2018-07-03

The Effects of E-cigarette Vapor Exposure, and the Role of a7 Nicotinic Acetylcholine Receptors and Micro-RNA, on the Regeneration Potential of Periodontal Ligament Derived Stem Cells

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By

Carlos Miguel Carballosa

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

August 2018

UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
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(August 2018)

Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Herman S. Cheung.
No. of pages in text. (120)

Cigarette smoking is the leading cause of preventable death worldwide. Electronic cigarettes (ECs) are marketed as a "safer" alternative to cigarette smoking due to a significant reduction in the number of toxic chemicals delivered to the user; however, ECs are still believed to cause major health concerns due to the concentrated delivery of toxic chemicals. Stem cells participate in tissue restoration and are often considered in therapies used to treat cigarette-smoking diseases. The oral cavity, the initial site of EC vapor exposure, contains various stem cell populations like periodontal ligament derived stem cells (PDSLCs) that are involved in the development, maintenance, and repair of oral tissues. PDLSCs are critical for maintaining healthy teeth and regenerating damaged oral tissues from destructive diseases such as periodontitis. PDLSCs can be easily isolated following natural tooth loss or routine dental procedures and are thus an ideal source of therapeutic stem cells for periodontal regeneration. However, by residing in the oral cavity, PDLSCs are exposed to and thus extremely susceptible to the effects of EC vapor. The precise effects of EC vapor on stem cell biology are unknown. Nicotine has been shown to negatively impact the regenerative capabilities of PDLSCs, but EC vapors contain other non-nicotine compounds whose effect, either alone or in tandem with nicotine, remains

unknown. Others suggest that the nicotinic effects are mediated through α7 nicotinic acetylcholine receptors (nAChRs) on the PDLSC surface; yet, it is still unknown whether nicotine-containing ECV cause detrimental effects to PDLSCs through similar mechanisms.

To study these effects, I propose using an automated smoking robot and a recent-generation EC device to produce vapor from 0mg/ml nicotine and 36mg/ml nicotine e-liquids (non-flavored, 50%/50% w/v PG/VG) and a liquid impinger to extract vapor into cell culture media so that it can be added directly into PDLSC cultures. The effects of exposure on the proliferation, migration, and osteogenic differentiation potential of PDLSC were investigated in order to determine the harms of use on dental stem cell regeneration potential. The involvement of α7 nAChRs and microRNA (miRNA) expression were also investigated in order to understand the molecular mechanisms regulating this effect. It was predicted that nicotine-containing ECVs would inhibit PDLSC proliferation, migration and osteogenic differentiation through α7 nAChRs and upregulated miRNA. It was concluded that exposure to nicotine-containing EC vapors significantly undermines the proliferation and osteogenic differentiation potential of PDLSCs. These effects were mediated in part by α7 nAChRs and e-liquid humectants and potentially by upregulated miRNAs that are involved in regulating cell proliferation, migration, and osteogenic differentiation.

Dedicated to my mom, dad, sisters, family, fiancé, and fiancé's family –
Thank you for all of your love and support.

To my mentor Dr. Herman S. Cheung –
Thank you for your mentorship, support, and friendship. You are a wonderful example of what a mentor should be. I will cherish everything that you taught me and I am forever grateful for all of your guidance.

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List of Abbreviations

α-BTX	Alpha bungarotoxin
ACAN	Aggrecan
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
BGLAP	Bone Gamma-Carboxyglutamate (Gla) Protein
BMDD	bone mineral density distribution
BMP2	Bone morphogenetic protein-2
BSA	Bvine Serum Albumin
CCM	Complete culture media
COL1A1	Type 1 Collagen- Alpha 1
COL1A2	Type 1 Collagen- Alpha 2
COPD	Chronic Obstructive Pulmonary Disorder
CPC	cetylpyridinum chloride
CS	Cigarette smoking
CSE	Cigarette Smoke Exrtact
CVD	Cardiovascular Disease
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMEM	Dulbecco's modified Eagle medium
EC	Electronic-cigarette
ECV	Electronic Cigarette Vapor Extract
ENDS	Electronic Nicotine Delivery System
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated protein kinases
FBS	Fetal Bovine Serum
FA	Formaldehyde
FDA	U.S. Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
gRNA	Guide RNA
hAMSC	Human adipose-derived MSCs
hBMSC	Human bone marrow stromal stem cells
hBMMSC	Human bone marrow-derived MSCs
HBSS	Hank's Balanced Salt Solution
hESC	Human embryonic stem cells
hGF	Human gingival fibroblasts
HSC	hematopoietic stem cells
hSDF	Human skin dermal fibroblasts
HUVEC	Human umbilical vein endothelial cells
IBSP	Bone sialoprotein
LCMS	Liquid chromatography-mass spectroscopy
MECA	Mecamylamine
miRNA	MicroRNA
MGV	Mean Gray Value
MSC	Mesenchymal stem cells

nAChR	Nicotinic acetylcholine receptors
NO	Nitric Oxide
NRT	Nicotine replacement therapies
OCN	Osteocalcin
OEM	Osteogenic exposure media
OM	Osteogenic media
OPN	Osteopontin
PDL	Periodontal Ligament
PDLSCs	Periodontal ligament derived stem cells
PG	Polyethylene Glycol
PTK2	Protein tyrosine Kinase 2
qPCR	Real time, quantitative polymerase chain reaction
ROI	Region of Interest
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcription-polymerase chain reaction
RUNX2	Runt-Related Transcription Factor 2
SPP1	Osteopontin
TM	Transmembrane
UTR	Untranslated region
VG	Vegetable Glycerin

List of Publications

Portions of this thesis have been previously published in peer reviewed academic journals.

Chapter 2 Background:

Parts of the Nicotine Subchapter and the entire The deleterious effects of cigarette smoking and nicotine usage on mesenchymal stem cell function and implications for cell-based therapies Subchapter, including Table 2-2, were taken from:

> *The deleterious effects of cigarette smoking and nicotine usage and mesenchymal stem cell function and implications for cell based therapies.* Jordan M. Greenberg, **Carlos M. Carballosa**, Herman S. Cheung. Feb 2017. Stem Cells and Translational Medicine, AlphaMed Press. September 2017. (6)9:1815-1821.

The entire Nicotine's Influence on Musculoskeletal Healing Subchapter was taken from:

> *Nicotine's Influence on Musculoskeletal Healing: A Review Featuring nAChRS and miRNA.* **Carlos M. Carballosa**, David J. Fernandez-Fidalgo, Herman S. Cheung. Global Journal of Medical Research: H, Orthopedic and Musculoskeletal System, Global Journals Inc. 2014. (14)1: 1-11.

The entire Expression and function of nicotinic acetylcholine receptors in stem cells Subchapter, including Figure 2-2 and Table 2-3, were taken from:

> *Expression and function of nicotinic acetylcholine receptors in stem cells.* **Carlos M. Carballosa**, Jordan M. Greenberg, Herman S. Cheung. AIMS Bioengineering, AIMS press. July 2016. (3)3: 245-263.

All of these articles were collaborative efforts between myself, my peers Jordan Greenberg and David Fernandez-Fidalgo, and my mentor Dr. Herman Cheung. All text and figures were prepared by myself and Mr. Greenberg. Conceptual design, editing, and final review were done by all authors.

Chapter 1 Significance

Cigarette smoking (CS) is the leading cause of preventable death in the US[1]. Every year millions of people try to quit CS with the help of nicotine replacement therapies (NRTs). These therapies are FDA approved and, under proper use, are safe ways to help smokers overcome their nicotine addictions. The electronic cigarette (EC) is a new NRT that is being marketed as a "safer alternative" to CS; however, these devices have not been fully regulated by the FDA and are still believed to cause major health concerns due to the concentrated delivery of toxic chemicals like nicotine and flavoring agents[2; 3]. Despite these concerns, EC use continues to be on the rise. In the U.S. alone within the last 5 years, EC use has doubled amongst adults[4] and tripled amongst adolescents[5]. The effect of EC vapors on human health has been previously studied[6; 7; 8]; however, the immediate and residual effects of these vapors on oral health and stem cell biology remain entirely unknown.

Adult stem cells are currently being considered as a therapeutic approach for the regeneration of tissues damaged by periodontal disease[9]. The oral cavity, the initial site of EC vapor exposure, contains various stem cell populations that are involved in the development, maintenance, and repair of oral tissues[10]. Periodontal ligament derived stem cells (PDLSCs) are one such population. PDLSCs are multipotent and have been shown to give rise to tooth supporting structures such as alveolar bone, periodontal ligament, and cementum[10]. In addition to their regenerative capabilities, PDLSCs can be easily isolated following natural tooth loss or routine dental procedures and are thus an ideal source of therapeutic stem cells for periodontal regeneration[10; 11]. A recent clinical trial using autologous PDLSCs as a therapeutic approach for the regeneration of

periodontal bony defects confirmed the safety of this approach for human use[9]. However, by residing in the oral cavity, PDLSCs are exposed to and extremely susceptible to the detrimental effects of EC vapor. EC chemicals like nicotine readily accumulate in the saliva due to nicotine "ion-trapping"[12]. In fact, salivary nicotine has been measured to be almost 87 times higher than in the blood plasma[13]. Our group has recently shown that nicotine significantly inhibits PDLSC regeneration potential[14]. Some suggest that these nicotinic effects are mediated through the activation of nicotinic acetylcholine receptors (nAChRs) present on the stem cell surface; however, the detrimental effects of nicotine are still partially observed even after the deactivation of nAChRs[15]. These results suggest that there are additional mediators behind these effects. Understanding the potential effects of EC use on PDLSC biology is therefore crucial for determining whether EC use can render PDLSCs ineffective for future therapeutic applications regarding periodontal regeneration.

Periodontitis is an inflammatory disease of the oral cavity that, if left untreated, leads to the deterioration of tooth supporting structures and culminates in eventual tooth loss. Periodontitis has been associated with increased incidences of cardiovascular disease[16] and stroke[17] and is extremely prevalent in cigarette smokers[18]. Nicotine has also been associated with higher risks of periodontitis due to its ability to augment alveolar bone loss[19]. ECs commonly contain nicotine in their e-liquids; therefore, it is possible that EC use may increase the risk of periodontitis. The detrimental effects of periodontitis on oral health can be avoided if the disease can be diagnosed at the onset in order to provide early preventative dental care. Current diagnostic techniques, however, are only capable of detecting advanced forms of the disease; therefore, a need for early

detection still exists in order to improve clinical management. One potential approach is to identify the presence of biomarkers in saliva that are associated with the early onset of periodontitis. The detection of microRNAs (miRNAs), for example, has proven useful for the early prognosis of numerous diseases, including cancer[20]. Interestingly, miRNAs can be measured in the saliva[21; 22] and are differentially expressed in human periodontal-diseased and normal gingival tissues[23]. Our group has recently shown that PDLSC miRNA expression is significantly altered following nicotine and cigarette smoke exposure, with certain miRNAs actually targeting several aspects of PDLSC regeneration potential[14; 24]. The use of miRNAs as salivary biomarkers for the early detection of periodontitis is therefore a novel approach that has the potential to improve the prognosis of periodontal diseases like periodontitis. This approach would allow for earlier preventative care, thereby reducing the overall prevalence of this disease. These methods can be converted into a new diagnostic technique for future commercial application. Ultimately, the findings obtained from this application can be used to assess the harm that ECs pose to oral health, identify potential root causes and new diagnostic techniques for the screening of destructive periodontal diseases like periodontitis, and further encourage tobacco use reduction.

This project introduces several innovative aspects, on a conceptual, technical and practical level. At the conceptual level, this project innovates by changing the common belief that nicotine is "only" the major addictive substance in cigarette smoke and EC vapor to nicotine being a major player responsible for adverse effects of cigarette smoking and EC vaping. In addition, the effects of EC vapors on stem cells in the oral cavity have not been explored. This proposal will directly address the novel concept that repair

mechanisms in the oral cavity are depressed by EC vapor through the action of nicotine changing the miRNA composition of stem cells. At the technical level, this project innovates by using sophisticated vaping (VC-1) and smoking robots (VC-10) that will allow us to mimic real-life smoke and vapor conditions. In addition, a liquid chromatography mass spectroscopy method was developed to determine the concentration of nicotine in collected extracts so that all experiments were conducted in physiological ranges of exposure. At the practical level, this project innovates by providing solid scientific data that will help understand possible adverse effects of nicotine-containing vapors on stem cells in the oral cavity and by identifying novel biomarkers (i.e. miRNA) that can be used as a prescreening method for the early detection of oral diseases associated with cigarette and EC use. Therefore, this project is highly relevant to dental health.

Chapter 2 Background

Cigarette Smoking

Cigarette smoking (CS) is the leading cause of preventable death worldwide[1]. In the United States alone, CS has claimed the lives of more than 20 million people since the release of the inaugural Surgeon General Report on Smoking and Health in 1964[25]. Exposure to CS toxins significantly increases the risk of cardiovascular disease, respiratory disease, and cancer. According to the 2014 Surgeon General's Report on Smoking and Health, CS is a major risk factor for the development of cardiovascular disease (CVD), the leading cause of death in the United States[25]. Smoking increases the risk of atherosclerosis, stroke, and coronary heart disease and, on average, smokers are two to three times more likely to suffer from myocardial infarction and ischemic heart disease compared to non-smokers[25; 26]. In addition to CVD, CS has been shown to severely affect the respiratory system. Asthma and chronic obstructive pulmonary disorder (COPD) are two common respiratory complications caused by chronic smoking[25]. In fact, COPD is the third leading cause of death in the US, with almost 80% of cases being smoking-related[25]. The most deadly respiratory disease caused by CS, however, is lung cancer-the leading cancer killer in the US[25]. Cigarette smoke is composed of more than 7,000 chemicals, 69 of which are known carcinogens[25]. In addition to exposing themselves, smokers expose millions of others to cigarette smoke toxins via 'second-hand'[27; 28; 29] and, more recently identified, 'third-hand' smoke[30; 31]. Exposure to these chemicals significantly increases the risk of various types of cancers (reviewed in[32]); however, despite knowing these facts and seeing them first-hand, the prevalence of CS among U.S. adults remains high at 22.0%[33]. Every year 68% of Americans try to quit smoking[34],

however a recent study determined that it may take smokers more than 30 attempts before they are actually able to do so[35]. Although surprising, the high failure rates and number of attempts needed to quit are to be expected because smokers have a strong addiction to nicotine, a common chemical found in cigarettes and all tobacco products. To aid in their quitting efforts, many tobacco users turn to alternative forms of nicotine delivery.

Nicotine Replacement Therapies

Nicotine replacement therapies (NRT) are Food and Drug Administration (FDA)-approved products that deliver nicotine at controlled rates to help cigarette smokers and other tobacco users overcome their nicotine addictions. By gradually reducing nicotine dose over time, these products help smokers wean off of nicotine and avoid the severe withdrawal symptoms associated with abrupt quitting[36]. NRTs come in a variety of forms like patches, lozenges, gums, nasal sprays, and inhalers with each product having their own unique way of delivering nicotine. **Nicotine patches** are adhered to the skin and deliver nicotine transdermally. They are easy to use, discrete, and only need to be applied once a day. They can be purchased over the counter and do not require a prescription. Also available over the counter, **lozenges** and **gums** deliver nicotine orally. Lozenges deliver nicotine as they dissolve, whereas gums deliver nicotine as they are chewed. Although both are discrete and easy to use, they are not long-lasting and require multiple doses throughout the day therefore making them easier to abuse. **Nasal sprays** and **inhalers** deliver nicotine via the respiratory tract and are prescribed to heavily addicted patients who need rapid nicotine delivery. Nasal sprays deliver nicotine in a liquid through the nose where it can be rapidly absorbed through nasal blood vessels. Inhalers, on the other hand, deliver nicotine through a vapor which the user must puff or inhale. These products closely resemble

cigarettes in both shape and use and are therefore said to help smokers also overcome the "act" of smoking. These products are tightly controlled in production and have strict guidelines for use, unlike **electronic cigarettes**, which are similar to inhalers but considered alternative tobacco products.

Electronic Cigarettes

The electronic cigarette (EC) is a relatively new device that has been adopted as the "safer" alternative to cigarette smoking due to a significant reduction in toxic chemical delivery[37]. The device was originally created by Hon Lik, a Chinese pharmacist, as a consequence of his inability to quit smoking with the assistance of the then current nicotine replacement therapies. Lik was determined to engineer a new "cigarette-like" device that appealed to his smoking habits and helped him quit smoking by delivering addiction-satisfying doses of nicotine in a safe and efficient manner. Lik eventually designed a device that met these specifications and, by the turn of the 21st century, patented the "electronic atomization cigarette"[38]. Since then, ECs have gain rapid popularity among active and former cigarette users, as well as non-cigarette users thanks to their simple design, convenience, and improved "safety". In fact, EC use in the U.S. alone has doubled amongst adults from 2010-2011[4] and tripled amongst adolescents (middle and high school students) from 2013-2014[5].

The EC, also commonly referred to as the e-cig or electronic nicotine delivery system (ENDS), was initially designed to closely resemble the size, shape, and use of a typical cigarette. The purpose of this design was to allow for a smoother transition between cigarette and EC use and ultimately a smoother transition to cigarette cessation. As depicted in Lik's patent application, the original EC contained a variety of electrical

components that were housed within a "cigarette-shaped rod" (Figure 2-1). Table 2-1, below, gives a brief description of each of the EC components. Together, these components convert a nicotine-containing solution, or "e-liquid", into a vapor that can be easily inhaled by the user. The "vaping" process is initiated once the user begins to inhale on the EC mouthpiece. The differential pressure created upon inhalation activates the sensor, which in turn activates the electronic circuit board and the atomizer. Simultaneously, outside air is drawn into the atomizer cavity through the opposite end of the EC. The flow of air produces droplets from the e-liquid bottle that trickle down into the atomizer cavity. There, the e-liquid is quickly heated by the atomizer and vaporized into smaller droplets. These droplets are mixed with incoming air to create an aerosol that can then inhaled by the user. The aerosol contents however will vary depending on the type of EC used (i.e., model) and the formulation of the e-liquid.

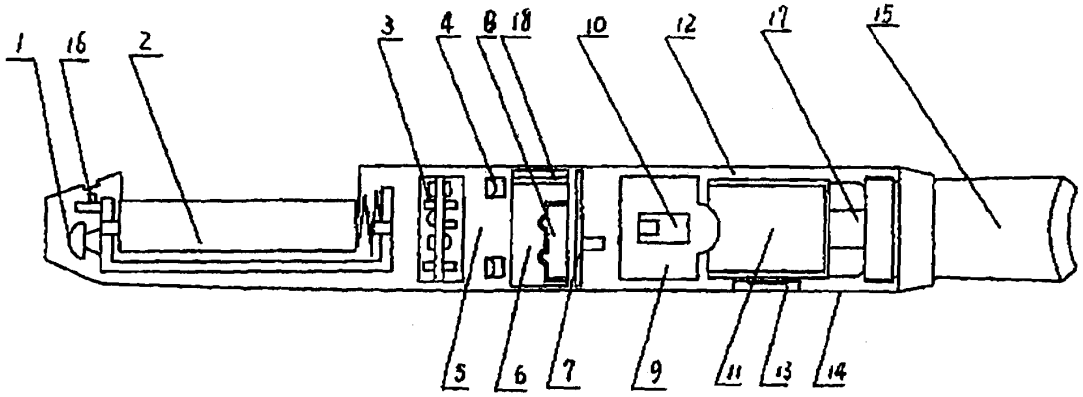

Figure 2-1: Electronic Atomization Cigarette Schematic. This schematic from Lik's original U.S. Patent application shows all the internal components of the first EC.

Table 2-1: Figure 2-1 Key. Identifying and Describing the Internal Components of the Electronic Atomization Cigarette Schematic in Figure 2-1

Component	Description
1. Light Emitting Diode (LED)	When the user inhales, the LED lights up to simulate a burning cigarette. The LED is also used as an indicator of proper function.
2. Rechargeable Battery	The battery powers all of the EC's electrical components.
3. Electronic Circuit Board	Contains electronic switching circuit and the high frequency generator.
4. Air Inlet	A space within the EC by which outside air can flow into the normal pressure cavity.
5. Normal Pressure Cavity	Fills with outside, atmospheric air upon inhalation. Provides a differential pressure upon inhalation (negative pressure).
6. Sensor	Recognizes the pressure difference that is created upon inhalation. Produces an actuating signal that initiates the operation of the circuit board.
7. Vapor-Liquid Separator	Separates the e-liquid contents from the freshly generated EC vapor.
8. Negative Pressure Cavity	Cavity in which a negative pressure is created via inhalation through the mouthpiece.
9. Atomizer	Heating element responsible for vaporizing the EC e-liquid.
10. Atomization Cavity	Surrounded by the atomizer, this cavity is where air, from the normal pressure cavity, and e-liquid droplets, from the e-liquid bottle, are mixed together and heated.
11. E-Liquid Bottle	E-liquid reservoir.
12. Aerosol Passage	A space within the EC by which the aerosol, created from the heating of the e-liquid in the atomization chamber, can flow towards the gas vent and mouthpiece.
13. Retaining Ring	Locks e-liquid bottle in place.
14. External Shell Wall	Protects internal components.
15. Mouthpiece	The contact point between the user and the EC. In a typical cigarette, the mouthpiece would correspond to the filtered end.
16. Micro-switch	Operates the sensor.
17. Gas Vent	An opening to allow for pressure equalization.
18. Air Passage of Sensor	An opening for the continual passage of air from the air inlet towards the atomization cavity.

Throughout the years the EC has undergone numerous modifications to enhance its function and capacity to deliver concentrated doses of nicotine. First-generation ECs closely resembled the size and shape of a regular cigarette. They were disposable devices intended for a single use (e.g., 200-300 puffs) and so contained a non-rechargeable battery and pre-filled e-liquid tank. Second-generation devices, however, addressed these limitations. Compensating size, these devices included bigger, rechargeable batteries and often re-fillable e-liquid tanks. These two features reduced waste, made devices last longer, and allowed users the ability to freely switch between e-liquid cartridges of different

flavors and nicotine strengths. Now, a much more advanced form of these second-generation devices exists. Third-generation ECs are essentially modifiable versions of its predecessor. These devices are equipped with digital screens that allow users the ability to modify battery voltage and wattage in an effort to personalize the vaping experience.

Regardless of generation, all ECs contain e-liquid. E-liquids are comprised of three ingredients: humectants, flavoring agents, and nicotine[39]. Humectants form the base of all e-liquids and are therefore the most common chemical found across brands. It is the heating of these solvents that produces the characteristic vapor of ECs. Propylene glycol and glycerol are the two compounds routinely used to create e-liquids. Both are FDA-approved and widely used in the food[40] and pharmaceutical industries[41]. These two compounds are often both present in e-liquids, but users can modify their ratio to alter the characteristics of the vapor produced (i.e., thicker cloud, sweeter taste, less of a "throat hit"). E-liquids also come in a variety of flavors. These additives can be used to improve a user's vaping experience. A recent study showed that flavor additives can be used to enhance the sweetness, pleasantness, and bitterness or EC vapor[42]. Flavored e-liquids are easily created by soaking ingredients in PG/VG for weeks or adding flavoring extracts to PG/VG batches. The flavor profiles can be simple or complex depending on how many flavoring agents are added into the e-liquid mixture. With thousands of e-liquid flavors available to date, users have a wide variety of options to choose from and are therefore more likely to try and continue vaping. In addition to humectants and flavoring agents, e-liquids can also contain nicotine, which further reinforces EC use.

Nicotine

Nicotine is the quintessential compound responsible for an individual's addiction to cigarettes and other tobacco-containing products[43]. E-liquids can contain anywhere from 0 to 36mg/ml nicotine[44], therefore EC use can also be addicting. Although present in both cigarettes and ECs, the concentration and the methods for the delivery of nicotine are unique to each product.

The most common method for nicotine delivery is through the burning, or combustion, of tobacco during cigarette smoking. In this method, nicotine is transported via tiny tar droplets that are inhaled as part of the particulate phase of cigarette smoke[43; 45]. Typical cigarette rods contain an average of 13-19mg of nicotine[46]; however, the total nicotine yielded from each cigarette depends on the user's smoking technique (i.e., duration, interval between puffs, total puffs)[47]. When smoked according to the parameters of ISO 3308:2012, the international standard for achieving reproducible cigarette smoking conditions across all scientific labs, a typical cigarette rod yields approximately 1 mg of nicotine[48]. Experienced smokers, on the other hand, can yield an average of 2.29mg nicotine/cigarette[49]. ECs deliver nicotine via an aerosol that is created from the vaporization of an e-liquid. As previously mentioned, e-liquid nicotine concentrations range anywhere from 0-36mg/mL. As is the case with cigarettes, only a portion of the nicotine in e-liquids, approximately between 20-80%, is actually aerosolized and delivered to the user[50; 51]. Recent studies have shown that these inefficiencies in nicotine yield can be affected by factors such as EC battery voltage, e-liquid nicotine concentration, puff protocol, and user experience[52; 53]. Therefore, similar to cigarettes, a vaper can easily modify nicotine yields by simply altering their smoking habits.

Internalization of smoking and vaping-toxins occurs through the respiratory tract. For specific compounds like nicotine, the subsequent absorption into specific tissues is largely dependent on tissue pH[43]. Nicotine is a weak base (pKa of 8.0)[43] and is therefore more readily absorbed in slightly basic conditions where it is less "ionized"[43]. This is partially why nicotine is readily absorbed through the lungs[43]. In addition to being at a slightly basic pH, the lungs offer a large surface which further facilitates nicotine absorption. After diffusing through the alveoli and into the surrounding vasculature, nicotine is quickly spread throughout the body via cardiovascular circulation. Within seconds, nicotine reaches the brain and begins to stimulate dopaminergic reward regions[54; 55; 56]. This immediate effect is one of the major reasons for nicotine addiction and abuse liability because, at this pace, the brain can ascertain internal nicotine concentrations and quickly modify a user's smoking behavior in order to ensure nicotine yields that satisfy addiction levels[57]. Nicotine is also readily absorbed in the mouth, the initial site of smoke and vapor exposure. The oral cavity is a slightly acidic environment. Others have reported that the pH of the mouth ranges from 6.7 to 7.3, with an average of 6.7[58]. Flue-cured cigarette smoke is also slightly acidic (pH 5.5–6.0), thus the two conditions do not make for efficient absorption in the oral cavity[43]. However, recent reports suggest that cigarette smoke may be more alkaline than originally thought[43], thereby improving oral nicotine absorption. EC liquids, on the other hand, are characterized by a slightly more basic pH[59]. Therefore, nicotine delivered from these devices is believed to be more readily absorbed in the mouth. Although the slightly acidic conditions of the mouth are not ideal for nicotine absorption, nicotine readily accumulates in the saliva due to nicotine "ion-trapping"[12]. In fact, salivary nicotine has been measured to be

almost 87 times higher than in the blood plasma[13]. Table 2-2 provides peak nicotine concentration measurements taken from the saliva and blood plasma of smokers after cigarette or EC use. These results show that despite differences in nicotine delivery between cigarettes and ECs, experienced EC users can achieve plasma and salivary nicotine concentrations similar to those achieved during cigarette smoking.

Table 2-2: Peak mean nicotine concentrations measured in blood plasma and saliva after cigarette and EC use. The variation in mean nicotine values across studies is due to differences in study methods.

Measurement Location	Peak Nicotine Concentrations (μM)				Ref.
	N	Cigarette Smoker Mean (SD)	*N*	Ecig Vaper Mean (SD)	
Blood Plasma (Venous)	N.R.	0.092 (N.R.)	N/A	N/A	[13]
	6	0.147 (0.017)	N/A	N/A	[60]
	10	0.115 (0.038)	N/A	N/A	[61]
	24	0.180 (0.067)	23	0.138 (0.047)	[62]
	10	N.R.	11	0.152 (0.072)	[63]
	N/A	N/A	13	0.118 (0.014)	[64]
	N/A	N/A	16	0.105 (0.110)	[65]
Saliva	N.R.	8.605 (N.R.)	N.R.	5.301	[66]
	N.R.	8.013 (N.R.)	N/A	N/A	[13]
	12	14.478 (7.775)	N/A	N/A	[67]
	36	1.276 (N.R.)	N/A	N/A	[68]
	42	1.073 (N.R.)	N/A	N/A	[69]
	122	0.398 (0.021)	N/A	N/A	[70]

Legend: N - number of subjects; N/A - Not Applicable. N.R.; - Not Reported or Mentioned.

Nicotine's Influence on Musculoskeletal Healing

The wide distribution and absorption of nicotine throughout the body results in many of the tissues being extremely susceptible to the effects of nicotine exposure. Since this dissertation focuses on the effects of nicotine-containing EC vapors on the wound healing and osteogenic differentiation potential of stem cells, the following sub-sections will aim to summarize the findings of recent scientific experiments investigating the effect of nicotine on musculoskeletal healing and stem cell biology.

Nicotine and Musculoskeletal Healing

Healing, in general, is a complex process orchestrated by several role players whose ultimate goal is to efficiently restore damaged tissue to its original state. The basic mechanisms behind wound and skeletal healing and the effects of nicotine on these processes have previously been reviewed[71; 72; 73]; however, the aim herein is to present recent and human-only-based research. In order to do so, the following filters and search titles were used when gathering potential publications on the PubMed database (http://www.ncbi.nl-m.nih.gov/pubmed): Publication Dates: 5 Years; Species: Human; Title: (Wound Healing OR Skin OR Soft Tissue OR Blood Vessels AND Nicotine) OR (Bone OR Fracture Fixation OR Fracture Healing OR Osteoblast OR Osteoclast AND Nicotine).

Wound Healing

Two of the major role players involved in the wound healing process are fibroblasts and endothelial progenitor cells. Fibroblasts, which produce extracellular matrix as well as collagen, and endothelial progenitor cells, which give rise to the endothelial cells that help form new capillaries, are simultaneously recruited by activated macrophages and cell mediators to the site of injury in order to replace damaged tissues (reviewed in[72]). Although efficient, these cells can become ineffective when exposed to outside factors such as nicotine. Therefore, current therapies, which aim to facilitate regeneration, use chemical agents and growth factors to enhance the number and function of fibroblasts and endothelial progenitor cells.

Fibroblast-Based Studies

In 2010, Choi et al. observed that nicotine increased the expression of early growth response-1 (EGR-1) in cultured human skin dermal fibroblasts (hSDFs)[74]. The increased expression of EGR-1, which encodes a protein involved in collagen production and skin wound repair, is suggested by Choi et al. to improve the function of hSDFs, which, in turn, will facilitate the wound healing process. In a later study, Silva et al. investigated the effects of nicotine on the viability and migration potential of human gingival fibroblasts (hGFs)[75]. The researchers observed that nicotine had little to no effect on cell viability and cell death, but did stimulate cell migration. Ultimately, however, Silva et al. concluded that the effect of nicotine on hGFs was not enough to significantly affect the healing potential of these cells. Tinti & Soory, investigating the oxidative effects of nicotine on hGFs and human periosteal fibroblasts, determined that the detrimental effects of nicotine oxidation on wound healing could be reversed by the anti-oxidant glutathione[76].

Endothelial-Based Studies

While studying the in vitro effect of nicotine on human umbilical vein endothelial cells (HUVECs), Y.J. Park et al. observed that nicotine exposure augmented the proliferation, migration and angiogenic potential of HUVECs[77]. In 2011, H.S. Park et al. investigated the acute and chronic effects of nicotine on the proangiogenic activity of HUVECs[78]. The group looked at the effect of nicotine on several factors including: production of nitric oxide (NO), expression of endothelial nitric oxide synthase (eNOS), cell viability, migration potential and morphology and the results from these experiments can be summarized into two relatable conclusions. The first conclusion is that nicotine, regardless of exposure time, has an effect on the angiogenic activity of HUVECs. This

result was supported by the variation in values between non-exposed and exposed groups for all factors. The second conclusion is that the degree of this nicotinic effect is dependent on exposure time. H.S. Park et al. showed that the production levels of NO and eNOS were significantly higher in acute vs. chronic exposed HUVECs. The migratory function and tubular formation (number and length of circles) of acutely exposed HUVECs was also significantly better when compared to the chronic exposed groups.

Combined Studies (Fibroblast and Endothelial Cells)

In 2011, Laytragoon-Lewin et al. investigated the effects of pure nicotine on human derived fibroblasts and endothelial cells[79]. The researchers showed that, compared to the control, nicotine exposure increased the proliferative capacity and altered the morphology of both cell types. In addition, the researchers evaluated nicotine's effect on the expression of 96-well-defined genes common to both cell types, which were grouped into 5 categories: Cell Cycle and DNA Damage, Apoptosis and Cell Senescence, Signal Transduction and Adhesion, Angiogenesis, and Invasion and Metastasis. Surprisingly, nicotine caused a differential expression in 80% of endothelial and 73% of fibroblast genes investigated within an hour of exposure.

Skeletal Healing

The dose dependent effect of nicotine is well known and has been recently demonstrated in many of the cells that comprise the skeletal tissues. The process of bone fracture healing is very similar to the process of wound healing. It can be divided into three phases: reactive phase, reparative phase and remodeling phase. During the reactive phase, blood vessels surrounding the fracture site constrict to prevent further bleeding. At the same time, extravascular blood cells form a clot, known as a hematoma, in the fracture site.

All the cells within the clot undergo apoptosis, allowing for the migration and proliferation of fibroblast cells within the clot, forming granulation tissue. The fibroblasts create a provisional extracellular matrix for the migration and proliferation of cells necessary for the formation of new bone. Once this phase is complete, the reparative phase begins with the migration, differentiation and proliferation of precursor cells from the periosteum, a connective tissue membrane covering the bone. These precursor cells include mesenchymal stem cells, which differentiate into chondrocytes and osteoblasts, which are responsible for the formation of new cartilage and new bone, respectively. During this phase, various preliminary bone structures are formed by chondrocytes and replaced by osteoblasts[80]. Finally, during the remodeling phase, the preliminary bone structure is reinforced with compact bone. It can take anywhere from 3 to 5 years for the newly formed bone to achieve its original strength[80]. The time frame in which wound healing and bone fracture healing take place depends on a patient's age and general condition, which includes a patient's exposure to nicotine.

Chondrocyte and Bone Marrow Stromal Cell-Based Studies

In 2012, Ying & Cheng et al. demonstrated that nicotine, at concentrations of 0.154μM (25ng/ml), 0.308μM (50ng/ml), and 0.617μM (100ng/ml), caused significant increases in the cellular proliferation and collagen type II expression/production of human derived chondrocytes[81]. That same year, Ying & Zhang et al. used a different set of cells, human bone marrow stromal stem cells (hBMSCs), to investigate the effects of a higher nicotine dose on the proliferation and collagen type II expression of these cells[81]. In this study, Ying & Zhang et al. observed significant enhancements of both qualities at lower nicotine doses (1μM), but significant impairments at higher doses of (10μM). In addition,

Ying et al. also investigated the effect of nicotine on the expression/ production of aggrecan; however, no significant changes were noted. A 2013 study conducted by Shen et al. also investigated the effects of nicotine on the hBMSCs derived from the iliac crest[82]. Similar to Ying & Zhang et al.'s results, Shen et al. observed that low doses of nicotine (0.308μM [50ng/ml] and 0.617μM [100ng/ml]) caused significant and sustained increases in the proliferation of hBMSCs, significant increases in alkaline phosphatase (ALP) activity, and significant and sustained increases in the expression of ALP and collagen type I. In addition to significantly decreasing all of these effects, higher doses of nicotine (6.17μM [1000ng/ml]) significantly inhibited cell-mediated calcium deposition, osteocalcin (OCN) expression, and bone morphogenetic protein-2 (BMP-2) expression.

Periodontal Ligament Cell-Based Studies

The increased incidences of alveolar bone degenerating diseases, such as periodontitis, have been well documented in smokers and tobacco users alike[83; 84; 85]. The oral cavity is the initial site of toxic exposure for all tobacco-containing products and many nicotine containing products (e-cigarettes, nicotine gums, and nicotine lozenges). During their use, nicotine remains in the oral cavity for extended periods of time causing a rapid increase in concentration. As a result, the tissues of the oral cavity are extremely susceptible to the effects of nicotine exposure. A 2009 study by H. Lee et al., investigating the effects of nicotine on periodontal ligament (PDL) cells, showed that nicotine downregulated the expression of osteoblastic differentiation markers ALP, OCN, and Osteopontin (OPN)[86]. In order to prevent additional cytotoxic effects, nicotine decreased the expression of osteoprotegerin while simultaneously increasing the expression of receptor activator of nuclear factorkappa B ligand and the production of transcription factor

NF-E2-related factor-2 and heme oxygenase-. A study by S.I. Lee et al. demonstrated that nicotine exposure promotes endoplasmic reticulum stress and facilitates extracellular matrix degradation via downregulation of extracellular matrix molecules, such: as collagen type I, elastin, and fibronectin; and upregulation of matrix metalloproteinases[84].

Cigarette Smoking, Nicotine, and Mesenchymal Stem Cells

All of the cells mentioned in the previous section originate, in one way or another, from tissue-restricted stem cells. These postnatal stem cells, also known as adult stem cells, reside in their respective tissue niches where they provide support for tissue maintenance and regeneration. To help them achieve these functions, stem cells rely on several characteristic traits including proliferation, migration, differentiation, and paracrine signaling. These abilities can also be harbored for stem-cell based therapies in where tissues-restricted stem cells are extracted from their niches and re-implanted into other sites of need. In fact, recent translational approaches using mesenchymal stem cells (MSCs), for example, have proven to be very effective for the treatment of a variety of injuries and diseases. However, compromising MSC function, through infectious or genetic diseases for example, can lead to ineffective clinical outcomes. Accordingly, these conditions are often included as exclusion criteria in patient recruitment for stem cell-based therapies. However, environmental risk factors, such as cigarette smoking and nicotine use, can also compromise MSC function, leading to inefficacious outcomes. Recent data have demonstrated that cigarette smoking and nicotine exposure can negatively affect MSC regeneration potential (i.e., proliferation, migration, and differentiation). The following sections review recent studies regarding the deleterious effects of cigarette smoking and nicotine usage on MSC and serve to provide evidence as to why cigarette smoking and

nicotine usage deserve further consideration as exclusion criteria when designing future stem cell-based trials and therapies.

Effect on Proliferation

The ability to self-renew is one of the defining characteristics of stem cells and vital to their translational efficacy. To highlight the role that cigarette smoking may play on proliferation, Wahl et al. exposed human adipose-derived MSCs in vitro to CSE at various concentrations. At levels of 5% and 10%, MSCs displayed no viability; however, under 1% showed no significant difference in cell viability. This study highlights that above specific thresholds cigarette smoking can be very toxic to MSC populations[87]. These data, however, are only helpful in the empirical sense, as determining what concentrations specific compounds in extract are found in is unknown.

In vitro, human umbilical cord blood cells exposed to 0.5–1.5 mg/ml nicotine (3–9 mM) showed dose-dependent decreases in proliferation and increases in apoptosis ($p<.05$ for all concentrations)[88]. Significant decreases in proliferation have also been observed at concentrations as low as 0.1–10 μM ($p<.05$)[14; 89]. Evaluating changes in proliferation in vivo can be challenging; however, decreased proliferation rates have also been observed in MSCs isolated from chronic smokers without in vitro exposure to any additional smoke or nicotine after isolation. These cells showed a 2.53-fold decrease in proliferation compared with nonsmoker derived control cells. Decreased proliferation was still observed even after sub-culturing cells 3–5 times, suggesting that the effects of nicotine exposure may be permanent or last for several generations of daughter cells[24]. This result is very important for translational approaches as it highlights the need to gain a full history of nicotine usage for all potential stem cell donors. Contrarily, several groups have reported

an increase in cellular proliferation after nicotine exposure. Shen et al. showed that human MSCs exposed to 50–100 nM nicotine for 7 days showed significant increase in the cell number ($p<.05$); however, 1 μM nicotine significantly decreased cell concentration ($p<.01$)[82]. Kim et al. report that 1 μM to 100 μM nicotine was not sufficient to alter proliferation in vitro, but that doses between 1 and 2 mM increased proliferation, and over 5 mM decreased proliferation of human alveolar bone marrow-derived MSCs[90]. Although the trend in this study is consistent with data presented by others the nicotine concentration used appears to be at minimum 1,000x higher compared with other studies.

There have been several theories to partially explain how nicotine alters proliferation of MSCs. One study suggests that the decrease in proliferation is dependent on the generation of reactive oxygen species. Culturing nicotine-exposed cells with the antioxidant vitamin C, for example, increases the rate of proliferation compared with 1 μM nicotine-exposed controls[82]. Other groups suggest that the decrease in proliferation may be a result of changes in the cell cycle induced by nicotine. Nicotine has been shown to induce an increase in the ratio of G0/G1 phase cells[88]. As G0 is considered the quiescent phase of the cell cycle, the increased ratio of cells in this phase may partially explain the observed decrease in proliferation induced by nicotine exposure[91].

Effect on Migration

In addition to their self-renewing capabilities, MSCs also exhibit the ability to undergo cell migration. Much like their embryonic counterparts, which migrate extensively during early embryogenesis to achieve proper organogenesis[92], MSCs migrate toward sites of injury and promote postnatal wound healing through the release of growth factors and cytokines and through direct differentiation[93]. This form of directed migration,

termed “stem cell homing,” is unique to MSCs and allows populations of resident or transplanted MSCs to achieve targeted delivery to diseased areas[94].

The migratory potential of MSCs, however, can be affected by cigarette smoking. Zhou et al. were the first to report that cigarette smoking inhibited the targeted migration of transplanted MSCs to the uterus in an in vivo rat model[95]. An in vitro wound closure assay using MSCs derived from human smoker PDL yielded similar results[24]. On average, smoker PDLSCs migrated 12% slower than those isolated from nonsmokers ($p<.05$) resulting in decreased wound closure potential after 24 hours[24]. The authors of this study mention that the isolated cells were cultured in non-cigarette smoke media for several weeks prior to analysis. Therefore, the results also suggest that the effects of cigarette smoke exposure on MSCs could be irreversible, even after long periods of recovery, for example, cessation.

But just like proliferation potential, the effect of cigarette smoke exposure on MSC migration is dose-dependent. The migratory potential of human adipose-derived MSCs (hAMSCs), for example, was unaffected in vitro by CSE concentrations of less than 1%, but severely affected by concentrations greater than 5%[87]. In a separate study, the in vitro exposure to 100% CSE induced the epithelial to mesenchymal transition of breast epithelial cells and, thus, promoted MSC migration and invasion (i.e., metastasis)[96]. Given the results from the first set of CSE exposures, one would expect that the use of 100% CSE would be extremely detrimental to cell migration. However, the compared studies used different cigarettes and methods for the collection of CSE, and thus the percent extracts are not identical.

Individual cigarette smoking compounds, like nicotine, can also affect MSC migration. Schraufstatter et al. showed that 1μM nicotine significantly increases the spontaneous migration of human bone marrow-derived MSCs (hBMMSCs) in chemokine free cultures by more than 40%[97]. In chemokine-supplemented cultures, however, the addition of 1μM nicotine significantly decreased MSC migration[97]. These effects were reversed by alpha bungarotoxin pretreatments, suggesting that nicotine's effects are likely mediated by α7 nAChRs. Ng et al. have also shown that hBMMSCs treated with 1μM of nicotine experienced a 60% reduction ($p<.05$) in both migration distance and speed when compared with non-treated controls[14]. MSC derived from human PDL suffered a similar fate, but with reductions of only 38% ($p<.05$)[14]. Moreover, nicotine also downregulated protein tyrosine kinase-2 (PTK2) gene expression in both MSC populations ($p<.01$) and significantly upregulated PTK2-targeting microRNA miR-1305 expression in PDLSCs. These results are interesting because they allude to another possible mechanism behind the effects of nicotine on MSC migration.

Effect on Differentiation

MSCs are routinely considered for many regenerative medicine applications because of their ability to form a variety of cell types[98]. Transplanted cells, however, must be screened to avoid infectious or genetic diseases that can interfere with MSC function. Often overlooked is the presence of external factors such as cigarette smoking that can affect MSC differentiation potential and render cells ineffective for transplantation. The following sections aim to summarize how cigarette smoking and nicotine exposure affect the three main lineages of MSC differentiation.

Adipogenic Differentiation

Wahl et al. demonstrated that in vitro exposure to 0.5% CSE did not significantly affect the adipogenic differentiation potential of hAMSCs after 21 days as evident by similar adipogenic marker expression (i.e., PPARg, ADIPOQ, and LEP) and Oil Red O staining between treated and non-treated hAMSCs[87]. On the other hand, Ng et al. demonstrated that MSCs derived from cigarette smoker PDLs experience increased lipid production compared with nonsmokers even after several weeks of in vitro culture with non-exposed medias[24].

Given such contrasting results and limited availability of references, it is difficult to determine the effect smoking has on MSC adipogenic differentiation. The former study models typical smoking exposure conditions in vitro with extract; however, additional analysis with regard to the measurement and concentration of smoking-related toxins was not performed. Therefore, it is impossible to determine if 0.5% CSE falls within the physiological ranges of toxic smoke exposure to reflect in vivo conditions. The results from the latter study, however, are much more indicative of in vivo conditions since the MSCs were extracted from actual smoking and non-smoking donors. Even so, the claim that smoking promotes the adipogenic differentiation potential of MSCs cannot be made due to a lack of exposure normalization between donors and small sample size of this study. Therefore, the effect of cigarette smoking on the adipogenic differentiation of MSCs remains inconclusive.

Chondrogenic Differentiation

Wahl and Ng also investigated the effects of cigarette smoking on MSC chondrogenic differentiation. Wahl et al. demonstrated that chondrogenic induced

hAMSCs exposed to 0.5% CSE caused an initial upregulation in aggrecan (ACAN) (6-fold, $p<.0001$) and chondrogenic transcription factor SOX9 (3-fold, $p<.01$) gene expression after 7 days of induction compared with non-treated controls in vitro[87]. Expression of both markers decreased and was comparable to non-treated control levels by 21 days. Collagen type II alpha I gene expression was consistently below non-exposed control levels and Alcian Blue staining was slightly decreased in CSE-treated hAMSCs after 21 days; however, no statistical difference was observed. Similar Alcian Blue results were observed by Ng et al.'s group in MSCs isolated from smoker PDLSCs after 14 days of differentiation[24]. The consistency of these results suggest that cigarette smoking is capable of undermining MSC chondrogenic differentiation; but, as previously mentioned, additional experiments with greater sample size and quantifiable levels of smoke exposure must be conducted in order to establish a more representative outcome.

Nicotine has also been shown to affect MSC chondrogenic differentiation potential. Rat BMMSCs treated with 25, 50, and 100μM nicotine experienced dose-dependent decreases in ACAN and COL2A1 gene expression ($p<.01$ across all concentrations) and Alcian Blue staining (15, 51, and 95% reduction; $p<.01$) after 4-weeks of chondrogenic induction compared with non-exposed MSCs in vitro[99]. It should be noted that the nicotine concentrations used in this study are at least 2.5 times higher than the most extreme concentrations experienced by smokers (around 10μM in saliva)[66; 67] and therefore non-representative of actual use. More physiological concentrations were investigated by Ying et al. in a separate in vitro study using human BMMSCs. Ultimately, only the 10μM nicotine inhibited Alcian Blue and sulfated glycosaminoglycan staining ($p<.05$) after 14 days, whereas 0.1 and 1μM (physiological concentrations of blood and saliva,

respectively), had minimal effect[81]. 10μM nicotine also downregulated COL-1 and COL-X ($p<.05$) gene expression, but showed minimal effect on ACAN and COL-2 expression. Less concentrated nicotine doses resulted in similar outcomes, except for the expression of COL-2, which was upregulated ($p<.05$) throughout the 21-day differentiation protocol. hAMSCs show yet a different response to in vitro nicotine with 100 ng/ml (0.61μM) causing a two-fold increase ($p<.05$) in ACAN gene expression, but no change in COL-1 or COL-X after 14 days of chondrogenic induction[100].

Although it is difficult to compare this results due to differences in differentiation protocol and cell source, it is easy to see that physiological doses of nicotine can negatively impact MSC chondrogenic differentiation potential. The inability to effectively produce aggrecan, an integral extracellular matrix proteoglycan, or collagen for example, could render MSCs ineffective for current stem cell-based therapies aimed at treating cartilage repair in debilitating diseases like osteoarthritis.

Osteogenic Differentiation

The osteogenic differentiation potential of MSCs is well known and has been extensively studied. Accordingly, MSCs have been routinely considered in therapies for metabolic bone diseases[101; 102; 103; 104], and fracture fixation[105; 106]. Cigarette smoking, however, can deteriorate bone health and inhibit normal and reparative bone formation. Compared with non-smokers, cigarette smokers are more likely to experience osteoporosis[107; 108; 109] and delayed healing times following skeletal fracture[110; 111; 112]. These delays are partly due to the inefficient osteogenic differentiation of MSCs[24; 87].

MSCs derived from smoker PDL have been shown to exhibit an overall reduction in calcium deposition and alkaline phosphatase production compared with nonsmokers after 14 days of osteogenic differentiation in vitro[24]. In the same study, smoker MSCs also experienced a significant upregulation in the expression of RUNX2-targeting microRNA miR-1305-a correlation that hints at a possible mechanism for smoking induced effects[24]. 0.5% CSE studies, on the other hand, have been shown to have a nonsignificant effect on hBMMSC calcium deposition after 20 days of in vitro culture[87]. Although CSE did upregulate RUNX2 (2.5-fold) and osteocalcin (2-fold) gene expression ($p<.001$) after 14 days induction.

Nicotine exposure has also been associated with inefficient skeletal healing[113]. Nicotine is known to affect the osteogenic differentiation of MSCs in vitro[14; 15] and therefore likely contributes to these outcomes. Specifically, 1μM nicotine exposure significantly decreases inherent RUNX2, COL1A1, COL1A2, ALPL, and OCN gene expression in both hBMMSCs and hPDLSCs ($p<.05$) and significantly upregulates the expression of RUNX2-targeting miR-1305 by more than 120-fold ($p<.001$) after just 3 days of exposure[14]. In this same study, decreased Alizarin Red S and alkaline phosphatase staining confirmed these results. Zhou et al. observed similar results, and, in addition, demonstrated that the nicotinic effects were dose-dependent and mediated through α7 nAChRs[15].

It should be noted that the results from Ng et al.'s 1μM nicotine in vitro exposures showed similar trends with those of the smoker-derived MSC experiments. Even though cigarette smoke contains over 7,000 chemicals, the similarity of outcomes from the two

studies suggest that nicotine is one of the more potent inducers of the effects seen in cigarette smoke exposures.

Effect on Paracrine Signaling

In many cell-based therapies, although improvement in conditions are observed, engrafted cells may not be observed[114]. It is hypothesized that the improvements that are observed are a result of paracrine signaling by the transplanted stem cells. These factors promote angiogenesis, and decrease inflammation among other processes. In hAMSC's, which were exposed to 0.5% CSE for 48 hours in vitro, investigators studied the release profile of 36 different cytokines secreted by MSCs. Of those studied IL-6 and IL-8 showed significantly ($p<.05$) lowered amounts secreted by those exposed to CSE. Both factors play roles in inflammatory response and angiogenesis and their decreased secretion may lead to delayed wound healing[87].

In addition to their role in tissue regeneration, naive MSC's also perform a crucial role in the niche acting as players in the supporting role of hematopoietic stem cells (HSC's) which give rise to the cellular components of blood. Within the bone marrow niche HSC's colocalize next to MSC's that secrete factors such as Fibroblast Activation Protein. In models with MSC's removed anemia and bone marrow hypocellularity ensue[115]. Cigarette smoking has been shown to directly alter MSC's in the HSC niche. In MSC's that were isolated from mice that had been exposed to cigarette smoke for 9 months, aberrant gene expression changes were noted which span across several pathways of MSCs that are known to regulate HSC function which may lead to a loss of niche functionality[116].

Nicotinic Acetylcholine Receptors and Stem Cells

The effects of nicotine exposure on musculoskeletal health and stem cell biology have been extensively studied, but the mechanisms behind these effects are still unknown. Current research suggests that these effects are mediated by the nicotinic acetylcholine receptors (nAChRs). These receptors, which are activated in the presence of nicotine, undergo conformational changes that eventually alter the ionic permeability of their respective membranes. The results of these actions are linked to changes in cell proliferation, differentiation and microRNA expression. Since this dissertation focuses on the effects of nicotine-containing EC vapors on the periodontal ligament derived stem cells, a mesenchymal stem cell subpopulation, the following sections will review the expression and function of nAChRs in MSCs and PDLSCs. As stem cell-based therapies become more common in the clinic, understanding the effects mediated by the activation of these receptors will be crucial due to the continued use of tobacco products, which contain nicotine.

Nicotinic acetylcholine receptors

nAChRs belong to the cholinergic family of receptors and are a class of ligand-gated ion channels that respond to the neurotransmitter acetylcholine as well as other ligands such as choline and nicotine. Functional nAChRs are composed of 5 transmembrane subunits that are arranged together to form a transmembrane pore. These pentameric receptors are created from various combinations of the 16 different nAChR subunit types. All subunits share the same molecular structure: an extracellular domain, four transmembrane subunits (TM1-TM4), and a cytoplasmic domain; however, each subunit has differences in its amino acid sequence[117]. Accordingly, subunits are classified as either α or non-α type based on the presence of a cysteine-cysteine residue

within the N-terminal domain near the entrance to TM1[117]. The Cys-Cys pair is only found on α subunits and is required for agonist binding. In total, there are 9 α subunits: α1-7, α9, α10, (α8 is identified in avian libraries but has not been observed in mammalian species) and 7 non-α subunits: β1-4, γ, δ, and ε[117; 118].

The affinity for ligands and the ion gating properties of the nAChR depends on the specific subunit composition of each receptor[117]. For instance, the binding affinity for nicotine, a typical nAChR agonist, is higher in nAChRs that contain the α4 subunit versus those that contain the α7[119]. This specificity is also observed for binding of receptor antagonists, which can competitively bind to nAChRs causing desensitization and inhibit activation. α-bungarotoxin (α-BTX), for example, binds specifically to α7 nAChRs[120], whereas dihydro-β-erythroidine binds specifically to α4 containing nAChRs[121]. On the other hand, mecamylamine (MECA) is a non-specific antagonist and therefore it binds to all nAChRs, regardless of subunit composition[122]. In addition to ligand affinity these receptors also vary in their ion gating properties. For example, nAChRs composed of α4 subunits are much less permeable to Ca^{2+} ions than the α7 homomeric nAChRs, which are considered to be the most permeable nAChR to Ca^{2+}[123]. The calcium permeability of the nAChR can, however, be modified. The addition of the α5 subunit to α3 nAChRs, for instance, greatly increases the permeability to calcium[124].

nAChRs are highly conserved among all species and are widely expressed throughout the body; however, they are predominantly found in muscle (muscular nAChRs) and neuronal (neuronal nAChRs) tissues. Muscular nAChRs are found in skeletal muscles where they mediate neuromuscular transmission at the neuromuscular junction. Neuronal types, on the other hand, are mainly found in the peripheral and central nervous

systems, but have been located in non-neuronal tissues including stem cells[125; 126]. Muscle nAChRs are heteropentameric (i.e. 5-diferent subunits) and consist of α1, β1, γ, and either δ or ε subunits in the 2:1:1:1 stoichiometric ratio[117]. Neuronal nAChRs, however, can exist as either hetero- or homopentamers (i.e. 5-identical subunits). Heteropentameric nAChRs are the more predominately found form of neuronal nAChR as they can exist in several different combinations. For example, studies show that α2-4 and/or α6 nAChR subunits typically assemble with β2 and/or β4 subunits, but they can also assemble with α5 or β3 subunits to make functional nAChRs[118]. Homopentameric nAChRs, on the other hand, are less diverse and can only be formed from α7, α9, and α10 subunits (Figure 2-2).

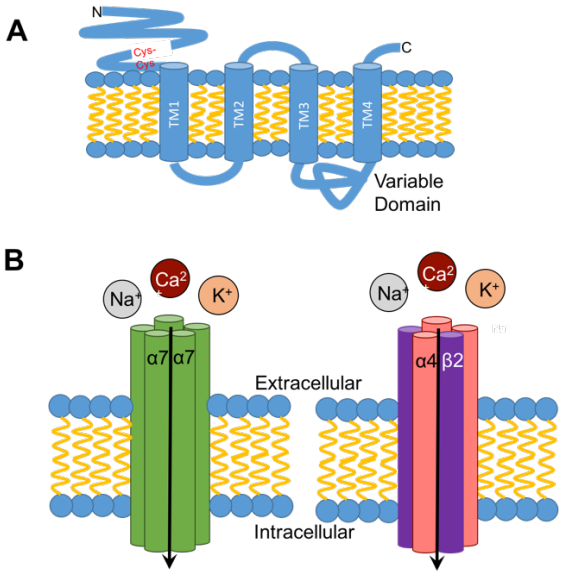

Figure 2-2: A: Subunit structure of nAChR showing the cys-cys pair that defines α-subtype nAChRs. B: A prototypical α7 homopentameric nAChR (left) and heteropentameric α4β2 nAChR (right).

nAChRs and Mesenchymal Stem Cells

Functional nAChRs have recently been identified on the surface of MSCs, a multipotent adult stem cell population capable of differentiating into various mesodermal

lineages like osteogenic, chondrogenic, and adipogenic lineages[98]. MSCs reside in stem cell niches and have been identified within various adult tissues like bone marrow[127], adipose tissue[128], and umbilical cord blood[129]. In addition to being readily available, MSCs exhibit immunosuppressive and homing properties[130] and are therefore an ideal therapeutic stem cell source[131].

nAChR Subunit Expression in MSC

Both α and β nAChR subunits have been identified in human MSCs. In 2009, Hoogduijn et al. were the first to identify the expression of α3, α5, and α7 nAChR subunits in hBMMSCs via reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry[132]. Although the authors did not observe expression in the other investigated subunits (i.e. α9, α10, β2 and β4), it is important to mention that this study was limited in scope as only a partial nAChR subunit panel was evaluated (mRNA expression for α2, α4, α6, and β3 was not conducted). A definitive conclusion on the full expression of nAChR subunits from this adult stem cell population therefore could not be made at the time.

A full nAChR subunit expression panel on hMSCs was, however, conducted by Schraufstatter et al. shortly thereafter. Initial analysis using RT-PCR indicated the expression of α1, α2, α3, α4, α5, α7, α9, β2, β3 and β4 subunits in hMSCs, but only the presence of α7, β2, and β4 subunits could be confirmed after further analysis with DNA sequencing, western blotting, and immunofluorescence[97]. Although these results were compared side-by-side to the previous Hoogduijn study, the precise origin (i.e., sex, age, location) of the Schraufstatter et al. hMSCs was never disclosed. Instead, cells were described as human MSCs from the Tulane Center for Gene Therapy. It is assumed that these cells were derived from the bone marrow; however, as previously mentioned, hMSCs

can be isolated from various sources therefore a comparison between current and previous BM-MSC data should be made with caution.

Most recently, Zablotni et al. confirmed the presence of several nAChR α-subunits on hMSCs derived from the long bone diaphyseal fractures of both men and women[133]. Interestingly, subunit expression was dependent on the sex and osteoporotic health of the donor. For instance, healthy male-derived hMSCs expressed the α3, α5, α7 and α9 nAChR subunits, whereas healthy female-derived hMSCs expressed α2, α5, α6, α7, α9, and α10 subunits. Osteoporotic-derived hMSCs, on the other hand, expressed the same subunits as healthy females with the additional expression of the α3 nAChR subunit. In addition to the variation in expression among patients of different sex and osteoporotic health, Zablotini et al. also observed that the expression of the specific subunits within each group was often not consistent for all patients. While the α7 subunit was expressed in all (4/4) healthy female patients, the α2 subunit was only expressed in half (2/4) of the healthy female patients. The authors suggest that these inconsistencies in expression are likely attributed to patient-specific conditions such as hormone levels, nutrition, and/or drug use. In a separate study, the expression of nAChR α-subunits was investigated after hMSCs were differentiated into osteogenic, chondrogenic, and adipogenic lineages (according to the protocols outlined). Interestingly, variations for some subunits were again observed between differentiated groups and within differentiated subjects. For example, while the α7 subunit remained mostly unchanged throughout all groups, the α3 subunit did not. In pre-differentiated male hMSCs, the α3 subunit was expressed in 3/4 patients; however, after differentiation the subunit expression was increased in osteoblasts (4/4), maintained in chondrocytes (3/4), and abolished in adipocytes (0/4). These differences in expression

suggest that nAChRs might be involved in the stem cell differentiation process. This suggestion is further strengthened by the widespread and maintained expression of the α7 subunit in all groups and differentiated states. Although Zablotni et al.'s results confirm previous observations of α-subunit expression in hMSCs, a western blot or other protein expression analysis is needed to confirm the actual translation of these genes into proteins that can then be assembled into functional nAChRs.

Functionality of hMSC nAChRs

Functional nAChRs, specifically those gating for the calcium ion (i.e. α7), have been identified on hMSCs[97; 132]. The functionality of these receptors can be determined via measurements of $[Ca^{2+}]_i$, which should increase upon proper activation of α7 nAChR. In an *in vitro* study, Hoogduijn et al. investigated the functionality of hMSC nAChRs by measuring changes in $[Ca^{2+}]_i$ via Fluo-3-AM imaging. Briefly, cultured hMSCs were loaded with Fluo-3-AM, a calcium-specific fluorescent probe, exposed to 1 μM nicotine, and immediately imaged under confocal microscopy. Increases in $[Ca^{2+}]_i$ were observed for almost half (22/50) of the hMSCs imaged, suggesting the presence of calcium-gating nAChRs like the homopentameric α7 receptor[132]. Additional studies by Hoogduijn et al. were conducted to determine whether receptor activation with nicotine affected the cAMP production or phosphorylation of extracellular signal-regulated protein kinases (ERK). After exposing hMSCs to 0.1 – 10μM nicotine, no significant changes in the production of cAMP when compared to non-nicotine treated cells was observed[132]. Nicotine (1μM) exposure did, however, increase the phosphorylation of ERK in hMSCs. When phosphorylated, ERKs are activated and capable of regulating cell proliferation and differentiation[134]. Along with migration, the processes of stem cell proliferation and

differentiation are critical for effective stem cell-based regeneration[135]. By mediating the effects that interfere with these processes, nAChRs are undoubtedly capable of influencing stem cell health and differentiation and regeneration potentials. Altogether, this data suggests that the observed nicotinic effects are predominantly regulated by the homopentameric α7 nAChRs. The other nAChR subunits identified in this study, α3 and α5, are incapable of forming homopentameric receptors with themselves[136] or heteropentameric receptors with each other (none have yet to be identified.) Moreover, since no β–subunit proteins were identified to form complexes with the identified α-subunit proteins it is very unlikely that the α3 and α5 subunits are involved in the formation of functional nAChRs in hMSCs.

The presence of functional homopentameric α7 nAChRs in hMSCs was also confirmed in separate *in vitro* studies conducted by Schraufstatter et al.. Briefly, cultured hMSCs were loaded with Fluo 4-AM, exposed to 2 μM nicotine, and immediately imaged using a fluorescent plate reader. hMSCs exposed to nicotine experienced a significant increase in $[Ca^{2+}]_i$ compared to non-nicotine-treated controls and calcium-chelated groups, thus confirming the presence of functional α7 nAChRs in hMSCs[97]. The researches acknowledged that one of the consequences of increased $[Ca^{2+}]_i$ is the increased vulnerability to cellular apoptosis. In further investigations, Schraufstatter et al. demonstrated that hMSCs exposed to 10 mM - 0.1 μM nicotine experienced a dose-dependent effect on cell viability, with doses above 1 μM resulting in significant trypan blue uptake and Annexin V staining. The toxic threshold dose of 1 μM nicotine was further investigated in order to determine its effect on the inherent homing characteristics of hMSCs. After 16 hours of exposure to nicotine, hMSCs exhibited significantly reduced

growth factor-mediated migration potential. Interestingly, this inhibition was ameliorated by 100 nM α-BTX pretreatments, thus providing further evidence that α7 nAChRs mediate these nicotine-induced effects. Our group has also previously shown that 1 μM nicotine is capable of inhibiting hMSC proliferation and migration potentials *in vitro*[14].

nAChRs and Periodontal Ligament Derived Stem Cells

Periodontal ligament derived stem cells (PDLSCs) are a subset of MSCs that reside in the periodontal ligament (PDL). Similar to MSCs, PDLSCs are capable of mesodermal lineage differentiation[11]. Moreover, these cells are capable of differentiating in various oral tissues, like cementum, alveolar bone, and periodontal ligament[10; 137], and are therefore critical to maintaining the overall health within the oral cavity. PDLSCs are also easily accessible after routine dental procedures (e.g., orthodontic extractions) and are thus an ideal source for therapeutic stem cells[138].

nAChR Subunit Expression in PDLSC

Both nAChR subunits and functional nAChRs have been identified on PDLSCs. Kim et al. were the first to confirm the expression of both α7 and β4 nAChR subunits and functional α7 nAChRs in PDLSCs through RT-PCR and nAChR antagonist studies, respectively[122]. The researchers' analyzed all nAChRs with the exception of the α9 and α10 subunits, which were not investigated at any level (rationale for exclusion not provided). Consequently, the presence or absence of these subunits could not be confirmed in PDLSCs. In a separate study, Zhou et al. confirmed the presence of functional α7 nAChRs on PDLSCs through RT-PCR, western blot, and nAChR-specific antagonist studies using α-BTX[15]; however, it should be noted that only the α7 nAChR subunit was examined in these studies. These results also confirmed the findings of his collaborators'

previous study demonstrating the expression of α7 and β4 nAChR subunits in PDL tissues[120].

Functionality of PDLSC nAChRs

To date, only the α7 and β4 nAChR subunit proteins have been confirmed in PDLSCs[15; 122]. Out of all the possible arrangements using these two subunits, only the homopentameric α7 and heteropentameric α7β4 conformations form functional nAChRs. The homopentameric α7 nAChR occurs naturally and has been previously identified in PDLSCs[15; 122]. The functional α7β4 heteropentameric nAChR, on the other hand, does not occur naturally (or has yet to be identified) and can only be created through forced experimental means[139]. Therefore, although both α7 and β4 subunits have been identified in PDLSCs, only the homopentameric α7 nAChR is believed to be present and functional in these cells.

PDLSC homopentameric α7 nAChRs have been shown to mediate nicotine-induced effects on cell viability[122]. In an *in vitro* study conducted by Kim et al., PDLSCs were exposed to a range of nicotine concentrations, between 0 and 10 mM, for 24 or 48 hours before being evaluated by MTT analysis for changes in cell viability. The results showed that nicotine caused a significant decrease in PDLSC viability after 24 and 48 hours of culture in concentrations greater than or equal to 0.1 mM. The reduction in cell viability could be explained by the observed increases in DNA fragmentation and by the increase in cells in subG1, the cell phase associated with apoptosis[140]. Moreover, PDLSCs treated with 10−2 M nicotine experienced an increase in the expression of p53, a pro-apoptotic tumor suppressor protein[141], after only 30 min.[122]. In order to confirm if these effects were regulated by nAChRs, Kim et al. measured the expression of several apoptotic

proteins in the presence or absence of α-BTX, the aforementioned α7-specific nAChR antagonist, and MECA, the non-specific nAChR antagonist (concentrations not provided). PDLSCs treated with nicotine (1μM - 10mM) for 48h experienced a decrease in the expression of Bcl-2[122], a prosurvival, anti-apoptotic protein from the mitochondrial pathway[142], and an increase in the expression of cleaved caspase-3, an activated pro-apoptotic enzyme[143]. These effects, however, were significantly reversed, to a similar extent, in PDLSCs pretreated with α-BTX and MECA. The similarity in data between α-BTX and MECA pretreatments further suggests that α7 is the predominant nAChR in PDLSCs.

In a separate *in vitro* study Zhou et al. demonstrated that homopentameric α7 nAChRs mediated the detrimental effects of nicotine on PDLSC osteogenic differentiation potential[15]. PDLSCs were pre-treated with 0 or 100 μM α-BTX and cultured in an osteo-inductive media (PDLSC culture media supplemented with 100nM dexamethasone, 50μg/mL ascorbic acid, and 5mM β-glycerophosphate) containing 0 or 0.1mM nicotine before being evaluated for evidence of osteogenic differentiation. After 3-weeks, PDLSCs cultured in osteo-inductive medias showed significant increases in osteogenic gene and protein expression (i.e., alkaline phosphatase, osteocalcin, bone sialoprotein, and runt-related transcription factor 2) and also showed evidence of mineralization through positive alizarin red S and alkaline phosphatase staining. We recently observed similar results, but at lower nicotine concentrations (1μM) and shorter differentiation periods (i.e. 2-weeks)[14]. PDLSCs treated with nicotine-containing osteo-media managed to show evidence of osteogenic differentiation, albeit at significantly lower levels than non-nicotine treated and α-BTX pre-treated groups[15]. These results suggest that the homopentameric

α7 nAChRs regulate, to an extent, the osteogenic differentiation potential of PDLSCs. In an additional study, Zhou et al. also confirmed that these receptors could regulate the Wnt/β–catenin pathway, which was previously shown in a separate study to be involved in PDLSC osteogenic differentiation[144]. PDLSCs exposed to 0.1 mM of nicotine showed an increase in the expression of active-β-catenin protein and a decrease in Wnt-related transcriptional factors DKK-1 and GSK-3β; however, all of these effects were reversed with α-BTX pretreatments[15] thereby providing further evidence of the role of homopentameric α7 nAChRs in PDLSC regeneration potential.

The reviewed studies confirm the widespread presence of nAChRs on MSCs (summarized in Table 2-3) and demonstrate the involvement and impact of these receptors on stem cell function. However, the detrimental effects of nicotine on stem cell regeneration potential are still partially observed even after nAChR inhibition[15], suggesting that there may be additional factors outside of nAChRs that mediate these effects.

MicroRNA

We were the first to report that nicotine exposure induces changes in global PDLSC microRNA (miRNA) expression[14; 24]. MiRNAs are small, non-coding RNAs (~22 nucleotides) that regulate gene expression through posttranscriptional modification of mRNAs[145]. miRNAs are expressed throughout the body and have been shown to affect cell viability, differentiation and even organ development by down-regulating the genes associated with these biological processes[146]. Each miRNA can target several genes, and therefore up-regulation of a single strand can affect various biological processes. A link

between the nicotinic effect and the miRNA expression has yet to be fully determined; however, there does appear to be a correlation between the two.

Table 2-3: Summary of stem cell nAChR expression from studies reviewed in section. Brackets denote which subunits were actually investigated in each respective study. N/A corresponds to a lack of any subunit investigation.

Mesenchymal Stem Cells					
Cell Source	**Species**	**Subunit mRNA Expression**	**Subunit Protein Expression**	**Functional nAChRs**	**Ref.**
BM-MSCs	Human	**α**: α3, α5, α7 **β**: N/A [α3, α5, α7, α9, β2 - β4]	N/A	α7	[132]
MSCs	Human	**α**: α1 - α5, α7, α9 **β**: β2 - β4 [α1 - α10, β1 - β4]	**α**: α7 **β**: β2, β4 [α3, α4, α7, β2, β4]	α7	[97]
BM-MSCs	Human (Male)	**α**: α3, α5, α7, α9 **β**: N/A [α2 - α7, α9, α10]	N/A	N/A	[133]
	Human (Female)	**α**: α2, α5 - α7, α9, α10 **β**: N/A [α2 - α7, α9, α10]	N/A	N/A	
PDLSC	Human	**α**: α7 **β:** β4 [α1 - α7, β1 - β4]	N/A	α7	[122]

miRNAs are synthesized in the nucleus, and, like mRNAs, are transcribed from genetic material. To become functional, most miRNAs must undergo further processing and be transported out of the nucleus (reviewed by [145]). Briefly, primary miRNA transcripts are recognized and cleaved into pre-miRNA hairpin structures by the endonuclease Drosha. The pre-miRNA hairpin structures are then exported out of the nucleus and into the cytoplasm by Exportin 5. Once in the cytoplasm, most pre-miRNA hairpins are recognized and processed into miRNA-duplexes by the endonuclease Dicer.

The duplexes are dissociated into two complimentary strands, but then only one of the strands combines with the Argonaut protein to create the silencing complex. With miRNA serving as the guide, the silencing complex is now capable of targeting mRNAs.

miRNAs-mRNA targeting depends on complementarity between the sequences of miRNA and sequences within the 3' untranslated region (UTR) of mRNAs. The extent of the base pairing between the two determines how the expression of the mRNA will be regulated. Extensive pairing between the two, although uncommon, leads to mRNA cleavage[147]. The degraded mRNA can no longer be translated and, therefore, its associated protein can no longer be assembled. Less extensive base pairing, specifically between the miRNA seed region (nucleotide positions 2-8) and the 3' UTR of mRNAs, also leads to mRNA degradation, but by a mechanism that shortens the poly-A tail and destabilizes the mRNA[147].

miRNA, nicotine, and PDLSCs

As previously mentioned, we were the first group to report that nicotine exposure induces global changes in PDLSC miRNA expression[14]. In these experiments, microarrays were used to determine changes in PDLSC miRNA expression in response to 0, 0.5 and 1μM nicotine treatments in vitro. Microarray analysis revealed that nicotine had a dose-dependent effect on miRNA expression, with 1μM nicotine causing the most significant change. In PDLSCs exposed to 1μM nicotine, 225 miRNAs were found to have a 2-fold difference in expression compared to non-exposed controls. 16 of these miRNAs (i.e., *hsa-miR-7, -18b, -30d, -137, -374b, -505, -543, -1305, -1914*, -1973, -3198, -3659, -210, -762, -1915,* and *-4281*) were considered to be differentially expressed as they had fold changes greater than 2 and p-values less than 0.05. These microarray results were

validated with qPCR. miRNA target gene lists and subsequent gene ontology analysis revealed that the differentially expressed miRNA might target genes associated with all three characteristics of stem cell regeneration (i.e. proliferation, migration, and differentiation).

Interestingly, similar patterns of miRNA expression were observed in vivo between smoker and non-smoker PDLSCs[24]. Exposed cells also experienced decreases in proliferation, migration, and osteogenic differentiation potential, further supporting the notion that miRNA and cigarette smoking influence PDLSCs regeneration potential. The results suggest that in vitro models using μM nicotine can potentially be used to predict in vivo patterns of miRNA expression. Out of the differentially expressed miRNA, only *hsa-miR-18b* and *hsa-miR-1305*, but not *hsa-miR-3198* showed similar directions of expression compared to nicotine treated PDLSCs in vitro. The results suggest that *hsa-miR-18b* and *hsa-miR-1305* may be related to the detrimental effects of cigarette smoking. In fact, gene ontology analysis revealed that miRNA targeted genes associated with cell proliferation and cell migration, which, again, are 2 out of the 3 major aspects of stem cell regeneration. A recent study by a separate lab also investigated the effect of nicotine and *hsa-miR-1305* expression on PDLSCs regeneration and found that nicotine exposure inhibited regeneration and upregulated *hsa-miR-1305* expression[148]. The authors claim that restoring the expression of *miR-1305* ameliorated the effects of nicotine, thereby providing further evidence of the important relationship between nicotine exposure, miRNA expression, and PDLSC regeneration potential.

Chapter 3 Materials and Methods

Cell Culture

Human PDLSCs were established previously[11]. The specific PDLSC line used for these experiments was harvested from the PDL of an 18-year-old male donor's impacted wisdom teeth. With their informed consent, samples were collected at Nova Southeastern University College of Dental Medicine (Davie FL) according to approved institutional review board protocols. Briefly, PDL was scrapped off of the lower-third of the molar and minced with a surgical blade. The chopped tissue was transferred into an Eppendorf tube containing digestion media (high glucose Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham MA), 10% v/v heat-inactivated fetal bovine serum (FBS; Atlanta Biologics, Flowery Branch GA), 1% v/v Penicillin-Streptomycin (PenStrep; Thermo Fisher Scientific), 1 mg/ml collagenase, and 0.6mg/mL protease) and was constantly agitated overnight in a 37C and 5% CO_2 incubator. The following day the mixture was strained through a 40-μm strainer and plated on collagen-coated 6-well plates in complete culture media (CCM) (DMEM, 10% v/v FBS, 1% v/v PenStrep, and 0.1% v/v Amphotericin B (Fungizone; Thermo Fisher Scientific). Plates were maintained at 37C and 5% CO_2 for 5 days. Culture media was replaced after 5 days in order to remove any non-adherent cells, and every 3 days thereafter. PDLSCs were passaged at 70% confluence with Trypsin/EDTA (Thermo Fisher Scientific) and either expanded or cryopreserved. PDLSCs between the 6-8th passage were used for experimentation.

Cigarette smoke and e-cigarette vapor production and extraction

Cigarette smoke and e-cigarette vapor were produced by automated smoking robots (VitroCell, Waldkirch Germany). Robots were programmed with specific puffing parameters that mimicked characteristic smoking and vaping conditions (Table 3-1). Cigarette smoke was produced by the VC-10- VitroCell's automated cigarette smoking robot (Figure 3-1). The VC-10 was programmed with the puffing parameters outlined in ISO 3308:2012 – *Routine analytical cigarette-smoking machine -- Definitions and standard conditions* [ISO 3308:2012] and smoke was derived from University of Kentucky 3R4F reference cigarettes (University of Kentucky, Lexington KY). E-cigarette vapor was produced by the VC-1- VitroCell's automated e-cigarette vaping robot (Figure 3-2). Since international standards for vapor production do not currently exist, the VC-1 was programmed with the puffing parameters observed in experienced e-cigarette users[50; 149]. eVic electronic cigarette (EC) devices (JoyTech, ShenZhen China) filled with 0 or 36mg/ml nicotine, 50%/50% (w/v) PG/VG, non-flavored e-liquid (American E-Liquids, Wauwatosa WI) were used. For vapor production, ECs were programmed at 3.3V. All machine generated smoke/vapor was diluted with 4.5 L-min^{-1} environmental air in order to mimic the conditions observed during normal smoking.

Table 3-1: Puffing parameters used for cigarette smoke and e-cigarette vapor production.

Protocol	Robot	Puff Volume	Puff Duration	Puff Frequency	Total Time
E-cigarette Vaping	VC-1	70mL	3s	20s	18 min.
Cigarette Smoking	VC-10	35mL	2s	30s	18 min.

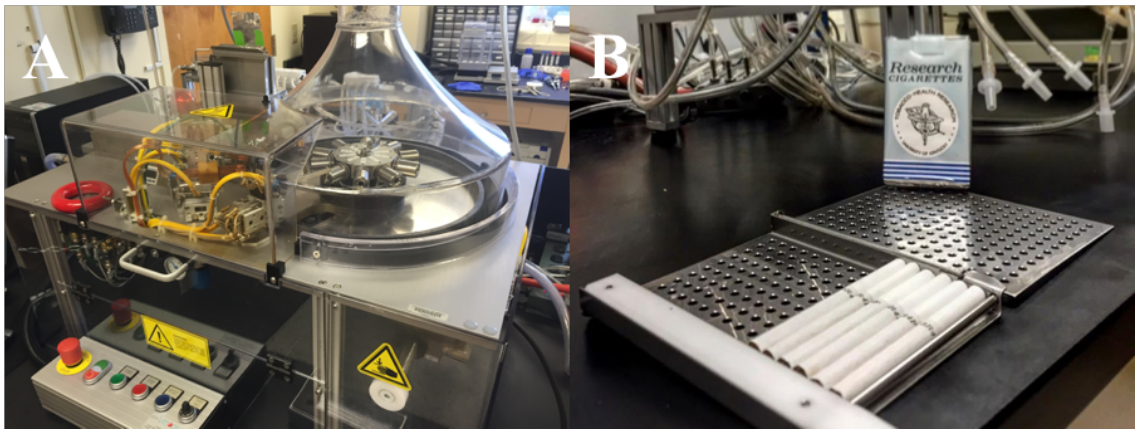

Figure 3-1: Images of the VC-10 and University of Kentucky 3R4F cigarettes. (A) Picture of the VC-10 smoking robot showing housing and control panel. (B) 3R4F cigarettes loaded into the VC-10 cigarette cassette.

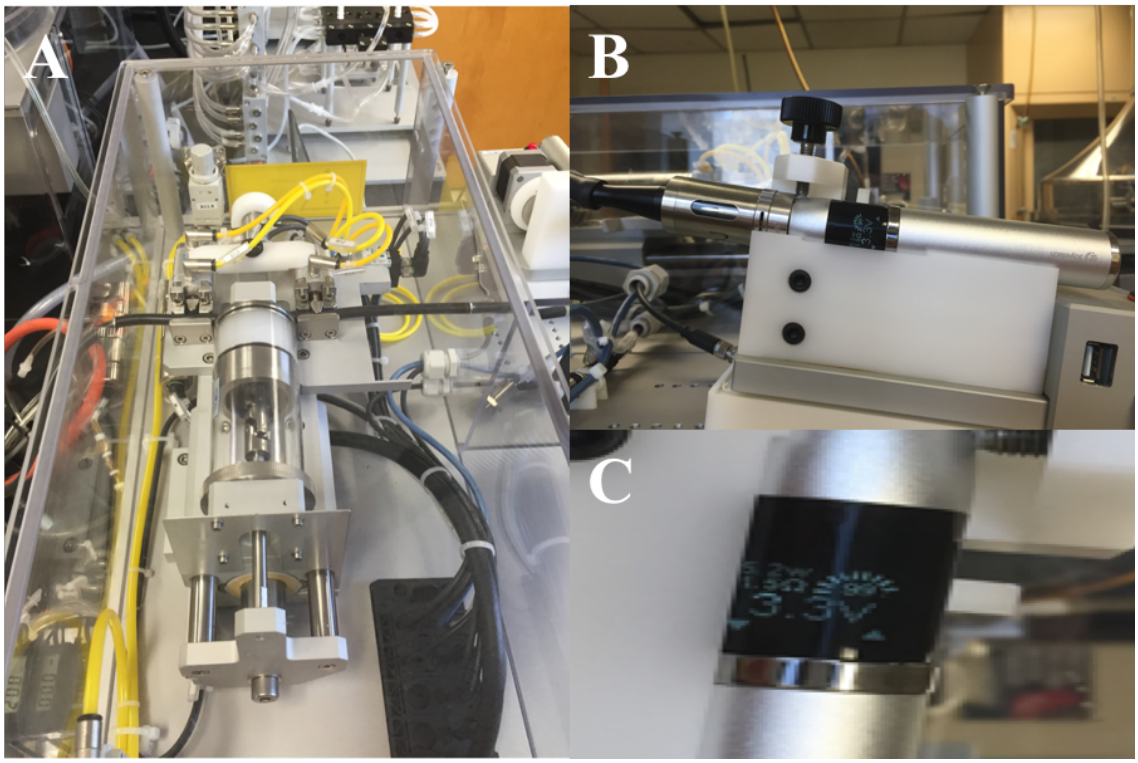

Figure 3-2: Images of the VC-1 and eVic electronic cigarette device. (A) Picture of the VC-1 vaping robot with vapor cloud in chamber. (B) eVic EC device setup. Mouthpiece is connected to robot inlet via tubing and the EC is secured onto a platform with a screw knob. The platform contains an automated push button device that is capable of pushing and holding down the EC vaping button. (C) Close-up image of eVic digital display showing the EC settings. The device was fully charged upon use and set to 3.3V.

Cigarette smoke and e-cigarette vapors were extracted into cell culture media via liquid impingers (Figure 3-3). Smoke and vapor was collected for 18 minutes, or the time it takes to smoke 3 cigarettes according to the ISO 3308 standard. In order to fully

submerge impinger nozzles, collector vessels were filled with 80mL of high glucose DMEM. Using silicone tubing, the inlet of the impinger was connected to the outlet of the robot and the outlet of the impinger was routed to the fume hood. Smoke was bubbled for the set time and collected extract was homogenized by mixing. The resultant solution was then aliquoted and stored at -80C until further use.

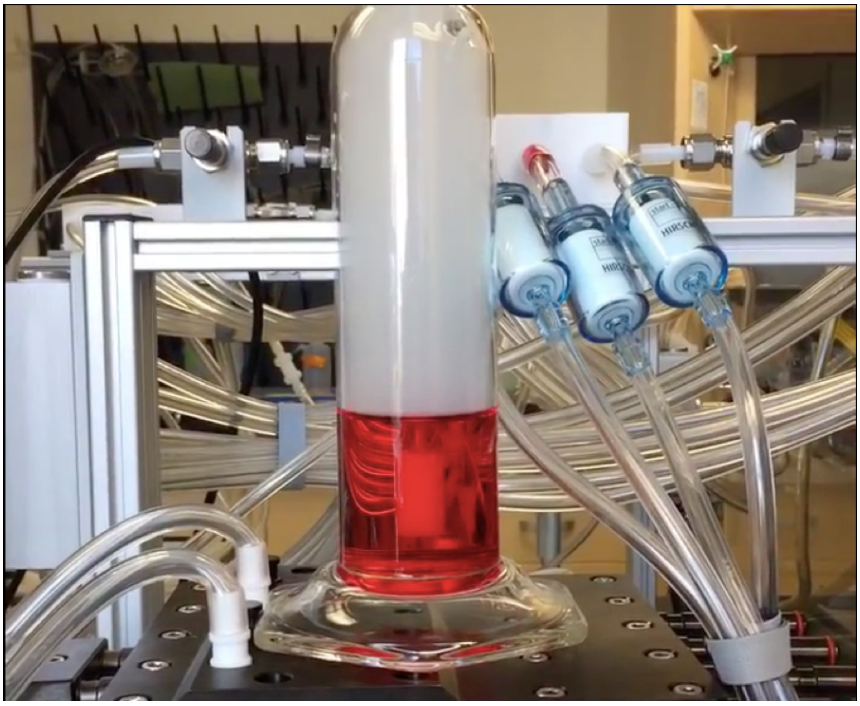

Figure 3-3: Picture of liquid impinger with vape cloud. DMEM media was used to fully submerge the impinger nozzle. After collection, the impinger was shaken to homogenize mixture.

Liquid Chromatography-Mass Spectroscopy (LCMS)

Liquid chromatography-mass spectroscopy (LCMS) was used to determine the nicotine concentration of the collected extracts. The developed methodology was based off a method first reported by Trehy et al.[150]. Briefly, nicotine standards were created with (-)-nicotine liquid (#N3876; Sigma Aldrich, St. Louis MO) and 50% methanol (#34885; Sigma)/water. Samples were diluted in 50% acetonitrile (#34851; Sigma)/water, fortified with 45nM nicotine-D3 (#N412425; Toronto Research Chemicals, Ontario Canada) and

transferred to the LC-MS/MS system. *Conditions for LC-MS/MS:* Sample injection (10 µL) and LC separation were performed by a Thermo Surveyor Plus HPLC system equipped with a HPLC column (Phenomenex Gemini NX-C18 150*4.6 mm, 3 µm) protected by a guard column. Mobile phase gradient was performed between 10 mM ammonium formate dissolved in Optima LCMS grade water with pH adjusted to 9 with ammonium hydroxide (aqueous) and Optima LCMS grade acetonitrile (organic). MS Detection was performed by an AB Sciex QTRAP 5500 triple-quadrupole mass spectrometer, equipped with a Turbo V™ ESI ionization source. The instrument was operated under MRM, positive mode.

Exposure Media

Based on the results of the LCMS, collected extracts were diluted with CCM to create exposure medias for cell culture that contained physiological doses of nicotine. Cigarette smoker saliva has been previously shown to contain between 0.4-14.5µM nicotine[66; 67; 68; 69; 70]. Similar results have also been documented in EC users, with one study reporting an average salivary nicotine concentration of 5.3µM[66]. To encompass this range, nicotine-containing ECV (i.e. 36mg/ml nicotine ECV) was diluted to 1 and 10µM nicotine with CCM. Since it was collected in the same manner, nicotine-free ECV (i.e. 0mg/ml nicotine ECV) was diluted in a similar fashion in order to isolate the effect of nicotine from the other vapor compounds. Due to the overwhelming number of chemicals and predicted toxicity, CSE media was only diluted to a 1µM nicotine concentration.

Cytotoxic Assay

The cytotoxic effects of ECVs and CSE was determined using a LIVE/DEAD Viability/Cytotoxicity kit (L3224; Thermo Fisher Scientific). PDLSCs suspended in CCM

were seeded onto 48-well plates at a density of $5x10^3$ cells/cm^2 and incubated overnight in a humidified atmosphere set to 37C and 5% CO_2. The following day, media was changed and new CCM supplemented with 1 and 10μM nicotine ECV and CSE extract was added. PDLSCs were returned to the incubator to be exposed to extract-containing media for 24h. After 24h, media was removed and wells were washed with PBS. PDSLCs were then incubated in Hank's Balanced Salt Solution (HBSS) containing 2μM calcein-AM for 20 minutes at room temperature. Wells were washed once to remove any excess calcein AM and fluorescent images were taken with the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific).

Immunohistochemistry

PLDSCs were seeded onto 8-chamber glass slides (Corning, Corning NY) at a density of 1.5x104 cells/cm2 and cultured in CCM until reaching an optimal confluence of ~70%. Cells were washed once with PBS and fixed in 10% neutral-buffered formalin for 10 min at room temperature. Cells were then washed twice to remove excess formalin and blocked for 1h in PBS buffer containing 0.05% v/v Tween-20 (Sigma) and 20 mg/mL Bovine Serum Albumin (BSA). For primary antibody treatment, PDLSCs were incubated overnight at 4C in a PBS solution containing 20 mg/mL BSA and α7 nAChR antibody (sc5544; Santa Cruz Biotechnology, Dallas, TX) at a 1:50 dilution. The following day, PDLSCs were washed three times with PBS to remove any excess and unbound primary antibody. For secondary antibody treatment, PDLSCs were incubated for 2h in a PBS solution containing 20 mg/mL BSA and AlexaFluor 546 secondary antibody (Abcam, Cambridge MA) at a 1:500 dilution. Cells were then washed twice with PBS to remove any excess and unbound secondary antibody. Cells were finally stained with DAPI and

protected with a glass coverslip. Slides were imaged using a Zeiss LSM 700 confocal microscope.

Fluo-4 AM Calcium Imaging

Agonist-induced activation of PDLSC α7 nAChRs was determined through calcium imaging with Fluo-4 (F14201; Thermo Fisher Scientific). PDLSCs suspended in regular culture media were seeded onto 6-well plates at a density of $5x10^3$ cells/cm^2 and incubated overnight in a humidified atmosphere set to 37C and 5% CO_2. The following day, cells were rinsed with HBSS and subsequently loaded with 10 μM Fluo-4 and 0.04% w/v Pluronic® F-127 (#P2443; Sigma) in HBSS for 20-min. at room temperature. The cells were then washed once with HBSS to remove any residual Fluo-4. Wells were re-filled with HBSS (Ca^{2+}/Mg^{2+}) and plates were covered for an additional 20-min. at room temperature to allow for de-esterification of internalized AM esters. A time-lapse imaging protocol was established within the EVOS FL Auto Cell Imaging System to capture fluorescent images every 5s, for 120s. In order to account for the 1:10 dilution that would occur upon addition of extract to cell culture media, 100μM and 10μM extracts were used to achieve final well concentrations of 10 and 1μM nicotine, respectively.

Acquired videos were analyzed with Fiji (Version 1.0, NIH). Upon opening, the AVI Reader was used to create a virtual stack and convert to grayscale. Using the Region of Interest (ROI) Manager, 13 ROIs were drawn on the first frame- 12 around cells and 1 around an area void of cells. The Mean Gray Value (MGV) of each ROI was then measured for each frame through the Multi Measure function within the ROI Manager. The MGV for each ROI was corrected by subtracting the background MGV of each frame from each individual cell ROI ($MGV_{\text{Corrected ROI (i,x)}} = MGV_{\text{Cell ROI (i)}} - MGV_{\text{Background ROI (x)}}$), where i

= cell ROI # (1-12) and x = frame number (1-25). All frame-specific corrected MGVs were then averaged to provide a final MGV for the respective time point.

ECV and CSE Exposure and α7 nAChR Inhibition

Nicotine-ECV and CSE were diluted to 10x stock concentrations and added directly to cell culture medias (1:10) to obtain a desired final concentration of either 1 or 10μM nicotine. 0mg/ml nicotine ECV was treated as nicotine-ECV and, therefore, added at similar volumes in order to isolate the effect of nicotine from the other vapor compounds. Inhibition of PDLSC α7 nAChRs was achieved with alpha bungarotoxin (αBTX), a specific, irreversible inhibitor of α7 nAChRs[120]. 10nM αBTX (#ab120542; Abcam) was added to cell culture media 30-min. prior to extract exposure. Cells were maintained in the incubator for the duration of the 30-min. pretreatment. Extract was then added directly to the culture media to obtain the respective final concentrations.

BrdU Cell Proliferation

Cell proliferation potential was analyzed using a BrdU Cell Proliferation ELISA Kit (#ab120542; Abcam). PDLSCs suspended in CCM were seeded onto a 96-well plate at a density of $5x10^3$ cells/well and incubated overnight in a humidified atmosphere set to 37C and 5% CO_2. Outer wells were not included in order to avoid edge effects. Media was replaced the following day with exposure media as previously detailed. Extract medias were replaced every 24h over a 48h exposure period. BrdU reagent was added to the wells and cells were incubated for an additional 24h at 37C and 5%CO_2. BrdU assay was then performed according to the manufacturer's protocol. Absorbance measurements were taken at OD=450nm with the Victor X4 multi-label plate reader (Perkin Elmer). Final

absorbance was calculated by averaging background-corrected group absorbance values (n=6).

ORIS Cell Migration

PDLSCs migration potential was examined via a cell exclusion migratory assay using the ORIS™ Cell Migration Assay Kit (#CMA1.101; Platypus Technologies, Maddison WA). PDLSCs suspended in regular culture media were seeded onto supplied 96-well plates at a density of $5x10^3$ cells/well and incubated overnight in a humidified atmosphere set to 37C and 5% CO_2. Outer wells were not included in order to avoid edge effects. Media was replaced the following day with exposure media as previously detailed. Extract medias were replaced every 24h over a 48h exposure period. To prepare the cell-free growth area, silicone stoppers were removed and wells were washed with HBSS to remove any unattached cells. In order to limit the effects of proliferation, 2% FBS supplemented culture media was added to the wells and migration was observed after 24h incubation.

PDLSC migration potential was determined by calculating wound closure percentage after 24h. To do so, wells were washed once with PBS and fixed in 10% neutral buffered formalin for 10-min. Cell cytoskeleton was subsequently stained with an Alexa Fluor 568 conjugated phalloidin probe (#A12380; Thermo Fisher Scientific) for 30-min. at room temperature. Wells were washed twice with PBS prior to imaging. The ORIS™ Detection Mask was attached to the bottom of the 96-well to capture fluorescent images containing only the area of migration. Images were captured using the EVOS FL Auto Cell Imaging System (ThermoFisher) and ImageJ (NIH) was used for image post processing. Briefly, fluorescent images were opened in ImageJ and the background was corrected using

a 50-pixel rolling ball radius (Process tab, Subtract Background). Fluorescently labeled cells were subsequently isolated from background by setting adjusting the threshold setting to 5-255 with dark background (Image tab, Adjust Threshold). Any remaining holes within the cell bodies were corrected with Fill Holes (Process tab, Binary tab, Fill Holes) and masks of the region were created (Process tab, Binary tab, Convert to Mask). Percent wound closure was then calculated by measuring Area Fraction (Analyze, Set Measurements) of labeled cells to non-labeled wound area (n=6).

Osteogenic Differentiation

Osteogenic differentiation was accomplished with the StemPro Osteogenesis Differentiation Kit (#A1007201; Thermo Fisher Scientific) according to the manufacture's protocol. Osteogenic media (OM) was created by adding the osteogenic supplement to the basal media and supplementing with 1% v/v PenStrep and 0.1% v/v Fungizone. Osteogenic exposure medias (OEMs) were then created by adding extracts directly to OM. OEMs were added on day 0 and replaced every third day. Concentrated extract was added directly to the culture plates, as described previously, on all other days in order to mimic daily exposure. 10nM αBTX pretreatments were also conducted as described previously.

Mineralization

PDLSCs suspended in regular culture media were seeded onto 24-well plates at a density of $30x10^3$ cells/well and incubated in a humidified atmosphere set to 37C and 5% CO_2. Osteogenic differentiation was initiated with OM once wells reached 100% confluence. OMs and extracts were added as previously detailed in osteogenic differentiation section. Mineralization potential was determined after 21d of osteogenic

stimulation through Alizarin Red Sulfate (ARS; #A5533; Sigma) staining. PDLSCs were washed once in PBS (Ca^{2+}/Mg^{2+}-free) and fixed in 10% neutral buffered formalin for 30-min. at room temperature. Wells were then washed once with de-ionized water (DI-H_2O) and stained with 2% w/v ARS staining solution (pH=4.2) for 5-min. at room temperature. The staining solution was removed and wells were washed 5 times with DI H_2O. Wells were photographed with a digital camera and ARS stain was quantified with cetylpyridinum chloride (CPC) according to previously published protocol[151]. Briefly, 10% w/v CPC (#C0732; Sigma) was prepared by dissolving 2g CPC in 20mL of 10mM sodium phosphate (#S7907; Sigma). The mixture was placed in a 60C oven for 10-min. in order to help the CPC dissolve. The solution was later added to the stained wells and the plate was rocked on a shaker for 30-min. at room temperate. If needed, stained plates were placed in 37C incubator for 15-min. to dissolve any remaining stain. The resultant dye-solution was transferred to a 96-well plate and read at 562nm using a SpectraMax M2e microplate reader (Molecular Devices).

Osteogenic Gene Expression

PDLSCs suspended in regular culture media were seeded onto 12-well plates at a density of $50x10^3$ cells/well and incubated in a humidified atmosphere set to 37C and 5% CO_2. Osteogenic differentiation was initiated with OM once wells reached 100% confluence. OMs and extracts were added as previously detailed in osteogenic differentiation section. Osteogenic gene expression was analyzed 7, 14, and 21 days after osteogenic stimulation. Briefly, total RNA was extracted from triplicate samples using TRIzol (#15596026; Thermo Fisher Scientific) and the manufacture's protocol. RNA quality and concentration was determined with the NanoDrop 2000 spectrophotometer

(Thermo Fisher Scientific). Samples were diluted with RNAse/DNAse-free water to concentration of to 100ng/µL and stored in -20C until needed for reverse transcription (RT). A High Capacity cDNA Reverse Transcriptase Kit (#4368814; Thermo Fisher Scientific) and GeneAmp PCR System 2700 (Applied Biosystems) were used to reverse transcribe 1µg of each RNA sample. Resulting cDNA was diluted with 80µL of RNAse/DNAse-free water and stored at -20C until further use.

Osteogenic gene expression was analyzed via real time, quantitative polymerase chain reaction (qPCR) using the MX3005P Real Time PCR machine (Applied Biosystems). qPCR was performed using 20ng of cDNA, SYBR Green PCR Master Mix (#4309155; Thermo Fisher Scientific), and specifically designed primers (Table 3-2). GAPDH was used as the reference gene for normalization.

Table 3-2: qPCR primers used for qPCR gene expression analysis

Gene		Sequence (5'→3')	Tm (C)	Accession #
Osteogenic	ALPL	F: AGGACGCTGGGAAATCTGTG	60	NM_000478.5
		R: CATGAGCTGGTAGGCGATGT		
	COL1A1	F: CGAGGCTCTGAAGGTCCC	60	NM_000088.3
		R: GCAATACCAGGAGCACCATTG		
	COL1A2	F: TCTGCGACACAAGGAGTCTG	60	NM_000089.3
		R: GCTGGGCCCTTTCTTACAGT		
	IBSP	F: CGATTTCCAGTTCAGGGCAGTA	60	NM_004967.3
		R: TCCATAGCCCCAGTGTTGTAGC		
	OCN	F: CCACCGAGACACCATGAGAG	60	NM_199173.5
		R: GCTTGGACACAAAGGCTGC		
	RUNX2	F: CAAGTGCGGTGCAAACTTTC	60	NM_001015051.3
		R: CTGCTTGCAGCCTTAAATGACT		
	SPARC	F: GAGAATGAGAAGCGCCTGGA	60	NM_003118.3
		R: GGAGAGGTACCCGTCAATGG		
	SPP1	F: TGTTGGTGGAGGATGTCTGC	60	NM_001251830.1
		R: AGTTTTCCTTGGTCGGCGTT		
Reference	GAPDH	F: TGTTGCCATCAATGACCCCTT	60	NM_002046.6
		R: CTCCACGACGTACTCAGCG		

Data was analyzed using the $2^{-\Delta\Delta C}{}_T$ method, where $\Delta\Delta C_T$ =

$$(C_{T,\text{Target Gene}} - C_{T,\text{GAPDH}})_{\text{Exposure Group}} - (C_{T,\text{Target}} - C_{T,\text{GAPDH}})_{\text{Control}}$$

The control is represented by the non-exposed, osteogenic differentiation group (i.e. osteogenic media only). The mean $2^{-\Delta\Delta C}{}_T$ was determined from the triplicate samples of each exposure group. The standard deviation (SD) of the mean was calculated by using the sum of the squares method of the triplicate C_T values (i=1-3), where SD =

$$SQRT((STDEV(C_{T,Target\ Gene,i})^2) + (STDEV(C_{T,GAPDH,i})^2))$$

For statistical analysis, ΔC_T values ($C_{T,Target} - C_{T,GAPDH}$) of the separate experimental groups were compared as recommended by Livak et al.[152].

MicroRNA

Changes in PDLSC global miRNA expression as a consequence of ECV exposure was determined via microarray analysis. PDLSCs suspended in regular culture media were seeded onto 6-well plates at a density of $5x10^4$ cells/well and incubated in a humidified atmosphere set to 37C and 5% CO_2. PDLSC exposure to ECV was initiated the following day by replacing regular culture media with media containing 1 μM nicotine ECV extract and returning the plate to the incubator for 24h. As a positive control, media containing 1 μM pure (-)-nicotine liquid (Sigma) was also used. Exposures were conducted for 72h during which media was changed every 24h in order to mimic daily nicotine exposure. Total miRNA was collected after 72h with the mirVana miRNA isolation kit (#AM1560; Thermo Fisher Scientific) according to the manufacturer's protocol. H_2O-eluted miRNA was quantified via NanoDrop spectrophotometer and stored at -20C until further use. GeneChip miRNA 4.0 Arrays (#AM1560; Affymetrix, Santa Clara CA) containing 30,424 mature miRNA probe sets were used to analyze PDLSCs miRNA expression. Expression was normalized against human-positive and negative chip controls using Robust Multi-

Array Average Normalization[153]. Significant miRNA expression over background was determined by detection p-values<0.05.

Microarray output data was used to identify differentially expressed miRNAs as a consequence of ECV exposure. Differential expression was classified as expression fold changes to control greater than +2 and detection p-values<0.05. The top-10 differentially expressed miRNAs were chosen for further analysis. To determine how miRNA expression could affect osteogenic differentiation potential, downstream mRNA targets of each miRNA were predicted with the TargetScan website (http://www.targetscan.org/vert_72/). miRNAs targeting more than 1 osteogenic gene were chosen for further validation in the osteogenic differentiation studies and gene enrichment and gene ontology analysis.

Expression validation of the miRNAs of interest was performed by qPCR using previously isolated RNA from osteogenic gene expression studies of 21d samples. 10ng of total RNA was reverse transcribed with the TaqMan MicroRNA Reverse Transcriptase Kit (#4366596; Thermo Fisher Scientific) and the appropriate TaqMan miRNA RT primers (Thermo Fisher Scientific). The resultant products were quantified by using the appropriate TaqMan miRNA Assays and TaqMan Universal Master Mix II, no UNG (#4440040; Thermo Fisher Scientific) on a Stratagene MX3005P Real-Time PCR Detection System (Agilent Genomics). Results were normalized to U6 expression.

For gene enrichment and gene ontology analysis, miRNA target-gene lists produced by TargetScan website were sorted according to cumulative weighted context score in order to identify the target genes with the greatest predicted repression. The top-3000 genes were then selected for gene enrichment and gene ontology analysis with Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics

Resources version 6.8 (https://david.ncifcrf.gov). Genes were grouped using the lowest stringency and significance was considered as enrichment scores greater than 1.3 and Benjamini corrected P-values<0.05.

CRISPR/Cas9 mediated knockout of α7 nAChR gene

PDLSC α7 nAChR gene knockout was performed with the CRISPR/Cas9 CHRNA7 Human Gene Knockout Kit from OriGene (#KN221382; OriGene Technologies, Rockville MD). 24 hours before transfection, $4x10^4$ PDLSCs in 1mL of transfection media (DMEM + 10% FBS) were seeded into each well of a 12-well plate to obtain a confluence of 50-70% by the following day. On the day of transfection, 2 guide RNA (gRNA) vectors and 1 scramble negative control vector were transfected using jetPRIME® transfection reagents (#114-01; Polyplus-transfection, Illkirch France). Briefly, 0.25µg of each gRNA vector and 0.25µg of the scramble control vector was diluted in three separate tubes containing 100µL of jetPRIME® buffer (Polyplus-transfection). 0.25µg of donor vector was then added to each tube. After vortexing, 1µL of jetPRIME® reagent (1:2 ratio) was added to each vial and the vials were incubated at room temperature for 10-min. The mixtures were added drop-wise to each respective well and the plate was gently rocked to distribute the complex evenly. Cells were incubated at 37C and 5% CO_2 for 4 hours, upon when transfection media was replaced with regular CCM. Cells were passaged 1:10 every three days. After 23 days, knockout efficiency was determined by fluorescent imaging and positive expression of green fluorescent protein (GFP).

Statistical Analysis

All data is presented as mean ± standard deviation. Statistical analysis was conducted with SPSS software (IBM SPSS Statistics 22). An unpaired, two-tailed Student's t-test was used for comparison between two groups. For comparison of three of more groups, a one-way analysis of variance (ANOVA) was used with post hoc Tukey Test. When necessary, a two-way ANOVA was used to understand the interaction between multiple factors. Statistically significant results were considered as $p<0.05$.

Chapter 4 Results

LCMS method for determining nicotine content of collected extracts

A LCMS method was developed to quantify the amount of nicotine in collected extracts so that they could be properly diluted to physiological doses at the time of experimentation. The LCMS method used was based on the original work done by Trehy et al.[150], but included modifications to incorporate different equipment and extraction techniques. Linear calibrations (R^2>0.99) were obtained for the target compound in the range of 2-800nM in all three channels used to monitor for nicotine (Figure 4-1) and the calibration was stable throughout the analytical sequence (deviation <15% from targeted concentration upon continuous calibration checks). After proper dilution, all samples fell within the dynamic range of the machine. Sample confirmation ratios lied within 3 standard deviations of the mean confirmation ratios in standards and quality control samples, thus confirming the identity of nicotine in the collected extract. Internal standard signal was constant throughout the analytical batch, thereby allowing for accurate quantification of nicotine in samples. Carryover between sample measurements was considered under control. Injection of a blank after the highest calibration standard produced a signal at or below 0.1% of that of the standard and a measurement below LOQ=2 nM which is the lowest calibration standard of this dataset and is assigned as instrumental limit of detection. Carryover tests performed after every calibration check were also negative.

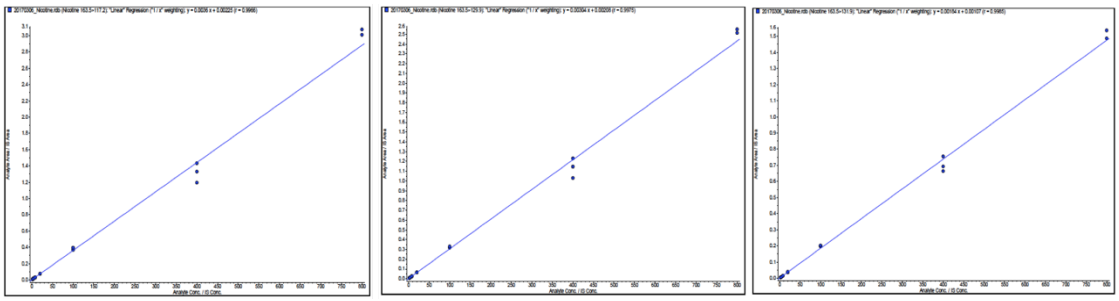

Figure 4-1: Calibration curves from all three channels used to monitor nicotine standards during LCMS. Calibration curves were linear with R^2>0.99 across all three channels.

In order to determine extract batch nicotine content, samples of collected extracts were analyzed using the developed LCMS method. Nicotine content varied dramatically between collected extracts (Table 4-1). A one-way ANOVA demonstrated that there was a statistically significant difference between group means. Extracts collected from 36mg/ml nicotine e-liquid vapors (ECV+) had the highest measured nicotine content at 248.7±6.4μM, followed by cigarette smoke extract (CSE) at 46.3±2.9μM and 0mg/ml nicotine e-liquid vapors (ECV-) at 2.9±0.1μM. Although trace amounts of nicotine were measured in the ECV- extracts, no nicotine was measured in the 0mg/ml nicotine e-liquid stock. Since the same machine was used to produce both 0 and 36mg/ml e-cigarette vapors, the small contamination of nicotine is likely due to residual nicotine in machine tubing that was left behind during the initial collection of the 36mg/ml nicotine e-cigarette vapors.

Table 4-1: Average nicotine content of collected e-cigarette vapor and cigarette smoke extracts as determined by LCMS.

Sample	Measured Nicotine (nM)	Average Nicotine (nM)	SD (nM)	Sample LLOQ	Sample ULOQ
ECV-_1	3,030				
ECV-_2	2,850	2,910[a,b]	±104	25	8,000
ECV-_3	2,850				
0mg/ml e-liq.	No Peak	-	-	5	1,600
CSE_1	44,300				
CSE_2	49,600	46,300[b,c]	±2879	250	80,000
CSE_3	45,000				
ECV+_1	256,000				
ECV+_2	244,000	248,666[a,c]	±6429	2,500	800,000
ECV+_3	246,000				

Legend: ECV-: 0mg/ml nicotine e-liquid extract; CSE: Cigarette smoke extract; ECV+: 36mg/ml nicotine e-liquid extract. SD: Standard Deviation; LLOQ: Lower Limit of Quantification; ULOQ: Upper Limit of Quantitation.
Statistics: Statistical analysis calculated by one-way ANOVA. Significance considered as $p<0.005$. Significance Symbols: (a) to CSE; (b) ECV+; (c) to ECV-.

For experimentation, extracts were diluted to physiological doses of nicotine (Table 4-2). 1 and 10μM nicotine concentrations were chosen as they encompass the range of previously reported values of salivary nicotine content in smokers and vapers. Extract from 0mg/ml nicotine was diluted in the same fashion as 36mg/ml extracts in order to isolate the effects of nicotine and study the effects of non-nicotine e-liquid compounds. The dilution percentages reflect the percent of stock solution in the respective concentrations.

Table 4-2: Extract stock dilution percentages corresponding to 1 and 10μM nicotine concentrations.

Extract	Extract Stock Conc. (μM)	Dilution	
		10μM	1μM
ECV+ (36mg/ml)	248.66	4.0%	0.4%
ECV- (0mg/ml)	2.91	4.0%	0.4%
CSE (3R4F)	46.30	23%	2.3%

PDLSCs remain viable after exposure to 1 and 10μM nicotine e-cigarette vapor extracts

The cytotoxicity of cigarette smoke and e-cigarette vapor (ECV) extracts were evaluated in order to ensure that exposed cells would remain viable during

experimentation. Based on the results of the LCMS, stock extracts were diluted with regular culture media to obtain 10 and 1μM nicotine supplemented media. Non-nicotine containing media was diluted like nicotine containing media in order to evaluate the effects of non-nicotine chemicals. PDLSCs were cultured in this media for 24h and viability was evaluated after with Calcein-AM. After 24h, PDLSCs remained viable and there were no detectable differences in viability between extract types (ECV- vs. ECV+ vs. CSE) and extract dose (1 vs. 10μM nicotine or 0.4 vs. 4% for ECV-) (Figure 4-2). As a reference, viable cells were not present in positive controls for 100% cell death. Experimental results obtained with these extract dilutions will therefore not be influenced by cell death.

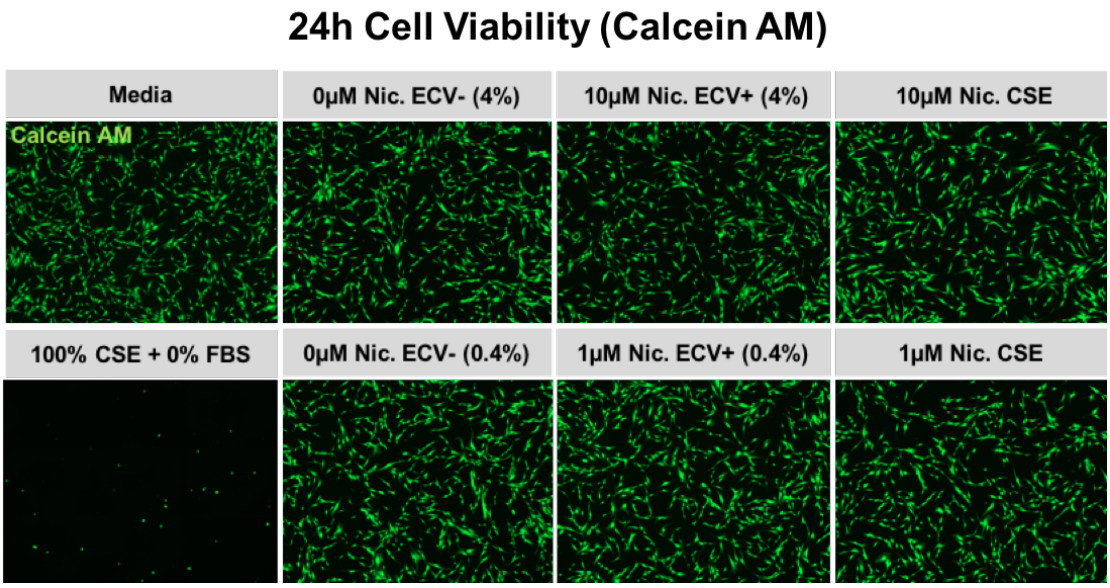

Figure 4-2: Calcein AM fluorescent images showing PDLSC viability after 24h culture in medias supplemented with nicotine-free ECV and 1 and 10μM nicotine ECV extract. No detectable differences in viability were noted between exposure groups except for positive control where no viable cells were present.

Nicotine-containing e-cigarette vapor extracts increase PDLSC intracellular Ca^{2+} levels upon stimulation

One of the pathways for nicotine interaction with cells occurs through α7 nAChRs. PDLSCs have been previously shown to express these receptors and nicotine alone has

been previously shown to activate them[15; 120]. However, it remained to be seen whether nicotine and non-nicotine containing ECV extracts elicited a similar response.

The presence of α7 nAChR subunits in PDLSCs was confirmed via immunohistochemistry (Figure 4-3). The ubiquitous expression of these receptors in all of the PDLSCs imaged suggests an important role in PDLSC biology. One of the hallmarks of α7 nAChR activation is the influx of extracellular calcium[117]. To test receptor functionality and response to cigarette smoke extract and ECV extract, PDSLCs were loaded with Fluo4-AM, a calcium-binding dye, and stimulated with varying doses of collected extract. Extract was added directly to the culture media 5s after initiating the time-lapse imaging sequence and fluorescent images were captured over a 2-min. period. As expected, increases in PDLSC fluorescence were only observed under nicotine stimulation (Figure 4-4). Cigarette smoke extract and ECV extracts diluted to 1 and 10μM nicotine caused rapid increases in PDLSC fluorescence after extract addition. Addition of nicotine-free ECV extracts, on the other hand, failed to cause a detectable increase in Fluo4 signal beyond baseline value as did addition of regular culture media. Therefore, although LCMS analysis detected trace amounts of nicotine in the collected stock of 0mg/ml vapor extract, the final diluted solutions used throughout these experiments did not contain enough nicotine to activate PDLSC α7 nAChRs. Thus, the potential contribution of nicotine to effects in these groups is negligible, and any effects seen in the 0mg/ml ECV groups can be predominantly attributed to the other components in the vapor.

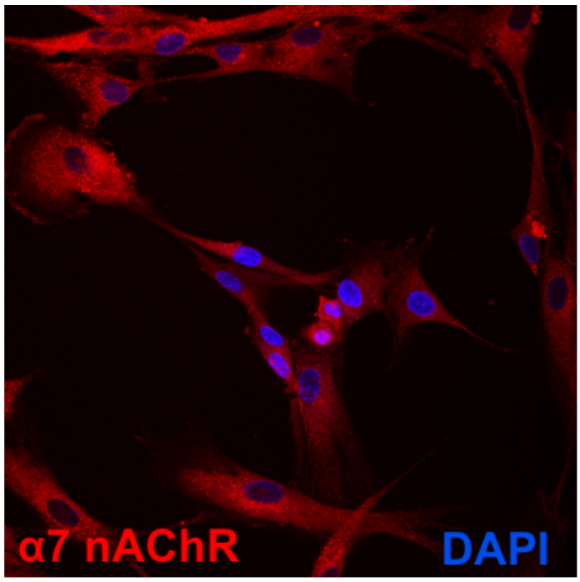

Figure 4-3: Immunohistochemistry image of PDLSCs showing the ubiquitous expression of α7 nAChRs (red) and their location in relation to the nucleus (blue).

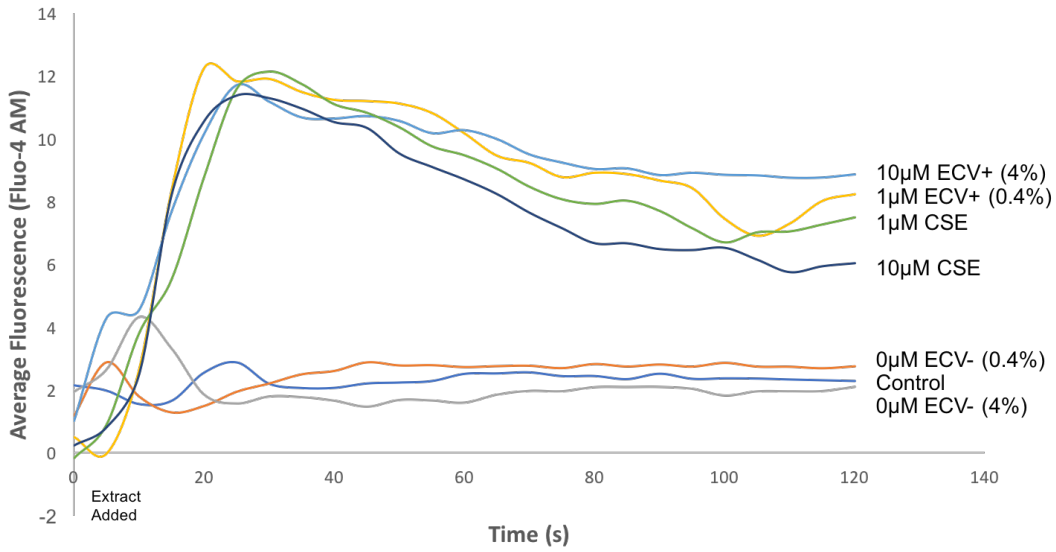

Figure 4-4: Average PDLSC fluorescence (Fluo-4 AM) over time in response to the addition of nicotine-free ECV, 1 and 10μM nicotine ECV, and cigarette smoke extracts to cell culture. Cultured PDLSCs loaded with Fluo4-AM and maintained in HBSS (Ca2+) responded to the addition of nicotine-containing extracts as evident by the rapid rise in intracellular Ca^{2+}. Nicotine-free ECV extracts and regular cell culture media did not elicit a detectable response above background.
Data presented as mean background-corrected fluorescence of 12 random PDLSCs (n=12).

E-cigarette vapors inhibit PDLSC proliferation in a dose-dependent manner through α7 nAChRs

One of the hallmark characteristics of stem cells is the ability to undergo controlled self-renewal. This process is critical for preserving undifferentiated stem cell populations

throughout life as they are often called upon in abundance to mediate wound healing and tissue regeneration. Stem cell proliferation is therefore a critical component of regenerative capability. The effect of ECV extract exposure on PDLSC proliferation potential was determined with a BrdU-incorporation assay. The exposure conditions for the assay were established by first measuring PDLSC proliferation under low and high serum concentrations (Figure 4-5). A 48h exposure followed by a 24h BrdU-incorporation period was sufficient enough to measure statistically significant differences in absorbance means between 1% and 10% FBS treated PDLSCs ($p<0.005$).

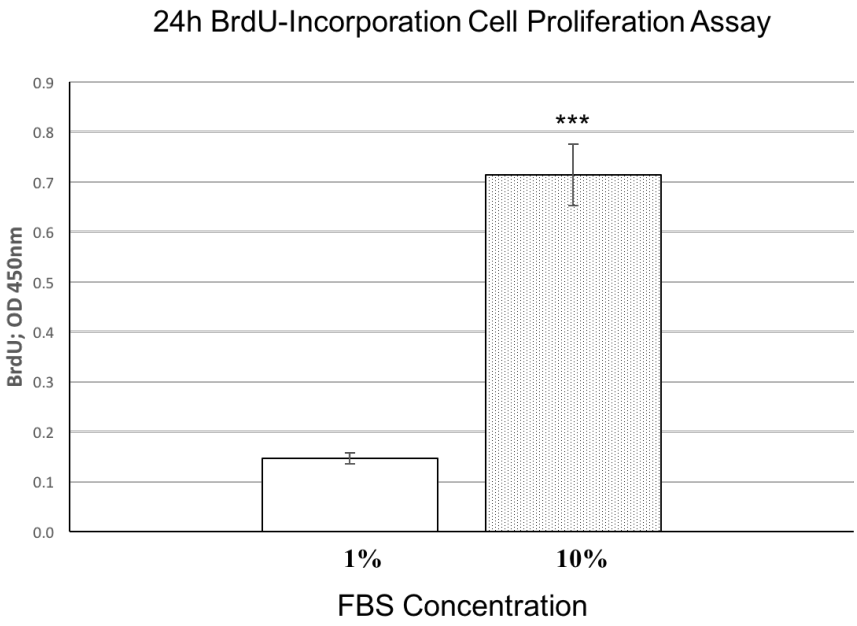

Figure 4-5: 24h BrdU-incorporation cell proliferation assay comparing PDLSC proliferation in 1% and 10% FBS-supplemented medias.
Data presented as mean ± SD of absorbance (n=4). Statistical analysis calculated by two-tailed unpaired Students's t-test. Significance considered as *p<0.05, **p<0.005, ***p<0.0005. Significance Symbols: (*) to control.

To determine how ECVs affect proliferation, PDLSCs were exposed to low and high doses of nicotine-free and nicotine containing ECVs (Figure 4-6). Only 1μM nicotine CSE was capable of significantly affecting normal rates of PDLSC proliferation as determined by a one-way ANOVA on BrdU absorbance results (control: 0.714 ± 0.031 vs.

CSE 0.826 ± 0.021, p=0.008). The observed 15% increase in proliferation was also significantly greater than that of PDLSCs exposed to 1μM nicotine ECV+ (0.826 ± 0.021 vs. 0.717 ± 0.045, p=0.01) suggesting that non-nicotine components in CSE are responsible for the increase in PDLSC proliferation.

Although there were no apparent differences in proliferation between control and ECV-exposed PDLSCs, there did appear to be an effect within ECV groups. A two-way ANOVA (pairwise comparison) showed a statistically significant difference in BrdU uptake between nicotine-free and nicotine-containing ECVs (0.780 ± 0.12 vs. 0.688 ± 0.013, p=2.63E-4) and between low and high doses of extract (0.755 ± 0.012 vs. 0.713 ± 0.013, p=0.034). The results suggest that the effect on PDLSC proliferation between ECV groups is dependent on both extract-type and extract-dose. The Partial Eta Squared values of 0.716 for extract-type and 0.347 for extract-dose further suggest that the majority of the observed effect (~72%) is due to extract type or, rather, whether or not nicotine is present in ECVs.

In fact, pairwise comparisons within extract-types (i.e. ECV- vs. ECV+) revealed a significant difference in proliferation amongst nicotine-containing ECVs (i.e. 0.4% vs. 4% ECV+) (p=0.046). The results confirm the notion that nicotinic effects on PDLSC proliferation are dose-dependent, with higher concentrations being more inhibitory and thus more detrimental. Within extract dilution groups (i.e. 0.4% and 4%) significant differences in proliferation were detected between nicotine-free and nicotine-containing ECVs at low and high extract concentrations (p=0.008 and p=0.002, respectively). The data suggests that nicotinic e-liquids are therefore more detrimental to PDLSC proliferation than non-nicotinic e-liquids.

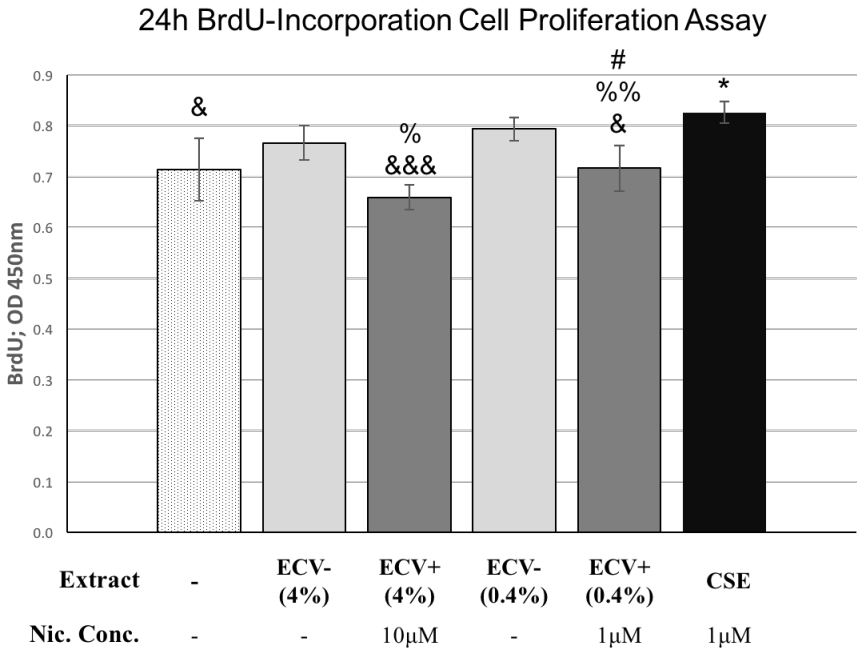

Figure 4-6: 24h BrdU-incorporation cell proliferation assay comparing PDLSC proliferation in regular culture media to nicotine-free ECV and 1 and 10μM nicotine ECV extract-supplemented medias.
Data presented as mean ± SD of absorbance (n=4). Statistical analysis calculated by one-way ANOVA with post hoc Tukey Test. Significance considered as *p<0.05, **p<0.005, ***p<0.0005. Significance Symbols: (*) to control; (%) to respective ECV-; (#) to respective 4% extracts; (&) to 1μM CSE.

The significant difference in proliferation potential between nicotine-free and nicotine-containing ECVs at both extract concentrations shows the potent effect of nicotine on PDLSC proliferation. To determine if nicotine-containing ECVs inhibit PDLSC proliferation through α7 nAChRs, PDSLCS were pretreated with αBTX, an α7-specific nAChR antagonist, prior to ECV exposure. A one-way ANOVA detected significant differences between ECV- and ECV+ exposed PDLSCs (p=0.034) and between ECV+ and ECV+ PDLSCs pretreated with αBTX (p=0.034) (Figure 4-7). Additionally, there was no significant difference between ECV- and ECV+ PDLSCs pretreated with αBTX (p=0.149). Taken together, the data confirms that nicotine-containing ECVs are capable of inhibiting PDLSC proliferation potential through α7 nAChRs.

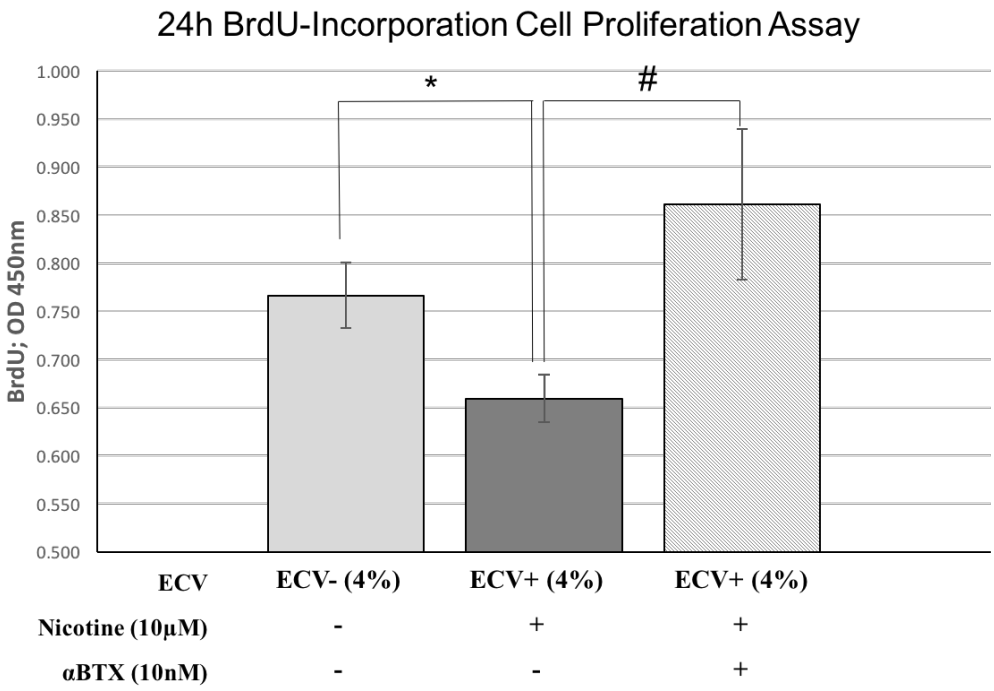

Figure 4-7: 24h BrdU-incorporation cell proliferation assay comparing PDLSC proliferation in nicotine-free ECV (4%) and 10µM nicotine ECV (4%) extract-supplemented medias with and without αBTX pretreatment. Data presented as mean ± SD of absorbance (n=4). Statistical analysis calculated by one-way ANOVA with post hoc Tukey Test. Significance considered as *p<0.05. Significance Symbols: (*) to control; (#) to 10µM ECV+.

E-cigarette vapor extracts appear to inhibit PDLSC migration potential

Stem cells are also capable of migration, an inherent characteristic that is critical for efficient wound repair. The effect of ECVs on PDLSC migration potential was evaluated with a cell exclusion assay. PDLSCs were exposed to ECVs for 48 hours and then allowed to migrate into a cell-free zone. Overall, exposure to ECV extracts appeared to inhibit PDLSC migration (Figure 4-8). Exposure to 1µM nicotine CSE also produced similar effects. However, a one-way ANOVA failed to detect any differences in migratory ability between groups. Therefore, it appears that acute exposure to neither 1 nor 10µM nicotine ECVs has a significant impact on PDLSC migration.

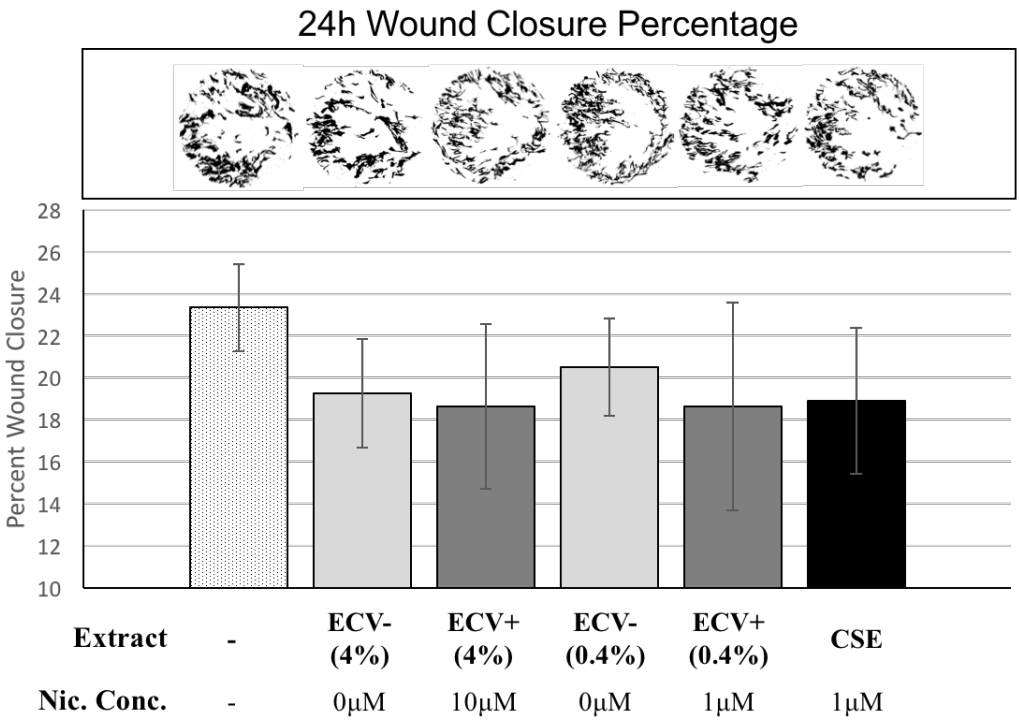

Figure 4-8: 24h wound closure assay showing the effects of ECV exposure on PDLSC migration potential. (A) PDLSCs were stained with phalloidin and fluorescent images of the exclusion area were taken after 24h of migration. (B) Percentage of wound area covered by migrating cells after 24h.
Data presented as mean ± SD of percent wound closure (n=6). Statistical analysis calculated by one-way ANOVA with post hoc Tukey Test. Significance considered as *p<0.05.

E-cigarette vapors inhibit PDLSC mineralization proliferation in a dose-dependent manner

PDLSCs are multipotent stem cells with a high capacity of osteogenic differentiation. Successful osteogenic differentiation results in tissue mineralization, which provides the necessary components for bone strength. To determine mineralization potential, PDLSCs were cultured in osteogenic media for 21d and calcium deposition was measured thereafter via Alizarin Red S staining. PDLSCs cultured in osteogenic medias exhibited profound calcium deposition at 21d (Figure 4-9). Mineralization did not occur in non-differentiated controls leading to a significant difference in Alizarin Red S staining between groups (p<5E-4). The outcomes of this assay prove the effectiveness of the

osteogenic differentiation protocol and demonstrate the tremendous mineralization potential of PDLSCs.

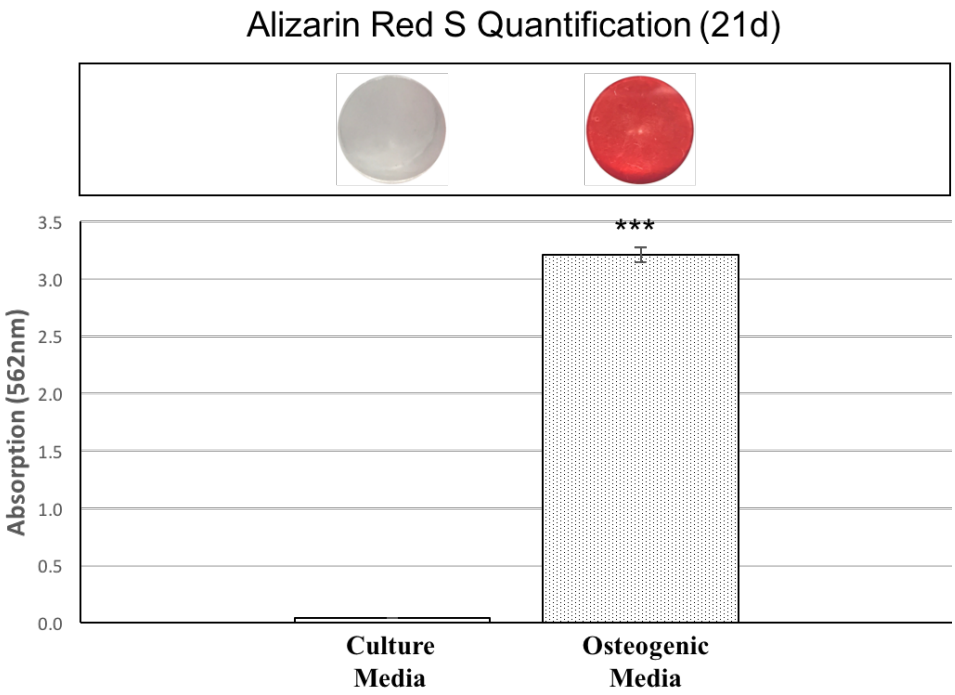

Figure 4-9: Alizarin Red S staining showing mineralization potential of PDLSCs after 21d of osteogenic differentiation. (A) Representative wells of PDLSCs stained with Alizarin Red S. (B) Alizarin Red S stain was dissolved with 10% CPC and the absorbance of each well was read at 562nm.
Data presented as mean ± SD of absorption at 562nm (n=4). A two-tailed unpaired Students's t-test was used for statistical analysis. Significance considered as *p<0.05, **p<0.005, ***p<0.0005. Significance Symbols: (*) to non-differentiated control.

To determine the effect of ECV on PDLSC osteogenic differentiation potential, osteogenic medias were supplemented with ECVs and PDLSC mineralization was evaluated after 21d of differentiation. A one-way ANOVA was performed to compare differences in Alizarin Red S (ARS) staining between non-exposed and exposed PDLSCs. Significant differences in alizarin red s staining between control and ECV exposed groups were only observed at concentrated doses (4%) of ECV extract (Figure 4-10). At this concentration, ARS staining was reduced by 25% in ECV- groups (p=3.1E-4) and 31% in ECV+ groups (p=2E-5).

A two-way ANOVA was conducted in order to understand the interaction between extract type and dose on ARS staining of exposed PDLSCs. For ECV groups, there was no detectable difference in ARS staining between extract type (p=0.616), but a significant difference between extract dose (0.4%: 2.929 ± 0.251 vs. 4%: 2.292 ± 0.146, p=5.2E-5). These results suggest that ECVs inhibit PDLSC mineralization predominantly through exposure to non-nicotine chemicals in a manner that is highly dose-dependent. In fact, on Tests of Between-Subject Effects, extract dose had a Partial Eta Squared value of 0.757, indicating that dose was indeed responsible for the majority (~75%) of the observed effect on mineralization.

CSE, on the other hand, significantly inhibited PDLSC mineralization at both low (3.208 ± 0.065 vs. 2.300 ± 0.336, p=9E-5) and high (control 3.208 ± 0.065 vs. 2.070 ± 0.157, p=4E-6) concentrations of extract. Pairwise comparisons to CSE confirmed a significant difference in mineralization between CSE and ECV extracts (vs. ECV-: p=0.001, vs. ECV+: p=0.003). The mean absorbance of CSE groups was significantly lower than either that of the ECV extract groups (CSE: 2.185 ± 0.272 vs. ECV-: 2.639 ± 0.364 vs. ECV+: 2.581 ± 0.427) suggesting that exposure to cigarette smoke is more detrimental to PDLSC mineralization than e-cigarette vapor.

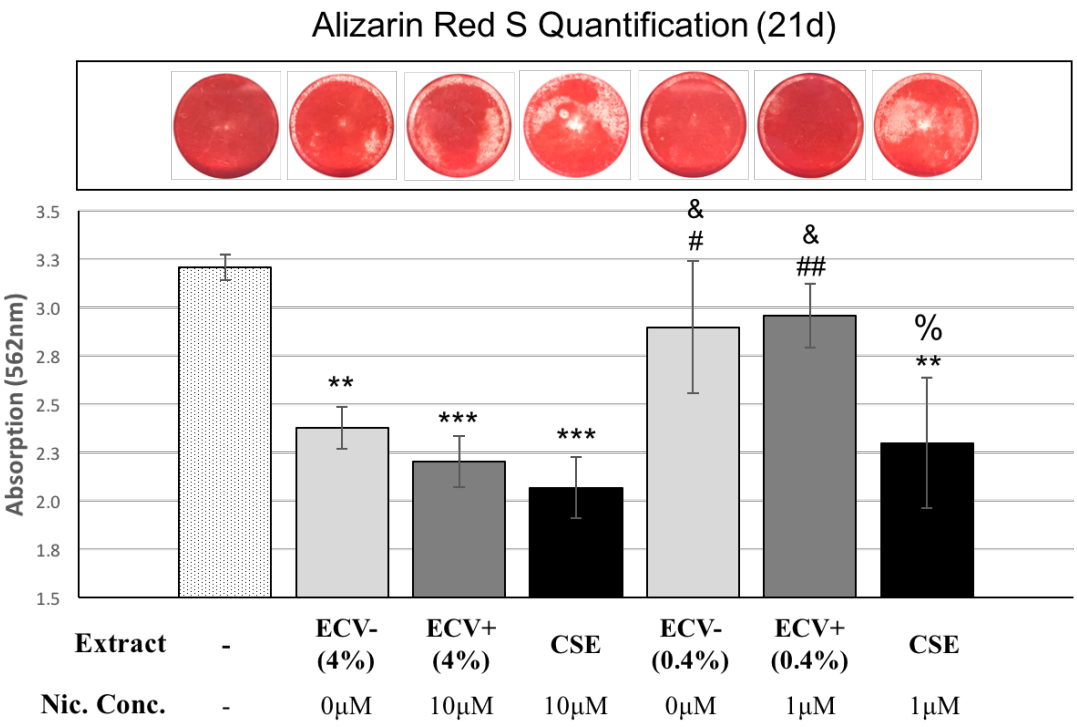

Figure 4-10: Alizarin Red S staining showing the effect of ECVs on mineralization potential of PDLSCs after 21d of osteogenic differentiation. (A) Representative wells of PDLSCs stained with Alizarin Red S. (B) Alizarin Red S stain was dissolved with 10% CPC and the absorbance of each well was read at 562nm.

Data presented as mean ± SD of absorption at 562nm (n=4). Statistical analysis calculated by one way analysis of variance (ANOVA). Significance considered as *p<0.05, **p<0.005, ***p<0.0005. Significance Symbols: (*) to control; (%) to respective ECV-; (&) to respective CSE; (#) to respective 4% extracts.

E-cigarette vapor delays characteristic osteo-gene expression of PDLSCs during osteogenic differentiation

Osteogenic differentiation is a multi-step process (i.e. proliferation, matrix maturation, and mineralization) characterized by specific patterns of osteogenic gene expression. For instance, early differentiation is characterized by elevated levels of transcription factors like RUNX2, which induce osteogenesis[154]. Late differentiation is characterized by decreased levels of mineralization inhibitors like Osteopontin (SPP1) and elevated levels of osteoblast-specific genes like bone sialoprotein (IBSP). Stem cell maturity markers like alkaline phosphatase (ALP) also decreased during differentiation[155]. To ensure that the differentiation protocol used modeled the proper

osteogenesis timeline, the osteogenic gene expression of non-differentiated and differentiated PDLSCs was compared over the three-week differentiation protocol (Figure 4-11).

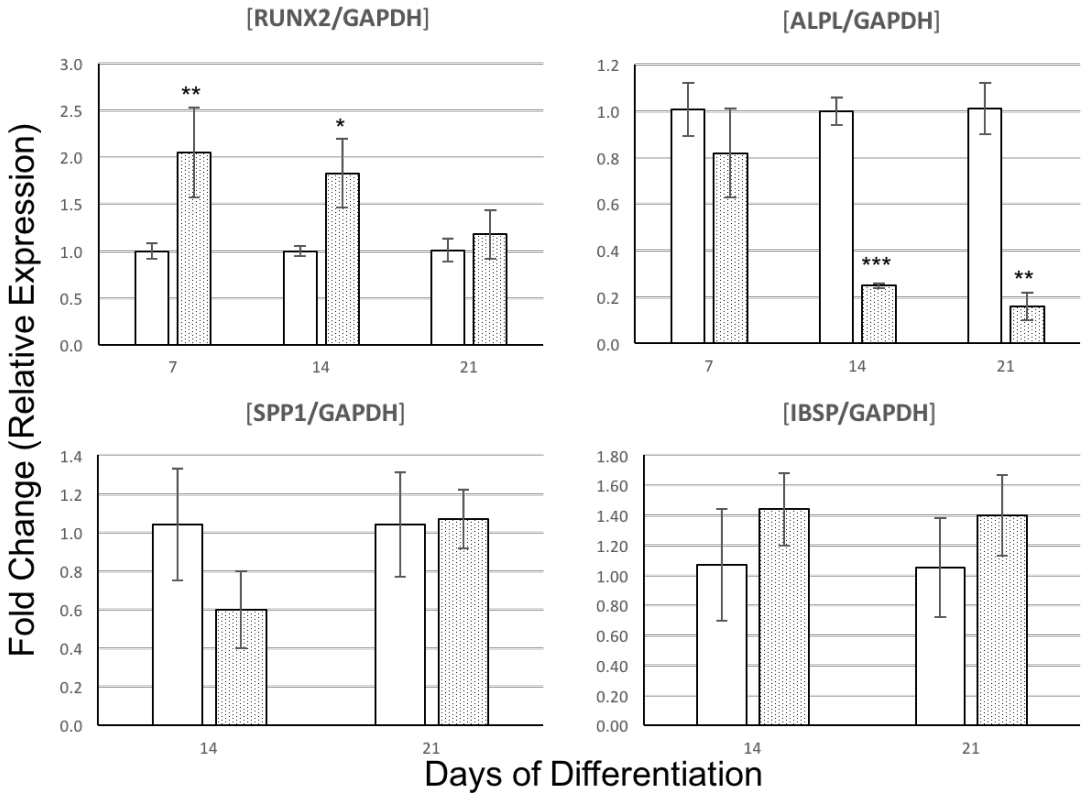

Figure 4-11: Gene expression analysis of PDLSCs during osteogenic differentiation showing differences between non-differentiated (white bars) and osteogenic differentiated (speckled bars) PDLSCs.
Data presented as mean ± SD fold change in mRNA expression relative to control (n=3). GAPDH was used as reference gene. A two-tailed Students T-Test was used for statistical analysis. Significance considered as *=$p<0.05$, **=$p<0.005$.

PDLSCs exposed to osteogenic media showed characteristic early- and late-stage osteogenic gene expression profiles. Expression of the RUNX2 gene, a key transcription factor required for early osteogenic differentiation, was significantly higher in differentiating PDLSCs on days 7 ($p<0.005$) and 14 ($p<0.05$). RUNX2 expression decreased over time, as is necessary for bone maturation. The expression of ALPL also followed a similar trend to RUNX2 expression. ALPL is a widely accepted marker of

undifferentiated stem cells[155]; therefore, the persistent level of expression through 21d in controls indicates non-differentiation, while the gradual decrease in osteogenic groups indicates differentiation and maturation. SPP1 and IBSP are genes associated with mineralization and late stage bone formation. SPP1 is a potential mineralization inhibitor[156], while IBSP is a major component of the bone matrix[157]. Compared to non-mineralizing controls, differentiating PDLSCs expressed lower levels of SPP1 and higher levels of IBSP at day 14, which, according to the osteogenesis time-line, coincides with early stage mineralization. SPP1 expression returned to baseline by day 21, while IBSP expression remained elevated in differentiating PDLSCs.

To determine the effect of ECV extract exposure on PDLSC osteogenic differentiation potential, PDLSCs were differentiated in osteogenic media supplemented with nicotine-free ECV extract (4%) or 10μM nicotine ECV extract (4%) and the expression of osteogenic genes were monitored for 21d. The relative fold changes in osteogenic gene expression compared to differentiated controls are displayed in Figure 4-12. Significant differences in expression were noted between nicotine-free and 10μM nicotine ECV extract exposure groups during middle- and late-stage osteogenic differentiation. 10μM nicotine ECV extract significantly inhibited COL1a1, COL1a2 ($p<0.05$) and IBSP ($p<0.05$) gene expression in PDLSCs at day 14. These decreases in expression during the early stages of mineralization suggest a possible delay in the osteogenic differentiation process. Such a delay would explain why maximal expression levels for these osteogenic genes occurred on day 21 instead of day 14, as they did for their nicotine-free counterparts. The significant difference in gene expression between these two groups could also be related to the differences seen in 21d mineralization potential.

Although no significant differences in expression were observed, comparing the expression of the other osteogenic genes seems to reveal additional signs of osteogenic delay. For example, day 7 RUNX2 gene expression was 30% higher in PDLSCs treated 10μM nicotine ECV extract than in non-exposed and nicotine-free ECV extract groups. RUNX2 gene expression typically peaks during the first few days of osteogenic differentiation, so the fact that PDLSCs are still expressing high levels of RUNX2 at this day suggests that 10μM nicotine ECV extract is causing a delayed shift in the RUNX2 expression timeline and, therefore, a delayed shift in osteogenesis. Delayed osteogenesis in these groups is also supported by a relatively higher level of ALPL gene expression at day 7, which indicates a more immature stem cell phenotype. Expression of osteoblast-specific markers like BGLAP, an indication of late-stage osteogenic differentiation and more mature bone formation, are also 20% lower in PDLSCs treated 10μM nicotine ECV extract than in non-exposed PDLSCs.

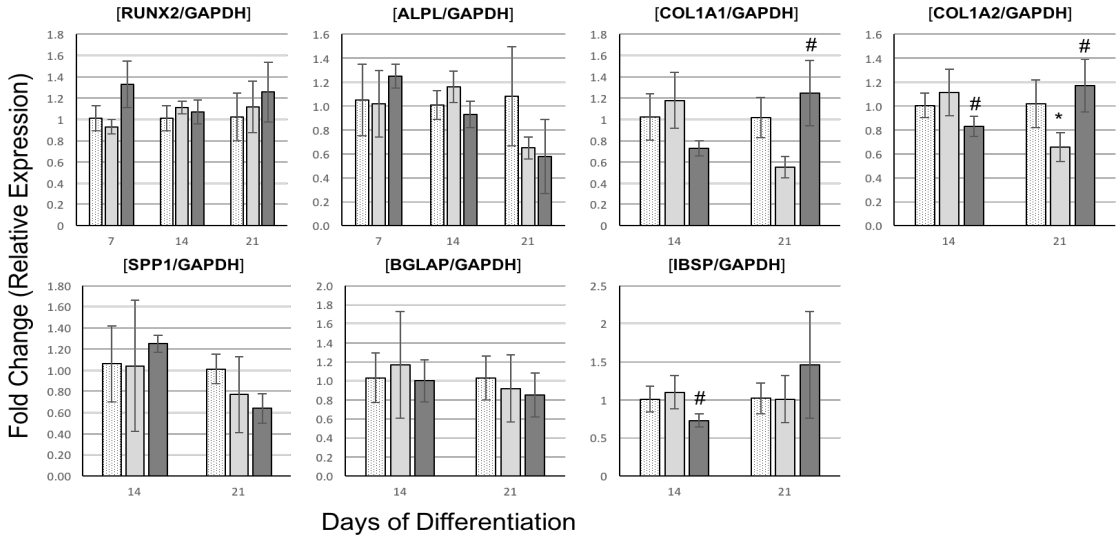

Figure 4-12: Gene expression analysis comparing the expression of osteogenic genes in non-differentiated PDLSCs (speckled bars), PDLSCs treated with nicotine-free ECV extract (4%) (light gray bar), and PDLSCs treated with 10μM nicotine ECV extract (4%) (dark gray bar).
Data presented as mean ± SD fold change in mRNA expression relative to control (n=3). GAPDH was used as reference gene. Statistical analysis calculated by one-way ANOVA. Significance considered as *=p<0.05, **=p=<0.005. Significance Symbols: (*) to control; (#) to 4% ECV.

E-cigarette vapor alters PDLSC global miRNA expression profiles

To determine whether ECVs alter PDLSC miRNA expression, the global miRNA expression profile of PDLSCs exposed to 1μM nicotine ECV extract in-vitro for 4 days was analyzed using microarrays. In total, 141 miRNAs, or roughly 5.5% of total chip-miRNA, were differentially upregulated (i.e., fold change > 2, detection p-value<0.05) between non-exposed and 1μM nicotine ECV extract exposed PDLSCs (Table 4-3), indicating that acute, low dose exposure to ECV extract is capable of altering PDLSC miRNA expression profiles. The increased number of upregulated miRNAs, although small, could result in drastic post-translational modification since every miRNA has several mRNA targets.

The effects of 1μM nicotine ECV extract on PDLSC miRNA expression were compared to those from 1μM (-)-nicotine liquid in order to highlight the effects of nicotine heating and non-nicotine ECV extract components on PDLSC miRNA expression. In a previous study, 1μM (-)-nicotine liquid was shown to differentially express 225 PDLSC miRNA (Ng et al. 2013). The top-10 differentially expressed miRNA identified in that study served as the basis of comparison for the following analysis (Table 4-4). The two studies showed some similar trends in miRNA expression. 70% of the miRNA (i.e., *hsa-miR-1914-3p, -374b-5p, -1973, -505-3p, 30d-5p, 18b-5p,* and *-137*) were upregulated in 1μM nicotine and 1μM nicotine ECV extract groups. *hsa-miR-505-3p, hsa-miR-30d-5p, hsa-miR-18b-5p,* and *hsa-miR-137* were differentially expressed in both groups and in both studies suggesting that these miRNAs are very likely upregulated in response to nicotine exposure. The relative expression of these miRNA depended on whether PDLSCs were treated with nicotine liquid or nicotine ECV extract. The expression of *hsa-miR-505-3p,*

Table 4-3: Differentially upregulated miRNA in PDLSC exposed to 1μM nicotine ECV for 4d. Differential upregulation classified as fold change (to non-exposed control) > 2 and chip miRNA-detection p < 0.05.

Transcript ID	Fold	p	Transcript ID	Fold	p	Transcript ID	Fold	p
miR-411-3p	+223.5	0	let-7g-5p	+4.4	0	miR-455-5p	+2.6	0
miR-342-5p	+100.0	0	miR-3135b	+4.3	0	miR-3615	+2.6	.02
miR-550a-3p	+89.0	0	miR-5195-3p	+4.3	0	miR-1226-3p	+2.6	.02
miR-29b-2-5p	+74.0	.01	miR-671-3p	+4.2	0	miR-30c-2-3p	+2.6	0
miR-148b-3p	+55.5	0	miR-505-3p	+4.2	0	miR-30e-5p	+2.6	0
miR-495-3p	+24.2	0	miR-106b-3p	+4.2	0	miR-27a-5p	+2.5	0
miR-15b-5p	+18.1	0	miR-29b-1-5p	+4.2	0	miR-4684-3p	+2.5	.01
miR-1296-5p	+16.4	0	miR-3937	+4.1	.01	miR-4286	+2.5	0
miR-30b-5p	+14.6	0	miR-18b-5p	+4.1	0	miR-484	+2.5	0
miR-20b-5p	+12.5	0	miR-539-5p	+4.0	0	miR-3200-3p	+2.5	.01
miR-140-5p	+11.5	0	miR-421	+4.0	0	miR-34b-3p	+2.5	.01
miR-195-5p	+11.1	0	miR-4800-3p	+4.0	0	miR-3192-5p	+2.4	.05
let-7f-5p	+10.4	0	miR-4442	+4.0	.02	miR-6819-5p	+2.4	0
miR-493-5p	+9.9	0	miR-532-3p	+4.0	0	miR-15a-5p	+2.4	0
miR-7162-3p	+9.5	0	miR-3609	+4.0	0	miR-1185-2-3p	+2.4	0
miR-146b-5p	+9.4	0	miR-412-5p	+3.9	0	miR-222-5p	+2.4	0
miR-299-5p	+8.9	0	miR-331-3p	+3.8	0	miR-10a-5p	+2.4	0
miR-151b	+8.6	0	miR-6831-5p	+3.7	0	miR-654-5p	+2.3	0
miR-411-5p	+7.8	0	miR-379-3p	+3.7	.03	miR-370-5p	+2.3	.03
miR-328-3p	+7.8	.01	miR-154-3p	+3.6	0	miR-23b-5p	+2.3	0
miR-7153-3p	+7.4	.03	miR-323a-3p	+3.5	0	miR-628-3p	+2.3	0
miR-1290	+7.2	0	miR-18a-5p	+3.5	0	miR-4304	+2.3	.01
miR-625-5p	+7.1	0	miR-212-5p	+3.5	.05	miR-342-3p	+2.3	0
miR-543	+6.8	0	miR-5004-5p	+3.3	.03	miR-18a-3p	+2.3	.01
miR-3117-3p	+6.7	0	miR-3909	+3.3	.02	miR-381-5p	+2.2	.01
miR-194-5p	+6.5	0	miR-1208	+3.3	.01	miR-652-3p	+2.2	0
miR-654-3p	+6.4	0	miR-376c-3p	+3.2	0	miR-296-3p	+2.2	.01
miR-1185-1-3p	+6.1	0	miR-299-3p	+3.2	0	miR-25-3p	+2.2	0
miR-22-5p	+6.0	0	miR-30d-5p	+3.2	0	miR-30a-3p	+2.2	0
miR-221-5p	+6.0	0	miR-505-5p	+3.2	0	miR-4478	+2.1	.03
miR-199b-5p	+5.9	0	miR-4736	+3.1	.01	miR-2110	+2.1	.01
miR-4669	+5.9	0	miR-34c-5p	+3.1	0	miR-629-3p	+2.1	0
miR-1973	+5.7	0	miR-1287-5p	+3.1	.01	miR-629-5p	+2.1	0
miR-4328	+5.5	.04	miR-4441	+3.0	0	miR-424-3p	+2.1	0
miR-181d-5p	+5.4	0	miR-501-3p	+3.0	0	miR-212-3p	+2.1	0
miR-5089-5p	+5.3	.05	miR-24-2-5p	+3.0	0	miR-146a-5p	+2.1	0
miR-30e-3p	+5.1	0	miR-31-3p	+2.9	0	miR-6076	+2.1	.01
miR-128-3p	+5.1	0	miR-550a-3-5p	+2.9	.01	miR-30b-3p	+2.0	.03
miR-487a-3p	+4.9	0	miR-92b-3p	+2.9	0	miR-497-5p	+2.0	.01
miR-3911	+4.9	0	miR-339-5p	+2.9	0	miR-500a-3p	+2.0	0
miR-6872-5p	+4.9	.04	miR-181c-5p	+2.8	0	miR-574-3p	+2.0	0
miR-8060	+4.9	.02	miR-92a-1-5p	+2.7	.02	miR-487b-3p	+2.0	0
miR-30c-5p	+4.8	0	miR-6075	+2.7	.01	miR-409-5p	+2.0	0
miR-376a-3p	+4.7	0	miR-4324	+2.7	0	miR-3926	+2.0	.05
miR-485-3p	+4.7	0	let-7e-3p	+2.7	0	miR-23c	+2.0	0
miR-329-3p	+4.6	0	miR-708-5p	+2.7	0			
miR-21-5p	+4.5	0	miR-29b-3p	+2.7	0			
miR-197-3p	+4.4	0	miR-378f	+2.6	0			

hsa-miR-30d-5p, hsa-miR-18b-5p was 70%, 14% and 58% higher, respectively, and the expression of *hsa-miR-137* was 36% lower in ECV extract treated PDLSCs compared to nicotine liquid treated PDLCs. Additional factors specific to ECV extract, like carrier chemicals and the formation of toxic aldehydes during e-liquid vaporization, could contribute to the observed differences in miRNA expression.

Table 4-4: Table comparing the effect of 1μM nicotine liquid vs. 1μM nicotine ECV on PDLSC miRNA expression with previously published list of top-10 differentially expressed miRNA in PDLSCs treated with 1μM nicotine liquid. Bolded miRNAs represent those that had a chip miRNA-detection p-value<0.05.

Top-10 Differentially Expressed miRNA from 2013 study (Ng, Carballosa et al. 2013)				
	2013[a]	2018[b]		
Transcript ID	**1μM Nic.:Control** Fold (p-value)	**1μM Nic.:Control** Fold (det. p-value)	**1μM Nic. ECV:Control** Fold (det. p-value)	**ECV:Nic.** Fold
miR-3198	+146.47 (3.90E-5)	0 (0.67)	0 (0.64)	1.00
miR-1305	+120.09 (3.26E-5)	-0.63 (0.55)	-0.63 (0.54)	1.00
miR-3659	+107.00 (0.001)	0 (0.42)	0 (0.38)	1.00
miR-1914-3p	+88.95 (0.002)	+1.23 (0.87)	+1.88 (0.76)	1.53
miR-374b-5p	+57.88 (0.001)	**+1.40 (0.01)**	+1.40 (0.17)	1.00
miR-1973	+50.20 (1.78E-5)	+1.2 (0.34)	**+5.70 (0)**	4.71
miR-505-3p	+46.42 (4.23E-5)	**+3.02 (0)**	**+3.18 (0)**	1.70
miR-30d-5p	+46.23 (1.78E-5)	**+3.18 (0)**	**+2.78 (0)**	1.14
miR-18b-5p	+36.9 (9.27E-5)	**+2.58 (0)**	**+4.07 (0)**	1.58
miR-137	+3.16 (0.006)	**+1.83 (0)**	**+1.17 (0)**	0.64

Legend: a: SurePrint G3 Human v16 miRNA Array Kit (Agilent), b: GeneChip™ miRNA 4.0 Array (Applied Biosystems)

Top-10 differentially upregulated miRNAs target several osteogenic mRNAs

Table 4-5 contains a list of the top-10 differentially upregulated miRNA from 1μM nicotine ECV treated PDLSCs compared to non-exposed controls. In order to determine the potential downstream effects of miRNA expression on PDLSC osteogenic differentiation potential, the osteogenic mRNA targets of each miRNA were identified with TargetScan, a miRNA-mRNA bioinformatics platform from MIT. Out of the ten upregulated miRNAs, 7 were predicted to target genes involved with osteogenesis[158]. Two miRNAs, *hsa-miR-342-5p* and *hsa-miR-29b-3p*, were predicted to target multiple osteogenic genes, therefore, these miRNAs were selected for further analysis in osteogenic

studies. *hsa-miR-20a-5p* was also chosen for further analysis as it has been cited multiple times in the literature as being involved in the osteogenic differentiation process[159; 160].

Table 4-5: Top-10 differentially upregulated miRNA in PDLSC exposed to 1μM nicotine ECV for 4d with predicted osteo-mRNA targets. Expression is relative to non-exposed PDLSCs. Differential upregulation classified as fold change > 2 and chip miRNA-detection p-value < 0.05. Target prediction conducted with TargetScan (MIT).

Top-10 Differentially Upregulated miRNA (fold >2, p<0.05)		
Transcript ID	**1μM Nic. ECV:Control** Fold (detection p-value)	**Predicted osteo-mRNA targets**
hsa-miR-411-3p	223.50 (0)	
hsa-miR-342-5p	100.00 (0)	RUNX2, BMP2, COL1A1
hsa-miR-550a-3p	89.00 (0)	COL2A1
hsa-miR-29b-2-5p*	74.00 (0.01)	COL1A1, COL1A2, ALPL
hsa-miR-148b-3p	55.50 (0)	
hsa-miR-495-3p	24.17 (0)	SPARC
hsa-miR-15b-5p	18.13 (0)	
hsa-miR-1296-5p	16.42 (0)	ALPL
hsa-miR-30b-5p	14.56 (0)	RUNX2
hsa-miR-20b-5p	12.47 (0)	BMP2

*hsa-miR-29b-2-5p is not a confidently identified miRNA in the TargetScan data base and therefore many of its predicted targets are false positives. hsa-miR-29b-3p is confidently identified and is the broadly conserved form of this miRNA. hsa-miR-29b-3p was differentially expressed in PDLSCs treated with 1μM nicotine (+2.66 fold to control, p=0) and also targeted more than one osteogenic gene (i.e. COL1A1 and SPARC). Therefore, it this form will be used instead of -5p.

E-cigarette vapor upregulates expression of miRNAs through mechanisms not involving α7 nAChRs

A general trend in the expression of the three miRNAs of interest was observed after 21d of osteogenic differentiation (Figure 4-13). Normal in-vitro PDLSC osteogenic differentiation was characterized by an upregulation in *miR-20a-5p* (p<0.005) and *miR-29b-3p* (p<0.05) expression and a downregulation of *miR-342-5p* expression (p<0.005). Introducing 10μM nicotine ECV into the osteogenic media resulted in an upregulation of at least 20% in the expression of all three miRNA of interest (this upregulation is consistent with the miRNA chip data, which also showed an upregulation of PDLSC miRNA after 4d of nicotine treatment). Inhibiting α7 nAChRs with αBTX did not reverse these effects, suggesting that the changes in miRNA expression are mediated through other nAChR sub-types or due to the non-nicotine chemicals found in ECV.

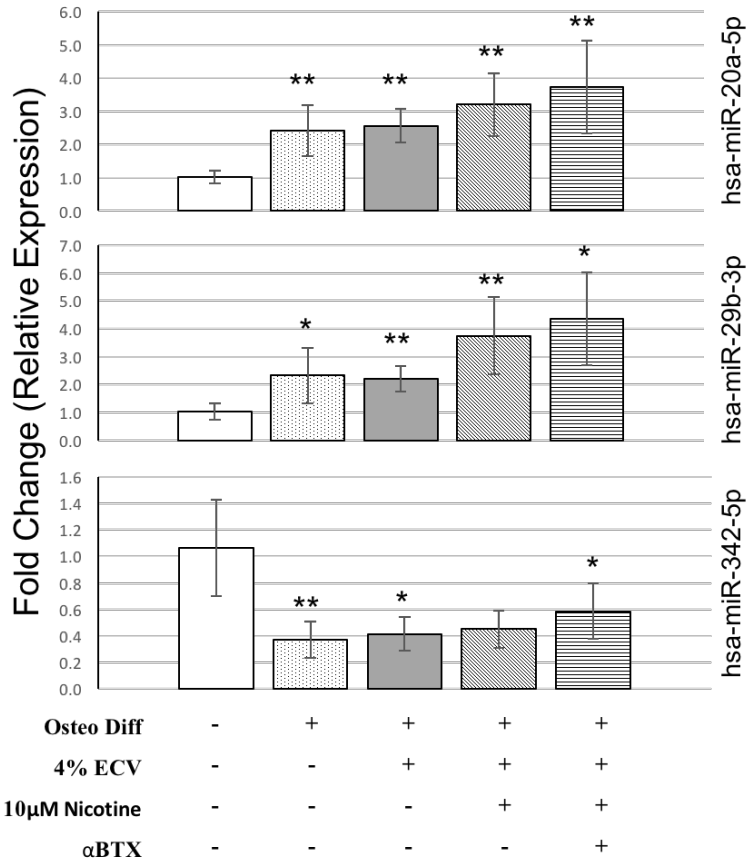

Figure 4-13: Taqman miRNA expression analysis of hsa-miR-20a-5p, -29b-3p, and -342-5p in PDLSCs after 21d of osteogenic differentiation and 4% ECV exposure.
Data presented as mean ± SD fold change in miRNA expression relative to non-differentiated control (n=3). U6 was used as reference. Statistical analysis calculated by one-way ANOVA. Significance considered as *=p<0.05, **=p=<0.005. Significance Symbols: (*) to non-differentiated control.

Chapter 5 Discussion

This project investigated the effect of EC vapor exposure on the osteogenic differentiation potential of PDLSCs, a resident stem cell population of the oral cavity- the initial site of EC vapor exposure. By comparing differences in potential between PDLSCs exposed to nicotine-free and nicotine-containing EC vapors, this project sought to determine the contribution of basic e-liquid chemicals (non-nicotine vs. nicotine) to the overall effect. The potential pathways and mechanisms behind these effects were also explored by investigating the roles of α7 nAChRs and miRNA expression during exposure. The results of these studies show that although EC are considered a safer alternative to cigarette smoking, their use could still be detrimental to human health. Additionally, the results show that α7 nAChRs only mediate the nicotinic-effects on PDLSC proliferation potential, but not migration, differentiation, or miRNA expression. It is possible that these receptors could mediate these effects under higher concentrations of nicotine or that the effects could be mediated by other nAChR subtypes. These results, therefore, merit further investigation in order to fully map out the regulatory network associated with the nicotine-induced effects.

Although not marketed as such, ECs used with nicotine-containing e-liquids are suitable nicotine replacement therapies. These devices deliver nicotine efficiently and curb smoking urge within several minutes of use. D'Ruiz et al. showed ECs filled with 1.6 and 2.4% nicotine (16mg/ml or 24mg/ml) significantly increased vaper plasma nicotine concentrations ($p<0.05$) and significantly curbed smoking urge within 5 min. of use ($p<0.001$)[62]. As such, these devices are an attractive alternative to cigarettes because

they can provide the nicotine content needed by the user in a way that simulates the act of smoking without the exposure to cigarette smoking toxins. Although there is a significant reduction in the amount of toxic exposure compared to cigarettes[37; 161], EC users are still at-risk due to the easy abuse of ECs and the fact that longer periods of use are required to satisfy nicotine cravings[150].

The current study investigated the effects of short-term EC vapor exposure, but given their addicting nature[162], EC use is more likely to be chronic than acute. The long-term health effects of EC use are not yet known. This is mostly due to the fact that ECs are a relatively new tobacco product and research regarding their safety is still in its infancy. Even so, understanding the implications of short- and long-term EC use may be difficult given that EC devices are ever-changing and e-liquid varieties are ever-growing. A 2014 report by Zhu et al. revealed that at the start of 2014 there were over 460 brands of ECs and over 7,760 e-liquid flavors[163]. Over a 17-month period, the authors observed an average increase of 10.5 new brands and 242 new flavors per month. With so many options, the possible combinations between EC device and e-liquid formulation are astronomical. However, this study aimed to determine the most likely (baseline) effects of EC exposure by producing vapor from a popular EC device (with default parameters) filled with the most basic of e-liquid formulations. In doing so, this study successfully begins to lay the groundwork for understanding the underlying measures of more complex vaping conditions (i.e. powerful devices with flavored e-liquids).

The JoyTech eVic EC was chosen for these studies because it is a popular and widely-available second-generation EC device. These devices have a rechargeable battery, allowing the same device to be used for repeat experiments, and can be programmed with

different voltages and/or refilled with different e-liquids to study different vaping conditions. For these experiments, the eVic was used with the default variable voltage setting of 3.3V. In order to isolate the effects of e-liquid carrier molecules from nicotine, e-liquids containing 0mg/ml and 36mg/ml nicotine were used. Non-flavored e-liquids were used as they constitute the base of all e-liquids. All e-liquids were purchased from the same company (AmericaneLiquid) to minimize variability in e-liquid batch composition. A 50%/50% w/v PG/VG e-liquid base was chosen in order to incorporate the available e-liquid base chemicals equally. The effects of cigarette smoke exposure were also investigated in order to gauge the severity of EC vapor damage. University of Kentucky 3R4F reference cigarettes were used in these studies since they are an accurate representation of modern-day cigarettes and are well documented in the literature and widely investigated across labs.

Two automated robots from VitroCell were used for smoke and vapor production. The smoking parameters of these two delivery devices are fully customizable; therefore, individualized puffing protocols can be inputted into the companion software in order to mimic the different smoking behaviors of cigarette and EC users. These unique abilities allowed us to closely model representative smoke and vapor exposure in vitro in a consistent and reproducible manner. The VC-10 was used for cigarette smoke production, while the VC-1 was used for EC vapor production. Cigarette smoke was generated according to International Organization for Standardization (ISO) 3308 (*Routine analytical cigarette-smoking machine -- Definitions and standard conditions*). These parameters are an established model for typical cigarette use and the standard protocol used by many investigators[164]. Adhering to these parameters assures compatibility with data generated

on other machines in other labs. The parameters of ISO 3308, however, are a poor representation of EC use[6]. At the time of these experiments, an ISO standard for the production of EC vapor had not yet been published (ISO/FDIS 20768: *Vapour products -- Routine analytical vaping machine -- Definitions and standard conditions* is currently under development). Therefore, in order to investigate a more characteristic model, the EC vaping protocol used was based off of the average smoking habits of experienced EC users[84; 86; 149].

These controlled conditions were used to study the effects of EC exposure on PDLSC regeneration potential. While they were ideal for a laboratory setting and provided a glimpse into the potential health effects of exposure, EC use is unique to each individual and the effects experienced by each person will depend on the specifics of their EC use. Factors like e-liquid composition [165; 166; 167], device used[161; 168], and vaping style[53; 167] influence the amount of toxic chemicals present in EC vapor. Accordingly, the severity of effects can vary between individuals and could be more serious than the ones reported in this project. A brief discussion of how each of these factors can contribute to EC toxicity is provided below.

Device composition

As previously mentioned EC devices are ever-changing[163]. Over the years these devices have gone from providing fixed power from weak, single use batteries to variable power from strong, rechargeable batteries. This increase in power production and the ability to modify the power output is highly desirable in the vaping community because it allows users to tailor the vapor profile (e.g. nicotine content, thickness/color of cloud, amount of vapor) and personalize their vaping experience. A survey of 4,421 experience

EC users revealed that 63.7% of users that started with a first-generation device switched to newer-generation device[169] and that only 5% of these users switched back. But these increases in power can actually make EC use more dangerous. In addition to over-heating the device and putting the user at risk of fires or explosions[170], higher battery power can increase the amount of toxic chemicals produced in EC aerosols[161; 168]. Ogunwale et al demonstrated that newer generation devices and higher battery output are consistent with higher detection levels of aldehydes in EC aerosols[168]. Formaldehyde levels, for example, were 1200% greater in newer generation devices compared to first generation devices and formaldehyde hemiacetal levels were 300% greater when output power was increased from 11.7W to 16.6W.

E-liquids

E-liquids are composed of three main ingredients: humectants, flavoring agents, and nicotine. Although this project only focused on the most basic of those (humectants and nicotine), each ingredient can contribute to toxic chemical formation in EC vapors.

Humectants

Humectants form the base of all e-liquids and are therefore the most common chemical found across brands. Propylene glycol (PG) and vegetable glycerin (VG) are the two most common humectants used in e-liquids. PG is routinely used in the pharmaceutical industry as a solvent for drug delivery systems[40] and VG is used as a sweetener in the food industry[41]. E-liquids can be composed of 100% PG, 100% VG, or a mixture of both. The relative ratio of both chemicals affects the type of vapor cloud produced and is therefore dependent on user preference. Both products are FDA-approved for safe use, however, when heated, as is the case during EC use, these products can form toxic

chemicals. Specifically, VG can form acrolein, whereas PG can form carbonyl compounds like formaldehyde and acetaldehyde[161; 171]. Formaldehyde (FA), for example, is classified as a probable human carcinogen by the U.S. Environmental Protection Agency[172] and has been associated with higher incidences of cancer[173]. According to Wang et al., the amount of FA exposure from EC use surpasses safety levels established by the EPA and can therefore cause substantial harm[171]. In this report, it was demonstrated that exposure to a 50%/50% w/v PG/VG EC vapor extract significantly inhibited PDLSC mineralization potential. This extract contained no nicotine and no flavoring agents therefore the inhibition in mineralization potential was solely due to the byproducts of aerosolized PG/VG. These results provide further evidence that aerosolized humectants are harmful to human health and may negatively impact stem cell differentiation. FA, one of the byproducts of PG, has been shown to affect the differentiation of human trophoblasts[174], the differentiation of natural killer cells[175], and the proliferation of human embryonic stem cells (hESCs)[176]. A study by She et al. showed that FA induced DNA strand breaks in mice bone marrow mesenchymal stem cells[177]. Together this data suggests that the effects of FA on differentiation and proliferation are potentially due to DNA damage. Additional studies comparing the effects of EC extract produced from e-liquids containing varying PG/VG ratios on stem cell regeneration potential could help elucidate the true impact of e-liquid humectants on stem cell regeneration potential.

Flavoring Agents

Although flavors were not covered in this project, it is worth noting that the majority of EC users vape flavored e-liquids. If purchased, non-flavored e-liquids are used

to create custom e-liquid blends with user-added flavoring agents. In either case, specific e-liquid flavoring agents can sometimes lead to additional cytotoxic effects during use[52; 165; 166; 178; 179]. Cinnamon flavors in particular have been shown to have drastic cytotoxic effects presumably due to the use of cinnamaldehyde for the flavoring agent[165]. Muthumalage et al. demonstrated that cinnamaldehyde alone and in tandem with e-liquid humectants significantly reduces cell viability and increases reactive oxygen species (ROS) in a dose-dependent manner[179]. In addition, cinnamaldehyde was shown to increase the secretion of inflammatory cytokine IL-8. The secretion of these cytokines in response to cinnamaldehyde was also demonstrated by Lerner et al., who showed an increase in IL-6 and IL-8 in human airway epithelial cells[178]. High concentrations of cinnamon flavored EC vapor extracts have also been shown to be cytotoxic to cultured cardiomyoblasts[52]. In addition to cinnamon, there have been several other flavors that have been shown to have cytotoxic effects[179]. Generally, these effects become more severe as more flavoring agents are added to the e-liquid[179]. The cytotoxic effect, however, may not be consistent across all cell types. A study by Bahl et al., for example, demonstrated that the cytotoxicity of flavored e-liquids was more apparent in stem cells than a terminally differentiated cell type[165]. This increased sensitivity in stem cells further supports the notion that PDLSCs may at risk during EC use.

Nicotine

Nicotine yield during EC use depends on several factors including e-liquid nicotine concentration[44], device used[180], puffing parameters[53; 167], and even e-liquid PG/VG content[161]. For users, this means that the amount of nicotine delivered per puff will depend on their personal preferences and their smoking techniques. For example, an

experienced user vaping a newer generation EC filled with a 36mg/ml nicotine e-liquid is expected to produce more nicotine per puff than a novice user who is vaping a first-generation device filled with 3mg/ml nicotine e-liquid. Researchers studying the effects of EC vapor exposure should keep these considerations in mind as they design experiments and compare their data across labs. This is especially important when collecting EC vapor extracts because the amount of nicotine in the stock solution can vary greatly depending on the reagents and puffing parameters used to collect the vapor. To account for this, researchers should analyze collected extracts using GC- or LCMS to determine the amount of nicotine content in the stock so that it can be diluted to physiological doses during experimentation. Yet, several early reports studying the cytotoxic effects of EC vapor with extract did not normalize their stock solutions to physiological doses of nicotine[167; 181]. Consequently, those results are difficult to analyze, taking away their scientific meaning.

To determine the additive effects of nicotine during EC vapor exposure, PDLSCs were exposed to EC vapor extract from 0mg/ml and 36mg/ml nicotine e-liquids and the effects between both groups were compared. To test physiological conditions, extract nicotine content was determined with LCMS and solutions were diluted to physiological concentrations of 1 and 10μM nicotine. By diluting 0mg/ml nicotine solutions in the same fashion, nicotine content was ensured to be the only variable. At the concentrations studied, these extracts were shown to be non-cytotoxic meaning that the experimental results obtained in this report were not influenced by cell death.

Proliferation

Nicotine-containing EC vapor extracts significantly inhibited PLDSC proliferation compared to nicotine-free extracts. These results are not surprising given that we and others

have shown that nicotine alone can significantly inhibit PDLSC proliferation[14; 15]. In the Zhou study, the effects of nicotine-containing EC extract on proliferation were reversed when PDLSCs were pretreated with αBTX[15], a specific antagonist of α7 nAChRs. α7 nAChRs are transmembrane ligand-gated calcium (Ca^{2+}) channels that undergo a conformational change in the presence of nicotine that results in a rapid influx of Ca^{2+} and a subsequent increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$)[132]. Cell proliferation is a process that is tightly regulated by calcium signaling[182] and increases in $[Ca^{2+}]_i$ are needed to promote cell proliferation[183]. However, nicotine exposure upregulates the expression of α7 nAChRs in PDLSCs[90], which, in turn, can lead to excess levels of $[Ca^{2+}]_i$. These superfluous amounts of $[Ca^{2+}]_i$ could disrupt normal calcium signaling mechanisms related to proliferation. Upregulation of α7 nAChRs, however, is time-dependent[184]. In rat airway smooth muscle cells, for example, maximal upregulation in α7 nAChR protein expression by 10μM nicotine occurs between 48-72h of exposure[184]. This phenomenon could explain why in this project non-significant decreases in proliferation between nicotine-EC and non-exposed PDLSCs weren't observed after 2 days of exposure and why significant differences in proliferation between nicotine and non-nicotine exposed PDLSCs weren't observed until day 4 in the Ng et al. study[14].

Migration

Our group was the first to show that nicotine increases the stiffness of MSCs and PDLSCs in a dose-dependent manner[185]. We predicted that this increase in stiffness could inhibit stem cell migration from the niche and through tortuous vasculature. Exposure to EC and CSE extracts appeared to inhibit PDLSC migration; however, significant differences to the non-exposed control were not observed. Significant

differences between exposure groups were also not detected. We and others have shown that longer periods of nicotine[14] and cigarette smoke exposure[24] can significantly inhibit PDLSC migration. Therefore, it is likely that significant decreases in migration could be observed if PDLSCs were exposed to EC vapor extracts for more than the 2 days of treatment used for these experiments.

Osteogenic Differentiation

Smoking is a potential risk factor for alveolar bone loss[186]. PDLSCs are a multipotent stem cell population that give rise to alveolar bone and actively participate in bone repair[187]. Bone repair is a multi-step process that culminates in bone remodeling[188]. Mineralization is a critical aspect of the remodeling phase and provides bone with its mechanical strength[189]. Compared to non-exposed controls, the mineralization potential of PDLSCs exposed to high concentrations of EC vapor is significantly decreased after 21d of exposure as evident by lower levels of Alizarin Red S staining. Moreover, the mineral deposition profile of the exposed PDLSCs is heterogeneous suggesting that EC exposure also appears to alter the local mineralization kinetics. The heterogeneity could lead to non-uniform bone mineral density distribution (BMDD) which could lead to an increase in the risk of bone fractures[190]. The overall decrease in mineralization potential could, however, be explained by a delay in the osteogenic healing process (remodeling phase) of these exposed cells. There is extensive data showing that healing from a traumatic bone injury takes longer in cigarette smokers than in non-smokers and that smokers are more likely to experience non-unions[111; 191; 192]. PDLSCs isolated from chronic cigarette smokers show a decreased osteogenic differentiation potential compared to PDLSC isolated from non-smokers[24]. In our

studies, a similar decrease in osteogenic differentiation potential was observed in PDLSCs exposed to cigarette smoke and EC vapor extracts. Together, this data suggests that EC vapors may act similar to cigarette smoke in delaying osteogenic healing.

EC vapors also affected the expression of certain osteogenic genes during differentiation. Significant differences were only observed in the expression of COL1A1, COL1A2, and IBSP genes between PDLSCs treated with nicotine-free and nicotine-containing vapors. COL1A1 and COL1A2 genes encode for alpha-1 and alpha-2 type 1 collagen protein. Together they form type-1 collagen, which is abundantly found in bone and supports bone structure and mechanics[193]. Collagen fibers, made up of alpha-1 and alpha-2 type 1 collagen molecules, are what is mineralized to form the initial components of bone[194]. IBSP, on the other hand, is a protein synthesized by osteoblasts and is highly expressed during early stages of mineralization[195]. In MSCs the mineralization process begins to occur toward the second week of differentiation[157]. During this time, the expression of mineralization genes like COL1A1, COL1A2, and IBSP should be upregulated. For PDLSCs exposed to nicotine-containing vapors, the expression of these genes was significantly downregulated at day 14. Significant upregulation in gene expression wasn't seen until day 21, suggesting that these cells could be experiencing a delay in the mineralization process. This explanation could help explain why PDLSCs exposed to nicotine-containing vapors had a slightly lower mineralization potential than PDLSCs exposed to nicotine-free vapors. However, the expression of these genes was mostly similar between the control and nicotine-free vapor treated PDLSCs, yet there was a significant difference in mineralization between both groups. Accordingly, gene

expression may not be the only factor regulating mineralization potential in PDLSCs exposed to EC vapor.

miRNA

Our group had previously shown that PDLSC miRNA expression is significantly altered following nicotine and cigarette smoke exposure, with certain miRNAs actually targeting several aspects of PDSC regeneration potential[14; 24]. In this study, microarray analysis revealed that nicotine-containing EC vapor exposures differentially upregulated 144 PDLSC miRNAs. 70% of the top-10 differentially expressed miRNA targeted at least 1 osteogenic gene, suggesting that miRNAs may be responsible for the detrimental effects that EC vapors have on the mineralization potential of PDLSCs. *Hsa-miR-29b-3p* and *hsa-miR-342-5p* were chosen for further validation in PDLSCs since they were differentially expressed and targeted more than 1 osteogenic gene, whereas *hsa-miR-20a-5p* was chosen since it role in osteogenic differentiation and calcification in other human cell types has been previously demonstrated in the literature[159; 160].

miRNA expression in PDLSCs was validated with 21d osteo-differentiated samples. Compared to non-differentiated PDLSCs, the expression of *hsa-miR-20a-5p* and *hsa-miR-29b-3p* was significantly upregulated, whereas the expression of *hsa-miR-342-5p* was significantly downregulated. This data suggests that these miRNAs might be involved in the osteogenic differentiation of PDLSCs. Given the stark differences in mineralization between non-differentiated and differentiated PDLSCs, the results also suggest that *hsa-miR-20a-5p, hsa-miR-29b-3p* and *hsa-miR-342-5p* may also be involved in mineralization. Exposure to 10μM nicotine EC vapor extract further upregulated the expression of these miRNA by 32.1% for *hsa-miR-20a-5p*, 61.2% for *hsa-miR-29b-3p*, and 21.6% for *hsa-*

miR-342-5p. Upregulation wasn't as drastic when nicotine-free EC vapor extracts were added, suggesting that nicotine is the primary chemical responsible for the upregulation of PDLSC miRNA during EC vapor exposure. Interestingly, αBTX pretreatments do not reverse nicotinic effects, indicating that PDLSC miRNA expression may not be modulated through α7 nAChRs, but rather by other receptor subtypes that have been identified in PDLSCs[122].

The function of *hsa-miR-20a-5p* (1181 target genes), *hsa-miR-29b-3p* (1064 target genes), and *hsa-miR-342-5p* (3000 target genes) in PDLSCs was not known. Gene enrichment and functional annotation analysis revealed that these miRNAs of interest could be involved in regulating cell proliferation, migration, and osteogenic differentiation (Table 5-1).

In addition to helping understand the molecular mechanisms of EC vapor exposure effects on PDLSCs, miRNA can be used as a diagnostic tool for the early detection of oral diseases like periodontitis. Periodontitis is an inflammatory disease of the oral cavity that, if left untreated, leads to the deterioration of tooth supporting structures and culminates in eventual tooth loss. Periodontitis has been associated with increased incidences of cardiovascular disease[16] and stroke[17] and is extremely prevalent in cigarette smokers[18]. Nicotine has also been associated with higher risks of periodontitis due to its ability to augment alveolar bone loss[19]. ECs commonly contain nicotine in their e-liquids; therefore, it is possible that EC use may increase the risk of periodontitis.

The detrimental effects of periodontitis on oral health can be avoided if the disease can be diagnosed at the onset in order to provide early preventative dental care. Current diagnostic techniques, however, are only capable of detecting advanced forms of the

Table 5-1: Functional annotation analysis for miRNAs of interest.

hsa-miR-20a-5p			
Functional Annotation	**Enrichment Score**	**Count**	**P**
Phosphoprotein	21.76	700	1.8E-42
Transcription regulation	4.51	232	2.3E-16
Zinc finger, FYVE-related	3.65	13	3.8E-04
Chromatin binding	3.11	47	3.0E-03
MAPK signaling pathway	2.86	31	5.5E-03
extrinsic component of membrane	2.83	16	9.8E-03
Differentiation	2.47	58	1.7E-02
intracellular receptor signaling pathway	2.38	12	5.8E-03
Chromatin regulator	1.76	34	1.6E-03
Apoptosis	1.64	48	2.3E-02
TGF-beta signaling pathway	1.52	15	1.1E-02
SMAD binding	1.52	11	1.6E-02
miRNA mediated inhibition of translation	1.52	7	1.4E-02
Translation regulation	1.5	17	3.8E-03
Cell Cycle	1.34	58	1.0E-02

hsa-miR-29b-3p			
Functional Annotation	**Enrichment Score**	**Count**	**P**
Calcium	4.6	64	3.7E-02
Collagen	4.19	26	1.2E-09
Transcription Regulation	3.21	168	4.6E-05
Cell Junction	2.25	59	1.1E-03
Microtubule	2.1	26	4.5E-02
Wnt Signaling pathway	1.53	20	1.4E-03
Translation Regulation	1.52	16	4.2E-03
Signaling pathways regulating pluripotency of stem cells	1.4	20	1.5E-03

hsa-miR-342-5p			
Functional Annotation	**Enrichment Score**	**Count**	**P**
Phosphoprotein	10.08	1387	1.0E-22
Cell junction	4.62	146	1.3E-06
sequence-specific DNA binding	3.07	111	1.2E-02
Transcription regulation	2.35	428	8.8E-09
Serine/threonine-protein kinase	1.99	79	8.8E-03
Cell adhesion	1.96	90	2.7E-02
Actin-binding	1.83	65	3.8E-04
Cell membrane	1.32	512	8.4E-02
cAMP	1.31	13	3.1E-02

disease; therefore, a need for early detection still exists in order to improve clinical management. One potential approach is to identify the presence of biomarkers in saliva that are associated with the early onset of periodontitis.

The detection of miRNAs, for example, has proven useful for the early prognosis of numerous diseases, including cancer[86]. Interestingly, miRNAs can be measured in the saliva[21; 22] and are differentially expressed in diseased vs. normal human gingival tissues[23]. The use of miRNAs as salivary biomarkers for the early detection of periodontitis is therefore a novel approach that has the potential to improve the prognosis of periodontal diseases like periodontitis. This approach would allow for earlier preventative care, thereby reducing the overall prevalence of this disease. These methods can be converted into a new diagnostic technique for future commercial application.

The use of adult stem cells is currently being considered as a therapeutic approach for the regeneration of tissues damaged by periodontal diseases like periodontitis[9]. The oral cavity, the initial site of EC vapor exposure, contains various stem cell populations that are involved in the development, maintenance, and repair of oral tissues[10]. Periodontal ligament derived stem cells (PDLSCs) are one such population. PDLSCs are multipotent and have been shown to give rise to tooth supporting structures such as alveolar bone, periodontal ligament, and cementum[10]. In addition to their regenerative capabilities, PDLSCs can be easily isolated following natural tooth loss or routine dental procedures and are thus an ideal source of therapeutic stem cells for periodontal regeneration[10; 11]. A recent clinical trial using autologous PDLSCs as a therapeutic approach for the regeneration of periodontal bony defects confirmed the safety of this approach for human use[9]. The success of this trial has opened the door for other

therapeutic applications, which, to date, are actively recruiting patients (clinicaltrials.gov). As the potential therapeutic use of dental cells begins to grow, so does the number of companies looking to store them for future use. The United States has 5 established dental stem cell banking companies: StemSave (New York, NY), Store-A-Tooth (Littleton, MA), The Tooth Bank (Brownsburg, IN), BioEden (Austin, TX), and the National Dental Pulp Laboratory (Newton, MA). Together, these companies have also opened around 40 international centers, making dental banking widely accessible around the world.

However, here we show that the regeneration potential of PDLSCs is significantly affected by EC vapor exposure and, therefore, PDLSCs exposed to EC vapors might not be suitable for biobanking as they might not have the same effectiveness as non-exposed cells for future therapeutic applications. A recent study by Barwinska et al. demonstrated that this was exactly the case for smoker-derived adipose stem cells (ASC), which were used in an approach to regenerate tissue vasculature after an ischemic event[196]. In this study, the authors found that human smoker-derived ASCs did not improve perfusion to ischemic areas, secreted less hepatocyte growth factor and stromal cell-derived growth factor 1, and secreted higher levels of proinflammatory cytokines compared to non-exposed ASC. ASCs isolated from cigarette exposed mice models showed similar results, confirming a decrease or loss in therapeutic potential following smoke exposure.

In addition to banking customers, stem cell-based therapeutic developers and study coordinators should also be cognizant of these detrimental effects as they could potentially impact the results of pre-developmental research or, even worse, outcomes of stem-based interventions in candidate patients. Accordingly, stem cell donors and recipients should be extensively screened prior to study initiations in order to determine the extent of toxic

exposure. Gathering information regarding daily consumption, concentration of e-liquid nicotine, and frequency or length of use will help distinguish between dangerous and safe levels of exposure in prospective patients. By incorporating this information with other inclusion/exclusion criteria, researchers can further hone in on the most ideal cells/candidates for research or transplantation.

Chapter 6 Future Considerations

By residing in the oral cavity, PDLSCs are extremely susceptible to the detrimental effects associated with toxic EC vapor chemical exposure. Understanding the molecular effects of vapor exposure could help elucidate the detrimental effects that EC use has on the regeneration potential of PDLSCs and could help explain the pathology of various oral diseases associated with smoking (and perhaps predict oral diseases associated with vaping). This study is the first to successfully investigate the effects of EC vapor exposure on PDLSCs. However, additional experiments should be conducted in order to confirm the results obtained in this report. To truly confirm whether α7 nAChRs mediate the nicotinic effect seen in PDLSCs, it is necessary to repeat these experiments with an α7 nAChR knockout model. We have shown that these models can be established in PDLSCs using CRISP/Cas9 genome editing (Figure 6-1).

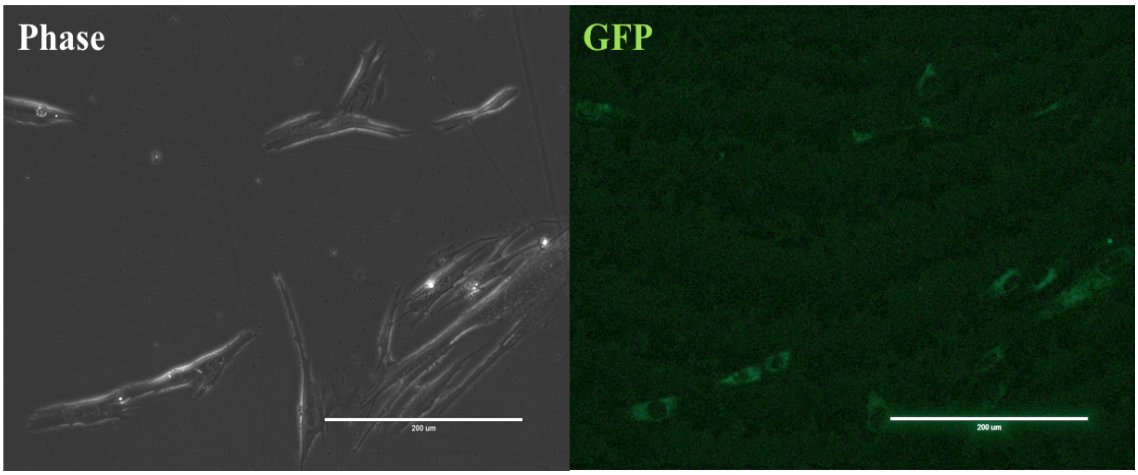

Figure 6-1: Immunohistochemical analysis of PDLSCs genetically engineered with CRISPR/Cas9 to knockout α7 nAChR expression. PDLSCs were transfected with α7 nAChR knockout vectors and imaged after 23d of culture. In successfully transfected cells the α7 nAChR gene was replaced with a GFP vector. PDLSCs that successfully integrated this gene passed on the genetic material to daughter cells which also stably express GFP.

To confirm the regulatory effects of the miRNA of interest, it would be necessary to repeat these experiments with PDLSCs that inhibit and overexpress each miRNA. However, given that exposure to EC vapor caused a differential expression in 144 miRNAs, it is unlikely that 1 miRNA will be solely responsible for the entire effect seen in PDLSCs. Thus, in addition to evaluating the effect of single miRNA inhibition and overexpression, the miRNA experiments should also be conducted with different combinations of miRNA overexpression. For example, the effects of PDLSCs overexpressing 1 miRNA should be compared to those overexpressing 2 and 3 miRNAs.

These experiments successfully laid the groundwork for studying the effects of EC vapor exposure on PDLSCs. Although PDLSCs behave very similar to other adult stem cell populations (namely MSCs), not all stem cells will respond to EC vapor in the same manner. Therefore, experiments using other stem cell sources merit further investigation. Future experiments should consider the effects of prolonged EC exposure since ECs contain nicotine and are likely to be abused like cigarettes. Additionally, future studies should look into the effects of cessation in order to determine the reversible and irreversible effects of EC use. In a recent study, our group determined that the regeneration potential of smoker-derived PDLSCs was diminished compared to non-smokers even after several weeks of culture in non-exposed conditions[24]. These results suggest that the effects of cigarette and EC use may be permanent and therefore require further investigation to determine long-lasting health effects.

The data from these experiments and the recommendations outlined in this section have been compiled into a grant application, which was recently submitted to the NIH. The grant will have three specific aims:

- **Aim 1:** To establish smoking suppression of stem cell potentials by miRNAs is a separate and independent pathway than via the α7 nAChR activation pathway.
- **Aim 2:** Determine the effects of EC and CS use on periodontal bone loss and the regenerative potential of mice dental pulp stem cells using an in-vivo animal model.
- **Aim 3:** Examination of the miRNA as biomarkers of smoking-induced periodontitis.

The α7 nAChR knockout studies described here are part of Task 1.1 of Aim 1 (Illustrated in Figure 6-2). The experiments conducted in this project are part of Task 1.2 of Aim 1. Aim 2 is focused on using a mice model to study the effects of EC use on periodontal bone loss and regenerative potential in vivo (Illustrated in Figure 6-3). Lastly, Aim 3 is focused on analyzing the salivary miRNA profiles of smokers and non-smokers to determine if there is any correlation between smoking-induced periodontal diseases and miRNA expression. Ultimately, the data gathered could be used to develop prescreening methods for the early detection of destructive oral diseases such as periodontitis

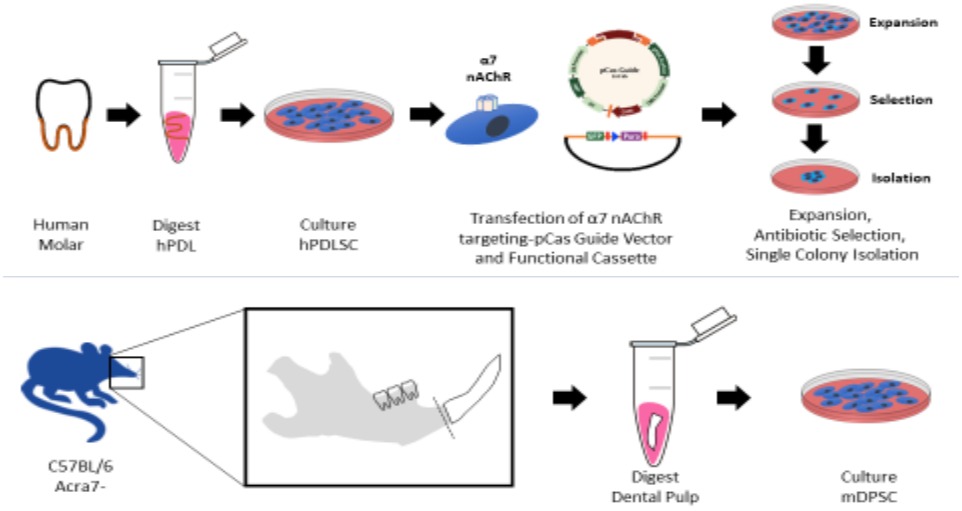

Figure 6-2: Illustrative overview of NIH Grant Aim 1, Task 1.1.

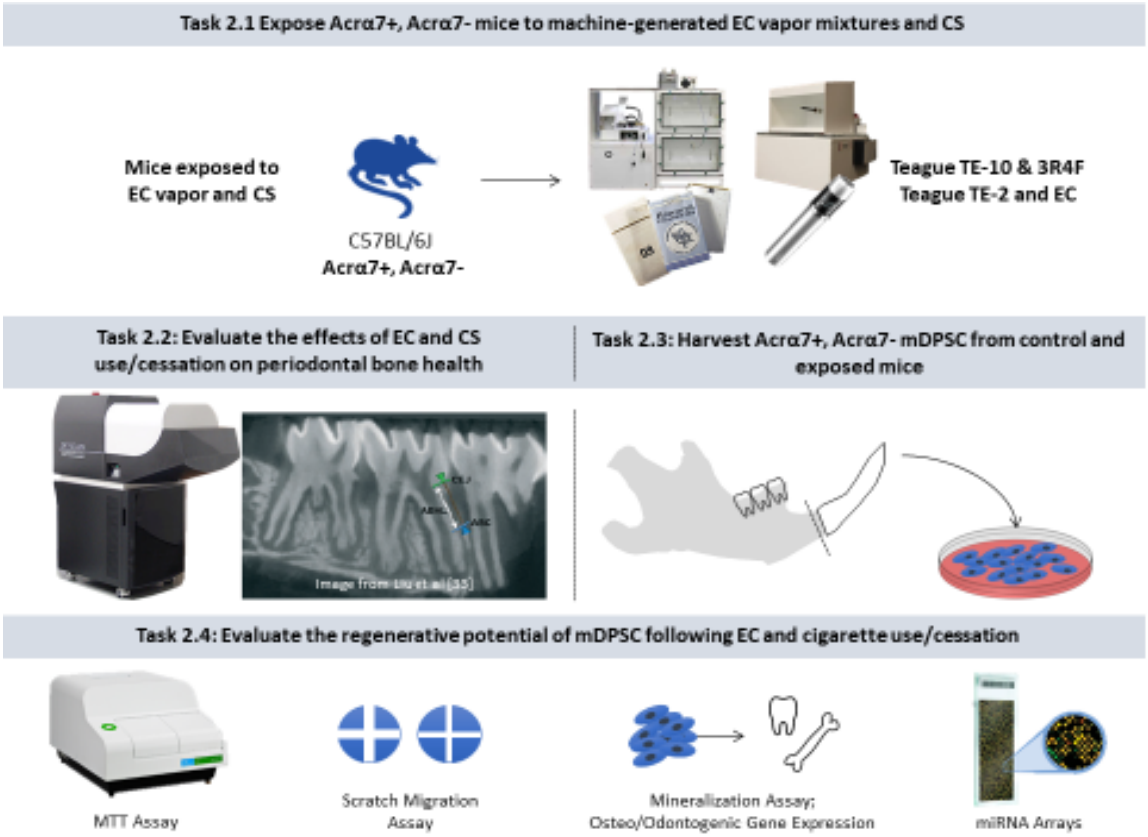

Figure 6-3: Illustrative overview of NIH Grant Aim 2.

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