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The Effects of Nicotine on Mesenchymal Stem Cell Function

Carlos M. Carballosa
University of Miami, ccarballosa@gmail.com

UNIVERSITY OF MIAMI

THE EFFECTS OF NICOTINE ON MESENCHYMAL STEM CELL FUNCTION

By

Carlos M. Carballosa

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Carlos M. Carballosa

Approved:

_____
Herman S. Cheung, Ph.D.
Knight Professor of Biomedical Engineering

_____
Dean of the Graduate School

_____
Chun Yuh Charles Huang, Ph.D.
Assistant Professor of Biomedical Engineering

_____
Guy A. Howard, Ph.D.
Research Professor of Medicine, Biochemistry and Molecular Biology

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This research focuses on the effects nicotine exposure has on Human, Bone Marrow Derived Mesenchymal Stem Cell (MSC) proliferation, migration and differentiation potential. Nicotine, the quintessential compound responsible for a smoker's addiction to cigarettes, is an organic compound, whose effects are either positive or negative depending on the dose of nicotine metabolized. In low concentrations, nicotine has been shown to induce angiogenesis and improve diseases such as Alzheimer's and Parkinson's. In high doses, however, nicotine has been shown to have detrimental effects including hindering both embryonic stem cell development and skeletal healing. Although much has been discovered about the effects of nicotine at the cellular level, information on its effect on MSC function are not fully understood. In order to evaluate such effects, MSC were cultured and exposed to physiological concentrations of nicotine similar to those experienced by chronic smokers. It is hypothesized that at such concentrations MSC are adversely affected, altering the proliferation, migration and differentiation potential of the MSC.

Four independent studies were conducted to evaluate the proliferation, migration and differentiation potential of MSC exposed to nicotine levels similar to those seen in the saliva of chronic smokers. Foremost, the proliferation rates of MSC treated in control

and nicotinic medias were analyzed via MTT assay. Absorbance readings were recorded over a 7-day period and correlated back to a relative rate of MSC proliferation. The proliferation rate of MSC is important in determining how quickly these cells become available to facilitate tissue repair in the case of traumatic injury. In such cases, and when needed, MSC emanate from the bone marrow and migrate toward a chemical signal. In order to assess the effectiveness of MSC migration in the presence of nicotine, the distance traveled and speed of various MSC were determined using a migration assay. Lastly, in order to determine nicotine's effect on differentiation potential, MSC were subjected to an osteogenic differentiation protocol. The effects on differentiation were determined via staining and gene expression studies.

Results from all studies clearly showed nicotine had an adverse effect on inherent MSC characteristics. MSCs exposed to nicotine containing media had a significant reduction in proliferation, overall distance traveled and average cell speed. Furthermore, down regulation was also evident in the genomic expression of PTK2 and RUNX2, gene associated with migration and osteoblast differentiation, respectively. Down regulation of the remaining osteogenic markers: BGLAP, ALPL, COL1A1 and COL1A2 were also seen in MSC treated with nicotine containing media. MSC treated with osteogenic differentiation media containing nicotine showed ineffective mineralization, but did not show a significant down regulation in the expression of the four osteogenic genes.

Although nicotine alone has shown to adversely effect the proliferation, migration and osteogenic potential of MSC, additional studies must be conducted to see how these results will change in the presence of the remaining cigarette smoke compounds. The most effective way to achieve such data would be to transfer these protocols to bone

marrow derived mesenchymal stem cells derived from animals exposed to a smoking chamber. Evaluating these cells after *in vivo* exposure would facilitate further studies to potentially elucidate the molecular pathways affected by cigarette smoke as a whole.

Dedicated to those who helped me see this day.
To my family, my laboratory friends, my roommates and my cells.

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CHAPTER 1 INTRODUCTION

Cigarette Smoke

With over 1.3 billion participants, cigarette smoking has increasingly become one of the most common lifestyle choices worldwide (Wipfli et al., 2009). A worldwide epidemic, cigarette smoking rapidly gained popularity among developed countries beginning in the early 1900s. In the United States, the average cigarette consumption among men and women rose from 1 cigarette a day in 1910 to 10 by 1950 (Peto et al., 1992). By 1960 over half of the male population in the United States actively participated in cigarette smoking (Mackay et al., 2006). It was not until the release of the Surgeon General's report, however, in 1964 that a steady decrease in cigarette smoking was seen among Americans (Wipfli et al., 2009). By 2008 the prevalence of American smokers dropped to less than 20% (MMWR Morb. Mortal. Wkly. Rep. 2008). More significantly however, was the drastic reduction in the smoking rates of American teens, which reached the lowest values since the early 1990s. Although these rates in smoking have decreased, the toxins and chemicals in cigarette smoke still affect a profound number of Americans and individuals worldwide.

Exposure to the toxins in cigarette smoke can either occur via 'mainstream' or 'sidestream' smoke (Ejaz et al., 2006). 'Mainstream' smoke, inhaled by the smoker during each puff of a filtered tip cigarette, is the most common and direct form of cigarette smoke exposure. Depending on the volume, number and duration of puffs, a cigarette smoker can alter the chemical composition of 'mainstream' smoke. On the other hand, 'sidestream' smoke originates from the lit end of the cigarette and is released into the environmental air. As a result, the smoker and individuals within the vicinity of

'sidestream' smoke are indirectly exposed to additional toxins, an action commonly called second hand smoking. These toxins, released from both 'mainstream' and 'sidestream' smoke, can be divided into volatile and particulate phases. The volatile phase, accounting for more than 90% of cigarette smoke, is composed of over 500 gases including nitrogen, oxygen, carbon monoxide, carbon dioxide, water, ammonia and hydrogen cyanide (Hoffmann et al., 2001). The particulate phase, on the other hand, is composed of over 3500 chemicals, which include anatabine, anabasine and nicotine (Sloan et al., 2010). The potential harms of all these chemicals have been extensively researched and as of recently 69 known carcinogens have been identified in cigarette smoke (Hoffmann et al., 2001). Exposure to these chemical combinations is detrimental to human health and as a result, it is imperative that smokers be informed of the side effects, complications and diseases associated with cigarette smoking.

The metabolization of these toxic substances has been attributed to an increased risk of cardiovascular and lung disease, and smokers are susceptible to higher incidences of atherosclerosis, microbial infections and cancers (Sopori, 2002). Metabolization of the toxic substances in cigarette smoke is also believed to delay ulcer (Maity et al., 2003) and wound healing (Sopori, 2002) by adversely affecting the migration of cells critical to the healing process (Wong et al., 2004). Although smokers have been made aware of the toxins and diseases resulting from cigarette smoking, addictive chemicals, such as nicotine, make it nearly impossible for them to quit smoking.

Nicotine

Nicotine, an organic compound found in all tobacco leaves, is the quintessential compound responsible for a smoker's dependence on cigarettes (Benowitz et al., 2009). On average, each cigarette contains 10 to 14 mg of nicotine. Smokers can vary their nicotine intake levels by modulating the frequency and volumes of puffs; however, approximately 1 mg of nicotine is actually absorbed systemically. A weak base, nicotine is absorbed more readily in basic conditions as opposed to acidic conditions. The slightly basic conditions and large surface area of the lungs, therefore, provide one of the most optimal conditions for nicotine absorption following inhalation. Once in the bloodstream, nicotine is quickly exposed to all organs through cardiovascular circulation. Average blood nicotine levels in chronic smokers have been shown to reach 19.0 +/- 11.3 ng per ml after the first cigarette and 22.9 +/- 11.2 ng per ml after the second cigarette (Herning, et al., 1983), correlating to 0.117 +/- 0.070 μM and 0.141 +/- 0.069 μM, respectively.

The effects of nicotine exposure are highly dependent on the dose of nicotine administered (Gullihorn et al., 2005). As a result, positive and negative effects of nicotine are common. Nicotine treatments have been shown to improve diseases such as ulcerative colitis (Wu et al., 2004), Alzheimer's (Chan et al., 2008) and Parkinson's (Takeuchi et al., 2009). On the other hand, nicotine has been shown to adversely effect embryonic stem cell development (Zdravkovic et al., 2008), as well as delay skeletal healing following traumatic injury (Ma et al., 2011).

Adult Stem Cells

Characteristic to all multicelluar organisms, stem cells are cells with the potential to promote and support tissue regeneration (Rios et al., 2010). These cells can be found in circulating blood, amniotic fluid and placental and adult tissues; however, regardless of origin, all stem cells are characterized as unspecialized, self-renewing cells capable of deriving multiple lineages. Different levels of stem cell potency, however, exist depending on the origin of the stem cell. Totipotent cells have the ability to derive a fully viable organism (Knoepffler et al., 2007), whereas pluripotent cells can only derive all three germ layers (Ulloa-Montoya et al., 2005). Multipotent cells are capable of deriving a general family of cells, while unipotent cells are restricted to self-replicating a terminal state (Knoepffler et al., 2007). Stem cells will maintain their potential for differentiation and proliferation until called upon by chemical signals at times of renewal (Rios et al., 2010). Depending on the status of development, different stem cells will be called upon to repair damaged tissues.

Although numerous stem cell populations can be examined, adult stem cells, and more specifically human bone marrow-derived mesenchymal stem cells, will be the primary focus for this research. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Stem Cell Therapy (ISCT) the following criteria define a mesenchymal stem cell: 1) Plastic adherence under culture conditions; 2) Expression of CD105, CD73 and CD90 and exclusion of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules; and 3) Capability of differentiating into osteoblasts, adipocytes and chondrocytes *in vitro* (Dominici et al., 2006). Viable mesenchymal stem cell populations have been isolated from bone marrow,

adipose tissue, skin, and bone periosteum (Ruiz et al., 2012). The mesenchymal stem cells present in the bone marrow are responsible for supporting the hematopoietic cell system through secretion of proteins, various growth factors, chemo- and cytokines (Bobis et al., 2006). Bone marrow derived mesenchymal stem cells only constitute about 0.01% of the cell population present in bone marrow. These mesenchymal stem cells can be isolated from the fractioned population by selection of cell-specific surface markers or by adherence to plastic cell culture plates. The immunosuppressive and homing characteristics of mesenchymal stem cells are desirable characteristics utilized for enhancing tissue regeneration applications. The lack of cell surface T-cell antigens allows these cells to be transplanted within species without eliciting an immune response. Following transplantation and appropriate stimuli, mesenchymal stem cells will often migrate towards sites of injury to initiate and support tissue regeneration. Mesenchymal stem cell based therapies can therefore utilize allogenic transplants to expedite tissue repair. Following traumatic bone injury, bone marrow-derived mesenchymal stem cells are signaled to migrate from within the bone marrow to initiate and support tissue repair. Analyzing the bone marrow-derived mesenchymal stem cell characteristics affected in the presence of nicotine could give insight as to why smokers experience inefficient wound healing.

CHAPTER 2 MATERIALS AND METHODS

Cell Culture

Previously isolated human bone marrow-derived mesenchymal stem cells (BM-MSC) (Cat #7500, ScienceCell Research Laboratories, Carlsbad, CA) were used for all experimental designs. Frozen in 10% DMSO (Sigma-Aldrich, St. Louis, MO), BM-MSC between passages 3 and 5 were thawed, resuspended, and then plated onto T-75 flasks supplemented with High Glucose Media (HGM) consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% Heat-Inactivated Fetal Bovine Serum (HI-FBS) (Invitrogen, Carlsbad, CA), 1% Penicillin/Streptomycin (Pen-Strep) (Invitrogen, Carlsbad, CA), and 0.1% Amphotericin B (fungizone) (ThermoScientific, Logan, UT). Cells were incubated at 5% CO_2 and 37° C and media was changed every other day. Upon reaching a desired confluency of 70%, the cells were detached in preparation for the actual plating of the experiment. Briefly, culture media was removed from all flasks and 500µl of Trypsin (Invitrogen, Carlsbad, CA) was added to each flask. Following 5 minutes of incubation, flasks were visualized under light microscopy for presence of floating cells. If cellular attachment was still evident, flasks were tapped lightly to facilitate the detachment of cells. Equal amounts HGM were added to each flask in order to counteract and dilute the Trypsin (Invitrogen, Carlsbad, CA). The seeding surface of each flask was washed twice with the Trypsin (Invitrogen, Carlsbad, CA)/high glucose media solution in order to shear off any cells that remained adhered to the flask surface. The cell solutions were collected and transferred to a 50 ml centrifuge tube (Corning Inc., Corning, NY). The sample was mixed via pipette and 10 µL of the homogenous sample was transferred to a

hemocytometer. The total number of cells collected is calculated by counting the number of cells present in 5 out of 9 hemocytometer segments under light microscopy. The remaining solution within the 50 ml tube is centrifuged for 10 minutes to create a pellet of cells. After centrifugation, the Trypsin (Invitrogen, Carlsbad, CA)/high glucose media mixture is removed via a fine glass-tip pipette, leaving behind the cell pellet. Depending on the number of cells calculated, enough high glucose media is added to resuspend the cells at 30,000 per ml. The high glucose media solution is mixed thoroughly in order to break up the cell pellet and evenly distribute the cells throughout the mixture.

Six different sets of media were used throughout the experiment: Control and Nicotine-containing DMEM, Control and Nicotine-containing HGM, and Osteogenic and Osteogenic+Nicotine HGM. Control DMEM consisted solely of DMEM (Invitrogen, Carlsbad, CA), whereas Control HGM consisted of DMEM (Invitrogen, Carlsbad, CA), 10% Heat-Inactivated Fetal Bovine Serum (HI-FBS) (Invitrogen, Carlsbad, CA), 1% Penicillin/Streptomycin (Pen-Strep) (Invitrogen, Carlsbad, CA), and 0.1% Amphotericin B (fungizone) (ThermoScientific, Logan, UT). Similarly, Nicotinic-containing DMEM and Nicotinic-containing HGM consisted of all the constituents of Control DMEM and Control HGM with the addition of nicotine (nicotine 99% [TLC], liquid, N0267; Sigma-Aldrich, St. Louis, MO) constituted in DMEM to create a 1 μM concentration. Osteogenic HGM contained all the constituents of Control HGM with the addition of Beta-Glycerophosphate (EMD Biosciences, Inc., La Jolla, CA) at 10 μM, 10 nM Dexamethasone (Sigma Aldrich, St. Louis, MI) at 10nM, BMP-2 (PeproTech, Rocky Hill, NJ) at 50ng/ml and Ascorbic Acid (Sigma Aldrich, St. Louis, MI) at 50μg/ml. Osteogenic+Nicotine HGM contained all the constituents of Osteogenic media with the

addition of nicotine (nicotine 99% [TLC], liquid, N0267; Sigma-Aldrich, St. Louis, MO) constituted in DMEM to create a 1 μM final concentration of nicotine.

MTT Proliferation Assay

A TACS® MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was used to evaluate the proliferation and viability of MSCs in the presence of nicotine, following the manufacture's protocol. Specifically, MSC were resuspended in Control HGM at 1 x 10^6 per ml and subsequently diluted to 1 x 10^4 per mL. Triplicate 100 μL cell samples were distributed within a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) to establish the following cell number gradient: 200,000; 100,000; 50,000; 25,000; 12,500; 6,250; 3,125; 1,562.5; 0. After 24 hours of incubation, 10 μL of MTT reagent (Trevigen, Gaithersburg, MD) was added to each well and then the cells were incubated for an additional 4 hours. After precipitate formation, 100 μL of MTT detergent (Trevigen, Gaithersburg, MD) was added to each well and the plate was then incubated for 2 more hours. Absorbance of each well was determined at 600 nm using an MRX Revelation plate reader (Dynex Revelation, Dynex Ltd., Billingshurst, UK). Triplicate values were averaged and the average blank value was subtracted from each. Absorbance vs. cell number/mL was plotted to establish the reference curve.

Using the above determined information, MSC were seeded at 1,500 cells per well on a 96-well plate (Becton Dickinson, Franklin Lakes, NJ) and analyzed daily over a 7-day incubation period. The plate was divided into two sections to analyze proliferation of MSC exposed to Control HGM and to Nicotinic-containing HGM. Each subset contained 4 MSC wells and 2 blank wells for each of the 7 days. Each well was supplemented with 100μL of Control HGM or Nicotinic-containing HGM. The protocol

used for MTT analysis was similar to that used to establish the reference curve. Plates were maintained in 5% CO_2 and 37° C incubation prior to MTT analysis. Initially, 10 μL of MTT reagent (Trevigen, Gaithersburg, MD) was added to each well and then the cells were incubated for an additional 4 hours. After precipitate formation, 100 μL of MTT detergent (Trevigen, Gaithersburg, MD) was added to each well and then the plates were incubated for yet another 2 hours. Absorbance of each well was then analyzed at 600 nm using an MRX Revelation plate reader (Dynex Revelation, Dynex Ltd., Billingshurst, UK). Following analysis, all remaining medias were removed via fine-tip glass pipettes and replenished with 100 μL of group specific media and the plate was returned for incubation. Medias changes were conducted daily in order to maintain a constant level of nicotine exposure within nicotine treated wells. The experiment was replicated in order to verify results.

Migration Assay

In order to evaluate the effect of nicotine on MSC motility a migration assay was conducted. MSC were seeded at 7,000 cells per 35 mm dish (Sarstedt Inc., Newton, NC). Control and nicotinic groups were established and supplemented with 2 mL of Control DMEM and Nicotinic-containing DMEM, respectively. Cells were incubated for a week at 5% CO_2 and 37° C and the respective medias were changed daily via fine-tip glass pipette suction. Daily media changes were conducted in order to maintain a constant elevated concentration of nicotine representative to chronic smoking. MSC were cultured in serum-deprived DMEMs (void of FBS) in order to avoid the presence of additional chemo-attractants that would stimulate cell migration during treatment. One day prior to migration analysis, Control HGM and Nicotine-containing HGM were prepared and

placed within the incubator to allow this media enough time to come into equilibrium with incubation conditions. On the day of evaluation, 200μL of 0.5 mg/mL Bovine Serum Albumin (BSA) (Boehringer Mannheim Corp., Indianapolis, IN) was added to both incubated HGMs in order to stimulate cell migration. A final media change was conducted, with the new Control- and Nicotine-containing HGM/BSA being added to each respective dish. Treated MSC were visualized using a Nikon Eclipse Inverted Microscope and Nikon NIS-Elements AR 3.2. Dishes were placed on a heated plate to maintain the cells at 37° C throughout the time-lapse image. Images were taken every 15 minutes over a two-hour period. A total of 5 MSC per group were chosen at random and their movements about the cell dish were traced and measured. The experiment was replicated in order to verify results.

Staining

In order to evaluate nicotine's effect on wound healing, specifically bone mineralization, MSC were subjected to an osteogenic differentiation protocol. MSC were seeded at 9,000 cells per well of a 4-well glass slide (Thermo Scientific, Rochester, NY). Cells were incubated at 5% CO_2 and 37° C and supplemented with Control HGM until reaching a confluency of approximately 70%. This confluency was chosen so that the MSC were crowded enough to interact with each other at the time of treatment. Once the desired confluency was reached, MSC were divided into control, nicotine, osteogenic and osteogenic+nicotine groups and treated with their respective medias daily over a 10-day period. Daily media changes were conducted in order to maintain a constant elevated concentration of nicotine representative of chronic smoking. After treatment, media were removed and each well was washed with Phosphate Buffer Saline (PBS) (Life

Technologies Corporation, Grand Island, NY). Wells were then fixed in 10% formalin (VWR, Radnor, PA) for 10 minutes and then washed again in PBS (Life Technologies Corporation, Grand Island, NY) before starting the Alizarin Red S staining protocol.

The Alizarin Red S staining protocol is utilized to visualize calcium deposits, an important constituent of human bone. The staining solution was prepared by first mixing 2 g of Alizarin Red S (BDH Chemicals LTD, Poole, England) in 100 mL of distilled water. Subsequently, 300 μL of 10% ammonium hydroxide (Sigma Aldrich, St. Louis, MI) was added to the solution to ensure the pH of the stain was within the recommended 4.1~4.3 range (IHCWorld). Adequate amounts of the Alizarin Red S stain were added to thoroughly cover each well. The stain was allowed to set for 5 minutes before removing the excess via fine-tip glass pipette and blotting. Wells were subsequently popped off and removed from the glass slide. Slides were then dehydrated in acetone (Sigma-Aldrich, St. Louis, MO) and then in a 1:1 Acetone (Sigma-Aldrich, St. Louis, MO):Xylene (VWR, Radnor, PA) solution. Samples were cleared in xylene and mounted using a synthetic mounting medium (Fisher Scientific Company, Fair Lawn, NJ). A 22x50 mm glass cover slip (General Scientific, Richmond, VA), outlined in nail polish, was added over the glass slide (Thermo Scientific, Rochester, NY) to preserve the samples. Stained slides were then visualized and imaged microscopically.

Osteogenic Differentiation

In order to supplement mineralization and migration data, specific osteogenic and migratory gene expression profiles were analyzed after MSC were subjected to an osteogenic differentiation protocol. MSC were seeded at 2,500 cells per well of a 6-well plate (Cellstar, INFO). Cells were incubated at 5% CO_2 and 37° C and supplemented

with Control HG-CCM until reaching a confluency of approximately 70%. This confluency was chosen so that the MSC were crowded enough to interact with each other at the time of treatment. Once the desired confluency was reached, MSC were divided into triplicate control, nicotine, osteogenic and osteogenic+nicotine groups and treated with their respective medias daily over a 10-day period. Daily media changes were conducted in order to maintain a constant elevated concentration of nicotine representative to chronic smoking.

mRNA Isolation

Following osteogenic differentiation treatment, MSC mRNA samples were collected from each well to analyze the MSC gene expression. First, media was removed and 500μL of Trizol (Invitrogen, Carlsbad, CA) was added to each well for 5 minutes. Wells were washed several times with the added Trizol (Invitrogen, Carlsbad, CA) to shear off any additional cells adhered to the plate. Collected Trizol (Invitrogen, Carlsbad, CA) samples were transferred into 5 ml autoclaved tubes with the addition of 100 μL of Chloroform (Sigma-Aldrich, St. Louis, MO). Tubes were shaken vigorously for 15 seconds and then submitted to centrifugation at 14,000 rpm and 4° C for 20 minutes. The aqueous phase of each tube was then transferred to new 5 ml tubes with the addition of 250 μL of 100% Isopropanol (Sigma-Aldrich, St. Louis, MO). Tubes were shaken and submitted to centrifugation at 14,000 rpm and 4° C for 20 minutes. The resulting aqueous phase was decanted and removed via pipette from each 5 mL tube, leaving the mRNA precipitate pellet behind. Once all the Isopropanol (Sigma-Aldrich, St. Louis, MO) was removed from each tube, 500uL of 75% ethanol (Sigma-Aldrich, St. Louis, MO) was added and each tube was shaken. Samples were centrifuged at 7,500 rpm and 4° C for 10

minutes. Ethanol (Sigma-Aldrich, St. Louis, MO) was decanted and removed via pipette and each sample was allowed to air dry for 10 minutes. Samples were resuspended in 20 µL of DNase-RNase-free water (Sigma-Aldrich, St. Louis, MO) and stored at -80° C.

Reverse Transcription (RT)

The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used to perform RT. Frozen mRNA was thawed, vortexed, centrifuged and quantified using the NanoDrop Spectrophotometer (Thermo-Fisher Scientific, Waltham, MA) and its associated ND-1000 software. Once quantified, 1 µg of each mRNA sample was precisely added to a 200µL PCR tube with enough DNase-RNase-free water (Sigma-Aldrich, St. Louis, MO) to make a 10µL mixture. The RT kit protocol was then followed to create enough master mix for all samples with the addition of a negative and two extra samples to cover for any inadvertent pipetting errors. Each master mix sample contained: 3.2µL of DNase-RNase-free water (Sigma-Aldrich, St. Louis, MO), 2.0µL of 10X RT Buffer (Applied Biosystems, Foster City, CA), 0.8uL of 25X dNTP mix (Applied Biosystems, Foster City, CA), 2.0µL of 10X Random Primers (Applied Biosystems, Foster City, CA), 1.0µL of Multiscribe RT (Applied Biosystems, Foster City, CA) and 1.0µL of RNase Inhibitor (Applied Biosystems, Foster City, CA). The master mix batch was subsequently vortexed, centrifuged and added in 10µL aliquots to each 200µL PCR

tube. Each tube was transferred to the GeneAMP PCR System 9700 Thermocycle (Applied Biosystems, Foster City, CA) and subjected to the settings listed in Table 2.1.

Table 2.1

Step	1	2	3	4
Temperature (°C)	25	37	85	4
Time (sec)	600	7200	5	∞

Table 2.1: Thermocycle settings for all RT samples.

Real-Time Polymerase Chain Reaction (PCR)

A total of six genes were analyzed in order to evaluate nicotine's effect on the migration and osteogenic MSC gene expression. The primers for the migration gene, PTK2, as well as the other 5 osteogenic genes, RUNX2, ALPL, BGLAP, COL1A1 and COL1A2, were created using the Ensemble Genome Browser and The Primer Basic Local Alignment Search Tool (Primer-BLAST) online resources. The primer specifications as well as the forward and reverse sequences are detailed in Table 2.2.

Table 2.2

Gene	Primer name	Sequence (5' > 3')	Length	Tm (℃)	Amplicon size (bp)	Reference
RUNX2	RUNX2-F	CAAGTGCGGTGCAAACTTTC	20	59.69	120	NM_001024630.3
	RUNX2-R	CTGCTTGCAGCCTTAAATGACT	22	60.43		
ALPL	ALPL-F	CTGCGCAGGATTGGAACATC	20	60.52	146	NM_000478.4
	ALPL-R	TGCCAATGGCCAGTACTAAGA	21	60.03		
BGLAP	BGLAP-F	CCACCGAGACACCATGAGAG	20	60.74	130	NM_199173.4
	BGLAP-R	CTTGGACACAAAGGCTGCAC	20	60.87		
COL1A1	COL1A1-F	AGACATGTTCAGCTTTGTGGAC	22	60.04	120	NM_000088.3
	COL1A1-R	GTGATTGGTGGGATGTCTTCG	21	59.9		
COL1A2	COL1A2-F	GGTTTCGGCTAAGTTGGAGG	20	59.46	199	NM_000089.3
	COL1A2-R	TCTTGTAAAGATTGGCATGTTGC	23	59.12		
PTK2	PTK2-F	ACGGAAGGGAGAATATGACAGA	22	59.3	196	NM_153831.3
	PTK2-R	GGTGGTTGGCTCACTATTGC	20	60.1		

Table 2.2: Osteogenic and Migratory Primers designed and optimized specifically for the gene expression study. The sequences for all osteogenic markers: RUNX2, ALPL (Alkaline Phosphatase), BGLAP (Osteocalcin), COL1A1 (Collagen 1A1) and COL1A2 (Collagen 1A2) and the migratory marker PTK2 (Protein Tyrosine Kinase 2) are included.

The SYBR kit (Applied Biosystems, Foster City, CA) was also used to perform qPCR analysis. A total of 13 samples (3 per group and 1 negative) were analyzed per gene. Each of these PCR samples contained 2μL of their respective RT product, 1μL of their respective primers, 1μL of DNase-RNase-free water (Sigma-Aldrich, St. Louis, MO) and 5μL of SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Each gene group was transferred to the Real Time PCR (Stratagene, Santa Clara, CA) and subjected to 40 cycles at the thermal settings listed in Table 2.3.

Table 2.3

Step	1	2	3	4	5	6	7
Temperature (°C)	95	95	60	72	95	60	95
Time (min)	10	30s	1	1	1	30	30

Table 2.3: Thermocycle settings for all Real Time PCR samples.

Real Time RT-qPCR was analyzed by first normalizing the genes against the GAPDH reference gene from each sample and then presented as a fold above control treated MSC. Triplicate samples for each group were analyzed and the experiment was replicated in order to verify results.

CHAPTER 3 RESULTS

MTT Proliferation Assay

In order to evaluate nicotine's effect on MSC proliferation an MTT assay was conducted. A reference curve was established by plotting the MSC absorbance corresponding to known MSC seeding densities (Figure 3.1).

Figure 3.1

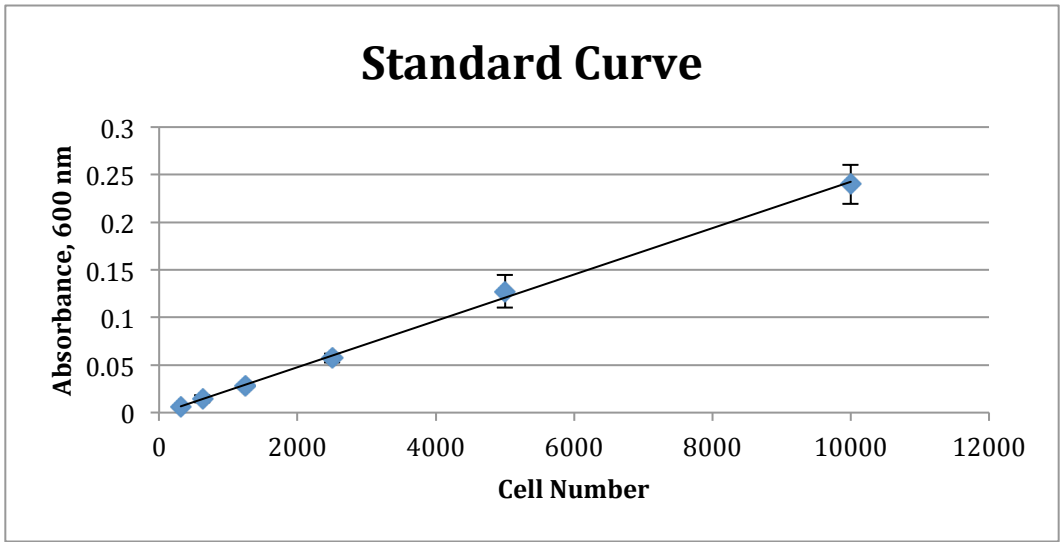

Figure 3.1: MTT proliferation standardized curve with best-fit line. Data is represented as Mean + Standard Error of the Mean, N=5.

The linear regression equation ($R^2 = 0.99857$) derived from the best-fit line shows a direct correlation between absorbance and cell number. This linear relationship can therefore be used to determine the relative proliferation rate of each treatment group. A higher absorbance value corresponds to a greater number of cells and an increased rate of proliferation.

A relatively steady increase in MSC absorbance was seen for both control and treated MSC groups for the first 4 days of treatment. However, from day 5 onward, the absorbance for MSC treated with control media is significantly higher than its nicotine

treated counterpart (Figure 3.2). Furthermore, MSC in the control group continued to see a steadily increase in absorbance (a measure of cell number), whereas MSC in the nicotinic group reached a plateau after day 5.

Figure 3.2

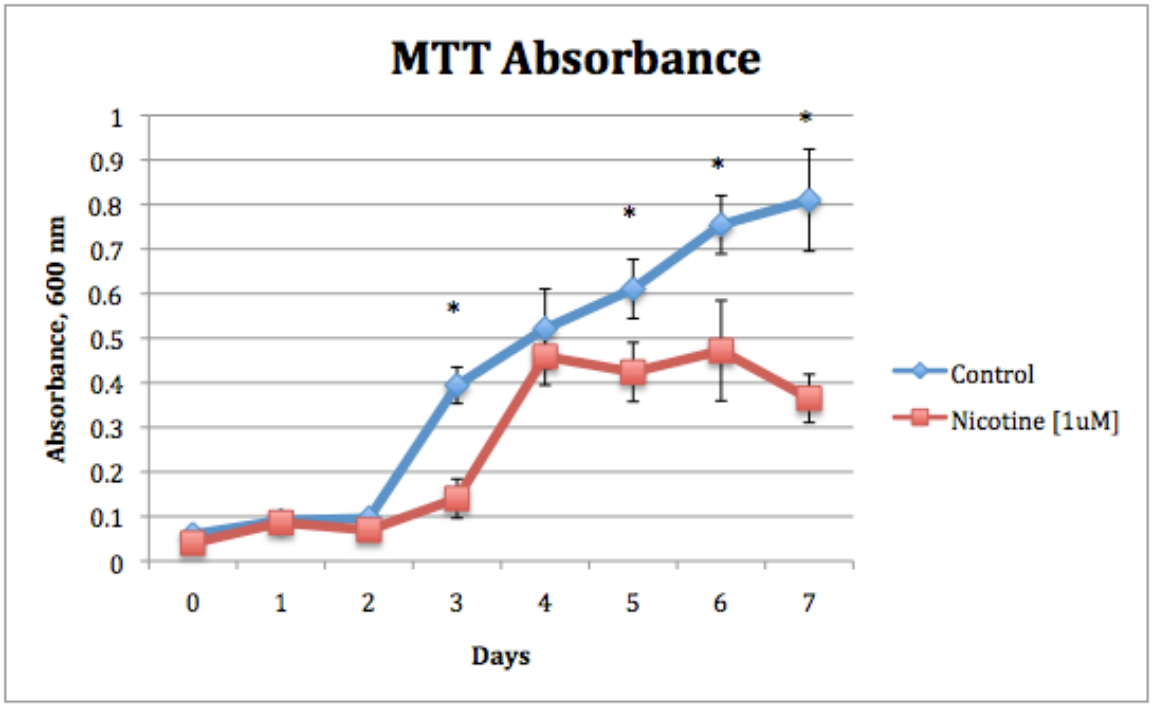

Figure 3.2: Average MSC MTT absorbance readings for MSC treated with Control media and 1μM Nicotinic media over a 7-day period. A significant difference in absorbance is observed on Day 3 (p=0.003), Day 5 (p=0.047), Day 6 (p=0.042), and Day 7 (p=0.011). Data is represented as the Mean + Standard Error of the Mean, N=4 (Significance = p<0.05).

Migration Assay

A migration assay was conducted in order to determine the effect of nicotine on the inherent migratory properties of MSC. After a 7-day serum-deprivation treatment, BSA supplemented Control and 1 μM Nicotine medias were introduced to each assay to stimulate cell migration. MSC were tracked via time-lapsed imaging and their total average distance was recorded (Figure 3). A significant difference (p=0.022) was observed in the average total distance traveled by the MSC in the control group, 37.71 +/-

4.43 μm, compared to the average total distance traveled by the MSC in the 1 μM nicotine treated group, 22.61 +/- 3.98 μm.

Figure 3.3

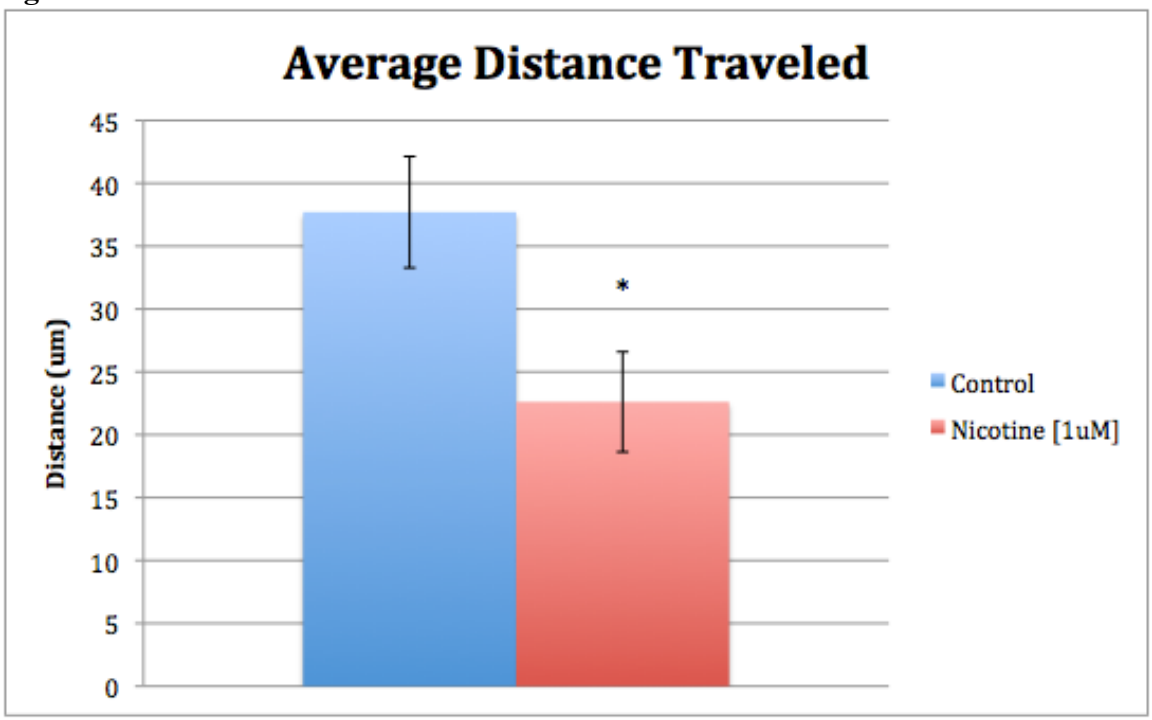

Figure 3.3: Average distance covered in 2 hours by MSC exposed to Control media and 1μM Nicotinic media both infused with BSA. A significant difference is observed between treatment groups ($p=0.022$). Data is represented as the Mean + Standard Error of the Mean, N=4 (Significance = $p<0.05$).

Average MSC speed was calculated based on the distance traveled and a similar significant difference was observed ($p=0.024$) (Figure 3.4). Control MSC reached an average speed of 5.19 +/- 0.61 nm/s, whereas Nicotine treated MSC only reached an average speed of 3.14 +/- 0.55 nm/s.

Figure 3.4

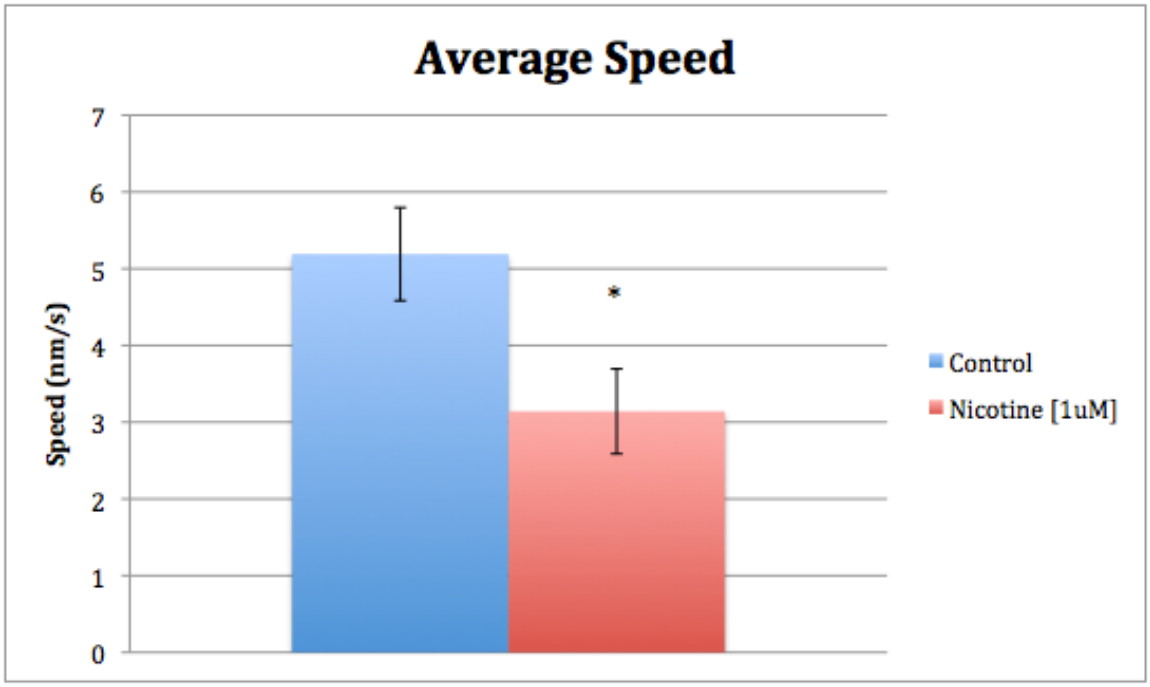

Figure 3.4: Average speed of MSC exposed to Control media and 1μM Nicotinic HG-CCM both infused with BSA. A significant difference is observed between treatment groups ($p=0.024$). Data is represented as the Mean + Standard Error of the Mean, N=4 (Significance = $p<0.05$).

Mineralization

In order to evaluate nicotine's effect on wound healing, specifically bone mineralization, MSC were subjected to an osteogenic differentiation protocol and stained for the presence of calcium. As predicted, MSC treated in Control media showed no positive signs of calcium staining (Figure 3.5). MSC directed toward an osteogenic phenotype via Osteogenic and Osteogenic+Nicotine media, however, both exhibited some degree of calcium deposition. MSC treated with Osteogenic media exhibited a greater, more evenly widespread amount of calcium deposits compared to MSC treated with Osteogenic+Nicotine media.

Figure 3.5

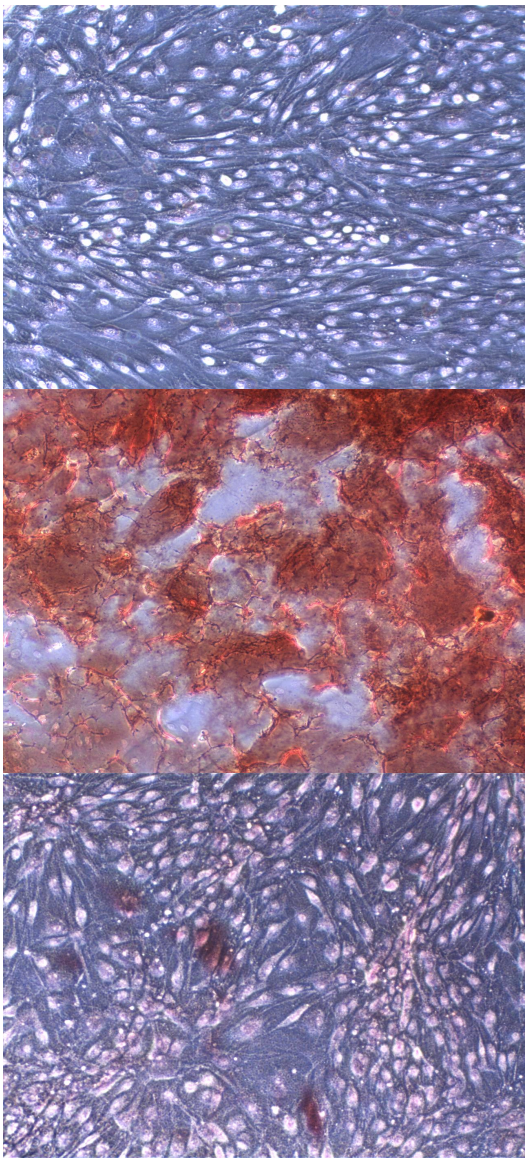

Figure 3.5: Alizarin Red staining for MSC treated with Control HGM (top), Osteogenic HGM (middle) and Osteogenic+Nicotine HGM (bottom). No positive staining for calcium was identified in the Control treated group. MSC treated with Osteogenic media (middle) exhibited a greater, more evenly widespread amount of calcium deposits compared to MSC treated with Osteogenic+Nicotine media (bottom).

Gene Expression

Genes encoding for migratory and osteogenic markers were analyzed in order to further verify nicotine's effect on the migratory and osteogenic differentiation potential of MSC. A significant decrease in gene expression was observed between the control and nicotine treated MSC for the following genes: Protein Adhesion Kinase 2 (PTK2), RUNX2, Collagen 1A1 (COL1A1), Collagen 1A2 (COL1A2), Alkaline Phosphatase (ALP) and Osteocalcin (BGLAP) (Figures 3.6-3.11). No significant decrease, was seen in the gene expression of MSC treated with osteogenic and osteogenic+nicotine medias.

Figure 3.6

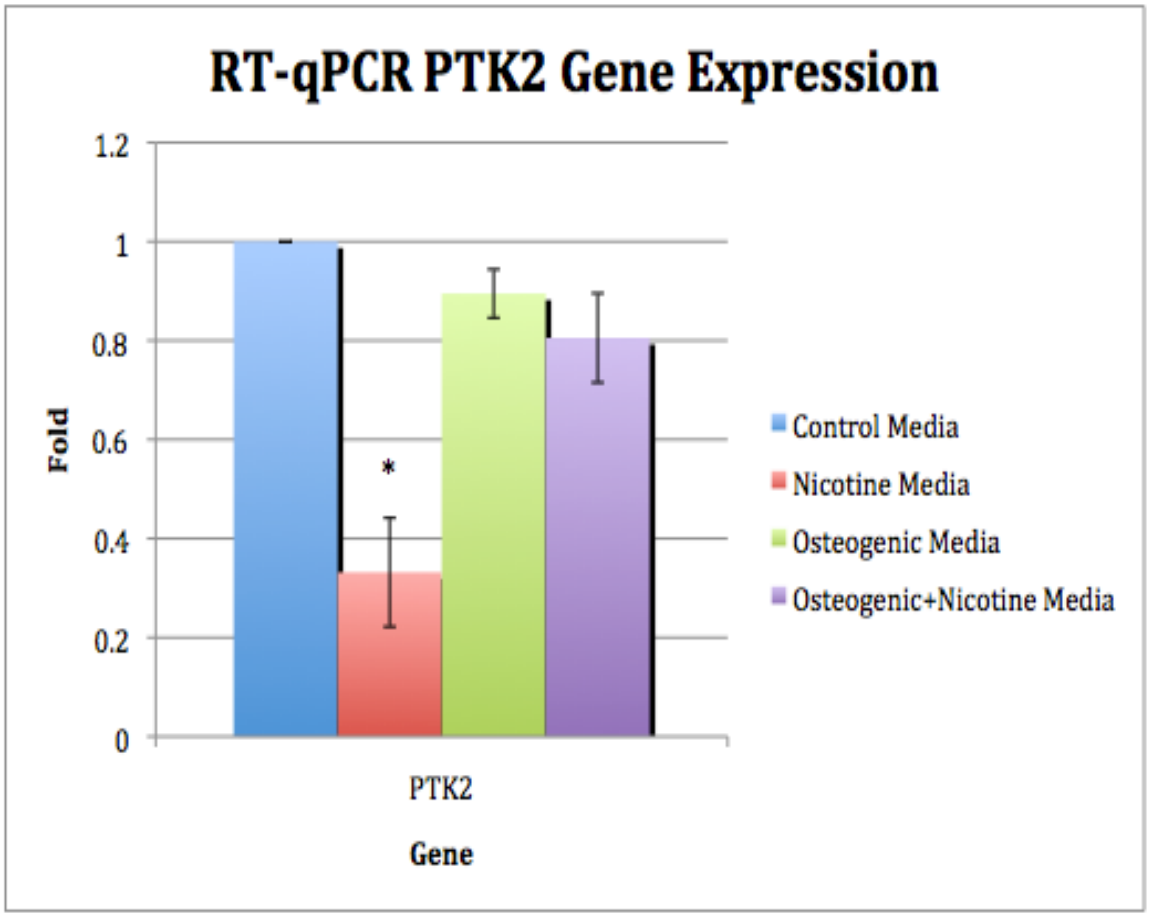

Figure 3.6: Expression of PTK2 for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. Significant decrease in fold factor was observed in the nicotine group ($p=0.004$). Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

Figure 3.7

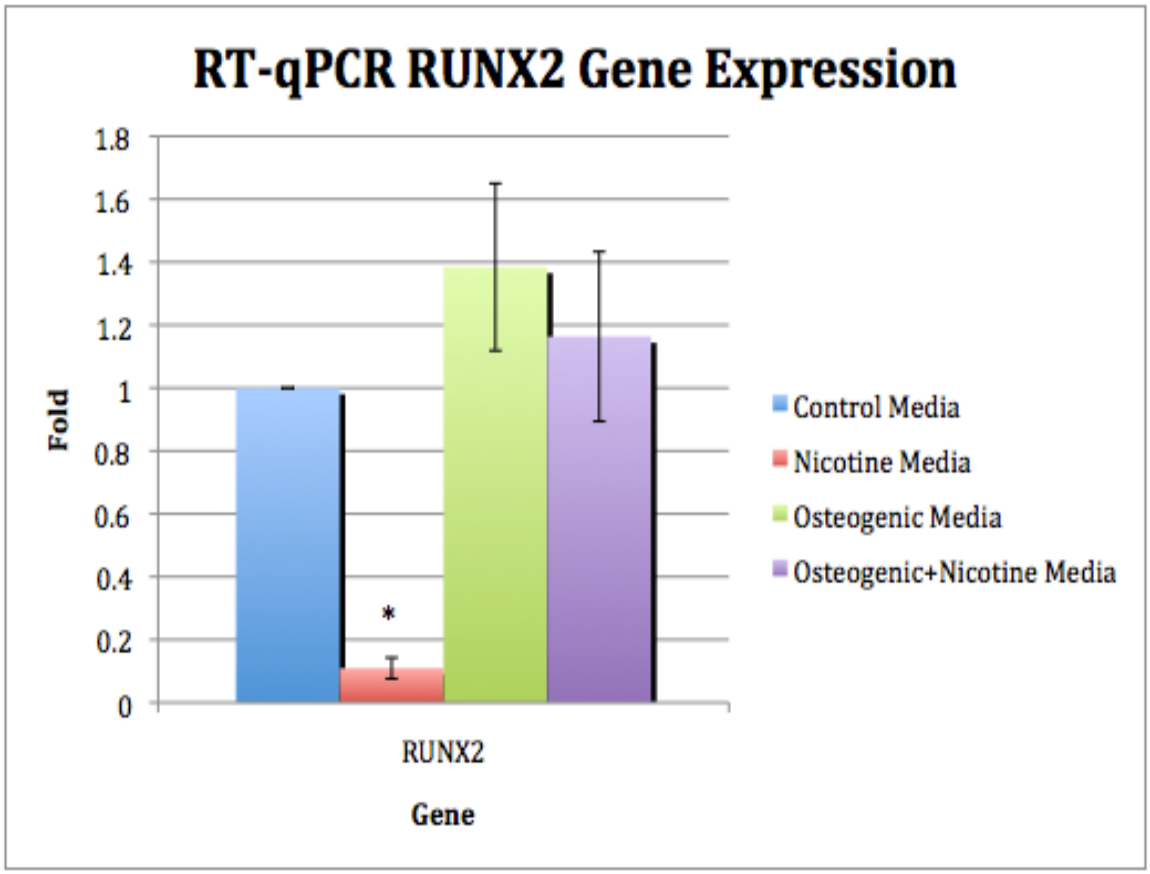

Figure 3.7: Expression of RUNX2 for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. Significant decrease in fold factor was observed in the nicotine group ($p<0.001$). Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

Figure 3.8

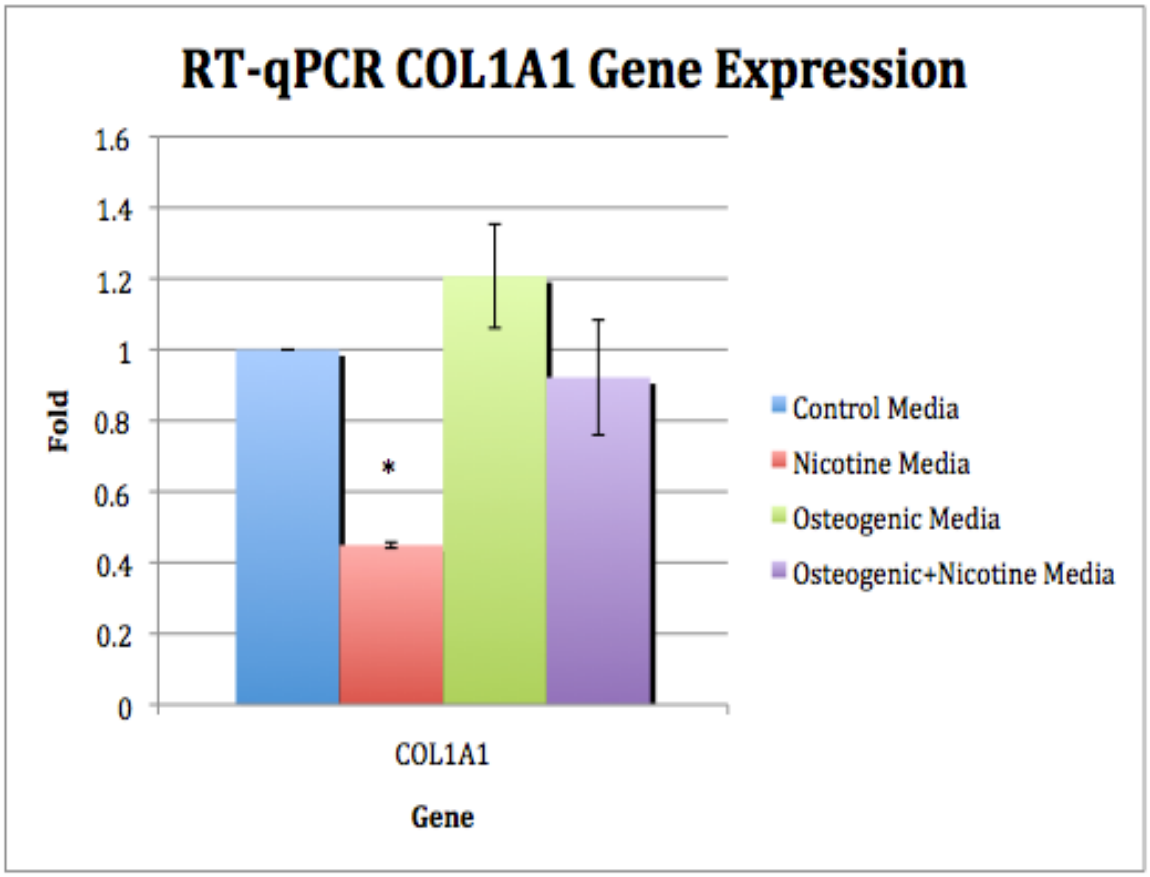

Figure 3.8: Expression of COL1A1 for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. Significant decrease in fold factor was observed in the nicotine group ($p<0.001$). Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

Figure 3.9

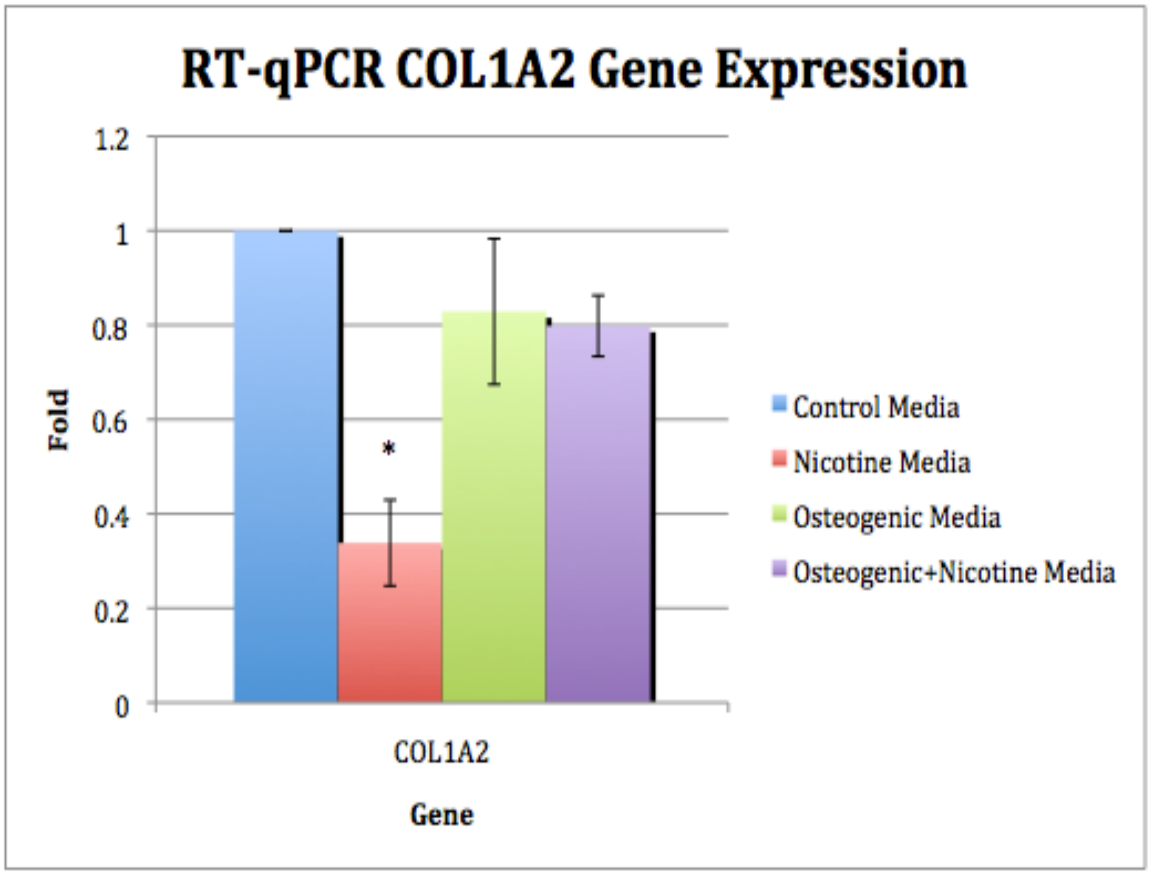

Figure 3.9: Expression of COL1A2 for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. Significant decrease in fold factor was observed in the nicotine group ($p=0.002$). Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

Figure 3.10

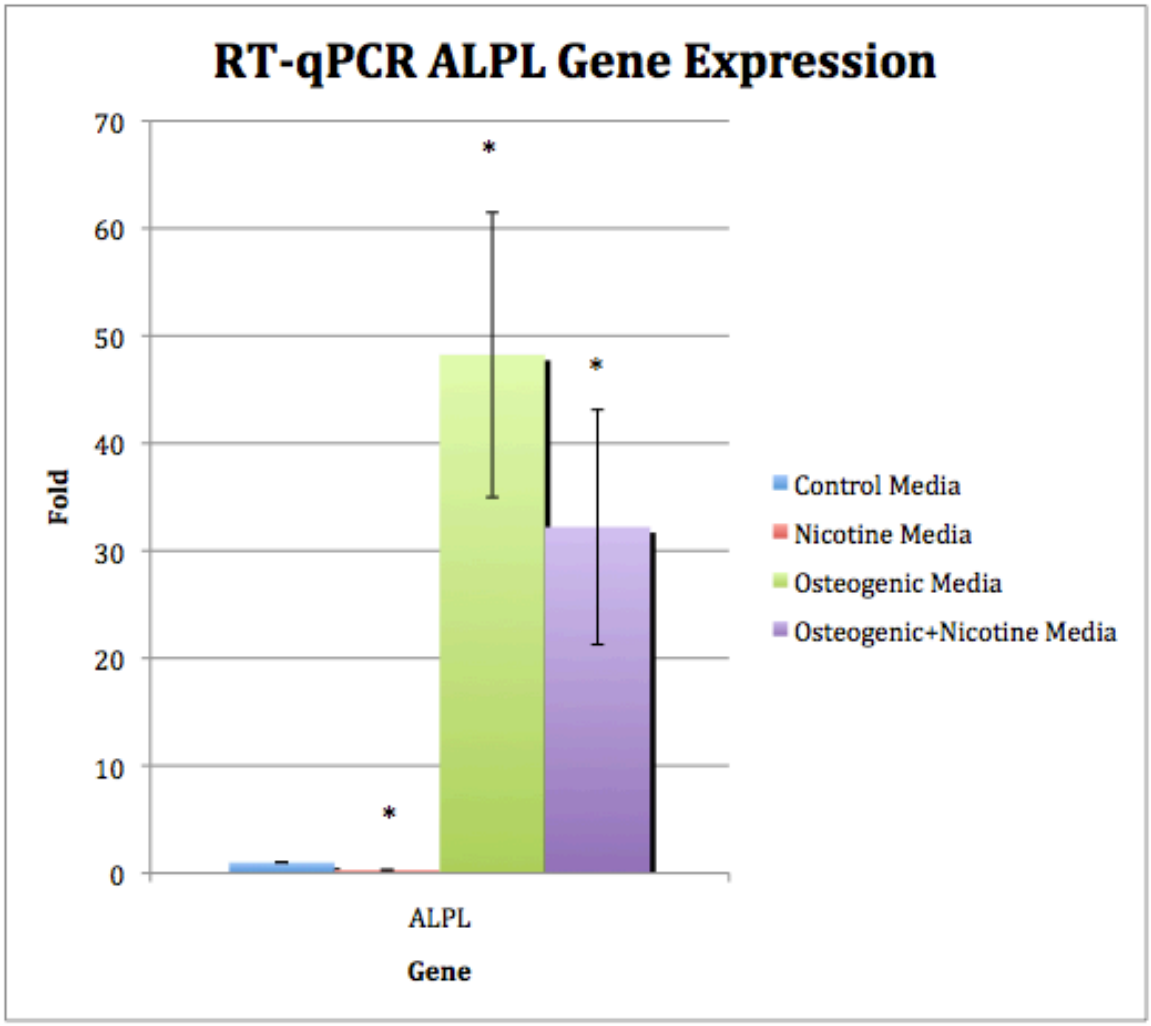

Figure 3.10: Expression of ALPL for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. A significant decrease was observed in the nicotine ($p<0.005$) group and a significant increase was seen in differentiation ($p=0.023$) and differentiation+nicotine groups ($p=0.034$). Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

Figure 3.11

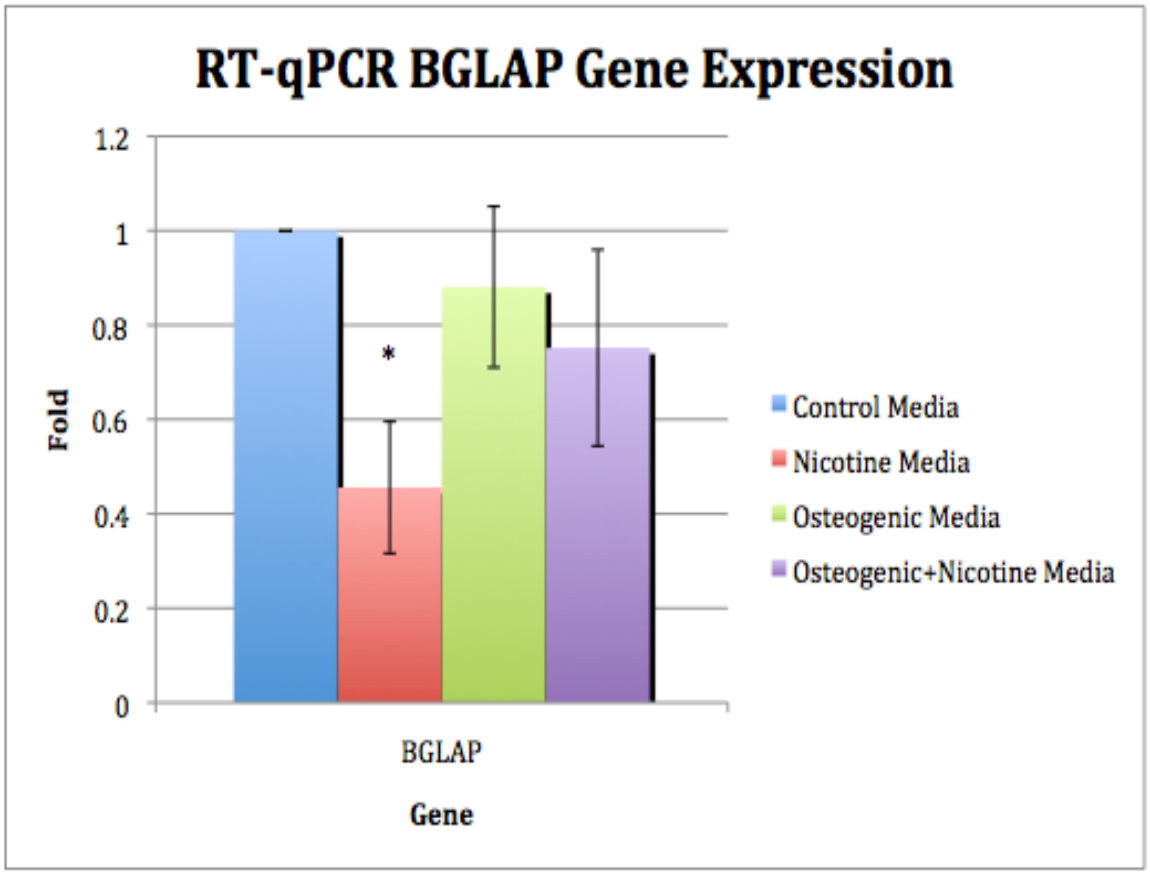

Figure 3.11: Expression of BGLAP for MSC treated with Control, Nicotine, Osteogenic and Osteogenic+Nicotine medias for a 10-day period. Genes were quantified and normalized against GAPDH. The fold factor and significance of each group is taken with respect to the control group. A significant decrease was observed in the nicotine ($p=0.023$) group. Data is represented as the Mean + Standard Error of the Mean, N=3 (Significance = $p<0.05$).

CHAPTER 4 DISCUSSION

The aim of this study was to determine what effect nicotine, in a peak concentration (1μM) similar to that seen in the blood plasma of chronic smokers, would have on the functionality of human, bone marrow-derived mesenchymal stem cells (MSC). In regards to the proliferation rate of MSC, our study found a significant decrease within the nicotine treated cells after a 4-day period. Untreated, control cells continued to show a steady increase in proliferation, while nicotine treated cells reached a plateau. The proliferation rate of MSCs are important in determining how quickly these cells become available to facilitate tissue repair in the case of traumatic injury.

When needed, such as in cases of traumatic injury, stem and progenitor cells from bone marrow and tissue-specific niches migrate toward a chemical signal via circulating blood. It is worthwhile to note however, that recent studies have shown a correlation between smoking and a reduction in the number of circulating progenitor cells (Michaud et al. 2005, Kondo et al. 2004). Although the results in the current study not take into account all the constituents found in cigarette smoke, results similar to these earlier reports were seen in MSC treated with just nicotine. Migration assays showed significant decreases in the average speeds and distances traveled by nicotine treated MSC. In addition, a reduction in the fold factor of the protein tyrosine kinase 2 (PTK2) gene, associated with cellular motility, was significant in MSCs treated with nicotine. This significant down regulation in the PTK2 gene could therefore provide a possible mechanism leading to the overall decrease in MSC migration in the presence of nicotine. The overall reduction in speed and distance would also be a cause for the reduced number of MSCs circulating within the blood. Furthermore, these results are significant in the

fact that MSC give rise to many of progenitor cells found in the circulation. Therefore, claims can be made that a reduction in circulating MSCs would also lead to the reduction in circulating progenitor cells.

The last goal of this study was to determine the effects nicotine had on the osteogenic differentiation potential of MSCs. MSC were subjected to an osteogenic differentiation protocol and treated with control and nicotine containing media. Mineralization of extracellular matrix (in vitro calcium deposits), evaluated via Alizarin red staining, was observed in both the regular and nicotine-infused differentiated samples, signifying the MSC commitment to the osteogenic lineage. The amount of staining observed, however, was considerably lower in MSC samples treated with the nicotine-infused media. Although calcium is not the only compound associated with bone formation, it is an integral component of bone composition. Its absence in bone generally leads to impaired bone integrity / strength, and is seen in diseases such as osteomalacia and osteoporosis (Cech, 2012). The decrease in mineralization observed in the nicotine treated groups can therefore be associated with a decrease in the osteogenic differentiation potential of MSCs.

In order to further verify the observations of the mineralization study, a gene expression study was conducted. MSC were subjected to an osteogenic differentiation protocol and the expression of five known osteogenic markers: RUNX2, COL1A1, COL1A2, ALPL and BGLAP were observed between treatment groups. A significant reduction in gene expression was observed for all genes treated with the nicotinic media. At the experimental nicotine concentration, the significant reduction in the MSC gene expressions, specifically that of RUNX2, a gene associated with osteoblast

differentiation, corroborate findings of delayed fracture healing times experienced by smokers (Sloan et al., 2010). With the exception of ALPL, no significant difference in the MSC osteogenic gene expression was observed between the osteogenic and osteogenic+nicotine treated groups when compared to the control. The significant increase in the ALPL gene expression was expected however, since the ALPL, alkaline phosphatase, gene is a critical component in the process of bone formation. The lack of variable expression within the osteogenic and osteogenic+nicotine treated groups could be attributed to the length of the differentiation treatment. Although smokers do experience a delay in fracture healing, their bones due eventually heal nevertheless. Reducing the osteogenic differentiation time might serve to show the immediate effects nicotine has on the osteogenic differentiation potential of smokers following traumatic injury. Moreover, a greater difference in the osteogenic gene expression of MSC treated with osteogenic and osteogenic+nicotine medias might be seen if MSC were first exposed to nicotine before being subjected to the osteogenic protocol. The vast difference in the expression between the control and nicotine treated MSC suggests this very possibility. In order to fully understand the effect of nicotine on the entire differentiation potential of MSCs, however, additional studies across all known potential lineages are needed.

CHAPTER 5 FUTURE CONSIDERATIONS

This study provides results with respect to the effects of nicotine on the proliferation, migration and differentiation potential of human, bone marrow-derived mesenchymal stem cells. An additional cell line needs to be evaluated using the same protocols in order to determine if nicotine has similar or additional effect son stem cell populations derived from outside the bone marrow. The cell line chosen will be derived from the periodontal ligament (PDL), a location known for harvesting neural crest derived stem cells (Huang et al., 2009). The cell line is being considered since it is located in one of the first major regions of cigarette smoke contact within a smoker. Epigenetic mi-RNA studies are also currently being conducted on both cell lines to supplement the gene expression data obtained in this study.

The effect on these MSC and PDL functions would undoubtedly be compounded if MSC and PDL were analyzed in the presence of all the chemical components found in cigarette smoke. These same protocols will be translated to an *in vivo* mouse model involving cigarette smoke, per se, in order to determine the effect of cigarette smoke on the proliferation, migration and differentiation potential of subsequently isolated mesenchymal and periodontal ligament derived stem cells. Preliminary studies have been initiated to evaluate the combinatory effect of benzo-a-pyrene, known cigarette smoke carcinogen, and nicotine on MSC and PDL functionality. As expected, preliminary data suggests that the benzo-a-pyrene/nicotine combination is more detrimental to the proliferation, migration and mineralization potential of MSC than nicotine alone. Validation of these preliminary results, together with results from additional experiments will facilitate elucidating the effect of cigarette smoke on the stem cell populations.

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