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ENAMEL MATRIX PROTEINS ENHANCE OSTEOBLAST SPREADING, PROLIFERATION AND DIFFERENTIATION ON TITANIUM SURFACES

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ABSTRACT

Modifications of implant surface topography and chemistry have proven a means to enhance osseointegration, a process that ensures the stability of bone-contacting devices including titanium dental implants. Enamel matrix derivative (EMD) extracted from developing porcine teeth, has been shown to promote regeneration of bone. The aim of the present study was to evaluate the effect of EMD on the attachment, proliferation and differentiation of osteoblasts on titanium surfaces *in vitro*. Pickled (smooth) and SLA (**S**and-blasted with **L**arge grit followed by **A**cid etching) titanium discs were coated with EMD or left uncoated. Primary rat calvarial osteoblasts were cultured on each surface 1 hour to 4 weeks. EMD significantly increased cell spreading and proliferation at time points ranging from 3 to 7 days on both topographies. Alkaline phosphatase activity was significantly increased on EMD-coated titanium compared with titanium alone. Moreover, there was a 4-fold increase in levels of mRNA encoding bone sialoprotein and osteocalcin in osteoblasts cultured on EMD-coated titanium surfaces compared with uncoated surfaces. We conclude that coating of titanium with EMD enhances the proliferation and differentiation of osteoblasts irrespective of the titanium substratum topography. Therefore, EMD may increase the speed and quality of osseointegration around bone contacting implants *in vivo*.

Keywords— Enamel matrix derivative (EMD), titanium implants, surface topography, surface chemistry, osseointegration, osteoblast

CO-AUTHORSHIP

Chapter 2 entitled "Enamel matrix proteins enhance osteoblast proliferation and differentiation on titanium surfaces" was written by R.J. Miron with suggestions from Drs. A. Molenberg, M. Dard, S.J. Dixon and D.W. Hamilton.

All studies were performed by R.M. Miron. All experiments were carried out in the laboratories of Drs. D.W. Hamilton, S.J. Dixon and S.M. Sims.

Donald Bocchinfusa was a summer student in the Hamilton lab in 2008 and assisted with experiments leading to the studies conducted in this thesis. His aid was greatly appreciated.

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LIST OF ABBREVIATIONS

α-MEM	α-minimum essential medium
ALP	alkaline phosphatase
ANOVA	analysis of variance
BMP	bone morphogenetic proteins
BSP	bone sialoprotein
cAMP	3',5'-cyclic AMP
Ca^{2+}	calcium
cDNA	complementary DNA
EMD	enamel matrix derivative
FBS	fetal bovine serum
FDA	food and drug administration
FGF	fibroblast growth factor
FHRIKKA	Phenylalanine –histidine –arginine –isoleucine –lysine –lysine –alanine
FPD	fixed partial dentures
HA	hydroxyapatite
HCl	hydrochloric acid
HF	hydrofluoric acid
HNO_3	nitric acid
H_2SO_4	sulfuric acid
IGF	insulin-like growth factor
Mg^{2+}	magnesium
MMP-20	matrix metalloproteinase-20
OC	osteocalcin
ON	osteonectin
OPN	osteopontin
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDL	periodontal ligaments
PO_4^{3-}	inorganic phosphate
PPARγ	peroxisome proliferator-activated receptor γ
PTH	parathyroid hormone

R_a	surface roughness
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
RCO	rat calvarial osteoblast
RGD	arginine –glycine –aspartic acid
RT-PCR	reverse transcription-polymerase chain reaction
Runx2	runt-related transcription factor 2
SD	standard deviation
SE	standard error of the mean
SLA	sand blasted with large particles followed by acid etching
Ti	titanium
TGFβ	transforming growth factor-β
VEGF	vascular endothelial growth factor

CHAPTER ONE

Introduction

1.1 Chapter Summary:

Tooth loss is a major clinical concern in North America. Approximately 70% of the population over the age of 40 is missing at least one tooth and half the population over the age of 65 has complete tooth loss (Meskin 2000). With an aging population, the need for improved treatment has become vital as certain modalities decrease masticatory efficiency and promote further tooth loss as well as periodontal degeneration (Schwartz et al. 1970, Bloom et al. 1992, Takala et al. 1994, Mojon 2003). Dental implants are currently the most reliable and desirable treatment option as they rarely affect surrounding healthy teeth and show a high rate of clinical success (Schmitt and Zarb 1993, Haas et al. 1995, Fagazzoto 2005). Essential to the success of dental implants is osseointegration, the direct bonding of bone to the surface of the implant material (Branemark 1969). To enhance osseointegration, modifications have been made to the surface of the implant specifically by altering surface topography or chemistry, or coating the surface with a 'biomimetic' protein. Enamel matrix derivative (EMD) is an FDA-approved product for the treatment and regeneration of periodontal structures, with studies demonstrating that EMD has the potential to influence fibroblast proliferation and differentiation *in vitro*. The use of EMD in clinical settings has been limited to periodontal regeneration and studies of the *in vivo* effects on bone have been limited to intrabony defects (Sculean et al. 2000). We hypothesized that precoating EMD on titanium surfaces would enhance osteoblast attachment, proliferation and differentiation *in vitro*, effects which may promote osseointegration *in vivo*.

1.2 Tooth Loss:

Tooth loss can be the result of severe trauma, or more commonly poor oral hygiene which can result in dental caries and periodontal degeneration (Schwartz et al. 1970, Bloom et al. 1992, Takala et al. 1994, Mojon 2003). In the United States, 70% of the population over the age of 40 is missing at least one tooth and this increases on average to 9 missing teeth in adults over the age of 60 (Meskin and Berg 2000). The average total edentulous rate (complete tooth loss) around the world is 20% at age 60 although wide variations exist globally (Mojon 2003). For example, in Canada 47% of people between the ages of 65 and 69 have complete tooth loss (Health Promotion Survey Canada 2008).

Dental caries are caused by bacterial infections that degrade the enamel and dentin layers of teeth. If left untreated, caries progress to pulpitis and necrosis of the pulp which often result in tooth loss (Nguyen and Martin 2008). Oral diseases, such as gingivitis, are caused by bacterial infections that affect the gingival tissues. In the early stages of gingivitis, bacteria become trapped between the gum and tooth producing a biofilm of plaque that cannot be removed easily (Brown and Loe 1993). At an early stage, gingivitis can be controlled by proper hygiene. If left untreated, the gum line begins to recede (pull away from the tooth) and pockets form that collect bacteria and tissue degradation products leading to severe infection. As the disease progresses (later termed periodontitis), bone loss can occur which may result in tooth loss if the tooth becomes unstable in the socket (Nguyen and Martin 2008).

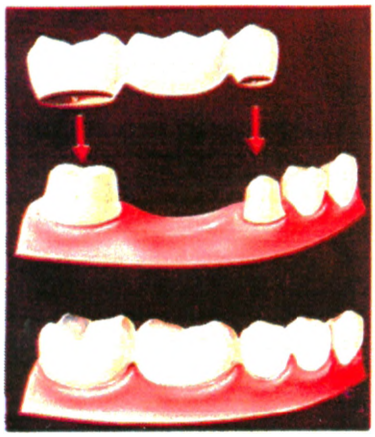
Gingivitis and periodontitis affects approximately 40% and 15% of the world's population respectively (Preshaw et al. 2004, Burt 2005) but varies considerably between

countries (Albandar and Rams 2000). In the United States, approximately 82% of the total population is affected by gingivitis on one or more teeth (Albandar 2002, Davies 2008). With an aging population, gingivitis and periodontitis pose serious threats to patient oral health (Morris et al. 2001). These diseases have also been linked to serious systemic pathologies including diabetes and cardiovascular diseases (Cabala et al, 2006, Rautemaa et al. 2007).

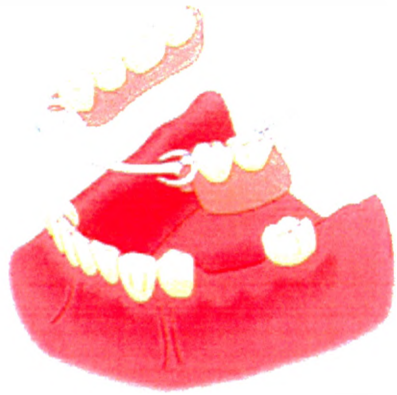
1.2.1 Treatment options:

There are numerous options to replace missing teeth including tooth supported fixed bridges, removable partial dentures, resin bonded fixed partial dentures, full dentures and dental implants (Fugazzotto 2009). Of these treatment modalities, each carry their advantages and disadvantages discussed briefly in figure 1.1.

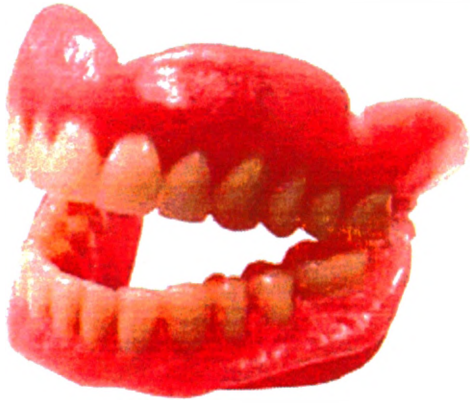
One of the most reliable although most expensive treatment option, is the single tooth implant (figure 1.1). Success rates have been reported as high as 95% by several investigators (Schmitt and Zarb 1993, Haas et al. 1995, Malevez et al. 1996, Fugazzotto 2005, Misch et al. 2005). The advantages of implants include a decreased risk of caries to adjacent teeth especially in comparison to fixed bridges, as well as improved access for cleaning the surfaces of adjacent teeth (Misch 2008). Bone mass is better maintained throughout the lifespan of the implant and patients are psychologically more pleased with the results from single tooth implants (Misch 2008).



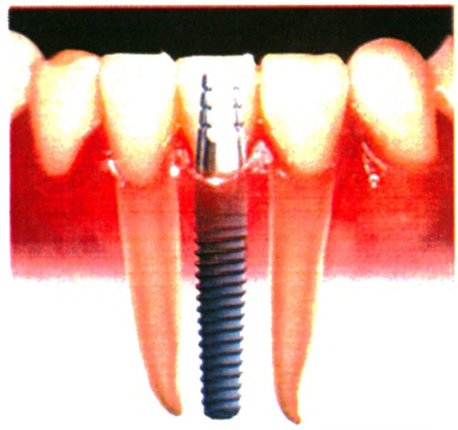
Tooth-supported fixed bridges



Removable partial denture



Full dentures



Dental Implants

Figure 1.1: Treatment modalities for missing teeth. Tooth supported fixed bridges are the most common choice to replace a single tooth with approximately 4 million produced yearly in the United States (Bloom et al. 1992, American Dental Association Survey Center 2004). Their main drawbacks are low survival rate (50% at 10 years), caries to adjacent teeth (24.3%) and mechanical problems (69.5%) on surrounding teeth (Walton et al. 1986, Goodacre et al. 2003). Removable partial dentures are less common because they are not as esthetically pleasing (Goodacre et al. 2003). Full dentures are commonly chosen by patients with complete tooth loss. Their main drawbacks are the reduction in occlusal forces which promotes bone loss in the jaw (Sutton et al, 2004, Carr and Laney 1987), increased masticatory difficulty (Joshipura et al. 1996) and low survival rates (60% at 4 years) (Tallgren 1972). Images from:
http://www.drkeithgilbert.com/images/bridge.jpg,
http://dentalsolutionscr.com/img/images/dentures3.gif
http://cyberanto.files.wordpress.com/2009/01/partial_denture.jpg
http://www.drlinhart.com/images/Implant_2.jpg

Although many studies show high implant success rates (Fugazzotto 2005, Misch et al. 2005), there is still a need to reduce healing times and improve integration into bone. Furthermore, the stability of dental implants varies considerably, depending on the region of the jaw in which they are placed (Esposito et al. 1998). For example, the posterior maxilla (back of the upper jaw) has the highest failure rate, estimated at around 20%, due to the presence of the sinus and soft quality of bone (Hutton et al. 1995, Esposito et al. 1998, 1999). Improved implant biocompatibility is therefore necessary, particularly for areas where bone mass is insufficient.

Failure of dental implants is caused by several factors which include low quality or quantity of bone, bacterial infection, epithelial down-growth and surgical procedures which result in improper osseointegration (Misch et al. 2008) (figure 1.2). With the market for dental implants estimated to increase at 12-15% per annum, additional research on osseointegration is necessary to decrease the number of failed procedures (Nergiz et al. 2009, Huynh-Ba et al. 2008). Furthermore, an understanding of bone biology is also vital for implant research as titanium surfaces must interact directly with adjacent bone (figure 1.2).

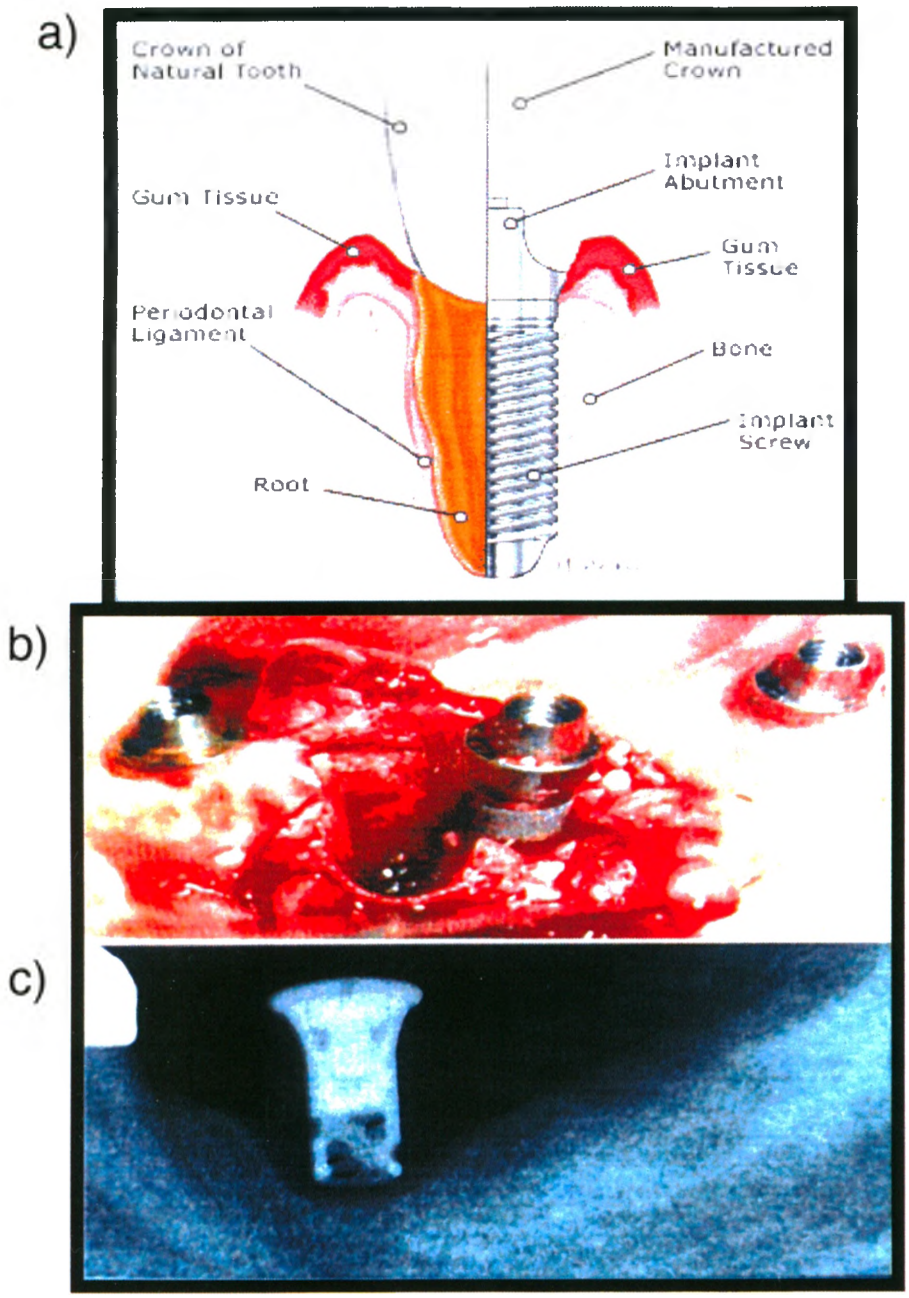


Figure 1.2: Osseointegration in dental implants. Differences between a natural tooth and prosthetic implant are shown in a). The root of a natural tooth is joined to bone through the periodontal ligament. During dental implant surgery, the periodontal ligament is completed removed and the titanium implant is in direct contact with bone. Osseointegration between the titanium implant and bone is vital for the success and longevity of the implant. Approximately 10-20% of implants are removed due to improper osseointegration shown in b). c) X-ray image of implant in b) which has failed. White area surrounding implant corresponds to bone and black area to gingival tissue and open space. When no bone attaches to the implant, the implant fails and must be removed. Image from www.chandigarhdentist.com.

1.3 Bone Biology:

1.3.1 Function of Bone:

The primary function of the skeletal system is to provide mechanical support, aid locomotion, protect internal organs, house blood forming marrow, as well as act as a reservoir for mineral ions such as calcium (Ca^{2+}), magnesium (Mg^{2+}) and phosphate (PO_4^{3-}).

Bone is categorized into 2 histologically defined types: dense bone, also known as cortical or compact bone; and spongy bone, known as cancellous trabecular bone (Reith et al. 1970, Baron 1999) (figure 1.3). During bone development, newly formed bone is woven bone. With remodeling, woven bone is replaced by lamellar bone, which can withstand larger mechanical forces and strains (Baron 1999).

Trabecular bone is composed of small beam-like structures that form the inner cavity of long bones (figure 1.3). Compact and trabecular bone share many features. They undergo mineralization in similar processes and form solid mineralized matrices with small canals and spaces which incorporate bone cells that continuously remodel bone (Baron 1999). Two distinct processes result in formation of bone: 1) endochondral and 2) intramembranous ossification.

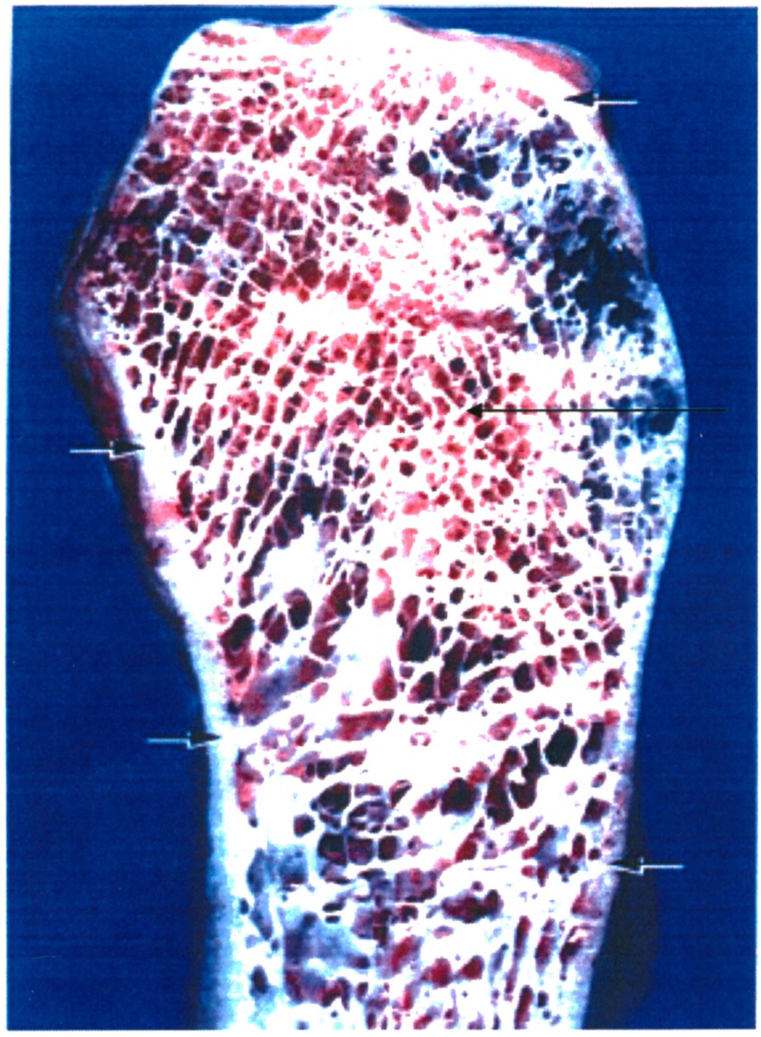


Figure 1.3: Histological definition of bone. Bone is composed of 2 histologically defined types. Trabecular bone is composed of small beam-like structures that form the inner cavity. Compact bone is found on the outer surface of bone and is greater in density and organization and is capable of withstanding larger mechanical and physical forces. Image modified from: Histology, a text and atlas, fifth edition, 2006

1.3.2 Endochondral and Intramembranous Ossification:

Endochondral ossification is the formation of bone from a cartilage template and is responsible for the formation of long bones in development and the longitudinal growth of long bones into adulthood (Brighton et al. 1973, 1986, Netter and Frank 1987). During endochondral development, chondrocytes form an embryonic cartilaginous scaffold that is then invaded by blood vessels, osteoclasts and bone forming osteoblasts, which deposit new bone (Zelzer et al. 2001, Mackie et al. 2008).

Intramembranous ossification results from condensation of mesenchymal stem cells. Flat bones found in the head and mandible region are formed through this mechanism. Unlike endochondral ossification, cartilage is not present during any stage of bone formation (Caplan 1988). Mesenchymal stem cells are recruited to form clusters where they proliferate and differentiate into osteoprogenitor cells (Caplan 1988, Bruder and Caplan 1989, Cowles et al. 1998). These progenitor cells differentiate into osteoblasts that ultimately produce an extracellular matrix that subsequently mineralizes (Caplan 1988, Bruder and Caplan 1989). Ultimately, both types of bone have similar properties with respect to composition.

1.3.3 Composition of Bone:

Bone matrix is composed of mineral, collagen, water and non-collagenous proteins. The most abundant component of bone is hydroxyapatite, which is composed of calcium phosphate $Ca_{10}(PO_4)_6(OH)_2$ (Glimcher 1998). Using atomic force microscopy and electron microscopy, it has been determined that hydroxyapatite crystals remain relatively small in young bone (9 nm * 6 nm * 2 nm) (Fratzl et al. 1996, Tong et al.

2003). This small size allows easy incorporation of foreign ions and increases the structural rigidity within collagen fibrils (Tong et al. 2003).

The second most abundant component of bone is collagen. Collagen provides the structural framework and allows dispersion of pressure, torsion, and tension resulting from movement and normal activity (Boskey et al. 1999). It also plays a role in osteoblast adhesion, proliferation, migration, differentiation and apoptosis (Von der Mark and Sorokin 2002). Two collagen types are found in bone, type I (95%) and type V (5%) (Niyibizi and Eyre, 1994). Type V collagen is a fibrillar forming collagen and is co-distributed with type 1 collagen to provide tensile strength and architectural stability to bone (Birk 2001).

All collagen molecules assemble into a triple helix, a coiled structure consisting of 3 polypeptide chains in the form of a right handed helix. Ascorbic acid is required for hydroxylation of proline and lysine during collagen biosynthesis and enhances collagen I mRNA levels and osteoblast differentiation *in vitro* (Chvapil and Ryan 1972, Pinnell 1985, Schwarz 1985).

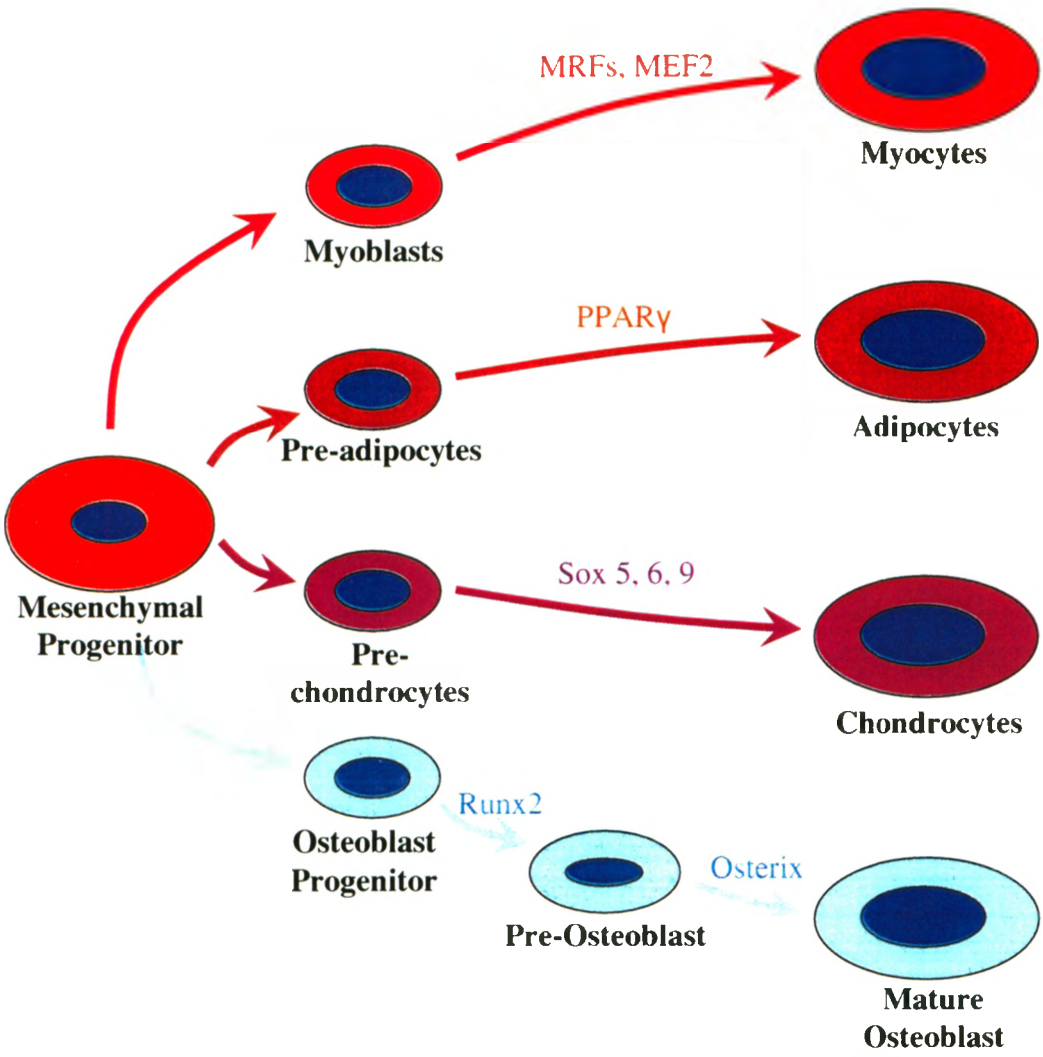
The other major components in bone are water and noncollagenous proteins (van der Rest and Garrone 1991, Niyibizi and Eyre 1994). Water accounts for approximately 5-10% of the weight of bone tissue and is necessary for cell hydration (van der Rest and Garrone, 1991). The remaining components in bone are the non-collagenous bone proteins including osteopontin (OPN), osteonectin (ON), bone sialoprotein (BSP), and osteocalcin (OC) (Niyibizi and Eyre 1994). The role of OPN is to increase osteoclast anchorage to the mineral matrix of bone, which is important for remodeling as well as to attract and regulate haematopoetic stem cells (Reinholt et al. 1990, Nilson et al. 2005).

ON is an extracellular matrix glycoprotein secreted by osteoblasts, plays a role in calcium release and collagen binding, and is required for bone mineralization (Termine 1988). The other non-collagenous proteins, OC and BSP, are considered the more important markers for osteoblast differentiation as they are expressed by mature osteoblasts (Harada and Rodan 2003) (figure 1.4).

1.3.4 Osteoblast Differentiation: Alkaline Phosphatase, Osteocalcin and Bone Sialoprotein:

Alkaline phosphatase (ALP) is considered an early marker of osteoblast differentiation (Tenenbaum 1987). *In vitro* studies have demonstrated that bone ALP is released from osteoblasts in an insoluble form attached to matrix vesicles (Fedde 1992, Anh et al. 1998), where it participates in the initiation of mineralization (Tenenbaum 1987, Bellows et al. 1991, Wennberg et al. 2000).

BSP and OC are late markers of osteoblast differentiation. BSP is a noncollagenous protein that is glycosylated, phosphorylated and sulfated (Ogata 2008). It has a molecular weight of 70-80 kDa and accounts for approximately 5 to 10% of the noncollagenous proteins of the bone extracellular matrix (Oldberg et al. 1988, Fisher et al. 1990). It contains an RGD (arginine, glycine aspartic acid) sequence through which osteoblasts can adhere (Oldberg et al. 1988). BSP binds preferentially to collagen type I and nucleates hydroxyapatite crystal formation *in vitro* (Ross et al. 1993, Hunter et al. 1994, Fujisawa et al 1995). The spatial deposition of BSP in the ECM of bone and the ability of BSP to nucleate hydroxyapaptite crystal formation, indicates a potential role for BSP in the initial mineralization of bone, dentin and cementum (Ogata et al. 2008).



	Osteoblast Progenitor	Pre-Osteoblast	Mature osteoblast
Role in development	Proliferation	Matrix Release	Mineralization
Osteogenic markers	TGFβ1 Runx2 Cfos/Cjun Collagen 1 (+)	ALP (++) Collagen 1 (++) Runx2 (?)	ALP (+++) Collagen 1 (++) Osteocalcin (+++) BSP (+++) Runx2 (?)

Figure 1.4: Stages of osteoblast differentiation. Mesenchymal progenitors give rise to myocytes, adipocytes and chondrocytes under the influence of MRFs and MEF2, PPARγ and C/EBPα, Sox 5,6,9, respectively (Tang et al 2003, Mueller et al. 2002, de Crombrugghe et al. 2000, Banerjee et al. 1997, Ducy 1997, Nakashima et al. 2002). The role of osteoprogenitor cells are to proliferate and provide an early pool for osteogenic cells. These cells express high levels of Runx2, TGFβ and collagen 1. As osteoprogenitor cells differentiate into pre-osteoblasts, they express high levels of ALP and begin to form a matrix. Mature osteoblasts express high levels of osteocalcin and bone sialoprotein and are fully capable of matrix mineralization. These cells also express high levels of ALP and collagen type I. Figure modified from Harada and Rodan 2003.

OC, also called bone gamma-carboxyglutamic acid protein, is the most abundant osteoblast-specific non-collagenous protein. OC has a single chain of 46-50 amino acids and 3 vitamin K-dependent γ-carboxyglutamic acid residues (GLA), which are involved in binding to calcium and hydroxyapatite (Hauschka et al. 1989, Weinreb et al. 1990, Boivin et al. 1990, Chenu et al. 1994). The precise function of osteocalcin remains unknown (Ducy et al. 2000).

These non-collagenous proteins play a crucial role in bone formation and bone turnover. Both OC and BSP contain RGD binding domains which are essential for osteoclast and osteoblast attachment to bone surfaces which later controls and regulates bone remodeling by both cell types (Oldberg et al. 1988, Hauschka et al. 1989, Weinreb et al. 1990, Fisher et al. 1990, Boivin et al. 1990, Chenu et al. 1994).

1.3.5 Cellular Component of Bone:

Bone is composed of 2 distinct cell types that have different parental lineages. Osteoblasts, the bone forming cells, are responsible for the synthesis of the bone extracellular matrix. Osteoclasts, the bone resorbing cells, are responsible for the degradation of old bone matrix through secretion of hydrochloric acid and hydrolytic enzymes. The two cell types exist to balance bone formation and resorption. On average, 2-3% of total bone is replaced yearly in healthy adults (Huiskes et al. 2000, Parfitt 2002).

1.3.6 Osteoblasts:

Osteoblasts are derived from mesenchymal stem cells that can also give rise to myoblasts, adipocytes and chondrocytes (Ducy et al. 2000) (figure 1.4). In order for a mesenchymal stem cell to differentiate into an osteoblast, the expression of 2 transcription factors is essential: Runt-related transcription factor 2 (Runx2) (Banerjee et al. 1997, Ducy et al. 1997) and osterix (Nakashima et al. 2002, Skillington et al. 2002).

The importance of Runx2 became apparent from the Runx2-null mouse, which has a cartilaginous skeleton and a complete absence of osteoblasts (Banerjee et al. 1997, Ducy et al. 1997). The osteoblasts from the knockout mice were incapable of entering the mineralization cycle required for bone formation (Ducy et al. 1997, Stricker et al. 2002). Runx2 heterozygous mice have a less aggressive phenotype, but still show defects in intramembranous ossification leading to the conclusion that Runx2 plays a crucial role in osteoblast differentiation.

The other essential transcription factor regulating osteoblast differentiation is osterix, a zinc-finger like containing protein which is induced by bone morphogenetic proteins (BMPs) (Nakashima et al. 2002, Skillington et al. 2002). Osterix knockout mice develop perfectly patterned skeletons that are composed entirely of cartilage. Osterix was determined to act downstream of Runx2; this was discovered through a lack of expression of osterix in the Runx2 knockout mice, while the expression of Runx2 is present in osterix knockout mice (Nakashima et al. 2002, Skillington et al. 2002). Runx2 is required for osterix expression and is considered by many to be the master gene for osteoblast differentiation because it is required for expression of non-collagenous

proteins such as BSP and OC (Ducy et al. 1997, Banerjee et al. 1997, Stricker et al. 2002, Nakashima et al. 2002, Skillington et al. 2002, Young et al. 2005).

Fully matured osteoblasts are characterized by their ability to synthesize osteoid, the organic phase of the bone matrix. This osteoid becomes mineralized by formation of hydroxyapatite (Burger and Klein-Nulend 1999). As an osteoblast becomes surrounded by its own matrix, it terminally differentiates into an osteocyte and plays a role in cell communication and the regulation of bone remodeling (Burger and Klein-Nulend 1999).

1.3.7 The Bone Remodeling Cycle:

There are 4 phases in the bone remodeling cycle: activation, resorption, reversal and formation (figure 1.5). Activation is defined as the conversion of previously quiescent bone surface into a remodeling surface. First, mononucleated osteoclast precursors penetrate the bone lining cell layer, fuse together and become multinucleated osteoclasts. This process is governed and tightly regulated by hormones such as RANKL and interleukins 1 and 6 (Roodman 1999, Burr 2002, Parfitt 2002A).

The resorption phase begins when mature osteoclasts secrete acid to dissolve the bone mineral and proteolytic enzymes such as cathepsin K to degrade the organic matrix (Littlewood-Evans et al. 1997). During resorption, a number of cytokines that attract osteoblasts are secreted and released from the bone matrix (Wright et al 1995). After resorption, osteoclasts undergo apoptosis.

The reversal phase of the cycle takes place when cytokines derived from the resorbed organic matrix recruit new osteoblast progenitors to proliferate and differentiate (Baron et al. 1980). Many of the signals are not known, but candidate molecules include

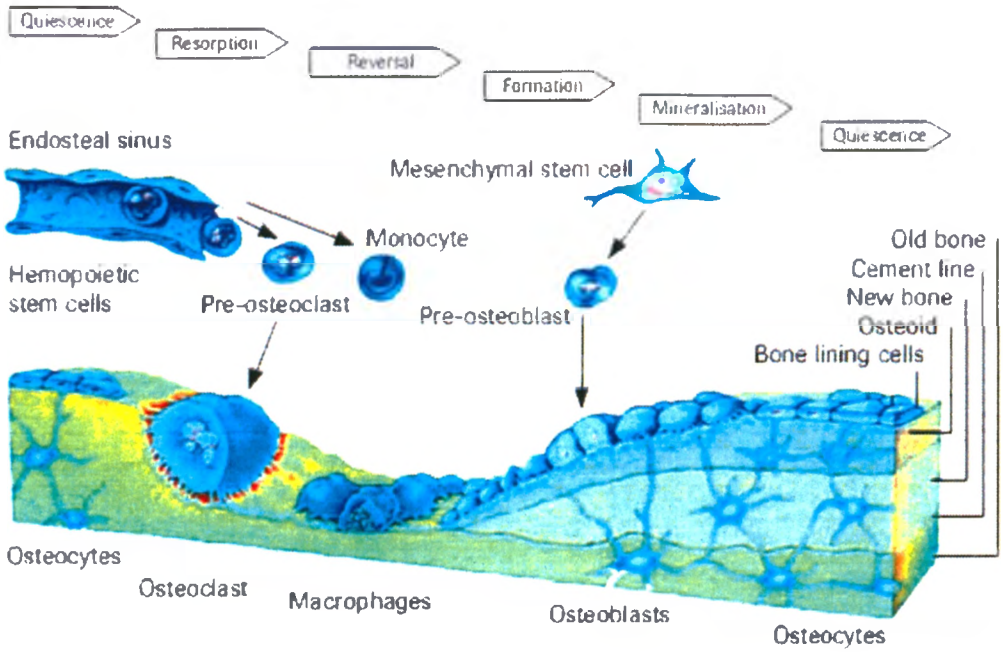


Figure 1.5: Bone remodeling cycle. Activation of the bone remodeling cycle begins when pre-osteoclasts from hemopoietic stem cells are recruited to the surface of old bone and fuse together forming multinucleated osteoclasts. These cells release acid and proteolytic enzymes such as cathepsin K for matrix degradation. When the area of bone is resorbed, osteoprogenitor cells from mesenchymal stem cells are recruited to the surface, proliferate and differentiate into osteoblasts. These osteoblasts synthesize the organic matrix and form new bone. A sub-population of osteoblasts become embedded in their own matrix and terminally differentiate into osteocytes. The remaining osteoblasts become bone lining cells or undergo apoptosis. Image modified from http://www.roche.com/pages/facets/11/bone_remodelling2.jpg

transforming growth factor beta (TGFβ), insulin-like growth factor (IGF) I and II, BMPs, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Bonewald and Mundy, 1990, Mohan and Baylink 1991, Fiedler et al. 2002).

In the formation phase of the bone remodeling cycle, osteoblasts initially synthesize the organic matrix followed by mineralization. Osteoblasts lay down osteoid matrix and become embedded in the bone. When formation is complete, inactive osteoblasts become flattened and elongated and form lining cells that regulate the flow of ions in and out of the bone extracellular fluid of bone (Civitelli et al. 1993, Smit and Burger 2000, Huiskes et al. 2000, Burger et al. 2003).

1.4 Dental Implants:

1.4.1 Osseointegration:

The ability of implants to integrate properly into bone relies heavily on aspects of the bone remodeling cycle. In order for an implant to be properly anchored in bone, bone apposition directly onto the implant surface is necessary. During dental implant surgeries, the periodontal ligament is destroyed, resulting in direct contact between titanium and living bone (figure 1.2). Osseointegration was defined by Branemark as "the direct structural and functional connection between living bone and the surface of a load-carrying implant" (Branemark 1969). Osseointegration involves a 3 step process: 1) osteoconduction (recruitment of osteogenic cells to the surface of the implant), 2) de novo bone formation, followed by 3) bone remodeling (Davies 1998, 2003).

To allow osseointegration to occur, dental implants are commonly loaded in a 2 step process (Adell et al. 1981, Collaert and Bruyn 1998). First, the intraosseous component is placed into the jaw bone for a period of 6 to 8 weeks to allow for osteoconduction and de novo bone formation followed by bone remodelling. Only thereafter is the transmucosal section and prosthetic crown attached to the new integrated implant (Adell et al. 1981). It would be of clinical significance if this 6-8 week period could be reduced; a time during which the patient has no use of the implant and occlusal forces are lost, which has an adverse impact on opposing teeth (McNeil 2000).

1.4.2 Implant Materials:

The ideal base materials for dental implants are metals as they are resistant to stress and corrosion, and have minimal potential toxicity to the patient (Lemons et al, 1975, 1986, 1992, Plenk et al. 1996). Many metals were initially studied for this application including tantalum, aluminium, nionium, nickel, zirconium and halfnium (Alberius et al. 1983, Johansson et al. 1991, Thomsen et al. 1997, Mohammadi et al. 2001, Kujala et al. 2003). A plethora of research resulted in titanium (Ti) and its alloys becoming the preferred implant material, as Ti promoted bone apposition directly onto the implant surface while remaining resistant to corrosion (Beder et al. 1956, Gross and Gold. 1957, Clarke et al. 1963, Branemark 1969, Lemons et al. 1976, 1987, Lucas et al. 1978, 1985). Further research showed that smooth titanium implants were not ideal and that rougher surfaces may be capable of mechanically interlocking with surrounding bone (Bobyn et al. 1980).

It was first recognized in the late 1980s that the surface topography of dental implants could enhance osseointegration (Bobyn et al. 1980, Buser et al. 1991, Wennerberg 1995, Cochran et al. 1996). *In vitro* research has further demonstrated that variations in surface topography could enhance bone deposition (Martin et al. 1995, Qu et al. 1996, 2007, Brunette and Chehroudi 1999, Chehroudi and Brunette 2002, Wennenburg 2003, Wieland et al. 2005, Hamilton 2007). Surface topography is a powerful modulator of osteoblast behaviors including adhesion, migration, signaling, proliferation and differentiation (Boyan et al. 2002, Schwartz et al. 2005, Zinger et al. 2004, Hamilton et al. 2006, Hamilton and Brunnette 2007). Several methods have been used to vary surface topography on titanium implants.

1.4.3 Titanium Plasma Spraying:

Titanium plasma spraying was first utilized on dental implants to increase surface roughness of dental implants. Titanium powders are injected into a plasma torch at high temperatures. These particles are projected on the surface of an implant and upon contact, condense and fuse together forming a 30 μm thick film with a pitted surface. The average roughness value (Ra) is approximately 7 μm (Bagno et al. 2004). Ti plasma spraying has been shown to increase tensile strength at the bone/implant interface (Buser et al. 1991). One significant disadvantage of this method is that titanium particles have been found in the bone adjacent to the implants, contaminating the tissue, which can lead to inflammation (Urban et al. 2000). This is of particular concern due to their potentially harmful local and systemic carcinogenic effects (Browne and Gregson 2000, Martini et al. 2003).

1.4.4 Acid Etching:

Ti surfaces can also be modified in the microscale range by etching with strong acids including hydrochloric acid (HCl), sulfuric acid (H_2SO_4), nitric acid (HNO_3) and hydrofluoric acid (HF). The procedure involves immersing titanium implants in acids and heating them above 100°C, which produces micro-roughness (Massaro et al. 2002). The pits produced range between 0.5 and 2 μm in diameter (Massaro et al. 2002, Zinger et al. 2004). Studies suggest that implants treated by acid etching are better able to bind fibrin which is thought to promote adhesion of osteogenic cells (Davies et al. 1998, Trisi et al. 2002). Acid etching also increases bone to implant contact levels and torque removal values (Buser et al. 2004).

1.4.5 Grit Blasting:

Another method employed to roughen the surface of titanium is blasting of the surface with hard ceramic particles. The particles (alumina, titanium oxide, calcium phosphate and corundum) are projected with compressed air through a nozzle at high velocities (Le Guehennec et al. 2007). Surface roughness can be controlled by varying the size of the particles. Wennerberg et al. have shown that when titanium implants are roughened by grit blasting with either TiO_2 or Al_2O_3, the bone-implant contact and the torque removal level drastically increase when compared to smooth implant (Wennerberg 1998). More recently, biocompatible, osteoconductive and resorbable materials such as calcium phosphates (hydroxyapatite (HA)) have been used as blasting particles (Novaes et al. 2002, Piatelli et al. 2002). These studies also showed an increase in roughness with higher bone to implant contact when compared to plasma sprayed and grit-blasted titanium surfaces alone (Novaes et al. 2002, Piatelli et al. 2002) although the clinical problems associated with HA coatings are numerous (Wheeler 1996, Chang et al. 1999, Lee et al. 2000, Tinsley et al. 2001).

1.4.6 SLA Surfaces:

SLA surfaces developed by Institut Straumann AG, combines two of the previously described methods to roughen the titanium surface. First, the implant is **S**and blasted with **L**arge particles followed by **A**cid etching in HCl/H_2SO_4. This creates a surface with 2 levels of roughness, large 20-40 um pits from the blasted particles with 1-2 um microtopographies super-imposed (Wieland et al. 2005, Kim et al. 2008). SLA surfaces have been shown to promote osteoblast differentiation and the formation of larger bone nodules at earlier time points *in vitro* compared to surfaces treated with grit

blasting or acid etching alone (Wieland et al. 2005). Furthermore, *in vivo* studies in canine mandibles and miniature pigs have shown that SLA surfaces exhibit greater bone to implant contact at earlier time points, and higher torque removal values when compared to surface treated with either acid etching or grit blasting alone (Cochran et al. 1998, Buser et al. 1998). See table 1 for comparison of surface properties between titanium dental implants.

1.4.7 Rough Surfaces:

Surface roughness has been extensively studied over the past 2 decades and the consensus is that strong correlations exist between increased roughness and increased rates of osseointegration (Martin et al. 1995, Qu et al. 1996, 2007, Brunette 1999, Chehroudi 2002, Wennenburg 2003, Wieland 2005, Hamilton 2007). Surface roughness can be sub-divided into three levels, based on the scale of alterations including macro-, micro- and nano-sized topographies (Buser et al. 1991, Gotfredsen et al. 1995, Wenneberg et al. 1995, 1998, Le Guehennec et al. 2007).

Any alterations made to the surface in the range of millimetres is defined as a macro level alteration (Le Guéhennec et al. 2007). This scale is related to implant geometry, with threaded screw and macro-porous surface treatments giving a larger surface area between the implant and bone surfaces. It is well recognized that macro level topographical changes improves both early fixation as well as long term mechanical stability of dental implants by increasing mechanical interlocking between the implant and bone (Buser et al. 1991, Gotfredsen et al. 1995, Wennerberg et al. 1995).

Table 1 – Surface Properties of Titanium Dental Implants

Type of implant	**Surface roughness (µm)**	**Contact angle (°)**	**References**
cpTi	R_a = 0.22+/- 0.01	55.4+/-4.1	Wieland et al. 2001, Bagno et al. 2004
Ti_6Al_4V	R_a = 0.23+/-0.01	56.3+/-2.7	Bagno et al. 2004
TPS	R_a = 5.21+/- 2.09	n.d.	Bagno et al. 2004
SLA	R_a = 3.97+/- 0.05	138.3+/-4.2	Wieland 2001, Bagno et al. 2004
SLA active	R_a = 3.97+/- 0.04	0	Buser et al. 2004
Plasma sprayed HA coating	R_a = 1.06+/- 0.21	57.4+/-3.2	Giavaresi et al. 2003
Biomimetic CaP	R_a = 1.83+/- 0.64	13.4+/- 0.2	Le Guehennec et al. 2007

Any alteration made in the range of 1-100 μm is defined as a micro-topographic alteration. This range of roughness has been shown to play a role at the cellular level (Wieland et al. 2005). The ability for rough surface implants to promote osseointegration has been shown in areas of anatomical limitations such as the maxilla where insufficient bone quantity often exists (Clayman 2006). These rougher surfaces have shown superior clinical outcomes when compared to smooth surfaces (Buser 1991, 1998, Cochran 1996, 1998, Wennerberg 2003).

The topographic profile of dental implants in the nano range plays an important role in adsorption of proteins and adhesion of osteoblasts (Brett et al. 2004). Nano scale and micro-machined topographical alterations have been shown to influence cell attachment and cell spreading *in vitro* (Hamilton et al. 2005, 2007).

1.4.9 Chemical Modifications to Dental Implants:

Recent advances involve modification of the surface chemistry of the titanium implant, creating a "biomimetic" surface to enhance integration with the surrounding bone (Schuler et al. 2006B). Bioactive molecules, including carbons, polymers and composites can directly enhance bone formation surrounding the implant (Lacefield 1998, Morra 2006). Furthermore, peptide sequences known to influence cell attachment, such as RGD, have been shown to directly influence osteoblast attachment and spreading on titanium surfaces *in vitro* (Schuler et al. 2006A). Alterations in surface chemistry have not been fully utilized in any clinical setting due to their large production costs, molecular complexity and questionable *in vivo* stability (Schuler et al. 2006B). A relatively new dental material, Emdogain®, has been extensively studied and is capable of inducing the regeneration of periodontal structures (Hammarstrom 1997, Sculean et al.

1999, 2000, Cochran et al. 2003). However, the influence of Emdogain® on bone formation around implants is uninvestigated.

1.4 Enamel Matrix Derivative (EMD):

1.5.1 Composition of EMD:

The commercial product Emdogain® is an enamel matrix derivative (EMD) extracted from porcine teeth. The major component of EMD is amelogenin which comprises 90% of the organic phase of the enamel matrix (Brookes et al. 1995). The remaining components of EMD include proline-rich non-amelogenins such as enamelins, tuftelin, amelin and ameloblastin (Schwartz et al, 2000; Parkar and Tonetti 2004; Shimizu et al. 2004, Carinci et al. 2006).

1.5.2 *In Vitro* Studies:

To elucidate possible biological activity of EMD, investigators have employed *in vitro* studies to observe the effects of EMD on gingival fibroblasts, periodontal ligament (PDL) cells and osteoblasts (Gestrelius et al. 1997, Haase et al. 2001, Yoneda et al. 2003, Schwarz et al. 2004, Hägewald et al., 2004, He et al. 2005, Jiang et al. 2006, Pischon et al. 2006, Carinci et al. 2006, Weishaupt et al. 2008). In one of the first *in vitro* studies, Gestrelius et al. found that EMD increased proliferation rates of periodontal ligament (PDL) cells and promoted mineralized nodule formation, although no significant differences in cell migration or attachment were observed (Gestrelius et al. 1997). It has since been hypothesized that EMD contains many growth factors including TGFβ and BMPs that may induce osteoblast, fibroblast and PDL cell proliferation and/or

differentiation (Kawase et al. 2001, 2002, Palioto et al 2004, Suzuki et al. 2005, Takayama et al. 2005, Nagano et al. 2006, Johnson et al. 2009).

1.5.3 *In Vivo* Studies – EMD persistence:

Hammarstrom et al. were the first to demonstrate that EMD could result in regeneration of the acellular extrinsic fiber cementum in a monkey model (Hammarstrom et al. 1997). Following this study, a plethora of research examining EMD's ability to promote periodontal regeneration followed. During these studies, Gestrelius et al. showed that EMD adsorbs to hydroxyapatite, collagen and denuded dental roots. Using rat and pig models, it was shown that a detectable amount of EMD remained visible on the root surface 2 weeks post surgery and that it forms insoluble spherical complexes (Gestrelius et al. 1997). They also observed using scanning electron microscopy of extracted teeth that EMD-treated teeth had significantly greater colonization by fibroblasts than control teeth after 2 weeks. In similar experiments using immunohistochemical analysis, it was found that EMD was still present 4 weeks after application on extracted rat molars that were transplanted to the abdominal wall (Hamamoto et al. 2002) and 4 weeks following periodontal surgery (Sculean et al. 2002). Thus, EMD persisted on several surfaces up to 4 weeks post application and therefore may be useful clinically following single application.

1.5.4 *In Situ* Studies:

Clinical studies have also shown the benefits of EMD as a treatment option for periodontal diseases including periodontitis and intrabony defects (Pontoriero 1999,

Sculean et al. 1999, 2000, 2007). Histological analysis revealed periodontal regeneration and new connective tissue attachment to root surfaces 6 months post surgery (Yukna and Mellonig 2000). Sculean et al. explained that "based on the available evidence from human histological studies, it may be concluded that the application of EMD in conjunction with periodontal surgery may promote formation of new cementum, PDL and bone in intrabony and recession defects" (Sculean et al. 2003).

1.5.5 EMD and bone growth:

Most of the research to date has focused on the influence of EMD on fibroblast and PDL cell proliferation and differentiation. Boyan et al. were the first to test the effects of EMD on osteoblasts and concluded that it may have osteoconductive properties (Boyan et al. 2000). This was further supported *in vivo* by Kawana et al. using a perforated femur model (Kawana et al. 2001). They drilled cylindrical holes in rat femurs and filled the defects with either EMD or its carrier, polyglycolic acid (PGA). EMD significantly increased bone volume fraction of the trabeculae in as little as 7 days post surgery (Kawana et al. 2001).

1.6.1 Rationale:

To date, there have been no *in vivo* studies testing the effectiveness of EMD around dental implants. Furthermore, *in vitro* studies have tested EMD's efficacy when added directly into cell culture media; a situation that does not mimic dental implant procedures. Strong evidence has demonstrated that EMD has the capability to promote bone growth *in vivo* following a single treatment with EMD (Kawana et al. 2001),

however, the application of precoating EMD on a surface remains unknown. Furthermore, when EMD is applied to rat and pig root surfaces, it remained detectable on the surfaces 2 weeks post re-implantation (Gestrelius et al. 1997). We hypothesized that by precoating EMD directly on titanium surfaces, EMD would modulate osteoblast morphology, proliferation and differentiation.

1.6.2 Hypothesis and Objectives:

EMD precoated on titanium surfaces will increase the speed and quantity of bone formation.

The research addressed the following specific objectives:

1. To characterize the influence of EMD on the attachment, proliferation and differentiation of primary rat calvarial osteoblasts (RCOs).
2. To investigate the influence of EMD in combination with titanium substratum topography on time-dependent formation of mineralized bone by RCOs.

1.7 References:

Adell R, Lekholm U, Rockler B, Brånemark PI. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surg. 1981 Dec;10(6):387-416.

Albandar JM. Global risk factors and risk indicators for periodontal diseases. Periodontol 2000. 2002;29:177-206.

Alberius P. Bone reactions to tantalum markers. A scanning electron microscopic study. Acta Anat (Basel). 1983 Apr;115(4):310-8.

Anh DJ, Dimai HP, Hall SL, Farley JR. Skeletal alkaline phosphatase activity is primarily released from human osteoblasts in an insoluble form, and the net release is inhibited by calcium and skeletal growth factors. Calcif Tissue Int. 1998 Apr;62(4):332-40.

Bagno A, Di Bello C. Surface treatments and roughness properties of Ti-based biomaterials. J Mater Sci Mater Med. 2004 Sep;15(9):935-49.

Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem. 1997 Jul 1;66(1):1-8.

Baron R. Anatomy and ultrastructure of bone. Primer on the metabolic bone diseases and disorders of mineral metabolism. M.J. Favus, editor. Lippincott Williams & Wilkins, Philadelphia, PA 1999. 3-10.

Baron R, Vignery A, Tran Van P. The significance of lacunar erosion without osteoclasts: studies on the reversal phase of the remodeling sequence. Metab. Bone Dis. Rel. 1980 2S, 35-40.

Beder OE, Eade G: An investigation on tissue tolerance to titanium metal implants in dogs, Surgery 39: 1956, 470.

Bellows CG, Aubin JE, Heersche JN. Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. Bone Miner. 1991 Jul;14(1):27-40.

Birk DE, Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. Micron. 2001 Apr;32(3):223-37.

Bloom B, Gaft HC, Jack SS National Center for Health Statistics. Dental Services and Oral Health. United States, 1989 Vital Health Stat 10 (183). DHHS Pat No (PAS) 93-1511. Washington, DC, 1992, US Government Printing Office.

Bobyn JD, Pilliar RM, Cameron HU, Weatherly GC. The optimum pore size for the fixation of porous-surfaced metal implants by the ingrowth of bone. Clin Orthop Relat Res. 1980 Jul-Aug;(150):263-70.

Boivin G, Morel G, Lian JB, Anthoine-Terrier C, Dubois PM, Meunier PJ. Localization of endogenous osteocalcin in neonatal rat bone and its absence in articular cartilage: effect of warfarin treatment. Virchows Arch A Pathol Anat Histopathol. 1990;417(6):505-12.

Bonewald LF, Mundy GR. Role of transforming growth factor-beta in bone remodeling. Clin Orthop Relat Res. 1990 Jan;(250):261-76.

Boskey, A.L., Wright, T.M., and Blank, R.D. Collagen and bone strength. J Bone Miner Res. 1999 Mar;14(3):330-5.

Boyan BD, Weesner TC, Lohmann CH, Andreacchio D, Carnes DL, Dean DD, Cochran DL, Schwartz Z. Porcine fetal enamel matrix derivative enhances bone formation induced by demineralized freeze dried bone allograft in vivo. J Periodontol. 2000 Aug;71(8):1278-86.

Boyan BD, Bonewald LF, Paschalis EP, Lohmann CH, Rosser J, Cochran DL, Dean DD, Schwartz Z, Boskey AL. Osteoblast-mediated mineral deposition in culture is

dependent on surface microtopography. Calcif Tissue Int. 2002 Dec;71(6):519-29. Epub 2002 Sep 18.

Brånemark P-I. Vital microscopy of bone marrow in rabbit. Scand J Clin Lab Invest 1959; 11(Suppl.38):1-82.

Brånemark PI, Adell R, Breine U, Hansson BO, Lindström J, Ohlsson A. Intra-osseous anchorage of dental prostheses. I. Experimental studies. Scand J Plast Reconstr Surg. 1969;3(2):81-100.

Brett PM, Harle J, Salih V, Mihoc R, Olsen I, Jones FH, Tonetti M. Roughness response genes in osteoblasts. Bone. 2004 Jul;35(1):124-33.

Brighton CT, Hunt RM. Histochemical localization of calcium in the fracture callus with potassium pyroantimonate. Possible role of chondrocyte mitochondrial calcium in callus calcification. J Bone Joint Surg Am. 1986 Jun;68(5):703-15.

Brighton CT, Sugioka Y, Hunt RM. Cytoplasmic structures of epiphyseal plate chondrocytes. Quantitative evaluation using electron micrographs of rat costochondral junctions with special reference to the fate of hypertrophic cells. J Bone Joint Surg Am. 1973 Jun;55(4):771-84.

Brookes SJ, Robinson C, Kirkham J, Bonass WA. Biochemistry and molecular biology of amelogenin proteins of developing dental enamel. Arch Oral Biol. 1995 Jan;40(1):1-14.

Brown LJ, Löe H. Prevalence, extent, severity and progression of periodontal disease. Periodontol 2000. 1993 Jun;2:57-71.

Browne M, Gregson PJ. Effect of mechanical surface pretreatment on metal ion release. Biomaterials. 2000 Feb;21(4):385-92.

Bruder SP, Caplan AI. Cellular and molecular events during embryonic bone development. Connect Tissue Res. 1989;20(1-4):65-71.

Brunette DM, Chehroudi B. The effects of the surface topography of micromachined titanium substrata on cell behavior in vitro and in vivo. J Biomech Eng. 1999 Feb;121(1):49-57. Review.

Burger EH, Klein-Nulend J. Mechanotransduction in bone--role of the lacuno-canalicular network. FASEB J. 1999;13 Suppl:S101-12.

Burger EH, Klein-Nulend J, Smit TH. Strain-derived canalicular fluid flow regulates osteoclast activity in a remodelling osteon--a proposal. J Biomech. 2003 Oct;36(10):1453-9.

Burr DB. Targeted and nontargeted remodeling. Bone. 2002 Jan;30(1):2-4.

Burt DB. Position paper. Epidermiology of periodontal diseases. Journal of Periodontology 76, 2005, 1406-1419.

Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res. 1991 Jul;25(7):889-902.

Buser D, Nydegger T, Hirt HP, Cochran DL, Nolte LP. Removal torque values of titanium implants in the maxilla of miniature pigs. Int J Oral Maxillofac Implants. 1998 Sep-Oct;13(5):611-9.

Buser D, Broggini N, Wieland M, Schenk RK, Denzer AJ, Cochran DL, Hoffmann B, Lussi A, Steinemann SG. Enhanced bone apposition to a chemically modified SLA titanium surface. J Dent Res. 2004 Jul;83(7):529-33.

Cabala A, Chomyszyn-Gajewska M, Drozdz W. Periodontitis and systemic disease relationships. Przegl Lek. 2006; 63(9): 773-777.

Caplan AI. Bone development. Ciba Found Symp. 1988;136:3-21.

Carinci F, Piattelli A, Guida L, Perrotti V, Laino G, Oliva A, Annunziata M, Palmieri A, Pezzetti F. Effects of EMD on osteoblast gene expression. Oral Dis. 2006 May;12(3):329-42.

Chang YL, Lew D, Park JB, Keller JC. Biomechanical and morphometric analysis of hydroxyapatite-coated implants with varying crystallinity. J Oral Maxillofac Surg. 1999 Sep;57(9):1096-1109.

Chehroudi B, Brunette DM Subcutaneous microfabricated surfaces inhibit epithelial recession and promote long-term survival of percutaneous implants. Biomaterials. 2002 Jan;23(1):229-37.

Chenu C, Colucci S, Grano M, Zigrino P, Barattolo R, Zambonin G, Baldini N, Vergnaud P, Delmas PD, Zallone AZ. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. J Cell Biol. 1994 Nov;127(4):1149-58.

Chvapil M, Ryan JN Diverse effect of 1,10-phenanthroline and 2,2'-dipyridyl on the synthesis of collagen and noncollagenous proteins in in vivo and in vitro systems. Biochim Biophys Acta. 1972 Jun 26;273(1):208-11.

Civitelli R, Beyer EC, Warlow PM, Robertson AJ, Geist ST, Steinberg TH. Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. J Clin Invest. 1993 May;91(5):1888-96.

Clayman L. Implant reconstruction of the bone-grafted maxilla: review of the literature and presentation of 8 cases. J Oral Maxillofac Surg. 2006 Apr;64(4):674-82.

Cochran DL, Nummikoski PV, Higginbottom FL, Hermann JS, Makins SR, Buser D. Evaluation of an endosseous titanium implant with a sandblasted and acid-etched surface in the canine mandible: radiographic results. Clin Oral Implants Res. 1996 Sep;7(3):240-52.

Cochran DL, Schenk RK, Lussi A, Higginbottom FL, Buser D. Bone response to unloaded and loaded titanium implants with a sandblasted and acid-etched surface: a histometric study in the canine mandible. J Biomed Mater Res. 1998 Apr;40(1):1-11.

Cochran DL, King GN, Schoolfield J, Velasquez-Plata D, Mellonig JT, Jones A. The effect of enamel matrix proteins on periodontal regeneration as determined by histological analyses. J Periodontol. 2003 Jul;74(7):1043-55.

Collaert B, De Bruyn H. Comparison of Brånemark fixture integration and short-term survival using one-stage or two-stage surgery in completely and partially edentulous mandibles. Clin Oral Implants Res. 1998 Apr;9(2):131-5.

Cowles EA, DeRome ME, Pastizzo G, Brailey LL, Gronowicz GA. Mineralization and the expression of matrix proteins during in vivo bone development. Calcif Tissue Int. 1998 Jan;62(1):74-82.

Davies JE Mechanisms of endosseous integration Int J Prosthodont. 1998 Sep-Oct;11(5):391-401.

Davies JE Understanding peri-implant endosseous healing J Dent Educ. 2003 Aug;67(8):932-49.

Davies RM. Toothpaste in the control of plaque/gingivitis and periodontitis. Periodontol 2000. 2008;48:23-30.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. Nature. 1996 Aug 1;382(6590):448-52.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell. 1997 May 30;89(5):747-54.

Ducy P, Schinke T, Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science. 2000 Sep 1;289(5484):1501-4.

Esposito M, Hirsch JM, Lekholm U, Thomsen P. Biological factors contributing to failures of osseointegrated oral implants. (I). Success criteria and epidemiology. Eur J Oral Sci. 1998 Feb;106(1):527-51.

Esposito M, Hirsch J, Lekholm U, Thomsen P. Differential diagnosis and treatment strategies for biologic complications and failing oral implants: a review of the literature. Int J Oral Maxillofac Implants. 1999 Jul-Aug;14(4):473-90.

Fedde KN. Human osteosarcoma cells spontaneously release matrix-vesicle-like structures with the capacity to mineralize. Bone Miner. 1992 May;17(2):145-51.

Fiedler J, Röderer G, Günther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. J Cell Biochem. 2002;87(3):305-12.

Fisher LW, McBride OW, Termine JD, Young MF. Human bone sialoprotein. Deduced protein sequence and chromosomal localization. J Biol Chem. 1990 Feb 5;265(4):2347-51.

Fratzl P, Schreiber S, Klaushofer K. Bone mineralization as studied by small-angle x-ray scattering. Connect Tissue Res. 1996;34(4):247-54.

Fugazzotto PA. Success and failure rates of osseointegrated implants in function in regenerated bone for 72 to 133 months. Int J Oral Maxillofac Implants. 2005 Jan-Feb;20(1):77-83.

Fugazzotto PA. Evidence-based decision making: replacement of the single missing tooth. Dent Clin North Am. 2009 Jan;53(1):97-129, ix.

Fujisawa R, Nodasaka Y, Kuboki Y. Further characterization of interaction between bone sialoprotein (BSP) and collagen. Calcif Tissue Int. 1995 Feb;56(2):140-4.

Fukae M, Tanabe T, Uchida T, Lee SK, Ryu OH, Murakami C, Wakida K, Simmer JP, Yamada Y, Bartlett JD. Enamelysin (matrix metalloproteinase-20): localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. J Dent Res. 1998 Aug;77(8):1580-8.

Gestrelius S, Andersson C, Johansson AC, Persson E, Brodin A, Rydhag L, Hammarström L. Formulation of enamel matrix derivative for surface coating. Kinetics and cell colonization. J Clin Periodontol. 1997 Sep;24(9 Pt 2):678-84.

Giavaresi G, Fini M, Cigada A, Chiesa R, Rondelli G, Rimondini L, Aldini NN, Martini L, Giardino R. Histomorphometric and microhardness assessments of sheep cortical bone surrounding titanium implants with different surface treatments. J Biomed Mater Res A. 2003 Oct 1;67(1):112-20.

Glimcher MJ. The nature of the mineral phase in bone: biological and clinical implications. In Metabolic Bone Disease and Clinically Related Disorders, Academic Press, San Diego, 1998, pp.23-50.

Gotfredsen K, Wennerberg A, Johansson C, Skovgaard LT, Hjørting-Hansen E. Anchorage of TiO2-blasted, HA-coated, and machined implants: an experimental study with rabbits. J Biomed Mater Res. 1995 Oct;29(10):1223-31.

Gross PP, Gold L: The compatibility of Vitallium and austanium in completely buried implants in dogs, Oral Surg 10:769, 1957.

Haas R, Mensdorff-Pouilly N, Mailath G, Watzek G. Brånemark single tooth implants: a preliminary report of 76 implants. J Prosthet Dent. 1995 Mar;73(3):274-9.

Haase HR, Bartold PM. Enamel matrix derivative induces matrix synthesis by cultured human periodontal fibroblast cells. J Periodontol. 2001 Mar;72(3):341-8.

Hägewald S, Pischon N, Jawor P, Bernimoulin JP, Zimmermann B. Effects of enamel matrix derivative on proliferation and differentiation of primary osteoblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Aug;98(2):243-9.

Hamamoto Y, Kawasaki N, Jarnbring F, Hammarström L. Effects and distribution of the enamel matrix derivative Emdogain in the periodontal tissues of rat molars transplanted to the abdominal wall. Dent Traumatol. 2002 Feb;18(1):12-23.

Hamilton DW, Riehle MO, Monaghan W, Curtis AS. Articular chondrocyte passage number: influence on adhesion, migration, cytoskeletal organisation and phenotype in response to nano- and micro-metric topography. Cell Biol Int. 2005 Jun;29(6):408-21.

Hamilton DW, Wong KS, Brunette DM. Microfabricated discontinuous-edge surface topographies influence osteoblast adhesion, migration, cytoskeletal organization, and proliferation and enhance matrix and mineral deposition in vitro. Calcif Tissue Int. 2006 May;78(5):314-25.

Hamilton DW, Brunette DM. The effect of substratum topography on osteoblast adhesion mediated signal transduction and phosphorylation. Biomaterials. 2007 Apr;28(10):1806-19.

Hamilton DW, Chehroudi B, Brunette DM. Comparative response of epithelial cells and osteoblasts to microfabricated tapered pit topographies in vitro and in vivo. Biomaterials. 2007 May;28(14):2281-93.

Hammarström L, Heijl L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins. J Clin Periodontol. 1997 Sep;24(9 Pt 2):669-77.

Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. Nature. 2003 May 15;423(6937):349-55.

Hauschka PV, Lian JB, Cole DE, Gundberg CM. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol Rev. 1989 Jul;69(3):990-1047.

He J, King Y, Jiang J, Safavi KE, Spangberg LS, Zhu Q. Enamel matrix derivative inhibits TNF alpha induced apoptosis in osteoblastic MC3T3-E1 cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2005 Jun;99(6):761-7.

Health Promotion Survey Canada: Statistics Canada, 1990, record number 3828. Available at www.statcan.ca. Accessed July 14, 2008.

Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD. Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. Nature. 2000 Jun 8;405(6787):704-6.

Hutton JE, Heath MR, Chai JY, Harnett J, Jemt T, Johns RB, McKenna S, McNamara DC, van Steenberghe D, Taylor R, et al. Factors related to success and failure rates at 3-year follow-up in a multicenter study of overdentures supported by Brånemark implants. Int J Oral Maxillofac Implants. 1995 Jan-Feb;10(1):33-42.

Huynh-Ba G, Friedberg JR, Vogiatzi D, Ioannidou E. Implant failure predictors in the posterior maxilla: a retrospective study of 273 consecutive implants. J Periodontol. 2008 Dec;79(12):2256-61.

Jiang J, Goodarzi G, He J, Li H, Safavi KE, Spångberg LS, Zhu Q, EMD-gel stimulates proliferation of odontoblasts and osteoblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006 Nov;102(5):698-702.

Johansson CB, Albrektsson T. A removal torque and histomorphometric study of commercially pure niobium and titanium implants in rabbit bone. Clin Oral Implants Res. 1991 Jan-Mar;2(1):24-9.

Kawana F, Sawae Y, Sahara T, Tanaka S, Debari K, Shimizu M, Sasaki T. Porcine enamel matrix derivative enhances trabecular bone regeneration during wound healing of injured rat femur. 2001 Dec 1;264(4):438-46.

Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN) rapidly stimulates phosphorylation of the MAP kinase

family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. J Periodontal Res. 2001 Dec;36(6):367-76.

Kawase T, Okuda K, Yoshie H, Burns DM. Anti-TGF-beta antibody blocks enamel matrix derivative-induced upregulation of p21WAF1/cip1 and prevents its inhibition of human oral epithelial cell proliferation. J Periodontal Res. 2002 Aug;37(4):255-62.

Kim H, Choi SH, Ryu JJ, Koh SY, Park JH, Lee IS. The biocompatibility of SLA-treated titanium implants. Biomed Mater. 2008 Jun;3(2):25011.

Kujala S, Ryhänen J, Danilov A, Tuukkanen J. Effect of porosity on the osteointegration and bone ingrowth of a weight-bearing nickel-titanium bone graft substitute. Biomaterials. 2003 Nov;24(25):4691-7.

Lacefield WR Current status of ceramic coatings for dental implants. Implant Dent. 1998;7(4):315-22.

Le Guéhennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. Dent Mater. 2007 Jul;23(7):844-54.

Lee JJ, Rouhfar L, Beirne OR. Survival of hydroxyapatite-coated implants: a meta-analytic review. J Oral Maxillofac Surg. 2000 Dec;58(12):1372-80.

Lemons JE Biomaterial considerations for dental implants part I: metals and alloys. Oral Implantol. 1975;5(4):503-15.

Lemons J, Natiella J Biomaterials, biocompatibility, and peri-implant considerations. Dent Clin North Am. 1986 Jan;30(1):3-23.

Lemons JE, editor: Quantitative characterization and performance of porous implants for hard tissue application, ASTM STP 953, Philadelphia, 1987, American Society for Testing and Materials.

Littlewood-Evans A, Kokubo T, Ishibashi O, Inaoka T, Wlodarski B, Gallagher JA, Bilbe G. Localization of cathepsin K in human osteoclasts by in situ hybridization and immunohistochemistry. Bone. 1997 Feb;20(2):81-6.

Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol. 2008;40(1):46-62.

Malevez C, Hermans M, Daelemans P. Marginal bone levels at Brånemark system implants used for single tooth restoration. The influence of implant design and anatomical region. Clin Oral Implants Res. 1996 Jun;7(2):162-9.

Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J Jr, Dean DD, Cochran DL, Boyan BD Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). J Biomed Mater Res. 1995 Mar;29(3):389-401.

Martini D, Fini M, Franchi M, Pasquale VD, Bacchelli B, Gamberini M, Tinti A, Taddei P, Giavaresi G, Ottani V, Raspanti M, Guizzardi S, Ruggeri A. Detachment of titanium and fluorohydroxyapatite particles in unloaded endosseous implants. Biomaterials. 2003 Mar;24(7):1309-16.

Massaro C, Rotolo P, De Riccardis F, Milella E, Napoli A, Wieland M, Textor M, Spencer ND, Brunette DM. Comparative investigation of the surface properties of commercial titanium dental implants. Part I: chemical composition. J Mater Sci Mater Med. 2002 Jun;13(6):535-48.

McNeill C. Occlusion: what it is and what it is not. J Calif Dent Assoc. 2000 Oct;28(10):748-58.

Meskin L, Berg R. Impact of older adults on private dental practices, 1988-1998. J Am Dent Assoc. 2000 Aug;131(8):1188-95.

Millenium Research Group report: US Markets for Dental Implants 2006, USDI 06, June, 2006.

Misch CE, Contemporary implant dentistry, third edition 2008, pp. 4-25

Mohammadi S, Esposito M, Cucu M, Ericson LE, Thomsen P. Tissue response to hafnium. J Mater Sci Mater Med. 2001 Jul;12(7):603-11.

Mohan S, Baylink DJ. Bone growth factors. Clin Orthop Relat Res. 1991 Feb;(263):30-48.

Mojon P: The world without teeth: demographic trends. In Feine JS, Carlsson GE, editors: Implant overdentures: the standard of care for edentulous patients, Carol Stream, Ill, 2003.

Morris AJ, Steele J, White DA. The oral cleanliness and periodontal health of UK adults in 1998. Br Dent J 2001: 191: 186-192.

Morra M, Biochemical Modification of Titanium Surfaces: Peptides and ECM proteins. European Cells and Materials Vol 12. 2006: 1-15.

Nagano T, Oida S, Suzuki S, Iwata T, Yamakoshi Y, Ogata Y, Gomi K, Arai T, Fukae M. Porcine enamel protein fractions contain transforming growth factor-beta1. J Periodontol. 2006 Oct;77(10):1688-94.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002 Jan 11;108(1):17-29.

Nergiz I, Arpak N, Bostanci H, Scorziello TM, Schmage P. Stability of loaded and unloaded implants with different surfaces. Int J Oral Maxillofac Implants. 2009 Mar-Apr;24(2):289-98.

Netter J, Frank H., Musculoskeletal system: anatomy, physiology, and metabolic disorders. Summit, New Jersey: Ciba-Geigy Corporation ISBN 0914168886, 1987 p. 130-39.

Nguyen DH, Martin JT. Common dental infections in the primary care setting. Am Fam Physician. 2008 Mar 15;77(6):797-802.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J.,Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J. & Haylock, D.N. (2005) Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood, 106, 1232–1239.

Niyibizi C, Eyre DR. Structural characteristics of cross-linking sites in type V collagen of bone. Chain specificities and heterotypic links to type I collagen. Eur J Biochem. 1994 Sep 15;224(3):943-50.

Novaes AB Jr, Souza SL, de Oliveira PT, Souza AM. Histomorphometric analysis of the bone-implant contact obtained with 4 different implant surface treatments placed side by side in the dog mandible. Int J Oral Maxillofac Implants. 2002 May-Jun;17(3):377-83.

Ogata Y. Bone sialoprotein and its transcriptional regulatory mechanism. J Periodontal Res. 2008 Apr;43(2):127-35.

Oldberg A, Franzén A, Heinegård D. The primary structure of a cell-binding bone sialoprotein. J Biol Chem. 1988 Dec 25;263(36):19430-2.

Palioto DB, Coletta RD, Graner E, Joly JC, de Lima AF. The influence of enamel matrix derivative associated with insulin-like growth factor-I on periodontal ligament fibroblasts. J Periodontol. 2004 Apr;75(4):498-504.

Parfitt AM. Physiologic and pathogenetic significance of bone histomorphometric data. In: "Disorders of Bone and Mineral Metabolism," FL Coe and MJ Favus (eds),

Second Edition, 2002A, pp.469-485, Lippincott Williams and Wilkins, Philadelphia.

Parfitt AM. Targeted and nontargeted bone remodeling: relationship to basic multicellular unit origination and progression. Bone. 2002B Jan;30(1):5-7.

Piattelli M, Scarano A, Paolantonio M, Iezzi G, Petrone G, Piattelli A. Bone response to machined and resorbable blast material titanium implants: an experimental study in rabbits. J Oral Implantol. 2002;28(1):2-8.

Pinnell SR Regulation of collagen biosynthesis by ascorbic acid: a review. Yale J Biol Med. 1985 Nov-Dec;58(6):553-9.

Pischon N, Zimmermann B, Bernimoulin JP, Hägewald S Effects of an enamel matrix derivative on human osteoblasts and PDL cells grown in organoid cultures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006 Oct;102(4):551-7. Epub 2006 Jul 20.

Pontoriero R, Wennstrom J, Lindhe J. The use of barrier membranes and enamel matrix proteins in the treatment of angular bone defects. A prospective controlled clinical study, J Clin Periodontol. 1999 Dec; 26(12):833-840.

Preshaw PM, Seymour RA, Heasman PA. Current concepts in periodontal pathogenesis, Dent Update. 2004 Dec; 31(10):570-578.

Qu J, Chehroudi B, Brunette DM. The use of micromachined surfaces to investigate the cell behavioural factors essential to osseointegration. Oral Dis. 1996 Mar;2(1):102-15.

Qu Z, Rausch-Fan X, Wieland M, Matejka M, Schedle A. The initial attachment and subsequent behavior regulation of osteoblasts by dental implant surface modification. J Biomed Mater Res A. 2007 Sep 1;82(3):658-68.

Rautemaa R, Lauhio A, Cullinan MP, Seymour GJ. Oral infections and systemic disease-an emerging problem in medicine. Clin Microbiol Infect. 2007 Nov;13(11):1041-7.

Reith, EEJ, and Ross MJ. Atlas of Descriptive Histology," Second Edition. Harper and Row, Publishers, Hagerstown MD,1970, pp.23-40.

Reinholt FP, Hultenby K, Oldberg A, Heinegård D. Osteopontin--a possible anchor of osteoclasts to bone. Proc Natl Acad Sci U S A. 1990 Jun;87(12):4473-5.

Roodman GD. Osteoclast function in Paget's disease and multiple myeloma. Bone. 1995 Aug;17(2 Suppl):57S-61S.

Roodman GD. Cell biology of the osteoclast. Exp Hematol. 1999 Aug;27(8):1229-41.

Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem. 1993 May 5;268(13):9901-7.

Schmitt A, Zarb GA. The longitudinal clinical effectiveness of osseointegrated dental implants for single-tooth replacement. Int J Prosthodont. 1993 Mar-Apr;6(2):197-202.

Schuler M, Owen GR, Hamilton DW, de Wild M, Textor M, Brunette DM, Tosatti SG. Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: a cell morphology study. Biomaterials. 2006A Jul;27(21):4003-15.

Schuler M, Trentin D, Textor M, Tosatti SG. Biomedical interfaces: titanium surface technology for implants and cell carriers. Nanomed. 2006B Dec;1(4):449-63.

Schwartz NL, Whitsett LD, Berry TG, Stewart JL. Unserviceable crowns and fixed partial dentures: life-span and causes for loss of serviceability. J Am Dent Assoc. 1970 Dec;81(6):1395-401.

Schwarz RI Procollagen secretion meets the minimum requirements for the rate-controlling step in the ascorbate induction of procollagen synthesis. J Biol Chem. 1985 Mar 10;260(5):3045-9.

Schwarz F, Rothamel D, Herten M, Sculean A, Scherbaum W, Becker J. Effect of enamel matrix protein derivative on the attachment, proliferation, and viability of human SaOs(2) osteoblasts on titanium implants. Clin Oral Investig. 2004 Sep;8(3):165-71.

Schwartz Z, Nasazky E, Boyan BD. Surface microtopography regulates osteointegration: the role of implant surface microtopography in osteointegration. Alpha Omegan. 2005 Jul;98(2):9-19.

Sculean A, Reich E, Chiantella GC, Brecx M. Treatment of intrabony periodontal defects with an enamel matrix protein derivative (Emdogain): a report of 32 cases. Int J Periodontics Restorative Dent. 1999 Apr;19(2):157-63.

Sculean A, Donos N, Brecx M, Reich E, Karring T. Treatment of intrabony defects with guided tissue regeneration and enamel matrix proteins. An experimental study in monkeys. J Clin Periodontol. 2000 Jul; 27(7):466-72.

Sculean A, Windisch P, Keglevich T, Fabi B, Lundgren E, Lyngstadaas PS. Presence of an enamel matrix protein derivative on human teeth following periodontal surgery. Clin Oral Investig. 2002 Sep;6(3):183-7.

Sculean A, Windisch P, Keglevich T, Gera I. Histologic evaluation of human intrabony defects following non-surgical periodontal therapy with and without application of an enamel matrix protein derivative. J Periodontol. 2003 Feb;74(2):153-60.

Sculean A, Schwarz F, Becker J, Brecx M, The application of an enamel matrix protein derivative (EMD) in regenerative periodontal therapy: a review. Med Princ Pract. 2007;16(3):167-80.

Shimizu E, Nakajima Y, Kato N, Nakayama Y, Saito R, Samoto H, Ogata Y. Regulation of rat bone sialoprotein gene transcription by enamel matrix derivative. J Periodontol. 2004 Feb;75(2):260-7.

Skillington J, Choy L, Derynck R. Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol. 2002 Oct 14;159(1):135-46.

Smit TH, Burger EH. Is BMU-coupling a strain-regulated phenomenon? A finite element analysis. J Bone Miner Res. 2000 Feb;15(2):301-7.

Steinemann SG. Titanium--the material of choice? Periodontol 2000. 1998 Jun;17:7-21.

Stricker S, Fundele R, Vortkamp A, Mundlos S. Role of Runx genes in chondrocyte differentiation. Dev Biol. 2002 May 1;245(1):95-108.

Suzuki S, Nagano T, Yamakoshi Y, Gomi K, Arai T, Fukae M, Katagiri T, Oida S. Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-{beta}.J Dent Res. 2005 Jun;84(6):510-4.

Takala L, Utriainen P, Alanen P. Incidence of edentulousness, reasons for full clearance, and health status of teeth before extractions in rural Finland. Community Dent Oral Epidemiol. 1994 Aug;22(4):254-7.

Takayama T, Suzuki N, Narukawa M, Tokunaga T, Otsuka K, Ito K. Enamel matrix derivative stimulates core binding factor alpha1/Runt-related transcription factor-2 expression via activation of Smad1 in C2C12 cells. J Periodontol. 2005 Feb;76(2):244-9.

Tenenbaum HC. Levamisole and inorganic pyrophosphate inhibit B-glycerophosphate induced mineralization of bone formed in vitro 1987. Bone Min. 3, 13-26.

Termine JD. Non-collagen proteins in bone. Ciba Found Symp. 1988;136:178-202.

Thomsen P, Larsson C, Ericson LE, Sennerby L, Lausmaa J, Kasemo B. Structure of the interface between rabbit cortical bone and implants of gold, zirconium and titanium. J Mater Sci Mater Med. 1997 Nov;8(11):653-65.

Tinsley D, Watson CJ, Russell JL. A comparison of hydroxylapatite coated implant retained fixed and removable mandibular prostheses over 4 to 6 years. Clin Oral Implants Res. 2001 Apr;12(2):159-66.

Tong W, Glimcher MJ, Katz JL, Kuhn L, Eppell SJ. Size and shape of mineralites in young bovine bone measured by atomic force microscopy. Calcif Tissue Int. 2003 May;72(5):592-8. Epub 2003 May 6.

Trisi P, Lazzara R, Rao W, Rebaudi A. Bone-implant contact and bone quality: evaluation of expected and actual bone contact on machined and osseotite implant surfaces. Int J Periodontics Restorative Dent. 2002 Dec;22(6):535-45.

Urban RM, Jacobs JJ, Tomlinson MJ, Gavrilovic J, Black J, Peoc'h M. Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip or knee replacement. J Bone Joint Surg Am. 2000 Apr;82(4):457-76.

van der Rest M, Garrone R. Collagen family of proteins. FASEB J. 1991 Oct;5(13):2814-23.

Von der Mark, K., and Sorokin, L. Adhesive Glycoproteins, In Connective Tissue and its Heritable Disorders (P.M. Royce, and B. Steinmann eds.), 2002 pp. 293-328, Wiley-Liss, New York.

Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J Bone Miner Res. 1990 Aug;5(8):831-42.

Weishaupt P, Bernimoulin JP, Trackman P, Hägewald S. Stimulation of osteoblasts with EMD increases the expression of specific mineralization markers. 2008 Aug;106(2):304-8.

Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millán JL. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res. 2000 Oct;15(10):1879-88.

Wennerberg A, Albrektsson T, Andersson B, Krol JJ. A histomorphometric and removal torque study of screw-shaped titanium implants with three different surface topographies. Clin Oral Implants Res. 1995 Mar;6(1):24-30.

Wennerberg A, Hallgren C, Johansson C, Danelli S. A histomorphometric evaluation of screw-shaped implants each prepared with two surface roughnesses. Clin Oral Implants Res. 1998 Feb;9(1):11-9.

Wennerberg A. Implant design and surface factors. Int J Prosthodont. 2003 16 Suppl:45-51.

Wheeler SL. Eight-year clinical retrospective study of titanium plasma-sprayed and hydroxyapatite-coated cylinder implants. Int J Oral Maxillofac Implants. 1996 May-Jun;11(3):340-50.

Wieland M, Textor M, Spencer ND, Brunette DM. Wavelength-dependent roughness: a quantitative approach to characterizing the topography of rough titanium surfaces. Int J Oral Maxillofac Implants. 2001 Mar-Apr;16(2):163-81.

Wieland M, Textor M, Chehroudi B, Brunette DM. Synergistic interaction of topographic features in the production of bone-like nodules on Ti surfaces by rat osteoblasts. Biomaterials. 2005 Apr;26(10):1119-30.

Wright KR, McMillan PJ, Hughes DF, Boyce BF (1995). Calcium deficiency/repletion in rats as an in vivo model for the study of osteoclast apoptosis. J. Bone Miner. Res. 10, S328.

Yoneda S, Itoh D, Kuroda S, Kondo H, Umezawa A, Ohya K, Ohyama T, Kasugai S. The effects of enamel matrix derivative (EMD) on osteoblastic cells in culture and bone regeneration in a rat skull defect. J Periodontal Res. 2003 Jun;38(3):333-42.

Young DW, Pratap J, Javed A, Weiner B, Ohkawa Y, van Wijnen A, Montecino M, Stein GS, Stein JL, Imbalzano AN, Lian JB. SWI/SNF chromatin remodeling complex is obligatory for BMP2-induced, Runx2-dependent skeletal gene expression that controls osteoblast differentiation. J Cell Biochem. 2005 Mar 1;94(4):720-30.

Yukna RA, Mellonig JT. Histologic evaluation of periodontal healing in humans following regenerative therapy with enamel matrix derivative. A 10-case series. J Periodontol. 2000 May;71(5):752-9.

Zelzer et al., 2001 E. Zelzer, D.J. Glotzer, C. Hartmann, D. Thomas, N. Fukai and S. Soker et al., Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2, Mech. Dev. **106** (1/2) (2001), pp. 97–106.

Zinger O, Anselme K, Denzer A, Habersetzer P, Wieland M, Jeanfils J, Hardouin P, Landolt D. Time-dependent morphology and adhesion of osteoblastic cells on titanium model surfaces featuring scale-resolved topography. Biomaterials. 2004 Jun;25(14):2695-711.

CHAPTER TWO

Enamel Matrix Proteins Increase Spreading, Proliferation and Differentiation of Osteoblasts Cultured on Titanium Surfaces

2.1 Abstract:

Modifications of implant surface topography and chemistry have proven a means to enhance osseointegration, a process that ensures the stability of bone-contacting devices, including titanium dental implants. Enamel matrix derivative (EMD) derived from developing porcine teeth, has been shown to promote regeneration of bone. The aim of the present study was to evaluate the effect of EMD on the attachment, proliferation and differentiation of osteoblasts on titanium surfaces *in vitro*. Pickled (smooth) and SLA (roughened) titanium discs were coated with EMD or left uncoated. Primary rat calvarial osteoblasts were cultured on each surface from 1 hour to 4 weeks. EMD significantly increased cell spreading and proliferation at time points ranging from 3 to 7 days on both topographies. Alkaline phosphatase activity was significantly increased on EMD-coated titanium compared with titanium alone. Moreover, there was a 4 fold increase in levels of mRNA encoding bone sialoprotein and osteocalcin in osteoblasts cultured on EMD-coated titanium surfaces compared with uncoated surfaces. We conclude that coating of titanium with EMD enhances the proliferation and differentiation of osteoblasts irrespective of the titanium substratum topography. Therefore, EMD may increase the speed and quality of osseointegration around bone contacting implants *in vivo*.

Keywords: Enamel matrix derivative (EMD), Emdogain, titanium implants, surface topography, surface chemistry, osseointegration.

2.2 Introduction:

Osseointegration is the direct structural and functional connection that exists between living bone and the surface of a material such as Titanium (Ti) (Branemark et al. 1969). Defined as a 3 step process, it involves recruitment of osteogenic cells to the surface of implant, followed by *de novo* bone formation and finally bone remodeling (Davies 1998). To facilitate osseointegration, devices such as Ti dental implants are most commonly loaded in a 2-step process (Esposito et al. 2007), with placement of the intraosseous component into the jawbone for a period of 6 to 8 weeks without loading (Esposito et al. 2007). Only thereafter is the transmucosal section and abutment placed. It would be of great clinical significance if the 6-8 week period required for implant integration could be reduced; a time in which the patient has no use of the implant and occlusal forces are lost which is detrimental for opposing teeth (McNeill 2000).

Ti and its alloys are the preferred dental implant material as they are highly resistant to corrosion and promote bone apposition directly on the surface of the implant (Branemark et al. 1969). Attempts to further improve the osseointegration of Ti and its alloys have primarily focused on altering the surface topography of the implant to enhance osteoblast adhesion, proliferation and mineralization (Davies 1998, Schwartz et al. 2005, Le Guehennec et al. 2007). Rough surfaces (produced by processes such as micro-machining, plasma spraying, particle-blasting and acid etching) in particular have been a successful strategy for osseointegration as osteoblasts respond favourably to such features (Le Guehennec et al. 2007), a phenomena termed rugophilia (Rich et al. 1981). SLA (**S**and-blasted, **L**arge grit, **A**cid-etched) surfaces currently employed on Institut Straumann AG implants have been shown to improve osteoblast adhesion, migration,

signaling, proliferation and differentiation *in vitro* and *in vivo* (Boyan et al. 2002, Wieland et al. 2005, Franchi et al. 2007). Despite the relative success of SLA dental implants, osseointegration remains a clinical problem in the maxilla where insufficient bone mass is common (Clayman 2006), as well as in patients with systemic conditions such as diabetes (Fiorellini & Nevins 2000) and metabolic bone diseases (Beikler & Flemmig 2003).

The addition of tissue-specific proteins to the surface of biomaterials can strongly enhance the desired cell response (Salgado et al. 2004, Du et al. 2005, Ku et al. 2005). Calcium phosphate (Schliephake et al. 2006), collagen (Hilbig et al. 2007) and bone morphogenetic proteins (Wikesjo et al. 2008) have enhanced osteoblast differentiation on Ti *in vitro* and *in vivo* when compared to Ti alone. Furthermore, peptide sequences known to influence cell attachment, such as RGD or FHRIKKA, have been shown to directly influence osteoblast attachment and spreading on Ti surfaces *in vitro* (Schuler et al. 2006, Schuler et al. 2009). However, many of these approaches to changing surface chemistry are not yet utilized in the clinical setting primarily due to questions over *in vivo* stability and resistance to wear (Schuler et al. 2006).

An alternate approach to chemically modifying Ti surfaces is to coat the Ti directly from an applicable protein solution. Of particular relevance in dentistry is the commercially available product Emdogain®, which has been extensively studied for regeneration of the periodontal ligament and gingival tissues (Sculean et al. 2006). Emdogain® is an enamel matrix protein derivative (EMD) extracted from developing porcine teeth, the major component of which are amelogenins, a family of hydrophobic proteins that account for more than 90% of the total protein content (Gestrelius et al.

1997). The remaining components of EMD include enamelins, such as proline-rich enamelin, sheathelin and tuftelin (Carinci et al. 2006).

The direct effects of EMD on bone regeneration have primarily been limited to intrabony defects in the periodontium (Sculean et al. 1999). *In vivo* treatment of perforated rat femurs with EMD significantly increased newly formed bone in 7 days when compared to untreated perforated femurs (Kawana et al. 2001). *In vitro* studies with human, rat and mouse osteoblasts showed increased proliferation and/or differentiation in the presence of EMD (Schwartz et al. 2000, Jiang et al. 2001, Hagewald et al. 2004, Schwarz et al. 2004, Carinci et al. 2006). However, *in vitro* studies to date have tested the efficacy of EMD by adding it to cell culture media; a situation that does not closely mimic the dental implant setting. One previous study has shown that osteosarcoma cells increase proliferation on Ti coated with EMD, but this study was very limited in scope and did not assess differentiation (Schwarz et al. 2004). We hypothesize that pre-coating Ti with varying topographies with EMD will result in attachment of proteins to the surface that will directly influence primary osteoblast attachment, subsequent spreading, proliferation and differentiation.

2.3 Methods:

2.3.1 Titanium Surfaces and EMD Coating:

Smooth pickled Ti (PT) and roughened SLA topographies were fabricated at Institut Straumann A.G. Briefly, 15mm discs were punched from grade 2 unalloyed Ti sheets. PT surfaces were prepared using dilute nitric acid to clean the surface, followed by washing in reverse osmosis purified water. SLA surfaces were prepared by blasting the Ti with corundum particles, followed by etching with HCl/H_2SO_4. PT and SLA topographies are shown in fig. 1A.

EMD was prepared according to Institut Straumann AG standard operating protocols. 30 mg 1 vial of EMD was dissolved in 3 ml of 4°C sterile 0.1% acetic acid. For experiments, stock EMD was diluted 100X in 0.1 M carbonate buffer at 4°C giving a working solution of 100 μg/ml. 1ml of EMD solution was poured onto each 15 mm Ti disc in 24 well culture dishes and incubated overnight at 4°C. Following incubation, Ti structures were rinsed with 1ml phosphate buffered saline (PBS) twice at 4°C. To confirm attachment of EMD proteins to PT and SLA surfaces after washing, protein was extracted from the surfaces using extract protein 150 μl of RIPA buffer (Sigma-Aldrich, Oakville, ON, CAN). Protein levels were quantified using the BCA protein assay kit (Pierce, Rockford, Il), and 5 mg of total protein was mixed in a 1:2 ratio with Laemelli buffer and 2-mercaptoethanol and boiled for 5 min to denature. Protein samples were run on a 15% polyacrylamide gel using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), with 5 μg of EMD used as a control. Protein was visualized as previously described (Goldberg & Warner 1997) by staining the gel with the cationic dye Stains-all (ICN Biochemicals, Cleveland, OH) for 2 h, followed by silver

nitrate staining for 30 min. A representative gel is shown in fig 1B.

2.3.2 Osteoblast Isolation and Differentiation:

Rat calvarial osteoblasts (RCOs) were obtained from newborn rat calvariae and cultured as previously described (Hamilton et al. 2006). Frontal, parietal and occipital bones were dissected and rinsed in α-MEM (Gibco, Grand Island, NY). Minced tissue was digested twice for 15 min each in a mixture of collagenase/trypsin (3:1; both purchased from Sigma-Aldrich) and the second digestion plated in tissue culture flasks using α-MEM supplemented with antibiotics (100 μg/ml penicillin G, Sigma-Aldrich; 50 μg/ml gentamicin, Sigma-Aldrich; 3 mg/ml amphotericin B, Gibco, Grand Island, NY and 15% fetal bovine serum, Cansera, Rexdale, ON). Osteoblasts were removed from the tissue culture plastic using a trypsin solution [0.25% trypsin (Gibco), 0.1% glucose, citrate-saline buffer (pH 7.8)]. At seeding on Ti surfaces, α-MEM medium was supplemented with 50 μg/ml ascorbic acid and 2 mM β-glycerophosphate to promote cell differentiation. For experiments lasting up to 28 days, medium was replaced twice weekly.

2.3.3 Immunofluorescence:

Osteogenic cells were plated at a density of 10,000 cells per structure in a 24 well plate. At 30 min, 2 and 4 h, cells were fixed in 4% buffered formalin, followed by three 5 min washes in PBS. Samples were then stained with phalloidin-FITC (Sigma-Aldrich), and phosphotyrosine (pTyr PY99) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, followed by a goat anti-mouse IgG conjugated to Texas Red at a dilution of 1:200 in

0.5% PBS/Bovine Serum Albumin (BSA) (Molecular Probes Inc./Invitrogen). The dilution of each antibody was titrated to determine the optimal concentration. Images were captured from each surface on an Axiophot microscope (Zeiss) using a Fast1394 digital camera (QICAM) and Northern Eclipse software (EMPIX). The planar area of the cells was measured using Northern Eclipse Software. A minimum of fifteen cells from each treatment group were counted, with 3 replicates per experiment, and three independent experiments performed.

2.3.4 Adhesion and Proliferation Assays:

Cells were seeded on structures in 24 well plates at a density of 5,000 cells per well. Cell number was measured by staining cells with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) at 2, 4 and 8 h for cell adhesion and 1, 3, 5 and 7 days for cell proliferation assays. At each time point, Ti structures were placed into 4% paraformaldehyde for 5 min then stained with DAPI and images captured on an Axiophot fluorescence microscope. Ten fields of view were captured per sample and nuclei were counted using Northern Eclipse software (Empix Imaging Inc, Mississauga, ON). Three independent experiments were performed each with 3 replicates for each condition. Data were analyzed for statistical significance using 2-way analysis of variance (ANOVA) with Bonferroni test.

2.3.5 Alkaline Phosphatase Activity:

Alkaline Phosphatase activity was monitored using a fast violet B salt kit (procedure No. 85, Sigma Aldrich). Briefly, 1 fast violet B salt capsule was dissolved in 48ml of distilled water and 2 ml of naphtol AS-MX phosphate alkaline solution. Osteoblasts were fixed by immersing in a citrate-buffered acetone solution (2 parts citrate, 3 parts acetone) for 30 s and rinsed in deionized water for 45 s. Surfaces were then placed in AP stain for 30 min and protected from light. Following 2 min of rinsing in deionized water, slides were treated with Mayer's hematoxylin solution for 10 min. All images were captured using pre-determined light intensity at the same magnification. Image Pro Plus thresholding software was used to generate percent stained values for each field of view. Data from 3 independent experiments were analyzed for statistical significance using 2-way ANOVA with Bonferroni test.

2.3.6 Real Time RT-PCR analysis:

Total RNA was isolated using TRIZOL reagent and RNAeasy Mini kit (QIAGEN) at time points 1, 7, 14, 21 and 28 days. Primer and probe sequences for genes encoding Runx2, bone sialoprotein (BSP) and osteocalcin (OC) were designed from PrimerDesigner software as previously described (Panupinthu et al. 2008). Following TRIZOL extraction, real-time RT-PCR was performed using 15µl final reaction volume of TaqMan's One step Master Mix kit (Applied Biosystems). 40ng of total RNA was used per sample well. Each sample contained pooled mRNA from TRIZOL extractions collected from 3 Ti surfaces. All samples were assayed in triplicate and 3 independent experiments were performed. The ??Ct method was used to calculate gene expression

levels relative to GAPDH and normalized to control cells (PT with no EMD). Data were log-transformed prior to analysis by one-way ANOVA with Bonferroni test, using Graphpad Software v. 4 (Graphpad Software, La Jolla, CA).

2.4 Results:

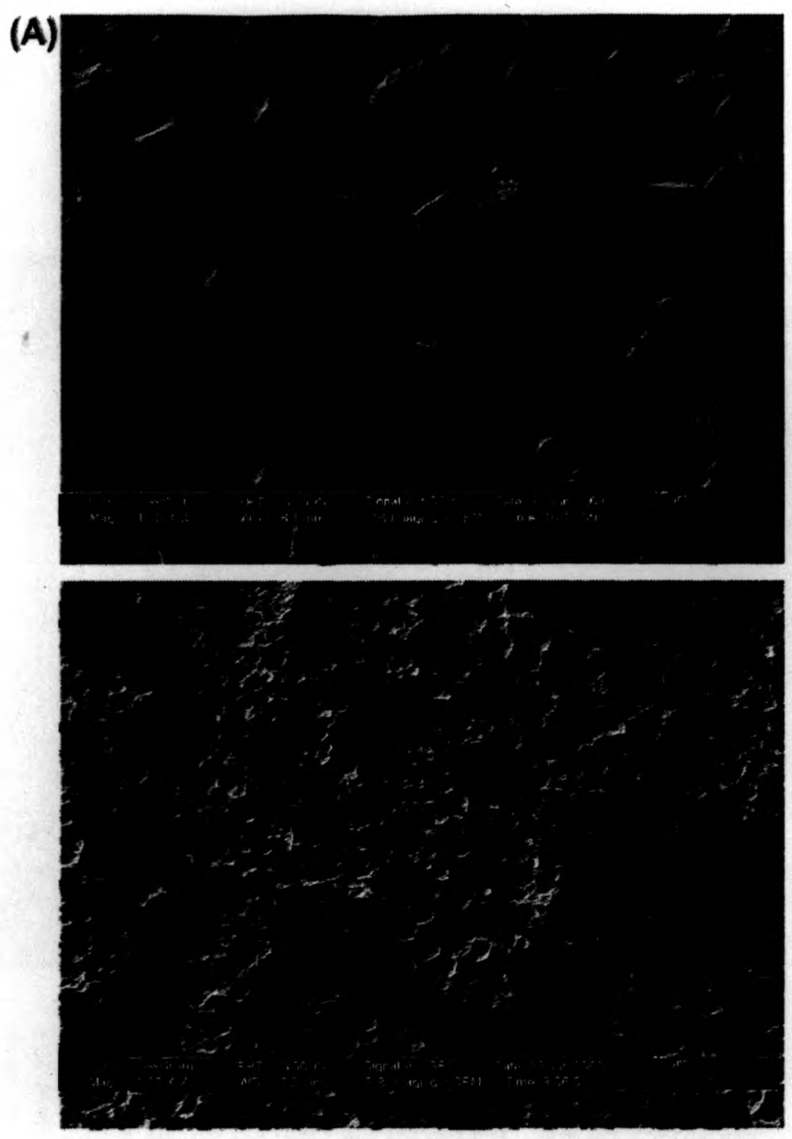
2.4.1 Osteoblast Attachment and Morphology:

RCO attachment was similar on both PT and SLA, with or without EMD (Fig. 2). Thirty minutes post seeding, osteoblasts were attached on all surfaces, but minimal spreading was observed (Fig. 3A). After 2 h, RCOs began to spread on all surfaces with focal adhesions visible on all surfaces, with the most prominent adhesions observed on EMD-treated PT and SLA. At 4 h, osteoblasts seeded on PT surfaces appear well spread and intense phosphotyrosine staining was evident at focal adhesions sites. Osteoblasts grown on SLA surfaces formed numerous microspikes, but showed less intense phosphotyrosine staining at focal adhesions (Fig. 3A). EMD significantly increased spreading of RCOs at both 2 and 4 h on PT (Fig. 3B). In contrast, the planar area of RCOs on SLA surfaces precoated with EMD were not significantly different than uncoated SLA surfaces. There was a significant difference in planar area of RCOs seeded on control and EMD-PT surfaces when compared to SLA surfaces with or without EMD.

2.4.2 Osteoblast Cell Number:

EMD significantly enhanced cell numbers on PT and SLA surfaces at 5 and 7 days post seeding ($p<0.05$) (Fig. 4). Osteoblast number was greater on control PT surfaces than on control SLA surfaces at 3, 5 and 7 days (Fig. 4). Interestingly, at 24 h post-seeding, RCOs began to aggregate on both PT and SLA surfaces pre-coated with EMD (Fig. 5B, 5D) when compared to Ti alone (Fig. 5A, 5C). Early cell clustering may be indicative of early bone nodule formation and osteoblast maturation.

Figure 1: SEM images of the topographies employed in this investigation, pickled and SLA Ti (A). To assess attachment of EMD to each type of topography, protein analysis was performed as described in section 2.1. In (B), a representative gel can be seen showing that EMD binds to both PT and SLA surfaces. Band at 20 kDa is amelogenin, the major component of EMD.


(A)

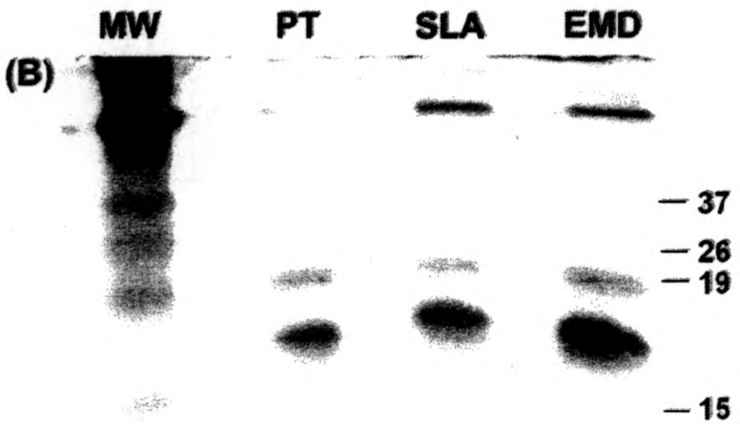

MW
PT
SLA
EMD
(B)
37
26
19
15

Figure 2: EMD does not significantly influence RCO adhesion on PT or SLA titanium surfaces. DAPI staining of cells on A) PT control, B) PT EMD, C) SLA control, and D) SLA+EMD at 4 hours post seeding. No significant differences were observed for RCO attachment levels between EMD-treated surfaces versus control surfaces at all time points (E). Data is shown as mean ± SE. n = 4 samples from 2 independent experiments with 10 fields of view analyzed per sample.

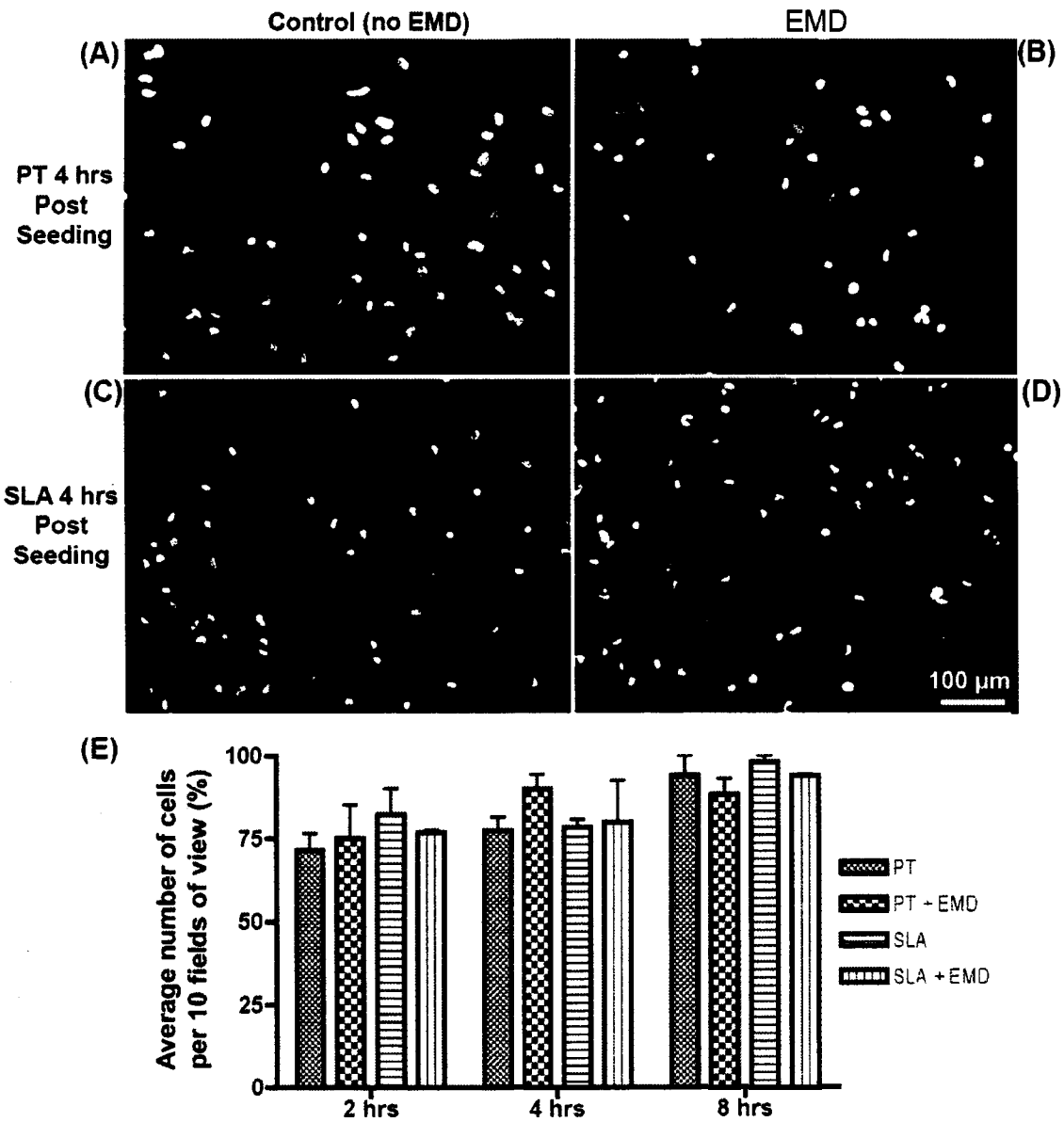
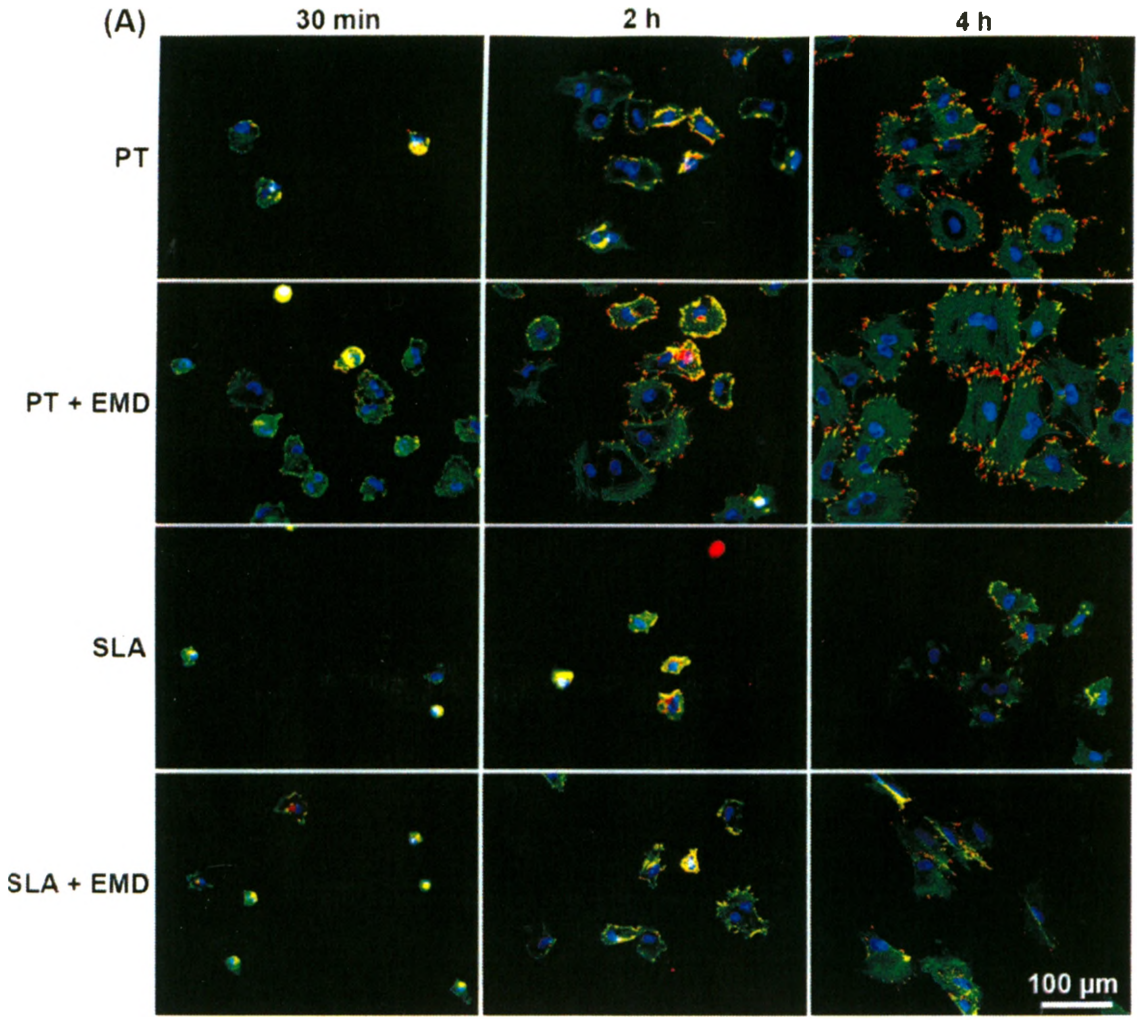

Control (no EMD)
EMD
(A)
(B)
PT 4 hrs Post Seeding
(C)
(D)
SLA 4 hrs Post Seeding
100 µm
(E)
Average number of cells per 10 fields of view (%)
100
75
50
25
0
2 hrs
4 hrs
8 hrs
PT
PT + EMD
SLA
SLA + EMD

Figure 3: EMD promotes initial cell spreading of RCOs on both PT and SLA titanium surfaces. At time points 30 min, 2 h and 4 h, cells were stained for phosphotyrosine PY99 (red), F-actin (green) and nuclei (blue). RCOs attached to PT surfaces were well-spread and radial in shape by 2 hours and continued to spread at 4 hours. On SLA surfaces, RCOs formed microspikes and were more polygonal in shape. B) EMD significantly enhances spreading of osteoblasts seeded on PT surfaces at 2 and 4 hours. Data presented is the average of 5 fields of view from 9 samples from independent experiments ± SE. Treatment groups were compared using two-way ANOVA with post-hoc testing performed with Bonferroni ($p<0.05$). * denotes significant difference between EMD treated surface and respective control surfaces and # denotes differences between PT and SLA.


(A)
30 min
2 h
4 h
PT
PT + EMD
SLA
SLA + EMD
100 µm

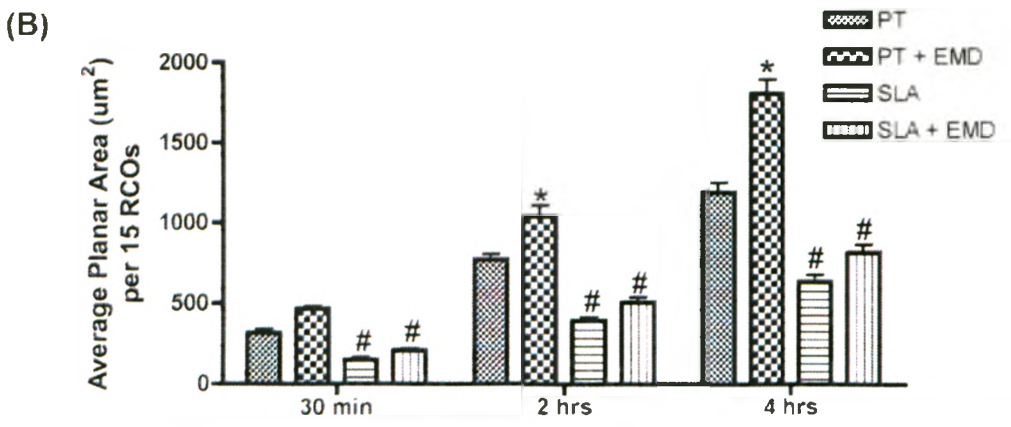

(B)
PT
PT + EMD
SLA
SLA + EMD
Average Planar Area (um²) per 15 RCOs
2000
1500
1000
500
0
30 min
2 hrs
4 hrs
*
#

Figure 4: EMD significantly increased cell number of RCOs on PT and SLA surfaces. At 5 days post seeding, RCOs were stained with DAPI: A) PT control, B) PT+EMD, C) SLA control, and D) SLA+EMD. E) EMD significantly increased cell numbers on PT at 5 days and SLA surfaces at both 5 and 7 days post seeding. In addition, RCOs seeded on control PT surfaces had increased cell numbers when compared to control SLA surfaces at 3, 5 and 7 days post seeding. Data presented is the average of 10 fields of view from 9 surfaces from 3 independent experiments ± SE. Treatment groups were compared using two-way ANOVA with post-hoc testing performed with Bonferroni ($p<0.05$). * denotes significant difference between EMD treated surface and respective control surfaces, # denotes differences between PT and SLA surfaces, Data ± SE).

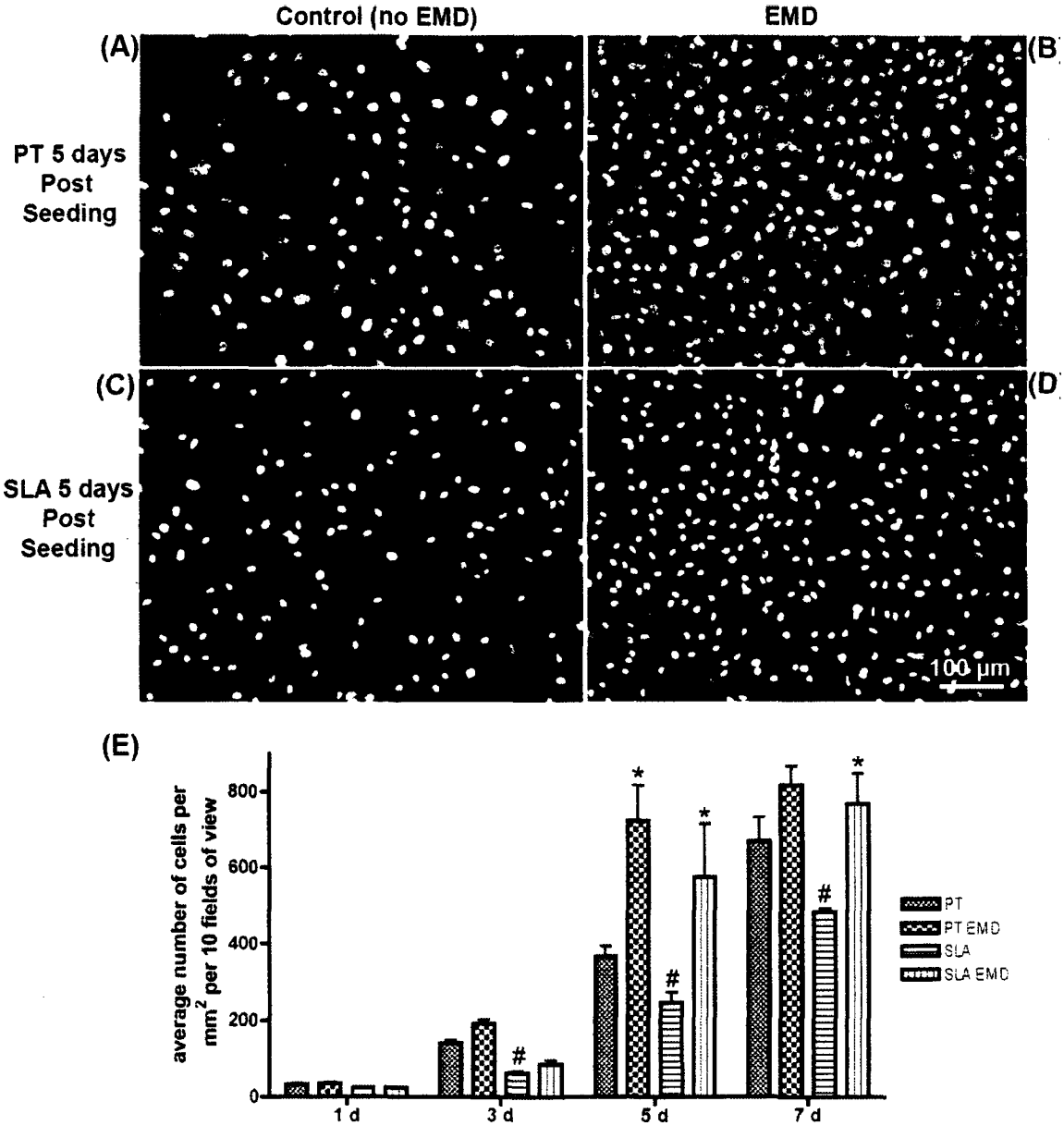
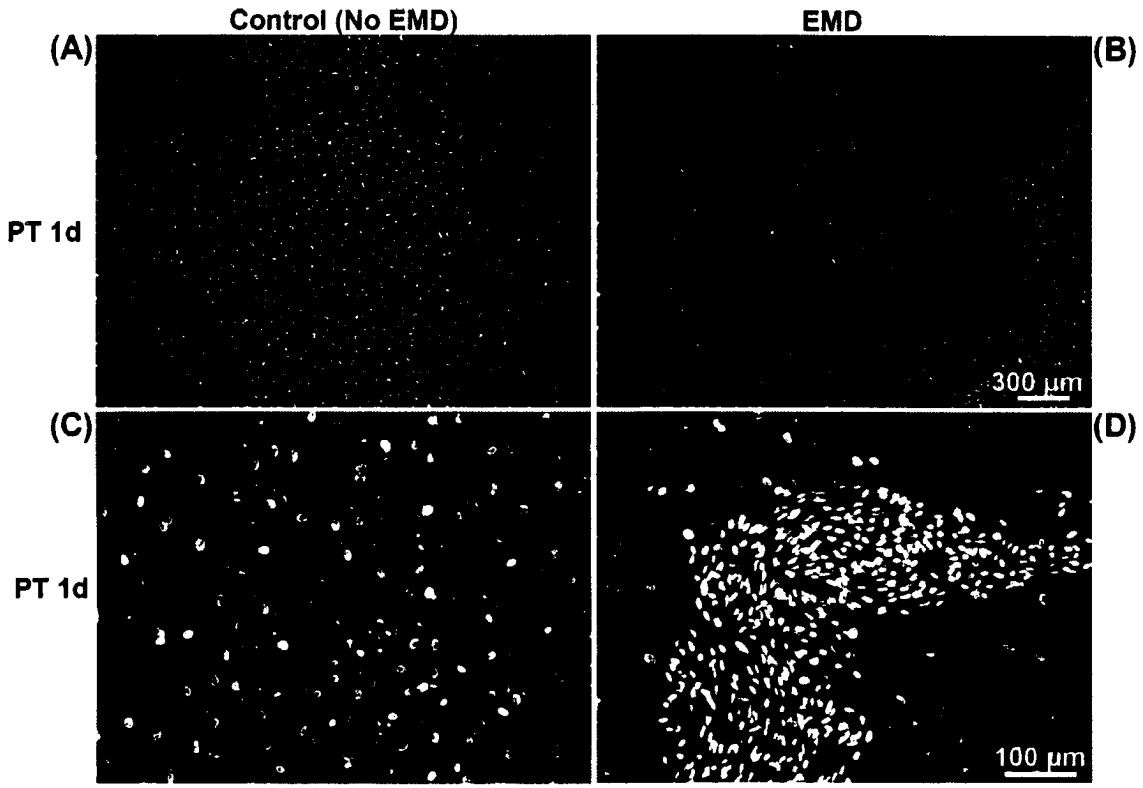

Control (no EMD)
EMD
(A)
(B)
PT 5 days Post Seeding
(C)
(D)
SLA 5 days Post Seeding
100 µm
(E)
average number of cells per mm² per 10 fields of view
0
200
400
600
800
1 d
3 d
5 d
7 d
PT
PT EMD
SLA
SLA EMD

Figure 5: EMD promotes clustering RCOs on PT surfaces. ON PT control surfaces, cells were evenly distributed as shown with DAPI staining (A, C), but on PT+EMD cell clumping was apparent after 1 day (B, D).


Control (No EMD)
EMD
(A)
(B)
PT 1d
300 μm
(C)
(D)
PT 1d
100 μm

2.4.3 Alkaline Phosphatase Activity:

RCOs seeded on EMD-coated surfaces showed significantly more alkaline phosphatase activity at all times points when compared to uncoated PT and SLA surfaces (Fig. 6). After a period of 1 week, alkaline phosphatase activity was 3 fold greater on PT surfaces and 2 fold on SLA surfaces compared to Ti alone. Significantly greater alkaline phosphatase activity was also observed on control SLA surfaces versus control PT surfaces at both 2 and 3 weeks (Fig. 6E).

2.4.4 Quantification of RCO Differentiation:

2.4.4.1 mRNA analysis:

RCOs were assessed for Runx2, osteocalcin (OC) and bone sialoprotein (BSP) gene expression at time points ranging from 1 day to 4 weeks (Fig. 7). Analysis of Runx2 gene expression showed no significant increase in mRNA levels at any time point on any surface, with or without EMD (Fig. 7A), although a time-dependent decrease in Runx2 mRNA levels was evident on all surfaces at 1 week. OC and BSP mRNA levels showed significant increases on EMD-coated surfaces at 1, 2, 3 and 4 weeks post seeding when compared to uncoated surfaces (Fig. 7B, 7C). Uncoated SLA surfaces showed significantly greater osteocalcin mRNA levels at both 3 and 4 weeks than uncoated PT surfaces. Up to 4 fold increases in BSP mRNA levels were observed on EMD surfaces at 1 to 4 weeks post seeding on both PT and SLA surfaces when compared to uncoated surfaces (Fig. 7C). Uncoated SLA surfaces also showed significant increases in BSP expression at 3 weeks when compared to uncoated PT surfaces (Fig. 7C).

Figure 6: EMD significantly increased alkaline phosphatase activity of RCOs on both PT and SLA surfaces. At time points 1, 2, 3 and 4 weeks, RCOs were fixed and stained for alkaline phosphatase: A) PT control at 1 week b) PT+EMD at 1 week c) PT no EMD at 3 weeks d) PT+EMD at 3 weeks. 10 fields of view per sample were captured and percentage area of staining calculated ± SE (results from 3 independent experiments). 3 fold increases in alkaline phosphatase activity were observed on all EMD treated surfaces (B). Treatment groups were compared using two-way ANOVA with post-hoc testing performed with Bonferroni ($p<0.05$). * denotes significant difference between EMD treated surface and respective control surface, # denotes differences between PT and SLA surfaces.

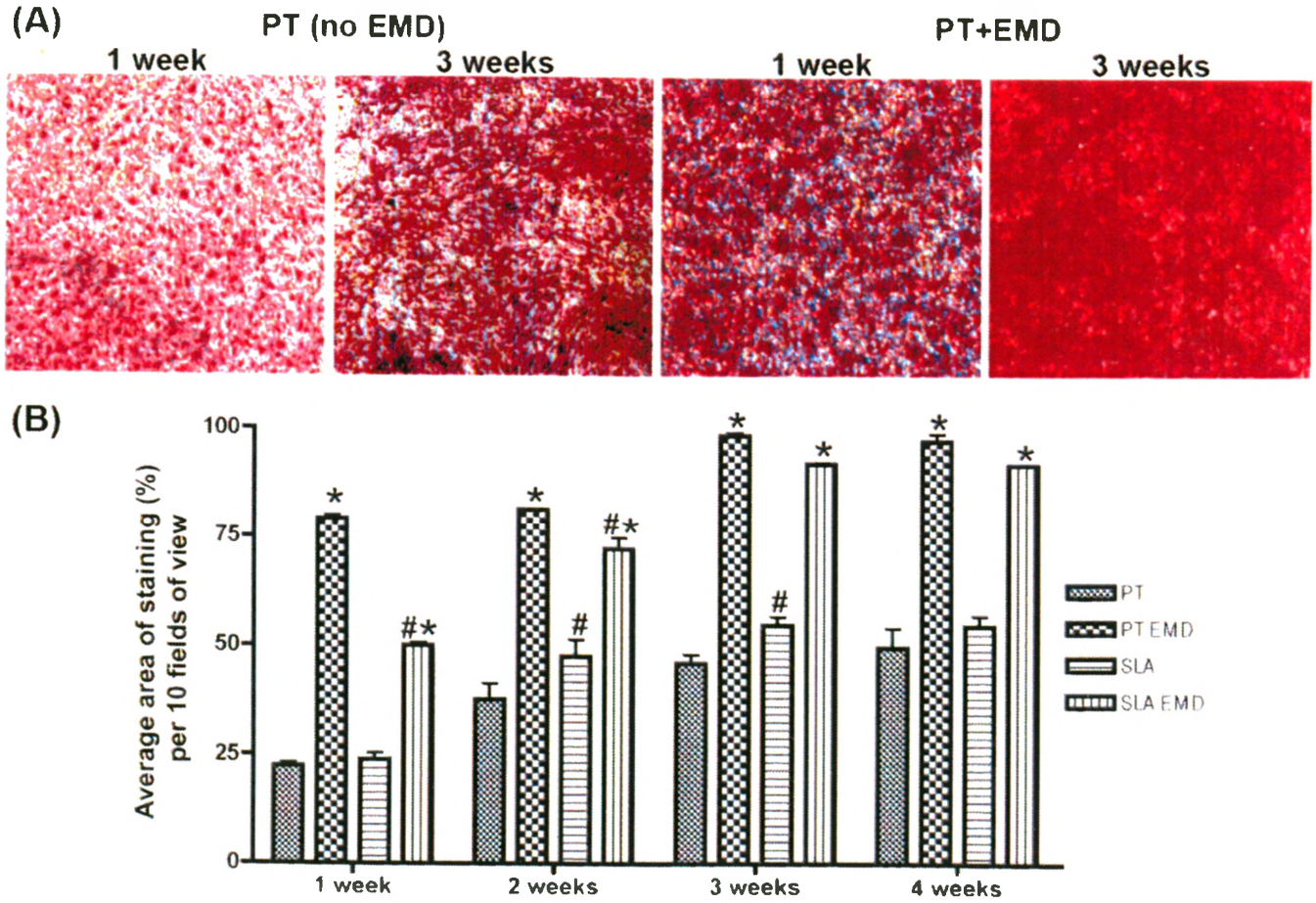
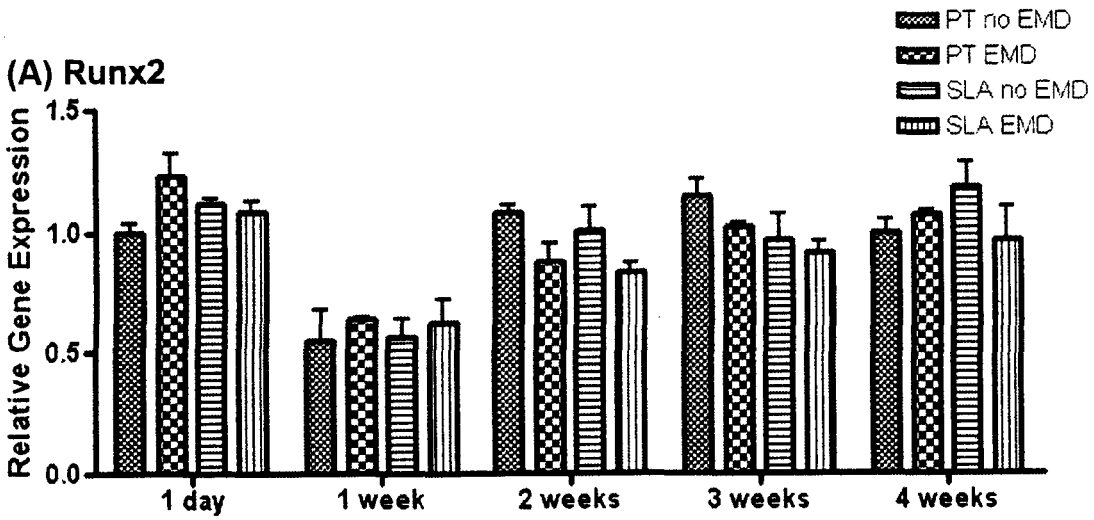
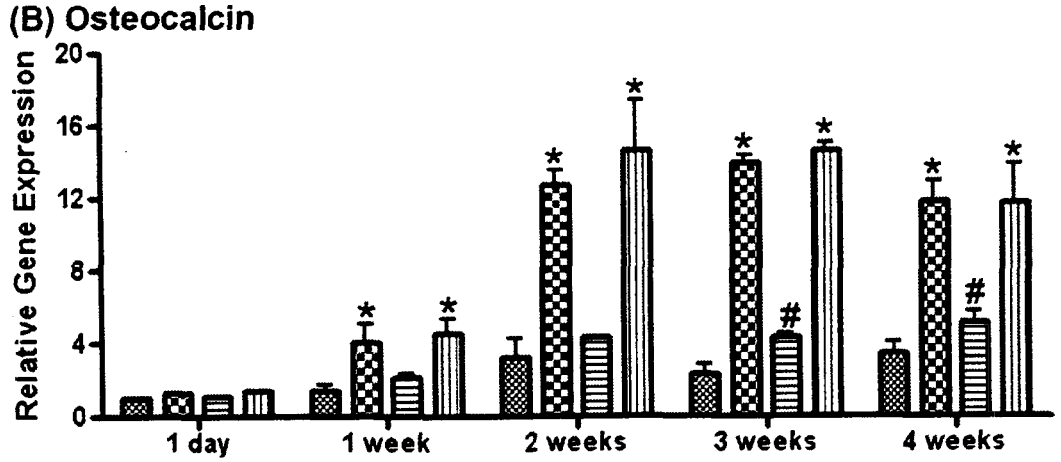
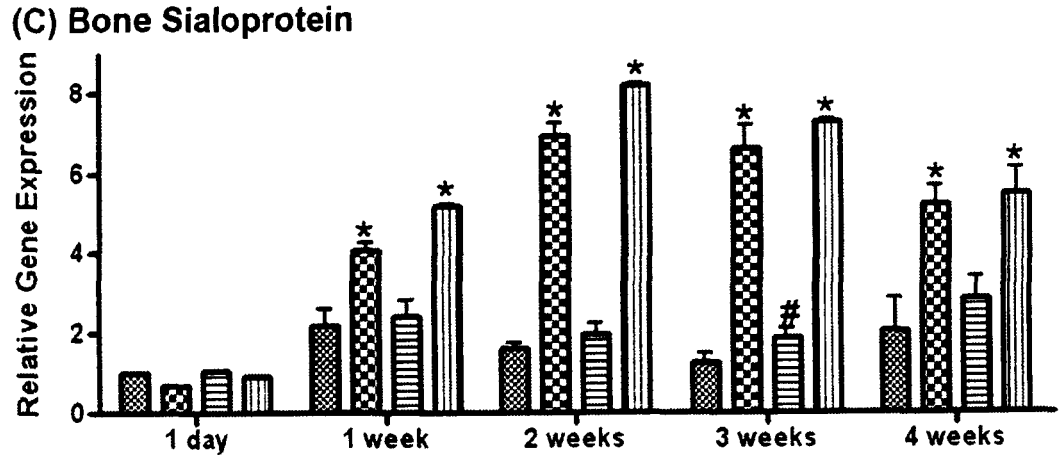

(A)
PT (no EMD)
PT+EMD
1 week
3 weeks
1 week
3 weeks
(B)
Average area of staining (%) per 10 fields of view
100
75
50
25
0
1 week
2 weeks
3 weeks
4 weeks
PT
PT EMD
SLA
SLA EMD

Figure 7: EMD increases mRNA levels of osteoblast differentiation markers. After 1 day, and 1, 2, 3 and 4 weeks post seeding, mRNA was extracted and realtime PCR performed using specific primers for Runx2, osteocalcin and bone sialoprotein. A) Levels of Runx2 were not significantly altered between EMD coated and control surfaces. When surfaces were pre-coated with EMD, up to 4 fold increases in gene expression were observed for B) osteocalcin and C) bone sialoprotein at time points on both PT and SLA surfaces ($p<0.05$). * denotes significant difference between EMD treated surface and respective control surface, # denotes differences between PT and SLA surfaces. All experiments were normalized to levels of GAPDH followed by normalization to mRNA levels at day 1 from PT control surfaces. Data shown is the average value from 3 independent experiments (3 replicates per experiment) ± SE.


(A) Runx2
PT no EMD
PT EMD
SLA no EMD
SLA EMD
Relative Gene Expression
1.5
1.0
0.5
0.0
1 day
1 week
2 weeks
3 weeks
4 weeks


(B) Osteocalcin
Relative Gene Expression
20
16
12
8
4
0
*
#
1 day
1 week
2 weeks
3 weeks
4 weeks


(C) Bone Sialoprotein
Relative Gene Expression
8
6
4
2
0
*
#
1 day
1 week
2 weeks
3 weeks
4 weeks

2.4.4.2 Runx2 Immunocytochemistry:

At 2 weeks, Runx2 was found to localize to the nucleus in RCOs plated on uncoated PT and SLA surfaces (Fig. 8). On EMD treated PT and SLA, Runx2 was found primarily in the cytoplasm at 2 weeks, with low levels in the nucleus. At 4 weeks, Runx2 was found outside the nucleus in RCOs plated on all surfaces independent of treatment with EMD.

2.4.4.3 Osteocalcin:

At 2 weeks, uncoated PT surfaces showed high levels of osteocalcin staining in the cytoplasm of the cell (Fig. 9). On SLA control surfaces, osteocalcin was deposited outside the cell in the extracellular matrix. When surfaces were precoated with EMD, osteocalcin was greater both in the cytoplasm and extracellular space on both PT and SLA surfaces. At 4 weeks, osteocalcin was observed in the extracellular matrix on all surfaces with greater amounts observed on EMD-treated surfaces. SLA surfaces showed patterns of extracellular deposition that were evenly dispersed across the entire surface when compared to control PT surfaces, where staining localized to more discrete nodules.

Figure 8: EMD influences localization of transcription factor Runx2. At 2 and 4 weeks, RCOs were labeled with specific antibodies to Runx2. At 2 weeks, Runx2 was found to localize to the nucleus on both PT and SLA control surfaces (white arrows). On EMD treated surfaces at 2 weeks, Runx2 localized to the cytoplasm with little staining observed in the nucleus. At 4 weeks, Runx2 was found in the cytoplasm on all surfaces.

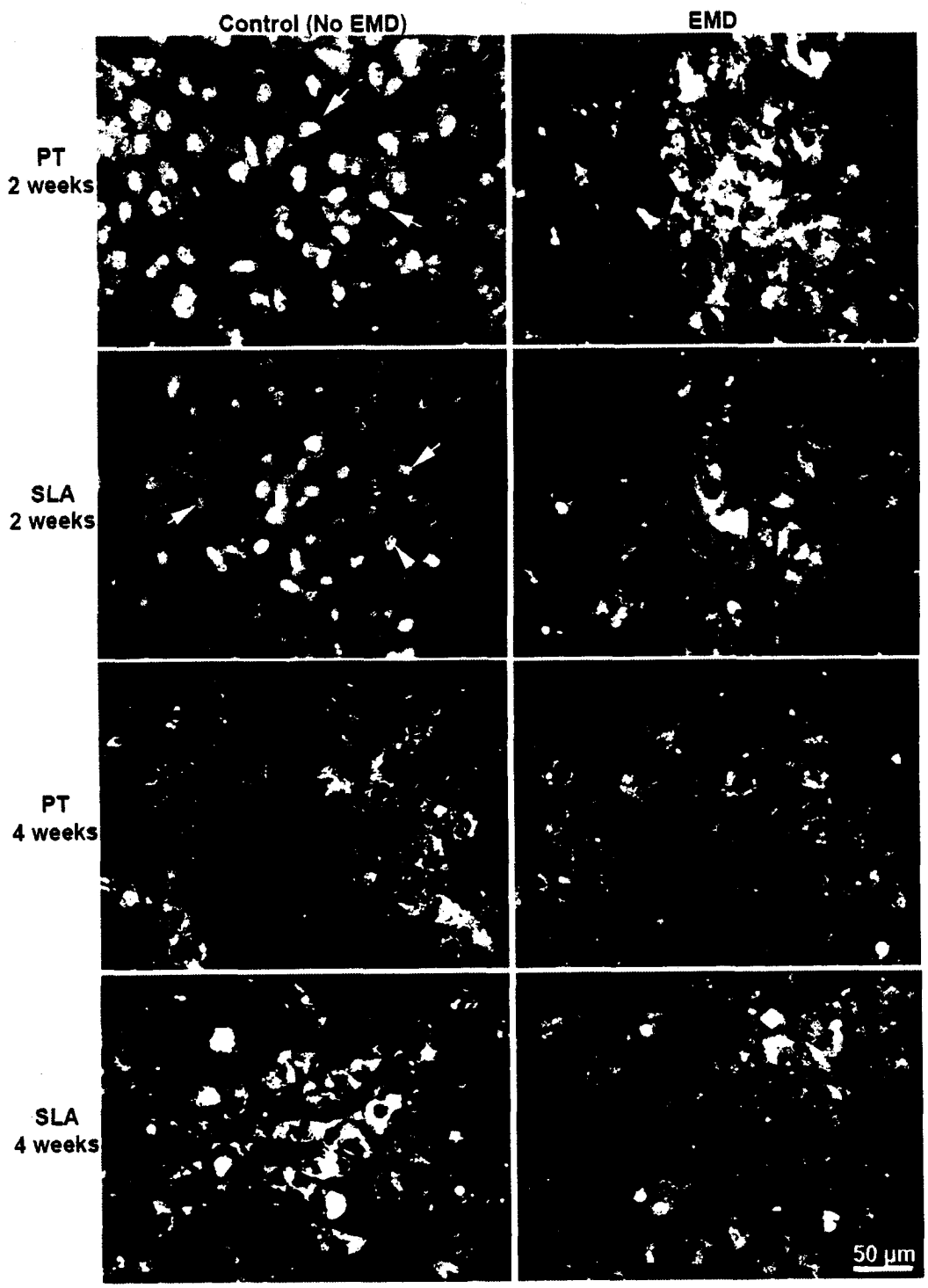
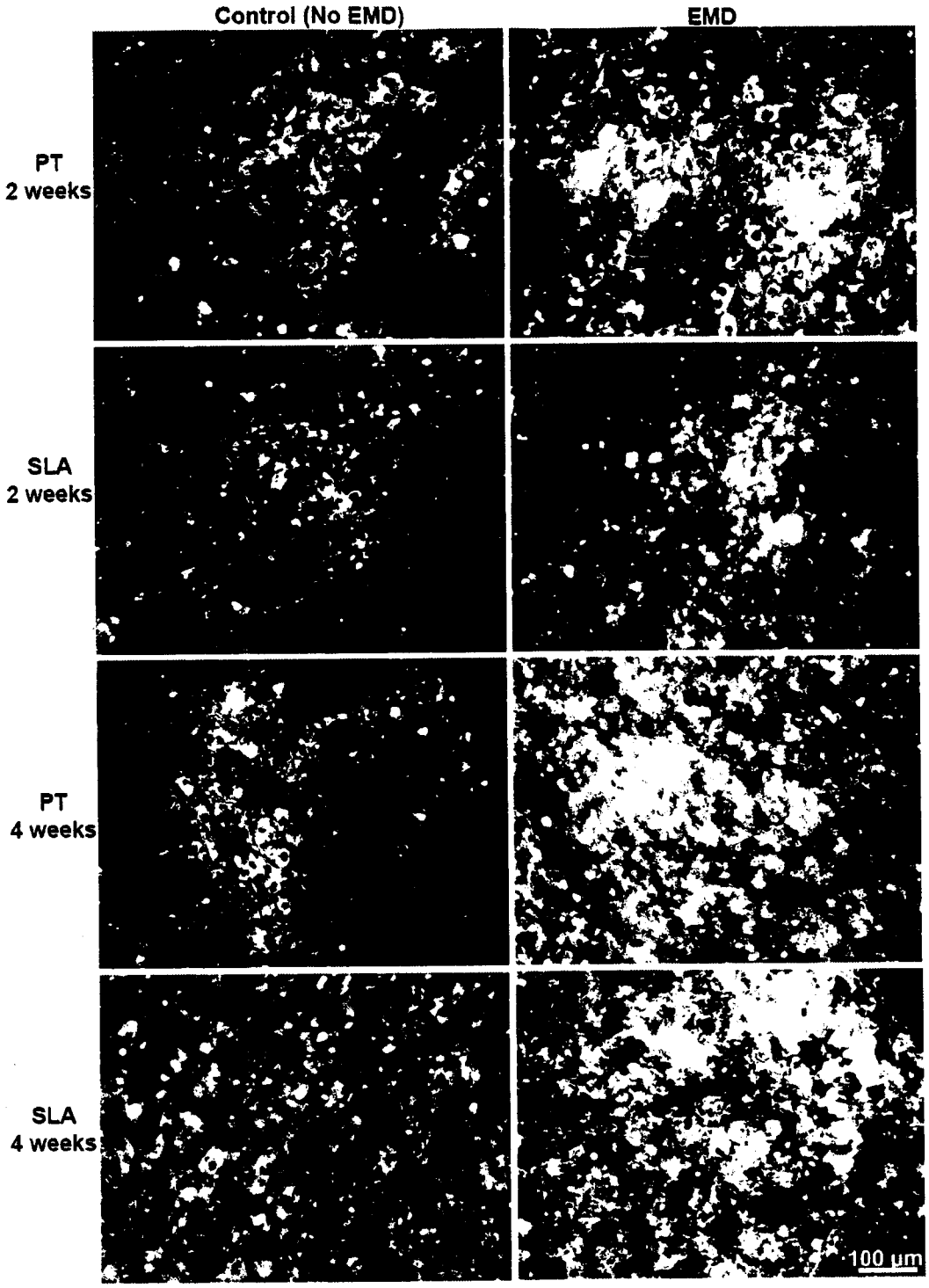

Control (No EMD)
EMD
PT
2 weeks
SLA
2 weeks
PT
4 weeks
SLA
4 weeks
50 µm

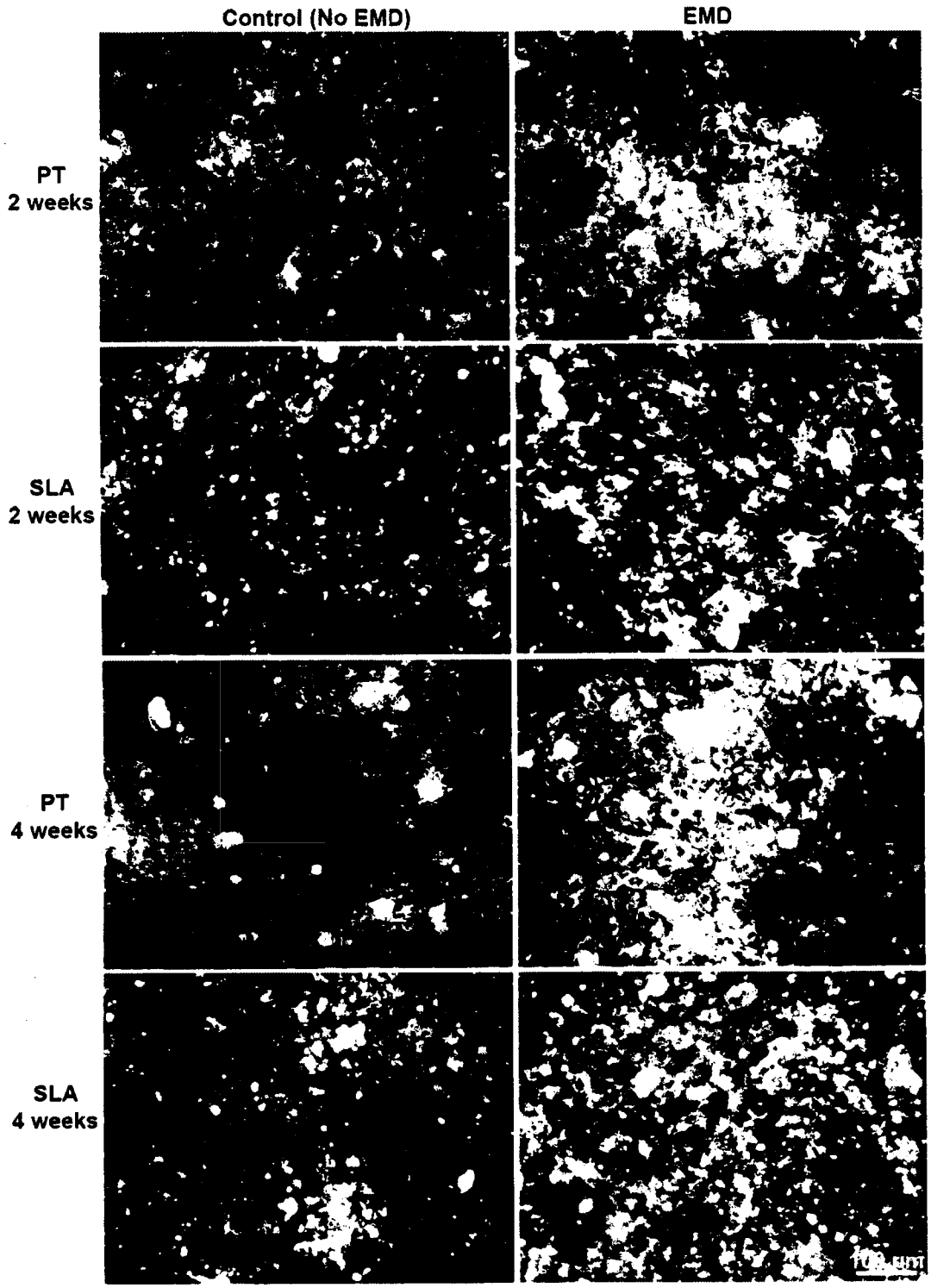
Figure 9: EMD increases extracellular matrix deposition of osteocalcin on PT and SLA surfaces. At 2 and 4 weeks, RCOs were labeled with specific antibodies to osteocalcin. RCOs seeded on EMD treated PT and SLA secreted higher levels of osteocalcin into the extracellular matrix when compared to control PT and SLA at both 2 and 4 weeks.


Control (No EMD)
EMD
PT
2 weeks
SLA
2 weeks
PT
4 weeks
SLA
4 weeks
100 µm

2.4.4.4 Alizarin Complexone:

Low levels of alizarin complexone staining were observed on uncoated PT surfaces at both 2 and 4 weeks (Fig. 10). The uncoated SLA surfaces showed patterns of alizarin complexone incorporation more localized then the PT control at both 2 and 4 weeks, with higher intensity observed at 4 weeks. The surfaces treated with EMD showed greater alizarin complexone incorporation on both PT and SLA surfaces versus control. Alizarin complexone labeled areas were much larger and more pronounced on PT EMD surfaces whereas the SLA EMD surfaces showed patterns that were dispersed more evenly across the entire surface.

Figure 10: EMD increases mineral deposition on both PT and SLA surfaces assessed through alizarin complexone (AC) incorporation. On PT and SLA at 2 weeks, AC labeling was low in intensity, but was increased by the presence of EMD. At 4 weeks, higher levels of labeling was evident on PT and SLA control surfaces (compared with 2 weeks), but higher AC incorporation was evident on surfaces pre-coated with EMD.


Control (No EMD)
EMD
PT
2 weeks
SLA
2 weeks
PT
4 weeks
SLA
4 weeks

2.5 Discussion:

In the past 15 years, many different types of topographical features have been added to dental implant surfaces with the aim of speeding the attachment of bone to the intraosseous component. Among these, it was shown that SLA surfaces enhanced bone apposition (Cochran et al. 2007) and increase torque removal values (Li et al. 2002), with a 99% success rate following 6 weeks of healing (Cochran et al. 2002, Bornstein et al. 2003). Although there is little dispute that SLA surfaces enhance osseointegration and primary stability, there is a wide variation of options with regard to surface chemistries and surface chemical treatments that, when combined with SLA, could speed or enhance bone formation (Schuler et al. 2006). To reduce healing times as well as increase the amount and quality of bone, research has now been focused on the role of growth factors, ECM proteins and cytokines in combination with substratum topographies in regulating osteoblast behaviour (Ratner 2001, Liu et al. 2006, Kim et al. 2008). In the present study, we assessed whether or not pre-coating PT and SLA Ti surfaces with EMD influenced osteoblast attachment, spreading, proliferation and differentiation.

No significant difference in attachment of RCOs was observed on surfaces pre-coated with EMD, although greater spreading was detected on PT EMD-coated surfaces, but not on EMD-coated SLA surfaces. This may be due to the inability of osteoblasts to spread on SLA compared to PT (Fillies et al. 2005, Wieland et al. 2005), as the roughness of SLA limits where the cells can form adhesions, a process that is unaffected by the presence of EMD. Previous studies have shown that osteoblasts cultured on SLA coated poly(L-lysine)-graft-poly(ethylene glycol)-RGD (arginine-glycine-aspartic acid) have a larger footprint and form more adhesions, but overall surface roughness was still the

determining factor dictating the level of cell spreading (Schuler et al. 2006). Britland et al. demonstrated using model topographies (microgrooves) that a transition point exists beyond which depth of topography is more important than surface chemistry for guiding cell response when the two are conflicting (Britland et al. 1996). As the roughness value (R_a) value of SLA surfaces is 4.33 (Wieland et al. 2005) and the transition depth identified by Britland is around 2 μm, it is likely that protein coatings will not have a large effect on cell spreading on rough topographies such as SLA. Furthermore, the major component in EMD, amelogenin, does not contain an RGD binding domain (Yamada et al. 1991). Although amelogenin has previously been reported to promote cell adhesion through an as yet unknown mechanism (Hoang et al. 2002), it is unlikely to over-ride the topographical features on SLA, but appears to enhance spreading on smoother PT.

Many investigators have demonstrated that EMD added to cell culture media increases osteoblast proliferation on tissue culture plastic (Schwart et al. 2000, Jiang et al. 2006). One previous study has assessed the influence of EMD and SLA on osteosarcoma cell behaviour (Schwarz et al. 2004), showing that proliferation was dependent on EMD concentration. In the present study, we observed that EMD increased osteoblast proliferation when pre-coated on Ti surfaces with the highest increase in cell number observed on EMD-coated PT surfaces, not SLA. An unexpected observation in our study was of osteoblasts forming clusters on surfaces treated with EMD after periods of 24 hours. Cluster formations were more evident on the PT surfaces, most likely due to osteoblasts migrating more on PT surfaces due to the smooth topography that does not constrain the cells migratory ability. However, the possibility also exists that EMD may contain chemokinetic proteins or may stimulate cells to produce chemotactic factors that

attract other cells. It has been previously hypothesized that EMD can stimulate angiogenesis, possibly through chemotaxis (Yuan et al. 2003). Interestingly, cell clustering has been previously reported on SLActive [hydrophilic (contact angle 0)] surfaces, with suggestions that these clusters may be indicative of early nodule formation and osteoblast maturation (Qu et al. 2007).

EMD has been previously shown to influence osteoblast differentiation, although using a wide range of cell models (MG-63, MC3T3-E1, Kusa/A 1, 2T9 cells) growing on standard tissue culture plastic (Yoneda et al. 2002, Hagelwald et al. 2004, He et al. 2004, Weishaupt et al. 2008). EMD has been shown to increase ALP activity (He et al. 2004, Reseland et al. 2006), mineral nodule formation (Wu et al. 2002) as well as markers for osteoblast differentiation such as BSP and OC (Weishaupt et al. 2008). In the present study, Runx2 (osteocalcin transcription factor) mRNA levels remained unchanged, but at the protein level, Runx2 nuclear localization was evident only at 2 weeks on uncoated Ti surfaces, with cytoplasmic localization evident in Ti-EMD surfaces. It was reported by Komori that Runx2 is necessary for early osteoblast differentiation but, at later time points, inhibits immature osteoblasts from differentiating into mature osteoblasts and osteocytes (Komori 2006). Therefore, it appears that EMD accelerates the differentiation process by promoting a mature phenotype at earlier time points than Ti alone does. This was confirmed at the mRNA and protein level as EMD precoated on PT and SLA significantly increased ALP activity as well as mRNA levels of BSP and OC. Using alizarin complexone incorporation and osteocalcin labeling with antibodies, we also demonstrated that these increased mRNA levels translated into increased mineral formation. Interestingly, EMD promoted osteoblast differentiation on both PT and SLA,

suggesting that EMD stimulates osteoblast differentiation irrespective of the underlying substratum topography. Therefore, although rough surfaces such as SLA promote osteoblast differentiation in comparison with smooth PT (Wieland et al. 2005), biochemical signaling appears to override topographical cues to speed osteoblast differentiation.

The results from this study demonstrate that EMD pre-coated onto Ti significantly increases osteoblast proliferation, differentiation and mineral formation. Two possibilities exist to explain how EMD regulates osteoblast behaviour. Firstly, it is possible that EMD pre-absorbed to Ti initiates intracellular signaling in osteoblasts that result in enhanced proliferation and differentiation at later time points. We have previously shown that topographies that promote osteoblast differentiation enhance early phosphorylation of intracellular signaling molecules including focal adhesion kinase, Src and extracellular signal-regulated kinase 1/2 (Hamilton & Brunette 2007). However, it is also possible that EMD persists on the Ti surface, directly mediating osteoblast behaviour over the entire period of the experiments. Interestingly, it has been shown that EMD adsorbs to hydroxyapatite, collagen and to denuded tooth roots, where it persists for up to 2 weeks post surgery, as an insoluble complex (Gestrelius et al. 1997). In similar experiments using immunohistochemical analysis, it was found that EMD was still present 4 weeks after application to extracted rat molars transplanted into the abdominal wall of the same animals (Hamamoto et al. 2002). The persistence of EMD on Ti would likely result in the continued presence of associated growth factors and cytokines. In this regard, EMD is thought to contain bone morphogenetic proteins (Takayama et al. 2005, Saito et al. 2008) and transforming growth factor β (Kawase et al. 2002). Indeed, recent research has

highlighted the bioactivity of different EMD fractions that were separated based on molecular weight (Johnson et al. 2009). Identifying the active proteins in each EMD fraction will further enhance our knowledge of the factors important in bone formation and remodeling.

2.6 Conclusions:

We have shown that EMD enhances osteoblast differentiation on Ti surfaces, in a topography-independent manner. Although the idea of pre-coating EMD onto implant surfaces is still in its infancy, the results from this study emphasize that it could prove to be an important tool for enhancing bone formation around dental implants. Furthermore, EMD coating represents a simplistic approach for "engineering" bone around metals such as Ti. As such, EMD could also be suitable for a wide range of other applications including repair of bone defects, as well as a coating for orthopedic implants, as EMD has the ability to control osteoblast proliferation and differentiation on Ti surfaces.

2.7 Acknowledgments:

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2.8 References:

Beikler T, Flemmig TF. Implants in the medically compromised patient. Crit Rev Oral Biol Med. 2003;14(4):305-16.

Bornstein MM, Lussi A, Schmid B, Belser UC, Buser D. Early loading of nonsubmerged titanium implants with a sandblasted and acid-etched (SLA) surface: 3-year results of a prospective study in partially edentulous patients. Int J Oral Maxillofac Implants. 2003 Sep-Oct;18(5):659-66.

Boyan BD, Bonewald LF, Paschalis EP, Lohmann CH, Rosser J, Cochran DL, et al. Osteoblast-mediated mineral deposition in culture is dependent on surface microtopography. Calcif Tissue Int. 2002 Dec;71(6):519-29.

Branemark PI, Adell R, Breine U, Hansson BO, Lindstrom J, Ohlsson A. Intra-osseous anchorage of dental prostheses. I. Experimental studies. Scand J Plast Reconstr Surg. 1969;3(2):81-100.

Britland S, Morgan H, Wojiak-Stodart B, Riehle M, Curtis A, Wilkinson C. Synergistic and hierarchical adhesive and topographic guidance of BHK cells. Exp Cell Res. 1996 Nov 1;228(2):313-25.

Carinci F, Piattelli A, Guida L, Perrotti V, Laino G, Oliva A, et al. Effects of Emdogain on osteoblast gene expression. Oral Dis. 2006 May;12(3):329-42.

Clayman L. Implant reconstruction of the bone-grafted maxilla: review of the literature and presentation of 8 cases. J Oral Maxillofac Surg. 2006 Apr;64(4):674-82.

Cochran DL, Buser D, ten Bruggenkate CM, Weingart D, Taylor TM, Bernard JP, et al. The use of reduced healing times on ITI implants with a sandblasted and acid-

etched (SLA) surface: early results from clinical trials on ITI SLA implants. Clin Oral Implants Res. 2002 Apr;13(2):144-53.

Cochran D, Oates T, Morton D, Jones A, Buser D, Peters F. Clinical field trial examining an implant with a sand-blasted, acid-etched surface. J Periodontol. 2007 Jun;78(6):974-82.

Davies JE. Mechanisms of endosseous integration. Int J Prosthodont. 1998 Sep-Oct;11(5):391-401.

Du C, Schneider GB, Zaharias R, Abbott C, Seabold D, Stanford C, et al. Apatite/amelogenin coating on titanium promotes osteogenic gene expression. J Dent Res. 2005 Nov;84(11):1070-4.

Esposito M, Grusovin MG, Willings M, Coulthard P, Worthington HV. The effectiveness of immediate, early, and conventional loading of dental implants: a Cochrane systematic review of randomized controlled clinical trials. Int J Oral Maxillofac Implants. 2007 Nov-Dec;22(6):893-904.

Fillies T, Wiesmann HP, Sommer D, Joos U, Meyer U. [Osteoblast reaction on SLA and microgrooved implant surfaces]. Mund Kiefer Gesichtschir. 2005 Jan;9(1):24-8.

Fiorellini JP, Nevins ML. Dental implant considerations in the diabetic patient. Periodontol 2000. 2000 Jun;23:73-7.

Franchi M, Bacchelli B, Giavaresi G, De Pasquale V, Martini D, Fini M, et al. Influence of different implant surfaces on peri-implant osteogenesis: histomorphometric analysis in sheep. J Periodontol. 2007 May;78(5):879-88.

Gestrelius S, Andersson C, Johansson AC, Persson E, Brodin A, Rydhag L, et al. Formulation of enamel matrix derivative for surface coating. Kinetics and cell colonization. J Clin Periodontol. 1997 Sep;24(9 Pt 2):678-84.

Goldberg HA, Warner KJ. The staining of acidic proteins on polyacrylamide gels: enhanced sensitivity and stability of "Stains-all" staining in combination with silver nitrate. Anal Biochem. 1997 Sep 5;251(2):227-33.

Hagewald S, Pischon N, Jawor P, Bernimoulin JP, Zimmermann B. Effects of enamel matrix derivative on proliferation and differentiation of primary osteoblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Aug;98(2):243-9.

Hamamoto Y, Kawasaki N, Jarnbring F, Hammarstrom L. Effects and distribution of the enamel matrix derivative Emdogain in the periodontal tissues of rat molars transplanted to the abdominal wall. Dent Traumatol. 2002 Feb;18(1):12-23.

Hamilton DW, Wong KS, Brunette DM. Microfabricated discontinuous-edge surface topographies influence osteoblast adhesion, migration, cytoskeletal organization, and proliferation and enhance matrix and mineral deposition in vitro. Calcif Tissue Int. 2006 May;78(5):314-25.

Hamilton DW, Brunette DM. The effect of substratum topography on osteoblast adhesion mediated signal transduction and phosphorylation. Biomaterials. 2007 Apr;28(10):1806-19.

He J, Jiang J, Safavi KE, Spangberg LS, Zhu Q. Emdogain promotes osteoblast proliferation and differentiation and stimulates osteoprotegerin expression. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004 Feb;97(2):239-45.

Hilbig H, Kirsten M, Rupietta R, Graf HL, Thalhammer S, Strasser S, et al. Implant surface coatings with bone sialoprotein, collagen, and fibronectin and their effects on cells derived from human maxillar bone. Eur J Med Res. 2007 Jan 31;12(1):6-12.

Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL. Amelogenin is a cell adhesion protein. J Dent Res. 2002 Jul;81(7):497-500.

Jiang J, Fouad AF, Safavi KE, Spangberg LS, Zhu Q. Effects of enamel matrix derivative on gene expression of primary osteoblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001 Jan;91(1):95-100.

Jiang J, Goodarzi G, He J, Li H, Safavi KE, Spangberg LS, et al. Emdogain-gel stimulates proliferation of odontoblasts and osteoblasts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006 Nov;102(5):698-702.

Johnson DL, Carnes D, Steffensen B, Cochran DL. Cellular effects of enamel matrix derivative are associated with different molecular weight fractions following separation by size-exclusion chromatography. J Periodontol. 2009 Apr;80(4):648-56.

Kawana F, Sawae Y, Sahara T, Tanaka S, Debari K, Shimizu M, et al. Porcine enamel matrix derivative enhances trabecular bone regeneration during wound healing of injured rat femur. Anat Rec. 2001 Dec 1;264(4):438-46.

Kawase T, Okuda K, Yoshie H, Burns DM. Anti-TGF-beta antibody blocks enamel matrix derivative-induced upregulation of p21WAF1/cip1 and prevents its inhibition of human oral epithelial cell proliferation. J Periodontal Res. 2002 Aug;37(4):255-62.

Kim TI, Jang JH, Kim HW, Knowles JC, Ku Y. Biomimetic approach to dental implants. Curr Pharm Des. 2008;14(22):2201-11.

Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem. 2006 Dec 1;99(5):1233-9.

Ku Y, Chung CP, Jang JH. The effect of the surface modification of titanium using a recombinant fragment of fibronectin and vitronectin on cell behavior. Biomaterials. 2005 Sep;26(25):5153-7.

Le Guehennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. Dent Mater. 2007 Jul;23(7):844-54.

Li D, Ferguson SJ, Beutler T, Cochran DL, Sittig C, Hirt HP, et al. Biomechanical comparison of the sandblasted and acid-etched and the machined and acid-etched titanium surface for dental implants. J Biomed Mater Res. 2002 May;60(2):325-32.

Liu Y, Li JP, Hunziker EB, de Groot K. Incorporation of growth factors into medical devices via biomimetic coatings. Philos Transact A Math Phys Eng Sci. 2006 Jan 15;364(1838):233-48.

McNeill C. Occlusion: what it is and what it is not. J Calif Dent Assoc. 2000 Oct;28(10):748-58.

Panupinthu N, Rogers JT, Zhao L, Solano-Flores LP, Possmayer F, Sims SM, et al. P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis. J Cell Biol. 2008 Jun 2;181(5):859-71.

Qu Z, Rausch-Fan X, Wieland M, Matejka M, Schedle A. The initial attachment and subsequent behavior regulation of osteoblasts by dental implant surface modification. J Biomed Mater Res A. 2007 Sep 1;82(3):658-68.

Ratner BD. Replacing and renewing: synthetic materials, biomimetics, and tissue engineering in implant dentistry. J Dent Educ. 2001 Dec;65(12):1340-7.

Reseland JE, Reppe S, Larsen AM, Berner HS, Reinholt FP, Gautvik KM, et al. The effect of enamel matrix derivative on gene expression in osteoblasts. Eur J Oral Sci. 2006 May;114 Suppl 1:205-11; discussion 54-6, 381-2.

Rich A, Harris AK. Anomalous preferences of cultured macrophages for hydrophobic and roughened substrata. J Cell Sci. 1981 Aug;50:1-7.

Saito K, Konishi I, Nishiguchi M, Hoshino T, Fujiwara T. Amelogenin binds to both heparan sulfate and bone morphogenetic protein 2 and pharmacologically suppresses the effect of noggin. Bone. 2008 Aug;43(2):371-6.

Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. Macromol Biosci. 2004 Aug 9;4(8):743-65.

Schliephake H, Scharnweber D, Roesseler S, Dard M, Sewing A, Aref A. Biomimetic calcium phosphate composite coating of dental implants. Int J Oral Maxillofac Implants. 2006 Sep-Oct;21(5):738-46.

Schuler M, Owen GR, Hamilton DW, de Wild M, Textor M, Brunette DM, et al. Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: a cell morphology study. Biomaterials. 2006 Jul;27(21):4003-15.

Schuler M, Trentin D, Textor M, Tosatti SG. Biomedical interfaces: titanium surface technology for implants and cell carriers. Nanomed. 2006 Dec;1(4):449-63.

Schuler M, Hamilton DW, Kunzler TP, Sprecher CM, de Wild M, Brunette DM, et al. Gomparison of the Response of Gultured Osteoblasts and Osteoblasts Outgrown From Rat Calvarial Bone Ghips to Nonfouling KRSR and FHRRIKA-Peptide Modified Rough Titanium Surfaces. Journal of Biomedical Materials Research Part B. 2009;In press.

Schwartz Z, Carnes DL, Jr., Pulliam R, Lohmann CH, Sylvia VL, Liu Y, et al. Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. J Periodontol. 2000 Aug;71(8):1287-96.

Schwartz Z, Nasazky E, Boyan BD. Surface microtopography regulates osteointegration: the role of implant surface microtopography in osteointegration. Alpha Omegan. 2005 Jul;98(2):9-19.

Schwarz F, Rothamel D, Herten M, Sculean A, Scherbaum W, Becker J. Effect of enamel matrix protein derivative on the attachment, proliferation, and viability of human SaOs(2) osteoblasts on titanium implants. Clin Oral Investig. 2004 Sep;8(3):165-71.

Sculean A, Reich E, Chiantella GC, Brecx M. Treatment of intrabony periodontal defects with an enamel matrix protein derivative (Emdogain): a report of 32 cases. Int J Periodontics Restorative Dent. 1999 Apr;19(2):157-63.

Sculean A, Windisch P, Dori F, Keglevich T, Molnar B, Gera I. Emdogain in regenerative periodontal therapy. A review of the literature. Fogorv Sz. 2007 Oct;100(5):220-32, 11-9.

Takayama T, Suzuki N, Narukawa M, Tokunaga T, Otsuka K, Ito K. Enamel matrix derivative stimulates core binding factor alpha1/Runt-related transcription factor-2 expression via activation of Smad1 in C2C12 cells. J Periodontol. 2005 Feb;76(2):244-9.

Weishaupt P, Bernimoulin JP, Trackman P, Hagewald S. Stimulation of osteoblasts with Emdogain increases the expression of specific mineralization markers. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008 Aug;106(2):304-8.

Wieland M, Textor M, Chehroudi B, Brunette DM. Synergistic interaction of topographic features in the production of bone-like nodules on Ti surfaces by rat osteoblasts. Biomaterials. 2005 Apr;26(10):1119-30.

Wikesjo UM, Qahash M, Polimeni G, Susin C, Shanaman RH, Rohrer MD, et al. Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2: histologic observations. J Clin Periodontol. 2008 Nov;35(11):1001-10.

Wu CL, Shu R. [The studies on osteoblasts' mineralization and attachment affected by enamel matrix proteins]. Shanghai Kou Qiang Yi Xue. 2002 Jun;11(2):135-7.

Yamada KM. Adhesive recognition sequences. J Biol Chem. 1991 Jul 15;266(20):12809-12.

Yoneda S. [The effects of enamel matrix derivative (EMD) on osteoblastic cells]. Kokubyo Gakkai Zasshi. 2002 Sep;69(3):207-14.

Yuan K, Chen CL, Lin MT. Enamel matrix derivative exhibits angiogenic effect in vitro and in a murine model. J Clin Periodontol. 2003 Aug;30(8):732-8.

CHAPTER THREE

GENERAL DISCUSSION

3.1 Summary and Conclusions:

Objective #1: To investigate the ability of EMD to promote osteoblast adhesion when coated on various surface topographies.

In this study, no significant differences in osteoblast attachment were observed on PT and SLA surfaces precoated with EMD when compared to uncoated PT or SLA surfaces. Although RCOs attached to all topographies in similar numbers, osteoblasts seeded on EMD-coated PT surfaces exhibited greater spreading as determined by measurements of cell planar area. No significant difference in spreading was observed on SLA surfaces coated with EMD.

Objective #2: To investigate the ability of EMD to promote osteoblast proliferation.

EMD increased osteoblast numbers at 5 and 7 days post seeding on SLA surfaces and at 5 days post seeding on PT surfaces. Furthermore, EMD promoted cell clustering which may be indicative of early bone nodule formation and cell-cell communication.

Objective #3: To investigate the effect of EMD on osteoblast differentiation.

EMD increased mRNA levels of osteocalcin and bone sialoprotein as quantified by real-time PCR. Consistent with these data, EMD also increased alkaline phosphatase activity and calcium mineral deposition as shown by alizarin complexone staining. EMD also changed cellular localization of the transcription factor Runx2 at 2 weeks post seeding on both PT and SLA surfaces from the nucleus to the cytoplasm. These data demonstrate that EMD promotes osteoblast differentiation on Ti surfaces irrespective of topography.

3.2 Contributions to the Current State of Knowledge and Suggestions for Future Studies:

General significance — Previous studies have demonstrated that addition of EMD to cell culture media enhances proliferation and differentiation of many cell types including osteoblasts and periodontal ligament fibroblasts (Kawase et al. 2001, 2002, Iwata et al. 2002, Parkar and Tonetti 2007, Johnson et al. 2009). However, the effect of precoating EMD on osteoblast behavior and maturation on varying titanium surface topographies was unknown. In this study, we report that EMD increases osteoblast spreading, proliferation and differentiation when coated on titanium surfaces of varying topographies *in vitro*. Future experiments will focus on how EMD-coated Ti implants respond *in vivo*. The results described here suggest that EMD will speed the formation of bone around implants.

Possible mechanism of EMD — Amelogenin, the major component of EMD, has previously been shown to increases osteoblast attachment by serving as an extracellular adhesion molecule. Though amelogenin lacks an RGD integrin binding site, it has been shown to promote cell adhesion (Hoang et al. 2002). Furthermore, several reports have confirmed that other molecules contained in EMD do contain integrin binding regions (Lyngstadaas et al. 2001, Suzuki et al. 2001). For example, Suzuki et al. found that osteoblasts attach to BSP-like molecules in EMD through $\alpha v\beta 3$ integrins (Suzuki et al. 2001). They subsequently demonstrated increases in osteoblast intracellular cAMP and secretion of TGFβ-1, IL-6 and PDGF downstream of integrin binding (Suzuki et al.

2001). Importantly, each of these factors plays a prominent role in the regulation of osteoblast differentiation.

Role of TGFβ in the osteoblast lineage — TGFβ-1 is a growth factor known to increase proliferation, inhibit apoptosis and enhance differentiation of osteoblasts *in vitro* (Centrella et al. 1994, Attisano et al. 1996). In the present study, we have shown that EMD increases osteoblast proliferation when precoated on titanium surfaces. Interestingly, preliminary data obtained using an osteogenic PCR super array (SABiosciences, Frederick, MD, USA) showed 10-fold greater TGFβ1 mRNA in osteoblasts on EMD-coated surfaces than on uncoated ones (appendix 1). Thus, it is likely that the enhanced proliferation induced by EMD is indirect, caused by the release of cytokines and growth factors such as TGFβ1 in response to osteoblast adhesion to EMD proteins on titanium.

The role of TGFβ1 has been well established in the regulation of osteogenesis. During early stages of bone formation, TGFβ1 functions to recruit and stimulate the proliferation of osteoprogenitor cells, providing a pool of pre-osteoblasts (Robey et al. 1987, Centrella et al. 1994). Kawase et al. observed that EMD induced rapid translocation of SMAD2 into the nucleus and suggested that it may be caused by upregulation of TGFβ1 (Kawase et al. 2001, 2002).

Role of cell-cell communication in osteoblast lineage — It has previously been suggested that osteoblast clustering on titanium surfaces is an early sign of condensation (Qu et al. 2007). Cell-cell interaction molecules, such as cadherins (Calcium-dependent

adhesion molecules) and connexins, are important for cellular communication and vital to osteoblast differentiation (Okazaki et al. 1994, Cheng et al. 1998, Ferrari et al. 2000, Lecanda et al. 2000). During the process of osteoblast maturation, progenitor cells express two types of cadherins, N-cadherin and cadherin 11 (Okazaki et al. 1994, Cheng et al. 1998, Ferrari et al. 2000, Schiller et al. 2001). Cadherin molecules ensure that cells are properly bound to one another and cells expressing N-cadherins tend to cluster with other N-cadherin-expressing cells (Duguay et al. 2003). Since EMD results in osteoblast clustering, it is conceivable that EMD upregulates cadherins. Furthermore, cadherins promote the formation of gap junctions, which permit the propagation of ions, growth factors, metabolites and second messengers between adjoining cells (Lecanda et al. 2000). EMD enhances osteoblast clustering and we hypothesize that EMD upregulates cell communication proteins such as Cx43, fibronectin and cadherins, all of which are vital for osteoblast maturation.

Summary of effect of EMD — Numerous studies have shown that EMD affects multiple signaling pathways in osteoblasts (Suzuki et al. 2001, Okubo et al. 2003, Lee et al. 2008). EMD promotes osteoblast cell spreading through $\alpha v\beta 3$ integrin (Suzuki et al. 2001). EMD enhances osteoblast secretion of TGFβ1, which is required for osteoblast recruitment and proliferation (Okubo et al. 2003). In the present study, EMD was found to enhance ALP activity, increase expression of markers for osteoblast differentiation and stimulate extracellular matrix mineralization. EMD has also been shown to promote the production of IL-6 and VEGF, which are responsible for the stimulation of angiogenesis, which may in turn contribute to more rapid and complete healing around an implant

(Parkar and Tonetti 2004, Rausch-fan et al. 2008). These multiple effects suggest that EMD may be suitable for clinical use as a coating for titanium implant materials.

In vivo investigations — Although it is well established that EMD affects osteoblast proliferation and differentiation *in vitro*, pre-clinical *in vivo* research is needed prior to use in humans. Future experiments could include implanting PT and SLA titanium rods precoated with or without EMD into the femurs of mature New Zealand white rabbits. At time points 3, 6 and 12 weeks, microCT imaging could be employed to quantify area of newly formed bone around each rod.

Future studies of protein fractions in EMD — The mechanisms through which EMD mediates effects at the cell and tissue level remain unclear. In an attempt to characterize the proteins found in EMD, Mumulidu et al. used high performance liquid chromatography to fractionate EMD into three major components: a 20 kDa, 12 + 9 kDa and 5 kDa fractions (Mumulidu et al. 2007). Amelogenin (20 kDa), the major component in EMD (representing 90% of its total protein content) is an adhesion molecule that was initially thought to be responsible for the effects observed in EMD (Hoang et al. 2002). However, further analysis has revealed that the 5 kDa component of EMD contains many bioactive molecules (Mumulidu et al. 2007). More recently, Johnson et al. found that each of the 3 fractions stimulates different cellular mechanisms (Johnson et al. 2009). They found that the proteins in fractions II and III affect proliferation and angiogenic activities in microvascular cells and that fraction I was associated with the recruitment and differentiation of osteoprogenitor cells. Iwata et al., using ST2 pre-osteoblast-like-

cells, found that fractions I and II stimulated the highest ALP activity (Iwata et al. 2002). Johnson et al. suggested that BMPs may be one of the key components of EMD (Johnson et al. 2009). Preliminary results from our PCR superarray showed that osteoblasts cultured on EMD-Ti had 15 fold greater BMP4 mRNA levels than uncoated surfaces (appendix 1). Previously, Parkar and Tonetti (2004) showed in their gene array analysis of PDL cells that EMD upregulated BMP4; one of the 3 BMPS known to be inhibited by noggin (Iwata et al. 2002).

Final Summary — In this thesis, we have shown that EMD has the ability to increase cell spreading on PT surfaces. Osteoblasts seeded on EMD-coated surfaces exhibit greater rates of proliferation when compared to uncoated surfaces. EMD also promotes upregulation of BSP and OC, both of which are required for osteoblast differentiation, changes cellular localization of the transcription factor Runx2 and increases calcium deposition as assessed by alizarin complexone staining. Although EMD contains many proteins and growth factors that remain uncharacterized, the results from this thesis suggest that it is suitable for clinical application and may promote osseointegration around dental implants.

3.3 References:

Attisano L, Wrana JL. Signal transduction by members of the transforming growth factor-beta superfamily. Cytokine Growth Factor Rev. 1996 Dec;7(4):327-39.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL. Transforming growth factor-beta gene family members and bone. Endocr Rev. 1994 Feb;15(1):27-39.

Cheng SL, Lecanda F, Davidson MK, Warlow PM, Zhang SF, Zhang L, Suzuki S, St John T, Civitelli R. Human osteoblasts express a repertoire of cadherins, which are critical for BMP-2-induced osteogenic differentiation. J Bone Miner Res. 1998 Apr;13(4):633-44.

Duguay D, Foty RA, Steinberg MS. Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. Dev Biol. 2003 Jan 15;253(2):309-23.

Ferrari SL, Traianedes K, Thorne M, Lafage-Proust MH, Genever P, Cecchini MG, Behar V, Bisello A, Chorev M, Rosenblatt M, Suva LJ. A role for N-cadherin in the development of the differentiated osteoblastic phenotype. J Bone Miner Res. 2000 Feb;15(2):198-208.

Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL. Amelogenin is a cell adhesion protein. J Dent Res. 2002 Jul;81(7):497-500

Iwata T, Morotome Y, Tanabe T, Fukae M, Ishikawa I, Oida S. Noggin blocks osteoinductive activity of porcine enamel extracts. J Dent Res. 2002 Jun;81(6):387-91.

Johnson DL, Carnes D, Steffensen B, Cochran DL. Cellular effects of enamel matrix derivative are associated with different molecular weight fractions following separation by size-exclusion chromatography. J Periodontol. 2009 Apr;80(4):648-56.

Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. J Periodontal Res. 2001 Dec;36(6):367-76.

Kawase T, Okuda K, Yoshie H, Burns DM. Anti-TGF-beta antibody blocks enamel matrix derivative-induced upregulation of p21WAF1/cip1 and prevents its inhibition of human oral epithelial cell proliferation. J Periodontal Res. 2002 Aug;37(4):255-62.

Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH, Civitelli R. Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol. 2000 Nov 13;151(4):931-44.

Lee AZ, Jiang J, He J, Safavi KE, Spangberg LS, Zhu Q. Stimulation of cytokines in osteoblasts cultured on enamel matrix derivative. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008 Jul;106(1):133-8.

Lyngstadaas SP, Lundberg E, Ekdahl H, Andersson C, Gestrelius S. Autocrine growth factors in human periodontal ligament cells cultured on enamel matrix derivative. J Clin Periodontol. 2001 Feb;28(2):181-8.

Mumulidu A, Hildebrand B, Fabi B, Hammarström L, Cochran DL, Dard M, Lemoult S Purification and analysis of a 5kDa component of enamel matrix derivative. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 Oct 1;857(2):210-8.

Okazaki M, Takeshita S, Kawai S, Kikuno R, Tsujimura A, Kudo A, Amann E. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. J Biol Chem. 1994 Apr 22;269(16):12092-8.

Okubo K, Kobayashi M, Takiguchi T, Takada T, Ohazama A, Okamatsu Y, Hasegawa K. Participation of endogenous IGF-I and TGF-beta 1 with enamel matrix derivative-stimulated cell growth in human periodontal ligament cells. J Periodontal Res. 2003 Feb;38(1):1-9.

Parkar MH, Tonetti M. Gene expression profiles of periodontal ligament cells treated with enamel matrix proteins in vitro: analysis using cDNA arrays. J Periodontol. 2004 Nov;75(11):1539-46.

Qu Z, Rausch-Fan X, Wieland M, Matejka M, Schedle A. The initial attachment and subsequent behavior regulation of osteoblasts by dental implant surface modification. J Biomed Mater Res A. 2007 Sep 1;82(3):658-68.

Rausch-fan X, Qu Z, Wieland M, Matejka M, Schedle A. Differentiation and cytokine synthesis of human alveolar osteoblasts compared to osteoblast-like cells (MG63) in response to titanium surfaces. Dent Mater. 2008 Jan;24(1):102-10.

Robey PG, Young MF, Flanders KC, Roche NS, Kondaiah P, Reddi AH, Termine JD, Sporn MB, Roberts AB. Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-beta) in vitro. J Cell Biol. 1987 Jul;105(1):457-63.

Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA. Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone. 2001 Apr;28(4):362-9.

Suzuki N, Ohyama M, Maeno M, Ito K, Otsuka K. Attachment of human periodontal ligament cells to enamel matrix-derived protein is mediated via interaction between BSP-like molecules and integrin alpha(v)beta3. J Periodontol. 2001 Nov;72(11):1520-6.

APPENDIX AND CURRICULUM VITAE

Appendix A: PCR super-array analysis of RCOs seeded on surfaces treated with and without EMD – preliminary data.

50 000 rat calvarial osteoblasts were seeded on PT and SLA surfaces and cultured in 10% FBS and 1% antibiotics. At time points 1 and 14 day, total RNA was isolated using TRIZOL reagent and RNAeasy Mini kit (QIAGEN). Following TRIZOL extraction, real-time RT-PCR was performed using 15µl final reaction volume using CyberGreen MasterMix (Biosciences) and plated on PCR super-array plates according to manufacture protocol. Each sample contained mRNA pooled from 3 Ti surfaces.

		Fold Regulation (comparing to control group)					
	Gene Symbol	pol EMD (1 day)	SLA no EMD (1 day)	SLA EMD (1 day)	pol EMD	SLA no EMD	SLA EMD
A01	alpha-2-HS-glycoprotein	-3.47	-2.14	-2.25	-1.19	-2.31	2.43
A02	alkaline phosphatase	3.47	5.93	3.7	5.21	2.38	2.43
A03	ameloblastin	-7.38	-4.55	-4.78	-2.54	-1.48	1.14
A04	annexin A5	2.29	3.72	4.03	1.07	6.05	2.73
A05	biglycan	2.17	3.25	2.22	-1.06	3.6	2.54
A06	bone morphogenetic protein 1	1.32	1.19	1.19	-1.11	-1.55	1.62
A07	bone morphogenetic protein 2	-1.62	3.19	4.48	-1.51	8.5	4.73
A08	bone morphogenetic protein 3	-4.17	-2.57	-2.7	-1.43	-2.78	2.02
A09	bone morphogenetic protein 4	-1.27	-1.5	-2.69	15.99	2.01	13.48
A10	bone morphogenetic protein 5	2.66	1.19	2.9	1.63	1.23	5.44
A11	bone morphogenetic protein 6	1.14	-1.88	2.43	1.44	-2.7	2.48
A12	bone morphogenetic protein receptor Ia	2.08	1.24	1.4	13.86	1.13	12.43
B01	bone morphogenetic protein receptor	2.26	1.03	1.82	1.34	2.31	5.12

	Ib thrombospondin						
B02	receptor	1.22	-1.75	-1.61	1.52	6.13	1.59
B03	cadherin 11	2.07	1.46	1.49	4.81	6.61	7.85
B04	collagen, type X, alpha 1	-1.12	-2.6	1.06	-1.38	-1.41	1.39
B05	collagen, type XI, alpha 1	1.73	-3.03	-1.08	4.99	-1.43	3.94
B06	collagen, type XII, alpha 1	1.59	1.92	1.51	6.02	1.43	5
B07	collagen, type XIV, alpha 1	1.13	1.08	1.08	14.03	1.77	6.43
B08	collagen, type I, alpha 1	1.93	2.2	2.28	2.07	1.24	7.34
B09	collagen, type I, alpha 2	1.55	-1.26	-1.48	6.18	-2.03	4.15
B10	collagen, type II, alpha 1	3.24	9.68	4.89	3.54	2.26	31.86
B11	collagen, type III, alpha 1	-1.28	-1.07	1.13	2.78	-1.69	5.92
B12	collagen, type IV, alpha 1	2.89	2.34	2.77	3.53	1.28	11.76
C01	collagen, type IV, alpha 2	3.45	1.11	-1.35	1.74	4.06	-1.63
C02	collagen, type V, alpha 1	5.65	1.89	2.13	-1.37	3.85	1.32
C03	collagen, type VI, alpha 1	1.48	1.41	1.66	-1.26	2.66	2.77
C04	collagen, type VI, alpha 2	1.11	1.81	1.94	-1.5	1.39	1.82
C05	collagen, type VII, alpha 1	4.75	-3.29	1.37	-4.81	-4.16	-1.01
C06	cartilage oligomeric matrix protein	-1.49	1.3	1.69	1.75	-1.84	2.46
C07	colony stimulating factor 2	-1.21	-2.14	4.6	1.72	2.44	8.78
C08	colony stimulating factor 3	-3.74	-7.08	-2.25	-3.95	-7.66	1.42
C09	cathepsin K	-1.37	-1.3	1.13	2.8	-2.46	3.31
C10	dentin matrix acidic phosphoprotein	29.76	5.37	14.54	3.21	2.43	19.62

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C11	epidermal growth factor	-3.55	-1.99	-2.14	1.3	2.53	5.85
C12	enamelin	-1.18	-2.14	-2.25	-1.19	-2.07	19.27
D01	fibroblast growth factor 1	3.27	-2.57	-1.35	-1.45	3.73	2
D02	fibroblast growth factor 2	7.02	1.5	2.52	-2.36	2.38	-2.96
D03	fibroblast growth factor 3	-3.47	-2.14	-1.55	1.25	3.79	6.55
D04	fibroblast growth factor receptor 1	1.06	-2.21	-1.59	1.78	-1.13	2.37
D05	fibroblast growth factor receptor 2	-2.68	-2.6	-41.12	4.26	1.01	4.39
D06	FMS-like tyrosine kinase 1	1.31	1.03	1.68	-2.55	-2.45	1.13
D07	fibronectin 1	1.09	1.26	1.48	1.81	1.1	4.47
D08	growth differentiation factor 10	-4.99	2.36	-1.42	39.59	4.31	21.3
D09	intercellular adhesion molecule 1	-2	1.4	-1.03	8.07	1.45	7.57
D10	insulin-like growth factor 1	-1.77	-2.1	-2.06	16.8	3.16	24.93
D11	insulin-like growth factor I receptor	-1.15	-1.28	-1.64	1.02	-1.26	4.71
D12	integrin, alpha 2	1.38	-2.59	1.31	10.48	-1.43	10.44
E01	integrin alpha 2b	-33	-1.56	-3	-1.83	-1.06	-3.92
E02	integrin alpha 3	1.83	-1.32	-1.1	-6.58	1.87	-1.91
E03	integrin, alpha M	-1.1	-1.38	-1.2	1.68	4.26	-1.18
E04	integrin, alpha V	2.08	1.28	-1.04	2.1	2.3	4.34
E05	integrin beta 1	1.9	-2.24	-1.03	1.35	1.11	1.71
E06	matrix metallopeptidase 10	1.3	-4.36	2.49	-16.66	-21.29	-12.17
E07	matrix metallopeptidase 2	-2.32	-2.08	-1.8	5.19	-1.81	3.03
E08	matrix metallopeptidase	-2.32	-1.6	-2.12	1.65	-4.16	1.07

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E09	matrix metallopeptidase 9	2.09	-2.21	1.06	3.42	2.17	132.99
E10	msh homeobox 1	-2.41	-2.9	2.47	2.96	-3.14	4.68
E11	nuclear factor kappa Beta 1	-1.32	-1.58	1.16	2.89	-1.33	6.1
E12	platelet-derived growth factor alpha	1.79	1.27	2.08	3.36	1.05	12.75
F01	phosphate regulating gene	1.63	-1.46	2.59	1.46	4.56	2.43
F02	runt-related transcription factor 2	4.03	2.17	2.86	1.23	3.01	1.95
F03	scavenger receptor class B	2.1	1.1	1.07	-2.75	1.72	1.81
F04	serpin peptidase inhibitor	2.28	-1.8	1.99	2.47	1.98	2.88
F05	SMAD family member 1	-1.47	-1.94	-2.26	1.49	-2.54	1.84
F06	SMAD family member 2	-1.06	-1.57	-1.11	2.03	-1.17	1.93
F07	SMAD family member 3	-1.43	1.11	-1.32	3.15	-2.02	3.18
F08	SMAD family member 4	1.11	1.4	4.05	2.38	-1.53	3.17
F09	sclerosteosis	-3.47	-1.01	2.87	-1.19	5.16	7.13
F10	SRY (sex determining region Y)-box 9	-1.51	1.55	2.19	4.41	1.82	12.15
F11	tuftelin interacting protein 11	-2.5	-2.09	-1.86	1.7	-3.4	3.24
F12	transforming growth factor, beta 1	1.31	1.12	2.36	1.57	-3.89	2.55
G01	transforming growth factor, beta 2	2.26	2.36	2.1	-1.47	1.9	1.28
G02	transforming growth factor, beta 3	3.53	1.81	1.98	1.8	4.82	4.3
G03	transforming growth factor,	1.49	1.08	2.1	-3.1	1.05	-5.17

	beta receptor 1 transforming growth factor,						
G04	beta receptor 2 transforming growth factor,	-1.15	1.49	1.1	2.5	2.61	3.8
G05	beta receptor 3 tumor necrosis	-1.39	-1.42	1	3.09	1.68	2.73
G06	factor	-9.65	-5.95	-6.25	2.45	-2.1	1.04
G07	tuftelin 1	-2.39	-1.2	1.24	2.91	1.03	5.18
G08	twist homolog 1 vascular cell adhesion	-2.21	-1.71	-1.46	3.04	-2.09	4.16
G09	molecule 1 vitamin D	-4.49	-1.44	-2.88	16.58	1.86	8.37
G10	receptor vascular endothelial	-1.89	-1.84	-3.03	4.72	-2.39	3.72
G11	growth factor A vascular endothelial	2.49	1.17	4.21	1.08	-2.46	3.56
G12	growth factor B glucuronidase,	-1.67	1.14	1.37	4.77	-1.77	5.86
H01	beta hypoxanthine guanine phosphoribosyl	-1.29	1.9	1.77	1.11	1.03	-1.12
H02	transferase 1 heat shock protein 90kDa alpha (cytosolic), class	1.4	1.1	1.26	1.39	1.52	2.88
H03	B member 1 glyceraldehyde-3-phosphate	1.18	1.23	-1.36	-1.53	-1.3	1.25
H04	dehydrogenase	-1.01	1.08	-1.23	-6060.15	1.37	1.54
H05	actin, beta	-1.28	-2.57	-1.64	-1.01	-1.19	-3.21