# CURRICULUM VITAE

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## Education:

**University of Maryland School of Dentistry** – Baltimore, Maryland
Post-Graduate Specialty Residency in Prosthodontics *July 2018*
Masters of Science (M.S) in Biomedical Sciences *December 2018*

**University of Pennsylvania School of Dental Medicine** – Philadelphia, PA
Doctor of Dental Medicine (D.M.D) *June 2015*

**Duke University** – Durham, N.C.
Bachelor of Science (B.S.) in Biology and Bachelor of Arts (B.A.) in Chemistry
Minor in Earth and Ocean Science *May 2011*

## Awards & Grants:

**Stanley D. Tylman Research Grant** *May 2016*

- Received competitive research grant sponsored by the American Academy of Fixed Prosthodontics supporting postdoctoral student research in fixed prosthodontics

## Research:

**Osteoblast and Dental Implant Surface Research** *Summer 2015-Present*

- Working on Master's dissertation at the University of Maryland School of Dentistry
- Investigating osteoblast adherence, proliferation, and differentiation on various dental implant surfaces.
- Prepare osteoblast (hFOB 1.19) cell cultures and perform RNA extraction, cDNA synthesis, and qRT-PCR analysis

**Oral Implantology Research** *Spring 2014-Fall 2015*

- Served as a Research Assistant working for Dr. Thomas Balshi and Dr. Glenn Wolfinger
- Studied the effect of immediately loaded implants concurrently placed in full mouth rehabilitations retrospectively
- Conducted database searches and statistical analysis of patients to determine the survival rates of concurrently placed maxillary and mandibular full mouth rehabilitations

**Oral Medicine Research** *Summer 2012*

- Helped out as a Research Assistant for Dr. Andres Pinto
- Completed database searches on Peripheral Neuropathy
- Performed comparative pricing analysis of various Biomedical Diagnostic Techniques

**Self-Healing Polymer Research** *Fall 2009- Spring 2011*

- Worked as an Undergraduate Researcher in Dr. Stephen Craig's Lab
- Experimented on Mechanical Stress Induced, Self-Healing Polymers, and Carbon Nanotubes

- Achieved proficiency with the NMR and Gel Permeation Chromatography (GPC)

**Oral Scientific Presentations**:

Choe RH, Rivera EM, Driscoll CF, "A Retrospective Analysis of Implants Placed Via Computer-Guided Template-Based (NobelGuide™) Surgery," Academy of Osseointegration, 2017 Annual Meeting, Orlando, FL, March 2017.

Choe RH, Driscoll CF, "Digital Dentistry: Cost-Benefit Considerations for the Dental Practitioner," American College of Prosthodontists, 2016 Annual Session, San Diego, CA, October 2016.

Choe RH, Balshi TJ, Wolfinger GJ, Balshi SF, "A Retrospective Analysis of Immediately Loaded Implants Concurrently Placed in Full Mouth Rehabilitations," Greater New York Academy of Prosthodontics, GNYAP 61st Scientific Meeting, New York, NY, December 2015.

Choe RH, Balshi TJ, Wolfinger GJ, Balshi SF, "A Retrospective Analysis of Immediately Loaded Implants Concurrently Placed in Full Mouth Rehabilitations," American College of Prosthodontists, 2015 Annual Session, Orlando, FL, October 2015.

**Publications:**

Lenhardt JM, Ong MT, Choe R, Evenhuis CR, Martinez TJ, Craig SL, (2010). Trapping a diradical transition state by mechanochemical polymer extension. Science. 329 (5995):1057-60.

**Teaching Experience**:

**Y2 Academy (now INTO Prep) –** Upper Darby, PA *Fall 2014-Summer 2015*

- Tutored in individual and small group settings for English, SAT I, SAT II Math, high school and AP Biology
- Instructed classes with an average size of 15 students
- Taught new material and reinforced materials students learned at school during individual tutoring sessions

**Preclinical Tutor** *Spring 2012- Spring 2015*

- Tutored dental school students in 1st and 2nd year preclinical lab

**Teaching Assistant** *Fall 2009- Spring 2011*

- Worked as an Undergraduate Teacher Assistant for General Chemistry Recitation
- Led recitation sessions with approximately 30 students per course
- Reviewed and re-taught the materials the students learned in lecture

**Peer Tutor** *Fall 2008- Spring 2011*

- Tutored undergraduate students one-on-one in General Chemistry and Organic Chemistry

**Skills & Achievements:**

- *Clinical Skills*: CDCA licensed dentist experienced in full mouth rehabilitation, dental implant surgery, and treatment of various medically complex patients including TMD and cancer patients.
- *Computer*: Proficient with Macintosh and Windows operating systems, Keynote, Microsoft Office, Meshmixer and dental implant/design software (3shape Dental Designer and Implant Studio, NobelClinician, Anatomage, and Blue Sky Bio)
- *Language*: Fluent in Korean and moderate proficiency in Spanish
- *Interests*: Achieved the title of Instructor and 3rd degree black-belt in Taekwondo; NTRP 4.5-5.0 level tennis player

## ABSTRACT

Title of Thesis: Osteoblast Attachment, Proliferation and Differentiation on Implant Surfaces

Robert Choe, Master of Science, 2018

Thesis Directed by: Radi Masri, DDS, MS, PhD

**Introduction.** Countless numbers of implants with different surface treatment techniques exist commercially around the world. Cell adhesion, proliferation and differentiation on all these different implant surfaces have different effects at the microscopic level.

**Purpose.** The purpose of this project is to expand on previous osteoblast attachment studies and investigate the osteoblast attachment, proliferation, and differentiation on five different types of implant surfaces: (1) Machined surface of surface roughness (Sa) of 0.3 – 0.4 μm; (2) Anodized yellow surface 0.3 – 0.5 μm; (3) anodized yellow surface 0.8 μm; (4) SLA surface of 1.6 μm; and (5) Biodenta surface treated (BST) surface of 1 μm. BST surface (Biodenta Swiss AG, Berneck, Switzerland) is anodized during the manufacturing process to have an amorphous titanium oxide layer. This process allows the BST surface to have its surface roughness enhanced via open porosity to promote osteoconduction.

**Materials and Method.** Human fetal osteoblast stem cells were cultured for two weeks on the five different types of implant surfaces. Three genes were examined: Collagen Type 2 (COL1A2), Runt-related transcription factor 2 (RUNX2) and Osteocalcin (BGLAP) because of their role as genetic markers of osteoblast attachment, proliferation, and differentiation. The quality of the mRNA was checked with a spectrophotometer and cDNA was synthesized for

quantitative RT-PCR. Genes were analyzed by means of melting curve analysis and relative quantitative ΔΔCt calculation to determine fold change.

**Results.** The anodized 0.3 – 0.5 μm surface displayed up-regulation of COL1A2 (n-fold of +1.589) but down-regulation of RUNX2 and BGLAP (n-fold of -1.109 and -2.624, respectively). However, the anodized 0.8 μm surface presented opposite trends for all three genes. RUNX2 and BGLAP were both up-regulated (n-fold of +1.406 and +1.778, respectively) while, COL1A2 exhibited the greatest down-regulation trend with respect to all surfaces (n-fold of -4.801). The BST specimens were the only surface type to exhibit up-regulation for all three genes. Of the three genes, COL1A2 and RUNX2 displayed the greatest fold change for the BST surface (n-fold of +2.462 and +2.688, respectively).

**Conclusions.** mRNA expression of RUNX2, COL1A2, and BGLAP in human fetal osteoblast stem cells, cultured on five different surfaces, indicated various expression profiles. The expression of COL1A2 and BGLAP, characteristics of a more mature osteogenic phenotype, were exhibited on the rougher surface implants. Following this trend, the BST surface expressed the most mature osteogenic phenotype in this study.

# Osteoblast Attachment, Proliferation and Differentiation on Implant Surfaces

by
Robert Choe

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2018

## DEDICATION

*Dedicated to (in order of appearance in my life):*

*Chong Suk Choe (mother), Bup Song Choe (father), and Samuel Hyungjun Choe (brother)*

## ACKNOWLEDGEMENTS

I would like to thank Dr. Radi Masri, my thesis advisor, and Dr. Carl F. Driscoll, my program director, for their support throughout this entire study. I appreciate all of Dr. Negar Homayounfar input in the design of this study as well as my thesis. I am grateful for the opportunity to be part of such a unique research study.

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## INTRODUCTION

A retrospective look back through history reveals that the notion of replacing a tooth with an implant has been around for several millennia. From gold ligature wires, shells and ivory to endosseous root-formed metals and allotransplatation of teeth from cadavers, humans have vastly explored the concept of implanting various biomaterials to substitute a tooth.[1] The scientific breakthrough to help further mankind's ultimate dream to replace a tooth came in 1952, when P-I Branemark serendipitously discovered the phenomenon of bone adhering to titanium chambers that he had installed for vital microscopic studies of the bone marrows collected from rabbit fibulas. Branemark perceived the significance of the discovery and proceeded to pursue further experimentations to elucidate this biological process that he later termed osseointegration.[2] Various innovative materials and designs were attempted before the community settled on endosseous root form implants.[1] Present day, modern dentistry attempts to restore the patient to normal function while achieving optimal esthetic results, no matter how dire the condition of the patient's craniofacial complex. Research investigating surface treatment methods to decrease osteointegration period of implants has been a focus implant research. As studies advance, the materials, structures and surface compositions of existing implants options to perfect the already impressive clinical success rate of over 95% over a five-year period.[3]

### *Biological Basis of Osseointegration*

Osseointegration is comparable to direct fracture healing, a process of primary healing in which the bone fragments merge through bone formation without intermediate fibrous or

fibro-cartilage tissue.[4] However the fundamental difference that distinguishes this process from direct fracture healing is that the concept of osseointegration describes the result where there is direct bone contact with a loaded implant surface at the microscopic level.[5] This peri-implant osteogenesis leading to osseointegration can be subdivided into two mechanisms: Distance osteogenesis describes the formation of peri-implant bone from the host bone cavity towards the implant, while contact osteogenesis refers to the formation of bone from the implant to the healing bone.[6] Contact osteogenesis can be further subdivided into three distinct phases of osteoconduction, de novo bone formation and bone remodeling of the established interfacial osteoid matrix.[7]

Osseointegration involves a robust yet delicate interplay between bone resorption and bone formation at the molecular level. Upon the creation of the osteotomy site and placement of the implant, a blood clot with large amounts of erythrocytes, neutrophils and macrophages imbedded within a fibrin network forms in 2 hours.[7] In four days, fibroblast-like mesenchymal cells surround the vascular structures and newly formed woven bone can be identified in 1 week.[7] In 2 weeks, new bone formation is very dynamic all around the implant, as the newly formed osteoid matrix extends from the parent bone into the pitch region that is rich in vascularity, spindle-shaped cells, few leukocytes, and collagen fibers.[7] In 4 weeks, bone remodeling remains intense as woven bone is seen with parallel-fibered and lamellar bone.[7] By 12 weeks, most of the woven bone is replaced with lamellar bone that is surrounded by bone marrow containing adipocytes, vessels, collagen fibers and mononuclear leukocytes.[7] The duration of the entire process takes at least 4 months in humans to complete, and the treatment outcomes

in dental implantology have been determined to be significantly dependent on implant surface topographies that optimize the biological mechanisms involved in contact osteogenesis.[6,8]

The combination of genetic, molecular, cellular, and biochemical signals has established a progressive expression program of cell growth and phenotypic genes that support osteoblast differentiation. Osteoblast development have genetic markers that are expressed at different phases of proliferation, matrix maturation and mineralization. Runt-related transcription factor 2 is a key transcription factor upregulated in actively proliferating immature osteoblasts before the onset of osteoblast differentiation.[76] Type I collagen is a protein involved in the development of the extracellular matrix, which is also expressed during the later phase of proliferation. Lastly, osteocalcin are proteins involved in the mineralization and regulation of the mature ECM.[77]

***Dental Implant Material and Design***

Along with an ever-expanding market for dental implants, many different materials and designs of implants have come and gone.[1] Branemark promoted the use of screw-shaped implants made of pure titanium due to the fact that the design introduced minimal tissue injury during surgery and the material demonstrated favorable reaction with the human body.[2] As a result, other screw-shaped root-formed endosteal implants were introduced.[8-9] At the beginning of the 21st century, there were 2000 implants from 80 different implant manufacturers identified throughout the world.[10] However, even with the abundance of available implant options for the dental practitioner, literature shows that

titanium has withstood the test of time and continues to be the predominant material of choice for implant dentistry.

Titanium possesses ideal properties that make them optimal material for dental implants. When titanium and its alloys are exposed to air, a layer of titanium oxide is formed, which plays an important role in corrosion resistance, biocompatibility and osseointegration.[11] They are biocompatible due to the formation of oxide levels at the molecular level that promotes chemical stability and impedes the corrosion process in the oral environment.[12] The optimal strength and lower modulus of elasticity enables titanium based implants to better distribute occlusal forces and ultimately resist forces that can lead to fracture or failure.[13] There are four grades of commercially pure (CP) titanium and two titanium alloys that are commonly used in the manufacture of dental implants: The four grades of cp titanium increase in tensile and yield strength from grade I to IV; the two alloys consist of Ti-6Al-4V, which possess high tensile and yield strength, and 'extra low interstitial' (ELI) Ti-6Al-4V that has slightly improved ductility over the former alloy due to the low levels of dissolved oxygen in the interstitial sites in the metal.[13] Titanium alloys possess great strength that helps resist occlusal forces without fracture and a lower modulus of elasticity that helps dissipate forces to the bone.[14]

Like all metals, when titanium is exposed to atmosphere, oxide layers form on its surface. The nature of this oxide layer depends on the metal itself and the conditions where the oxidization occurred. Freshly cut titanium during the machining process is exposed to

the atmosphere and an oxide layer of greater than 10 Å is formed in less than a millisecond; this oxide layer achieves a thickness on the order of 50-100 Å within a minute.[12] The oxide layer is responsible for the chemical properties and the implant-tissue interface. Titanium forms various stable oxides TiO, $TiO_2$, $Ti_2O_3$ with $TiO_2$ being the most common one.[12]

***Ideal Implant Surface Characteristics: Surface Composition***

Cell proliferation and attachment are the initial phases of the osteogenesis between the bone and the implant. Cell-implant interactions depend upon the implant's surface topography, chemistry and surface energy.[12] The chemical composition of the implant surface often differs from that of the bulk material due to surface treatment methods and impurities trapped on the implant surface. Due to the fact that the surface of a dental implant is the only part that is in contact with the bio-environment, the uniqueness of the surface directs the response and affects the mechanical strength of the implant/tissue interface. The surface treatment on the implant is required to increase the functional surface area of the implant-bone interface, in order to enhance adherence of platelets and fibrin to implant surface, improve primary stability, and expedite bone deposition and osseointegration.[15] These treatments are designed to manipulate surface roughness, surface charge, surface energy, and surface composition to affect the interaction of implants with cells and tissues and ultimately affect the success of dental implants.[15] Implant surfaces with varying surface chemistry, surface topography and roughness are commercially available.

### *Ideal Implant Surface Characteristics: Surface Energy*

The surface energy of a biomaterial is determined by surface-charge density and the net polarity of the charge. The surface energy of a dental implant is known to be a key factor that guides bone cell adhesion and early stage bone mineralization in the bone-implant interface. A surface with net positive or negative charge may be more hydrophilic when compared to an electrically neutral surface.[16] Pure titanium surfaces exhibit high surface energy due to the oxide layer that grows spontaneously at the room temperature.[12] The implant surface energy is an important factor in regulating osteogenesis, which is assessed by determining the wettability of a surface by measuring contact angle. There are two mechanisms that explain the effect of potential surface-charge on osseointegration properties of titanium dental implants: (1) by forming an apatite layer and (2) by attracting certain types of proteins with desirable reactions from bone-forming cells.[17] Higher surface energy has been hypothesized to be desirable due to enhancing the interaction between the implant surface and biologic environment, which enables the implant to handle more loads than the adjacent tissue elements.[18] On the other hand, low surface energy implant materials are characterized by fibrous capsule interface between the implant and the bone. Such surfaces, at the time of insertion retain these poor adhesive layers for a longer period of time.[19]

### *Ideal Implant Surface Characteristics: Corrosion Resistance*

When an implant is introduced into the body, complex reactions occur at the oxide and the bio-environment interphase where the oxide layer grows, as ions diffuse outward from the metal and inward from the environment.[12] Therefore, the oxide layer that forms

in the body is different than the one that forms in the air. CP titanium and titanium alloys are easily passivated to form a stable $TiO_2$ layer that makes it corrosion resistant [20]. However, changes on the surface of the metal implant and in surrounding tissues can be seen overtime. Stresses from time, temperature and a corrosive environment can induce mechanical and environmental effects on a metal, changing its properties and the properties of the surface oxide.[20] Titanium alloys are extremely resistant to corrosion fatigue, making them the metal of choice when high corrosion fatigue strength is desirable. While rare in titanium alloys, localized corrosion can sometimes be seen on the metal surface due to the incorporation of contaminants introduced during the manufacturing process in the form of crevices and pitting corrosion.[21]

***Ideal Implant Surface Characteristics: Biocompatibility***

The criteria for any potential metallic material to have excellent corrosion resistance include ease of oxidation, strong adherence of formed oxide layer to the substrate, density of oxide layer, and protectiveness of formed oxide layer.[20, 22] Pure titanium is a highly reactive metal and will react within microseconds to form an oxide layer that stabilizes it, even in a biological system including chemical and mechanical environments.[12] Inert surface oxide film makes titanium a highly biocompatible material, with low surface ion release from its surface into the surrounding environment. Importantly, the human body does not reject the oxide surface that forms.[23] When implanted, titanium releases corrosion products, mainly in the form titanium oxide or titanium hydro-oxide, into the surrounding tissue and fluids, even though a thermodynamically stable oxide film is present. An increase in oxide thickness, as well

as incorporation of elements from the extra- cellular fluid (P, Ca, and S) into the oxide, changes the oxide stoichiometry, composition, and thickness.[22] This titanium passivating layer not only produces good corrosion resistance, it also allows physiological fluids, proteins, and hard and soft tissue deposit directly on the layer.[20]

***Implant Shape and Surface Topographies***

The efforts by implant manufacturers to gain a competitive edge over rival implant companies by altering surface characteristics for quicker osteogenesis and improved bone-implant interface allowed the scientific community to observe that controlled surface properties were an essential quality for the establishment of osseointegration.[5, 24] Mechanical, topographic and physicochemical properties all affect the surface quality of implants, which in turn plays an essential part in cell-surface interaction since surface properties can be altered to promote cell attachment, proliferation, and differentiation via the surface modification techniques.[3, 25-27] However, comparisons of the various implant surface types have been difficult due to heterogeneity of all the studies due to different study criteria and definitions of surface characterizations.[27-28]

Efforts have been made to identify and standardize surface parameters. Wennerberg and Albrektsson have determined that three dimensional evaluations of implant surfaces, such as Sa values that measure the surface area, are preferable and more informative than two dimensional ones like Ra that only measure one profile view of the implant.[29] Smooth implants have a Sa value lower than 0.5 μm whereas minimally rough implants have a Sa of 0.5 to 1.0 μm, represented by turned NobelBiocare (Branemark) implants and acid-

etched implants.[29] Moderately roughened surfaces vary between 1.0 and 2.0 μm and include most modern implants, such as the Astra Tech TiOblast™, OsseoSpeed™ surfaces, Nobel Biocare TiUnite, Straumann SLA and Dentsply Cellplus designs.[29-30] Lastly, rough implants possess a Sa above 2.0 μm and are exemplified by plasma sprayed devices, an example of which is the Dentsply Frialite implant.[29]

Recent studies comparing surface properties and cell adhesion on dental implants have utilized new surface topography parameters for measuring cell adhesion.[31-32] These studies found cell adhesion was affected by implants with different surface treatments – cells differentiated more quickly on roughened implant surfaces, but the advantages of certain surface treatment techniques over others have yet to be conclusively established in the literature. Therefore, further studies are needed to elucidate the impact of these various surface treatment techniques on cell adhesion of cells involved in osseointegration.

***Machined Surfaces***

Machined surfaces are smooth/turned surfaces, described by Branemark, were the prototypical endosteal implants that preceded the modern implants.[2] Machined surfaces are relatively 'smooth' on the microscopic level. Present day, machined surfaces are considered to have minimally rough surfaces with Sa 0.5 - 1.0 μm.[29] A considerable disadvantage regarding the morphology of this implant surface is that the surface defects provide resistance to bone interlocking that impedes the process of osseointegration along the surface grooves.[33] The success rates of turned surfaces were found to be

successful when the two-stage surgical protocol Branemark described was followed.[2, 34] More recent studies suggest significantly higher bone-implant contact of rough surface implants over machined surface implants.[35-36]

***Hydroxyapatite Coating (HA)***

A hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) surface coating on an implant can be considered bioactive because of the sequence of events that happen during the formation of a calcium phosphate rich layer on the surface through a solid solution ion exchange - the layer with calcium phosphate will gradually be developed via octacalcium phosphate that is incorporated in the developing bone.[33] Hydroxyapatite coated implants have shown to increase the rate of peri-implant osteogenesis, provide better primary stability, increase the surface area of bone contact and the amount of lamellar bone, and exhibit greater strength to the coronal bone compared strength displayed with titanium implants.[37-38] High survival rates for hydroxyapatite coated implants were found in a 12-year follow-up study with a 93.2% survival rate for HA implant.[39]

There are some concerns regarding HA coated implants that includes microbial adhesion, osseous breakdown and coating failure.[40] A study on fatigue resistance of Ti-6Al-4V with hydroxyapatite film thickness ranging from 0-150 μm found that samples with 150 μm film thickness had significantly decreased fatigue resistance while 25-50 μm coatings had had no observable delamination during fatigue tests.[41] The hydroxyapatite coatings with 75-150 μm thickness delaminated after the initiation of the first fatigue crack in the substrate.[41] The disadvantage of hydroxyapatite coated surfaces

is the risk of cracking, flaking or scaling on insertion, especially when they under shear stress during insertion into dense cortical bone.[42]

However, these implants could be beneficial in grafted bone or type IV bone where more rapid bone implant contact is needed. Even though signs of degradation were also observed, it was demonstrated that bone reaction to the chemical composition of HA-coated implants was more profound than the implant surface roughness.[43] Additionally, another study reported the formation of bone at the bone-implant interface and the implant surface for hydroxyapatite coated implants 18 months after loading.[44] Short implants may also be an indication for HA coated implants.

***Plasma Sprayed Surfaces***

Plasma spraying process involves the injection of powdery forms of titanium into a plasma torch at elevated temperatures. These properties increase the surface area microscopically by 6-10 times and the functional load-bearing area by 25-30%. Plasma spraying allows the implant to have a coating thickness of approximately 40-50 micrometers.[45] The plasma-spraying method has several drawbacks, including poor long-term adherence of the coating to the substrate material, uneven thickness of the deposited layer, and dissimilarities in composition of the coating.[1] The surface layer may undergo resorption and delaminate to cause eventual loosening of the titanium particles.[1] The detachment of the plasma sprayed titanium particles after implant insertion could be related to the shear forces between the implant surface and the bone during implant placement, which may ultimately lead to implant failure.[46] Although, in one study that

evaluated the removal torque machined, acid etched, and plasma sprayed implants found significant differences between acid etched compared to machined surfaces and plasma sprayed versus machined surfaces. [47] However, the differences between acid etched and plasma sprayed were not statistically different.[47]

### *Anodized Surfaces*

Anodization is the process of altering the surface topography and surface composition by increasing surface roughness, surface area, and thickness of the titanium oxide layer.[48] Anodized surfaces are prepared by applying a voltage to an immersed titanium surface in an electrolyte solution. This moderately rough surface improves osteoconductive properties that has been reported to enhance osteoblast cell adhesion to titanium implants and speed up osseointegration of the implant.[49] A primate study evaluated the oxidized implant surfaces placed in the posterior maxilla of monkeys and found the mean bone-to-implant contact to be 74%, which suggested that this oxidized surface retains a considerable osteoconductive potential promoting a high level of implant osseointegration.[50] Additionally, a human study on anodized versus and machined surfaced implants verified that the bone-to-implant contact to be significantly higher in anodized implants.[51] A thicker oxide layer, which increases surface roughness and changes the surface morphology in terms of porosity and crystal structure, may be responsible for the improved osseointegration.[52] A ten-year follow-up of immediately loaded implants with porous anodized surfaces reported a cumulative 65.26% success rate and 97.96% survival rate.[53] In a randomized clinical trial, anodized implant survival

rates were reported to be higher than machined implants (95.5% and 85.5% respectively).[54]

### *Acid-Etched Surfaces*

Strong acids are used for roughening the surface of titanium implants. The acid etching of implant surfaces is generally completed by utilizing hydrochloric acid, hydrofluoric acid, sulfuric acid, nitric acid or a combination of these acids.[55] The initial surface roughness, type and duration of acid used, and bath temperature all affect the acid etching process.[53] The process of acid etching removes the surface oxide layer and some layers of the underlying material.[56] More of the material surface layer is removed as the acid concentration, temperature and treatment time are increased. The process of acid etching provides homogeneous irregularities, enlarged surface area and enhanced cell adhesion to implant surfaces.[57] Some distinct advantages of this technique are that no particles are encrusted in the surface along with a lower surface energy and reduced possibility of contamination. Acid etching facilitates osteoblastic retention and allows them to migrate toward the implant surface, titanium implants roughened through acid etching have demonstrated rapid osseointegration with long-term success.[58]

Dual acid-etching is an enhanced etching technique that roughens up the implant surface by immersing the titanium implants for several minutes in a mixture of concentrated HCl and $H_2SO_4$ heated above 100 °C.[33] This process improves the process of osteoconduction through the attachment of osteogenic cells and fibrin, which ultimately leads to direct bone formation.[59] The specific topography achieved by dual acid etching of implants has

been hypothesized to enable the surface to attach to the fibrin scaffold that promotes the adhesion of osteogenic cells and increases bone apposition.[60] This fibrin adhesion guides osteoblastic migration along the surface.[61] Studies have reported higher bone implant contact and less bone resorption with dual acid etched surfaces compared to machined implants.[62]

***Sandblasted Surfaces***

Sandblasting implant surfaces aim to increase implant surface roughness by using gritting agents such as aluminum or titanium oxide. Titanium dioxide particles with sizes of 0.25 – 0.50 μm are used to grit-blast the machined implants for rough surface finish.[55] One study evaluated the interface between bone and titanium implants blasted with $Al_2O_3$ particles of different sizes, ranging from 25 – 250 μm, only to find that all the sandblasted surfaces exhibited more bone in contact with the implant surface compared with turned surfaces after 12 weeks of integration.[63] In a human study, sandblasted implants achieved enhanced osseointegration similar to results shown in animal studies.[51, 64-65] An *in vitro* study measuring gene expression for osteoblast proliferation and differentiation of machined titanium, micro-sandblasted (0.5 μm), and macro-sandblasted (3.0) μm showed that macro-sandblasted surfaces are more favorable than micro-sandblasted surfaces for osteoblast differentiation. In a ten-year prospective study of $TiO_2$ grit blasted implants demonstrated a success rate of 96.9%, which was significantly better than unblasted, machined implants.[66]

Although grit-blasting has several advantages, there is evidence of adverse effects, such as surface contamination and distortion of blasted work piece depending on type of blasting media, technique and intensity. One study found that despite low grinding speeds and water-cooling, the abraded surfaces were contaminated by abrasive constituent elements, which may result from the surface reactivity and hardness.[67] The resulting contamination negatively influences the titanium's resistance to corrosion and its biocompatibility.[67]

***Sandblasted and Acid-Etched Surfaces (SLA)***

The SLA implant surface is produced after sandblasting with large grit particles of 250–500 μm followed by etching with acids. Macrostructures resulting from the sandblasting is supplemented by the micro-irregularities from acid etching.[68] This resultant surface exhibits uniformly scattered gaps and holes, but appears to be less rough than plasma sprayed surfaces.[25] The depth and distribution of irregularities, the morphology of the formed cavities, and the presence of contaminating elements derived from the treatment procedure appear to play an important role in cell behavior.[69] A histomorphometric study displayed that modified SLA surfaces showed significantly more bone apposition than standard SLA surfaces after two weeks healing.[70] Both surface types showed the same apposition after four weeks, with an increasing apposition between two and four weeks, which suggests that the acid-etched modified implants may be beneficial patients undergoing early loading implant techniques.[70] Another study evaluated the pattern of bone formation in sandblasted acid etched and machined implants and found that the bone implant contact in sandblasted and acid etched sites were significantly greater than

in machined sites.[71] Longer-term studies substantiate this observation, as the bone to implant contact have shown to be significantly higher for sandblasted acid etched surfaces over plasma sprayed surfaces.[71] Additionally, sandblasted and acid etched surfaces also presented with better osteoconductive properties and a higher capability to induce cell proliferation than plasma- sprayed surfaces. Long term success and survival rates of SLA surfaces showed a success rate of 97% and a survival rate of 95.1%.[72]

***Biodenta Surface Treatment***

The Biodenta implant was designed with features that make it a good implant choice for many clinical applications, as the design allows for high levels of initial stability and a reliable prosthetic platform for most restorative situations. As mentioned previously, significant surface roughness is crucial in providing an effective surface for bone implant contact, cell proliferation and good mechanical properties. Osteointegration in alveolar bone involves an interplay of mechanical force and biological surface bonding force. Numerous studies have shown that titanium surface have been modified to enhance osseointegration. In order to achieve optimal osseointegration, Biodenta implants utilize a proprietary anodization process during the manufacturing. Biodenta Surface Treatment (BST) creates an amorphous titanium oxide layer and the surface roughness enhanced through open porosity. This treatment process is designed to enhance the osteoconductive process on its surface.

**PURPOSE**

The purpose of this project is to expand on previous osteoblast attachment studies and

investigate the osteoblast attachment, proliferation, and differentiation on five different types of implant surfaces: (1) Machined surface of 0.3 – 0.4 μm; (2) Anodized yellow surface 0.3 – 0.5 μm; (3) Anodized yellow surface 0.8 μm; (4) SLA surface of 1.6 μm; and (5) BST surface of 1 μm. A new implant surface is introduced in this project. BST implants (Biodenta Swiss AG, Berneck, Switzerland) are anodized during the manufacturing process to have an amorphous titanium oxide layer. This process allows the BST surface to have its surface roughness enhanced via open porosity and promotes osteoconduction. Differences between surface roughness and osteoblast adhesion will be measured in the study.

## HYPOTHESES

### *Null Hypotheses*

1. When treated *in vitro*, there is no significant difference in the mRNA expression levels in regards to osteoblast proliferation and differentiation among the five different implant surfaces.
2. There are no significant correlations between surface roughness of implants and osteoblast adhesion, proliferation, and differentiation on all five implant surfaces.

### *Research Hypotheses*

1. *In vitro*, SLA (1.6 μm) surface will exhibit with significance the greatest level of osteoblast proliferation and the most amount of differentiation among all the surfaces. BST (1.0 μm) and anodized yellow (0.8- 1.0 μm) surfaces will have the next highest level of osteoblast proliferation and differentiation. Anodized yellow

(0.3- 0.5 μm) and machined surfaces (0.3-0.4 μm) will have the least amount of osteoblast proliferation and the least amount of differentiation.

2. There is a positive correlation between the surface roughness and osteoblast proliferation and differentiation with respect to the five different implant surfaces, when considered together or separately.

## MATERIALS AND METHODS

### *Implant Specimens*

Five different types of implant surfaces were tested in this experiment (Figure 1): (1) Machined surface with Sa 0.3 – 0.4 μm (negative control group); (2) anodized yellow surface with Sa 0.3 – 0.5 μm; (3) anodized yellow surface with Sa 0.8 μm; (4) SLA surface with Sa 1.6 μm (positive control group); and (5) BST surface of 1 μm. Miniature implant specimens provided by Biodenta Swiss AG (Berneck, Switzerland) were used, and had the following dimensions: The screw head was 5 mm in diameter and 2 mm in height; the shank was 1.3 mm in length and 0.75 mm in diameter at its connection to the screw head, which then tapers to a 2 mm diameter at its connection to the thread; the thread length is 4.7 mm with a major diameter of 2.0 mm and a pitch of 0.04 mm. The diagrammatic representation of a miniature implant specimen is shown in Figure 2. The surface treatment and sterilization of the specimens were performed by Reutschi Technology AG (Muntelier, Switzerland).

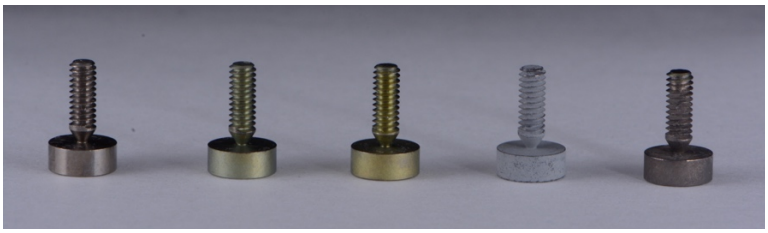

**Figure 1 Five different surface treated miniature implant specimens from left to right: (1) Machined 0.3 - 0.4 μm; (2) Anodized 0.3-0.5 μm; (3) Anodized 0.8 μm; (4) SLA 1.6 μm; and (5) BST 1.0 μm**

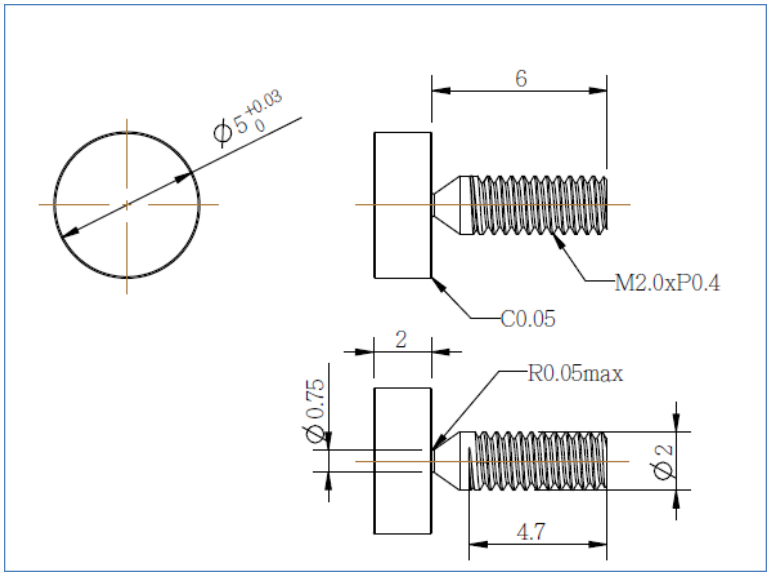


**Figure 2 Dimensions of the miniature implant specimens**

### *Cell Culture*

An immortalized human fetal-osteoblast cell line (hFOB 1.19; American Type Culture Collection, Manassas, VA, USA; Cat. No. CRL-13372™) was used for the study. These cells provide a homogenous, rapidly proliferating model system for studying normal human osteoblast differentiation with the expression of osteoblast-specific phenotypic marker and synthesis of matrix proteins [73-74]. The cells were cultured according to the ATCC recommendations at 33.5°C in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. D5796) supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin and used during the 3rd passage of the cells. Fourteen screws of each implant surface being tested were laid on the bottom of two wells of a 12-well plate. Media for the wells was replaced every 3 days. After culturing the cells for 2 weeks the cells were trypsinized and seeded directly onto the screws. Two wells of a 12-well plate were seeded without the implant specimens to serve as control. The cells were incubated for 14 days, maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin that was changed every 3 days at 37°C with 5% $CO_2$ and 95% air. The cells were cultured for 14 days to make sure the osteoblast cells reached confluence of at least 80% and underwent sufficient growth and differentiation to express the genetic markers expressed during matrix maturation.[73, 75]

### *Cell Collection and Storage*

The spent media culture media was aspirated and 3 mL of PBS was used to wash the cells. The PBS was aspirated and the wash process was repeated twice more to ensure

the cells were washed thoroughly. Trypsin-EDTA (3 mL) was added into each well and the dish was incubated 3 minutes to detach the osteoblasts from the screws. Once the incubation period was completed, 3 mL of media was added to each well and triturated to detach any remaining cells on the screws. The loose osteoblast cells were harvested into a 15 mL centrifuge tube and centrifuged for 5 minutes at 1000 RPMs. The osteoblasts were checked for vitality and integrity using a Nikon TMS inverted phase-contrast microscope (Nikon, Tokyo, Japan; Cat. No. TMS 3155). The supernatant was aspirated and 120 μL ultrapure water and 30 μL binding buffer from the High Pure MiRNA Isolation Kit (Roche Diagnostics, Mannheim, Germany; Cat. No. 05 080 576 001) was added to the centrifuge tube. The contents were transferred over to a 1.5 mL microcentrifuge tube. The cell culture and collection process were conducted three times to accumulate sufficient quantities of osteoblasts for RNA extraction and cDNA synthesis.

***RNA Extraction and cDNA Synthesis***

Total RNA was extracted using the High Pure miRNA Isolation Kit (Roche Diagnostics, Mannheim, Germany; Cat. No. 05 080 576 001) according to the manufacturer's instructions. Afterwards, an assay was performed in accordance with the manufacturer's instructions. The culture medium was used as a background reference. Absorbance values were measured in the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Subsequently, 0.631 μg total RNA was used from each sample as template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA; Cat. No. 4368814) with a

reaction volume of 50 μL.

***RT-PCR***

The expression levels of 3 cellular markers was quantified using RT-PCR: Runt-related transcription factor 2 (RUNX2), type I collagen (COL1A2), and osteocalcin (BGLAP). The cells were cultured as described above then collected for RT-PCR. Primers for all SYBR assays were designed using Primer 3 [78]. Melting curve analysis was performed to ensure single-product amplification for all primer pairs.

***RT- PCR Analysis***

Quantitative RT-PCR was performed on the ABI 7900HT Fast Real Time PCR System (Applied Biosystems) using assays specific for each gene of interest. For SYBR assays, each reaction well contained 5 μL of *Power* SYBR Green PCR Master Mix (Applied Biosystems; Cat. No. 4367659), cDNA equivalent to 20 ng of total RNA and 400 nM each of forward and reverse amplification primers in a reaction volume of 10 μL. Cycling conditions will be as follows: 95°C for 10 minutes for polymerase activation, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data analysis was performed using Sequence Detection System software from Applied Biosystems version 2.4. The experimental cycle threshold (Ct) was calibrated against the endogenous control product GAPDH. All the amplifications were carried out with the normalization of gene expressions against the GAPDH control reagent. The quantification of gene expression was expressed as the fold change being performed using the relative quantitation method ΔΔCt calculation, where the fold change of the target was given as $2^{-(\Delta\Delta Ct)}$ [79]. The positive

control group acted as the "calibrator" to which the samples were compared. The relative quantification (RQ) value of the calibrator group was set to 1 since it did not vary.

**RESULTS**

In this *in vitro* study, the influence of various implant surface treatments on osteoblast adhesion, proliferation and differentiation were examined. After culturing the osteoblasts for 2 weeks, the osteoblasts were collected for RNA extraction and cDNA synthesis. The absorbance values were measured to confirm the quantity and integrity of the RNA. The concentrations and absorbance values of all five samples are listed in Table 1.

Quantitative RT-PCR and corresponding melting curve analyses were performed for all the samples. The Ct values were gathered for all samples except for the machine surface sample, which resulted in undeterminable values. The average Ct values for are listed in Table 2 for the remaining four implant specimens.

With GAPDH being the control reagent for each gene, the remaining samples of the four implant surfaces were analyzed for relative gene expression by utilizing the mathematical model to calculate fold change [79]. Differences were searched among the remaining four implant surfaces, with the SLA surface (positive control) as the 'calibrator' for the relative quantification of gene expression. Table 3 through Table 6 presents the ΔΔCt values and related mean fold change (n-fold) of down- or up-regulation for the four surfaces: anodized yellow surface with Sa 0.3 – 0.5 μm; anodized yellow surface with Sa 0.8 μm; SLA surface with Sa 1.6 μm (positive control group); and BST surface of 1.0

μm.

The anodized 0.3 – 0.5 μm surface displayed up-regulation of COL1A2 (n-fold of +1.589) but down-regulation of RUNX2 and BGLAP (n-fold of -1.109 and -2.624, respectively). However, the anodized 0.8 μm surface presented opposite trends for all three genes. RUNX2 and BGLAP were both up-regulated (n-fold of +1.406 and +1.778, respectively) while, COL1A2 exhibited the greatest down-regulation trend with respect to all surfaces (n-fold of -4.801). The BST implant specimen was the only surface type to exhibit up-regulation for all three genes. Of the three genes, COL1A2 and RUNX2 displayed the greatest fold change for the BST surface (n-fold of +2.462 and +2.688, respectively).

| *Surfaces* | *Machined* | *Anodized* | *Anodized* | *SLA* | *BST* |
|---|---|---|---|---|---|
| *(Sa)* | *(0.3 - 0.4 μm)* | *(0.3 - 0.5 μm)* | *(0.8 μm)* | *(1.6 μm)* | *(1.0 μm)* |
| *Conc. (ng/μl)* | **91.5** | **80.6** | **126.3** | **119.9** | **78.9** |
| $A_{260}/A_{280}$ | **1.85** | **1.98** | **2.00** | **1.91** | **1.96** |

**Table 1 Absorbance values $A_{260}/A_{280}$ ratios for all implant surfaces**

| *Surfaces* (Sa) | *Anodized* (0.3 - 0.5 μm) | *Anodized* (0.8 μm) | *SLA* (1.6 μm) | *BST* (1.0 μm) |
|---|---|---|---|---|
| *RUNX2* | **26.622 ± 0.017** | **29.805 ± 0.171** | **29.397 ± 0.151** | **25.932 ± 0.006** |
| *COL1A2* | **21.229 ± 0.290** | **24.161 ± 0.314** | **24.822 ± 0.345** | **20.448 ± 0.120** |
| *BGLAP* | **32.179 ± 4.286** | **33.781 ± 2.814** | **33.711 ± 0.302** | **29.211 ± 0.194** |

**Table 2 Average Ct values for anodized (0.3 – 0.5 μm), anodized (0.8 μm), SLA and BST surfaces**

| | *Δdt* | *ΔΔdt* | *RQ* | *n-Fold* | *Neg. Error* | *Pos. Error* |
|---|---|---|---|---|---|---|
| ***RUNX2*** | **10.683** | **0.149** | **0.902** | **-1.109** | **0.102** | **0.114** |
| ***COL1A2*** | **5.291** | **-0.669** | **1.589** | **+1.589** | **0.331** | **0.418** |
| ***BGLAP*** | **16.241** | **1.392** | **0.381** | **-2.624** | **0.362** | **7.072** |

**Table 3 Results of qPCR of the expression of 3 genes obtained in the analysis of the Anodized (Sa 0.3-0.5 μm)**

| | *Δdt* | *ΔΔdt* | *RQ* | *n-Fold* | *Neg. Error* | *Pos. Error* |
|---|---|---|---|---|---|---|
| ***RUNX2*** | **10.043** | **-0.491** | **1.406** | **+1.406** | **0.178** | **0.202** |
| ***COL1A2*** | **8.230** | **2.263** | **0.208** | **-4.801** | **0.042** | **0.530** |
| ***BGLAP*** | **14.019** | **-0.830** | **1.778** | **+1.778** | **1.525** | **10.737** |

**Table 4 Results of qPCR of the expression of 3 genes obtained in the analysis of the Anodized (Sa 0.8 μm)**

| | *Δdt* | *ΔΔdt* | *RQ* | *n-Fold* | *Neg. Error* | *Pos. Error* |
|---|---|---|---|---|---|---|
| ***RUNX2*** | **10.535** | **0.000** | **1.000** | **+1.000** | **0.100** | **0.112** |
| ***COL1A2*** | **5.960** | **0.000** | **1.000** | **+1.000** | **0.215** | **0.273** |
| ***BGLAP*** | **14.849** | **0.000** | **1.000** | **+1.000** | **0.190** | **0.233** |

**Table 5 Results of qPCR of the expression of 3 genes obtained in the analysis of SLA surface (Sa 1.5 μm)**

| | *Δdt* | *ΔΔdt* | *RQ* | *n-Fold* | *Neg. Error* | *Pos. Error* |
|---|---|---|---|---|---|---|
| ***RUNX2*** | **10.143** | **-0.391** | **1.311** | **+1.311** | **0.045** | **0.047** |
| ***COL1A2*** | **4.660** | **-1.300** | **2.462** | **+2.462** | **0.213** | **0.233** |
| ***BGLAP*** | **13.422** | **-1.427** | **2.688** | **+2.688** | **0.349** | **0.401** |

**Table 6 Results of qPCR of the expression of 3 genes obtained in the analysis of the BST surface (Sa 1.0 μm)**

## DISCUSSION

In this study, immortalized human fetal-osteoblast cells were cultured on five different surface-treated miniature implant specimens and their contribution on expressions of 3 genes involving osteogenesis were investigated. Research efforts over the past few decades have attempted to improve the physicochemical properties of the implant surface in order to decrease healing time, improve the quality of the bone-implant anchorage, and enable more predictable outcomes in poor-quality implant sites. Several new methods were introduced in the early 1990s to roughen the surfaces of titanium implants such as acid etching, sandblasting, sandblasting and acid etch, anodization.[1] At first, the main advantage appeared to be improved mechanical anchorage.[47, 80] However, it soon became evident that the significant improvement in implant anchorage of microrough surfaces was not entirely due to the increased bone implant contact from mechanical anchorage, as various studies demonstrated that different surface treatments resulted in different interfacial breakage strength.[47] These studies helped to gain insight into the biological aspect of osseointegration.

The growth and differentiation of osteoblast lineage cells can be divided into three main periods of proliferation, extracellular matrix maturation, and extracellular matrix mineralization. Skeletal development and bone remodeling require deft control of gene activation and suppression, as each of these periods is characterized by a specific up-regulation or down-regulation of specific genes.[73] A broad array of regulatory signals is involved in governing the commitment of osteoprogenitor stem cells to the bone cell lineage, proliferation and differentiation of osteoblasts, and the maintenance of the bone

phenotype in osteocytes residing in a mineralized bone extracellular matrix.[4] The requirements for short-term and sustained developmental expression of bone-related genes are managed by promoter regulatory elements. The extent to which genes are transcribed is determined by the spatiotemporal orchestration of protein interactions that control assembly, organization and activity of the regulatory machinery for physiological responsiveness.

***Runt-related transcription factor 2 (RUNX2)***

Runx transcription factors contribute to the regulatory mechanism that plays a key role throughout osteoblast differentiation and modulate expression of target genes at key developmental osteogenesis transitions. Irregular expression of Runx proteins can cause disturbances in transcriptional and post-transcriptional regulation associated with problems in skeletogenesis and skeletal disease. RUNX2 transcription, mRNA, and proteins have all been found to be upregulated during the cessation of cell growth in osteoblast cells that do not express mature bone phenotypic gene expression.[76] RUNX2 is principally linked to osteoblast proliferation and differentiation and is critical for regulation of skeletal genes and skeletal development.[81-83] It is initially observed when pluripotent mesenchymal stem cells are committed to the bone cell lineage prior to expression of the osteoblast phenotype. RUNX2 suppresses the growth in proliferating immature osteoblasts without activating mature bone phenotypic genes in the mesenchymal progenitor cells, further indicating that it has a biological function preceding osteoblast maturation.[84] The requirement of RUNX2 to have stringent control of osteoblast proliferation has been demonstrated in the lab to substantiate this process.[85]

However, RUNX2 expression also increases during osteoblasts differentiation validating the involvement in maturation of the osteoblast. RUNX2-mediated activation of RUNX2 expression in osteoblasts reflects autologous control.[84]

***Collagen Type I (COL1A2)***

Collagen represents a major class of proteins present in the various connective tissues in a structural capacity. Type I collagen is the most prevalent protein in the bone, as it makes up to 95% of total bone collagen and 80% of the whole bone protein.[86-87] Synthesized by osteoblasts as procollagen $\alpha 1$ and $\alpha 2$, type I collagen is considered an early marker of bone growth and osteoblastic differentiation based on its foundational role as the framework of the bone matrix, upon which the noncollagenous proteins position themselves to lead to mineralization.[88] The expression of collagen Type I is described to be an early yet unspecific indicator of de novo bone formation, as COL1A2 expression was enhanced at 2 and even 4 weeks into bone formation while a general decline was found for the collagen type I after 12 weeks.[89]

***Osteocalcin (BGLAP)***

Osteocalcin is a noncollagenous protein that is a unique product of osteoblasts and odontoblasts. As the most abundant noncollagenous protein in bone, osteocalcin is mainly incorporated into the bone matrix where it is bound to hydroxyapatite.[90] Only a limited amount of osteocalcin is released into circulation in the body and the serum concentration is a sensitive marker of bone formation that correlates with histomorphometric indices of bone formation.[90-92] Since osteocalcin indicates the

mineralization process of osteogenesis implemented by the calcifying osteocytes in the collagen layer, its appearance during bone maturation comes after the development of the extracellular matrix. Osteocalcin represents a specific marker for pre-osteoblast activity, and it is expressed after 3 weeks of de novo bone formation that reaches its peak expression at 5 weeks.[89] The BGLAP gene encoding for osteocalcin is expressed efficiently in osteoblast and odontoblasts.[93] Transcription of the gene is temporarily regulated depending on the phase of osteogenesis and on osteoblast development, as the BGLAP gene is inactive during proliferation but active during differentiation.[94] The role for osteocalcin during mineralization of the extracellular matrix has yet to be identified during *in vivo* loss and gain-of-function experiments.[95-96] However, carboxylated osteocalcin is considered to be an chemoattractant activator in cells with bone resorption capabilities.[97-98] Interestingly, osteocalcin-deficient mice exhibit increased bone formation that suggests the notion that there is a central role for carboxylated osteocalcin in regulating bone remodeling.[95]

In the present study, the gene expression of cells cultured on the five surfaces were evaluated and compared. Expression of all three genes from machined surface specimens could not be determined due to indeterminable Ct results from the RT-PCR. The SLA surface implant specimen was denoted as the positive control to compare another microrough surface treatment process to the anodized specimens – anodized 0.3 – 0.5 μm, anodized 0.8 μm, and BST 1.0 μm. The anodized 0.3 – 0.5 μm surface displayed up-regulation of COL1A2 (n-fold of +1.589) but down-regulation of RUNX2 and BGLAP (n-fold of -1.109 and -2.624, respectively). The results suggest that the

development of the extracellular osteoid matrix is progressing while bone maturation and mineralization processes are not induced. However, when there was an increase in the surface microroughness to 0.8 μm surface, the genes displayed opposite trends. RUNX2 and BGLAP were both up-regulated (n-fold of +1.406 and +1.778, respectively) while, COL1A2 exhibited the greatest down-regulation trend with respect to all surfaces (n-fold of -4.801). This suggests that the increase in surface microroughness stimulated some increased tendencies of osteoblasts to mature and mineralize. The BST implant specimen was the only surface type to exhibit up-regulation for all three genes. Of the three genes, COL1A2 and RUNX2 displayed the greatest fold change for the BST surface (n-fold of +2.462 and +2.688, respectively). The gene expression results of the BST surface, having the greatest surface roughness of the three anodized samples, seemed to indicate the most maturing of the osteoblasts and its corresponding matrix, with respect to the other four implant specimens.

When a dental implant is placed in the osteotomy site, the degree of cellular activity of the peri-implant bone is determined both by surgical technique and surface properties: surface morphological characteristics of the dental implant affects primary mechanical stability, biocompatibility, cell activity, and the ability to achieve secondary mechanical stability.[99] Studies on the biological influences of different surface types tend to suggest major strides in improving the osteointegration process. However, despite the progressive steps in bettering the knowledge and technology of dental implants, there are still questions surrounding the molecular mechanism governing the osteogenic phenomenon around titanium dental implants.

It has been well established in the literature that the osteogenic process involves a complicated regulatory network comprising of physiological stimuli, several molecular signals, and transcription factors. In 2010, Wennerberg and Albrektsson suggested that surface topography influenced bone response at the micrometer level for implant surfaces, especially moderately rough surface with average height deviation from 1 to 2 μm.[29] However, the advantages of nano-roughness of implant surfaces to peri-implant bone response at the nanometer level is considered debatable to date, even if some indications exist.[100] When human osteoblasts cells come in contact with modified titanium surface, early osteoblast adhesive response has been accelerated to varying degrees.[31] The results from this study also suggest that different titanium surfaces might induce some difference in terms of gene expression.

**LIMITATIONS**

This study, being conducted under *in vitro* conditions, is not reflective of the complex peri-implant bone complex during osteointegration process. In our study, osteoblasts were grown in media on five different surface treated implant specimens under a controlled environment. However, osteoblasts are just one of the many cells involved in the bone formative processes in the body. Also, the samples used in this study were provided by a single manufacturer and may not be an accurate representation of commercially available implants.

The PCR method used in this study relied on mRNA extracted from the samples collected in order to obtain DNA. One of the potential risks using this technique is that the RNA template is prone to rapid degradation if not handled properly.[101] Even when handled properly, there may be some discrepancy between the true quantity and actual quantity of the protein measured and analyzed in PCR (i.e. absolute quantitation vs. relative quantitation, latter of which was used in this study).

Lastly, due to the problems culturing the osteoblasts on the miniature implant specimens, a triplicate of the study could not be performed. As a result, the gene expressions of all three genes in this study may not properly reflect the expression pattern seen under normal osteogenesis conditions in the body.

**FUTURE RESEARCH**

In this study, the initial stages of osteoblast adhesion, proliferation, and differentiation has been studied *in vitro*. Increasing the sample size or using commercially available dental implants will be recommended for this type of *in vitro* culture study, in order to prevent further problems collecting cells for RT-PCR.

Studies that examine osteoblasts behavior *in vivo* for all implant surfaces investigated in this study are still needed. It will be beneficial to examine the osteoblasts interactions to implant surfaces with histological and animal studies in the near future.

More recently, methods have been developed to improve clinical performance by improving the wettability of the implant surfaces and facilitating osteoconductivity creating biomimetic nanoenhanced implant surfaces. Further studies are needed to examine the osteogenic response to the newly developed implant surfaces.

## CONCLUSIONS

In the present experimental model, mRNA expression of RUNX2, COL1A2, and BGLAP in human fetal osteoblast stem cells, cultured on five different surfaces, indicated differences in expression compared to the control groups. The expression of COL1A2 and BGLAP, characteristics of a more mature osteogenic phenotype, were generally exhibited on the rougher surface implants. Following this trend, the BST surface expressed the most mature osteogenic phenotype in this study. Overcoming the limitations and considering the changes suggested, future studies may produce clinically meaningful results.

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