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Effects of Antidepressants on Human Mesenchymal Stem Cell Differentiation on Clinically Relevant Titanium Surfaces

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EFFECTS OF ANTIDEPRESSANTS ON HUMAN MESENCHYMAL STEM CELL DIFFERENTIATION ON CLINICALLY RELEVANT TITANIUM SURFACES

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biomedical Engineering at Virginia Commonwealth University.

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List of Abbreviations

5-HT	5-hydroxytryptamine
5-HTT	Serotonin transporter
BMP2	Bone morphogenetic protein 2
DDC	Decarboxylase
EC	Enterochromaffin cells
MDD	Major Depressive Disorder
mSLA	Modified SLA
M-CSF	Macrophage-Colony Stimulating Factor
MOAI	Monoamine Oxidase Inhibitors
MSCGM	Mesenchymal Stem Cell Growth Media
OCN	Osteocalcin
OPG	Osteoprotegerin
PT	Pretreatment
RANKL	Receptor Activator of Nuclear Factor Kappa-B Ligand
RUNX2	Runt-related transcription factor 2
SLA	Sandblasted and Acid Etched
SNRI	Selective Norepinephrine Reuptake Inhibitors
SSRI	Selective Serotonin Reuptake Inhibitors
TCA	Tricyclic Antidepressants
VMAT	Vesicular Monoamine Transporter

Abstract

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Selective Serotonin Reuptake Inhibitors (SSRIs) are the most frequently prescribed class of drugs worldwide and are implemented in the treatment of depression and other psychiatric disorders. SSRIs relieve depressive symptoms by modulating levels of the neurotransmitter serotonin in the brain. SSRIs block the function of the serotonin transporter, thereby increasing concentrations of extracellular serotonin. However, serotonin levels in the neurons of the brain only account for 5% while the remaining 95% is present outside the brain. Serotonin receptors and transporter are located on bone resident cells (mesenchymal stem cells (MSCs)), osteoblasts and osteoclasts, and serotonergic activity is believed to affect bone homeostasis. Consequently, alterations in serotonin levels by SSRI treatment have the potential to alter bone formation and remodeling. Clinical reports correlate increase risk of bone fractures and delayed bone healing with SSRI use. Metallic implants are commonly used as orthopedic and dental implants to fix bony defects. Surface modifications have been used to increase the level of bone to implant contact by controlling the differentiation of MSCs into an osteoblastic linage and facilitate bone production. However, it is not known if SSRIs can affect MSCs osteoblastic differentiation and bone remodeling signaling in response to microstructured biomaterials. The aims of this study were: 1) Investigate the effects of SSRIs on MSCs differentiation on microstructured titanium (Ti), 2) Determine the effects of SSRIs

on bone remodeling signaling and osteoclast activation, and 3) Elucidate the effects of SSRIs on serotonin receptors and their effect on bone remodeling. To investigate this, human MSCs were grown on tissue culture polystyrene (TCPS), smooth Ti (PT) or microstructured Ti (SLA) surfaces under exposure to therapeutic concentrations of commonly prescribed antidepressants (SSRIs (fluoxetine, sertraline, paroxetine), Selective Norepinephrine Reuptake Inhibitor (SNRI) (duloxetine) and other regularly prescribed antidepressants (bupropion)) during differentiation toward osteoblasts. Osteoblastic differentiation was assessed in MSCs after treatment with the drugs (0.1μM, 1μM, 10μM) by alkaline phosphatase activity and osteocalcin levels. Antidepressant treatment decreased levels of MSC differentiation markers on microstructured Ti surfaces. Furthermore, treatment dose-dependently decreased protein levels secreted by MSCs which are important for bone formation (BMP2, VEGF, Osteoprotegerin), and increased those involved in bone resorption (RANKL). To determine the effect of SSRIs on bone remodeling signaling and osteoclast activation, human osteoclasts were either directly exposed to antidepressants or conditioned media obtained from MSCs treated with antidepressants on Ti surfaces, after which, enzymatic tartrate-resistant acid phosphatase (TRAP) activity was assessed. Antidepressants increased TRAP activity both directly and through treated MSCs, with the highest levels evident after treatment with conditioned media from MSCs on microstructured Ti surfaces. To elucidate the effects of serotonin receptors and their effect on bone remodeling, receptors were pharmacologically inhibited. Surface roughness decreased gene expression of HTR2A, HTR1B, and HTR2B, and antidepressant treatment increased their expression. Inhibition of HTR2A decreased RANKL protein levels, while inhibition of other serotonin receptors had no effect on RANKL or OPG levels. These studies suggest that antidepressants inhibit MSCs differentiation on microstructured Ti surfaces and increase levels of proteins associated with bone resorption. Additionally, our results showed that RANKL is regulated by serotonin receptor HTR_{2A}. Taken together, our results suggest that antidepressants have a negative effect on osteoblastic differentiation, compromising bone formation and enhancing bone resorption, which can be detrimental to patients under orthopedic and dental treatment.

CHAPTER 1 INTRODUCTION

1.1. MAJOR DEPRESSIVE DISORDER

Depression is a globally threatening psychiatric disorder, affecting approximately 350 million people worldwide and rapidly becoming the leading cause of disability as rates continue to rise. [1]. Within the United States, the World Health Organization estimates that depression prevails in over 20% of the population, with a lifetime prevalence of about 15-20% [1]. In the adolescent population, the predominance of depression is reported to be as high as 8.3% [12]. Although no difference in rates are evident prior to puberty, among adolescence, however, rates are two to three times greater in females than males. This trend carries over into adulthood, as depression is twice as common in adult women when compared to men [12].

The illness is diagnosed by health care providers as "Major Depressive Disorder (MDD)," according to a set criteria of symptoms interrupting routine personal or occupational function, usually lasting longer than two weeks [1]. The disorder is thought to be caused by lower than normal neuronal serotonin production and synaptic availability [41]. MDD is chronic in nature, as an estimate of 80% of diagnosed individuals were reported to be prescribed an antidepressant for at least 12 months [28]. Currently, making the diagnosis of MDD is not based on a diagnostic examination, but rather a set of variable symptomatic criteria. Symptoms may be as mild as an unhappy mood, feelings of low self-esteem or decreased interest in activities once enjoyed, but can be as severe as diminished appetite and recurrent suicidal thoughts or actions. Regardless of the severity, its common practice to prescribe treatments for all cases of depression.

1.2. PHARMACOLOGICAL TREATMENTS OF MDD

There are several treatment options for managing depressive symptoms. Psychological treatments are available through health care providers in the form of cognitive behavioral therapy and interpersonal psychotherapy [1]. However, pharmacological manipulation of the serotonergic system in the form of antidepressant medications has proven to be the most common and effective strategy for managing depression, and therefore, will be the focus of this work. Certain classes of antidepressants are especially effective in treating depression due to their selective

pharmacological activity at specific action sites in the neurons of the brain. Despite this efficacy, however, they are known for their delayed onset of therapeutic action. Clinical symptoms of depression are generally not profoundly improved until 2-4 weeks of continuous pharmacological treatment, depending on the type of antidepressant [38]. Various types of antidepressants may differ in composition and efficacy, but all agents partake in some degree of modulation to the serotonergic system, however, at diverse selectivity.

1.2.1. TRICYCLIC ANTIDEPRESSANTS (TCA)

Many types of antidepressant medications are currently used for managing depression. Early generation antidepressants include Tricyclic Antidepressants (TCA) such as amitriptyline, clomipramine, imipramine and doxepin [12] and [27]. TCA treat depression by improving cholinergic, noradrenergic and/or serotonergic signaling in the brain [12]. Regardless of their success in resolving depressive symptoms, TCA act on adrenaline, choline and histamine receptors, which lead to presentation of undesirable side effects. Side effects such as drowsiness, dizziness, dry mouth and weight gain led to their infrequent prescription [12].

1.2.2. MONOAMINE OXIDASE INHIBITORS (MAOI)

Monoamine oxidase inhibitors (MOAI) are a second type of early generation antidepressants. Many MOAI include phenelzine, tranylcypromine, isocarboxazid, selegiline and pargyline [12]. MOAI treat depression by inhibiting monoamine oxidases, which are catabolic enzymes responsible for serotonin degradation in the neurons of the brain. Inhibition of monoamine oxidase enzyme activity reduces serotonin degradation and prolongs its presence within presynaptic neurons of the brain for more efficient signaling with post synaptic neurons. Despite their efficacy, these drugs are also infrequently prescribed due to their non-specific interactions and associations with many adverse, and sometimes fatal, cardiac effects [12].

1.2.3. OTHER ANTIDEPRESSANTS

Antidepressants are usually grouped by class due to their mechanism of action, however, some other types, such as trazodone and bupropion, do not belong to a specific category. These other medications are also frequently prescribed. Treatment of MDD using these other antidepressants is similar in mechanism as those described above, however, these types are less selective for the neurotransmitter serotonin. Bupropion and trazodone are a target for other types

of neurotransmitters involved in modulation of mood in the brain, either separately or in addition to serotonin. These drugs are also very effective in treating depression.

1.2.4. SELECTIVE NOREPINEPHRINE REUPTAKE INHIBITORS (SNRIs)

Newer generation antidepressants, including Selective Norepinephrine Reuptake Inhibitors (SNRIs) have reliably shown to be effective in treating depressive symptoms and have much fewer side effects, partially due to their selectivity and specificity for the serotonin and norepinephrine neurotransmitters [12]. These medications treat depression by modulating levels of serotonin as well as norepinephrine in the brain. Medications in the SNRI class include duloxetine and venlafaxine [27].

1.2.5. SELECTIVE SEROTONIN REUPTAKE INHIBITORS (SSRIs)

Selective Serotonin Reuptake Inhibitors (SSRIs) are currently the most widely prescribed family of medication in the treatment of depression, and thus, are the focus of this work. According to data obtained from the Prescription Pricing Authority, SSRI prescriptions drastically increased by 45% between the years 2000 and 2005, rapidly becoming the most prescribed class of antidepressants on the market [28]. This class of drugs include well-known medications like fluoxetine, sertraline, paroxetine, fluvoxamine and citalopram, which are generic for Prozac, Zoloft, Paxil, Luvox and Celexa, respectively. Not only are SSRIs prescribed for treatment of MDD, but also for other psychological disorders, such as anxiety, and are consistently being prescribed for treatment of depression in expectant mothers [1].

1.2.5.1. SSRI MECHANISM OF ACTION

SSRIs relieve depressive symptoms by modulating levels of the neurotransmitter serotonin in the neurons of the brain. A depressed mood corresponds with reduced serotonin neurotransmission within synaptic spaces [2]. Lower levels of serotonin weaken the signal transduction from one neuron to the next. One way to potentate this signal is to block the reuptake of extracellular serotonin molecules by the presynaptic neuron from the synapse. The serotonin transporter (5-HTT), also known as SERT, is a monoamine membrane transporter protein [2]. Its function is to transport extracellular serotonin from the synaptic spaces into presynaptic neurons. SSRIs have high affinity for 5-HTT. The binding of SSRIs to 5-HTT is very efficient in blocking the reuptake of serotonin back into the presynaptic neuron. Impeding 5-HTT controls, and

prolongs, the duration of serotonergic activity (figure 1). This action will permit the presence of higher levels of serotonin and strengthen the signaling transduction from presynaptic to postsynaptic neurons, thereby treating depression.

Although very effective in treating depression due to their high selectivity and potency for 5-HTT, which enables them to increase serotonin levels in the brain, SSRIs increase systemic serotonin levels as well. High peripheral serotonin concentrations may have an impact on other cells within the body, such as bone cells. Mounting evidence links SSRI use with decreased bone mineral density, increased risk of fracture and dental implant failure [26] and [36]. Recent research suggests serotonin may be a substantial regulator involved in bone metabolism, and such effects are thought to be linked to serotonergic signaling.

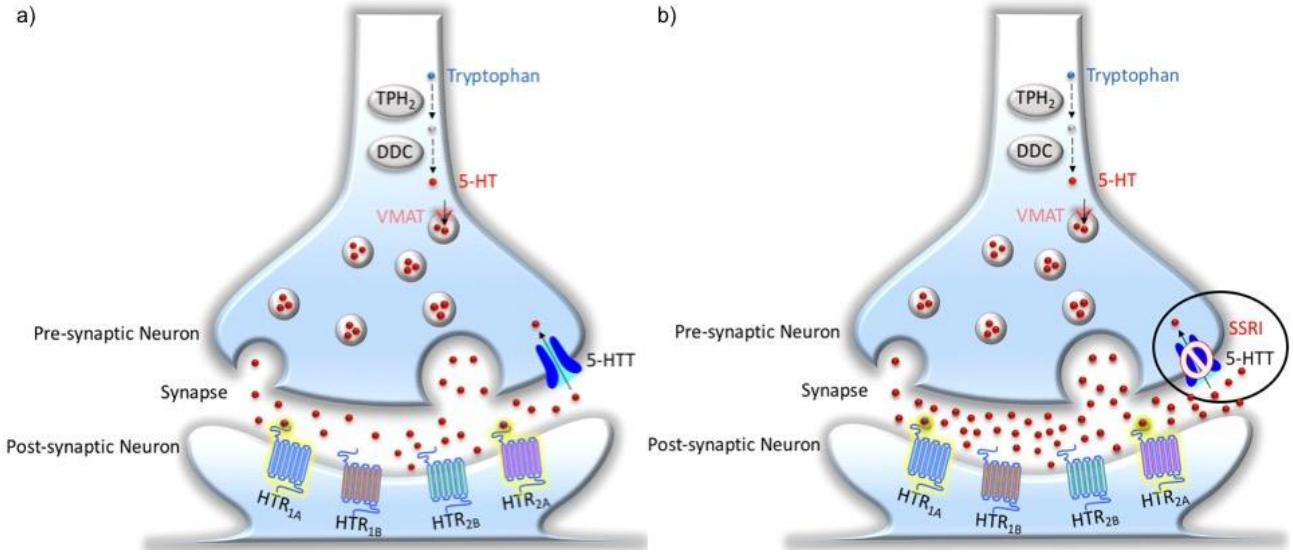

Figure 1: SSRI Mechanism of Action in the Neurons of the Brain. a) Normal physiological function. b) SSRI-mediated inhibition of the serotonin transporter (5-HTT).

1.3. SEROTONIN PRODUCTION AND FUNCTION

Serotonin, also referred to as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter and is well known as a mood regulator. It is responsible for mediating several of functions throughout the body, many of which include appetite, intestinal functions, sleep behavior and blood pressure regulation [22] and [27]. 5-HT is produced within the presynaptic neurons in the central nervous system as well as at other peripheral locations. Its production is carried out in a two-step biochemical process. The first step in synthesis involves hydroxylation of its precursor

amino acid, L-tryptophan, into L-5-hydroxytryptophan. This is a rate-limiting reaction and is facilitated by an enzyme called tryptophan hydroxylase (TPH) [13]. There are two isoforms of the TPH enzyme, TPH_1 and TPH_2 [13]. Within the neurons of the brain, the TPH_2 isoform is the one responsible for serotonin production. The second reaction in the production process involves a decarboxylation of the product obtained in the first reaction, which is achieved by an L-amino acid decarboxylase (DDC) enzyme (figure 1) [13]. Upon production, 5-HT is readily transported into secretory vesicles via vesicular monoamine transporters (VMAT) where it remains stored in presynaptic neurons [39].

In addition to synthesis within the raphe neurons of the brain stem, 5-HT is also synthesized throughout the gastrointestinal tract. Enterochromaffin cells (EC) lining the gut are the major source of peripheral 5-HT. EC are responsible for approximately 95% of circulating 5-HT levels in humans, and the remaining 5% is produced in the brain stem [6]. In the gut, 5-HT production takes place within the EC in a similar mechanism to central production, however, via TPH_1. Once it is produced, 5-HT molecules may be released from the base of EC in response to external stimuli at the apical region of the cell [39]. When produced peripherally, the majority of serotonin in the gut is transported inside platelets within the blood for storage [11]. Platelets also possess 5-HTT on their membranes and are able to utilize it for 5-HT uptake.

Depending on its site of synthesis, 5-HT has diverse functions. In the brain stem, 5-HT behaves as a neurotransmitter, where it is responsible for mood regulation [5]. Peripherally, however, it behaves as a hormone, signaling many cells as it travels through the blood stream [6]. Under normal physiological conditions, 5-HT cannot unreservedly cross the blood-brain barrier, and therefore, its function at one location should be thought of as independent of the other [5] and [6].

1.3.1. SEROTONIN AND BONE BIOLOGY

Although poorly understood, increasing evidence proposes 5-HT to be a substantial factor in the regulation of bone quality and metabolism. 5-HT mediates its effects via membrane bound receptors within the $5\text{-}HTR_1$ and $5\text{-}HTR_2$ family, some of which are found on all major bone cell types, including, osteoblasts, osteoclasts and osteocytes [4] and [6]. Additionally, direct serotonin synthesis through TPH_1 by bone cells has been documented [44]. The presence of serotonergic

receptors and 5-HTT on bone cells, in addition to their ability to synthesize serotonin, suggests an important role for the neurotransmitter in bone metabolism, and therefore, complex cellular mechanisms may be affected by the excessive stimulation of these receptors with continual SSRI use.

Increasing indications within the literature regarding the link between SSRI use and increased risks of fractures and markers of bone resorption are evident [26]. Studies investigating the effects of serotonergic signaling on bone quality utilized mice with a knockout gene for 5-HTT. Their results revealed substantial decreases in bone density and architecture [37]. Furthermore, reports of SSRI bioaccumulation in the bone marrow are evident, and at much higher concentrations than those detected in the blood or neurons of the brain [36]. Serotonin produced within the central nervous system, however, seems to have the opposite effect, favoring bone mass accrual. The sympathetic nervous system is a known modulator of bone formation and resorption, favoring a decrease in bone mass accrual [27]. Serotonin produced centrally constrains such sympathetic output, enhancing bone mass accrual [27]. Furthermore, recent research involving mice with a Tph2-knockout, which is the enzyme responsible for 5-HT production in the brain, showed a reduction in number of osteoblasts, rate of bone formation and bone volume [27]. This suggests that serotonin may affect bone metabolism differently, depending on its origin.

1.3.2. SEROTONERGIC SIGNALING IN BONE

The majority of systemic 5-HT responsible for effects on bone is produced peripherally and kept inside platelets in the blood. Platelets also express 5-HTT, and are able to uptake extracellular 5-HT molecules from the blood and store it inside dense granules. Stored 5-HT molecules may be released upon activation or lysis of platelets. SSRI use may also raise 5-HT concentrations by blocking 5-HTT located on the platelets, inhibiting uptake of molecules from the surrounding space. This results in higher systemic 5-HT levels.

Serotonin exerts its multitude of functions by signaling through its numerous receptors. The monoamine behaves as a hormone in the blood, as well as locally, in an autocrine and/or paracrine manor. It exhibits its effects through complex signaling mechanisms involving many of the serotonin receptors (5-HTR) located on plasma membranes of various cell types. Numerous serotonin receptors have been discovered, ranging between 5-HTR_1 to 5-HTR_7 [22]. However, the

focus of this work will be on those within the 5-HT_1 and 5-HT_2 families, as these are the ones known to be involved in modulating serotonergic effects on bone.

Recent studies confirm the functionality of serotonin signaling in bone, with serotonin production by the EC of the gut being the most responsible for mediating skeletal effects. Peripheral serotonin found in the blood may bind to its HTR_{1B} receptor located on osteoblasts and inhibit cAMP response element binding (CREB) protein. This protein is a transcription factor responsible for both positive and negative regulation of gene transcription. Serotonin signaling via HTR_{1B} has been shown to inhibit Creb expression, and as a result, prevent osteoblastic proliferation [14].

1.3.2.1. 5-HT_1 AND 5-HT_2 RECEPTORS

Serotonin receptors are composed of 7 different subfamilies, ranging from HTR_{1-7} [21], however, the focus of this work will be on those expressed in bone and involved in bone metabolism. The 5-HT_1 receptor family includes 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}. Receptors within the 5-HT_1 family are G-protein coupled receptors of the G_i class. They are coupled to two specific effector systems, where they are involved in inhibition of adenylyl cyclase activity, as well as opening of potassium (K^+) channels [20]. Within the 5-HT_1 family, this work will focus on the 5-HT_{1A} and HTR_{1B} receptors as they have been located on bone cells and their functions are involved in modulating bone metabolism. Many of the 5-HT_{1A} receptors can be found within the hippocampus, hypothalamus, amygdala, as well as in the serotonergic cell body regions of the central nervous system [20].

Serotonergic neurons utilize various mechanisms for self-control and regulation, one of them involving the 5-HT_{1A} receptor, an inhibitory, auto-receptor that is responsible for suppressing serotonergic activity. These receptors are activated by interactions with local serotonin. The binding of serotonin to 5-HT_{1A} initiates the opening of K^+ channels, which leads to hyperpolarization of the cell membrane and ultimately, inhibition of cell firing [38]. Effects of antidepressants are more closely associated with the 5-HT_{1A} receptor. It has been shown that long term treatment with antidepressants increases serotonergic transmissions via mechanisms mediated by the 5-HT_{1A} receptor in the hippocampal regions of the brain [38]. Continuous antidepressant use downregulates 5-HT_{1A} receptors in the neurons of the brain, and as a result,

enhances firing of 5-HT neurons for improved serotonergic transmission, promoting an anti-depressive effect.

The 5-HT_2 receptors include 5-HT_{2A}, 5-HT_{2B} and 5-$HT_{2C.}$ They are located in the prefrontal cortex, hypothalamus, throughout the spinal cord, the choroid plexus, and the cerebral cortex [38] and [20]. All receptors within this family are responsible for stimulating phosphoinositide-specific phospholipase C (PI-PLC) [20]. These receptors are therefore thought to be involved in modulations of emotional states, cognitive functions and serotonergic activity [20]. Unlike the inhibitory effects of the 5-HT_{1A} receptor, 5-HT_{2A} activation results in an increase in pyramidal activity within the prefrontal cortex [38].

1.4. BIOMATERIALS

Biomaterials are synthetic resources commonly used in clinical applications to aid in the healing and regeneration of damaged or diseased tissues. The main goal of a biomaterial is to integrate with the body and reinstate normal tissue functioning post disease or injury. Biomaterials applied in dental and orthopedic applications are utilized in the replacement of damaged hard tissues in events of atrophy, trauma or disease [18]. Dental implants are surgically anchored in bones of the jaw or skull in order to support tooth prosthetics. Successful biomaterials encourage healing post implantation by promoting new tissue formation while minimizing undesirable biological responses.

Implant location and function determine a biomaterial's requirements. In order for these materials to be successful in bone applications, they must exhibit excellent biocompatibility and provide great load-bearing capacity [17]. Dental applications require a biomaterial to possess high yield and fatigue strength to overcome cyclic loading forces present during mastication. Due to their suitable biomechanical properties, the most commonly used biomaterials for orthopedic and dental applications are metals. Pure titanium (Ti) as well as some of its alloys, predominately titanium, aluminum and vanadium (Ti6Al4V), are the most commonly used metals for dental applications [17].

1.5. OSSEOINTEGRATION

The success of biomaterials in dental implantology is largely dependent on interactions at the interface between the material's surface and the host bone. When an implant is placed, newly

formed bone must establish a firm and direct connection with the surface of the implanted material in order for it to be well secured and functional [7]. This process is referred to as osseointegration. An implant is acknowledged as successfully osseointegrated by the lack of relative movement at the connection between its surface and the bone. Implant characteristics, such as material type, surface topography and chemical composition can greatly influence this process. Other factors, such as the quality of the host bone or pharmacological agents are also great contributors to the success of this process.

Implant failure due to inadequate osseointegration may be due to impaired healing responses, infections, such as peri-implantitis, or mechanical overloading [3]. Osseointegrated failures associated with the inability to properly heal are usually evident within the first few weeks or months post implantation [3]. Such early failures may be a result of the inability to of the implant to successfully osseointegrate with the surrounding bone as a result of poor bone formation or quality, causing mechanical instability and ultimate failure. Peri-implantitis related failures are usually evident in the second year after implantation [3].

1.6. TITANIUM

Some of the most widely used biomaterials for dental implant applications are composed of either pure or alloyed titanium (Ti) [17]. Commercially pure Ti has been used for many years in dental and orthopedic applications due to its corrosion resistance, high strength yet low modulus of elasticity and excellent biocompatibility [17] and [16]. Upon air exposure, Ti is able to spontaneously form a stable oxide layer on its surface [19]. This surface oxide film production is what allows the material to remain biologically inert and resistant to corrosion after implantation [19]. Increased biocompatibility elicits a favorable biological response to the implanted material, promoting bone formation and faster osseointegration.

A dental implant's success is highly dependent on the implanted material's ability to encourage osseointegration. Once a dental implant is placed, it is initially minimally stabilized by frictional forces as it becomes interlocked between the existing bone within the jaw. For the implant to be successful, it must be firmly fixed by establishing direct contact with the surrounding bone tissue during the following weeks after implantation. The surrounding host bone tissue undergoes remodeling, where some bone is resorbed and replaced by newly formed bone. As the

surrounding bone is remodeled, newly formed bone gets deposited on the implant's surface, allowing for direct contact with the material. Although Ti has proven to be one of the best materials for such applications, dental implant failure remains a dilemma. Topographical modifications performed at the surface of the material are commonly used and can further enhance its clinical efficacy in dental applications.

1.6.1. TOPOGRAPHICAL MODIFICATIONS

A material's surface topography has a crucial influence on cellular responses, and therefore is a great contributor to the general success or failure of a dental implant after it has been introduced in a host. In order to enhance the body's biological response to an implant, topographical modifications can be applied at the implant surface. Altering material surface properties allows for control of cellular responses surrounding an implant, ultimately attaining efficacious clinical results. Since the majority of a dental implant's success largely depends on cell-material interactions at its surface, topographical alterations can be done to enhance its success while still maintaining its desirable bulk material properties. Applying a superficial treatment to increase the material's roughness and surface area allows for greater cell-implant adhesion. Increasing the surface roughness of an implant expands its surface area that is adjacent to bone tissue and encourages cellular attachment and proliferation. These events aid in improving the osseointegration process [29]. Modifications to a material's surface can be made by utilizing additive or subtractive approaches [29]. Additive methods, such as a plasma sprayed hydroxyapatite or a calcium phosphate coating, involve applying a treatment to cover the material's surface. Alternatively, subtractive methods involve either the removal of a portion of the material's top surface or applying physical deformations to create a roughened microtopography.

1.6.1.1. SANDBLASTING, LARGE-GRIT AND ACID ETCHING

In order to improve a dental implant's mechanical anchorage, numerous surface treatment techniques are performed to enhance the biological response of the material. Subtractive techniques, such as sandblasting and acid etching (SLA), are utilized to achieve roughened surfaces. Modifications to increases the surface energy (modified SLA/mSLA) are also used to enhance implant success (figure 2). SLA surfaces are produced by subjecting Ti implants to large-

grit sand elements followed by submersion in a heated, strong acid [29]. Such treatments not only clean the implant surfaces, but also create a micro-roughened superficial texture. mSLA surfaces are prepared from the same SLA technique, however, under nitrogen conditions to prevent hydrocarbon contamination and maintain hydrophilicity.

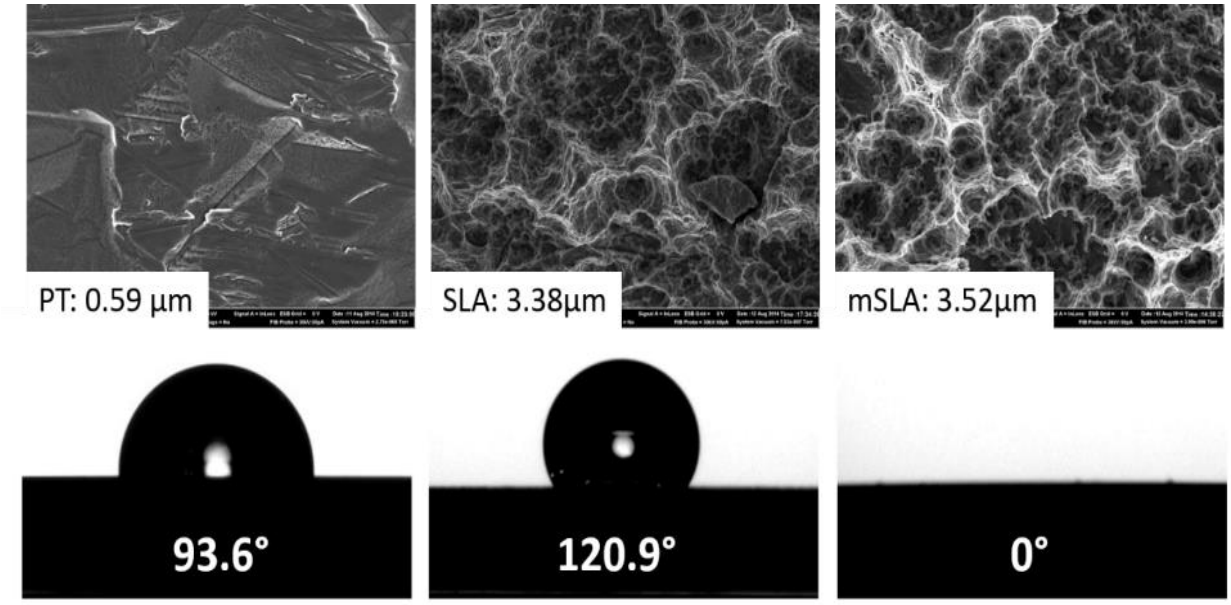

Figure 2: Quantitative scanning electron microscopy images and contact angle analyses of Ti surface topography. Images were taken at 5kx magnification for PT, SLA and mSLA surfaces with the corresponding wettability as measured by contact angle values.

Prior studies have shown that utilizing techniques to create rough surface topographies with high energy, such as SLA and mSLA surfaces, have proven to enhance cell attachment and osteoblastic lineage cell differentiation in comparison to smoother topographies [16] and [24]. By increasing the surface roughness, the material's surface area also increases, thereby allowing a larger area for protein-cell-material interaction and improving cellular attachment and adhesion [29]. Furthermore, SLA surfaces mimic the normal physiological structure of remodeled bone. Thus, utilizing a topography that is most similar to that of the natural state provides for better contact between the implant's surface and the surrounding bone and ultimately an improved healing response.

1.7. INITIAL INTERACTIONS BETWEEN CELLS AND MATERIALS

Moments after an implant is introduced in the body, it makes contact with the host blood, where a sequence of cascading healing processes are initiated, beginning with protein adsorption.

Proteins present in the blood interact with and become adsorbed to the material's surface until a monolayer is quickly formed. Inhabiting cells on a material's surface do not actually attach to the surface directly, but rather to the layer of adsorbed proteins. Once the proteins are attached to the surface of the material, cells can make contact with these proteins and attach as well. This chemical bond formation at the cell-material interface is what promotes the healing process by facilitating implant fixation and reduced loosening.

The composition and arrangement of proteins adsorbed at the implant surface is regulated by the material's surface properties, such as chemical composition and microstructured topography [32]. Furthermore, this arrangement of the adsorbed proteins also influences the lineage progression of the attached cells by enhancing integrin binding [8]. Osteoprogenitor cells initiate attachment to the adsorbed layer of proteins on an implanted material via integrins. Integrins are transmembrane receptors, composed of α and β subunits, acting as bridges for cell-protein interactions [8] and [30]. The binding of integrins to the extracellular matrix (ECM) proteins induces mechanical stresses in the cytoskeleton of the cell, which stimulates intracellular signaling pathways involved in gene expression and osteogenic cell lineage differentiation [8].

This process is critical for initiating osseointegration of the implant with the surrounding host bone. Studies supported this by showing that osteoblasts grown on roughened, microstructured Ti surfaces increased integrin gene expression when compared to smooth Ti and TCPS [30]. Thus, material surface properties do not only regulate protein adsorption on the implant's surface, but also influences cellular attachment, adhesion and differentiation.

1.8. MESENCHYMAL STEM CELL DIFFERENTIATION

The majority of the bones making up the mammalian skeleton, with the exception of the calvaria and other flat bones, originate from mesenchymal progenitors. Mesenchymal stem cells (MSCs) are known to be self-proliferative and multipotent. Given the proper stimuli, MSC are capable of differentiating into any of the following lineages: adipocyte, chondrocyte or osteogenic [15], [23] and [31]. MCSs reside in the bone marrow and the periosteum on the outside surface of bone, and in the presence of osteogenic supplements, are capable of differentiating into an osteoblastic lineage [31]. However, prior studies have demonstrated that rough, microstructured Ti surfaces alone were successful in differentiating human MSC into osteoblasts, without the

addition of osteogenic supplements [16]. Upon differentiation, osteoblasts form bone by creating and depositing bone matrix.

MSCs are initially recruited from the bone marrow to the implant site through various signaling factors secreted by platelets and immune cells. The cells travel via blood and through the clot to reach the implant's surface, where they bind to the adsorbed proteins on the implant surface via integrins and begin differentiating into an osteoblastic lineage. Differentiation of MSCs into an osteoblastic lineage involves complex cell-cell and cell-protein communication, which greatly contributes to an implant's success. Multiple soluble factors produced and secreted by local and distal progenitors are required for the survival of these cells, as well as healing and regeneration surrounding the implant. Newly differentiated osteoblasts on the surface of the material allows the implant to become better integrated with the surrounding host bone.

When MCSs are grown on microstructured Ti surfaces, without exogenous addition of osteogenic supplements, produce markers known to be expressed by osteoblasts during bone formation [8]. Initially, cells produce alkaline phosphatase (ALP) during early stages of differentiation and levels decline as the cells continue to later stages [34]. Expression of runt-related transcription factor 2 (RUNX2) by osteoprogenitor cells is vital for bone formation. This protein belongs to the RUNX family and is a master transcription factor in regulating osteoblastic differentiation [25]. RUNX2 expression is upregulated in preosteoblast cells and is often measured as an early marker of osteoblastic differentiation [34]. ALP activity also reaches a peak prior to matrix mineralization and is a reliable measurement of early osteoblastic differentiation [40]. During later differentiation stages, Runx2 expression declines and osteoblasts produce and secrete osteoclacin (OCN), also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP) [34] and [35]. This is a non-collagenous protein that is vital for bone mineralization, and it is promoted by the initial presence of Runx2 [34]. OCN is typically measured as a late marker of osteoblastic differentiation.

1.9. LOCAL FACTOR PRODUCTION

MSC differentiation is heavily regulated in an autocrine and paracrine fashion by transcription and growth factors secreted from surrounding osteogenic cells at a distal location and present within the microenvironment. The secreted biochemical stimuli are vital for influencing

MCSs in their journey to becoming osteoblasts, or bone forming cells. Osteogenic factor production is a key element in bone formation, osseointegration and implant success. Many of these molecules include osteogenic factors such as bone morphogenetic protein 2 (BMP2), which is a potent osteoinductive agent important for stimulating MSC differentiation towards an osteoblast [33].

Bone formation requires access to blood supply, and for that reason, osteogenic cells produce and secrete factors like vascular endothelial growth factor (VEGF), an important agent for angiogenesis, or blood vessel formation [33]. Previous studies illustrated the capability of microstructured Ti surfaces in inducing greater levels of osteogenic factor production by MSC in comparison to smoother Ti topographies or tissue culture polystyrene (TCPS) surfaces [8]. Osteogenic lineage cells grown on such surfaces had greater production of osteogenic factors such as BMP-2 and angiogenic growth factor production of VEGF was also enhanced [8] and [30]. The microenvironment maintained by distal osteogenic cells is also important for proper bone formation. Local factor production of other important proteins by osteogenic cells regulate the bone remodeling process. This process is vital for preserving bone density and quality. Even slight misregulation in this process can have substantial effects on the normal bone physiology.

1.10. BONE REMODELING

The skeletal system is a highly dynamic organ that is responsive to various stimuli, and as a result, is continuously renewed. Bone tissue is in a constant state of renovation, as controlled by two types of specialized cells: osteoblasts and osteoclasts. This process, known as bone remodeling, involves the removal of superficial cortical and trabecular bone by osteoclasts, followed by its subsequent replacement with new bone matrix deposited by osteoblasts [25]. MCSs are the precursors for osteoblasts, while osteoclasts are giant, multinucleated cells originating from hematopoietic cells of the monocyte and macrophage lineage [21] and [13]. In the presence of macrophage-colony stimulating factor (M-CSF) and receptor activator for NF-KB ligand (RANKL), osteoclast precursors are known to differentiate into mature osteoclasts [45]. M-CSF promotes development and expression of receptor activator of NF-KB (RANK), a receptor for RANKL that is located on the surface of osteoclasts.

Bone remodeling is sensitive to various stimuli and can be altered by the mechanical forces of walking or systemic hormonal fluctuations [22]. Continuously renewing mineralized tissue ensures proper growth, maintenance and repair of the skeletal system. Under normal physiological conditions, tissue formation precedes at a similar rate as matrix resorption, and in a site-specific manner, in order to maintain adequate bone quality. Thus, osteoblasts and osteoclasts must simultaneously coordinate their activities to balance formation with resorption. Mineralized bone is resorbed by the osteoclasts, creating resorption pits. These resorbed areas become replaced with newly formed bone matrix by the osteoblasts [30]. In order for this process to be tightly controlled, the specialized cells produce and secrete local factors for communication with one another.

1.10.1. OPG, RANK AND RANKL

Bone remodeling is largely regulated by RANK, RANKL and its decoy receptor, osteoprotegerin (OPG) [25] and [22]. Local regulation of these secreted factors is vital for modulating bone remodeling and balancing the rate of bone formation with resorption. Osteoblastic lineage cells produce RANKL throughout their differentiation process. RANKL is an essential protein involved in the formation, activation and function of osteoclastic cells [25]. Stimulation of RANK by RANKL on osteoclasts promotes osteoclastogenesis and osteoclastic activity. The binding of RANKL to its receptor, RANK, on the surface of osteoclast precursors promotes the fusion and formation of a multinucleated osteoclast. RANKL/RANK interactions also activate and initiate bone resorption on mature osteoclasts [25]. Bone resorption is marked by higher levels of osteoclastic activity, which relates to increased production of tartrate-resistant acid phosphatase (TRAP). Osteoclasts differentiated from the monocyte lineage are known to produce and secrete TRAP enzymes on the surface of the bone matrix. Mature osteoclasts participate in bone remolding by increasing this enzymatic activity at their ruffled borders on the surface of bone, creating various resorption pits. In order to regulate osteoclastic activity, osteoblastic lineage cells also produce and secrete OPG as a means for overriding bone resorption. OPG binds to RANKL in order to prevent its binding to RANK on the osteoclast surface, thereby reducing osteoclastic activation and resorption [25]. Therefore, bone remodeling is controlled by the relative concentrations of these proteins locally, which are used as a form of communication between

osteoblasts and osteoclasts. Imbalances in secreted RANKL/OPG ratios by osteogenic cells disrupts baseline levels and has the potential to affect the quality of bone.

1.11. SSRI USE AND DENTAL IMPLANTS

SSRIs are the most effective in treating depression due to their high selectivity and potency for 5-HTT, which enables them to increase serotonin levels in the brain in much higher concentrations when compared to other types of antidepressants. However, SSRIs increase systemic serotonin levels as well, and elevated peripheral serotonin concentrations may have a detrimental impact on other cells in the body, such as bone cells. Recent research suggests serotonin as a substantial regulator involved in physiological control of bone mass, and such effects are thought to be linked to serotonergic signaling in bone. Taking into account SSRI influence on peripheral serotonin concentrations, chronic use may severely affect bone remodeling and quality, ultimately reducing implanted biomaterials' success.

Although dental implants are very successful in establishing a firm connection with the host bone, implant failures remain evident in patients compromised by disease, old age or chronic prescription use. Implant success is not only dependent on secured stabilization, but also on the quality of the recipient's bone which surrounds the implanted material. Mounting evidence links SSRI use with decreased bone mineral density, increased risk of fracture and dental implant failure as compared to nonusers [3, 26 and 36]. Failures were shown to occur between the first 4 and 18 months post implantation [3]. Excessive peripheral serotonergic signaling may disrupt maintenance of skeletal remodeling processes required for preservation of healthy bone quality and adaptation to mechanical stimuli, and therefore, may be the probable cause of dental implant failure.

The use of dental implants is becoming increasingly popular in the United States, as more than 5 million are placed per year, and numbers are expected to continue increasing by an annual rate of 15% [43]. These statistics are alarming when taken in consideration with the overwhelming depression rates and SSRI use. Bioaccumulation of SSRIs in the bone marrow is also a major concern, as the drugs are known to sequester in those locations at much greater concentrations than those in the blood or brain. Due to the chronic nature of MDD, it is standard practice for newly diagnosed patients to be prescribed an antidepressant, typically an SSRI, for at least 12 months

[28]. However, most patients continue this regimen for much longer periods, and in some cases, throughout their lifetime. Thus, SSRI use may have deleterious effects on the healing and regeneration capability of progenitor cells recruited from reservoirs in the bone marrow for new bone formation surrounding an implanted biomaterial.

1.12. SPECIFIC AIMS

In order to be successful, an implanted material must osseointegrate with the surrounding host bone to establish a firm connection. Since this process is highly dependent on the quality of host bone, recipients taking medications affecting bone metabolic mechanisms, such as antidepressants, increase their risk of osseointegrated implant failure. The **main objective** of this research is to understand how antidepressants can affect bone formation by MSC differentiation and local protein production in the microenvironment by these cells on clinically relevant Ti biomaterials commonly used in dental applications. Studies in this work utilize a novel *in vitro* model for human osteoblastic differentiation from early MSC precursors using only Ti surface characteristics to induce differentiation into mature osteoblasts. This model was used as a tool to investigate the effects of antidepressants on dental implant failure by assessing their effect on bone formation by osteoblastic differentiation and bone resorption by osteoclastic TRAP activity, and how these effects can be modulated by the implant's surface characteristics. The **main *hypothesis*** *is that antidepressants will prevent human MSC differentiation, decrease local protein production associated with bone formation and quality and increase proteins involved in bone resorption surrounding Ti biomaterials, ultimately delaying the osseointegration process and diminishing dental implant success.*

Specific Aim 1: Investigate the effects of SSRIs on MSC differentiation on microstructured Ti. Antidepressants exhibit their therapeutic effects by modulating extracellular levels of serotonin. It has been shown that serotonin is involved in bone metabolism and all major types of bone cells possess serotonin receptors and the transporter. Microstructured Ti surfaces enhance MSC differentiation by increasing local angiogenic and osteogenic factor expression and production. However, effects of antidepressants on MSC differentiation during interactions with Ti surfaces have yet to be determined. The **objective** of this aim will be to assess the effects of antidepressant treatment on human MSC differentiation by measuring early and late osteoblastic

differentiation markers and local factor production by cells cultured on smooth (PT) or microstructured (SLA) Ti and compared to TCPS surfaces. The ***hypothesis*** *for this aim is that human MSC differentiation will be enhanced by Ti surface roughness, however, treatment with antidepressants will prevent differentiation, with SSRIs having the most detrimental effects on osteoblastic differentiation in comparison to other classes of antidepressants*. Levels of proteins associated with bone formation, modulators of bone remodeling, angiogenesis and markers of osteoblastic differentiation will be measured. Since antidepressants modulate levels of serotonin, the effects of serotonin treatment on MSC differentiation and local factor production will also be investigated.

Specific Aim 2: Determine the effects of SSRIs on bone remodeling signaling and osteoclast activation. Osteoclastic activity is initiated by the binding of RANKL to its receptor, RANK, on the osteoclast surface. Higher osteoblastic secretions of RANKL increase production of TRAP by osteoclasts and promote bone resorption. Misregulation of the RANKL/OPG ratio in the microenvironment has negative consequences on the quality of bone and ultimately osseointegration of implants. The **objective** of this aim is to understand how direct exposure of osteoclast precursors to antidepressants, or exposure to factors in the microenvironment in conditioned media obtained from MSCs treated with antidepressants, can affect osteoclastic activity. The ***hypothesis*** *is that treatment of osteoclasts with antidepressants, or conditioned media from MSCs treated with antidepressants, will increase osteoclastic TRAP activity.* For this aim, MCS-F and RANKL-treated osteoclast precursors will be directly exposed to antidepressants or conditioned media and TRAP enzymatic activity will be assessed.

Specific Aim 3: Elucidate the effects of SSRIs on serotonin receptors and their effect on bone remodeling. Bone cells utilize serotonin in metabolic processes and express serotonin receptors and serotonin transporters on their cell membranes. However, whether surface characteristics of Ti biomaterials can modulate MSC gene expression of serotonin receptors has not been determined. The **objective** of this aim will be to confirm if surface roughness and wettability can alter expression of serotonin receptors by human MSCs, and how this expression can be modulated with antidepressant treatment. Additionally, the role of MSC serotonin receptors in the production of OPG and RANKL will be assessed. The ***hypothesis*** *for this aim is that surface*

characteristics will alter MSC gene expression of serotonin receptors, as well as OPG and RANKL protein production, and treatment with antidepressants will modulate these effects. For this aim, mRNA levels of MSC serotonin receptors on smooth or rough Ti surfaces with and without serotonin or antidepressant treatments will be examined and compared to TCPS surfaces. To determine whether serotonin receptors are involved in OPG and RANKL production, MSCs will be grown on smooth or rough Ti surfaces and treated with specific inhibitors for each serotonin receptor in the presence of physiological concentrations of serotonin. Protein levels for OPG and RANKL will be measured.

CHAPTER 2 METHODS, MATERIALS AND RESEARCH DESIGN

2.1. SPECIFIC AIM 1: INVESTIGATE THE EFFECTS OF SSRIS ON HUMAN MSC DIFFERENTIATION ON MICROSTRUCTURED TI

Studies performed in aim 1 are to determine the differentiation capability of human MSCs on various Ti surfaces under exposure to most frequently prescribed antidepressants within the SSRI class (fluoxetine, sertraline and paroxetine), the SNRI class (duloxetine) as well as other antidepressants not belonging to a specific category (trazodone and bupropion). Since antidepressants increase extracellular serotonin concentrations in the body, the effects of serotonin treatments on MSC differentiation was also assessed as a positive control. Cells not treated with serotonin or antidepressants were a negative control. The hypothesis is that treatment with antidepressants will prevent MSC differentiation.

2.1.1. AIM 1.1: DETERMINE THE EFFECTS OF ANTIDEPRESSANT TREATMENT ON MSC DIFFERENTIATION WHILE CULTURED ON SMOOTH VS. ROUGH TI SURFACES

The purpose of aim 1.1 is to examine whether antidepressant treatment prevents osteoblastic differentiation of MSCs, and how this is affected by increases in surface roughness. For these studies, clinically relevant Ti surfaces utilized in dental implant applications were used to assess differentiation capability. All surfaces used in these studies were generated by producing 15mm diameter cut outs from grade 2 unalloyed Ti sheets of 1mm thickness obtained from Institut Straumann AG (Basel, Switzerland). Disks were cut out into 15mm to ensure an accurate fit in the wells of a 24 well tissue culture plate. Smooth, pretreatment (PT) surfaces were created by treating the disks with acetone for degreasing purposes, then processing in a 55°C 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution for 30 seconds. Rough (SLA) surfaces were created by sand blasting and acid etching PT surfaces with 0.25-0.50mm corundum grit and HCl/H_2SO_4, respectively.

Human bone marrow-derived MCSs (Lonza, Walkersville, MD) were commercially obtained. Cells with passages between 4 and 5 were plated at a 10,000 cells/cm^2 density in the wells of a 24 well plate and cultured in 0.5 mL per well of Mesenchymal Stem Cell Growth

Medium (MSCGM, Lonza). PT and SLA disks were placed in the wells of a 24 well plate (n=6) and MSCs were cultured on the surface of the disks (figure 3). Cells were cultured on TCPS, PT or SLA surfaces at 37°C with 5% CO_2 and 100% humidity. MSCs were grown for 7 days in MSCGM and in the absence of exogenous supplements. In prior studies, MSCs have been shown to produce an osteoblastic phenotype after 7 days of culture on microstructured Ti surfaces, without the addition of osteoblastic differentiation supplements [31]. The same model of

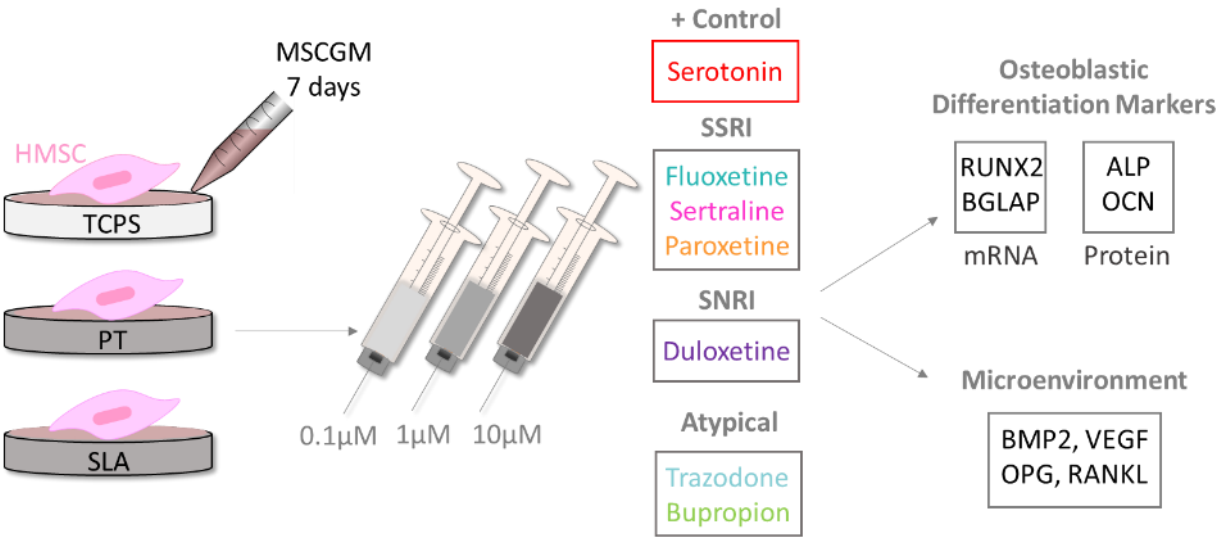

Figure 3: Specific Aim 1 Research Design. Human MSCs were cultured on TCPS, PT or SLA surfaces in MSCGM for 7 days throughout differentiation while being exposed to 0.1µM, 1µM or 10µM concentrations of antidepressants within the SSRI or SNRI categories, as well as other types of commonly prescribed antidepressants. Gene expression and protein levels for early and late osteoblastic differentiation markers and production of proteins in the microenvironment associated with bone formation and quality were assessed.

differentiation was adapted for MSC differentiation studies in this aim and in all upcoming studies in this work.

For pharmacological treatments, cells were treated with either an antidepressant or serotonin. Agents including bupropion, duloxetine, fluoxetine, serotonin, sertraline, paroxetine and trazodone (Cayman Chemical) were reconstituted in DMSO (Sigma) to stock concentrations of 1mM or 10mM and stored at -20°C. Human MSC were cultured as described above and media was changed after the first 24 hours post plating, then again every 48 hours for the remainder of the 7 days. Cells were treated with either 1µM or 10µM concentrations of media containing either an SSRI (fluoxetine, sertraline and paroxetine), an SNRI (duloxetine), another antidepressant

(trazodone and bupropion) or serotonin for 7 days throughout their differentiation. Pharmacologically treated cells were compared to cells cultured with media containing 10μM of DMSO as the control. After 7 days, all cells were incubated with fresh MSCGM for 24 hours, without any pharmacological treatment. After 24 hours, conditioned media was collected, cells were harvested from each surface and lysed. ALP activity (early osteoblastic differentiation marker) was assessed and normalized to total protein content in each well. An Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure protein levels for OCN (late osteoblastic differentiation marker) (AlfaAesar). OCN protein levels were normalized to total DNA content in each well using a Quant-iT$_{TM}$ PicoGreen dsDNA Assay Kit (Life Technologies) in cell lysates, measured according to the manufacturer's instructions.

In a second study investigating effects of antidepressants on osteoblastic gene expression, MSCs were cultured for 7 days and treated with either an antidepressant or serotonin, as described above. After 7 days, all cells were incubated with fresh MSCGM for 12 hours, without any pharmacological treatment. Cell supernatants were collected after 12 hours, cells were harvested and RNA was extracted using the TRIzol® (Life Technologies) extraction technique. NanoDrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts) was utilized to quantify mRNA. To convert RNA to complementary DNA (cDNA), reverse transcription polymerase chain reaction (RT-PCR) was performed on 750ng of RNA using a High Capacity Reverse Transcription cDNA Kit (Life Technologies). Real-time quantitative polymerase chain reaction (qPCR) was performed for analysis of RUNX2 (early) and OCN (late) osteoblastic differentiation genes using Power SYBR® Green Master Mix (Life Technologies) and primers specific to each gene of interest in StepOnePlus Real-Time PCR Systems (Life Technologies). A standard curve was generated using human MSCs cultured on TCPS and all gene expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.1.2. AIM 1.2: DETERMINE THE EFFECTS OF ANTIDEPRESSANT TREATMENT ON MSC LOCAL FACTOR PRODCUTION OF BMP2, OPG, RANKL AND VEGF PROTEIN LEVELS IN THE MICROENVIRONMENT ON TI SURFACES

The purpose of aim 1.2 is to determine if antidepressant treatments affect MSC protein production in the microenvironment and if these effects can be modulated by increases in surface roughness. MSCs were cultured as described above. Treatments of either 1μM, or 10μM

concentrations of media containing either an SSRI (fluoxetine, sertraline and paroxetine), an SNRI (duloxetine), other types of antidepressants (trazodone and bupropion) or serotonin were given to cells for 7 days throughout differentiation. Pharmacologically treated cells were compared to cells cultured with media containing 10μM of DMSO as the control. After 7 days, all cells were incubated with fresh MSCGM for 24 hours, without any pharmacological treatment. After incubation in fresh MSCGM for 24 hours, conditioned media was collected. ELISA (PeproTech) was used to measure secreted protein levels in the media for BMP-2 (osteogenic marker), RANKL (osteoclast activator) and VEGF (angiogenic factor) according to the manufacture's protocol. Secreted protein levels for OPG (osteoclast inhibitor) (DuoSet ELISA) were also measured. All secreted protein levels were normalized to total DNA content within each well using a Quant-iTTM PicoGreen dsDNA Assay Kit (Life Technologies) in cell lysates, according to the manufacturer's instructions.

2.2. SPECIFIC AIM 2: DETERMINE THE EFFECTS OF SSRIS ON BONE REMODELING SIGNALING AND OSTEOCLAST ACTIVATION

Studies performed in aim 2 are designed to assess whether effects of antidepressants on MSC production of the microenvironment can affect osteoclastic TRAP activity. In these studies, osteoclast precursors will be either directly exposed to antidepressants or conditioned media obtained from MSCs treated with antidepressants. The hypothesis is that treatment of osteoclasts with antidepressants, or conditioned media from MSCs treated with antidepressants, will increase osteoclastic TRAP activity.

2.2.1. AIM 2.1: DETERMINE THE DIRECT EFFECTS OF ANTIDEPRESSANTS ON OSTEOCLASTIC ACTIVITY

Studies in aim 2.1 will be performed to explore whether directly treating osteoclasts with antidepressants affects their osteoclastic TRAP activity. Primary human CD14+ monocytes isolated from peripheral blood were obtained commercially (StemCell Technologies). Approximately 50,000 cells were plated per well in a collagen-coated 48 well plate and cultured in .250mL per well of Roswell Park Memorial Institute (RPMI) medium 1640 (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin. Cells were

differentiated to osteoclasts through RPMI media treated with 20ng/mL and 50ng/mL of human M-CSF and RANKL (Peprotech) supplements for 7 days and media was changed every 3 days.

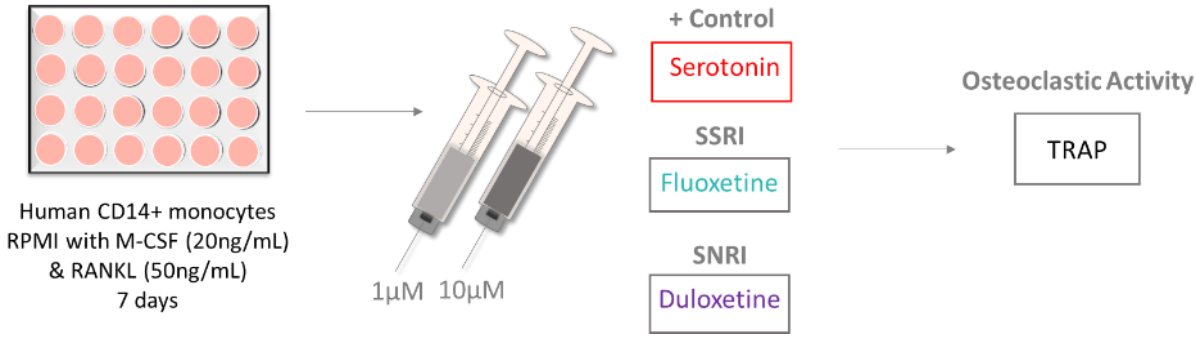

Figure 4: Aim 2.1 Research Design. Primary human CD14+ monocytes were cultured on collagen I coated surfaces in RPMI supplemented with 20ng/mL human M-CSF and 50ng/mL RANKL for 7 days while being exposed to 1μM or 10μM concentrations of antidepressants within the SSRI or SNRI categories. Osteoclastic resorption was assessed by measuring TRAP activity.

Cells were treated with 1μM or 10μM concentrations of RPMI media containing an SSRI (fluoxetine or sertraline), an SNRI (duloxetine), or serotonin for 7 days (figure 4). Pharmacologically treated cells were compared to cells cultured with media containing 10μM of DMSO as the control. Osteoclastic TRAP activity was assessed by measuring total acid phosphatase activity in cell lysates, which was quantified using an Acid Phosphatase Colorimetric Assay Kit (Cayman Chemical), according to the manufacturer's instructions. Colorimetric changes were measured at a wavelength of 405nm and TRAP activity was normalized to protein levels per well.

2.2.2. AIM 2.2: DETERMINE THE EFFECTS OF CONDITIONED MEDIA FROM MSCS TREATED WITH ANTIDEPRESSANTS ON OSTEOCLASTIC ACTIVITY

Studies in aim 2.2 will determine if exposing osteoclasts to the microenvironment generated by MSCs after their treatment with antidepressants has the potential to affect osteoclastic TRAP activity. For these studies, human MSCs and monocytes were cultured simultaneously and as previously described (figure 5). MSCs were plated on PT, SLA or TCPS surfaces and treated with MSCGM containing either an SSRI (fluoxetine and sertraline), an SNRI (duloxetine), or serotonin in 1μM or 10μM concentrations for 7 days throughout differentiation. Media was changed in the first 24 hours post plating, then again every 48 hours for the remainder of the 7

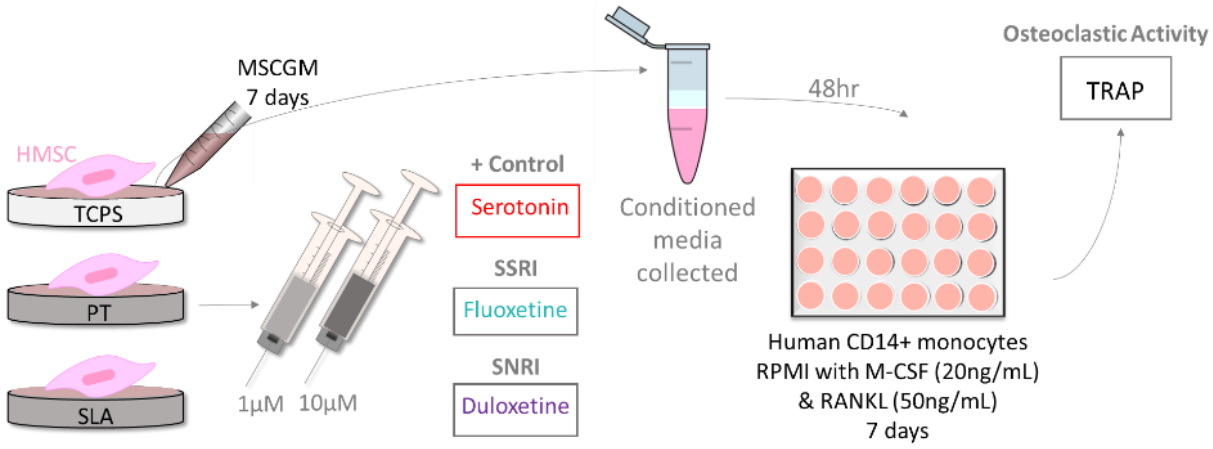

Figure 5: Aim 2.2 Research Design. MCS-F and RANKL-stimulated CD14+ monocytes were exposed to conditioned media collected from MSCs treated with concentrations of 0μM, 1μM or 10μM of fluoxetine, sertraline, duloxetine or serotonin while cultured on TCPS, PT or SLA surfaces.

days. After 7 days, MSCs were incubated with fresh Dulbecco's Modification of Eagle's Medium (DMEM) (VWR) for 24 hours, without any pharmacological treatment. Conditioned media was collected on day 7 and used to treat osteoclasts.

Monocytes were simultaneously plated on collagen-coated surfaces as previously described and treated with RPMI media supplemented with 20ng/mL and 50ng/mL of human M-CSF and RANKL during the same 7 days as the MSCs. On day 7, RPMI media was discarded, and cells were treated with .250mL per well of the conditioned media collected from the MSCs for 48 hours, then cells were harvested and osteoclastic TRAP activity was assessed in cell lysates using Acid Phosphatase Colorimetric Assay Kit as previously described in the prior study and TRAP activity was normalized to protein levels per well.

2.3. SPECIFIC AIM 3: ELUCIDATE THE EFFECTS OF SSRIS ON SEROTONIN RECEPTORS AND THEIR EFFECTS ON BONE REMODELING

The main purpose of aim 3 is to confirm if surface characteristics can alter expression of serotonin receptors by human MSCs, and how this expression can be modulated with antidepressant treatments. In addition, the role of serotonin receptors in the production of OPG and RANKL by MSCs will also be assessed to investigate if receptor behavior affects processes involved in bone remodeling. The hypothesis for this aim is that surface characteristics will alter

MSC gene expression of serotonin receptors, as well as OPG and RANKL protein production, and treatment with antidepressants will further modulate these effects.

2.3.1. AIM 3.1: DETERMINE THE EFFECTS OF TI SURFACE CHARACTERISTICS ON HUMAN MSC SEROTONIN RECEPTOR GENE EXPRESSION

Studies in aim 3.1 will be performed to study the effects of surface roughness and wettability on serotonin receptor gene expression. For this studies in this aim, human MSCs were cultured on PT (smooth), SLA (rough and hydrophobic), or mSLA (rough and hydrophilic) Ti surfaces and compared to those on TCPS. Cells were cultured in MSCGM for 7 days as previously

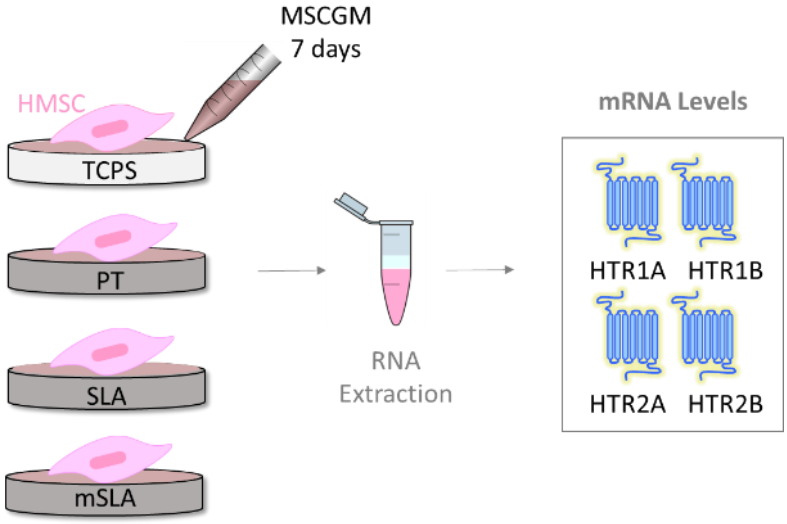

Figure 6: Aim 3.1 Research Design. Human MSCs were cultured on TCPS, PT, SLA or mSLA surfaces in MSCGM for 7 days throughout differentiation. Gene expression of HTR1A, HTR1B, HTR2A and HTR2B serotonin receptors was assessed by qPCR.

described. Cell supernatants were collected after 12 hours of incubation, cells were harvested and RNA was extracted using the extraction technique described above. Levels of human MSC mRNA were quantified and cDNA was obtained by preforming RT-PCR. For gene expression analysis, qPCR was performed for the following serotonin receptor genes: HTR1A, HTR2A, HTR1B and HTR2B. A standard curve was generated using human MSCs cultured on TCPS and all gene expression was normalized to the expression of GAPDH (figure 6).

2.3.2. AIM 3.2: EXAMINE THE EFFECTS OF ANTIDEPRESSANTS AND TI SURFACE CHARACTERISTICS ON HUMAN MSC SEROTONIN RECEPTOR GENE EXPRESSION

Experiments performed in aim 3.2 were to determine if antidepressants affect serotonin receptor expression by human MSCs, and how this expression is affected by surface roughness

when the cells are cultured on rough vs. smooth Ti surfaces. Human MSCs were cultured and grown as previously described. Briefly, cells were exposed to 0.1μM, 1μM or 10μM concentrations of MSCGM containing antidepressants within the SSRI family (fluoxetine, sertraline or paroxetine), SNRI family (duloxetine), other antidepressants (trazodone or bupropion) or serotonin while plated on PT or SLA surface and compared with those on TCPS (figure 7). Serotonin treatments were used as the positive control while treatments with 10μM concentrations of DMSO

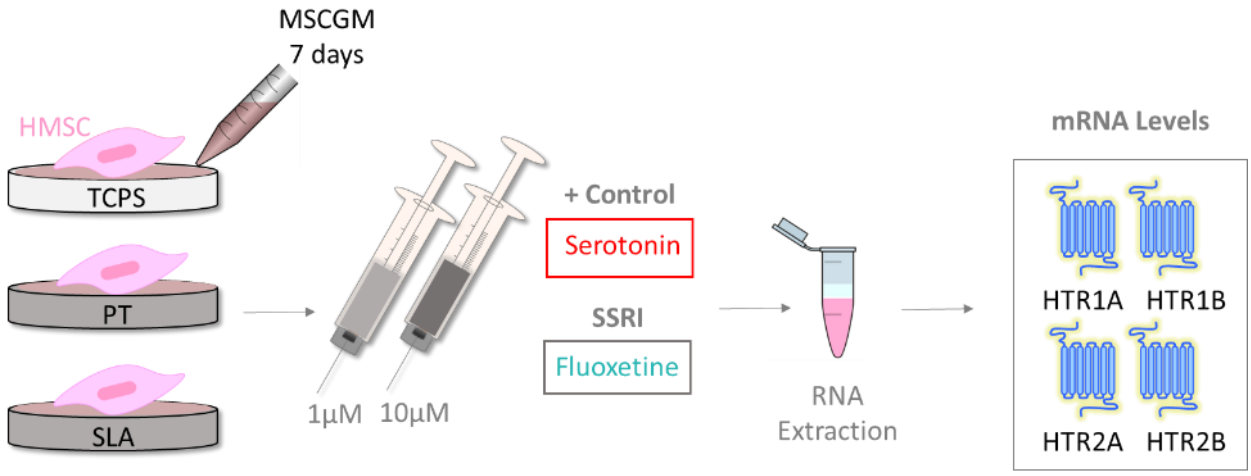

Figure 7: Aim 3.2 Research Design. Human MSCs were cultured on TCPS, PT or SLA surfaces in MSCGM for 7 days throughout differentiation while being exposed to 1μM or 10μM concentrations of serotonin or antidepressants within the SSRI or SNRI categories, as well as other antidepressants. Gene expression of HTR1A, HTR1B, HTR2A, HTR2B serotonin receptors was assessed by qPCR.

in media were used as the no treatment control. Cell supernatants were collected after 12 hours of incubation, cells were harvested and RNA was extracted using the extraction technique described above. Levels of human MSC mRNA were quantified and cDNA was obtained by preforming RT-PCR. For gene expression analysis, qPCR was performed for the following serotonin receptor genes: HTR1A, HTR2A, HTR1B and HTR2B. A standard curve was generated using human MSCs cultured on TCPS and all gene expression was normalized to the expression of GAPDH.

2.3.3. AIM 3.3: DETERMINE THE EFFECTS OF SEROTONIN RECEPTOR INHIBITION ON HUMAN MSC PRODUCTION OF OPG AND RANKL ON TI SURFACES

The purpose of aim 3.3 is to determine the effect of serotonin receptors in modulating bone remodeling processes by human MSC production of OPG and RANKL. For this aim, serotonin

receptors on human MSCs will be pharmacologically blocked using specific inhibitors while the cells are plated on smooth vs. rough Ti surfaces and OPG and RANKL protein levels will be measured. Human MSCs were cultured as previously described on PT or SLA surfaces and compared to those on TCPS and grown in the presence of MSCGM containing 1μM concentrations of the following serotonin receptor inhibitors: WAY-100635 (HTR_{1A}), RH-34 (HTR_{2A}), SB-224289 (HTR_{1B}), RS-127445 (HTR_{2B}) (Cayman Chemical), or a combination of all 4. Pharmacologically treated cells were compared to cells cultured with media containing 1μM of DMSO as the control. After 7 days, all cells were incubated with fresh MSCGM for 24 hours, without any pharmacological treatment. All cells were incubated with fresh MSCGM on day 7 for 24 hours, without any pharmacological treatment. After 24 hours, conditioned media was collected, cells were harvested from each surface and lysed. OPG and RANKL secreted protein levels in the media were assessed with ELISA and normalized to total DNA content of cell lysates in each well.

CHAPTER 3 RESULTS

3.1. SPECIFIC AIM 1: INVESTIGATE THE EFFECTS OF ANTIDEPRESSANTS ON HUMAN MSC DIFFERENTIATION ON TI SURFACES

DNA Quantification on Surfaces

The response of human MSCs to Ti substrates and the TCPS control during exposure to serotonin or antidepressants was assessed by quantitative DNA analysis. Cells were cultured on smooth (PT) or rough (SLA) Ti surfaces in the presence or absence of serotonin or other categories of antidepressants within the SSRI or SNRI class. Additional types of commonly prescribed antidepressants that are not selective for serotonin were used for comparison. Serotonin treatment was used as the positive control. Effects of serotonin or antidepressants were compared to cells cultured in media not containing any treatment. Treatment concentrations of 1μM or 10μM were chosen. The lower concentration is representative of therapeutic levels present in the blood for patients taking SSRIs. Since SSRIs are known to sequester in the bone marrow at concentrations much higher than those in the brain or blood, treatment with the 10μM concentration is intended to represent these conditions.

Serotonin

In the no treatment groups for all experiments, DNA content was significantly lower in human MSCs cultured on SLA surfaces, but not different on PT, in comparison to TCPS (figure 8). Treatment with 1μM of serotonin significantly increased DNA content in comparison to the no treatment groups on TCPS and PT surfaces (figure 8a). There was no difference between serotonin treatment and the no treatment control on SLA surfaces.

SSRIS

Cells were grown in the presence on fluoxetine, sertraline or paroxetine. Similar to serotonin, treatment with 1μM of fluoxetine also increased DNA content in comparison to the no treatment groups on TCPS and PT. Treatment with 10μM decreased it in comparison to the no treatment control (figure 8b). There was no difference between the 1μM and the control on SLA, however, treatment with 10μM showed the most significant decreases in DNA content in comparison with TCPS, PT, the 1μM concentration and the no treatment control.

There was no difference between the control and the 1µM concentration of sertraline on TCPS and PT, however, increasing the concentration to 10µM significantly decreased DNA content in comparison to the control (figure 8c). There was no difference between sertraline treatments and the no treatment control on SLA surfaces.

Paroxetine treatment at 1µM concentration had no significant difference when compared with the no treatment control on TCPS and PT (figure 8d), however, increasing the concentration to 10µM significantly decreased DNA content in comparison to the no treatment control. On SLA surfaces, paroxetine treatment dose-dependently decreased DNA content in comparison to the no treatment control, with the 10µM concentration having the most significant decreases in DNA content in comparison with TCPS, PT and the 1µM concentration.

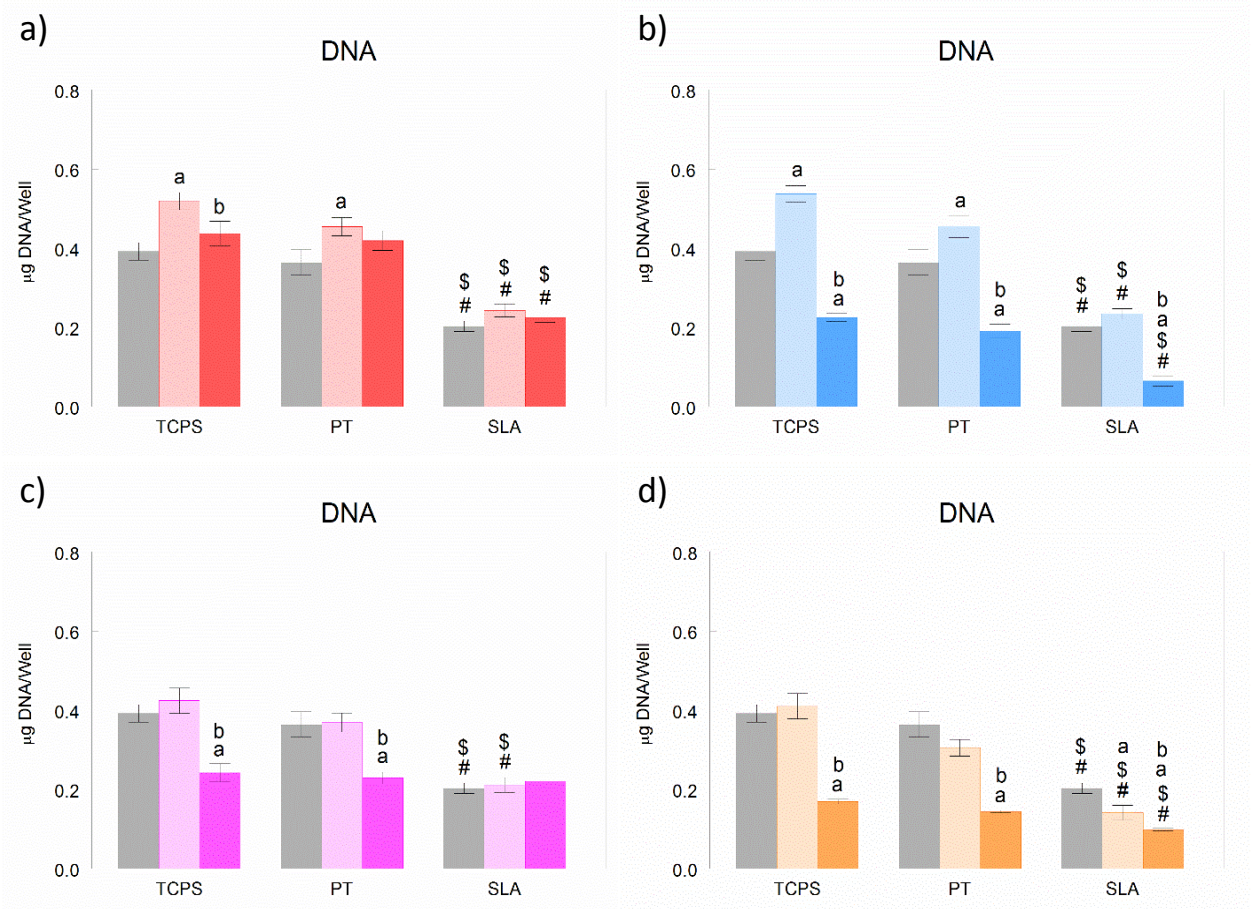

Figure 8: Human MSC DNA content after treatment with serotonin or SSRIs. Cells were treated with 1µM or 10µM concentrations of a) serotonin, b) fluoxetine, c) sertraline or d) paroxetine and cultured on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

SNRI

Duloxetine, an SNRI, had no difference in DNA content between the no treatment control and the 1µM concentration on TCPS and PT surfaces (Figure 9). Increasing the dose to 10µM significantly decreased DNA content on TCPS in comparison to the no treatment control and the 1µM concentration, with further decreases on PT surfaces. There was no difference in DNA content on SLA surfaces after duloxetine treatment in comparison to the no treatment control.

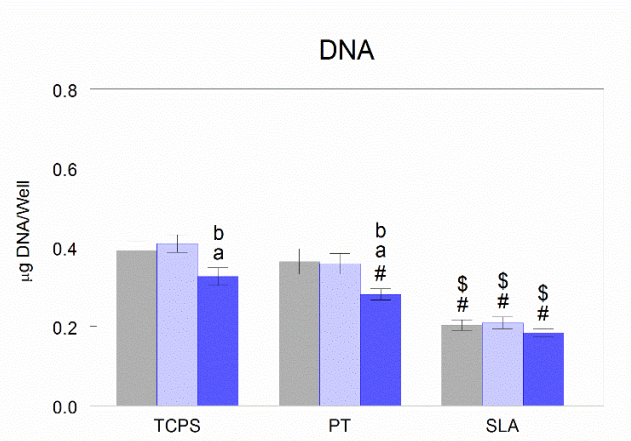

Figure 9: Human MSC DNA content after treatment with an SNRI. Cells were treated with 1µM or 10µM concentrations of duloxetine and cultured on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

Other Antidepressants

Trazodone was similar to duloxetine in that there was no significant difference between the no treatment control and the 1µM concentration on TCPS and PT surfaces, but treatment with 10µM significantly decreased DNA content on TCPS, with further decreases on PT (figure 10a). DNA content was lower on all SLA surfaces in comparison to TCPS and PT, however, no significant differences were apparent between treatment and no treatment controls.

There was no difference in DNA content after treatment with bupropion in comparison to the no treatment control on TCPS (figure 10b). Treatment with 1µM and 10µM concentrations of bupropion decreased DNA content in comparison to the no treatment control on PT, with further decreases on SLA surfaces, however, there were no significant differences between each dose.

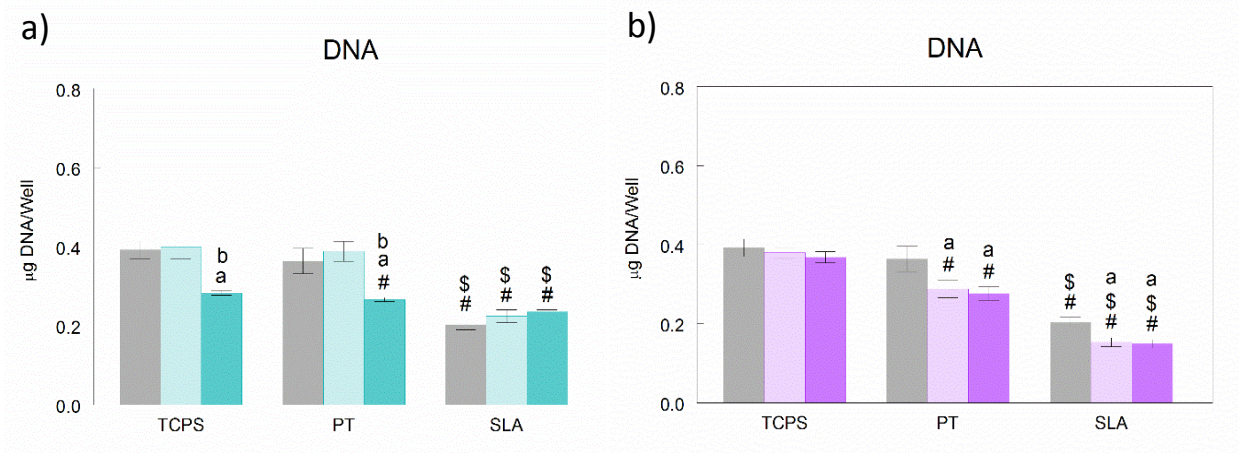

Figure 10: Human MSC DNA content after treatment with other types of antidepressants. Cells were treated with 1μM or 10μM concentrations of a) trazodone or b) bupropion and cultured on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0μM, b vs. 1μM.

3.1.1. AIM 1.1: DETERMINE THE EFFECTS OF ANTIDEPRESSANT TREATMENTS ON HUMAN MSC DIFFERENTIATION WHILE CULTURED ON SMOOTH VS. ROUGH Ti SURFACES

To determine the effects of antidepressant treatment on bone formation and dental implant osseointegration, MSC differentiation potential was assessed on Ti substrates. Cells were cultured on smooth (PT) or rough (SLA) Ti substrates and compared to those on TCPS during treatments of either serotonin or antidepressants. MSC differentiation was measured by analyzing alkaline phosphatase specific activity as an early marker of osteoblastic differentiation, and OCN protein levels as a late marker. The hypothesis is antidepressant treatment will prevent human MSC differentiation, which can lead to delayed osseointegration.

Effects of Serotonin on MSC Differentiation

Alkaline phosphatase activity was highest prior to any treatment with serotonin in human MSCs plated on SLA surfaces when compared to TCPS and PT (figure 11a). There was no significant difference in enzyme activity after treatment with 1μM in comparison to the no treatment control. Enzyme activity increased with the addition of serotonin at the 10μM concentration in comparison with the 1μM treatment on TCPS and with further increases on PT surfaces, in comparison to the no treatment control. On SLA surfaces, however, this effect was reversed. Serotonin treatment dose-dependently decreased enzymatic activity, with the lowest levels evident at the 10μM concentration in comparison to the no treatment control.

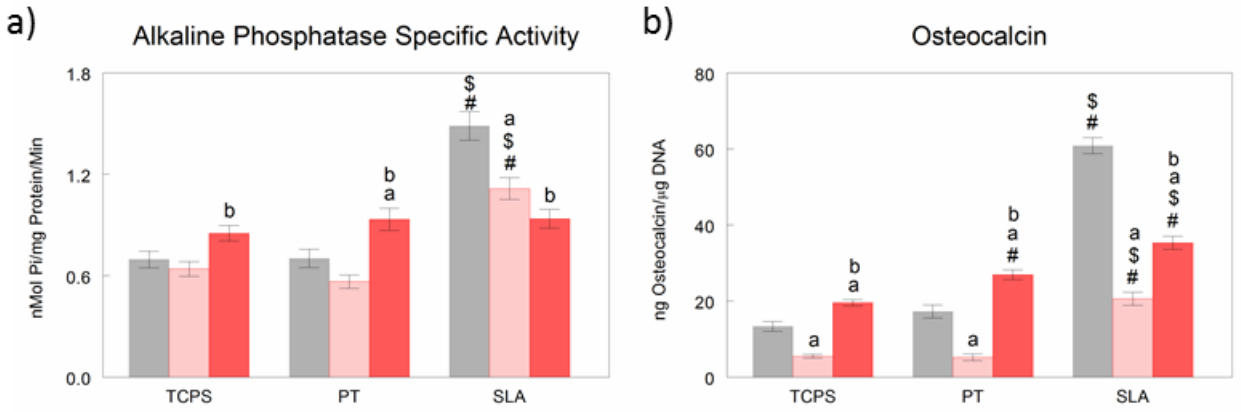

Figure 11: Effects of serotonin on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of serotonin on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

The effect of serotonin on late osteoblastic differentiation was assessed by measuring secreted levels of OCN by human MSCs throughout differentiation. Prior to any treatments, levels of OCN increased with the increasing surfaces roughness, with the highest increases evident on SLA (figure 11b). Treatment with 1µM of serotonin significantly decreased OCN protein levels on all surfaces in comparison to their no treatment controls. This effect was rescued when the dose was augmented to 10µM, however. Protein levels were higher on TCPS, with further increases on PT surfaces, in comparison to their no treatment controls. All serotonin treatments decreased OCN protein levels on SLA surfaces in comparison to the no treatment control, with the most decreases at the 1µM concentration.

Effects of SSRIs on MSC Differentiation

Effects of SSRIs fluoxetine, sertraline and paroxetine on early human MSC differentiation were assessed by measuring enzymatic activity for alkaline phosphatase. Enzymatic activity was highest prior to treatment with antidepressants in human MSCs plated on SLA surfaces when compared to TCPS and PT (figures 12, 13, 14, 15, 16 and 17). In general, treatment with all types of SSRIs decreased alkaline phosphatase activity in a dose-dependent manor on SLA surfaces, with the lowest levels evident at the 10µM concentration, in comparison to the no treatment control (figures 12, 13 and 14). Fluoxetine treatment did not affect enzymatic activity on TCPS in comparison to the no treatment control (figure 12a). Only the 10µM dose decreased enzyme activity when compared with the lower dose on PT surfaces and TCPS. Treatment with 1µM

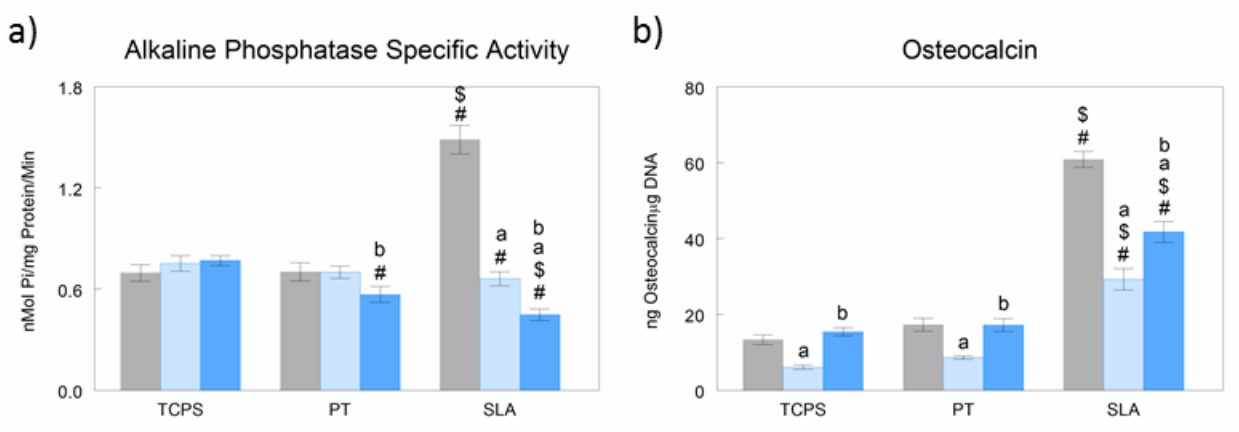

Figure 12: Effects of SSRI fluoxetine on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase specific activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of fluoxetine on TCPS, PT or SLA surfaces. # $P<0.05$ vs. TCPS, $ vs. PT. a $P<0.05$ vs. 0µM, b vs. 1µM.

concentrations of fluoxetine decreased OCN protein levels on all surfaces in comparison to the no treatment control (figure 12b). The 10µM treatment increased levels on TCPS and PT when compared with the 1µM dose, but was not statistically significant against the no treatment control. All concentrations of fluoxetine decreased OCN protein levels on SLA surfaces in comparison to the no treatment control, with the lowest decreases seen after treatment with the 1µM concentration.

Alkaline phosphatase activity was lowest after treatment with 10µM concentration of sertraline on TCPS and PT surfaces in comparison to the 1µM concentration and their no treatment control (figure 13a). There was no difference in enzymatic activity between the 1µM and 10µM treatments on TCPS and PT surfaces. Both 1µM and 10µM sertraline treatments decreased OCN protein levels on TCPS in comparison to the no treatment control (figure 13b). Sertraline effects were more robust on Ti surfaces, as evident by the significantly lower decreases, in a dose-dependent manor, in protein levels on PT and SLA surfaces.

Paroxetine treatment at the 10µM concentration decreased alkaline phosphatase activity on TCPS in comparison to the 1µM concentration, but was not statistically significant when compared to its no treatment control (figure 14a). Treatment with the 1µM concentration increased enzymatic activity in comparison to the no treatment control, however, when the dose was augmented to 10µM, activity decreased in comparison to the 1µM on PT surfaces. There was no difference

between paroxetine treatments on OCN protein levels on TCPS and PT in comparison to the no treatment control (figure 14b), though, both concentrations equally decreased levels on SLA surfaces in comparison to the no treatment control.

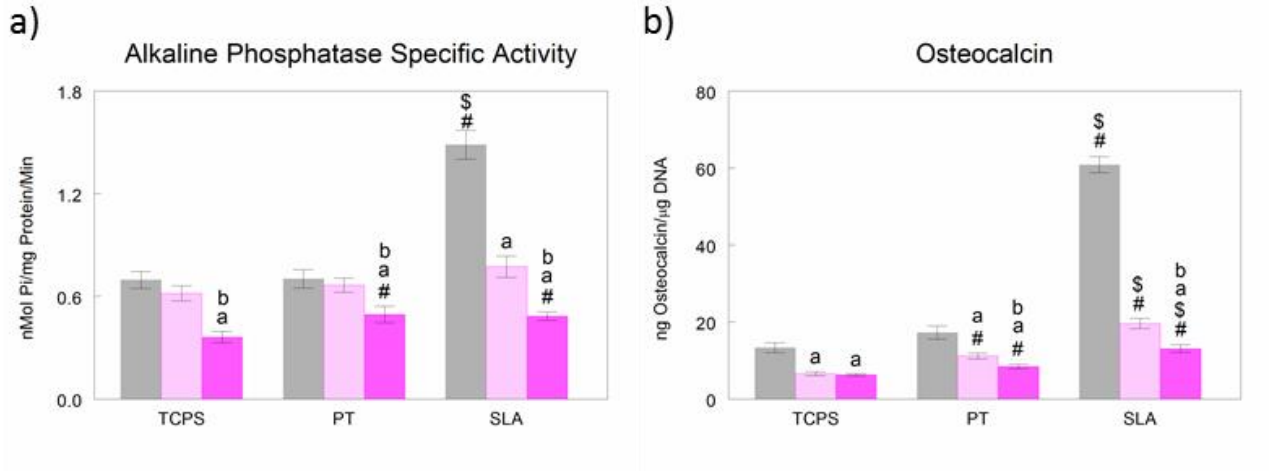

Figure 13: Effects of SSRI sertraline on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of sertraline on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

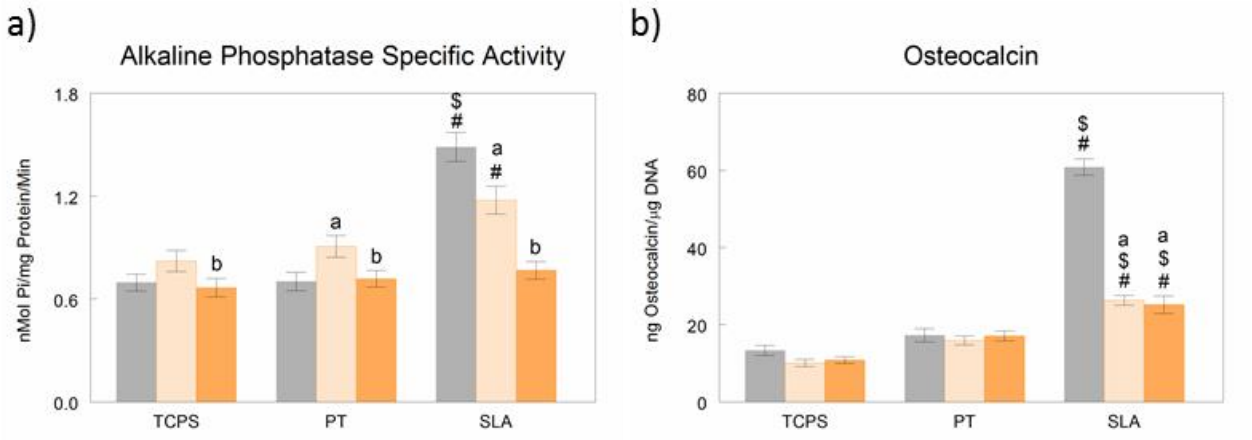

Figure 14: Effects of SSRI paroxetine on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of paroxetine on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

Effects of SNRIs on MSC Differentiation

To determine how SNRIs affect human MSC differentiation in comparison to SSRIs and serotonin, MSCs were treated with duloxetine at the same doses and early and late differentiation was assessed. There was no statistical difference in enzymatic activity between duloxetine

treatments at either concentration on TCPS or PT surfaces in comparison to the no treatment control (figure 15a). On SLA, there was no statistical difference between the 1μM concentration and the no treatment control, yet both were higher than levels on TCPS and PT surfaces. There was also no difference between the 1μM and 10μM concentrations on SLA, but the 10μM concentration decreased activity the most in comparison to the no treatment control. Treatment with 1μM concentration of duloxetine decreased OCN protein levels on all surfaces in comparison to the no treatment control (figure 15b). Raising the dose to 10μM increased protein levels in comparison to the 1μM dose on Ti surfaces in comparison to TCPS, with the most increases seen on SLA.

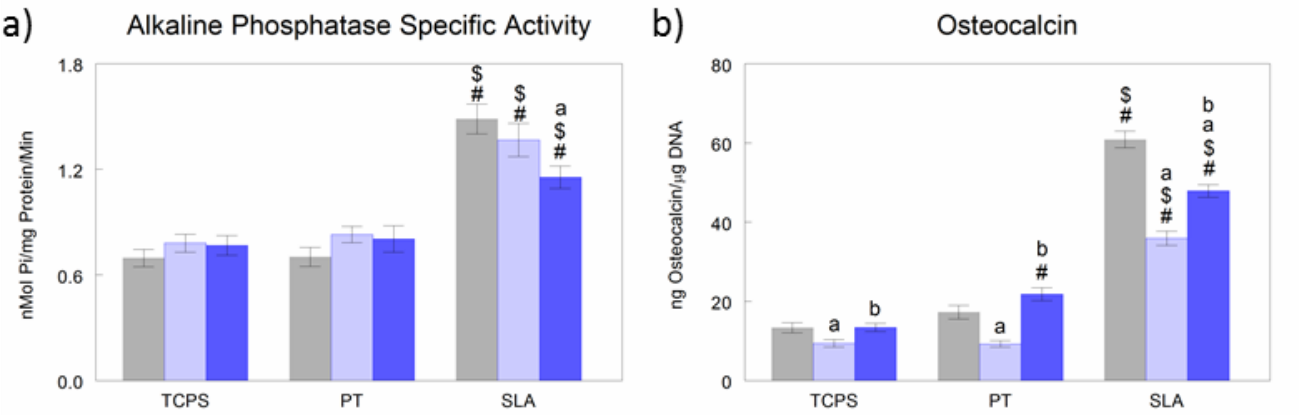

Figure 15: Effects of SNRI duloxetine on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1μM or 10μM concentrations of duloxetine on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0μM, b vs. 1μM.

Effects of Other Antidepressants on MSC Differentiation

Trazodone and bupropion are two antidepressants that do not belong to a specific category. Their effects on cell differentiation was also compared with serotonin and other types of antidepressants. There was no significant difference between both, trazodone and bupropion treatments, on alkaline phosphatase activity for human MSCs cultured on TCPS surfaces (figures 16a and 17a). Only the 10μM concentration of trazodone decreased enzymatic activity on PT in comparison to the no treatment control (figure 16a), but no differences were evident with either bupropion treatments on the same surfaces (figure 17b). Trazodone treatment dose-dependently decreased enzyme activity on SLA surfaces in comparison to the no treatment control, with the most significant decreases seen after treatment with the 10μM concentration (figure 16a). There

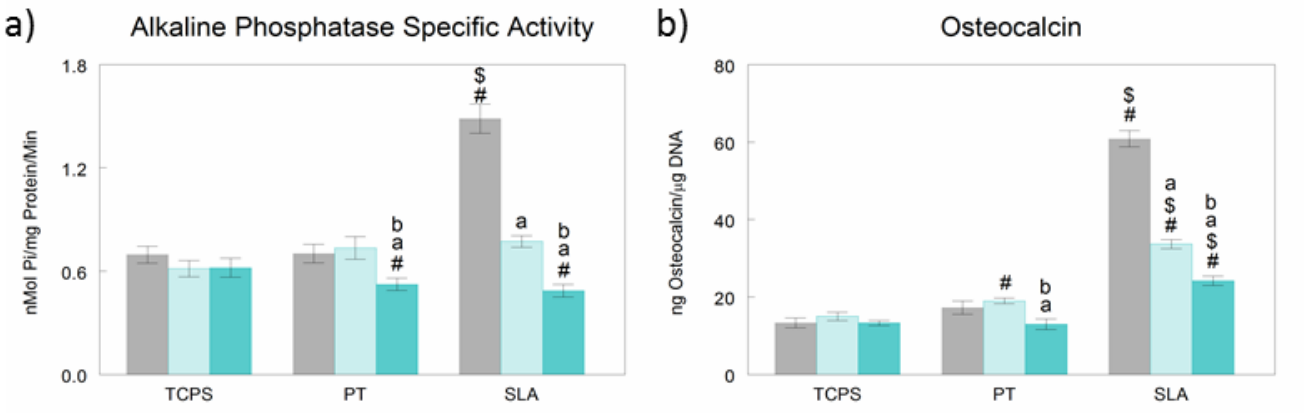

Figure 16: Effects of trazodone on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of trazodone on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

was no significant difference between the 1µM dose of bupropion and the no treatment control on SLA surfaces, however, the 10µM concentration decreased enzyme activity in comparison to the 1µM concentration and the no treatment control (figure 17b).

Both Trazodone and bupropion treatments were similar in that they had no significant effects on OCN protein levels in comparison to the no treatment controls on TCPS surfaces (figures 16b and 17b). On PT surfaces, treatment with 1µM of trazodone slightly increased protein levels in comparison to the same concentration on TCPS, but this effect was not statistically significant

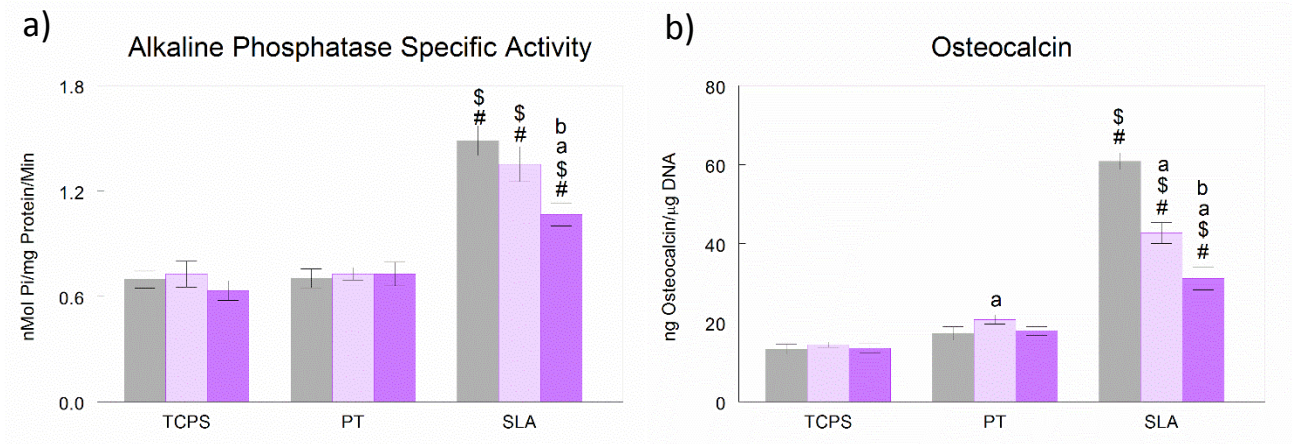

Figure 17: Effects of bupropion on early and late osteoblastic differentiation. Human MSC a) alkaline phosphatase activity and b) OCN protein levels after treatment with 1µM or 10µM concentrations of bupropion on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT. $P < 0.05$ a vs. 0µM, b vs. 1µM.

when compared to its no treatment control (figure 16a). Increasing the trazodone dose to 10μM on PT, however, decreased protein levels in comparison to the 1μM concentration and the no treatment control. Only the 1μM treatment of bupropion increased protein levels on PT surfaces in comparison to the 10μM and the no treatment control (figure 16b). On SLA surfaces, both trazodone and bupropion treatments dose-dependently decreased OCN protein levels in comparison to TCPS and PT, with the highest concentrations having the lowest amount of protein (figures 16b and 17b). Overall, decreases in early and late differentiation markers were significantly less robust with treatments of serotonin or any type of antidepressant on TCPS or PT surfaces in comparison to SLA.

Antidepressants and Ti Surface Characteristics Modulate Gene Expression of Osteoblastic Differentiation Markers

For further investigation of the effects of antidepressants on bone formation, human MSC gene expression of early and late osteoblastic differentiation markers were assessed on various Ti surfaces. The effects of surface roughness and wettability on osteoblastic gene expression were assessed first, prior to addition of antidepressant or serotonin treatment. mRNA expression levels of genes important for early (RUNX2) and late (BGLAP) bone development for human MSCs were assessed by qPCR. Cells were grown on smooth and hydrophobic (PT), rough and hydrophobic (SLA), or rough and hydrophilic (mSLA) Ti surfaces, throughout differentiation and compared with cells on TCPS as the control. Expression of RUNX2 and BGLAP increased on Ti

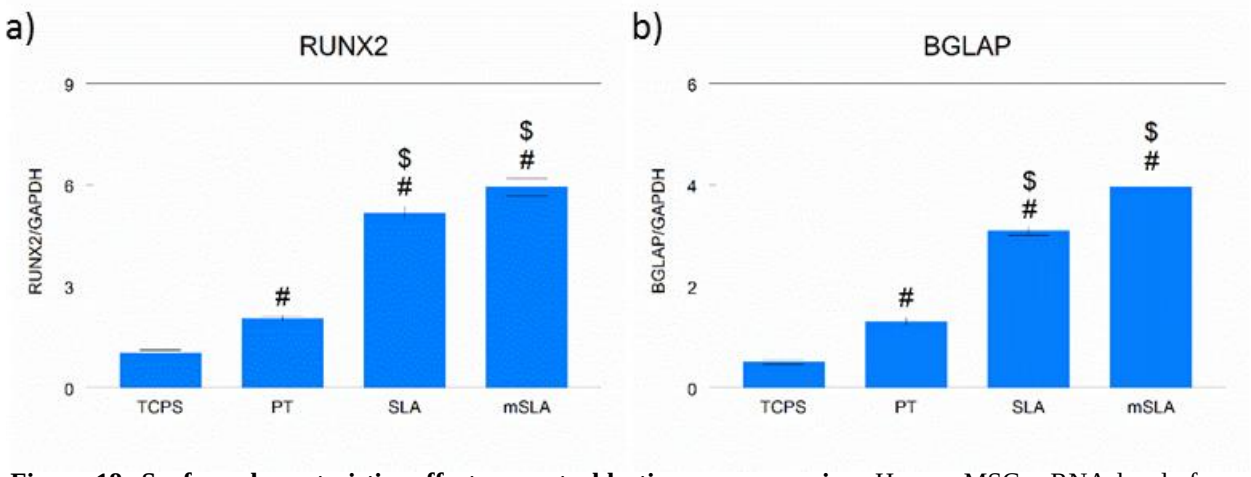

Figure 18: Surface characteristics effects on osteoblastic gene expression. Human MSC mRNA levels for RUNX2 and BGLAP cultured on TCPS, PT, SLA or mSLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT, % vs. SLA.

substrates in comparison to TCPS, with the highest increases evident on rough and rough and hydrophilic substrates (figure 18). There were no significant differences in mRNA levels between rough (SLA) and rough and hydrophilic (mSLA) substrates.

Effects of Serotonin and Surface Characteristics on Osteoblastic Gene Expression

Once the surface characteristics effects on gene expression were established, effects of antidepressants were assessed. In order to determine the effects of serotonin on bone formation on Ti surfaces, mRNA expression levels of RUNX2 and BGLAP for human MSCs were assessed by qPCR after treatment with 0.1µM, 1µM or 10µM concentrations of serotonin. Since no differences in mRNA levels for osteoblastic differentiation markers were seen in MSCs cultured on hydrophilic (mSLA) vs. hydrophobic (SLA) rough substrates, cells were grown on smooth (PT) or rough (SLA) Ti surfaces for the remainder studies to compare the effects of rough vs. smooth substrates on differentiation in the presence of serotonin. As previously shown, higher levels of early osteoblastic differentiation marker and transcription factor, RUNX2, and late marker, BGLAP, were evident on PT with further increases on SLA surfaces in comparison with TCPS prior to serotonin treatment (figure 19).

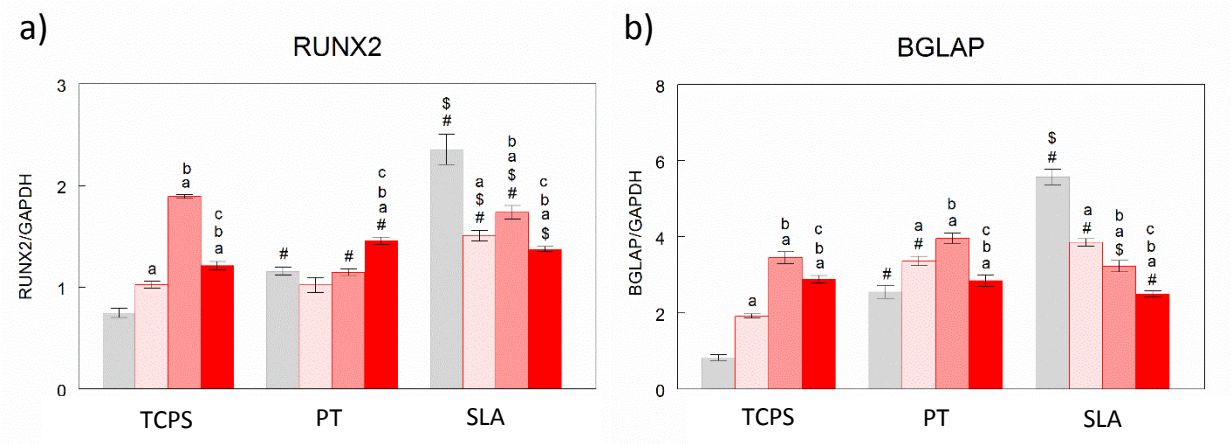

Figure 19: Effects of serotonin on gene expression for osteoblastic differentiation. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0µM, 0.1µM, 1µM or 10µM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 0.1 µM, c vs. 1µM.

Treatment with serotonin increased mRNA levels on TCPS in comparison to the no treatment control, with the highest levels seen after treatment with the 1µM concentration (figure 19a). Only the highest concentration, 10µM, on PT surfaces showed significant increases in mRNA

levels in comparison to the lower concentrations and the no treatment control. On SLA surfaces, however, all serotonin treatments decreased RUNX2 mRNA levels in comparison to the no treatment control, with the most significant decreases apparent after treatment with the 10µM concentration.

Serotonin treatment at all concentrations increased mRNA levels for the late osteoblastic differentiation marker, BGLAP, on TCPS and PT surfaces in comparison to their no treatment control (figure 19b). The highest expression was apparent after treatment with the 1µM concentration, while the lowest was after 10µM on both TCPS and PT surfaces. On SLA surfaces, however, this effect was reversed. Serotonin treatments dose-dependently decreased mRNA levels in comparison to the no treatment control, with the 10µM concentration having the lowest levels.

Effects of SSRIs and Surface Characteristics on Osteoblastic Gene Expression

To determine how MSC differentiation is affected under fluoxetine, sertraline or paroxetine exposure in comparison to serotonin on Ti surfaces, mRNA levels for the same early and late

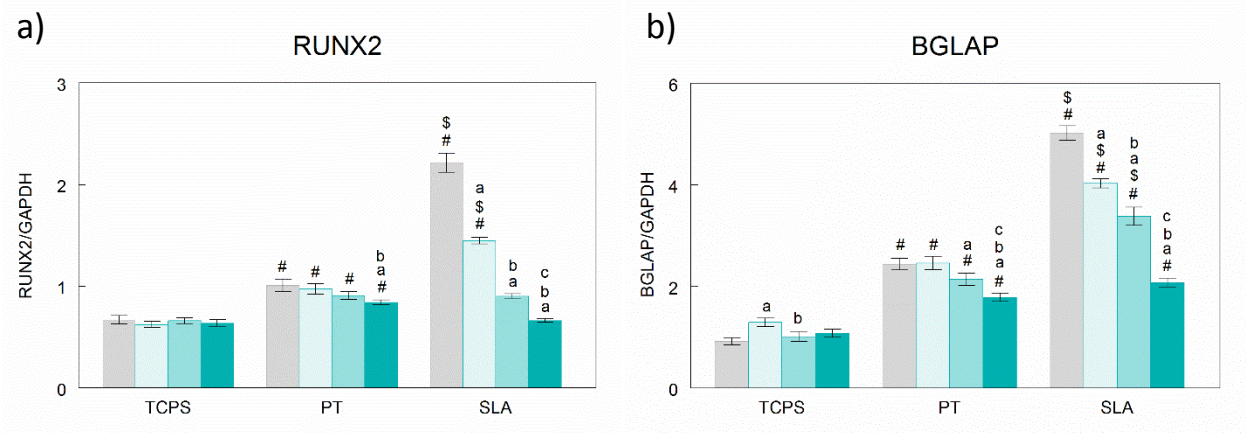

Figure 20: SSRI fluoxetine inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0µM, 0.1µM, 1µM or 10µM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 0.1 µM, c vs. 1µM.

osteoblastic differentiation markers were assessed. Prior to any SSRI treatment, RUNX2 and BGLAP expression increased on PT surfaces, with further increases on SLA (figures 20, 21 and 22). There was no difference between levels of RUNX2 mRNA after treatment with all concentrations of fluoxetine in comparison to the no treatment control on TCPS (figure 20a). Only the 10µM concentration decreased mRNA levels when compared with the no treatment control on

PT surfaces. In a similar manor, mRNA levels for BGLAP were only slightly elevated after treatment with the 0.1µM concentration of fluoxetine on TCPS in comparison to the no treatment control (figure 20b). On PT surfaces, the 1µM and 10µM concentrations decreased mRNA levels in comparison to the no treatment control. However, mRNA levels for both RUNX2 and BGLAP displayed the same dose-dependent response on SLA surfaces, where all concentrations of fluoxetine decreased levels, with the most significant decreases evident at the highest concentration of 10µM when compared with the no treatment control.

Since treatment with the 1µM and 10µM concentrations of serotonin or fluoxetine exhibited the most statistically significant changes in mRNA levels for markers of differentiation, they were the concentrations of choice and were used for the remaining osteoblastic differentiation assessments. Sertraline treatment had no effect on mRNA levels for BGLAP, and only slightly decreased levels at the 10µM concentration for RUNX2 levels on TCPS surfaces in comparison to the no treatment control (figure 21). On Ti surfaces, however, effects were more robust. Sertraline

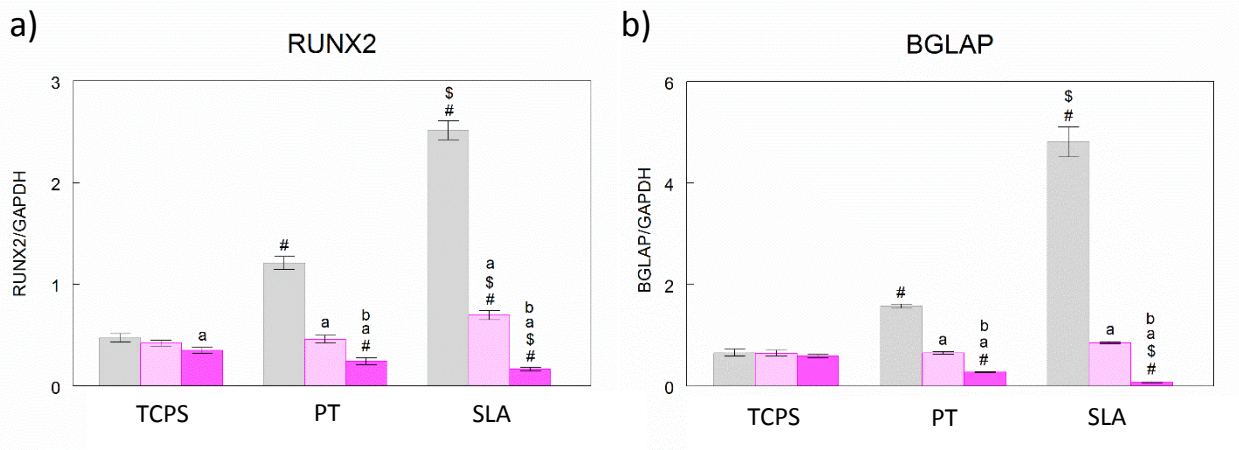

Figure 21: SSRI sertraline inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0µM, 0.1µM, 1µM or 10µM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 0.1 µM, c vs. 1µM.

treatment exhibited dose-dependent decreases in RUNX2 and BGLAP mRNA levels on Ti surfaces in comparison to TCPS, with the 10µM concentration having the most significant decreases. Dose-dependency effects on mRNA levels for both RUNX2 and BGLAP were more robust on rough (SLA) Ti surfaces in comparison to smooth (PT) and TCPS, with the 10µM

concentration having the most significant decreases on SLA in comparison to all treatment and no treatment groups.

Paroxetine increased mRNA levels for BGLAP on TCPS, and at the 10µM concentration for RUNX2, when compared to the no treatment control (figure 22a and b). This effect was reversed, however, on Ti substrates. Treatment with paroxetine decreased levels for RUNX2 and BGLAP on PT and SLA surfaces in comparison to the no treatment control, with the lowest decreases evident after treatment with the 1µM concentration. Levels were higher when the concentration was increased to 10µM in comparison to the 1µM treatment, but were still significantly lower than the no treatment control.

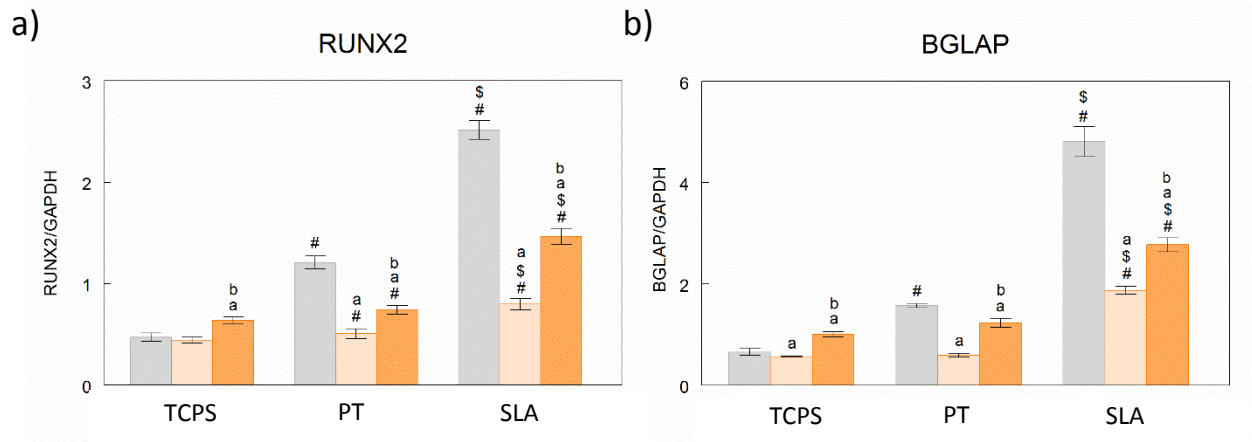

Figure 22: SSRI paroxetine inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0µM, 0.1µM, 1µM or 10µM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 0.1 µM, c vs. 1µM.

SNRI and Surface Characteristics Effects on Osteoblastic Gene Expression

Duloxetine treatment had no significant effect on RUNX2 or BGLAP mRNA levels on TCPS in comparison to the no treatment control (figure 23a and b). However, significant differences were evident with the same treatment on microstructured Ti substrates. Treatment with duloxetine dose-dependently decreased levels of early (RUNX2) and late (BGLAP) osteoblastic differentiation markers on PT and SLA surfaces in comparison to the no treatment control, with the lowest levels evident after treatment with the highest concentration of the drug.

Other Antidepressant and Surface Characteristics Effects on Osteoblastic Gene Expression

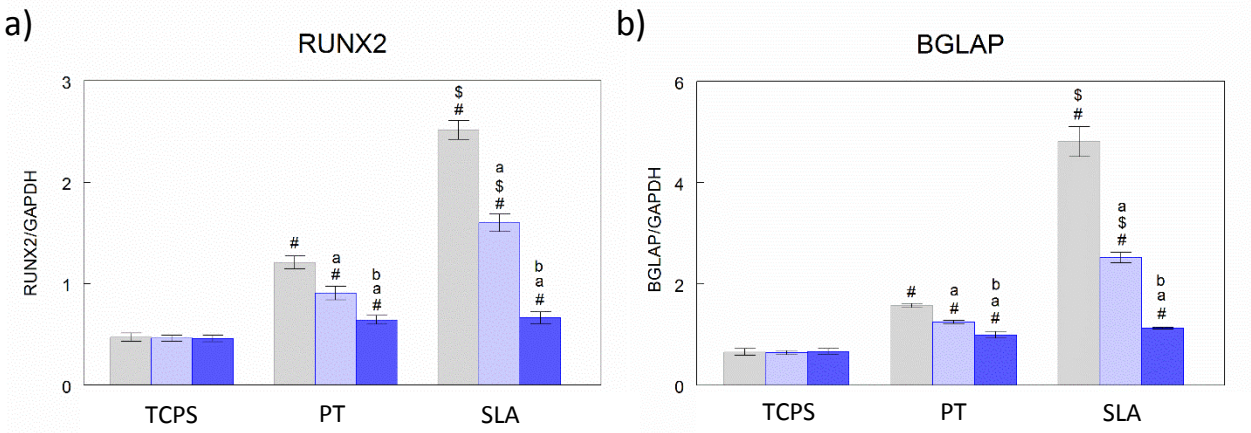

Figure 23: SNRI duloxetine inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0μM, 0.1μM, 1μM or 10μM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 0.1 μM, c vs. 1μM.

Trazodone and bupropion treatment had no effect on mRNA levels for RUNX2 or BGLAP on TCPS in comparison to the no treatment control (figures 24 and 25). Treatment with trazodone and bupropion decreased levels of RUNX2 on PT surfaces in comparison to the no treatment control, with further decreases after treatment with 10μM of bupropion (figures 24a and 25a).

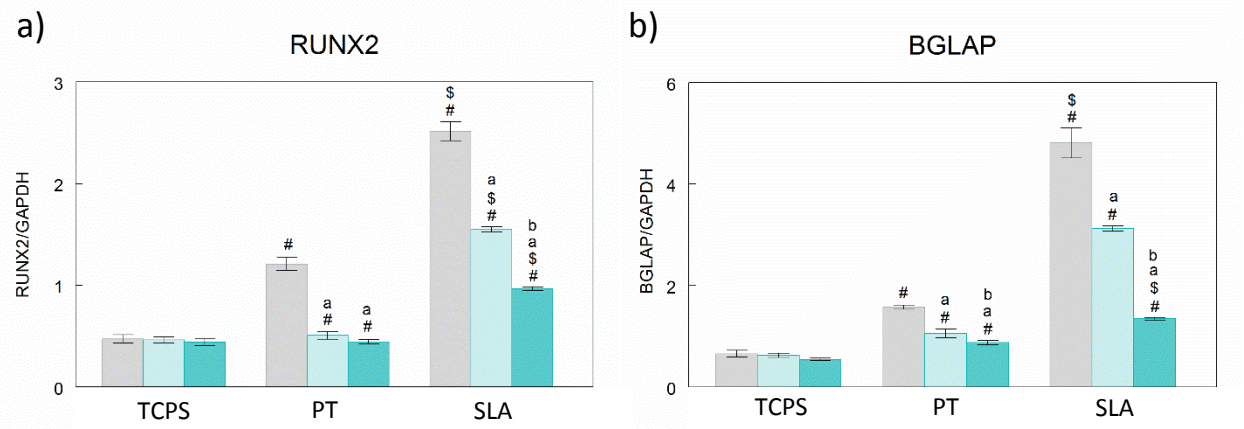

Figure 24: Trazodone inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0μM, 0.1μM, 1μM or 10μM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 0.1 μM, c vs. 1μM.

There was no significant difference in RUNX2 levels between each trazodone dose (figure 24a). There was a dose-dependent decrease for levels of BGLAP on PT surfaces, with the lowest levels evident after treatment with the highest concentration of trazodone (figure 24b). Bupropion also

decreased BGLAP levels on PT surfaces in comparison to the no treatment control, however with no significant difference in levels between each dose (figure 24b). Both trazodone and bupropion dose-dependently decreased RUNX2 and BGLAP mRNA levels on SLA surfaces in comparison to the no treatment control, with the lowest levels evident after treatment with the highest concentration of drug (figures 24b and 25b).

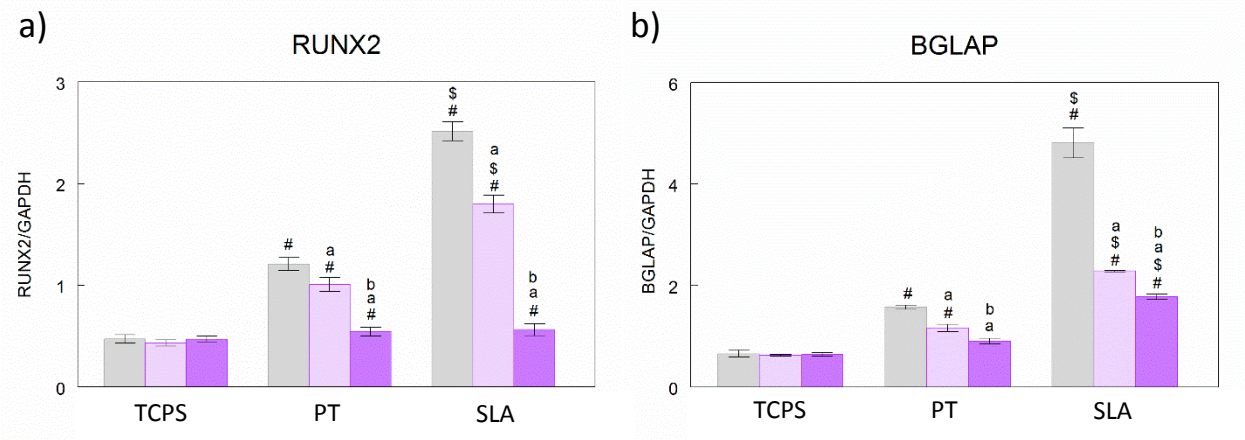

Figure 25: Bupropion inhibits gene expression for osteoblastic differentiation on microstructured Ti. Human MSC mRNA levels for RUNX2 and BGLAP after treatment with 0μM, 0.1μM, 1μM or 10μM concentrations of fluoxetine, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 0.1 μM, c vs. 1μM.

3.1.2. AIM 1.2: DETERMINE THE EEFFECTS OF ANTIDEPRESSANT TREATMENTS ON HUMAN MSC LOCAL FACTOR PRODCUTION OF BMP2, OPG, RANKL AND VEGF PROTEIN LEVELS IN THE MICROENVIRONMENT ON Ti SURFACES

In order to determine if antidepressant treatment has an effect on local factor production of important proteins in the microenvironment generated by human MSCs while cultured on Ti surfaces, cells were cultured on smooth or rough Ti surfaces and treated with various types of antidepressants. Cells were treated with 1μM or 10μM of serotonin, SSRIs (fluoxetine, sertraline or paroxetine), SNRIs (duloxetine), or other antidepressants (trazodone or bupropion). Treated cells were compared to the no treatment controls on each surface. To assess the effects of treatments on the microenvironment, secreted protein levels for BMP2, VEGF, OPG and RANKL were measured.

Serotonin Effects on the Microenvironment

Local factor production by human MSCs was assessed after treatment with 1µM or 10µM concentrations of serotonin. Human MSC protein levels for BMP2, VEGF and OPG all increased prior to any serotonin treatment on PT surfaces, with further increases on SLA, in comparison to TCPS (figure 26a, b and c). Treatment with serotonin had no significant effect on secreted BMP-2 levels on TCPS, however, all concentrations of serotonin decreased protein levels on PT and SLA surfaces in comparison to the no treatment control, with no significant differences between the 1µM and 10µM concentrations (figure 26a). Only the 10µM concentration of serotonin decreased VEGF protein levels on TCPS in comparison to the no treatment control, while the 1µM concentration had no significant effect (figure 26b). Both 1µM and 10µM treatments of serotonin decreased VEGF protein levels on PT and SLA surfaces in comparison to the no treatment control. No significant differences in protein levels were detected between the 1µM and 10µM

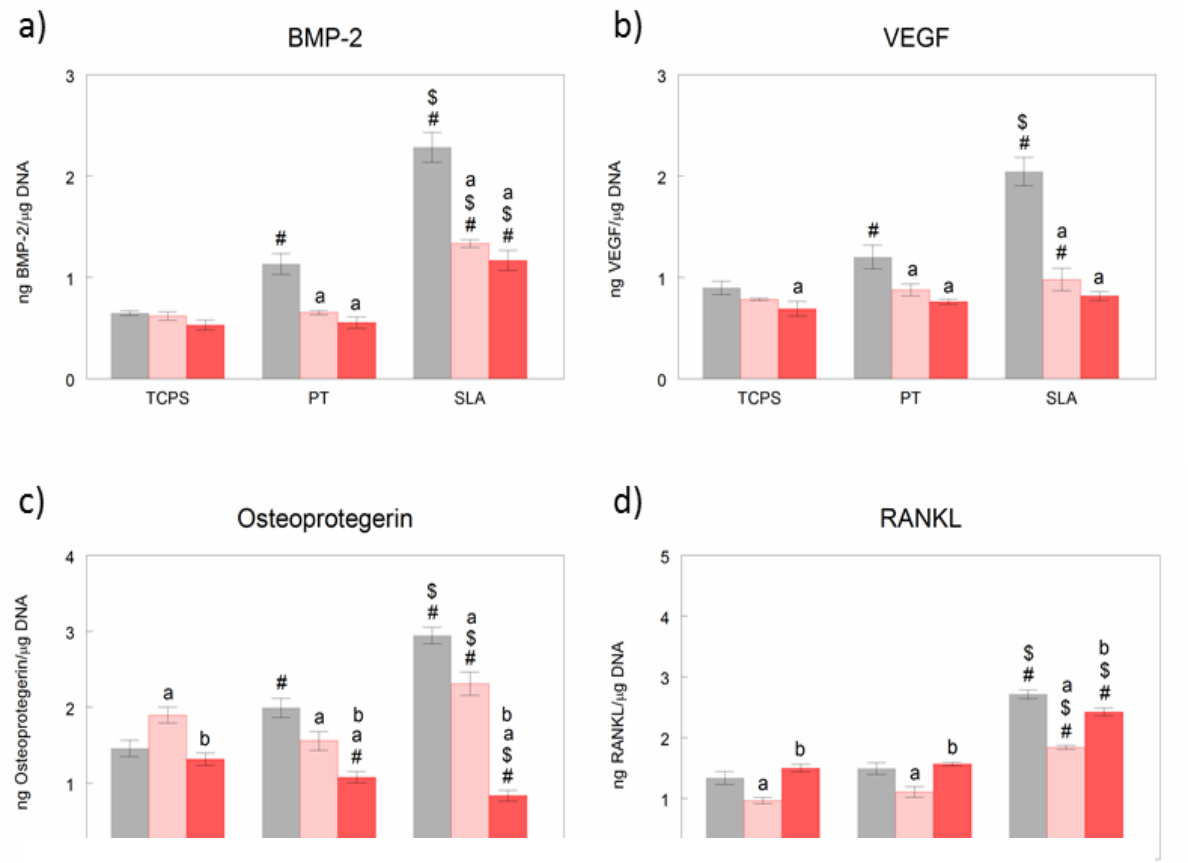

Figure 26: Effects of serotonin on MSC protein production. Human MSC protein levels for a) BMP2, b) VEGF, c) OPG and d) RANKL after treatment with 0µM, 1µM or 10µM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

concentration treatments on Ti surfaces. OPG protein levels increased after treatment with 1μM concentration of serotonin on TCPS in comparison with the no treatment control (figure 26c) and increasing the dose to 10μM decreased levels in comparison to the 1μM concentration, though with no significance difference when compared with the no treatment control. Serotonin treatment dose-dependently decreased OPG levels on PT in comparison to the no treatment control, with further decreases on SLA surfaces, in comparison to TCPS. The 10μM concentration seemed to have the more decreases in protein levels, with the most decreases evident on SLA surfaces. RANKL protein levels decreased after treatment with 1μM of serotonin on all surfaces in comparison with the no treatment control (figure 26d). Augmenting the concentration to 10μM slightly increased levels when compared with the 1μM concentration, but this effect was not statistically significant when compared with the no treatment control.

SSRI Effects on the Microenvironment

Effects of SSRI treatment on local factor protein production by human MSCs while plated on Ti surfaces was assessed after treatments with fluoxetine, sertraline or paroxetine at 1μM or 10μM concentrations. All protein levels secreted by human MSCs increased on Ti surfaces in comparison to TCPS prior to SSRI treatment (figures 25, 26 and 27). There was no difference in BMP2 protein levels after treatment with fluoxetine on TCPS when compared with the no treatment control, but on Ti surfaces, however, fluoxetine decreased protein levels in comparison to the no treatment control (figure 25a). There was no difference in BMP-2 levels between treatments with 1μM or 10μM concentrations on PT surfaces. Differences in doses was apparent on SLA surfaces, as fluoxetine exhibited a dose-dependent decrease in secreted protein levels, with the lowest levels evident after treatment with the 10μM concentrations in comparison to the no treatment control.

Fluoxetine treatment had no significant effect on secreted VEGF protein levels when compared to the no treatment control on TCPS and PT surfaces (figure 27b). Augmenting the dose to 10μM slightly increased protein levels compared to the 1μM concentration, but was still statistically insignificant when compared to the no treatment control. On SLA surfaces, 1μM and 10μM of fluoxetine decreased protein levels in comparison to the no treatment control. Effects of treating with the 10μM dose seemed to be slightly stimulatory in terms of protein secretion when compared to the 1μM dose.

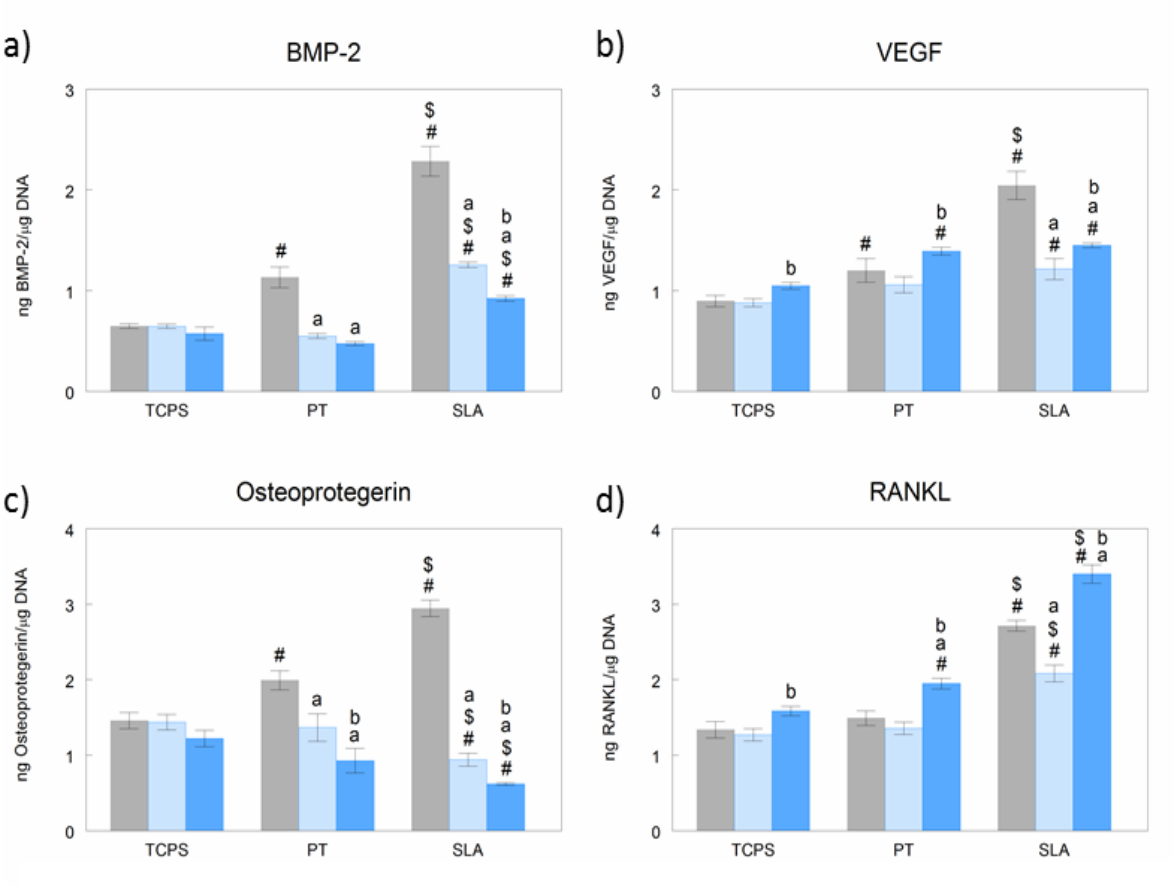

Figure 27: Effects of SSRI fluoxetine on MSC protein production. Human MSC protein levels for a) BMP-2, b) VEGF, c) OPG and d) RANKL after treatment with 0μM, 1μM or 10μM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 1μM.

There was no significant effect on OPG protein levels when treated with fluoxetine on TCPS in comparison to the no treatment control (Figure 27c). Fluoxetine dose-dependently decreased protein levels on PT and SLA surfaces in comparison to their no treatment controls, with the most significant decreases apparent after treatment with the 10μM concentration. There

was no difference in RANKL protein levels after 1μM of fluoxetine on TCPS and PT surfaces (figure 27d). The 10μM concentration slightly increased levels compared to the 1μM concentration on TCPS, with further increases on PT in comparison to the 1μM and the no treatment control. On SLA surfaces, fluoxetine decreased RANKL protein levels at the 1μM concentration compared to the no treatment control, but levels significantly increased after treatment with 10μM in comparison to TCPS, PT, 1μM and the no treatment control.

Sertraline treatment decreased BMP2 protein levels on all surfaces in comparison to the no treatment control (figure 28a). There was no significant difference in protein levels between the 1μM concentration and the no treatment on TCPS, however, augmenting the dose to 10μM significantly decreased levels in comparison to the 1μM and the no treatment control. Treatment with sertraline dose-dependently decreased protein levels on PT and SLA surface in comparison to the no treatment control, with the most significant decreases seen after treatment with the 10μM concentration. Dose-dependent decreases in VEGF protein levels were evident on all surfaces, with the lowest levels evident after treatment with the 10μM concentration of sertraline (figure 28b).

There was no difference in OPG protein levels between the 1μM dose and the no treatment control on TCPS, however, the 10μM concentration of sertraline significantly decreased levels (figure 28c). On PT surfaces, slight decreases in protein levels were detected after treatment with the 1μM concentration, with further decreases after the 10μM dose in compared to the no treatment control. The 1μM dose significantly decreased protein levels on SLA surfaces when compared with the no treatment control and protein levels actually failed to be detected after treatment with the 10μM dose of sertraline.

All treatments of sertraline increased RANKL production on TCPS, with further increases on Ti surfaces, when compared with the no treatment control (figure 28d). There was no difference in RANKL protein levels between the 1μM and the 10μM dose on TCPS in comparison to no treatment. On PT surfaces, augmenting the dose to 10μM further increased RANKL protein levels in comparison to the no treatment and the 1μM concentration. Treatment with 10μM of sertraline increased protein levels on SLA surfaces in comparison to the no treatment control, however, the

highest levels were evident after treatment with the 1μM dose when compared to the 10μM dose, TCPS and PT surfaces.

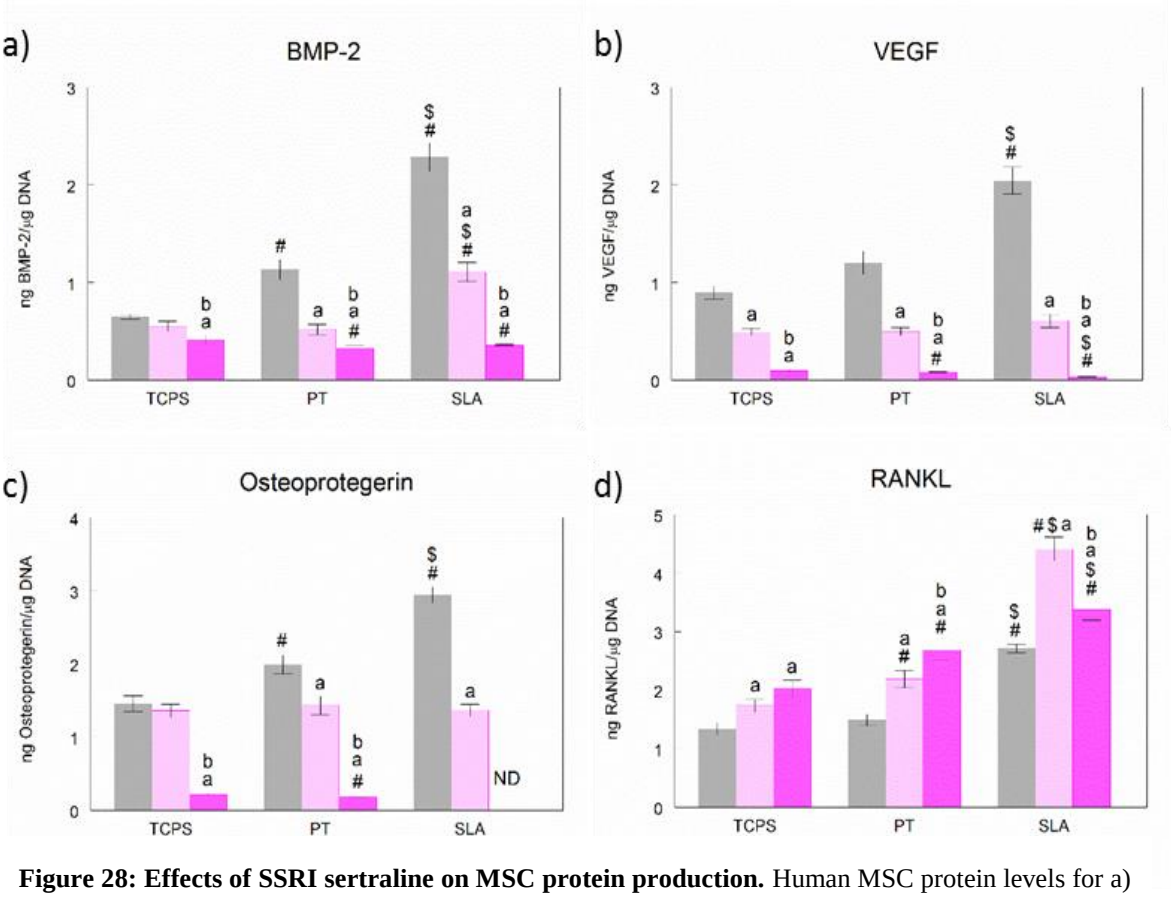

Figure 28: Effects of SSRI sertraline on MSC protein production. Human MSC protein levels for a) BMP2, b) VEGF, c) OPG and d) RANKL after treatment with 0μM, 1μM or 10μM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0uM, b vs. 1uM.

Paroxetine dose-dependently decreased BMP2 and VEGF protein levels on TCPS, PT and SLA surfaces in comparison to the no treatment control, with the lowest levels evident after treatment with the 10μM concentration (figure 29a and b). There was no difference in OPG protein levels between the 1μM concentration and no treatment control on TCPS and PT surfaces, but the 10μM dose significantly decreased levels when compared to the 1μM and no treatment control (figure 29c). Dose-dependent decreases in protein levels on SLA surfaces were evident with paroxetine treatment, with the lowest levels apparent with 10μM concentration treatments. Paroxetine slightly increased RANKL protein levels on TCPS and PT in comparison to the no treatment control, with no significant differences in levels after treatment with either the 1μM or

the 10μM dose (figure 29d). On SLA surfaces, however, both doses significantly increased RANKL production compared to no treatment, with the 1μM concentration having the highest protein levels in comparison to TCPS, PT, the 10μM concentration and the no treatment control.

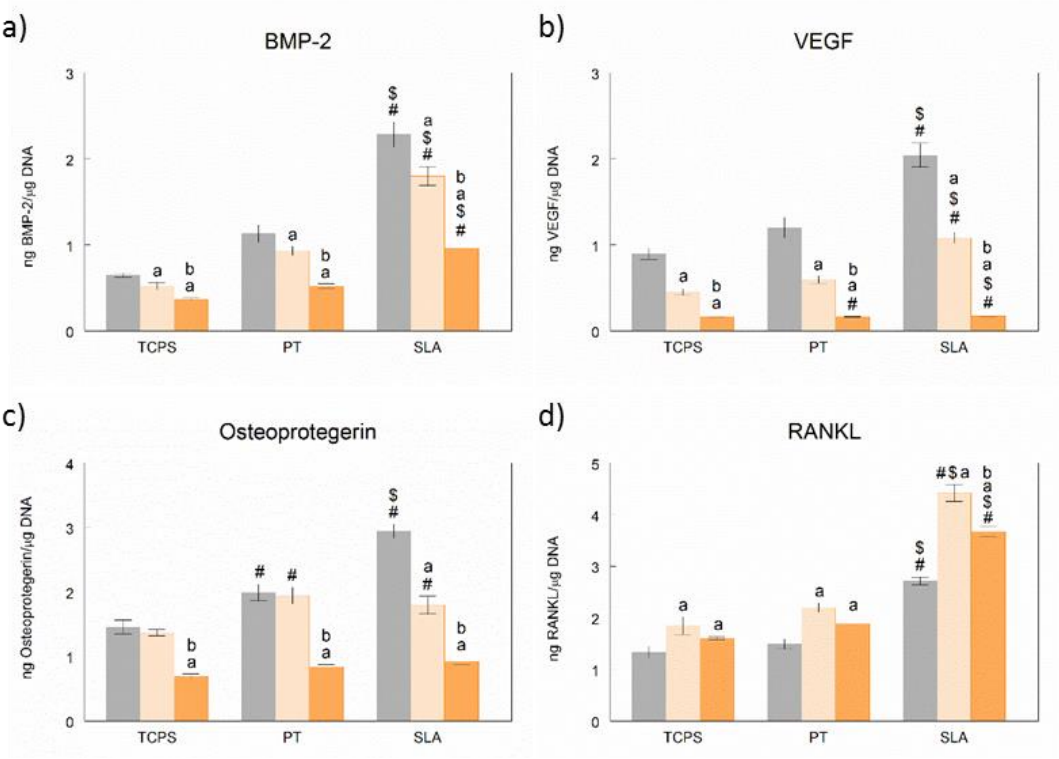

Figure 29: Effects of SSRI paroxetine on MSC protein production. Human MSC protein levels for a) BMP2, b) VEGF, c) OPG and d) RANKL after treatment with 0μM, 1μM or 10μM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0uM, b vs. 1uM.

SNRI Effects on the Microenvironment

Effects of SNRIs on the microenvironment production by MSC was assessed in in a similar manner as the SSRIs and serotonin. Antidepressants within the SNRI class are not only selective for serotonin, as they modulate levels of norepinephrine as well. MSCs were plated on PT or SLA surfaces and compared to those on TCPS after treatments with 1μM or 10μM concentrations of the SNRI duloxetine. All protein levels secreted by human MSCs increased on Ti surfaces in comparison to TCPS prior to SNRI treatment (figures 30). Treatment with duloxetine dose-dependently decreased BMP2 and VEGF protein levels on TCPS, PT and SLA surfaces in comparison to the no treatment control, with the lowest protein levels evident after treatment with

the 10μM concentration (figure 30a and b). There was no significant difference in OPG protein levels between treatments with the 1μM concentration of duloxetine and the no treatment control on TCPS and PT surfaces (figure 30c). However, increasing the dose to 10μM decreased protein levels on TCPS when compared to the no treatment control, with further decreases in comparison to the 1μM and the no treatment control on PT. Dose-dependent decreases in protein levels were evident on SLA surfaces, with the lowest levels of protein after treatment with the 10μM

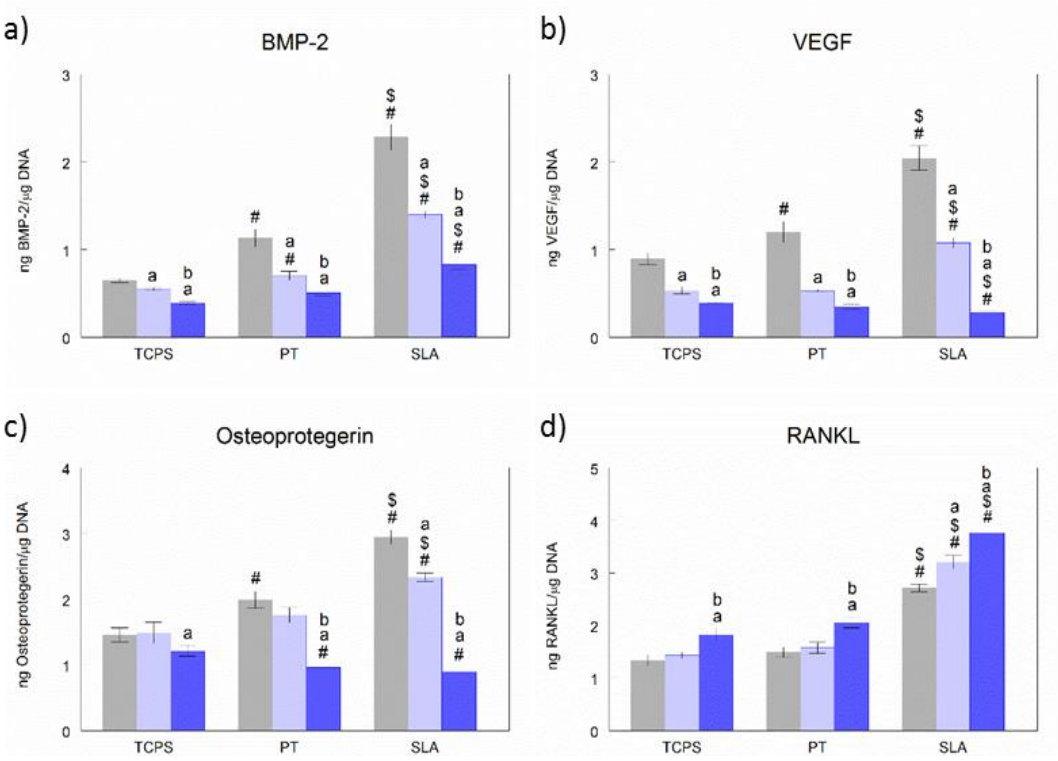

Figure 30: Effects of SNRI duloxetine on MSC protein production. Human MSC protein levels for a) BMP2, b) VEGF, c) OPG and d) RANKL after treatment with 0μM, 1μM or 10μM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 1μM.

concentration in comparison to the 1μM concentration and the no treatment control. Treatment with 10μM of duloxetine increased RANKL protein levels on TCPS and PT surfaces in comparison to the no treatment control, while the 1μM had no significant effects (figure 30d). On SLA, does-dependent increases in protein levels were apparent, with the highest levels seen after treatment with the 10μM concentration.

Other Antidepressant Effects on the Microenvironment

Trazodone and bupropion are commonly prescribed antidepressants that do not belong to a specific class. Their effects on microenvironment production by MSC on Ti surfaces was also assessed. All protein levels secreted by human MSCs increased on Ti surfaces in comparison to TCPS prior to any antidepressant treatment (figures 31 and 32). Addition of trazodone decreased BMP2, VEGF and OPG protein levels and increased RANKL levels on all surfaces (figure 31).

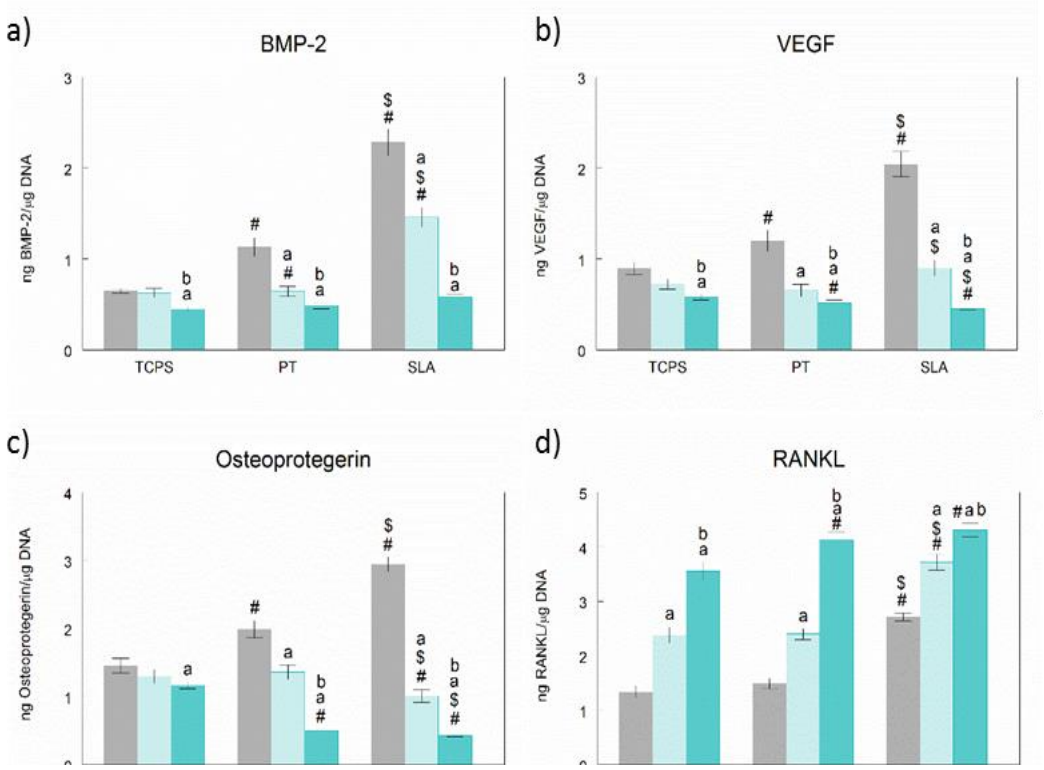

Figure 31: Effects of trazodone on MSC protein production. Human MSC protein levels for a) BMP-2, b) VEGF, c) OPG and d) RANKL after treatment with 0µM, 1µM or 10µM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

There was no difference in BMP2 and VEGF protein levels after treatment with 1µM of trazodone on TCPS surfaces in comparison to the no treatment control and increasing this concentration to 10µM decreased protein levels when compared to the 1µM and the no treatment control (figures 31a and b). Dose-dependent decreases in BMP2 protein levels were more evident on PT and SLA surfaces as trazodone concentrations increased (figure 31a). A similar effect was seen for VEGF as levels were even lower on Ti surfaces in comparison with no treatment (figure 31b). VEGF levels were lower on PT surfaces in comparison to TCPS, and further decreases on

SLA surfaces. There were no significant differences between the 1µM concentration and the no treatment control, but treatment with 10µM of trazodone decreased OPG protein levels on TCPS in comparison to the control (figure 31c). Trazodone treatment at 10µM concentrations dose-dependently decreased OPG levels on PT, with further decreases on SLA, when compared to the 1µM concentration and the no treatment control. The opposite effect was true for RANKL protein levels, as trazodone dose-dependently increased secreted levels with the incremental increases in concentrations on all surfaces when compared to the no treatment control (figure 31d). Protein levels were higher as trazodone concentrations increased on Ti surfaces in comparison to TCPS.

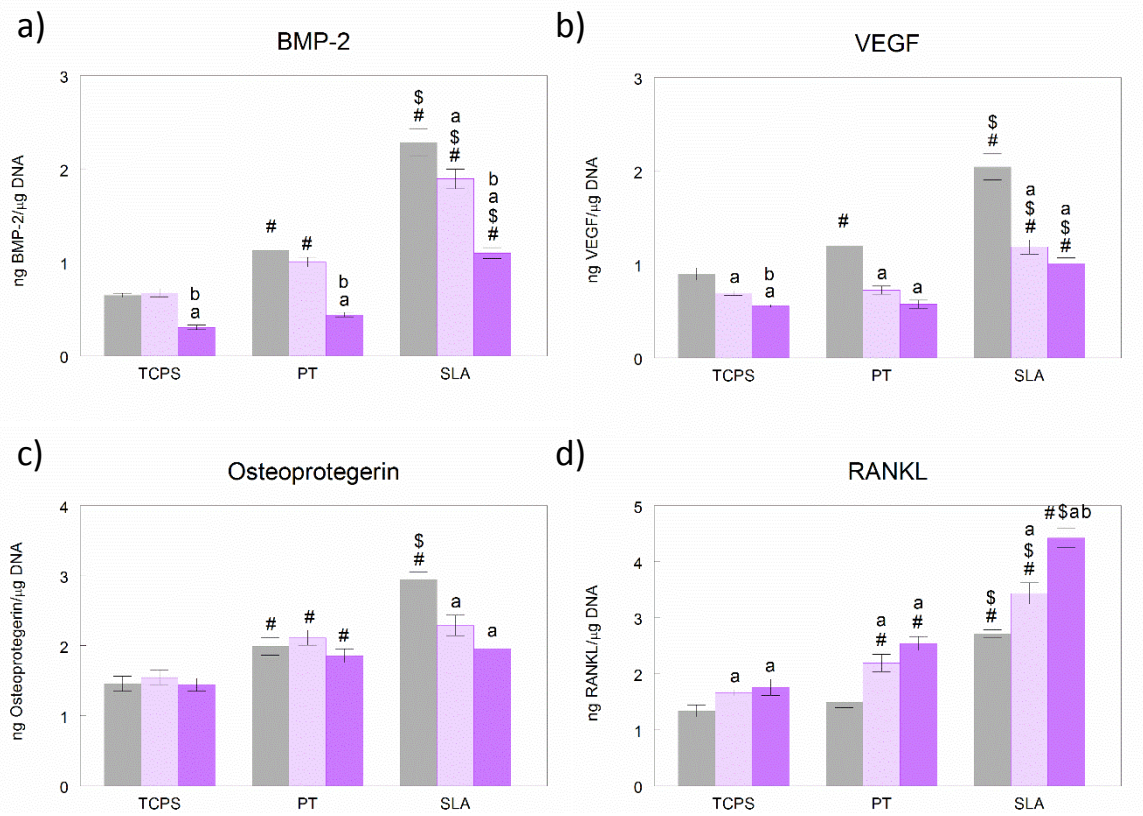

Figure 32: Effects of bupropion on MSC protein production. Human MSC protein levels for a) BMP2, b) VEGF, c) OPG and d) RANKL after treatment with 0µM, 1µM or 10µM concentrations of serotonin, cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

Treatment with bupropion does-dependently decreased BMP2 protein levels on PT and SLA surfaces in comparison to the no treatment control, with the 10µM concentration having the lowest levels (figure 30a). Only the 10µM dose decreased levels on TCPS in comparison to the

1μM and no treatment control. Treatment decreased VEGF levels on all surfaces in comparison to the control, with no difference in levels between concentrations on TCPS (figure30b). Bupropion treatment had no effect on OPG protein levels in comparison to the no treatment control on TCPS or PT surfaces (figure 30c). Treatment decreased levels on SLA surfaces in comparison to the no treatment control, with no significant difference in levels between the 1μM or 10μM concentrations. Both concentrations of bupropion increased RANKL protein levels on TCPS and PT surfaces in comparison to the no treatment control, with no significant differences in levels between each dose (figure 30d). Dose-dependent increases in RANKL levels were evident on SLA surfaces in comparison to the no treatment control, with the greatest levels seen after treatment with the 10μM concentration when compared with TCPS, PT, the 1μM concentration and the no treatment control.

3.2. SPECIFIC AIM 2: DETERMINE THE EFFECTS OF ANTIDEPRESSANTS ON BONE REMODELING SIGNALING AND OSTEOCLAST ACTIVATION

Results from aim 2 assess the effects of antidepressants on MSC production of the local microenvironment and how this can affect osteoclastic activity. Osteoclast precursors were treated with MCS-F (20ng/mL) and RANKL (50ng/mL) and exposed to various types and concentrations of antidepressants or serotonin, either directly or through conditioned media from human MSCs which were treated with antidepressants. Osteoclastic behavior was assessed by measuring TRAP activity.

3.2.1. AIM 2.1: DETERMINE THE DIRECT EFFECTS OF ANTIDEPRESSANTS ON OSTEOCLASTIC ACTIVITY

In order to determine the direct effects of SSRIs on osteoclastic TRAP activity in comparison to other antidepressants with lower selectivity for serotonin, SSRI fluoxetine was selected, as well as SNRI duloxetine were used for treatments and compared to treatment of serotonin. There was no difference in osteoclastic TRAP activity between 1μM treatments of serotonin and fluoxetine in comparison to the no treatment control (figure 33). However, treatment with a higher concentration of 10μM increased TRAP activity in comparison to the 1μM dose and the control. Duloxetine 1μM treatments slightly increased TRAP activity in comparison to the

control, however, activity significantly decreased when cells were treated with 10µM concentrations in comparison to the control.

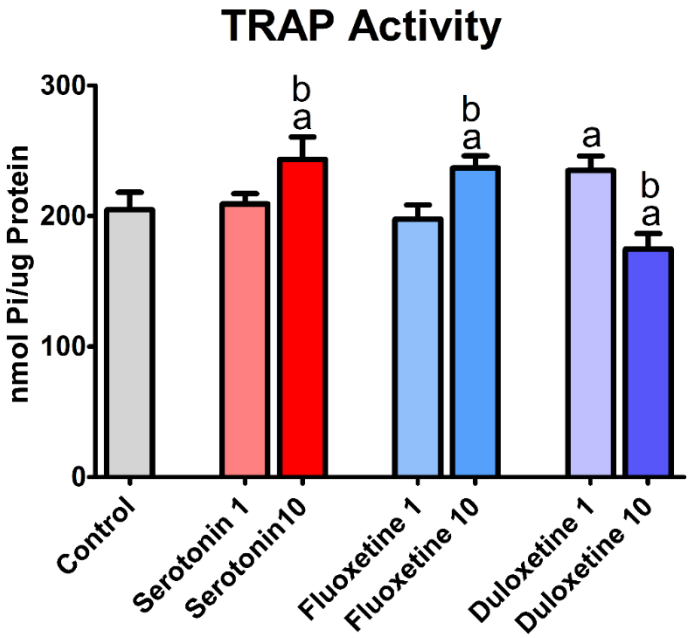

Figure 33: Direct effects of antidepressants on osteoclastic TRAP activity. MCS-F and RANKL-differentiated human monocytes were directly exposed to serotonin, SSRI fluoxetine and SNRI duloxetine at concentrations of 1µM or 10µM while cultured on collagen-coated TCPS. $P < 0.05$ a vs. 0µM, b vs. 1µM.

3.2.2. AIM 2.2: DETERMINE THE EFFECTS OF CONDITIONED MEDIA FROM MSCs TREATED WITH ANTIDEPRESSANTS ON OSTEOCLASTIC ACTIVITY

In order to further investigate if exposing osteoclasts to the microenvironment generated by MSCs after their treatment with antidepressants has the potential to affect TRAP activity, osteoclasts were treated with conditioned media obtained from MSCs cultured on Ti surfaces. Surface roughness effects on MSC microenvironment production during antidepressant treatments on TRAP activity were also assessed. MSCs were plated on TCPS, PT or SLA surfaces and treated with 1µM or 10µM concentrations of serotonin, fluoxetine, sertraline or duloxetine. After treatment, the conditioned media was collected and used to treat osteoclasts for 48 hours and TRAP activity was measured to determine osteoclastic activity.

In conditioned media obtained from MSCs not exposed to antidepressants or serotonin (control media), TRAP activity increased on Ti surfaces in comparison to TCPS, with no difference in activity between smooth or rough Ti surfaces (figure 34a, b and c). Overall,

antidepressants and serotonin conditioned media increased osteoclastic TRAP activity on all

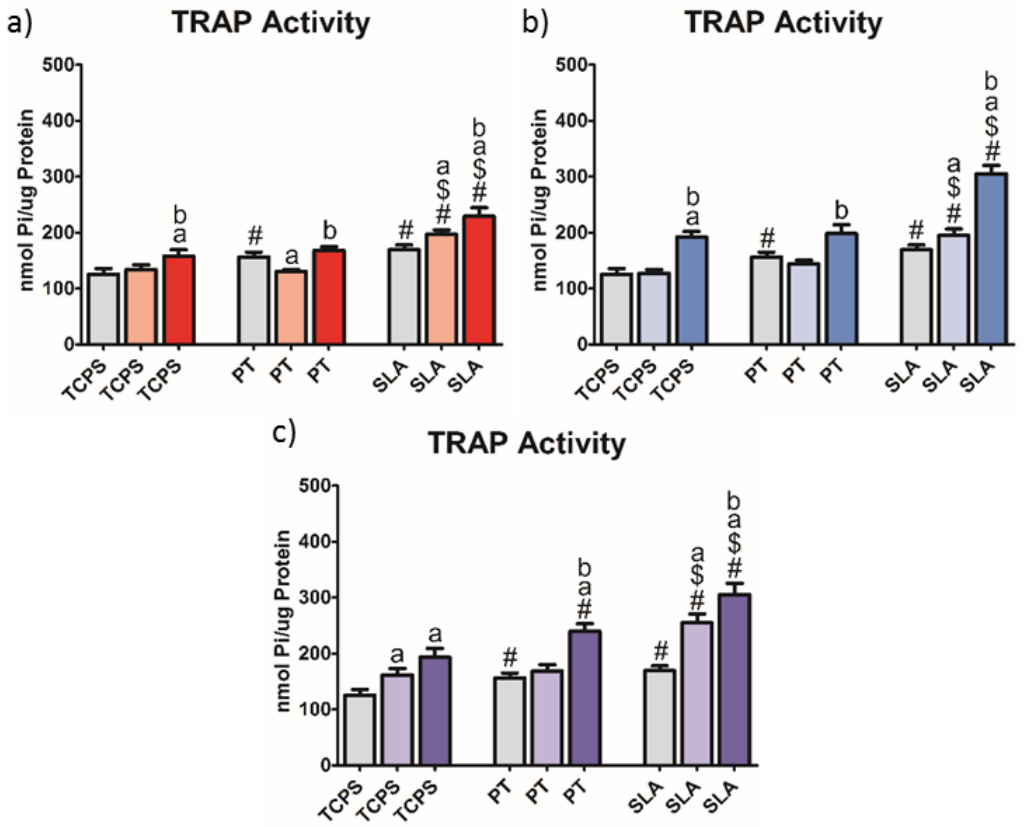

Figure 34: Effects of conditioned media on osteoclastic TRAP activity. MCS-F and RANKL-differentiated monocytes were exposed to conditioned media from MSCs treated with concentrations of 1μM or 10μM of a) serotonin, b) fluoxetine, and c) duloxetine while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 1μM.

surfaces, with the most significant increases evident on SLA. Serotonin and fluoxetine conditioned media increased TRAP activity on TCPS only at the 10μM concentration, with no significant increases on PT surfaces, in comparison to the control (figure 34a and b). On SLA surfaces, however, serotonin and fluoxetine conditioned media dose-dependently increased TRAP activity, with the highest levels after treatment with 10μM of conditioned media when compared to TCPS, PT, 1μM and the control. Conditioned media obtained from MSCs treated with duloxetine from all surfaces increased TRAP activity when compared with the control (figure 34c). Activity was highest for media obtained from the 10μM duloxetine treatments on PT and SLA surfaces in comparison to the control.

3.3. SPECIFIC AIM 3: ELUCIDATE THE EFFECTS OF SSRIS ON SEROTONIN RECEPTORS AND THEIR EFFECTS ON BONE REMODELING

Antidepressants exhibit their therapeutic effects *in vivo* by blocking 5-HTT and increasing extracellular concentrations of serotonin. Serotonin then interacts with its various receptors found on cells, initiating complex internal signal transduction pathways involved in gene expression. The goal for this aim is to determine how surface characteristics of Ti implants affect human MSC gene expression of serotonin receptors when cultured on microstructured Ti surfaces. Human MSCs were grown on smooth (PT), rough (SLA) or rough and hydrophilic (mSLA) Ti substrates throughout differentiation and mRNA levels for HTR1A, HTR2A, HTR1B and HTR2B were measured for using qPCR.

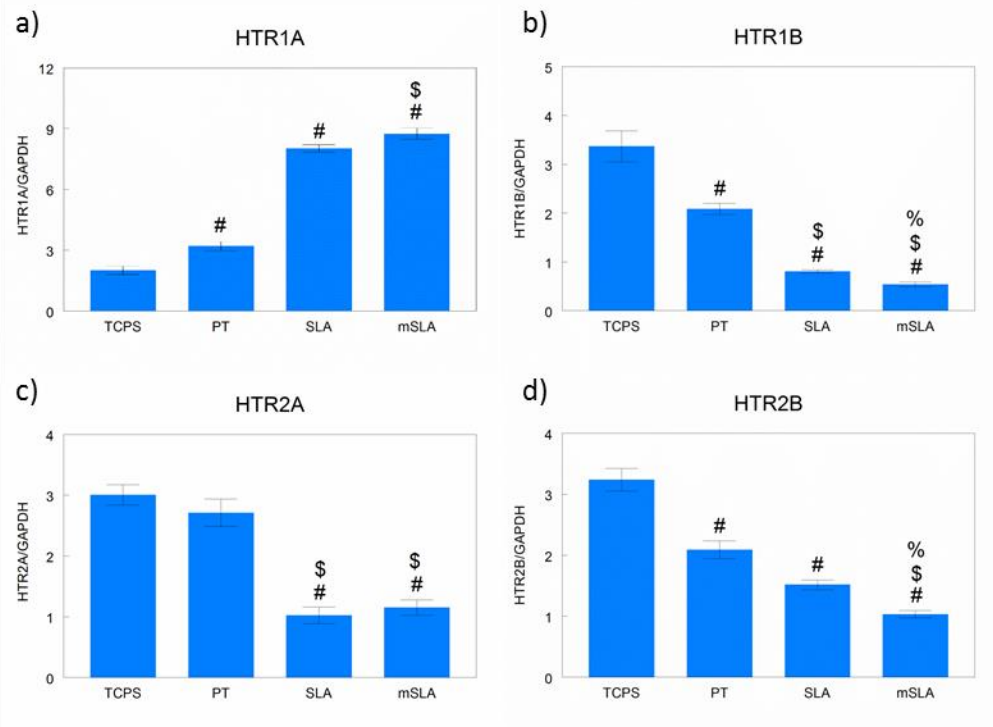

Figure 35: Surface characteristics modulate serotonin receptor gene expression. Human MSC gene expression of serotonin receptors a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B on TCPS, PT or SLA surfaces. $P < 0.05$ # vs. TCPS, $ vs. PT, % vs. SLA.

3.3.1. AIM 3.1: DETERMINE THE EFFECTS OF TI SURFACE ROUGHNESS AND WETTABILITY ON HUMAN MSC SEROTONIN RECEPTOR GENE EXPRESSION

Levels of mRNA for HTR1A receptor increased on PT in comparison to TCPS, with further increases on SLA and mSLA surfaces (figure 34a). There was no difference between mRNA levels for HTR2A on TCPS and PT surfaces, however, levels decreased on SLA and mSLA in

comparison to TCPS and PT (figure 34b). There was no significant difference in expression of HTR2A on SLA in comparison with mSLA surfaces. mRNA levels for the HTR2B receptor were lower on PT in comparison to TCPS, with further decreases on SLA and the most significant decreases evident on mSLA surfaces (figure 34c). HTR2B mRNA levels also decreased on Ti substrates in comparison to TCPS (figure 34d). Levels were lower on PT and SLA in comparison to TCPS, with further decreases on mSLA surfaces.

3.3.2. AIM 3.2: EXAMINE THE EFFECTS OF ANTIDEPRESSANT TREATMENT AND TI SURFACE CHARACTERISTICS ON HUMAN MSC SEROTONIN RECEPTOR GENE EXPRESSION

To further investigate receptor expression in the presence of antidepressants on microstructured Ti surfaces, human MSCs were treated with antidepressants or serotonin while cultured on smooth (PT), rough (SLA) or rough and hydrophilic (mSLA) Ti surfaces throughout differentiation and mRNA levels for HTR1A, HTR1B, HTR2A and HTR2B were assessed.

Serotonin Treatment Modulates Serotonin Receptor Expression

Prior to any treatment with serotonin, expression of HTR1A was higher on PT, with further increases on SLA surfaces, when compared with TCPS (figure 35a). Conversely, expression for HTR1B, HTR2A and HTR2B decreased on SLA surfaces in comparison to TCPS and PT (figure 35b, c and d). All concentrations of serotonin increased HTR1A receptor expression on TCPS and PT surfaces, in comparison to the no treatment control (figure 35a). On SLA surfaces, however, this effect was reversed as serotonin treatment dose-dependently decreased mRNA levels, with the 10μM concentration having the most significant decreases, in comparison to the no treatment control.

Treatment with serotonin at the lower concentrations increased mRNA levels for HTR2A and HTR1B on TCPS and PT surfaces, however, increasing the treatment concentration to 10μM significantly decreased levels in comparison to the no treatment control (figure 35b and c). All concentrations of serotonin increased levels for HTR2A and HTR1B on SLA surfaces in comparison to the no treatment control. Expression was sensitive to the dose, as the 10μM concentration seemed to have slightly lower expression levels when compared to the 1μM dose on SLA surfaces, though still higher than the no treatment groups.

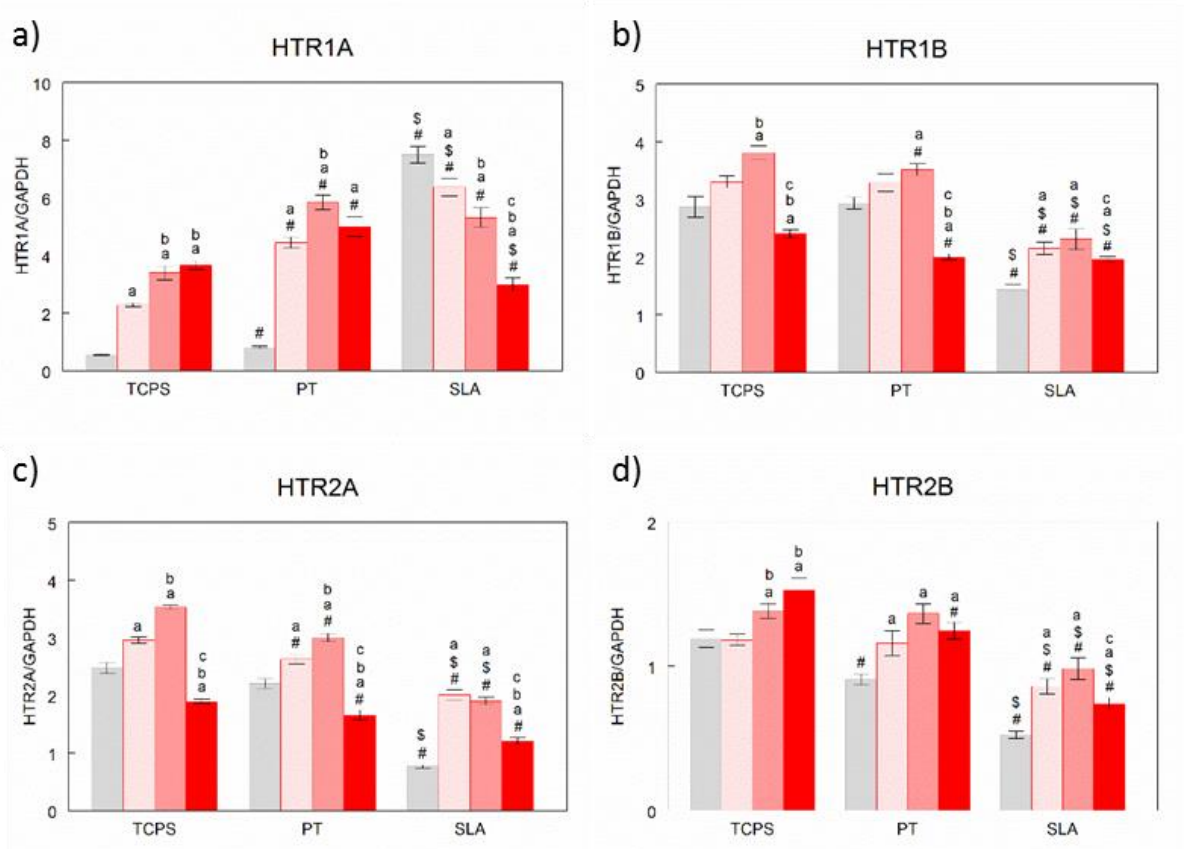

Figure 36: Effects of serotonin treatment on serotonin receptor gene expression. MSC gene expression of a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B was measured after treatment with 0.1µM, 1µM or 10µM of serotonin on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

SSRI Treatment Modulates Serotonin Receptor Expression

In order to determine the effects of SSRIs on human MSC serotonin receptor expression while cultured on microstructured Ti surfaces, cells were treated with various concentrations of the SSRIs fluoxetine, sertraline or paroxetine while cultured on smooth (PT) or rough (SLA) Ti surfaces throughout differentiation and mRNA levels for HTR1A, HTR1B, HTR2A and HTR2B were assessed. Prior to any SSRI treatment, expression of HTR1A was significantly higher on SLA surfaces when compared with TCPS or PT (figures 36a, 37a and 38a). On the contrary, expression for HTR1B, HTR2A and HTR2B was lowest on SLA surfaces in comparison to TCPS and PT (figures 36b-d, 37b-d and 38b-d).

Fluoxetine treatment does-dependently increased HTR1A expression on TCPS and PT in comparison to the no treatment control, with the highest levels seen after treatment with the 10µM concentration (figure 36a). This effect was reversed on SLA surfaces, as treatment decreased expression levels in comparison to the no treatment control, with the lowest expression after

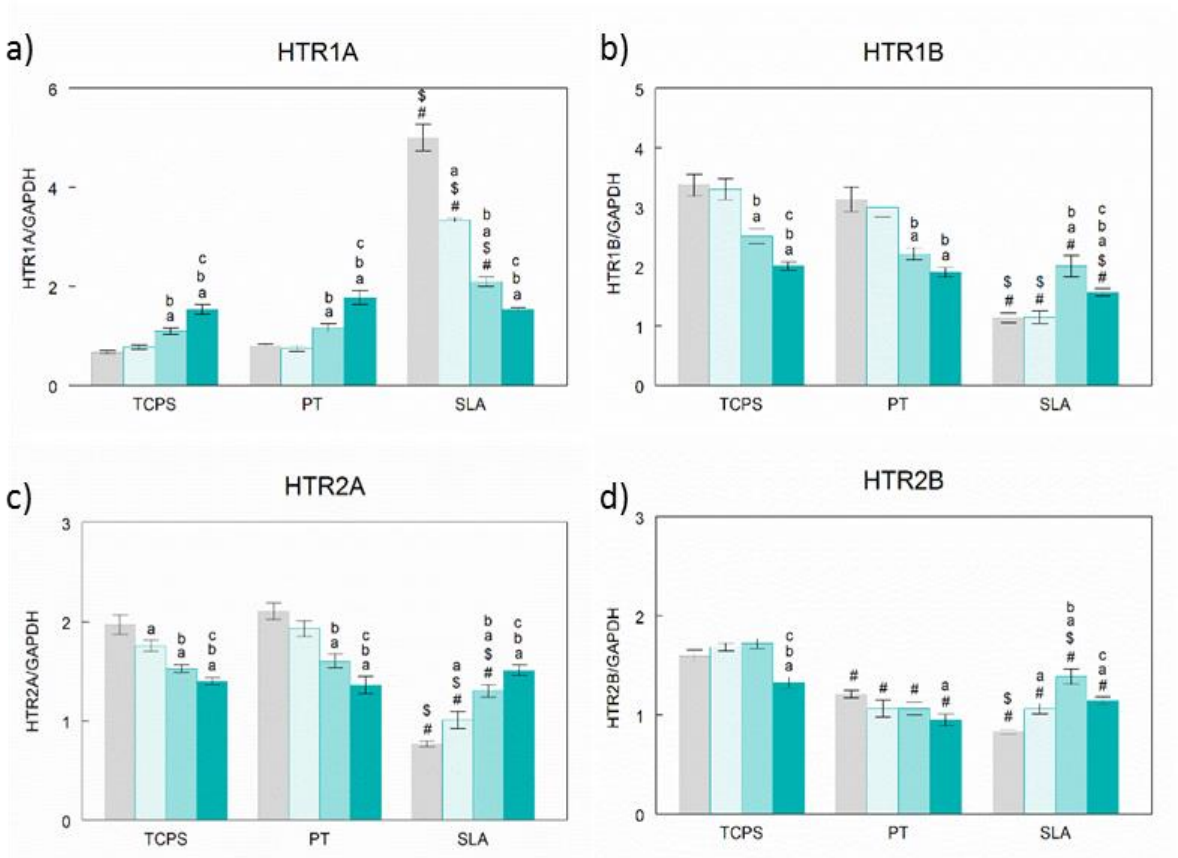

Figure 37: Fluoxetine (SSRI) modulates serotonin receptor gene expression. MSC gene expression of a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B was measured after treatment with 0.1µM, 1µM or 10µM of fluoxetine on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

treatment with the 10µM concentration. Contrary to HTR1A, mRNA levels for HTR2A, HTR1B and HTR2B decreased with fluoxetine treatment on TCPS and PT in comparison to the no treatment control (figure 36b, c, d). Effects were dose-dependent for HTR2A levels on TCPS and PT, with the 10µM dose having the lowest expression (figure 36b). There were no significant differences in HTR1B mRNA levels between the 0.1µM fluoxetine dose and the no treatment control on TCPS and PT, and also for HTR2A on PT surfaces. Only the 10µM concentration

decreased levels for HTR2B on TCPS and PT in comparison to the no treatment control (figure 35d).

On SLA surfaces, however, fluoxetine increased mRNA levels for HTR2A, HTR1B and HTR2B (figure 36b, c and d). Effects were dose-dependent for HTR2A expression, with the highest levels evident after treatment with the 10µM concentration. There were no significant differences between HTR1B mRNA levels after treatment with 0.1µM of fluoxetine in comparison to the no treatment control on SLA surfaces (figure 36c). A slight increase in HTR2B expression after 0.1µM of fluoxetine in comparison to the no treatment control was evident on SLA (figure 36d). Treatment with the 1µM and 10µM concentrations increased mRNA levels for HTR1B and HTR2B, with the highest expression evident after the 1µM dose in comparison to the no treatment control.

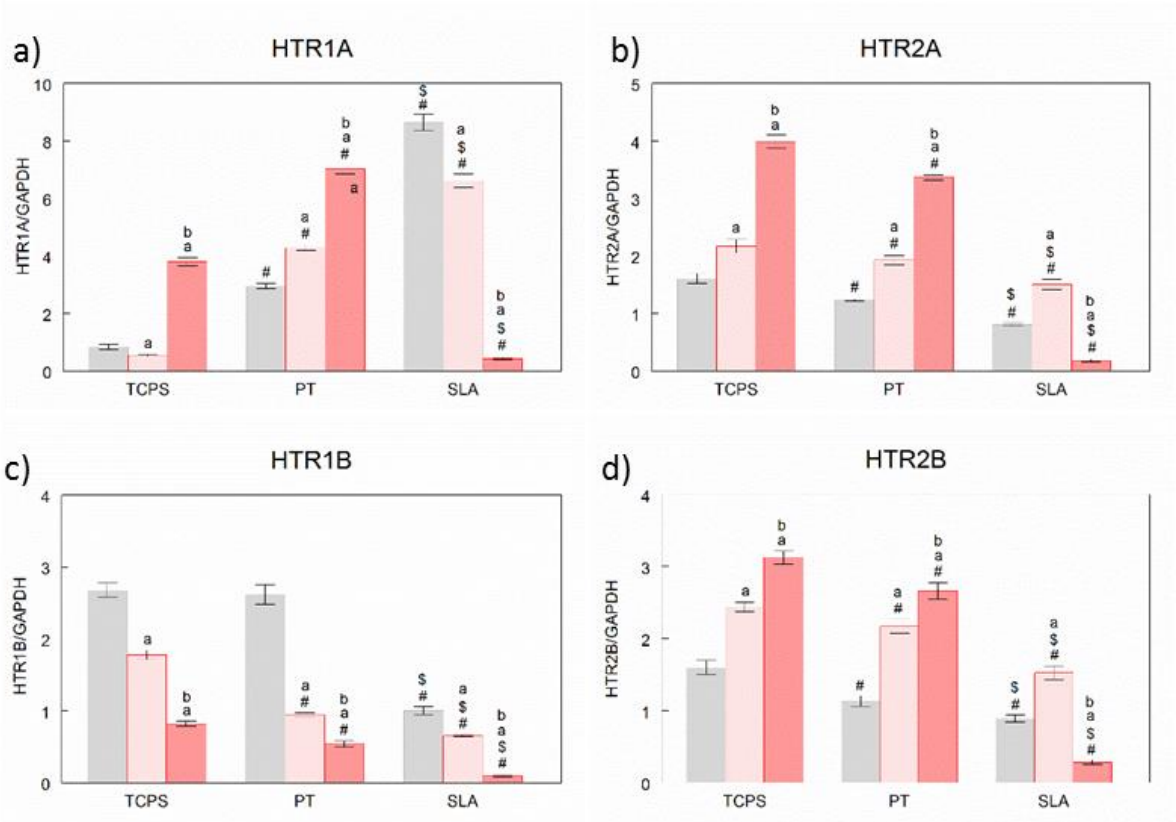

Figure 38: Sertraline (SSRI) modulates serotonin receptor gene expression. MSC gene expression of a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B was measured after treatment with 0.1µM, 1µM or 10µM of sertraline on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

Effects of sertraline and paroxetine were assessed as previously described for fluoxetine. Only the 1μM and 10μM concentrations were used for treatments, as no significant differences in serotonin receptor expression were evident for the 0.1μM concentration of fluoxetine. Sertraline and paroxetine treatments dose-dependently increased mRNA levels for the HTR1A and HTR2A receptors on TCPS and PT in comparison to the no treatment control, with the highest expression levels evident after treatment with the 10μM dose (figure 37a, b and 38a and b). Only the 1μM concentration of sertraline slightly decreased expression on TCPS when compared to the no treatment group (figure 37a). The opposite effect was seen on SLA surfaces. Levels of HTR1A mRNA dose-dependently decreased after sertraline and paroxetine treatment, with the lowest levels evident with the 10μM dose in comparison to PT, the 1μM concentration and the no treatment control (figure 37a and 38a). Treatment with sertraline at the 10μM concentration produced lower expression levels than paroxetine on SLA surfaces. Sertraline significantly decreased levels of mRNA for HTR1A compared to TCPS, PT, the 1μM and the no treatment control when cells were cultured on SLA. All paroxetine treatments increased levels of HTR2A on SLA surfaces in comparison to the no treatment control, with the highest expression evident after treatment with the 1μM concentration (figure 38b). Only the 1μM concentration of sertraline increased HTR2A mRNA levels in comparison to the no treatment control on SLA surfaces (figure 37b). Treatment with 10μM significantly decreased expression in comparison to TCPS, PT, the 1μM dose and the no treatment control.

All concentrations of sertraline dose-dependently decreased HTR1B mRNA levels on TCPS, PT and SLA surfaces in comparison to the no treatment control, with the lowest levels evident after treatments with the 10µM concentration (figure 36c). Expression levels were lower on PT surfaces after 10µM sertraline treatments in comparison to TCPS, with further decreases on SLA surfaces. Dose-dependent decreases for HTR1B levels were similar after paroxetine treatment, but only on TCPS and PT surfaces (figure 37c). On SLA surfaces, however, both concentrations of paroxetine increased HTR1B expression, with the 1µM having the highest levels in comparison to the no treatment control. Sertraline treatment dose-dependently stimulated HTR2B expression on TCPS and PT surfaces with the highest levels evident after treatment with the 10µM concentration, while paroxetine had the opposite effect (figure 36d and 37d). Only the 1µM dose of sertraline increased HTR2B levels on SLA surfaces in comparison to the no treatment

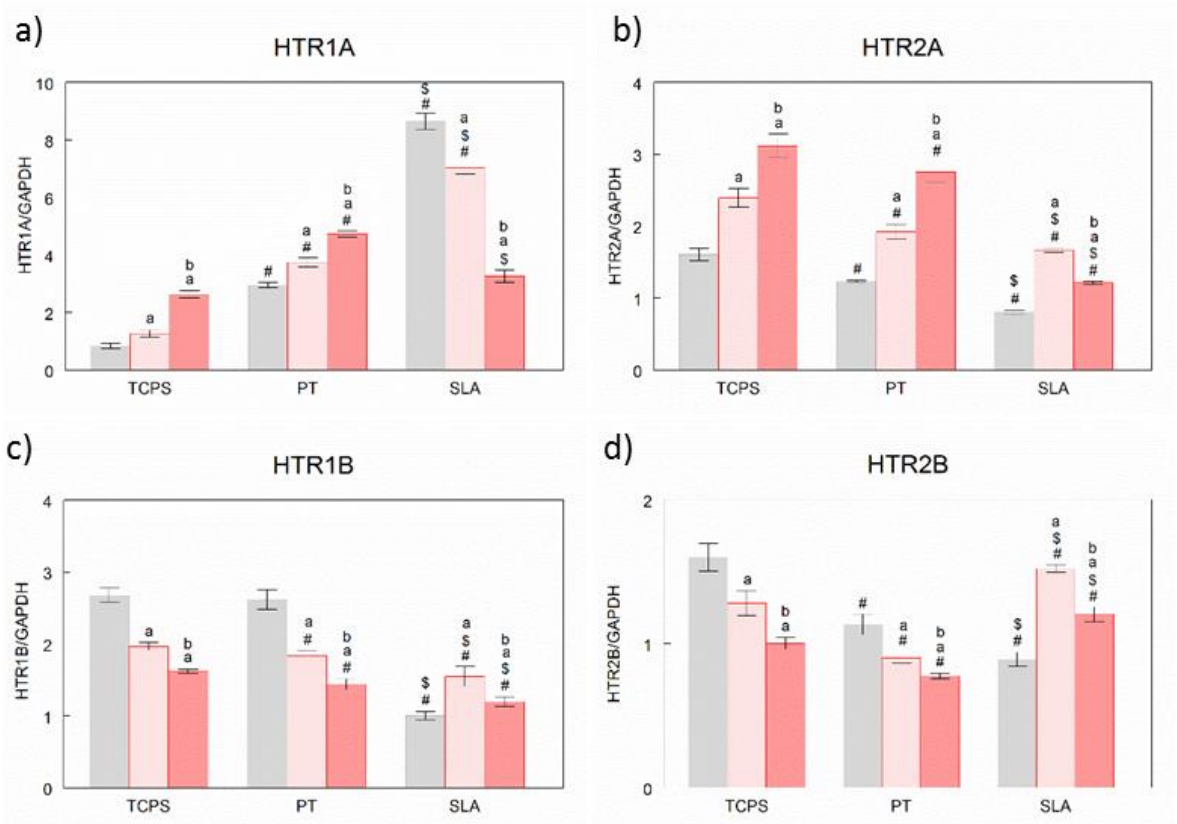

Figure 39: Paroxetine (SSRI) modulates serotonin receptor gene expression. MSC gene expression of a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B was measured after treatment with 0.1µM, 1µM or 10µM of paroxetine on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

control. Treatment with 10μM of sertraline had the most significant decreases in HTR2B expression when compared with TCPS, PT, the 1μM concentration and the no treatment group. Both the 1μM and 10μM paroxetine doses increased HTR2B mRNA levels on SLA surfaces in comparison to the no treatment control, with the 1μM dose having the highest expression levels.

SNRI Treatment Modulates Serotonin Receptor Expression

In order to assess the effects of antidepressants within the SNRI class on gene expression of serotonin receptors by human MSCs, cells were treated with 1μM or 10μM concentrations of duloxetine, a commonly prescribed SNRI. Dose-dependent increases of HTR1A expression was evident on TCPS and PT with an increase in duloxetine dose as compared to the no treatment control (figure 39a). This effect was reversed, however, on SLA surfaces. Duloxetine treatment decreased HTR1A expression with the increase in drug concentration when compared to the no treatment control. Treatment with 1μM of duloxetine increased mRNA levels of HTR2A on all surfaces in comparison to the no treatment control (figure 39b). Increasing the dose to 10μM had the lowest levels in comparison to the 1μM concentration and the no treatment control on TCPS and PT surfaces. Treatment with the 10μM concentration decreased HTR2A mRNA levels when compared to the 1μM treatments on SLA surfaces, but levels were still slightly higher than the no treatment controls.

Duloxetine treatment does-dependently decreased HTR1B expression levels on TCPS and PT in comparison to the no treatment control, however, expression increased on SLA surfaces by treatments, with the 1μM concentration having the highest levels in comparison to the 10μM and the no treatment control (figure 39c). Only the 10μM concentration significantly decreased HTR2B expression on TCPS in comparison to the no treatment control (figure 39d). Treatment with the 1μM dose of duloxetine increased expression in comparison to the no treatment control, while the 10μM concentration had no significant effects on PT surfaces. All treatment

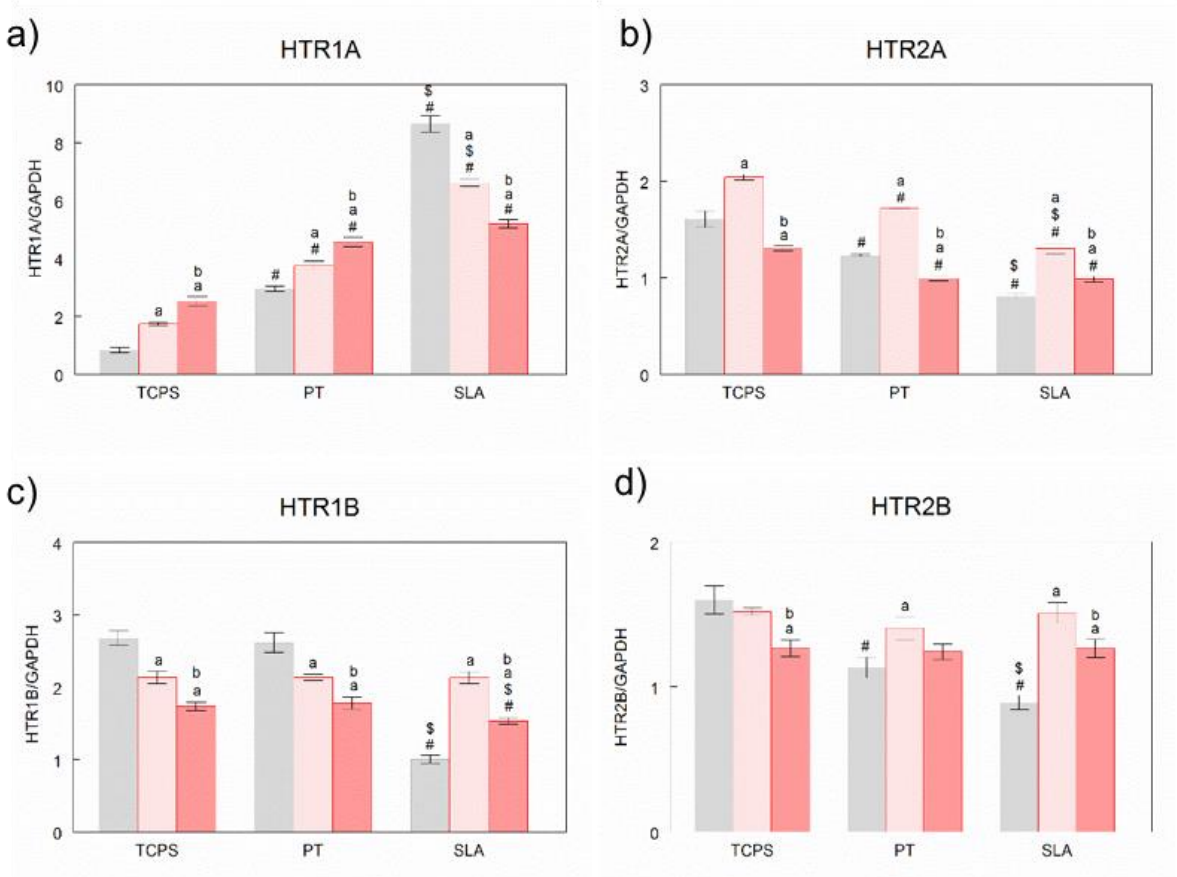

Figure 40: Duloxetine (SNRI) modulates serotonin receptor gene expression. MSC gene expression of a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B was measured after treatment with 0.1μM, 1μM or 10μM of duloxetine on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0μM, b vs. 1μM.

concentrations increased expression levels on SLA surfaces, with the highest levels evident after treatment with the 1μM concentration of duloxetine, when compared to the no treatment control.

Other Antidepressant Treatment Modulates Serotonin Receptor Expression

Trazodone and bupropion are also two very commonly prescribed antidepressants, but they do not belong to a specific class. Their effects on serotonin receptor expression by MSC was assessed in a similar way. Cells were cultured on PT or SLA surfaces and compared to those on TCPS while being treated with either 1μM or 10μM concentrations of trazodone or bupropion. Serotonin receptor expression was evaluated after treatments. Only the 10μM concentration of trazodone increased HTR1A receptor expression on TCPS when compared to the no treatment control (figure 40a). Treatment at the 1μM concentration had the highest expression levels, and

10µM treatments had the lowest, when compared to the no treatment control on PT surfaces. All treatments dose-dependently decreased HTR1A expression levels on SLA surfaces, with the 10µM concentration having the lowest levels, in comparison to the no treatment control. Expression levels for HTR2A were highest after treatment with 1µM of trazodone on TCPS, PT and SLA surfaces when compared to the 1µM concentration and the no treatment control (figure 40b). The 10µM treatments decreased expression on TCPS but there was no difference between the 10µM concentration and the no treatment control on PT surfaces. Both trazodone concentrations increased HTR2A expression levels on SLA surfaces in comparison to the no treatment control,

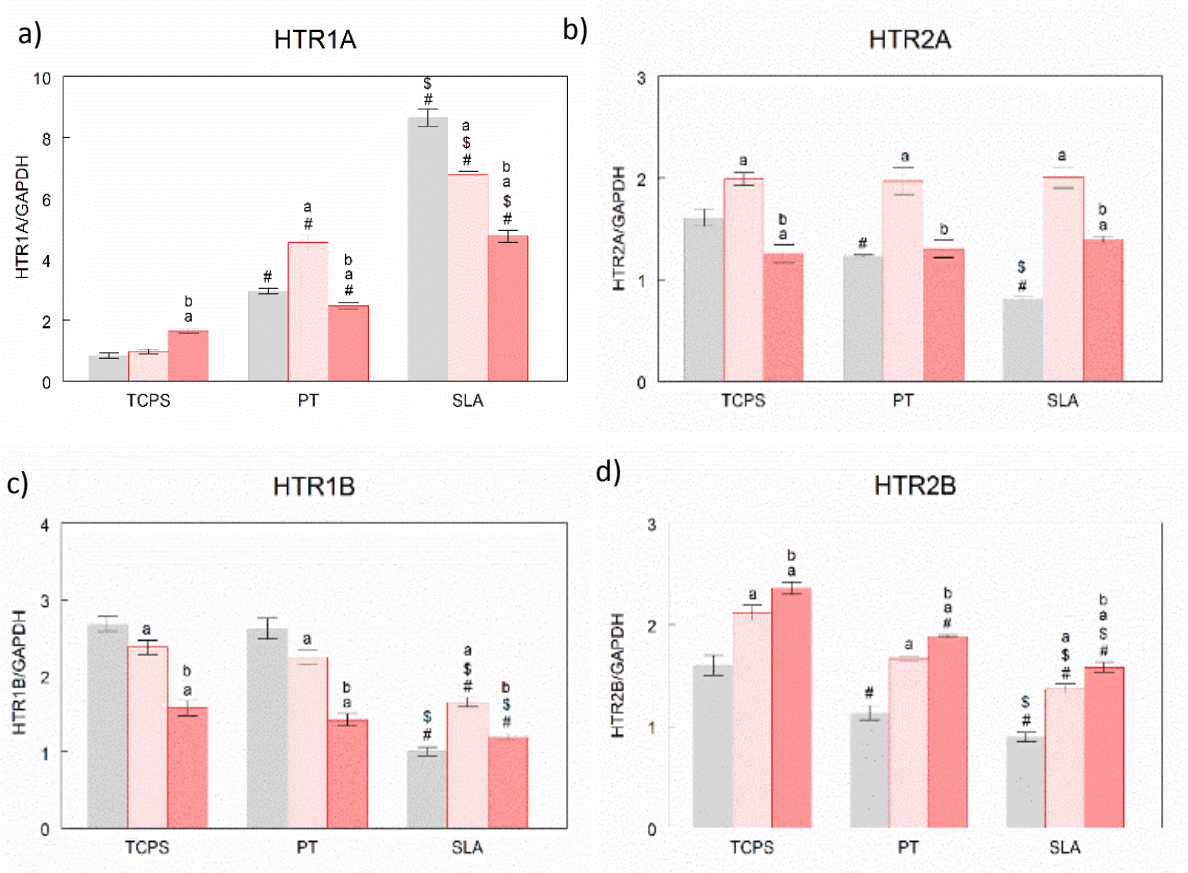

Figure 41: Trazodone modulates serotonin receptor gene expression. MSCs gene expression for a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B after treatment with 0.1µM, 1µM or 10µM of trazodone on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

with the 1µM concentration having the highest levels. Treatments with trazodone at either concentration was inhibitory for HTR1B expression levels on TCPS and PT surfaces, with the highest concentration having the lowest levels of mRNA in comparison to the no treatment control

(figure 40c). The reverse was true on SLA surfaces, as both concentrations increased expression, with the highest levels evident after treatment with the 1µM dose, when compared to the no treatment control. Expression of HTR2B dose-dependently increased after trazodone treatment on all surfaces in comparison to the no treatment control, with the highest levels evident after treatment with the 10µM concentration (figure 40d).

Bupropion treatments dose-dependently increased HTR1A expression levels on TCPS and PT in comparison to the no treatment control, with the highest levels evident after treatment with the 10µM concentration (figure 41a). On SLA surfaces, treatments decreased expression in comparison to the no treatment control, with the lowest seen after treatment with 1µM doses.

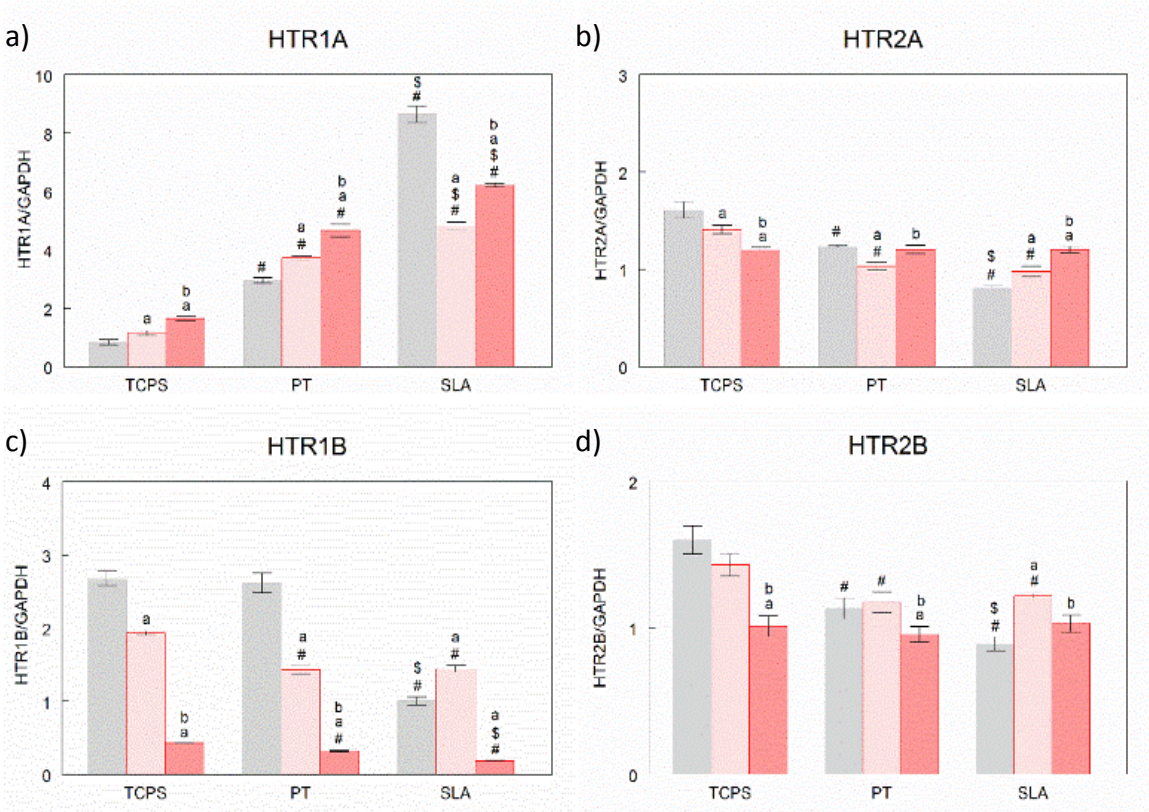

Figure 42: Bupropion modulates serotonin receptor gene expression. MSCs gene expression for a) HTR1A, b) HTR2A, c) HTR1B and d) HTR2B after treatment with 0.1µM, 1µM or 10µM of bupropion on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM, b vs. 1µM.

Expression of HTR2A dose-dependently decreased on TCPS, and increased on SLA surfaces, when compared to the no treatment control (figure 41b). Only the 1µM concentration decreased expression on PT surfaces, in comparison to the no treatment control. The 10µM dose increased

expression when compared to the 1μM, but there were no significant differences between the 10μM dose and the control. Expression of HTR1B also followed dose-dependent decreases on TCPS and PT surfaces in comparison to the no treatment control, with the lowest levels evident after treatment with the highest concentration (figure 41c). Expression increased after treatment with 1μM concentration of bupropion, however, increasing the dose to 10μM significantly decreased expression when compared to the 1μM concentration and the no treatment control on SLA surfaces. There was no difference in HTR2B expression between the 1μM concentration and the no treatment control on TCPS or PT surfaces, however, augmenting the dose to 10μM significantly decreased expression (figure 41d). Treatment with 1μM of bupropion increased expression on SLA surfaces in comparison to the no treatment control. The 10μM concentration lowered expression in comparison to the 1μM dose, but was not statistically significant when compared to the no treatment control.

3.3.3. AIM 3.3: INVESTIGATE THE EFFECTS OF SEROTONIN RECEPTOR INHIBITION ON HUMAN MSC PRODUCTION OF BONE REMODELING SIGNALING ON TI SURFACES

Studies in aim 3.3 were performed to investigate whether serotonin receptors play a role in human MSC local factor secretion of proteins involved in bone remodeling processes, and how these effects are modulated by Ti surface roughness. Human MSCs were treated with different types of serotonin receptor inhibitors specific for HTR_{1A}, HTR_{1B}, HTR_{2A} and the HTR_{2B} receptors while being cultured on PT or SLA surfaces and compared to those on TCPS. Cells were treated throughout differentiation for 7 days, then levels of secreted OPG and RANKL were measured. The hypothesis is that if a serotonin receptor is involved in bone remodeling processes, then it will alter OPG or RANKL protein production by the MSCs according to surface roughness.

Serotonin Receptor Inhibition

In order to examine whether the HTR_{1A} receptor is involved in modulating OPG and RANKL levels, human MSCs were treated with 1μM of WAY-100635, an HTR_{1A} receptor inhibitor. Prior to any inhibition, secreted levels of OPG and RANKL were higher on Ti surfaces in comparison to TCPS (figures 42, 43, 44, 45 and 46). Treatment with the HTR_{1A} inhibitor decreased OPG and RANKL protein levels on TCPS and PT surfaces when compared to the no

treatment control, with further decreases on SLA surfaces in comparison to the control, PT and SLA (figure 42).

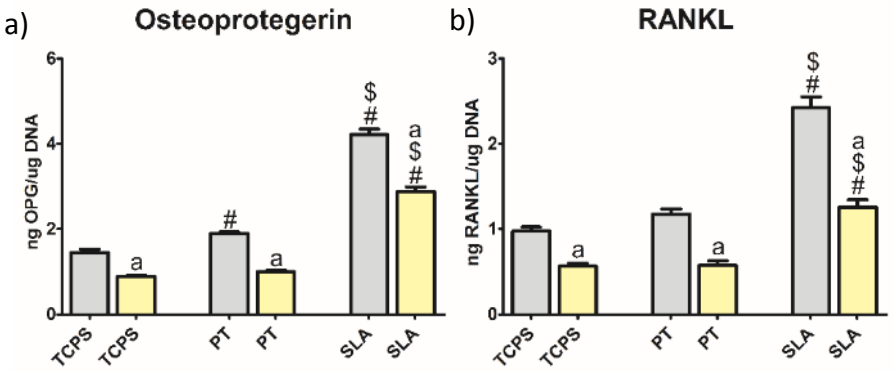

Figure 43: Effects of HTR_{1A} on bone remodeling. Human MSC secreted protein levels were measured for a) OPG and b) RANKL after inhibition of HTR_{1A} receptor with 1µM treatment of WAY-100635 while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM.

Human MSC HTR_{2A} inhibition was achieved by treatment with 1µM of RH-34, an HTR_{2A} inhibitor, for 7 days throughout differentiation on PT, SLA or TCPS surfaces. There were no significant differences in secreted OPG levels on TCPS, PT or SLA surfaces after HTR_{2A} inhibition when compared to the no treatment control (figure 43a). However, RANKL levels decreased in comparison to the no treatment control post HTR_{2A} inhibition all surfaces (figure 43b).

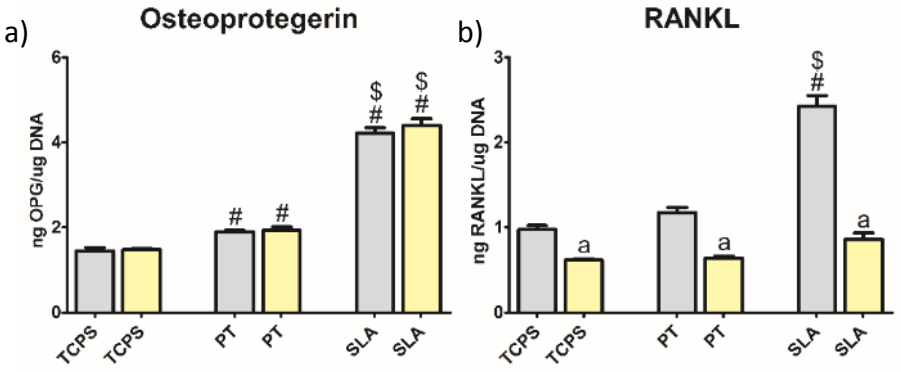

Figure 44: Effects of HTR_{2A} on bone remodeling. Human MSC secreted protein levels were measured for a) OPG and b) RANKL after inhibition of HTR_{2A} receptor with 1µM treatment of RH-34 while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM.

MSCs were treated in the same manner as above but with either SB-224289 or RS-127445, HTR_{1B} or HTR_{2B} inhibitors. Blocking the HTR_{1B} and HTR_{2B} receptors had no effect on secreted

OPG or RANKL protein levels on any of the surfaces (figures 43 and 44). Treatment with all of the inhibitors combined showed similar effects as those of the HTR_{1A} inhibition (figure 45). There were no significant differences in OPG protein levels after inhibition of all four of the serotonin receptors on TCPS in comparison to the no treatment control (figure 45a). However, on Ti surfaces, receptor inhibition decreased protein levels on PT in comparison to the no treatment control, with further decreases on SLA surfaces when compared to TCPS, PT and the no treatment control. Levels of secreted RANKL decreased on all surfaces in comparison to the no treatment control (figure 45b).

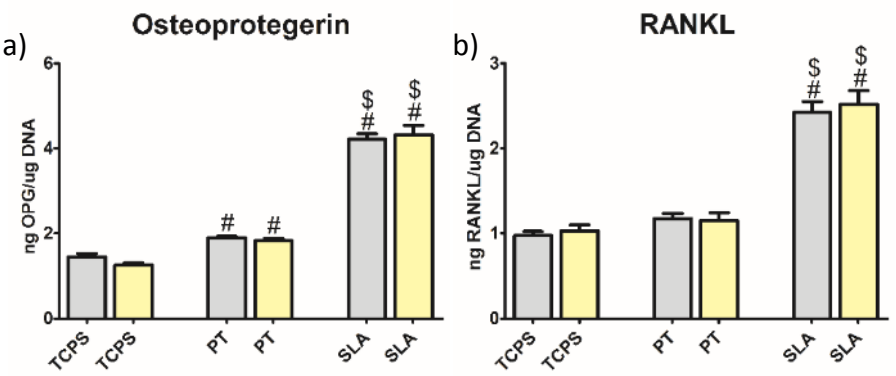

Figure 45: Effects of HTR_{1B} on bone remodeling. Human MSC secreted protein levels were measured for a) OPG and b) RANKL after inhibition of HTR_{1B} receptor with 1µM treatment of SB-224289 while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM.

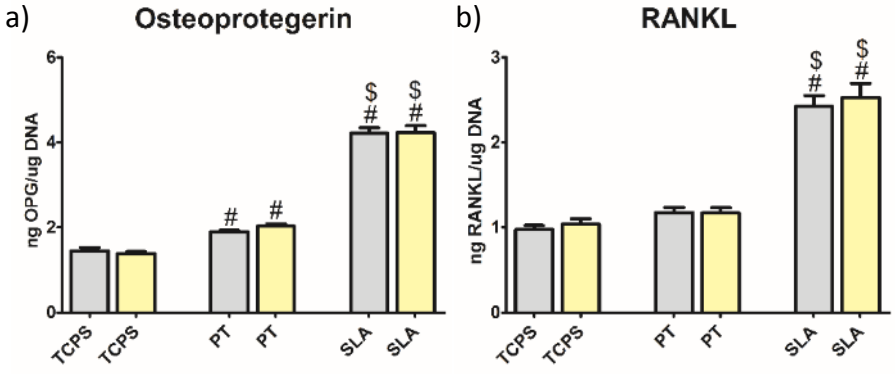

Figure 46: Effects of HTR_{2B} on bone remodeling. Human MSC secreted protein levels were measured for a) OPG and b) RANKL after inhibition of HTR_{2B} receptor with 1µM treatment of RS-127445 while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM.

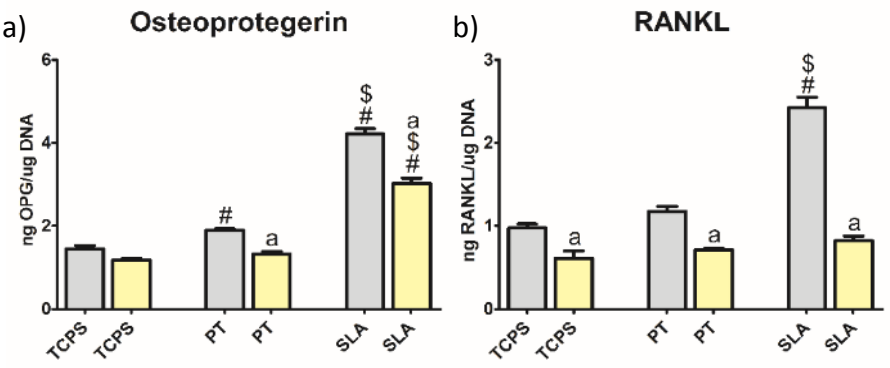

Figure 47: Effects of serotonin receptors inhibition on bone remodeling. MSC secreted proteins were measured for a) OPG and b) RANKL after inhibition of HTR1A, HTR1B, HTR2A and HTR2B serotonin receptors with 1µM treatment of all the inhibitors combined while cultured on TCPS, PT or SLA surfaces. Between surfaces: $P < 0.05$ # vs. TCPS, $ vs. PT. Per surface: $P < 0.05$ a vs. 0µM.

CHAPTER 4 DISCUSSION

The findings presented in this work demonstrate the detrimental effects of frequently prescribed antidepressants on bone formation and remodeling on clinically relevant Ti surfaces commonly used in dental applications. Antidepressants inhibited human MSC differentiation and decreased protein levels associated with bone formation while increasing those involved in bone resorption on microstructured Ti surfaces. The drugs also increased osteoclastic activity both directly and through treated MSCs, with the highest levels evident after treatment with conditioned media from MSCs on microstructured Ti surfaces. Our findings suggest that osteoclastic activity is mediated through increased RANKL production, which is regulated by serotonin receptor HTR_{2A}.

Clinically, the use of microstructured implants have higher success rates than smooth ones in that they have been shown to reduce healing time, improve mechanical stability and provide greater bone-implant contact [29]. *In vitro* studies have also demonstrated that microstructured Ti substrates increased human MSC differentiation without the addition of osteogenic supplements [31]. Topographical modifications at the implant surface created a roughened surface topography, which was adequate enough on its own to induce differentiation in human MSCs. However, despite the high success rate of rough implants in healthy recipients, patients taking antidepressant medications, especially those in the SSRI category, have an increased risk of dental implant failure, decreased bone mineral density and an increased risk of fractures [3].

Antidepressants achieve therapeutic effects by increasing extracellular concentrations of synaptic serotonin. This is facilitated by blocking the functionality of the serotonin transporter in the brain, as well as on various cells throughout the body. Higher levels of systemic serotonin may have detrimental effects on the quality of bone. Serotonergic functions have previously been thought to be restricted to the brain, however, serotonin has recently been shown to be important in bone metabolism. Peripherally-derived serotonin accounts for the majority of serotonin production in the body. Additionally, the neurotransmitter is unable to readily cross the blood-brain barrier, meaning that central serotonergic actions should be thought of as separate from

peripheral ones. Since a lesser amount of serotonin positively favors bone mass accrual in the brain in comparison to the majority that is produced peripherally, effects of peripheral serotonin are the most concerning in terms of bone metabolism.

Effects of serotonin may be modulated by various types and doses of antidepressants. Although there has been an increase in research efforts regarding the effects of SSRIs on bone, the capacity of bone formation during antidepressant exposure surrounding biomaterials has yet to be elucidated. Work done in this thesis demonstrates the *in vitro* capacity of bone formation on Ti substrates commonly utilized for dental implant applications under exposure of the most frequently prescribed categories of antidepressants. Bone formation was assessed by early and late osteoblastic differentiation markers and secreted local factors produced by MSCs on Ti surfaces. Ti surface roughness effects on these processes were also assessed during various concentrations of serotonin or antidepressant treatments. These studies provide valuable insight into the *in vivo* processes involved with antidepressant use with biomaterial applications.

Prescribed doses of SSRIs in humans vary between each drug. Fluoxetine, the most commonly prescribed SSRI in the U.S., is given in doses of 10, 20 or 40mg capsules in order to achieve a therapeutic range of around 0.5-2.5μM in the blood [11]. The 1μM concentration used in these studies was chosen as a low dose to simulate such therapeutic ranges while investigating the effects of the drugs on MSC differentiation. However, increasing evidence of SSRI bioaccumulation have been documented at much greater concentrations in the bone marrow in comparison to those in the blood [36]. Reports of fluoxetine levels being as high as 100μM in human bone marrow of patients taking the drug, and traces were still detected 3 months after the treatment was discontinued [42]. The 10μM concentration used in these studies was used to investigate the effects of the drugs at a higher dose. Such concentrations detected in the bone marrow are much greater than the highest concentration used in these studies, suggesting larger potential toxicity and greater decreases in MSC differentiation capacity with increased prescription doses and longer duration of treatments.

Quantification of human MSC DNA concentrations on Ti substrates prior to any serotonin or antidepressant treatment showed the cells interacting with and attaching to the substrates which they were cultured on. Prior to pharmacological treatment, DNA concentrations were lower on

microstructured surfaces than on TCPS or smooth Ti. Early and late osteoblastic differentiation marker expression of RUNX2 and BGLAP, respectively, was also higher on rough surfaces than smooth or TCPS. Similar results were established in prior studies [8], and collectively, these responses are indicative of MSC differentiation towards an osteoblastic lineage. Similar levels of DNA were evident after treatment with serotonin at all concentrations on each surface when compared to the no treatment controls, suggesting that serotonin treatment is not detrimental to cell survival. However, when the cells were treated with higher concentrations of antidepressants (10μM), DNA content was significantly decreased on all surfaces. Although we did not perform cytotoxic assays in these studies, decreased DNA levels after treatments with higher concentrations of antidepressants suggests that the cells are sensitive to the dose and were unable to survive with the increase in treatment concentration.

Serotonin has been shown to be an important regulator in bone metabolism. This was evident in these studies, as physiologically relevant concentrations of serotonin enhanced expression of RUNX2 and BGLAP osteoblastic differentiation markers when MSCs were plated on plastic or smooth Ti surfaces. Effects were dose-sensitive, as lower doses (1μM) seemed to induce greater expression of osteoblastic differentiation markers compared to higher ones (10μM). However, when cells were cultured on rough Ti surfaces in this work, all concentrations of serotonin affected MSC differentiation by decreasing expression of early and late osteoblastic markers. This was further confirmed by decreases in alkaline phosphatase specific activity (early marker of osteoblastic differentiation) as well as secreted OCN (late marker) protein levels in the media. These effects of serotonin treatment were only evident on rough Ti surfaces, and were not apparent on plastic or smooth Ti surfaces. Antidepressants, and higher concentrations of serotonin, impaired the osteoblastic differentiation potential of human MSCs and decreased protein levels important for the osteogenic and angiogenic environment. ALP and OCN are proteins produced and secreted by osteogenic cells and are essential for bone formation, and therefore, a decrease in their expression by elevated serotonin levels illustrates a decreased ability of MSCs to differentiate and form bone. These responses were exacerbated on rough Ti surfaces, as cells seemed to be sensitive to the surface roughness and the treatment dose.

Cells cultured on rough Ti surfaces were more susceptible to serotonin and antidepressant treatment than those on TCPS or smooth PT. These effects can be explained by the materials' surface characteristics. It has been shown that MSCs grown on microstructured Ti are more differentiated than those on smooth surfaces, as measured by increases in osteoblastic differentiation markers [31]. It is possible that cells undergoing differentiation, as induced by surface characteristics, are more affected by the drugs than those not in a differentiation state (on TCPS). Cells on rough surfaces may utilize certain signaling mechanisms which are critical to their differentiation process, and such signaling may be altered by serotonin or antidepressants, where as non-differentiated cells (cells cultured on TCPS) are not susceptible in the same manner.

Higher doses of serotonin or antidepressants exhibited greater inhibition of MSC differentiation and lower protein levels on rough surfaces compared with smooth or plastic, as measured by decreases in alkaline phosphatase specific activity and secreted OCN, OPG, BMP-2 and VEGF protein levels. However, not all antidepressants performed equally. Those within the SSRI family showed significantly lower levels of proteins associated with bone formation when compared to other types of antidepressants which are not as selective for serotonin, such as duloxetine (SNRI), trazodone or bupropion. Sertraline seemed to be the antidepressant that affected bone formation the most, as measured by having the most significant decreases in OCN and VEGF protein levels. Furthermore, secreted OPG protein levels by MSCs treated with the high dose of sertraline and cultured on rough Ti surfaces were so low they could not be detected. It is possible that the drug is affecting other vital protein production or secretion at this particular dose. Similar results were published by Fraher et al., where human adipose tissue-derived MSCs showed significant osteoblastic differentiation inhibition on plastic substrates by measuring decreases in ALP activity as well as RUNX2 mRNA levels after treatment with the same high dose (10μM) concentration of sertraline used in this work [4].

These findings are of important relevance, as most *in vitro* studies explore cellular processes using TCPS, however, it has been shown that cells are sensitive to surface characteristics such as roughness, chemistry and energy, and more importantly, modulate their functions accordingly. Thus, results obtained from TCPS surfaces are not the best representation of the potential *in vivo* cellular response surrounding rough Ti implant surfaces. Plastic substrates are not

the materials of choice for applications in healing and regeneration, and so, cellular effects during antidepressant exposure are more accurately represented on clinically relevant biomaterial surfaces.

It is necessary for osteogenic cells to control their microenvironment in order to successfully support the formation of new bone. Osteoblastic cells produce proteins in their local environment as a form of communication with other cells to promote osteogenesis, angiogenesis and regulate bone remodeling processes. Cells surrounding an implant can regulate themselves and those located distally via autocrine and paracrine means by secretions of BMP-2, and VEGF. Osteoblastic lineage cells also secrete OPG and RANKL for communication with osteoclastic cells and regulating bone remodeling process. Imbalances in secreted protein levels may affect osteogenic capability, bone quality and delay implant osseointegration. While the greatest production of BMP-2, OPG, RANKL and VEFG was evident on rough Ti surfaces, MSCs treated with antidepressants or serotonin had significant decreases in these protein levels. Higher doses of serotonin or antidepressants exhibited even lower protein levels on rough surfaces compared with smooth or plastic, as measured by decreases in secreted OPG, BMP-2 and VEGF protein levels. Conversely, RANKL production significantly increased on rough Ti surfaces after treatment with antidepressants.

Interestingly, not all antidepressants performed equally. Those within the SSRI family showed significantly lower levels of proteins important for bone formation when compared to other types of antidepressants which are not as selective for serotonin. Of all SSRIs used in these studies, sertraline seemed to be the drug that affects the highest bone formation, as measured by having the most significant decreases in OCN and VEGF and increases in RANKL protein levels. Furthermore, secreted OPG protein levels by MSCs treated with the high dose of sertraline and cultured on rough Ti surfaces were so low they could not be detected.

Serotonin and antidepressants had direct effects on osteoclastic activity by increasing levels of enzymatic TRAP activity when treated with higher concentrations of serotonin or fluoxetine. These results parallel the work done by Gustafsson et al. demonstrating expression of serotonin receptors and the transporter by osteoclasts [11] and Chabbi-Achengli et al., showing the importance of physiological serotonin levels in stimulating osteoclastic resorption [22]. Treatment

of osteoclasts with conditioned media from MSCs treated with antidepressants had higher TRAP activity when compared to the control. Osteoclasts were affected by factors present in the conditioned media as a result of MSC treatment with antidepressants, as measured by increases in TRAP activity, and is an indication that bone resorption may be increased. On the contrary, other types of antidepressants such as duloxetine, an SNRI, decreased TRAP activity at the high dose of treatment. Furthermore, when factors produced by MSCs in the conditioned media as a result of antidepressant treatment were used to treat osteoclasts, osteoclastic activity was also affected, as measured by significant increases in TRAP activity according to dose and the Ti substrate from which the conditioned media was collected from. The highest levels of TRAP activity were evident in cells treated with conditioned media where MSCs were grown on rough Ti surfaces.

Direct treatment of osteoclast precursors did not reflect the same effects on TRAP activity as indirect treatment by conditioned media. TRAP activity decreased when cells were directly treated with the high dose of duloxetine. However, when osteoclasts were exposed to conditioned media from duloxetine-treated MSCs at the same concentration, TRAP activity increased. An explanation for this may be that other factors, such as interleukins produced by the cells as a response to antidepressant treatment, could be contributing to TRAP activity, in addition to the increased RANKL production. Higher concentrations of extracellular serotonin can enhance production and secretion of interleukin 1 beta (IL-1β) and interferon gamma (IFN-γ) pro-inflammatory cytokines by cells possessing serotonin receptors [46].

Work done in these studies suggests that dental implant failure in individuals taking antidepressants could be caused in part by antidepressant-induced imbalances in the OPG/RANKL system. Misregulation in this signaling could have an effect on bone quality surrounding a Ti dental implant, which can have direct effects on its osseointegration and ultimate success. Prior to any drug treatment, OPG and RANKL protein production by osteogenic cells increased on microstructured Ti surfaces, and similar results were shown in prior studies [47]. Antidepressant treatment decreased OPG and further increased RANKL levels. The presence of excessive amounts of RANKL by antidepressant use may overwhelm the already decreased levels of OPG, increasing the chance for RANK to bind to RANKL and supporting osteoclastic bone resorption. This scenario could lead to excessive osteoclastic activity and greater bone loss surrounding an

implant. In this scenario, osteoblasts may continue to differentiate and deposit new bone matrix, however, the rate of differentiation will be severely affected by the drug, and matrix deposition may be much slower in comparison to the rate of resorption by the osteoclasts. Higher osteoclastic activity may leave many unfilled resorption pits, and overtime, bone quality will worsen. Since osseointegration of dental implants is dependent on the quality of bone, chronic antidepressant use may delay the osseointegration process, ultimately leading to implant failure.

Antidepressants elevate systemic concentrations of serotonin, which encompasses the majority of levels in the body. Serotonin then modulates its effects on bone through its various receptors. Work performed in these studies not only demonstrates MSC expression of serotonin receptors, but more importantly, cells modulated this expression according to surface characteristics. All receptor expression decreased with increasing surface roughness, with the exception of HTR1A, and treatment with serotonin or antidepressants further modulated these effects. It is suggested that HTR_{1A} may be involved in differentiation and this is inhibited by antidepressant use.

Prior studies indicate that the HTR_{2A} receptor is highly expressed in comparison to all other receptors and their isoforms [9] and [10]. With respect to these findings, serotonin may be modulating bone metabolism and exhibiting its actions mostly through this receptor. In these studies, human MSCs does-dependently increased expression of this receptor on rough surfaces in comparison to smooth or TCPS. In addition, when treated with fluoxetine, HTR_{2A} expression by MSCs increased incrementally with the increasing dose on rough Ti surfaces. Such results may be an indication that fluoxetine enhances expression of this receptor in bone with increasing doses. This may be remarkably detrimental on bone quality, given that a higher dose may further magnify these effects.

The potential role of each serotonin receptor was individually assessed in bone remodeling processes. Blocking HTR_{1B} and HTR_{2B} on MSCs with their specific inhibitors did not affect OPG or RANKL production, however, blocking HTR_{1A} significantly decreased OPG and RANKL protein levels in comparison to the control, but within similar amounts. HTR_{2A} inhibition only decreased RANKL protein levels. These results suggest that only HTR_{2A} is the receptor involved

in modulating the OPG/RANKL ratio and production by MSCs and their functions regulate signals produced to communicate and activate osteoclast activity.

Some limitations of this work are that all studies were completed *in vitro*. Although these studies demonstrate the negative effects of SSRIs on MSC differentiation and bone formation, *in vivo* investigations should also be performed in order to understand the full mechanisms of the role of serotonin and antidepressants in bone metabolism surrounding microstructured Ti implants. This thesis work describes *in vitro* effects of serotonin and antidepressants. However, such effects may not be the same when studied *in vivo*, as there are many confounding factors involved. For instance, inactivation of 5-HTT via an SSRI will enhance central and peripheral serotonin concentrations. However, negative feedback mechanisms may be activated in response to this peripheral serotonergic signaling, as well as other cells may be involved in these processes. Furthermore, studies performed for investigation of the effects of antidepressants on bone remodeling signals between MSCs and osteoclasts utilize conditioned media obtained from MSCs from only the last 24 hours of the experiment. This design fails to take into account the real-time interactions between each cell type throughout differentiation and in response to the drugs. In this case, factors produced by MSCs on microstructured Ti surfaces could play a role in mitigating osteoclastic TRAP activity. Osteoclasts may also produce factors in response to this signaling to further contribute to TRAP activity. Additionally, studies performed in this work utilize fixed concentrations of different types of antidepressants. The same concentrations of different drugs were used for treatment as a uniform way to accurately compare various drugs within different categorizes. However, not all antidepressants are equal in that many of them differ in chemical composition and efficacy and will not have the same toxicities.

Work in this thesis provides insight into the deleterious effects of antidepressant medication use on bone formation and remodeling signaling during interactions with microstructured Ti biomaterials. Antidepressants within the SSRI class exhibited the most negative effects on bone formation and remodeling signaling in comparison to antidepressants that are both serotonin and norepinephrine inhibitors. These findings are of great interest when taking into account the frequency of SSRI prescriptions and the increasing demands for microstructured Ti biomaterials in dental applications. Additionally, individuals with risk factors such as older age or

osteoporosis are at an even greater risk for implant failure. Clinicians should be aware of the type of antidepressant, the dose and the length of time a patient is under treatment and caution should be taken when considering a dental implant. Additionally, future directions for this work should target therapeutic compounds that specifically block HTR_{2A} signaling on MSCs to alleviate negative effects on bone. Other compounds may be considered as a synergistic treatment with antidepressants in order to salvage some of the deleterious effects on bone. This approach may be especially beneficial during dental implant applications.

CHAPTER 5 CONCLUSION

This thesis evaluated the *in vitro* effects of antidepressants on bone formation during interactions with clinically relevant microstructured Ti surfaces. This work indicates that antidepressants inhibit human MSC differentiation, decrease local factor production of proteins associated with bone formation and increases those involved in bone resorption. These effects were intensified by Ti surface characteristics, specifically rough, microstructured surfaces. It is suggested that these effects may be mediated through the presence of various serotonin receptors located on cell membranes of human osteogenic cells. Furthermore, cells are sensitive to Ti surface topography and modify serotonin receptor expression according to the surface roughness and antidepressant treatment further modulated these effects. Results in this work also suggest a role of antidepressants in regulation of bone remodeling, predominantly on microstructured Ti surfaces. These processes are vital for the quality of bone and are tightly associated with successful osseointegration of dental implants. As a result, work done in this thesis provides further insight in the understanding of bone formation and remodeling signaling surrounding microstructured Ti biomaterials in response to chronic prescription use and its overall effect on the ultimate success or failure of dental implants.

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