Biomimetic Coated Titanium Dental Implants For Improved Osseointegration

BY

JOSHUA M HASSAN

Specialty Certificate in Periodontics, University of Illinois at Chicago
College of Dentistry, 2017
D.M.D., Temple University Kornberg School of Dentistry, 2014
B.A., Yeshiva University, 2009

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Oral Sciences
in the Graduate College of the
University of Illinois at Chicago, 2017

Chicago, Illinois

Thesis Committee Members:

Sriram Ravindran, PhD-Advisor and Chair
Praveen Gajendrareddy, BDS, PhD-Periodontics
Luisa DiPietro, DDS, PhD-Periodontics

DEDICATION

This thesis is dedicated to my loving wife and daughters, Aviva, Sara and Jordyn Hassan, without whose support none of this would be possible

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Ravindran, for his support, confidence, encouragement and his continual patience. I would also like to thank Dr. Gajendrareddy for his time and support. Without either of these individuals I would not have been able to achieve this research goal. My appreciation for both Dr. Ravindran and Dr. Gajendrareddy cannot be understated.

I would also like to thank my thesis committee members, Dr. Gajendrareddy, Dr. Ravindran, and Dr. Luisa DiPietro for their assistance. Lastly, this thesis would have not been possible without the encouragement and flexibility of my clinical program directors, Dr. Gajendrareddy and Dr. Nares, and clinical manager, Sharon Arrigo, who were supportive of my research goals.

JMH

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

List of Figures

LIST OF ABBREVIATIONS

American Dental Association (ADA)
Alkaline Phopshatase (ALPL)
Bone to Implant Contact (BIC)
Bone Morphogenic Protein (BMP)
Bone Morphogenic Protein 2 (BMP 2)
Bone Morphogenic Protein 6 (BMP 6)
Bone Morphogenic Protein 9 (BMP 9)
B2M
Type Collagen 1 (COL-1)
Extracellular Matrix (ECM)
Frizzled Family (Fz)
Growth Differentiation Factor 10 (GDF10)
Guided Bone Regeneration (GBR)
Human Mesenchymal Stem Cells (HMSC)
Hydroxyapatite (HA)
Low Density Lipoprotein Receptor Related 5 (LPR5)
Low Density Lipoprotein Receptor Related 6 (LPR6)
Mesenchymal Stem Cells (MSC)
Periodontal Ligament Stem Cells (PDLSC)
Phosphate Buffered Saline (PBS)
Osteoprotegrin (OPG)
Osterix (OSX)
Arginylglycylaspartic acid (RGD)
Runt Related Transcription Factor 2 (Runx-2)
Recombinant Platelet Derived Growth Factor (Rh-PDGF)
Small Integrin-Binding Ligand N-Linked Glycoproteins (SIBLING)
Sand blasted, Large Grit, Acid Etched (SLA)
Titanium Plasma Sprayed (TPS)
Transforming Growth Factor (TGF)
Vascular Endothelial Growth Factor (VEGF)

SUMMARY

Hypothesis: An implant coating comprised of a natural extracellular matrix (ECM) of osteogenic MSCs has the potential to improve osseointegration by promoting increased MSC attachment, proliferation and differentiation.

Objective: To provide in vitro research that will assess the implant surface for the presence of key osteogenic proteins via immunohistochemical analysis, as well as Quantitative real-time PCR to document changes in gene expression levels of osteogenic growth factors, transcription factors, ECM proteins.

Methods: HMSCs were seeded on to titanium implants and cultured for specific periods of time in an osteogenic differentiation medium. The purpose of which was to identify the ideal minimal time interval and number of cells required to optimally coat implants with ECM and evaluated via fibronectin staining, a common ECM protein. The implants were decellularized using standardized methods leaving behind an ECM coating on surface. The coated implants were re-seeded with HMSCs for 2 weeks and underwent proliferation studies and PCR analyses to quantify proliferation and differentiation of stems cells into an osteogenic lineage. Uncoated implants seeded with HMSCS served as controls.

Results: The ideal time interval and number of cells required to optimally coat implants with ECM was 2 weeks with 200k cells. The presence of pro-osteogenic structural and functional proteins on the biomimetically coated titanium surfaces was verified using confocal microscopy. Results indicate that HMSCs show improved proliferation and differentiated into an osteogenic lineage compared to non coated control roughened surface implants. This was evidenced by a significant increase in proliferation rate and a statistically significant fold changes in expression levels of osteogenic genes such as specific transcription factors and growth factors.

Conclusions: Results provide proof of concept to support feasibility of generating biomimetic ECM on titanium dental implants and its ability to significantly enhance stem cell recruitment, attachment, proliferation and differentiation.

1. INTRODUCTION

1.1 Background

The early 1940's is the period where most historians identify as the initiation of the development of the modern day dental implant. This period is defined by the advent of the subperiosteal implant framework for totally edentulous patients, developed by Dr. Gustav Dahl in 1941. The idea was that this metal substructure was placed below the gingiva, sit on the jawbone and have vertical prongs that would project through the gingiva to retain the denture. Dahl's work was brought to the United States by Drs. Gershkoff and Goldberg in 1947-1948.[1, 2] Gershkoff and Goldberg produced a cobalt-chromium-molybdenum metal substructure for the implant with an extension to include the external oblique ridge, which was previously not in Dahl's design.[3] The subperiosteal implant design was further researched and elaborated upon by Dr. Lew in the 1950's. He was the first to utilize a direct impression method of the jawbone to receive the implant, thus allowing for fewer supports over the ridge crest.[1] (Figure 1)

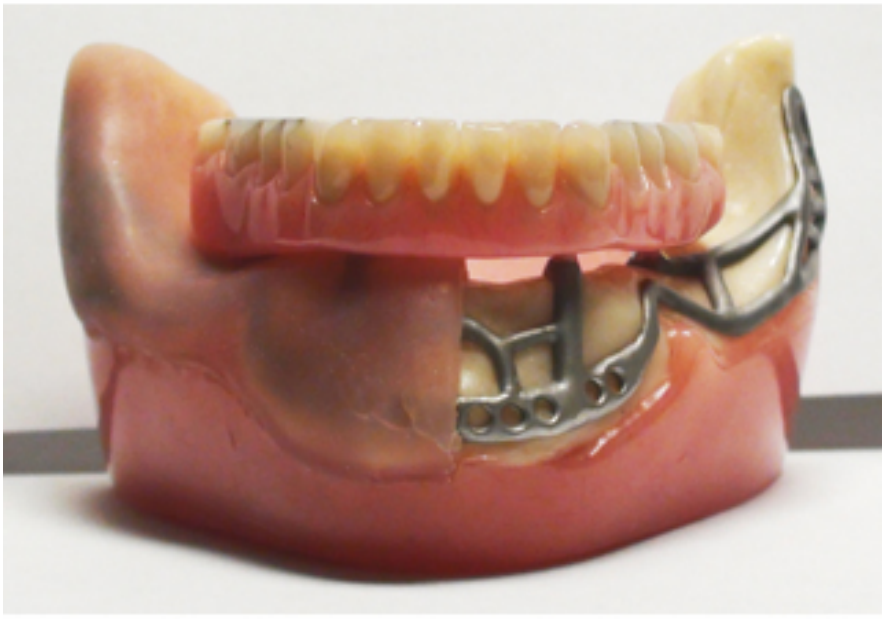


Figure 1: Subperiosteal implant

The predictability and practicality of using these subperiosteal implants was widely debated amongst dentists from the 1950's to the1970's, due to complications associated with this method. These complications included the exfoliation of the bone connecting the screws of the frame, and the exposures of the frame through mucosal perforations were problems. Numerous studies on these subperiosteal implants were conducted and showed some poor long-term results. For example, one retrospective study conducted in 1974 on 27 mandibular subperiosteal implants that were placed between 1952 and 1959 found that short-term survival rates (<5 years) of 96% but only 60% survival rates at 10 years.[4] Another 10-year study reported a 60% survival rate, and an even lower survival rate of 50% after 15 years. In addition to that, twenty-year studies showed a failure rate of 34% in the mandible.[5] Due to these low success rates subperiosteal implant frameworks remained controversial and eventually fell out of practice. The implant dilemma that bothered man since prehistoric times raged on and dentists continued to look for new solutions.

Almost 25 years after the development of the subperiosteal implant, in 1968, Dr. Small introduced a new dental implant concept called the transosteal implant. This implant "transverses" the entire mandible, from the top to the bottom, hence its name. [8] This implant was "inserted underneath the chin and flat bone plate was fixed under the skin against the inferior border of the mandible. Several threaded posts projected into the anterior mandible from the plate and two to four of these posts projected through the mucosa to fixate and stabilize the denture prosthesis." [6] (Figure 2)

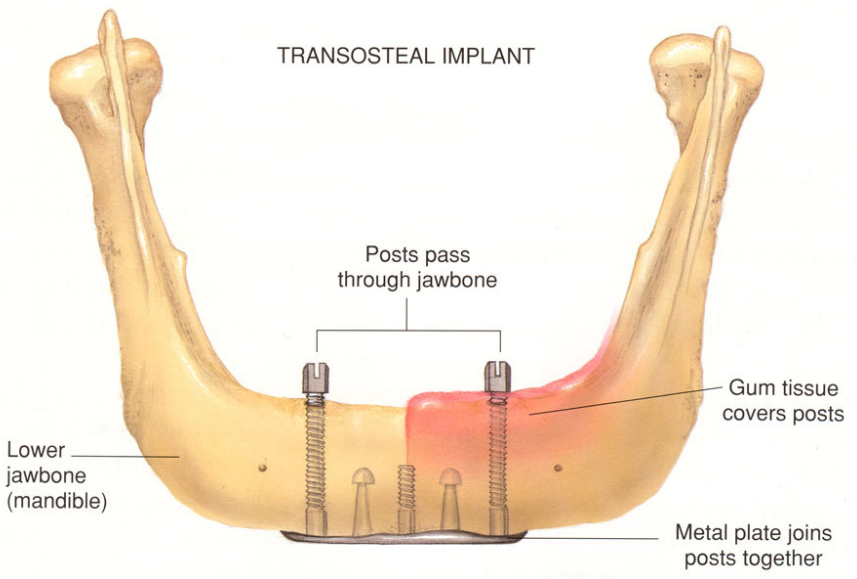
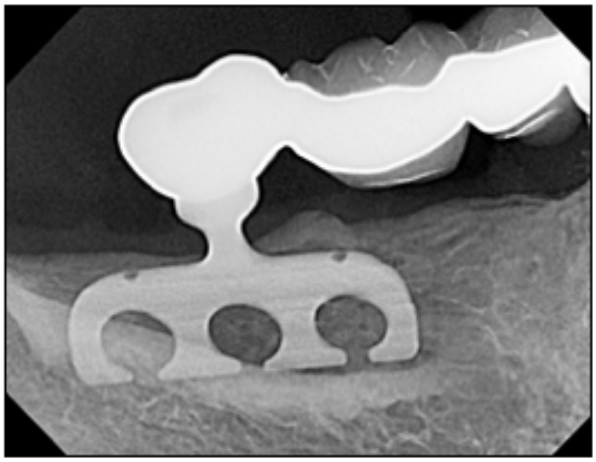


Figure 2: Transosseous implant

Transossteal implants consisted of titanium, or a gold alloy. Unfortunately, like other implants before it, the transosteal had several major disadvantages. One, the surgery could not be performed in an office like setting but instead needed to be executed under general anesthesia in a hospital setting. Secondly, since the implant went through the entire mandible it was quite an invasive procedure. Thirdly, these implants frequently had bleeding on probing, a technique that normally indicates disease as well as pronounced bone loss around the posts.[6] Due to these aforementioned issues, the transosteal implant is no longer used today and was eventually replaced by other implant designs.

In 1966, Dr. Linkow presented the "blade implant" around the same time as the transosteal implant.[1, 6] This is considered one of the first endosseous implants and was inserted intraorally in the bone by making a small groove in the alveolar bone and then tapping the implant into place. One or more pillars were attached to the fin-shaped blade, which anchored the restoration. (Figure 3A, 3B)



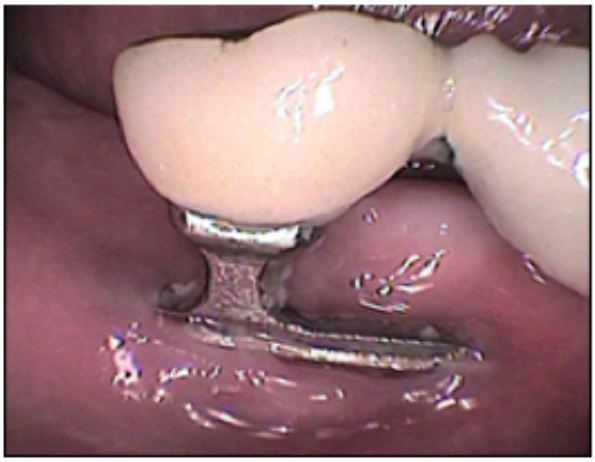


Figure 3A and #B: Blade implant

The success rate for this implant was also very low, under 50%, with exposure of the implant being a common complication. As a result, the major drawback with the blade implants was that they became loose, leading to infection and they had to be removed.[7, 8]

So, the question must be asked why did all these somewhat recent implants fail? Was technology not advancing in such ways that implants should have higher success rates? The answer was discovered by accident by Dr. Per-Ingvar Brånemark, the dentist considered the father of the modern day endosseous implant. While working on his thesis studying bone regeneration capacity/capabilities in rabbits, Dr. Brånemark fabricated a titanium chamber and placed it in a rabbit's femur to observe wound healing. On attempting to remove it, Dr. Brånemark found that the bone adhered and integrated to the implant. To remove the titanium chamber, Dr. Brånemark had to break the rabbit's femur. In fact, he found that it if a fractured occurred, it usually occurred between the bone and bone and rarely between the implant and bone. He developed many more experiments to confirm this finding but in the end, it was clear that the titanium integrated into the bone. Dr. Brånemark immediately thought of applying this discovery to dentistry, specifically its long ailing implant dilemma.[1, 6]

Brånemark's first implant patients were treated well before he published his work on implants. In Sweden in 1965, he placed the first endosseous implant on one of his patients with the prosthesis being an implant-supported bridge. Four implants were positioned in the patient's mandible, which integrated within six months, and then the prosthesis was attached. Forty years later, that prosthesis was still in place. [1, 6, 9] Then after extensive research, in 1978, Dr. Brånemark published his work on machined surfaced cylindrical titanium root-form endosseous implant to the world; termed endosseous because it sits in the bone and simulates a natural root. He called these pure titanium screws fixtures because they were fixed in the bone and fixated the artificial tooth. [1] In 1982, Dr. Brånemark's introduced his system to the United States and ADA (American Dental Association), both of which quickly approved the Brånemark system to be used for single tooth replacement, partially and fully edentulous implant supported bridges and implant retained overdentures. [1]

With Dr. Brånemark's discovery and implant system came the concept of "osseointegration", a term he personally coined. Dr. Brånemark was one of the first to realize that the clinical success of oral implants is related to their osseointegration. This biologic fixation is a prerequisite for implant supported/retained restorations and the implant's long-term success. After placement, dental implants interact with biological fluids and tissues. There are two types of response that bone has to implants after placement. The first type involves the formation of a fibrous soft tissue connection around the implant that leads to clinical failure due to lack of integration into hard tissue and proper biomechanical fixation. The second type of bone response is related to direct bone–implant contact (BIC) without an intervening connective tissue layer. [6, 10-12] This is what is known as osseointegration, a term explained by Brånemark as "a direct structural and functional connection between ordered, living bone, and the surface of a load carrying implant".[9]

Clinically, osseointegration is measured by "asymptomatic rigid fixation of an implant in bone with the ability to withstand occlusal forces". [12] The presence of this osseointegration again is why Dr. Brånemark's implants succeed where so many others failed. This was either something that was lacking in previous implant modalities or the implants were loaded to soon or with too much force to maintain rigid fixation. One must realize that before Brånemark's introduction of his protocol, dental implants were commonly loaded at placement because the thought at the time was that immediate bone stimulation would avoid crestal bone loss. However, in 1969 Brånemark showed that direct bone apposition at the implant surface was possible and lasts under loading with the stipulation that the implants were left to heal under the gingiva for 6 months.[9, 13]

Since Dr. Brånemark's system was introduced in 1982, advancements in biotechnology, biomaterials, implant science and nanotechnology have improved dentistry's understanding of the bone to implant contact interface. This has resulted in improved outcomes, increased success and survival rates and an expanded utilization of implants. Currently, the field of dentistry is using exclusively roughen surfaced implants (figure 4), which as unheard of in Dr. Brånemark's era of the 1970's and 80's, where machine smooth surfaced implants where used. [1]

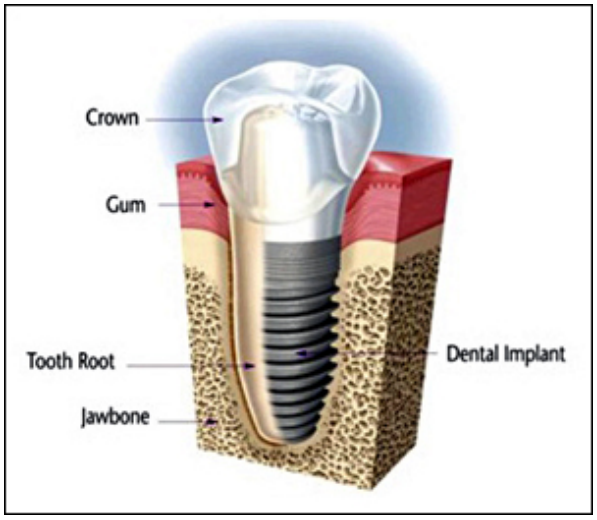


Figure 4: Modern day implant

The reason for this being is that numerous studies, including in vitro, animal and humans studies have showed clinical superiority of roughened surfaced implants (implants with textured pattern) compared to machined smooth surface implants (implants that lack texture). In 1999, a Cochran Review Meta-analyses was published comparing smooth surface implants to roughened surface implants. Meta-Analyses were performed on all implants in all locations, on implants placed only in the maxilla or the mandible, and, finally, on implants placed in the maxilla compared to implants placed in the mandible. The review concluded that rough surfaced implants had significantly higher success rates compared to implants with smoother surfaces except in the case of single tooth replacements where the success rates were comparable. In addition, the documented advantage of implants with a roughened surface in animal and in vitro experiments has been demonstrated in clinical cases and the magnitude of this advantage is significant for patient care.[14] Understanding the significantly improved performance of roughened surface implants, along with the development of hard tissue grafting for the sake of implant placement, yielded an industrial shift in dentistry to solely place roughned implants.

Nowadays, there are abundant materials available to aid in bone regeneration in the maxillofacial region to place implants in sites where it was previously thought it could not be done due to the little or poor quality residual bone. Therefore, the majority of cases of dental implant require some form of hard tissue grafting; whether it be Guided Bone Regeneration (GBR) or grafting at time of implant placement in order to safely place the implant. These materials include biologics like Bone Morphogenetic Protein (BMP) or bone substitute composite allografts or xenografts and even the patients' own autogenous bone. These graft types involve the key concepts of osteogenesis, osteoinduction, osteoconduction.[12]

In the past, for these grafting procedures, autogenous grafts were considered the gold standard but came with the obvious consequence of requiring a second site surgery as a donor site. This would increase the morbidity of the surgery and increased the risk of infection, not to mention being quite uncomfortable for the patient. Presently, with the advent of these allografts or xenografts and their high

success rates comparable or better to autogenous bone, the dentist can avoid a donor site and its associated morbidity by using allografts or xenografts. However, there is a significant shortcoming to bone regeneration with allografts or xenografts, that being, that grafted bone is softer than natural bone and has a slower cell turnover rate. Therefore, two problems could exist with grafting treatments; the first being that since the bone has a slower turnover rate there is a delay in osseointegration and a longer wait time to load the implants. Secondly, when placing implants in grafted bone ideal primary stability is not always achieved, which also can lead to delayed osseointegration and lower bone to implant contact. [10, 11] These downsides to grafting serve as another reason for the field of dentistry to modify implant surfaces to improve osseointegration.

Furthermore, the prevailing school of thought today is that the rate and quality of osseointegration in dental implants, which is in large part responsible for its success, is directly related to the surface properties of the implant; namely the surface area. Thanks to modern technologies, several different types of roughened implant surfaces are currently commercially available for clinical use. The primary aim of this is to increase the functional surface area of the bone to implant interface, so that more of the implant area can become integrated, which consequently allows occlusal stresses to be more effectively transferred. Additionally, roughened implants have been found to promote bone apposition by enhancing cellular activity via: regulating the growth rate and metabolic action of osteoblasts, influencing cytokine and growth factor production by osteoblasts and increased osteoblast cell propagation. Therefore, the uniqueness of the roughened surface directs the host response and affects the mechanical strength of the implant to bone interface. Thus, demonstrating that the structure of the implant influences the interaction between the metal and the living tissue.[1, 13, 15]

Therefore, changing the surface composition the implant will enhance and expedite bone to implant interaction and osseointegration.[10]As a result, roughened surface implants decrease the healing time needed for osseointegration in autogenous or grafted bone thereby allowing for the implant to be loaded in a shorter amount of time. This decreases the wait time the patient has to endure before receiving

his or her prosthesis, which is ideal for everyone involved.[1] For these reasons most commercial dental implants have a roughened surface.

Roughened surface treatments can be classified as additive, mechanical and chemical electrochemical, vacuum, thermal and laser treatments.[1, 10, 12, 13] These treatments are not mutually exclusive and they can be combined to enhance the roughened surface, e.g. the SLA implant is sand blasted and acid etched.

Additive treatments include Titanium Plasma Spraying (TPS) or Hydroxyapatite (HA) materials. With regards to HA, Hydroxyapatite (HA) is a bioactive mineralized material that bone and teeth are composed of. Therefore, it has the potential to form a strong bond between the bone and the implant. Plasma spraying (PS) allows the implant to have a coating thickness of approximately 40-50 micrometers. This thermal spray process involves the injection of powdery forms of titanium or HA into a plasma torch at elevated temperatures and high velocities. These particles eventually condense, fuse together and harden into a coating material on the implant surface. [1, 10, 16] The advantages of PS include simplicity, low cost, low substrate temp and rapid deposition. However, PS is not without its problems. With regards to the HA coating, it may degrade and resorb over time and eventually de-bond. Ultimately, PS has poor bond strength between coatings, which results in poor long-term adherence of the coating to the implant. On top of that, PS can have uneven thickness of deposited layer as well as inconsistencies in in the composition of the coating. Despite all of this, 3-D topography has demonstrated increased tensile strength at the bone to implant interface. Additionally, it has been shown that there are no clinical differences observed when comparing PS to SLA (Sand blasted, Large Grit, Acid Etched) implant surfaces. [10, 16]

Mechanical treatments include either deforming the implant surface by blasting or by removing some of the implant surface material by abrasion or cutting. The most common of these is grit blasting, which is the process of blasting the implants with hard ceramic particles that are propelled through a nozzle at a high velocity via compressed air. Different surface roughness can be achieved through blasting different size particles at the implant. After the blasting, the particle material is frequently

entrenched on the implant surface and residue normally remains even after ultrasonic cleaning, acid passivation and sterilization. The ideal blasting material should be biocompatible, easily cleansable, chemically stable and above all not interfere with the osseointegration between the bone and the implant. The most common grit blasting materials used are Alumina Oxide, Titanium Oxide or Calcium Phosphate. However, with respect to Alumina, it is insoluble in acid and therefore is very difficult to remove residual material, which could eventually leach into surrounding tissues and interfere with osseointegration. In contrast to Alumina Oxides, the Calcium Phosphates (which consist of HA) are reabsorbable and easily cleansable leaving behind a clean roughened implant surface. [10, 16]

Chemical surface treatment is basically acid etching the implant surface with a highly acidic acid. Etching with such strong acids like hydrochloric acid or sulfuric acid produces "micro pits" on titanium surfaces ranging from 0.5 to 2 μm in diameter. Placing the titanium implants through the process of dual acid etching produces micro-rough surfaces. Dual etching consists of placing implants in a heated mixture above 100^0 C of hydrochloric and sulfuric acid for several minutes. Acid etching appears to greatly enhance the potential for osseointegration especially in the earlier stages of healing after implant placement. [10, 16] Etching also has a positive effect on bone apposition with a larger percentage of direct bone to implant contact resulting in strong implant stability. One of the major advantages of this technique compared to the others is that there is no requirement to use another agent to remove residual material. This is due to the fact that acid etching creates a highly detailed surface texture that lacks residual particles or impurities. When comparing TPS surfaces to dual acid etching surfaces, studies have found a higher bone to implant contact with acid etched surfaces.[10, 16] Nevertheless, there are still drawbacks when it comes to dual etching; one being the phenomenon of "hydrogen embrittlement". After etching, the titanium can absorb hydrogen from the acids, which can create micro cracks on its surface. This could lead to reduced fatigue resistance and a reduction in the ductility of the titanium resulting in a possible higher occurrence of fractures in dental implants.[10]

The field of dentistry is always changing and evolving, even more so for the dental implant arena. Implants seem have become quite popular nowadays and more research then ever has been dedicated to

this area of study. Future trends with regards to surface roughness modifications are taking place at the nano-scale level to promote protein adsorption and cell adhesion. In addition, researchers are investigating the incorporation of biological drugs coated on implants for accelerating the healing process after implant placement; as well as investigating placing a biomimetic extracellular matrix (ECM) on titanium implants to promote osteoinduction to enhance osseointegration. The idea behind this is that a dental implant is a biologically inert material and an ECM bioactive coating will promote faster bone turnover around implant thus allowing for improved osseointegration by enhancing bone formation. This is something that has not been seen with previously bioactive coating materials.

This is important clinically for two reasons. One it improves the quality of osseointegration and two it reduces the time that is needed to wait for loading the implants, i.e. the time period that patients have to wait for their final crown delivery. Implants can be immediately placed and immediately loaded, but the number one predictor to their success, survival and overall clinical outcome is primary stability. If an implant is loaded when lacking primary stability, it is at much higher risk for failure compared to conventional loading.[17] Therefore, if the implant lacks primary stability, the current practice is to wait 2-3 months to load this implant. [17-19] This principle is based on the time it takes for the bone to form, remodel and mature in order to resist mechanical stimulation or loading. After placing an implant in an osteotomy, bone undergoes three phases: osteoconduction, de novo bone formation and bone remodeling. After the last phase of bone remodeling, the bone to implant interface is now comprised of maturing de novo bone formation.[20] Prior to surface modifications of implants, this time period of bone maturation was 6 months but with the advent of surface modification techniques described above it has allowed clinicians to shorten healing to 3 months prior to loading. However, this is still a significant time that a patient has to wait prior to crown fabrication and loading. The possibility of having an implant with a biomimetic coating of ECM that induces osteogenic differentiation could potentially significantly shorten this clinical waiting period by enhancing the rate and quality of osseointegration.

In the human body, cells are surrounded by their ECM which is collection extracellular structural proteins (primarily collagen) that provide support to surrounding cells. These proteins can bind growth

factors, or other cells to the matrix and transmit biochemical signals that influence cell to cell communication and cell migration, proliferation, and differentiation [21]. Due to this, ECM scaffolds have been and continued to be used in tissue regeneration research. ECM scaffold that is tissue specific has been shown to induce, direct and maintain lineage specific differentiation of stem cells[22-24]. In a cartilage regeneration model, it has been demonstrated that it is possible to achieve and maintain chondrogenic differentiation of HMSCs using biomimetic ECM scaffolds. When comparisons were made between osteogenic and chondrogenic biomimetic ECM scaffolds, it was determined that the scaffolds induce lineage specific differentiation without additional external stimuli[22]. ECM research has also been applied to the oral cavity. An odontogenic derived ECM scaffold stimulated PDLSCs (Periodontal Ligament Stem Cells) and HMSCs (human mesenchymal stem cells) to undergo odontogenic differentiation i.e. these cells formed a vascularized pulp like- tissue, without the need for exogenous growth factors [23]. This same principle has been applied to dental alveolar bone regeneration. Previous studies have demonstrated that the ECM of HMSCs cultured in osteogenic medium influence the differentiation of these stem cells toward an osteoblastic lineage. [23, 24] This study showed that ECM scaffold allowed for osteogenic differentiation of undifferentiated HMSCs as evidenced by significant changes expression levels of growth factors, transcription factors, proteases, receptors, and ECM proteins. [24]

Based on this ECM research, there is potential to possibly improve the rate and quality of dental implant osseointegration by coating implants with an ECM embedded with HMSCs. This ECM could potentially induce differentiation of these HMSCs into osteogenic lineage without the need for external intervention by means of growth factors or differentiating agents, thus promoting faster bone growth.

1.2 Hypothesis of Study

The objective of this *in-vitro* study is to coat titanium implant surfaces with bimimetic coating and if this coating enhances attachment, proliferation and osteogenic differentiation of HMSCs. This will be assessed by the presence of key osteogenic proteins via immunohistochemical analysis, as well as Quantitative real-time PCR to document changes in gene expression levels of osteogenic growth factors, transcription factors, proteases and receptors.

1.3 Significance

Proof of concept to support feasibility of generating biomimetic ECM on titanium dental implants and its effectiveness in promoting stem cell recruitment, attachment, proliferation and differentiation. This is clinically relevant for two reasons. One, it improves the quality of osseointegration and two it reduces the time that is needed to wait for loading the implants by enhancing the rate of osseointegration.

1.4 Expected Outcomes

We expect that HMSCs show improved proliferation and differentiated into an osteogenic lineage. And that there will be a significant increase in proliferation rate and significant fold changes in expression levels of osteogenic genes such as specific bone transcription and growth factors. We expect to generate an even coating of ECM on implant surfaced that will enhance MSCs attachment, proliferation and differentiation with respect to untreated controls.

2. METHODOLOGY

2.1 Standardization

Different concentrations (50,000, 100,000 and 200,000) of HMSCs were seeded onto dental roughened surface implants in 96 well plates in triplicates. 24 hours later implants were transferred to 6 well plates and cultured under osteogenic conditions (w/ osteogenic media) for 2 weeks. At the end of 2 weeks implants were decellularized using the following protocol: Implants were treated with buffer 1 (10mM sodium phosphate, 150mM sodium chloride, and 0.5% Triton X-100) for 30 min at 37°C in a tissue culture incubator. The buffer was then changed to buffer 2 (25mM ammonium hydroxide) and the implants were incubated for 20 min at 37°C. Finally, the implants were washed three times in Hanks balanced salt solution (HBSS) containing no calcium or magnesium. Implants were then subjected to three freeze–thaw cycles in liquid nitrogen and in a 37°C cell culture incubator, respectively. Finally, the implants were washed three times in HBSS and treated with DNAse (Gibco) at 37°C for 30 min to remove traces of DNA bound to the matrix. The implants were then washed four times in HBSS and stored at 4°C in HBSS containing 5% antibiotic– antimycotic cocktail (Gibco). This was repeated for all ECM implants used in this study.

After decellularization process of the ECM implants, immunohistochemical staining was performed using the following protocol. ECM implants were placed in Formaldehyde for 30 mins, then washed three times with PBS. Implants were incubated for 1 hour in PBS with 5% Bovine Serum Albumin (BSA). Then rabbit polyclonal anti fibronectin antibody (1/250, abcam) was added and implants were incubated for 24 hours. After incubation, PBS with 5% (BSA) was removed and implants were washed three times in PBS. Secondary antibody w/ red fluorescing die (Anti rabbit with TRITC) was added to each implant and underwent one hour incubation. After incubation, implants were washed three times with PBS. Implants were imaged using Zeiss LSM 710 confocal microscope.

2.2 Immunostaining for osteogenic proteins

Hematoxylin and eosin (H&E) staining and alizarin red staining was performed on ECM and control implants (as per previously published protocols)[23]. Presence of proteins was assessed using

secondary antibodies conjugated to fluorescent probes, followed by confocal microscopy. The following primary antibodies were used: mouse monoclonal anti tubulin antibody (1/1000, Sigma), mouse monoclonal anti bone morphogenetic protein 2 (BMP2) antibody (1/100, abcam), rabbit polyclonal anti transforming growth factor β1 (TGFβ) antibody (1/100, abcam), mouse monoclonal anti phosphorylated serine (pSer) antibody (1/100, abcam), mouse monoclonal anti osteocalcin antibody (1/100, abcam), rabbit polyclonal anti VEGF antibody (1/100, abcam). All fluorescently immunostained surfaces were imaged using a Zeiss LSM 710 META confocal microscope equipped with Zen imaging software.

2.3 Proliferation Experiment

60,000 HMSCs were seeded onto control and ECM coated implants in quadruplicates. 24 hours post seeding, the number of attached live cells was quantitatively measured using an MTT cell titer assay (Promega) as per manufacturer protocols. The number of cells present at various time points up to 10 days was also measured to obtain the proliferation rate.

2.4 In Vitro Differentiation of HMSCs

30,000 HMSCs were seeded onto 8 control and 8 ECM implants and cultured in standard growth media for 2 weeks. After 1 and 2 weeks, the RNA was isolated from both sets of samples using BioRad RNA isolation kit as per manufacturer protocol. After first strand synthesis (BioRad first strand synthesis kit), quantitative real time PCR (qPCR) was performed using gene specific primers. Table 1 lists the primer sequences used in this study. All expression data were normalized to housekeeping genes GAPDH and B2M. All experiment were performed in quaddruplicates and statistical significance was calculated with respect to uncoated surfaces using students t-test with greater than 95% confidence interval ($p<0.05$).

GENE	FORWARD	REVERSE
FGF2	5'-AGA AGA GCG ACC CTC ACA TCA – 3'	5' – CGG TTA GCA CAC ACT CCT TTG – 3'
BMP2	5' – ACT ACC AGA AAC GAG TGG GAA – 3'	5' – GCA TCT GTT CTC GGA AAA CCT – 3'
GDF10	5' – AGA TCG TTC GTC CAT CCA ACC - 3'	5' – GGG AGT TCA TCT TAT CGG GAA CA– 3'
PHEX	5' – GAG GCA CTC GAA TTG CCC T – 3'	5' – ACT CCT GTT TAG CTT GGA GAC TT – 3'
ALPL	5' – ACT GGT ACT CAG ACA ACG AGA T – 3'	5' – ACG TCA ATG TCC CTG ATG TTA TG – 3'
TGFB1	5' – CAA TTC CTG GCG ATA CCT CAG – 3'	5' – GCA CAA CTC CGG TGA CAT CAA – 3'
RUNX2	5' – TGG TTA CTG TCA TGG CGG GTA -3'	5' – TCT CAG ATC GTT GAA CCT TGC TA -3'
OSX	5' – CCT CTG CGG GAC TCA ACA AC – 3'	5' – AGC CCA TTA GTG CTT GTA AAG G – 3'
OCN	5' – AGC CCA TTA GTG CTT GTA AAG G – 3'	5' – CCC TCC TGC TTG GAC ACA AAG – 3'
OPN	5' – GAA GTT TCG CAG ACC TGA CAT – 3'	5' – GTA TGC ACC ATT CAA CTC CTC G – 3'
VEGFA	5' – AGG GCA GAA TCA TCA CGA AGT – 3'	5' – AGG GTC TCG ATT GGA TGG CA – 3'
COL1	5' – GAG GGC CAA GAC GAA GAC ATC – 3'	5' – CAG ATC ACG TCA TCG CAC AAC -3'
BMP9	5' – AGA ACG TGA AGG TGG ATT TCC – 3'	5' – CGC ACA ATG TTG GAC GCT G -3'
BMP6	5' - TGT TGG ACA CCC GTG TAG TAT - 3'	5' – AAC CCA CAG ATT GCT AGT GGC – 3'
GAPDH	5' – CAG GGC TGC TTT TAA CTC TGG - 3'	5' – TGG GTG GAA TCA TAT TGG AAC A -3'
B2M	5' – GAG GCT ATC CAG CGT ACT CCA – 3'	5' – CGG CAG GCA TAC TCA TCT TTT – 3'

Table 1: Gene Primer Sequence

3. RESULTS

3.1 Standardization

In our study, we determined that the optimal cell density required to generate an even ECM coating on the implant surface was 200,000 cells per implant. Figure 5A and 5B show the surface of a titanium implant coated with the biomimetic ECM coating, seeded with 50,000 HMSCs for two weeks, and subsequently stained for fibronectin, which is one of the most abundant ECM proteins that sequesters growth factors and allows for cell attachment to the ECM. The images are color coded for depth. Red indicates the top and blue the bottom along the z-axis. The staining showed an uneven pattern of distribution with multiple areas of breaks in the color coding indicating an uneven or even missing ECM deposition. This indicates an insufficient number of cells to form an even layer. Figures 5C and 5D show the results from culturing 100,000 HMSCs for two weeks. Although, the pattern is more uniform, there are still breaks and gaps in the staining indicating that there is not even distribution of the ECM. Figures 5E and 5F display the 200,000 HMSCs showing the uniform pattern on the crest and trough which indicate an even ECM deposition. Based on these observations, we concluded that the 200,000 cells and the 2 week culture as the ideal time frame. This condition was used to generate coated implants for the following experiments.

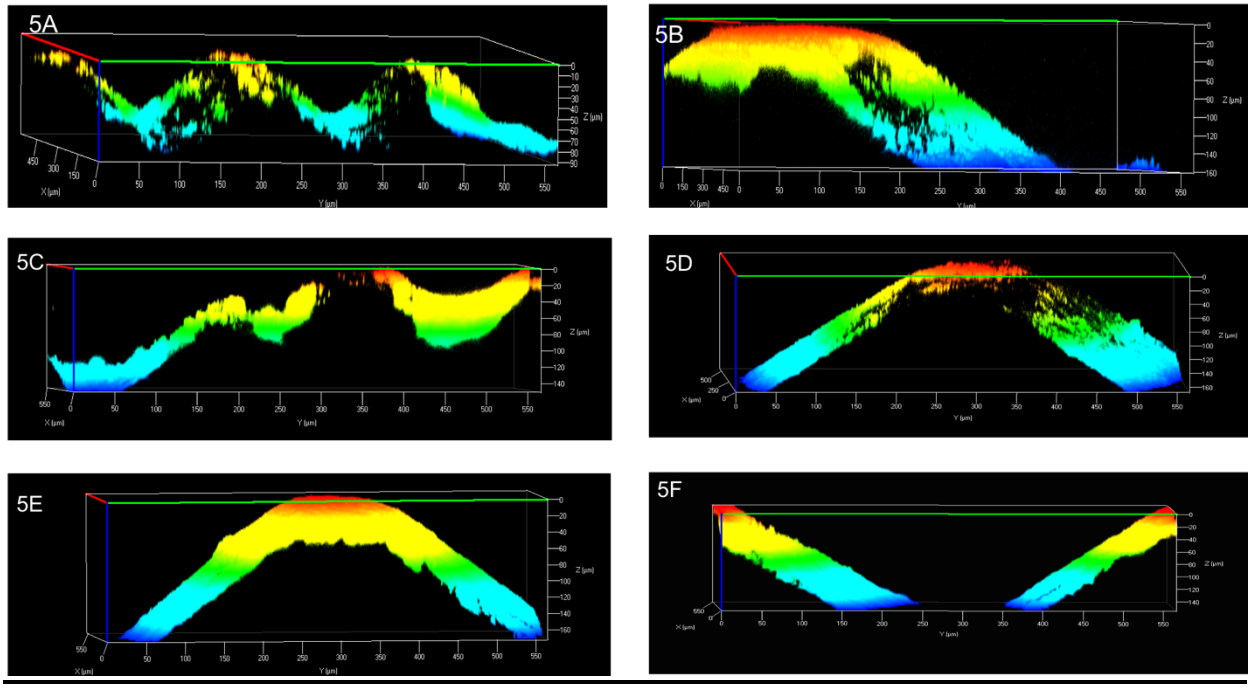


Figure 5: Images A- F are 3D reconstructions of titanium implant surfaces coated with our biomimetic ECM and immunostained for fibronectin. The reconstructions are from a z-stack of confocal images obtained using a Zeiss LSM 710 confocal microscope. The images are color coded for depth. Red indicates the top and blue the bottom. 5A and 5B have concentration of 50,000 cells, 5C and 5D have concentration of 100,000 cells, 5E and 5F have concentration of 200,000 cells. 5E and 5F show the uniform pattern on the crest and trough indicate an even ECM deposition.

3.2 Immunostaining for osteogenic proteins

Immunohistochemcial staining was performed to observe the presence of osteoinductive ECM proteins. Immunohistochemical analysis revealed the presence of BMP2 (Fig. 6A), TGFβ (Fig. 6B), VEGF, (Fig. 6C), Osteocalcin, (Fig. 6E). Figure 6D shows the presence of fibronectin and Figure 5F shows positive staining with anti-phosphorylated serine antibody. Figure 6G and 6H are rabbit and mouse secondary antibody controls. DAPI staining of the surfaces did not show any positive stain, indicating absence of cellular DNA (Fig. 6G). Tubulin was used as a negative control for the presence of intracellular proteins. A negative staining was observed (Figure 6H) indicating the absence of intracellular proteins.

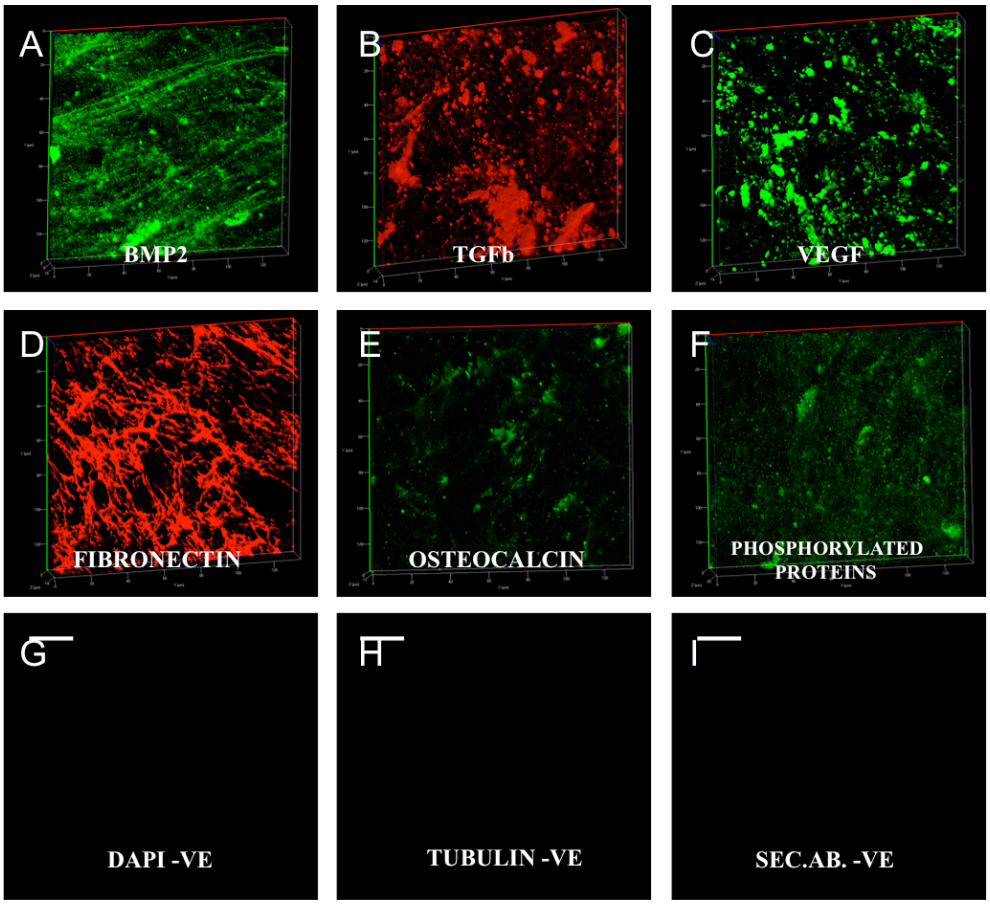


Figure 6: Images A to F are 3D reconstructions of titanium surfaces coated with our biomimetic ECM and immunostained for several matrix proteins that are important for promoting stem cell differentiation and bone growth *in vivo*. The reconstructions are from a z-stack of confocal images obtained using a Zeiss LSM 710 confocal microscope. Images G, H and I are controls that show the absence of DNA, intracellular proteins and non-specific staining respectively.

3.3 Proliferation of HMSCs

A proliferation experiment was performing using 60, 000 HMSCs seeded onto coated and uncoated titanium surfaces. It was found that the coated ECM implants induced a 72.84% increase in the proliferation rate of stem cells. Figure 7 shows a graphical representation that shows proliferation of HMSCs on the control and coated implants, which is a plot of absorbance versus time. An increase in absorbance correlates with an increase in cell number. A linear increase was assumed, since cells proliferate linearly, and straight line fit was performed. The slope of the lines give the rate of increased absorbance corresponding to the proliferation rate.

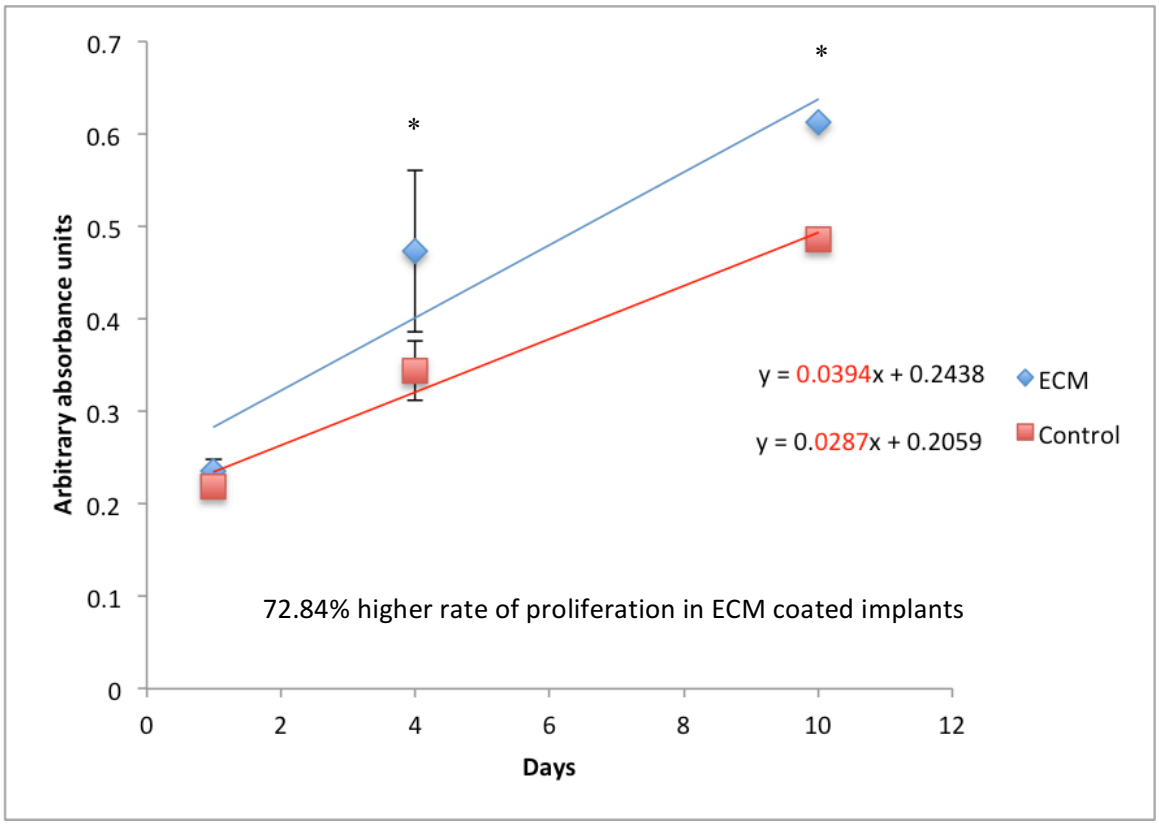


Figure 7: The red and blue lines are linear approximations of the rate of proliferation of HMSCs on control and ECM coated implants respectively. The red number in the corresponding equations shows the slope of the line indicating the rate of proliferation of the cells. The proliferation rate was 72.84% higher on the ECM coated implants. The star above the data points indicates statistical significance as measured by student's t-test with 95% confidence interval ($p<0.05$).

3.4 *In Vitro* Differentiation of HMSCs

30,000 HMSCs were seeded onto 8 control and 8 ECM implants. After 1 and 2 weeks RNA was isolated from both sets of samples and qPCR was performed as described in the methods sections. Tables 2 and 3 show the results from this experiment. It is evident from the presented data that there was a statistically significant increase in the expression levels of pro-osteogenic genes in cells that were cultured on ECM coated titanium surfaces as opposed to the control titanium surfaces. The data was normalized to the average of GAPDH and B2M Cq values. Fold change was calculated using the formula Fold change = $2^{(-\Delta\Delta Cq)}$. From these tables, expression of growth factors, which play key roles in osteogenesis, have an earlier expression than those in the control group. Majority of the growth and transcription factors had anywhere from 1- 1.5 cycle changes.

It is important to know that Osterix (OSX) was turned on after 1 week and BMP 9 was turned on after 2 weeks, meaning it was not amplified with 40 cycles of PCR for control implants. However, OSX and BMP 9 were expressed in the RNA from the coated implant samples. This is important because OSX is a potent transcription factor and BMP-9 is potent osteogenic growth factor.

Gene	Fold Change	P-Value (T-Test)
Runx2	3.87	0.003
ALPL	3.2	0.0005
OSX	TURNED ON	-
COL1	1.93	0.002
GDF10	1.47	0.0001

Table 2: Fold change in gene expression after 1 week of HMSC culture on ECM coated titanium surface as opposed to control titanium surface. Osterix (OSX) one of the key osteogenic transcription factors was not expressed in the control. n=4

Gene	Fold Change	P-value (t-test)
BMP2	2.25	0.061
BMP9	TURNED ON	-
Runx2	1.94	0.015
ALPL	1.8	0.091
OSX	3.64	0.008
COL1	3.61	0.007
TGFβ1	1.77	0.02
GDF10	3.93	0.057

Table 3: Fold change in gene expression after 2 week of HMSC culture on ECM coated titanium surface as opposed to control titanium surface. BMP9 one of the key osteogenic growth factors was not expressed in the control. n=4

4. DISCUSSION

The ECM is a network in which cells are connected to one another and this ECM directs cellular communication, behavior and tissue functionality[25]. As stated before, there are several reports that show the use of decellularized ECM as a biomimetic biomaterial for tissue regeneration of either dental pulp tissue or bone[23, 24, 26]. Furthermore, outside of dental applications, decellularized biomimetic matrices have been studied and for tissue engineering applications such as cartilage[22, 27], heart[28] and lung [29]. The advantage of these matrices over other types of biomaterials is that these matrices can induce a tissue specific environment, which does not rely on exogenous growth factors to induce cell differentiation. Consequently, in this study we have coated dental implants with an osteogenic ECM that has the potential to significantly enhance and expedite osseointegration. Our results show that the coated ECM implants contains pro-osteogenic factors and ECM proteins required for osseointegration.

4.1 Osteogenesis and Signaling Pathways

Bone mainly consists of an ECM, osseous tissue is comprised of 90% calcified ECM and 10% cells. The ECM composition is 88% protein, 30% organic collagen type I, and the remaining 12% is a combination of glycosminoglycans and lipids.[30] Furthermore, bone is constantly undergoing change; it is a dynamic tissue that is demineralizing and remineralizing or what is better known as bone remodeling. During remodeling bone is resorbed primarily through osteoclast activity and deposited through osteoblast activity. Our study focuses on osteogenic lineage and differentiation of HMSCs in a biomimetic environment. An osteoblast is a mononucleate cell that located either in bone marrow or periosteum that is responsible for forming osteoid tissue and the mineralization of osteoid.[31] Osteoblasts originate from MSCs which are enlisted from the local environment. Osteoblast differentiation is regulated by endocrine, paracrine and autocrine systems involving cytokines, hormones and growth factors which proceeds in a linear sequence from MSCs to preosteoblasts to osteoblasts (Figure 8).

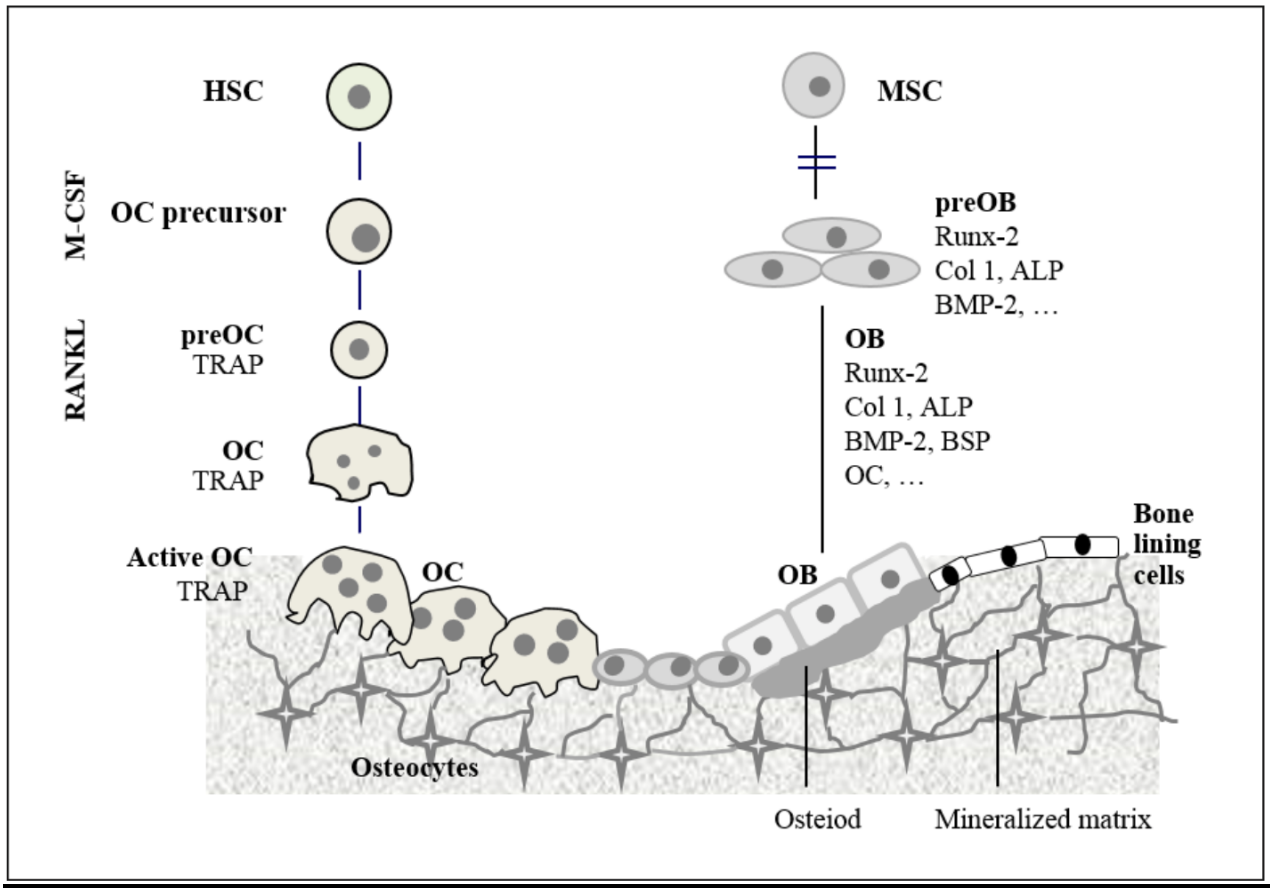


Figure 8: Simplified representation of the bone cells and their differentiation pathways, in a bone remodeling site.
HSC = hematopoietic stem cell; MCS = mesenchymal stem cell; OC = osteoclast; OB = osteoblast.
Active osteoclasts are characterized by a ruffled membrane, actin ring organization, expression of vitronectin receptors and cathepsin K (CATK), and are active on the bone resorption process. Active osteoblasts express characteristic osteogenic markers, being responsible for the osteoid synthesis and its subsequent mineralization. The intense cellular crosstalk develops within the frame of endocrine, paracrine and autocrine regulation.
Taken from: Fernandes, M.H. and P.S. Gomes, *Bone Cells Dynamics during Peri-Implantitis: a Theoretical Analysis.* J Oral Maxillofac Res, 2016. **7**(3): p. e6.

This pathway has a temporal sequence of events: first active cell proliferation accompanied by osteoid (organic matrix) formation, then matrix maturation and organization and finally matrix mineralization. [30]

There are two pathways involved with osteoblastic differentiation Bone Morphogenetic Protein (BMP)/Transforming Growth Factor Beta (TGFβ1) and wingless-type MMTV integration site (Wnt)/b-catenin signaling. These two pathways are involved in most of the osteoblastic recruitment, differentiation, and proliferation which will lead to bone deposition.[30, 32-34] BMPs form the largest group of the TGFβ superfamily and are incredible osteoinductive. This TGFβ/BMP signal pathway is regulated by intercellular ligand receptors (Smad proteins) that move into the nucleus of the cell and act as transcriptional regulators of several osteoblastic genes. The signaling pathway of Wnt/ b-catenin is heavily involved with osteoblast development and maturation. This pathway works starts with Wnt, a cysteine rich glycoprotein, binding to a low- density lipoprotein receptor-related protein 5 (LRP5) or LRP6, and one of the frizzled family member (Fz) that forms a membrane co-receptor complex. This will then lead to b-catenin being released and activated in the cytoplasm, which will eventually modulate gene transcription of osteoblastic genes, such as Runx-2. This (Wnt)/b-catenin pathway is also involved in expression Osteoprotegrin (OPG) gene, which inhibits osteoclastogenesis or bone resorption, and contributes to the survival of osteocytes and osteoblasts.[30] The purpose of individual growth and transcription factors will be discussed later.

4.2 ECM Proteins

Bone is composed of 2/3^{rds} of inorganic mineral (Hydroxyapatite) that is composed of calcium phosphate crystals. The other 1/3rd is organic material, mainly comprised of ECM. Bone's ECM is comprised of ~90% collagen type I (COL-1), ~5-10% non-collagenous proteins (NCPs), ~2% lipids and water.[35-37] These NCPs, contained SIBLING (Small Integrin-Binding Ligand N-Linked Glycoproteins) proteins, are acidic in nature containing high amounts of glutamic acid, aspartic acid, and phosphorylated serine/threonine residues. Further, SIBLINGs have a high affinity for binding calcium ions and hydroxyapatite crystal surfaces as a result of binding to COL-1 substructure of the ECM. Since these proteins are phosphorylated they serve as the source of inorganic phosphate needed for mineralization.

SIBLINGs acidic nature allows for nucleating hydroxyapatite crystallization and modulating crystal growth to obtain particular formations. [38]

Alkaline phosphatases (ALPL) facilitate the conversion of organic phosphorus, present in the SIBLING proteins of ECM, into it inorganic form that can bind calcium and start bone mineralization. [30, 39] This mineralization occurs on top of the COL-1 matrix that the SIBLING proteins are bonded on. Therefore, bone alkaline phosphatase is a good marker of bone synthesis. It is expressed at early stages of osteoblast differentiation when MSCs start to differentiate into preosteoblastic cells.

4.3 Fibronectin

One of the significant observations was the presence of fibronectin in the coating. It is one of the most abundant ECM proteins. Fibronectin been shown to bind several growth factors, and as a result presence of fibronectin can result in increased sequestration of growth factors *in vivo*. [26] In addition, fibronectin forms fibrils that favor cellular attachment. This works though fibronectin's sequence motif for integrin binding which is a tripeptide of Arginine-Glycine-Aspartic acid or Arginylglycylaspartic acid (RGD) peptide. Integrin's bind to the RGD peptide and through that complex cells can attach to the ECM.[25] Due to fibronectin's ability to attach cells to ECM via the RGD peptide, it is considered acceptable practice to evaluate scaffolds in tissue engineering via fibronectin staining.[22-24, 26, 40] In our study, the osteogenic ECM was coated on a dental implant and stained for fibronectin. The 200,000 HMSCs showed the most the uniform pattern on the crest and trough of the implants, which indicate an even ECM deposition. Therefore, the 200,000 HMSCs are needed to make an evenly coated ECM on the implant and the presence of the ECM fibronectin will allow for cellular attachment by mimicking in vivo conditions by binding Integrins through their RGD peptide motif.[25, 40]

4.4 Vascular Endothelial Growth Factor (VEGF)

Osteogenesis and angiogenesis are closely coupled sequences that occur throughout one's lifetime; during phases of growth and development or remodeling and repair. VEGF is a growth factor that plays a major role in both angiogenesis and osteogenesis. VEGF is a mitogen for vascular endothelial

cells and is a major mediator during angiogenesis promoting growth of vessels. With regards to bone development, it works as a facilitator in both intramembranous, such as formation of craniofacial bones, and endochondral, such as long bones, bone formation.[31] VEGF is involved in osteoblast and chondrocyte differentiation and osteoclast enlistment.

As stated above, bone is constantly undergoing remodeling with osteoblasts and osteoclasts being stimulated and unstimulated. VEGF has been shown to have chemoattractive effect on osteoblasts in a dose dependent relationship. In in vitro studies, VEGF increases nodule formation as well as alkaline phosphatase which then promotes differentiation of osteoblasts[31, 41]. Additionally, osteoblastic proliferation can be increased up to 70% in the presence of VEGF. VEGF has low levels of expression at the beginning of osteoblastic differentiation but is strongly increased at the end stage of differentiation with maximum expression occurring during mineralization.[31] As such, VEGF plays a vital role in bone formation and remodeling by stimulation of osteoblasts to differentiate and proliferation.

In our study, immunohistochemical analysis revealed the presence of VEGF on the ECM coated implants whereas the control implants, when stained, lacked the presence of VEGF. This confirms the fibronectin results that the ECM coated the experimental implants and provides an environment to promote osteogenesis.

4.5 Runt Related Transcription Factor -2 (RUNX2)

Runx-2 is a vital transcription factor that is responsible for HMSCs to differentiate into an osteoblastic cell lineage, and indirectly suppressing HMSCs from becoming adipocytes and chondrocytes. In fact, several experiments have shown that if the Runx-2 gene is "knocked-out" in mice then these mice will completely lack of both endochondral and intramembraneous bone formation due to the lack of osteoblastic differentiation.[30, 32-34] In addition, Runx-2 is a major regulator and controls the expression of bone matrix protein genes including Collagen Type 1 (Col-1), Osteocalcin, Osteopontin, Alkaline Phosphatase (ALPL) and bone sialoprotein at early stages of osteoblast differentiation.[30, 33] Runx-2 also plays a role in the regulation of Osterix (OSX), a critical transcription factor that is involved with late stage osteoblastic differentiation.[30, 33, 42] It has also been shown that Runx-2 interacts with BMPs to

stimulate osteoblastic gene expressions, as well as Runx-2 is crucial for the implementation and completion of BMP2 signaling for osteoblastic differentiation.[32] As stated before, Runx-2 is imperative for osteoblast differentiation, without it there is no bone formation.

Our results indicated an increase in Runx-2 gene expression when HMSCs were cultured on coated implants compared to uncoated controls. This result indicates the ability of the coating to promote enhanced MSC differentiation that can positively influence the rate and quality of osseointegration.

4.6 Osterix (OSX)

As stated previously, Runx-2 has a role in downstream regulation of OSX. OSX is a second transcription factor that mainly exerts its effects in late stage osteoblastic differentiation and eliminates the potential of the preosteoblasts to convert into chrondrocytes.[30] As demonstrated with Runx-2, OSX "knock out" mice also shows no bone formation because, in this case, preosteoblast cannot undergo differentiation into functional osteoblasts. OSX is thought to act downstream from Runx-2 due to the fact that MSCS from OSX "knock out" mice express Runx-2 but the inverse is not true: Runx-2 "knock out" mice do not express OSX.[33] In this way, Runx-2 regulates the gene responsible for determining osteoblastic phenotype and function. Our results also showed increase OSX gene expression in HMSCs on coated implants coupled with Runx-2 expression, the evidence points to improved differentiation.

4.7 Transforming Growth Factor Beta-1 (TGFβ1) Superfamily

Bone Morphogenetic protein (BMPs) are a family member of transforming growth factor beta (TGFβ) that stimulates bone formation. BMPs are regulators of proliferation and differentiation of MSCs and induce bone, cartilage, tendon/ligament and fat formation. By binding to two transmembrane receptors of serine-theronine kinase (termed Type I and Type II), BMPs launch their signaling cascade. These activated receptors then phosphorylate Smads (transcriptional factors) which then activate the expression of target genes.[43, 44]

BMP-2,6 and 9 have been shown to be the most osteogenic BMPs in both in vitro and in vivo studies.[44] In a study of 14 subclasses of BMPs and their effects on MSCs, it was found that BMP-2, 6

and 9 had the highest ability to induce both early and late bone formation markers in the forms of significantly increased amounts of Alkaline Phosphatase and Osteocalcin. BMP-2, 6 and 9 demonstrate the ability to stimulate MSCs to differentiate into an osteogenic lineage. Although BMP-2 and BMP-7 are marketed and sold commercially as recombinant growth factors to be used in bone regeneration procedures in medicine and dentistry, it may be that BMP-9 is more effective in inducing bone formation. Nude mice injected with BMP-9 showed histological evidence of earlier bone formation and more extensive ossification than mice with BMP-2,6 and 7.[44] Furthermore, BMP-2 and BMP-7 induce expression of Runx-2 and OSX, which stated before, are two vital transcription factors in osteoblastic differentiation. [30]

Signals of the TGFβ superfamily, which besides BMPs include Activin, GDFs, and TGFb, are required for bone formation. TGFβ-1 is seen in higher amounts immediately after bone fracture, suggesting that it plays a required role as well in osteoblastic differentiation and bone maintenance.[24, 39] GDF10 is upregulated after a bone fracture and is continually expressed throughout repair osteogenesis. GDF10 has also been shown to be notably expressed during embryogenesis in the development of the vertebrae and craniofacial bones.[24, 45]

4.8 Summary of Discussion

All of the above discussed genes were positively regulated by our coating signifying its effectiveness. Apart from fibronectin, growth and transcription factors like VEGF, BMP-2 and Runx-2 as well as others were also observed in early expression on ECM coated implants compared to the control implants. As stated earlier, these factors all play vital roles in osteoblastic differentiation and proliferation resulting in bone formation. This increased expression indicates that our ECM coating has an osteogenic potential that could potentially enhance the quality and rate of osseointegration.

This increased expression of ECM mediated osteogenic factors was seen without the need for external intervention by means of recombinant growth factors or differentiating agents. Clinical use of recombinant growth factors, such as Rh-BMP-2 or Rh-PDGF, requires high dosages that are physiologically irrelevant and may not result in any clinically significant bone gain or improved implant

survival compared to conventional treatments.[43, 46, 47] Furthermore, in some cases, dosages of BMP-2 reach near toxic amounts on a cellular level and bone formation is retarded which is entirely detrimental for clinical success.[43] Our ECM coating replicates the natural cellular environment by providing a complete array of proteins including growth factors in physiological relevant amounts.

5. CONCLUSION

Based on the results of this study we can conclude that:

1. We can generate an even coating of ECM on implant surfaces
2. The ECM contains pro-osteogenic factors
3. The ECM coated titanium surfaces promote enhanced cell proliferation
4. As PCR results show that the coated implants enhanced the osteogenic differentiation of naïve HMSCs

Based on these results we hypothesize that the coated implants may improve the rate and quality of osseointegration *in vivo*. The next step will be evaluation of coated and uncoated implants embedded in the long bone of small animals such as rats to measure improvements in rate and quality of integration.

CITED LITERATURE

1. Abraham, C.M., *Historical Perpective on Dental Implants, Their Surface Coatings and Treatments.* The Open Dentistry Journal, 2014. **8**(Suppl 1-M2): p. 50-55.
2. Ring, M., *A Thousand Years Dental Implants; a definitive history--part1.* Compendium of Contiuning education in Dentistry, 1995. **16**(10): p. 1060-1069.
3. Goldberg, N., *The implant lower denture.* Dental Digest, 1949. **55**(11): p. 490.
4. Bodine, R., *Evaluation of 27 mandibular subperiosteal implant dentures after 15 to 22 years.* J Prosthet Dent, 1974. **32**(2): p. 188-197.
5. Bodine, R., *Forty years of experience with subperiosteal implant dentures in 41 endentulous patients.* Journal of Prosthetic Dentistry, 1996. **75**(1): p. 33-44.
6. Moldovan *<Implant History Review.pdf>*. 2013.
7. Kapur, K., *Kapur KK, Veterans Administration Cooperative Dental Implant Study--comparisons between fixed partial dentures supported by blade-vent implants and removable partial dentures. Part I: Methodology and comparisons between treatment groups at baseline. .* J Prosthet Dent. , 1987. **58**(4): p. 499-512. .
8. Kapur, K., *Kapur KK. Veterans Administration Cooperative Dental Implant Study--comparisons between fixed partial dentures supported by blade-vent implants and removable partial dentures. Part II: Comparisons of success rates and periodontal health between two treatment modalities.* J Prosthet Dent. , 1989. **62**(6): p. 685-703.
9. Branemark, P., *Osseointegrated implants in the treatment of the edentulous jaw: Experience from a 10 yr paper.* Scand J Plast Reconstr Surg, 1977. **17**: p. 1-132.
10. Le Guehennec, L., et al., *Surface treatments of titanium dental implants for rapid osseointegration.* Dent Mater, 2007. **23**(7): p. 844-54.
11. Mendonca, G., et al., *Advancing dental implant surface technology--from micron- to nanotopography.* Biomaterials, 2008. **29**(28): p. 3822-35.
12. Tagliareni, J.M. and E. Clarkson, *Basic concepts and techniques of dental implants.* Dent Clin North Am, 2015. **59**(2): p. 255-64.
13. Krishna Alla, R., *Surface Roughness of Implants: A Review.* Trends Biomater. Artif. Organs, 2011. **25**(3): p. 112-118.
14. Cochran, D.L., *<Cochran Review- Smooth vs Roughened Surfaces.pdf>.* J Periodontol, 1999. **70**: p. 1523-1539.
15. Boyan BD, L.S., Wang L, et al. 2003; 6: 22-7, *Osteoblasts generate anosteogenic microenvironment when grown on surfaces with rough microtopographies.* Eur Cell Mater 2003. **6**: p. 7-22.
16. Parekh, R., *Surface Modificationsfor Endosseous Dental Implants.* Int J Oral Implantol Clin Res, 2012. **3**(3): p. 116-121.
17. Schwarz, F., et al., *Loading protocols and implant supported restorations proposed for the rehabilitation of partially and fully edentulous jaws. Camlog Foundation Consensus Report.* Clin Oral Implants Res, 2016. **27**(8): p. 988-92.
18. Gallucci, G., et al., *Consensus Statements and Clinical Recommendations for Implant Loading Protocols.* The International Journal of Oral & Maxillofacial Implants, 2014. **29**(Supplement): p. 287-290.
19. Romanos, G., et al., *Survival rate of immediately vs delayed loaded implants: analysis of the current literature.* J Oral Implantol, 2010. **36**(4): p. 315-24.

20. Davies, J.E., *Mechanism of Ednosseous Integration.* The international Journal of Prosthodontics, 1998. **11**(5).
21. Gil, F.J., et al., *Biomimetic treatment on dental implants for short-term bone regeneration.* Clin Oral Investig, 2014. **18**(1): p. 59-66.
22. Ravindran, S., *Biological and MRI characterization of biomemtic ECM scaffold for cartilage tissue regeneration.* Journal of Biomaterial, 2015. **71**: p. 58-70.
23. Ravindran, S., C.C. Huang, and A. George, *Extracellular matrix of dental pulp stem cells: applications in pulp tissue engineering using somatic MSCs.* Front Physiol, 2014. **4**: p. 395.
24. Ravindran, S., et al., *Biomimetic extracellular matrix-incorporated scaffold induces osteogenic gene expression in human marrow stromal cells.* Tissue Eng Part A, 2012. **18**(3-4): p. 295-309.
25. Gao, M. 2006 10/10/2006 [cited 2017 02/08/2017]; Available from: http://www.ks.uiuc.edu/Research/fibronectin/.
26. Ravindran, S., et al., *Biomimetically enhanced demineralized bone matrix for bone regenerative applications.* Front Physiol, 2015. **6**: p. 292.
27. Schwarz, S., et al., *Decellularized cartilage matrix as a novel biomatrix for cartilage tissue-engineering applications.* Tissue Eng Part A, 2012. **18**(21-22): p. 2195-209.
28. Moroni, F., *Decellularized Matrices for Cardiovascular Tissue Engineering.*
29. Petersen, T.H., et al., *Matrix composition and mechanics of decellularized lung scaffolds.* Cells Tissues Organs, 2012. **195**(3): p. 222-31.
30. Fernandes, M.H. and P.S. Gomes, *Bone Cells Dynamics during Peri-Implantitis: a Theoretical Analysis.* J Oral Maxillofac Res, 2016. **7**(3): p. e6.
31. Yang, Y.Q., et al., *The role of vascular endothelial growth factor in ossification.* Int J Oral Sci, 2012. **4**(2): p. 64-8.
32. Chen, G., C. Deng, and Y.P. Li, *TGF-beta and BMP signaling in osteoblast differentiation and bone formation.* Int J Biol Sci, 2012. **8**(2): p. 272-88.
33. Datta, H.K., et al., *The cell biology of bone metabolism.* J Clin Pathol, 2008. **61**(5): p. 577-87.
34. Hayrapetyan, A., J.A. Jansen, and J.J. van den Beucken, *Signaling pathways involved in osteogenesis and their application for bone regenerative medicine.* Tissue Eng Part B Rev, 2015. **21**(1): p. 75-87.
35. Boskey, A.L., *Bone composition: relationship to bone fragility and antiosteoporotic drug effects.* Bonekey Rep, 2013. **2**: p. 447.
36. He, G., et al., *Phosphorylation of phosphophoryn is crucial for its function as a mediator of biomineralization.* J Biol Chem, 2005. **280**(39): p. 33109-14.
37. Shi, S., *The Role of Type I Collagen in the Regulation of the Osteoblast Phenotype.* Journal of Bone and Mineral Research, 1996. **11**(8).
38. George, A., *Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition.* Chem. Rev, 2008. **108**: p. 4670-4693.
39. Blair, H.C., et al., *Osteoblast Differentiation and Bone Matrix Formation In Vivo and In Vitro.* Tissue Eng Part B Rev, 2016.
40. Jeschke, *RGD-Peptides for tissue engineering of articular cartilage.* Biomaterials, 2002: p. 3455-3463.
41. Street, J., *Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover.* Proc Natl Acad Sci U S A 2002. **99**(15): p. 9656-9661.

42. Xu, W.-L., *In situ release of VEGF enhances osteogenesis in 3D porous scaffolds engineered with Osterix-modified adipose-derived stem cells.* Tissue Eng Part A, 2017.
43. Kao, D., *The Negative Effect of Combining rhBMP-2 and Bio-Oss on Bone Formation for Maxillary Sinus Augmentation.* International Journal of Periodontics & Restorative Dentistry, 2012. **32**(1): p. 61-67.
44. Luu, H.H., et al., *Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells.* J Orthop Res, 2007. **25**(5): p. 665-77.
45. Zhao, R., *Characterization of GDF-10 Expression Patterns and Null Mice.* Developmental Biology, 1999. **212**: p. 68-79.
46. Bashutski, J.D. and H.-L. Wang, *Biologic Agents to Promote Periodontal Regeneration and Bone Augmentation.* Clinical Advances in Periodontics, 2011. **1**(2): p. 80-87.
47. L, S., *Recent Outcomes and PersDectives of the Application of Bone Morphogenetic Proteins in Implant Dentistry.* Clinical Implant Dentistry and Related Research, 2004. **4**(1): p. 27-32.

VITA

NAME:	Josh Hassan
EDUCATION:	B.A., Psychology, Yeshiva University, New York, NY 2008
	D.D.S., Temple University Kornberg School of Dentistry, Philadelphia, PA 2014
TEACHING:	Periodontics Department, University of Illinois-Chicago, Undergraduate Clinics, 2014-2017
HONORS	Chief Resident, University of Illinois-Chicago, Department of Periodontics, 2016-2017
	Student Ambassador, Pat Allen Seminar Program, 2016
	American Board of Oral Maxillofacial Pathologists Award, 2014
PROFESSIONAL MEMBERSHIP:	American Academy of Periodontology Chicago Dental Society Midwest Society of Periodontists