization and angiogenesis were upregulated at both surfaces (day7 vs. day3). Abundant upregulation of several differential markers of alternative activated macrophages was observed (eg. *MRC1*, *MSR1*, *MS4A4A*, *SLC38A6* and *CCL18)*. The biological processes involved with the inflammatory/immune response gene expression were concomitantly downregulated.

Conclusions: Gene regulation implicating collagen fibrillogenesis and extracellular matrix organization as well as the inflammatory/immune responses involving the alternative activated pathway are observed in implant adherent cells at early (3-7 days) after implantation. These gene expression events may indicate an important role for surface in modulating collagen fibrillogenesis as well as immunomodulation that play pivotal roles in altering bone accrual and biomechanical physical properties of the implant-bone interface.

Introduction:

Dental implants are of therapeutic benefit in the pursuit of alloplastic tooth replacement. The success of contemporary dental implants is largely attributed to the process of osseointegration. Osseointegration is a term defined as the direct structural and functional combination between the bone and the implant at the level of light microscopy [1]. The formed bone tissue microstructure is predominantly affected by the remodeling processes occurring in the early phase of peri-implant healing at the level of the bone-implant interface [2-4]. The implant surface serves as an intricate signaling cue for the attached cells playing an important role in altering the rate and quality of osseointegration [5-8]. The different physico/chemical features of the implant surface influence the biomolecular and cellular interactions with the implant [9-15]. Roughened surfaces enhance focal adhesion formation [16], promote cell commitment to the osteoblastic lineage and support greater expression of phenotype specific markers [17-19]. These changes result in greater bone formation evidenced by in-vivo histomorphometric and removal torque studies with improved bone anchorage of the roughened titanium implants than smoother surfaces [7,20]. In the dynamic research field of implants, a multitude of biomaterial modification approaches with nanoscale features have been pursued [21]. Various reports support that embellishment of the micron-rough surface with nanoscale features further enhances osteoblast differentiation [9,22-24], which could also promote stability and increase interfacial biomechanical locking with bone [25-28].

In contrast to the direct bone to implant contact representing osseointegration, fibrous encapsulation of implants leading to their failures remains a clinical challenge in situations where there is lack of primary implant stability, poor bone quality, or patients with compromised healing capacity such as those with a history of head and neck irradiation, uncontrolled diabetes, chronic periodontitis, smoking, etc.[29-31]. Fibrous encapsulation represents a repair mechanism of tissue directed by the cellular responses to the implanted device environment.

A comprehensive understanding of the molecular and cellular processes relevant to peri-implant healing is critical for achieving therapeutically relevant targets to positively influence implant osseointegration. Research devoted to deciphering the molecular mechanisms controlling implant-bone interface development is mostly interrogated through in vitro investigation in cell culture. A systematic review revealed approximately 90% of these studies focused on osteoblastic cell model able to differentiate under culture stimulation [32]. Key

observations indicated that adhesion to surfaces of increased topographic magnitude results in increased osteoinductive and bone matrix specific gene expression. Achieving optimum peri-implant healing requires an orchestration of the complex biological and molecular events of cell migration, proliferation, extracellular matrix (ECM) deposition, angiogenesis and remodeling. Apposition of a mature mineralized bone matrix is dependent on pivotal events of the early healing cascade. For example, the quality of the bone-implant interface may be determined by the hierarchical micro-architecture of its ECM constituents in which collagen plays a central role [33]. A recent in vitro investigation described the differential regulation of collagen biosynthesis in cells adherent to smooth and rough titanium surfaces [34]. This revealed greater collagen biosynthesis and enhanced expression of several collagen-modifying genes including prolyl hydroxylases, lysyl oxidases and lysyl hydroxylases within cells adherent to the roughened titanium substrates. Information about the role of the aforementioned genes and other potential collagen modifying genes in modulating implant integration in in-vivo models is still missing.

Additionally, among the many factors contributing to non-healing is impairment in the production of cytokines by local inflammatory cells and fibroblasts and reduced angiogenesis. Implant adherent macrophages and multinuclear cells are detected prior to bone formation at the surfaces of different materials inserted in bone [35]. However, their role in osseointegration remains poorly defined. Recent evidence suggests a positive influence of macrophages during bone healing in fracture models. Alexander et al [36] demonstrated that depletion of macrophages in Mafia transgenic mice is associated with significantly suppressed collagen type I bone matrix deposition and mineralization during bone healing in vivo in a tibia fracture model.

While in vitro studies can be a valuable tool and provide important insights into the biological mechanisms underlying osseointegration, they do not fully recapitulate the complex in-vivo microenvironment [37,38]. Various animal models have been used previously to evaluate the early molecular events at the implant surface interface. Most studies employed either qRT-PCR or immunohistochemistry [39]. However, these studies are limited in the number of genes that could be identified due to the non-global nature of these approaches. A recent investigation evaluated early molecular events (day4 vs day2) occurring at either a moderately roughened surface (AT-II) or a moderately roughened surface with super-imposed nanofeatures (AT-I) in a rat model using Affymetrix array [40]. Functionally relevant categories related to ossification, skeletal system development, osteoblast differentiation, bone development, bone mineralization and biomineral tissue development were

upregulated and more prominent with at AT-I (day 4 vs. day2) compared to AT-II. However, the bone turnover and resorption in a rat is several times faster than in a human [41]. In contrast to the rat model where early signs of new bone formation appear within 7 days after implantation and complete bone formation around a moderately roughened implant is achieved in 28 days [42- 44], osseointegration in human bone occurs at a slower pace and is achieved later [45]. Still, these timelines are significantly affected by the implant surface topography [46]. Understanding the kinetics of early phases of healing in human in vivo models of osseointegration should be more fully investigated. The aim of this study is to determine the early temporal molecular events associated with osseointegration in a human model in the implant adherent cells by assessing the whole genome expression profile at days 3 and 7 following the insertion of either a micro-roughened implant (TiOblast) or a moderately roughened with superimposed nanofeatures (Osseospeed).

Materials and methods:

Preparation of the model implants:

Forty-four screw-shaped mini-implants (2.2 x5.0 mm) were manufactured by AstraTech Dental (AstraTech, Molndal, Sweden) from grade IV commercially pure titanium. One sample group (n=22) was grit blasted with 75μm particles TiO2 (AstraTech AB TiOBlast^{TM}), while the other group (n=22) was blasted with TiO2 particles in a similar manner and further subjected to HF immersion treatment according to Osseospeed manufacturing procedure (AstraTech AB Osseospeed^{TM}). All samples were washed by sterile water and beta-sterilized according to standard protocols for manufacture of dental implants.

Previous studies on surface roughness, expressed in S_a-values according to the methods described by Wennerberg et al. [47] reported S_a-values ~ 1-1.2 μm for TiOBlast implants (TiO-moderately roughened) and 1.4-1.5 μm for the Osseospeed implants (OS). HF immersion produces discrete 50-200 nm nanofeatures on the OS surface (moderately roughened with superimposed nanofeatures). In addition, XPS analysis studies identified the presence of fluoride at approximately 1.0 % on the OS surface but not on the TiO surface [22,48].

Subjects' inclusion

The study protocol was approved by the Institutional Review Board (IRB) at University of North Carolina at Chapel Hill (IRB protocol #10-1963).

Eleven systemically healthy individuals who were either partially or completely edentulous were included in this study. The absence of at least 2 teeth, with adequate bone volume to allow placement of four mini-implants without impingement on vital anatomical structures (sinuses, nerves and adjacent teeth) and an edentulous period of at least 6 months were defined criteria for enrollment. Patients who were smokers, or with any systemic condition that could affect bone healing such as uncontrolled diabetes, history of radiotherapy in the head and neck region, taking corticosteroids or bisphosphonates, etc, were excluded. Included were 9 females and 2 males between the ages 47-69 with a mean age of 60.2.

Surgery:

Prior to surgery, informed written consents to participate in this study were obtained. Panoramic x-rays were made to assess the height of the alveolar bone as well as proximity to vital structures in the edentulous planned surgical sites. The radiographs were also screened for any pathology.

Following anesthesia, two separate crestal incisions approximately 6mm in length were made and full thickness mucoperiosteal flaps were raised. Each subject received two TiO and two OS implants. Two osteotomies were prepared per site to allow placement of one implant of each surface (1 TiO and 1 OS). This paired surgical design permitted the retrieval of the first 2 implants (1 of each surface) at the earlier time point without disturbing the healing of the other 2 implants. Drilling was completed using a 2.0 mm twist osteotomy drill under profuse irrigation with sterile saline. Single use osteotomy drills were used on each subject. All implants were placed by a self-tapping procedure and primary stability at the time of implant placement was insured. A submerged implant installation technique was used and the mucosal tissues were closed primarily with 4-0 chromic gut sutures.

Implants retrieval:

At 3 and 7 days following surgery, one surgical site was chosen at random, re-entered and the paired (One TiO and one OS) implants were removed by reverse threading. The implants were immediately rinsed in cold PBS and then placed into 1000 μl of Tri-reagent (Invitrogen, Carlsbad, CA). Cell lysates were snap frozen, and stored at -80°C until further use. Tissues were again reapproximated and closed with 4.0 chromic gut sutures.

RNA isolation and microarray hybridization:

Total RNA was isolated from the lysates using the standard Tri-reagent protocol and collected by ethanol precipitation. This was followed by purification using RNeasy MinElute Clean up kit (Qiagen, Valencia, CA, USA). RNA was assessed for quality and quantity using a bioanalyzer (Agilent, Santa Clara, CA, USA) and nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) respectively. Samples were processed and hybridized to the Affymetrix Human Gene 1.1 ST Array at the UNC core facility following the manufacturer's recommended protocol and reagents (Affymetrix, Santa Clara, CA). The Human Gene 1.1 ST Array Plate interrogates more than 27,000 well-annotated genes. The raw microarray data is available online at the NCBI-GEO ((http://www.ncbi.nlm.nih.gov/geo/) database, accession number GSE41446.

Microarray data analysis:

Data analysis was completed using GeneSpring software v.12.0 (Agilent Technologies, Santa Clara, CA). 2-Way ANOVAs statistical analysis was applied to determine differentially expressed genes among the various parameters (implant surface type and time points). Further analysis included pairwise comparisons of each implant surface independently at the different time points (day 7 vs. day 3). A p-value of 0.05 was set as the threshold for statistical significance. Exported gene lists included significant genes; fold changes and p-values for comparisons. These lists of genes were then condensed into organized classes of related biology using the GO Ontology Browser function in the GeneSpring. Significant GO ($p < 0.05$) was corrected for multiple sampling using the method of Benjamini and the Hochberg false discovery rate method [49].

Results:

Microarray

2-Way ANOVA was applied to determine differentially expressed genes among the various parameters (implant surface type and time points). No genes were identified (p-value<0.05) to be significantly different when comparing the 2 implants surfaces at each time point. Next, pairwise comparisons were made to determine the time-course dependent effects on gene expression profiles at either surface (TiO, OS) independently (day 7 vs. day3). 6450 genes were found to be differentially expressed (Fold change (FC) >1.1; p-value ≤0.05) at OS; of these, 2495 were upregulated, and 3955 were downregulated. On the other hand, 13,292 genes were found to be differentially expressed (FC>1.1; p≤0.05) at TiO; of these, 3262 were

upregulated and 10,030 were downregulated (Table 1). The identity of the top 35 genes differentially upregulated at OS implants surfaces (day 7 vs. day 3; p-value<0.05) with their fold regulation in OS and TiO is shown in table 2. Another table (table 3) was created to include list of top 35 differentially downregulated genes (p-value≤0.05) at Osseospeed (Day 7 vs. day3) with their fold regulation in OS and TiO.

Identification of significant gene ontologies associated with the different implant surfaces

GO analysis was used to identify functional categories overrepresented in the gene lists of the differentially upregulated and downregulated genes between day 7 and day 3 (fold change ≥2; p ≤0.05) independently at both implants surfaces.

GO terms upregulated at Osseospeed and TiOblast (day 7 compared with day 3)

The gene functional annotation identified at OS and TiO consisted of a large variety of processes. 97 terms satisfied p≤0.05 at OS and 216 terms satisfied p≤0.05 at TiO. Table 4 outlines the top 30 GO terms for both surfaces. Functionally relevant categories relevant to the extracellular region and matrix, collagen fibril organization and blood vessel development were clearly demonstrated at both surfaces. Gene lists of differentially expressed genes relevant to the extracellular matrix category, collagen fibril organization, ossification and blood vessel development are represented in Table 5.

In this study, a high number of genes encoding ECM components were upregulated including several collagens (*COL1A1, COL1A2, COLA3A1, COL6A1, COL6A3, COL12A1*), glycoproteins (*SPARC, ECM1, CHI3L1, IBSP, GPNMB*), and proteoglycans (*DCN, BGN, LUM, GPC4*). Increased levels of several genes involved in collagen biosynthesis, processing and post-translational modifications including *PLOD1, PLOD2, PLOD3* and *LOX* were also identified. Differential upregulation of heat shock protein 47 (*HSP47*) and (procollagen C-endopeptidase enhancer) *PCOLCE* was also evident. In addition, we observed an upregulation of several matrix metallopeptidases (MMPs) including *MMP2, MMP7, MMP8, MMP9, MMP12, MMP13 and MMP14*. Upregulation of *TIMP2, TIMP3 and A2M* was also identified. Furthermore, several phenotypical markers characteristic of osteoclast/bone resorption activity including cathepsin K (*CTSK*), acid phosphatase 5, tartrate resistant (*ACP5*) were increased at both surfaces.

In addition, when we analyzed the lists of the differentially upregulated genes on both surfaces, we identified a number of genes (table 6) that have been associated with the alternatively activated macrophage (M2) including the membrane receptors mannose receptor, C type 1 (*MRC1*), macrophage scavenger receptor 1(*MSR1*), membrane-spanning 4-domains, subfamily A member 4 (*MS4A4A*), the solute carrier family 38, member 6 (*SLC38A6*), and the chemokines *CCL18, CCL22.*

GO terms downregulated at Osseospeed, TiOBlast (day 7 compared with day 3)

Further functional annotation analysis of the differentially downregulated genes at both surfaces highlighted several categories in this domain. 97 terms satisfied $p \leq 0.05$ in both surfaces. The functional categories differentially regulated were the same for both surfaces. Table 7 outlines the top 30 terms. An overrepresentation of functional annotations associated with chemotaxis, immune response and inflammatory response was demonstrated. Involved were several chemokines (*CCL3, CCL4, CXCL1, CXCL2, CXCL5, CCL20*), interleukins along with their associated receptors and kinases (*IRAK2, IL1RAP, IL1B, IL1A, IL8*), defensins (*DEFA3|DEFA1|DEFA1B, DEFB4A*), *TREM1* and prostaglandin-endoperoxide synthase 2 (*PTGS2*).

Discussion:

This study evaluated the global transcription regulation in implant adherent cells to either micro-roughened (TiO) or nanostructured (OS) titanium implants at an early phase of osseointegration (day 3 and 7) in human. Comparative analysis of gene expression profiles at these early time points did not reveal significant differences between both surfaces. To further comprehend the early molecular events determining the process of osseointegration, analysis of the time course dependent changes (day 7 vs. day 3) on each surface was performed. 6450 genes were found to be differentially expressed at OS and 13,292 genes were found to be differentially expressed at TiO. A large number of genes upregulated at this early phase of osseointegration were related to the extracellular matrix and collagen fibril organization. The expression of several collagen encoding mRNAs including *COL1A1, COL1A2, COL3A1*, *COL6A1, COL6A2* and *COL12A1* was increased. Collagens comprise 90% of the organic content of bone [50]. Type I collagen constitutes the major type of collagen in bone [51] . Essential to collagen integrity and bone mechanical strength is regulation of fibrillogenesis by providing specific molecular bridges between collagen fibrils and other matrix components [52]. Type III and type XII collagen modulates the size of type I collagen and play a role in collagen fibril

formation [53,54]. Dysregulation of proper fibrillogenesis can ultimately affect bone strength and biomechanical properties. For instance, Col12a1 $^{-/-}$ mice exhibits skeletal abnormalities including shorter, more slender long bones with decreased mechanical strength [54]. HSP47 is the molecular chaperone that assists in the folding and/or assembly of procollagen [55,56] and it was also upregulated. It is essential for the maturation of collagen. Fibrils of type I collagen secreted from hsp47-/- cells are abnormally thin and are more sensitive to protease digestion [57]. Collagens types I to III are secreted as procollagen precursors containing N and C propeptides that are cleaved to yield mature triple helical monomers that associate into fibrils. BMP1, also known as procollagen C proteinase, and related metalloproteinases cleave the C propeptides [58]. Attenuated BMP1 function compromises osteogenesis leading to bone fragility due to improper collagen fibrils maturation [59]. The activity of BMP1 is potentiated by a secreted glycoprotein, designated the procollagen C-endopeptidase enhancer [PCOLCE/procollagen C-proteinase enhancer (PCPE)] [60]. An increased expression of *PCOLCE* was identified in the implant adherent cells implicating the role of collagen maturation in achieving implant integration. Other non-collagenous proteins involved in ECM assembly and cell matrix interaction were also differentially upregulated including various proteoglycans (eg. *DCN, BGN, LUM, GPC4*) and glycoproteins (e.g. *SPARC, ECM1, IBSP*). Several of the ECM related proteins differentially expressed are known to play a direct role in bone formation; lumican [61], SPARC [62], ECM protein1 [63,64] , periostin [65], biglycan [66], decorin [67] and IBSP [68]. Decorin, biglycan and lumican are secreted extracellular small leucine-rich proteoglycans (SLRPs) that modulate collagen matrix assembly and regulate fibrillogenesis [66,67]. An earlier report demonstrated the role of proteoglycans in the establishment of a bone-titanium integration where the addition of GAG degradation enzymes significantly reduced the interfacial strength of the titanium and the mineralized osteoblastic tissue culture [69]. Enhanced expression of several genes involved in the posttranslational modification of collagen was also observed. Included were the Lysyl hydroxylases (*PLOD1, PLOD2, PLOD3)* and the Lysyl oxidase (*LOX)*. These genes encode for proteins involved in catalyzing the hydroxylation and the oxidative deamination of specific lysine and/or hydroxylysine residues in collagen respectively [70-73]. These posttranslational modifications greatly influence the pattern of collagen cross-linking. The organization, density and the amount of cross-linking of collagen determine the mechanical strength and ultimately, hierarchical tissue organization .In addition, the type and quantity of cross-linking are crucial for proper mineralization [74,75]. This study highlights the role of various ECM related genes during

implant healing that might be crucial in the biological and biomechanical establishment of the bone-implant interface. These not only provide the structural support and the scaffold for mineralization, but also contain reservoirs of cell signaling motifs that may guide subsequent cellular adhesion and behavior [16].

In addition, increased expression of various MMPs (*MMP7, MMP8, MMP9, MMP13, MMP14*) occurred. MMPs are zinc-dependent endoproteases, which have the capacity to degrade a variety of extracellular components. They are also involved in bone resorption and remodeling by osteoclasts [76]. Establishment of osseointegration is a dynamic process; where there is a delicate interplay between bone resorption in contact regions and bone formation in contact free regions [77]. Several genes implicated with the osteoclastogenic bone resorption were upregulated including *CATK* and *ACP5*. Recent histological data in human implicated a potential role of osteoclasts in modulating the process of osseointegration, where no new bone formation was observed in areas with no prior resorptive processes visible [78,79]. The recruitment and invasion of cells during vasculogenesis also involve proteolytic activities and degradation of the extracellular matrix [80]. This interrelationship is highlighted by gene deletions in mice demonstrating the essential role of MMPs in the onset of angiogenesis in the fracture healing and bone formation [81-83]. Indeed, our bioinformatics analysis revealed an overrepresentation of the functional biological processes related to blood vessel development in cells adherent to the implant surface. The development of an elaborate vascular network maybe an important step for bone regeneration and successful integration of implants leading to turnover of necrotic bone that develops after implant placement. Precise regulation of MMP activity is crucial for preventing excessive ECM degradation that can hinder the formation of a functional bone-implant interface. Here, the array data identified differential expression of specific tissue inhibitors of metalloproteinases (TIMP2 and TIMP3) as well as the more broad-spectrum tissue inhibitor (A2M), which suggests the presence of tight mechanisms associated with the early remodeling processes. In contrast to most MMPs, MMP-12 (macrophage metalloelastase) which has potent extracellular remodeling properties [84], has also been shown to cleave and inactivate human ELR+CXC chemokines consistent with a role in controlling the duration of the inflammatory cellular infiltrate of PMNs [85]. MMP-12 is mainly expressed by macrophages [86] and its activity is one of the mechanisms to dampen inflammation and promote resolution.

The osseointegration process is a consequence for the coordinated interplay of several cell types. Implant adherent macrophages and multinuclear cells are detected prior to bone formation at the surfaces of different

materials inserted in bone [35]. While macrophages have an established role in wound healing through the activity of secreted cytokine, their potential influence of macrophages in modulating implant-tissue integration has been briefly described. A previous in vivo report demonstrated the preferential accumulation of macrophages on rough implant surfaces, which was associated with more rapid bone-like tissue production [87]. Furthermore, Cooper and coworkers established utilizing the murine macrophage cell line the influence of surface roughness on BMP2 expression, which directly affects osteoblasts cell maturation and bone formation [88-90]. In cell culture model, Hamlet et al suggested the potential role for downregulation of a macrophage's pro-inflammatory phenotype including cytokines and chemokines (eg. *Tnfα, Ccl2, Ccl5 Il-1ß, Il1α*) adjacent to nanoscale CaP-modified implants and chemically modified SLActive surfaces as a mechanism for the clinically proven superior osseointegration associated with these surface modifications [91,92]. Similarly in a human model, Bryington et al[48] demonstrated the influence of the presence of nanofeatures (OS) in mediating an immunomodulatory effect on macrophages by enhanced expression of IL-9 (a Th2-associated cytokine that reduces monocyte oxidative burst and TNF-α release) [93]) and IL-22 compared with micro-roughened surfaces (TiO).

The dynamic changes in the presence of polarized macrophages with pro-inflammatory (M1) and anti-inflammatory (M2) properties may affect the early healing processes to achieve implant-tissue integration. The classical 'M1' activation of monocytes (eg. LPS- Th1 cytokines-INF γ, IL-1B) leads to the production of the pro-inflammatory cytokines such as IL-1 β, IL-6 and TNF-α, whereas the alternative 'M2' macrophage activation (IL-4 activated or IL-13 activated-Th2 cytokines) exhibits an anti-inflammatory profile with the expression of IL-10. M2 macrophages are able to phagocytose without oxidant production [94] and their activation is associated with immunoregulation, matrix deposition and remodeling [95,96]. Omar et al [97] revealed surface specific signaling of human monocytes resulting in significantly greater expression of BMP2 and RUNX2 of hMSCs cultured with conditioned media at oxidized surfaces compared with machined surfaces. They implicated a role for classical activation of monocytes (LPS activation) with osteoblast differentiation of hMSCs. Their in vivo analysis of LPS coated implants demonstrated an important role for signaling of osteoclasts but involving the classically activated monocytes. How alternatively activated macrophages are engaged was not directly considered in this previous study. In the present report, however, abundant upregulation of genes related to acquisition of M2 properties [98,99] [100] was identified. The data set included

the membrane receptors mannose receptor, C type 1 (*MRC1*), macrophage scavenger receptor 1(*MSR1*), membrane-spanning 4-domains, subfamily A member 4 (*MS4A4A*), the solute carrier family 38, member 6 (*SLC38A6*), and the chemokines (*CCL18, CCL22).* Indirect and direct co-cultures of either M1 or M2 polarized human monocytes with hMSCs revealed their differential effects on the growth of hMSCs [101] where M2 macrophages and their associated cytokines supported the growth and proliferation of hMSCs, while M1 macrophages and their associated cytokines were detrimental to the growth and survival of hMSC. Hence, the type of macrophage cells (M2 vs. M1) may potentially impact the early process of osseointegration by harnessing the host tissue response towards enhanced survival and function of hMSCs repair cells. M2 macrophages release various chemokines including CCL18 [102], CCL22 [103] , the anti-inflammatory IL-10 and TGF β -1. CCL18 was suggested to play a role at the resolution stage of the inflammatory process as it upregulates IL-10, known for its capacity to inhibit production of Th1 cytokines. CCL18 can also induce an M2 spectrum macrophage phenotype even in the absence of IL-4 [104]. CCL18 activated macrophages can remove cellular debris and apoptotic cells in the absence of a respiratory burst. The development of the bone-implant interface requires the colonization of the implant surface with MSCs/osteoprogenitors cells and their subsequent proliferation and differentiation. Homing of MSCs to the implant site is essential. CCL22 is amongst the chemokines that serve as a migratory cue that drive transendothelial migration of MSCs [105]. Consistent with the notion where M2 activation stands at the cross road between inflammation and resolution, our array data indentified downregulation of several chemokines (*CCL3, CCL4, CXCL1, CXCL2, CXCL5, CCL20*), interleukins along with their associated receptors and kinases (*IRAK2, IL1RAP, IL1B, IL1A, IL8*), defensins (*DEFA3|DEFA1|DEFA1B, DEFB4A*), *TREM1* and prostaglandin-endoperoxide synthase 2 (*PTGS2*) [106-109]. Meanwhile, we also identified the differential upregulation of Chitinase 3-like 1 (*CH13L1*), also known as YKL-40. YKL-40 inhibits cellular responses induced by IL-1 and TNF-α [110] suggesting that the induction of YKL-40 feeds back to control local tissue responses. While a general picture of selection of M2 macrophages and diminished inflammation emerges in the context of the bone forming events at endosseous implants, further characterization of the roles of M2 macrophage is required.

Histomorphometric data using several animal models [26,111] demonstrates differential bone formation at the two surfaces annotated here (OS > TiO). Analysis of differential gene expression at this early time failed to identify significant differences between both surfaces. Moreover, upregulation of osteoblast differentiation

transcription factors was not demonstrated. In contrast, Bryington et al [48] were able to demonstrate a differential upregulation of OSX, a key transcription factor for osteoblast differentiation on the nanoscale featured implants compared with the microtopography TiOblast surface. A possible factor contributing to this difference is the age of the population included. In our study, the mean age was 60 years old as opposed to 36 year old in Bryington et al study. MSCs are susceptible to age-related changes. MSCs from older donors have an overall decline in their differentiation potential [112-114]. This implicates that modulation of osteogenic gene expression might have been delayed in our aging population. Hence, further investigations using later time periods may be needed to identify key elements essential for modulating the osteoblast differentiation. In support of this speculation, a previous study [115] using a trephine model of the peri-implant tissue in the retromolar area has identified sequential inflammatory (days 4), angiogenic (days 7) and osteogenic (days14) responses involved in the process of osseointegration. These repair responses were evaluated with SLA, SLActive implants. In addition, while this study incorporated the use of Affymetrix microarray, Bryington et al [48] utilized qRT-PCR to identify differential gene expression in implant adherent cells. Differences in the sensitivity of the platforms used for assessing mRNA gene expression might have contributed to the differences in the results obtained [116].

Our explanted model with implant adherent cells provides important information critical to driving the process of osseointegration at the implant-bone interface. One limitation to this approach is the scarcity of the RNA quantity on human explanted implants in this population that did not allow further validation of our microarray data using qRT-PCR.

Conclusions:

The present study investigated the global transcription regulation in implant adherent cells adherent to either a nanosurface or a moderately rough surface in a human model at early implantation times. In this study, novel biomolecular and cellular responses that underlie the process of osseointegration at this early phase were revealed. These genes exert a multitude of functions including ECM matrix organization, immune modulation, angiogenesis and skeletal remodeling. Numerous genes associated with the fibrillogenesis and cross-linking of the collagen matrix were identified. In addition, key markers for the alternatively activated ‘M2’ macrophage

were revealed. Further characterization of the roles of the genes identified is crucial to identifying potential targets for improving osseointegration.

Table 1: Number of genes differentially expressed (Day 7 vs. Day 3)

Comparison	Differentially expressed genes *	Upregulated genes*	Downregulated genes*	Differentially expressed (FC>2)
OS	6450	2495	3955	163
TiO	13292	3262	10,030	269

* Upregulated (>1.1) and downregulated (<1.1) genes with p-value <0.05. FC; fold change

Table 2:List of top 35 differentially upregulated genes at OS (Day 7 vs. day3) with their fold regulation and p-value in OS and TiO.

Gene symbol	Gene description	FC ([OS-Day7] vs [OS-Day3])	p-value	FC ([TiO-Day7] vs [TiO-Day3])	p-value
CTSK	cathepsin K	8.21	0.008	8.66	0.002
MMP12	matrix metallopeptidase 12 (macrophage elastase)	8.10	0.015	11.73	0.003
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	7.03	0.008	9.14	0.002
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)	6.91	0.014	9.73	0.004
TCTEX1D2| TM4SF19/// TM4SF19	Tctex1 domain containing 2 | transmembrane 4 L six family member 19///transmembrane 4 L six family member 19	5.67	0.011	7.53	0.002
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	4.92	0.013	8.72	0.003
LUM	lumican	4.84	0.019	5.53	0.002
COL6A3	collagen, type VI, alpha 3	4.50	0.013	4.79	0.002
COL3A1	collagen, type III, alpha 1	4.50	0.014	4.84	0.002
POSTN	periostin, osteoblast specific factor	4.47	0.014	4.13	0.003
COL1A1	collagen, type I, alpha 1	4.44	0.014	4.68	0.002
DCN	decorin	3.94	0.019	4.16	0.003
CHIT1	chitinase 1 (chitotriosidase)	3.87	0.033	7.50	0.007
ACP5	acid phosphatase 5, tartrate resistant	3.81	0.010	4.32	0.004
GM2A	GM2 ganglioside activator	3.77	0.013	4.86	0.002
PNLIP| COL1A2/// COL1A2	pancreatic lipase | collagen, type I, alpha 2///collagen, type I, alpha 2	3.75	0.015	3.73	0.002
COL12A1	collagen, type XII, alpha 1	3.41	0.015	3.16	0.003
TPM2	tropomyosin 2 (beta)	3.40	0.008	2.95	0.002
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	3.35	0.014	4.21	0.002
BGN	biglycan	3.29	0.016	4.20	0.002
LPL	lipoprotein lipase	3.17	0.026	3.48	0.008
GPNMB	glycoprotein (transmembrane) nmb	3.07	0.021	3.77	0.003
MSR1	macrophage scavenger receptor 1	3.05	0.022	2.78	0.006
C1QB	complement component 1, q subcomponent, B chain	3.04	0.016	3.49	0.004
MMP13	matrix metallopeptidase 13 (collagenase 3)	3.03	0.026	2.43	0.010
COL6A1	collagen, type VI, alpha 1	3.00	0.013	3.01	0.002
CDR1|YTHD C2	cerebellar degeneration-related protein 1, 34kDa | YTH domain containing 2	2.98	0.016	2.82	0.005
HIST1H3B	histone cluster 1, H3b	2.93	0.014	2.84	0.004
C1QC	complement component 1, q subcomponent, C chain	2.87	0.023	1.55	NS
LIPA	lipase A, lysosomal acid, cholesterol esterase	2.84	0.024	2.68	NS
CCL22	chemokine (C-C motif) ligand 22	2.82	0.020	5.76	0.006
BIRC5|EPR1	baculoviral IAP repeat-containing 5 | effector cell peptidase receptor 1 (non-protein	2.70	0.013	2.05	0.008

	coding)				
CCL18	chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	2.70	0.027	2.56	0.005
CKB	creatine kinase, brain	2.67	0.025	2.17	0.017
ATP1B1|NME7	ATPase, Na+/K+ transporting, beta 1 polypeptide | non-metastatic cells 7, protein expressed in (nucleoside-diphosphate kinase)	2.67	0.018	2.50	0.007

Table 3:List of top 35 differentially downregulated genes at OS (Day 7 vs. day3) with their fold regulation and p-value in OS and TiO.

Gene symbol	Gene description	FC ([OS-Day7] vs [OS-Day3])	p-value	FC ([TiO-Day7] vs [TiO-Day3])	p-value
DEFA3|DEFA1|DEFA1B	defensin, alpha 3, neutrophil-specific | defensin, alpha 1 | defensin, alpha 1B	-3.03	0.016	-2.80	0.014
SPRR2A|SPRR2B	small proline-rich protein 2A | small proline-rich protein 2B	-2.90	0.040	-3.00	0.014
SPRR2E	small proline-rich protein 2E	-2.75	0.037	-2.87	0.018
IL1B	interleukin 1, beta	-2.60	0.019	-3.58	0.008
KRT6C|KRT6B|KRT6A	keratin 6C | keratin 6B | keratin 6A	-2.58	0.038	-2.27	0.017
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	-2.35	0.021	-3.00	0.005
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-2.24	0.022	-2.80	0.013
LIPN	lipase, family member N	-2.23	0.036	-2.20	0.048
S100A2	S100 calcium binding protein A2	-2.19	0.039	-2.41	0.025
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	-2.18	0.045	-2.20	NS
CYTIP	cytohesin 1 interacting protein	-2.16	0.021	-4.67	NS
TMEM154	transmembrane protein 154	-2.15	0.022	-2.06	0.045
CSF3R	colony stimulating factor 3 receptor (granulocyte)	-2.12	0.032	-2.25	0.009
IL1A	interleukin 1, alpha	-2.11	0.033	-2.92	0.009
RGS2	regulator of G-protein signaling 2, 24kDa	-2.11	0.022	-2.00	NS
MIR222	microRNA 222	-2.06	0.027	-1.67	0.009
RNU11	RNA, U11 small nuclear	-2.02	0.025	-2.23	0.031
SPRR1B	small proline-rich protein 1B (cornifin)	-1.99	0.039	-1.94	0.016
NCF1|NCF1C|NCF1B	neutrophil cytosolic factor 1 | neutrophil cytosolic factor 1C pseudogene | neutrophil cytosolic factor 1B pseudogene	-1.98	0.034	-1.95	0.020
GPR97	G protein-coupled receptor 97	-1.98	0.029	-2.49	0.023
SPRR2D|SPRR2B///SPRR2B|SPRR2F|SPRR2D///SPRR2A|SPRR2B///SPRR2B	small proline-rich protein 2D | small proline-rich protein 2B///small proline-rich protein 2B | small proline-rich protein 2F | small proline-rich protein 2D///small proline-rich protein 2A | small proline-rich protein 2B///small proline-rich protein 2B	-1.97	0.041	-2.07	0.022
FPR1	formyl peptide receptor 1	-1.96	0.031	-2.04	0.030
IER3	immediate early response 3	-1.96	0.029	-2.27	0.030
IL1R2	interleukin 1 receptor, type II	-1.96	0.039	-1.74	ns
FOS	FBJ murine osteosarcoma viral oncogene homolog	-1.96	0.012	-1.22	0.038
SORL1	sortilin-related receptor, L(DLR class) A repeats-containing	-1.95	0.034	-1.71	0.020
IL1RAP	interleukin 1 receptor accessory protein	-1.92	0.036	-2.29	0.015
KCNJ15	potassium inwardly-rectifying channel,	-1.90	0.023	-2.00	0.021

	subfamily J, member 15				
TREM1	triggering receptor expressed on myeloid cells 1	-1.89	0.037	-2.59	0.017
DUSP1	dual specificity phosphatase 1	-1.87	0.020	-1.58	ns
ZFP36	zinc finger protein 36, C3H type, homolog (mouse)	-1.87	0.032	-1.99	ns
MIR223	microRNA 223	-1.85	0.030	-1.79	ns

Table 4: Top 30 GO terms for differentially upregulated genes at both surfaces (Day 7 vs. Day3). 98 terms satisfied p <0.05 at OS; 216 terms satisfied p < 0.05 at TiO.

GO Term - OS	p-value (OS)	Count in Selection (OS)	GO Term - TiO	p-value (TiO)	Count in Selection (TiO)
extracellular region part	3.59E-20	47	extracellular region part	2.89E-15	49
proteinaceous extracellular matrix	2.47E-16	27	proteinaceous extracellular matrix	1.61E-13	28
extracellular matrix	2.47E-16	28	extracellular matrix	1.61E-13	29
extracellular region	2.67E-14	57	extracellular region	4.79E-10	62
extracellular space	2.66E-10	30	extracellular space	7.65E-08	32
Extracellular matrix organization	2.66E-10	14	cytoplasmic part	7.65E-08	103
extracellular structure organization	2.66E-10	14	extracellular matrix part	8.92E-08	14
extracellular matrix part	8.31E-10	14	localization	1.05E-06	72
collagen fibril organization	3.59E-08	8	melanosome	2.17E-06	12
collagen	8.29E-07	8	pigment granule	2.17E-06	12
collagen metabolic process	9.39E-06	7	collagen fibril organization	1.82E-05	7
multicellular organismal macromolecule metabolic process	1.62E-05	7	establishment of localization	2.89E-05	63
multicellular organismal process	4.21E-05	66	cell surface	3.00E-05	18
multicellular organismal metabolic process	4.21E-05	7	mitochondrial inner membrane	3.65E-05	18
fatty acid binding	4.25E-05	6	transport	3.93E-05	62
pattern binding	1.09E-04	11	mitochondrial membrane	4.23E-05	21
polysaccharide binding	1.09E-04	11	mitochondrial envelope	8.55E-05	21
lipid binding	1.81E-04	17	organelle inner membrane	8.68E-05	18
tissue remodeling	3.31E-04	7	anatomical structure development	8.68E-05	59
cellular component organization	5.38E-04	43	regulation of plasma lipoprotein particle levels	8.68E-05	7
monocarboxylic acid binding	7.23E-04	6	multicellular organismal process	8.83E-05	83
platelet-derived growth factor binding	0.0018	4	blood vessel development	8.94E-05	15
carbohydrate binding	0.0018	14	vasculature development	1.21E-04	15
cellular component organization or biogenesis	0.0019	43	collagen	1.30E-04	7
extracellular matrix binding	0.0021	5	extracellular matrix organization	1.79E-04	10
fibrillar collagen	0.0021	4	extracellular structure organization	1.79E-04	10
ossification	0.0026	8	system development	2.20E-04	52
blood vessel development	0.0026	11	mitochondrial membrane part	2.29E-04	11
glycosaminoglycan	0.0026	9	mitochondrial part	2.37E-04	24

glycosaminoglycan binding	0.0026	9	mitochondrial part	2.37E-04	24
collagen biosynthetic	0.0026	3	cholesterol transport	2.48E-04	7

Table 5: list of differentially expressed genes in the functional categories related to ECM and collagen fibril organization, blood vessel development, ossification and osteoclastogenesis.

Extracellular matrix and collagen fibril organization					
Gene description	**Gene symbol**	**FC [OS-D7 vs OS-D3]**	**p-value**	**FC [TiO-D7 vs TiO-D3]**	**p-value**
periostin, osteoblast specific factor	POSTN	4.47	0.014	4.13	0.003
chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	4.92	0.013	8.72	0.003
collagen, type I, alpha 1	COL1A1	4.44	0.014	4.68	0.002
pancreatic lipase | collagen, type I, alpha 2///collagen, type I, alpha 2	PNLIP|COL1A 2///COL1A2	3.75	0.015	3.73	0.002
collagen, type III, alpha 1	COL3A1	4.50	0.014	4.84	0.002
collagen, type VI, alpha 1	COL6A1	3.00	0.013	3.01	0.002
collagen, type VI, alpha 3	COL6A3	4.50	0.013	4.80	0.002
collagen, type XII, alpha 1	COL12A1	3.41	0.015	3.16	0.003
coiled-coil domain containing 80	CCDC80	2.19	0.027	1.90	0.011
decorin	DCN	3.94	0.019	4.16	0.003
extracellular matrix protein 1	ECM1	2.01	0.011	1.86	0.003
glypican 4	GPC4	1.89	0.024	2.60	0.006
annexin A2	ANXA2	2.18	0.015	2.34	0.003
tenascin C	TNC	2.05	0.028	2.38	0.005
lectin, galactoside-binding, soluble, 1	LGALS1	1.90	0.013	2.02	0.002
lysyl oxidase	LOX	2.07	0.027	1.87	0.020
procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	PLOD1	1.77	0.013	1.90	0.002
procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	PLOD2	2.22	0.018	1.75	0.032
procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	PLOD3	1.45	0.021	1.53	0.006
lipoprotein lipase	LPL	3.17	0.026	3.48	0.008
lumican	LUM	4.84	0.019	5.53	0.002
matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	1.87	0.029	1.81	0.008
matrix metallopeptidase 7 (matrilysin, uterine)	MMP7	6.91	0.014	9.73	0.004
matrix metallopeptidase 8 (neutrophil collagenase)	MMP8	2.46	0.018	3.24	0.008
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	7.03	0.008	9.14	0.002
matrix metallopeptidase 12 (macrophage elastase)	MMP12	8.10	0.015	11.73	0.003
matrix metallopeptidase 13 (collagenase 3)	MMP13	3.03	0.026	2.43	0.011

matrix metallopeptidase 14 (membrane-inserted)	MMP14	2.35	0.013	2.51	0.002
	CD248	2.20	0.019	2.03	0.010
biglycan	BGN	3.29	0.016	4.20	0.002
solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3	1.75	0.028	2.16	0.008
secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	3.35	0.014	4.21	0.002
transforming growth factor, beta-induced, 68kDa	TGFBI	2.05	0.018	1.78	0.005
TIMP metallopeptidase inhibitor 2	TIMP2	2.09	0.013	2.11	0.002
TIMP metallopeptidase inhibitor 3	TIMP3	2.58	0.013	2.62	0.005
alpha-2-macroglobulin	A2M	2.49	0.020	2.87	0.006
calreticulin	CALR	1.72	0.024	2.02	0.005
procollagen C-endopeptidase enhancer	PCOLCE	2.50	0.017	2.12	0.006
serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	2.12	0.015	1.98	0.005
Ossification					
glycoprotein (transmembrane) nmb	GPNMB	3.07	0.021	3.77	0.003
collagen, type I, alpha 1	COL1A1	4.44	0.014	4.68	0.002
extracellular matrix protein 1	ECM1	2.01	0.011	1.86	0.003
integrin-binding sialoprotein	IBSP	2.00	0.034	1.96	0.023
ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1	2.13	0.037	1.94	0.006
Osteoclast-related genes					
cathepsin K	CTSK	8.21	0.008	8.66	0.002
acid phosphatase 5, tartrate resistant	ACP5	3.81	0.009	4.32	0.004
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	MMP9	7.03	0.008	9.14	0.002
matrix metallopeptidase 14 (membrane-inserted)	Mmp14	2.35	0.013	2.51	0.002
integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)		1.99	0.016	2.03	0.003
integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	1.83	0.047	1.84	0.017
Blood vessel development					
collagen, type I, alpha 1	COL1A1	4.44	0.014	4.68	0.002
pancreatic lipase | collagen, type I, alpha 2///collagen, type I, alpha 2	PNLIP|COL1A2///COL1A2	3.75	0.015	3.73	0.002
collagen, type III, alpha 1	COL3A1	4.50	0.014	4.84	0.002
endothelial PAS domain protein 1	EPAS1	2.01	0.015	2.09	6.43E-04
annexin A2	ANXA2	2.18	0.020	2.34	0.003
apolipoprotein E | high mobility group AT-hook	APOE|HMGA1///HMGA1	1.92	0.017	2.08	0.007

apolipoprotein E | high mobility group AT-hook 1///high mobility group AT-hook 1	APOE|HMGA1///HMGA1	1.92	0.017	2.08	0.007
apolipoprotein E | high mobility group AT-hook 1	APOE|HMGA1	2.33	0.020	2.65	0.005
interleukin 18 (interferon-gamma-inducing factor)	IL18	2.04	0.013	2.27	0.004
integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	ITGAV	1.99	0.016	2.03	0.003
lysyl oxidase	LOX	2.07	0.030	1.87	0.020
matrix metallopeptidase 14 (membrane-inserted)	MMP14	2.35	0.013	2.51	0.002
myosin IE	MYO1E	2.05	0.023	2.15	0.009
ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	ATP5B	1.82	0.018	2.34	0.004
tumor necrosis factor receptor superfamily, member 12A	TNFRSF12A	1.89	0.015	2.28	0.004

Table 6: list of genes differentially expressed relevant to alternative activation of macrophages pathway (M2)

Gene description	Gene symbol	FC [OS D7 vs D3]	p-value	FC [TiO D7 vs D3]	p-value
mannose receptor, C type 1 | mannose receptor, C type 1-like 1	MRC1|MRC1L1	1.79	0.025	1.54	0.017
macrophage scavenger receptor 1	MSR1	3.05	0.022	2.78	0.006
membrane-spanning 4-domains, subfamily A, member 4	MS4A4A	2.05	0.019	1.99	0.009
chemokine (C-C motif) ligand 22	CCL22	2.82	0.020	5.76	0.006
chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	2.70	0.027	2.56	0.005
solute carrier family 38, member 6	SLC38A6	2.14	0.022053376	2.78	0.002
transforming growth factor, beta-induced, 68kDa	TGFBI	2.05	0.018	1.78	0.005

Table 7: Top 30 downregulated genes at both surfaces.

GO ID	GO Term	P-value	Count in Selection
5051	chemotaxis	5.80E-16	20
19177	taxis	5.80E-16	20
5637	chemokine activity	4.18E-13	11
19226	chemokine receptor binding	7.63E-13	11
19080	response to chemical stimulus	1.06E-12	37
18862	locomotion	5.74E-12	21
3707	cytokine activity	1.16E-11	15
5065	immune response	1.18E-11	23
6918	response to external stimulus	1.55E-11	22
6924	response to wounding	3.60E-11	20
3708	cytokine receptor binding	5.20E-11	14
5062	defense response	5.20E-11	22
5064	inflammatory response	7.49E-11	16
1699	immune system process	7.77E-11	26
5061	response to stress	9.72E-11	33
1024	G-protein coupled receptor binding	4.83E-09	11
6920	response to biotic stimulus	2.38E-08	15
3985	extracellular space	8.88E-08	19
26637	response to other organism	8.88E-08	14
3949	extracellular region	1.69E-06	29
21158	extracellular region part	1.72E-06	20
12798	keratinocyte differentiation	4.11E-06	7
30186	cellular response to chemical stimulus	5.23E-06	15
6928	response to bacterium	6.52E-06	10
7201	epidermal cell differentiation	6.71E-06	7
13861	keratinization	9.03E-06	6
25864	response to stimulus	9.61E-06	48
26635	multi-organism process	9.75E-06	17
7176	tissue development	2.13E-05	16
18995	regulation of cell proliferation	2.13E-05	17

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