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Regulation of nAChRs and Stemness by Nicotine and E-cigarettes in NSCLC

by

Courtney M. Schaal

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
with a concentration in Cancer Biology
Department of Cell Biology, Microbiology, and Molecular Biology
College of Arts and Sciences
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DEDICATION

I dedicate this work to everyone who has inspired, encouraged, or helped me along the way; knowingly or unknowingly. From my first mentor at Moffitt who opened my eyes to the opportunities which led me here today, to everyone who has shaped both my professional and personal life since. Especially my family and those I consider family, who always support me in chasing my dreams. I also dedicate this work to the enumerate cancer patients and their families, and hope to one day make a significant impact in their lives.

“Nothing is impossible.”

ACKNOWLEDGMENTS

I would like to acknowledge my family, friends, and significant other for all of their love and support throughout my time in the PhD program and throughout my lifetime. I would not be the person I am, or where I am today without them.

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Lastly, and humorously, I have to thank my German Shepard, Mako, who keeps me sane, keeps me in shape, has been there waiting with a tail wagging through all of my long hours and trips, and has lived up to his name as man's best friend.

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ABSTRACT

Lung cancer is the leading cause of cancer-related death in both men and women, nationally and internationally and kills more people each year than breast, prostate, and colon cancers combined. Non-small cell lung carcinoma (NSCLC) is the most common histological subtype of lung cancer, and accounts for 85% of all cases. Cigarette smoking is the single greatest risk factor for lung cancer, and is correlated with 80-90% of all lung cancer deaths. Nicotine, the addictive component of tobacco smoke, is not a carcinogen and cannot initiate tumors itself; however, it is known to act as a tumor promoter, by enhancing the proliferation, migration, and invasion of cells *in vitro*, thus accelerating tumor growth and metastasis *in vivo*. Nicotine exerts is tumor promoting effects primarily by binding to, and activation of, nicotinic acetylcholine receptors (nAChRs), specifically the α7 subunit of nAChRs. While α7 nAChR is expressed in a wide array of cells, how its expression is regulated is not fully understood. Here we sought to elucidate the transcriptional regulation of α7 nAChR in NSCLC cells. We report that α7 nAChR expression is induced by nicotine in an autoregulatory feedforward loop, and that the α7 gene promoter is differentially regulated by E2F1 and STAT1 transcription factors at an overlapping binding site suggesting a competitive interplay. Depletion of E2F1 resulted in a reduced ability of nicotine to induce α7 nAChR, while depletion of STAT1 resulted in enhanced induction, suggesting that nicotine might use these two transcription factors to modulate the expression of α7 nAChR in a very precise fashion. More recently, nicotine has been implicated in promoting self-renewal of stem-like side-population cells from lung cancers. Cancer stem-like cells have been implicated in tumor initiation as well as the maintenance, drug resistance, dormancy, recurrence, and metastasis of various tumor types. We had previously shown that the embryonic stem cell transcription factor, Sox2, is indispensable for self-renewal of stem-like

cells from lung adenocarcinoma cell lines; hence we sought to determine whether nicotine enhances stemness of lung cancer stem-like cells through Sox2. We find that nicotine can induce the expression of Sox2 at the transcriptional level and this occurs through a nAChR-Src-Yap1-E2F1 signaling axis. Over recent years, electronic cigarettes (e-cigarettes) have emerged as healthy alternatives to traditional cigarette smoking as they do not contain tobacco; however, they do still contain nicotine. Our studies show that e-cigarette components can enhance tumor promoting properties of NSCLC cells similar to that observed with nicotine alone, and find that they can induce expression of Sox2 and mesenchymal markers as well as enhance migration and stemness of NSCLC cells. Taken together, these studies reveal novel molecular mechanisms by which exposure to nicotine, *via* cigarette smoke or e-cigarettes, could alter the oncogenic potential of NSCLC cells.

CHAPTER ONE:

INTRODUCTION

Note to Reader

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Lung Cancer and Smoking

Despite growing knowledge of the disease and advanced treatment options, lung cancer remains the leading cause of cancer-related deaths in the United States and globally, in both genders, accounting for more deaths than breast, prostate, and colon cancers combined (1). There will be an estimated 224,390 new cases and 158,080 deaths due to lung cancer in 2016, and the 5-year survival rate is a bleak 15%. There are two primary histological subtypes of lung cancer: small cell lung carcinoma (SCLC) comprising 15% of all lung cancers, and non-small cell lung carcinoma (NSCLC) which is the most common form, comprising 85% of all lung cancers (2). NSCLC can be further characterized into squamous cell carcinoma (SCC) comprising 25-30%, adenocarcinoma compromising 40%, and large-cell carcinoma of the lung comprising 10-15% of cases (2, 3). The standard of care for lung cancer patients is surgical resection for early stage disease (3, 4) (5); however, typically by the time a patient becomes symptomatic it is too advanced for surgery alone and has often metastasized, requiring adjuvant chemo or radiation therapies (6, 7). With the advances in understanding the molecular mechanisms underlying this disease, treatment decisions are now based not only on stage and histological subtype of disease, but also on the genetic alterations present (6). Common genetic alterations identified as drivers in lung cancer include mutations in *EGFR*, *KRAS*,

LKB1/STK11 genes, translocation of *EML4-ALK*, and loss of the tumor suppressors *TP53 and RB* (8). Cancers harboring some of these alterations can be effectively targeted with agents including gefitinib and erlotinib for *EGFR* mutations, and crizotinib for *EML4-ALK*. More recently, immunotherapies have emerged that target immune checkpoint molecules such as PD-1 and CTLA4 with agents like the anti-PD-1 antibody Nivolumab (Opdivo), which has shown clinical efficacy in some cohorts of lung cancer patients (9). Albeit these recent advances in therapeutic modalities, even if the patient responds to either targeted or traditional lines of therapy, they tend to relapse with the appearance of metastatic disease, resulting in mortality. All of this indicates a need to further study lung cancer and develop more efficacious, long lasting therapeutic modalities.

Smoking is the single largest and most preventable risk factor for lung cancer, and is responsible for 70% of all NSCLC cases and 90% of SCLC cases; although there is a subset of lung cancer patients who develop the disease without a history of ever smoking (8, 10). Tobacco smoke contains 6,000 known carcinogens of various classes such as polycyclic aromatic hydrocarbons, tobacco specific nitrosamines, and aldehydes which are capable of initiating tumorigenesis (8, 11). Tobacco specific carcinogens like NNK (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone and NNN (N-nitrosonornicotine), which are nicotine derivatives, act as mutagens primarily through the formation of DNA adducts resulting in mutation of vital genes like *KRAS*, *p53*, and *RB* (12). While there are multiple nitrosamines present in tobacco, NNK and NNN have proven to be the most prevalent and potent, and are classified as Group 1 carcinogens in humans (13). They are present in tobacco as pro-carcinogens which require metabolic activation to exert their functions, and are unique in that they not only cause deletion mutations through DNA adducts, but can also bind to receptors to promoter pro-tumorigenic effects (13, 14). NNN is produced by the nitrosation of nornicotine during the curing and processing of tobacco, as well as during the burning or smoking of tobacco (15). NNK is naturally synthesized in tobacco leaves when they are exposed to light; upon exposure, the

purrolidine ring in nicotine opens up converting nicotine into NNK (16). Most of the NNK and about half of the NNN found in tobacco smoke originates from burning (15, 16).

Cancers such as melanoma are known to have high mutational burden, and not surprisingly, smoking-related lung cancer is reported be one of them, with a mean somatic mutation rate of 8-10 mutations per 1 million base pairs compared to 0.8-1 mutations per million base pairs in never smokers (8, 17). Tobacco exposure is additionally correlated with frequent cytosine-adenine (C→ A) nucleotide transversions, substituting a purine for a pyrimidine or a pyrimidine with a purine, seen primarily in lung adenocarcinoma patients with a history of smoking as opposed to their non-smoking counterparts (17). Overall this represents a complex disease and confounding clinical challenge, and a deeper understanding of the underlying mechanisms of smoking-related lung cancers is warranted.

Nicotine, which is the addictive component of tobacco smoke, is generally thought to be non-carcinogenic, though a recent study has suggested that chronic exposure at high concentrations could initiate muscle sarcoma in A/J mice (18, 19). At the same time, nicotine has widely been shown to promote tumor growth and metastasis by inducing a number of tumor promoting properties including cell cycle progression, epithelial-to-mesenchymal transition (EMT), angiogenesis, migration, invasion, survival, and drug resistance in multiple tumor types (20-24). Additionally, nicotine has been shown to disrupt metabolic processes and induce the secretion of growth factors and cytokines altering the physiology of multiple organ systems (23, 25, 26). These observations suggest that nicotine contributes to the progression and metastasis of tumors in patients who continue to smoke.

Nicotine Mediated Tumor Promotion

The first set of large-scale epidemiological studies causatively linked smoking to lung cancer in 1950, which was then confirmed by the U.S. Surgeon General, the World Health Organization, and the Royal College of Physicians in London (26). By 1988, tobacco smoke had already been well established as causative agent for multiple cancer types including those of the respiratory

tract, upper digestive system, bladder, renal, liver, and pancreas with suspicion for those of the cervix, head and neck which has since been confirmed (13). The basis for this was linked to the nitrosamines NNN and NNK which were reported as being the strongest carcinogens present in tobacco and which had the ability to initiate tumors in rats, Syrian golden hamsters, and A/J mice; it is worth noting that NNK had strong propensity for inducing lung cancers (27). Initial reports investigating these nicotine derivatives found that while NNN and NNK could initiate tumors and promote proliferation, nicotine could not initiate tumors but had strong proliferative effects in neuroendocrine cells and lung tumors with neuroendocrine differentiation (28). Since then, numerous studies have reported the proliferative effects of nicotine on both normal cells as well as tumor cells in vitro and in vivo, primarily through driving cell cycle progression (21, 22, 29, 30). Nicotine has been shown to promote proliferation in various cell types of the lung including neonatal rat lung tissue, normal human lung and airway cells, NSCLC, and SCLC; in addition to other organ specific cell types such as smooth muscle and endothelial cells, gingival fibroblasts, normal murine splenocytes, normal breast epithelium and breast cancers, head and neck cancers, cervical cancers, pancreatic cancers, colon cancers, cells in the immune compartment including promyelocytic leukemia cells (22, 31-40). Conversely, nicotine has been shown to have a growth inhibitory effect on osteoblast and osteoblast-like cells which demonstrates a different role for nicotine in the bone compartment; the growth suppressive effect in the bone compartment is thought to have significant implications for the development of osteoporosis and bone loss associated with tobacco use (41, 42).

Shortly after the reports of nicotine's effects on proliferation, numerous studies found that nicotine could additionally enhance multiple aspects of tumor progression, which are summarized in Figure 1.1. Nicotine is a potent angiogenic agent in endothelial cells involved in both the physiological and pathological process of angiogenesis, a phenomenon important for the growth and progression of cancer (43). Studies found that nicotine could enhance angiogenesis in vitro and in vivo, in the context of multiple cancer types, primarily through VEGF

(43, 44). Nicotine has also been reported to enhance migration and invasion of lung, pancreatic, and breast cancer cells in vitro, and the metastasis of lung and pancreatic tumors in vivo; processes which ultimately lead to mortality of patients (45). Prior to metastasizing, cells are thought to undergo epithelial-mesenchymal transition (EMT), leading to the cells acquiring a more mesenchymal phenotype enabling them to more easily escape the primary tumor site, survive in the vasculature, and reach distal organs to recolonize as metastatic tumors (45). This process is also thought to be enhanced by nicotine as it acts to induce mesenchymal markers and phenotypes, while suppressing epithelial markers and phenotypes (46).

In addition to these effects, nicotine has been implicated in chemoresistance and survival of cancer cells. This is largely in part through inhibiting apoptotic pathways which will be discussed later, conferring a survival advantage to the cells; and also by impacting drug metabolism (47, 48). Tobacco smoke itself is known to alter drug metabolism through the polycyclic aromatic hydrocarbons (PAHs) present in tobacco, which induce key drug metabolizing enzymes which results in higher clearance of cancer therapeutic agents including erlotinib, gemcitabine, and taxanes (48, 49). In animal studies, nicotine has been found to induce similar drug metabolizing enzymes as PAHs; however, whether this occurs in humans in yet unknown (48). Interestingly, the enzyme CYP2A6 which is responsible for the metabolic inactivation of nicotine into cotinine is induced by tobacco smoke and possibly nicotine itself; polymorphisms in the CYP2A6 gene may correlate with lung cancer incidence and nicotine dependence (50). This raises the intriguing possibility that when CYP2A6 is enhanced in response to smoking, not only are therapeutic agents cleared more readily but nicotine is also metabolized at a faster rate resulting in the patient's need to smoke greater numbers of cigarettes to maintain the nicotine in their bloodstream, thus increasing the likelihood of lung cancer incidence.

More recently, nicotine has been shown to enhance stemness in a subset cancer cells known as cancer stem cells (CSCs) in lung, breast, head and neck, and pancreatic cancers (51-53)

(54-57). Tumors are traditionally thought to be a disease of clonal origin where a single transformed cell has the ability to partially differentiate into heterogenous tumor cell populations, thereby initiating and maintaining the tumor. More recently, growing evidence supports the cancer stem cell model which indicates that CSCs arise through a reprogramming mechanism and these cells are responsible for tumor progression and maintenance (58). These stem-like cells resemble traditional stem cells in that they are able to self-renew, divide asymmetrically, and are slow cycling (59), and have been implicated not only in tumor initiation, progression, and maintenance, but also in therapeutic resistance, dormancy, recurrence, and metastasis (59-61). Given these properties, understanding and targeting CSCs has become a promising area of cancer research with the concept being that targeting this resistant population in addition to the tumor bulk would be necessary for the complete eradication of the tumors.

Over the past decade or better, CSCs have been characterized and isolated based on specific cell surface markers that are differentially expressed in the CSCs. Various markers such as aldehyde dehydrogenase 1 (ALDH) and CD133 positivity are commonly used for cancers such as breast, colon, brain, pancreas, head and neck (62-65). For cancer types with no ubiquitously expressed marker, a side-population (SP) phenotype has been used to isolate stem-like cells. Side-population phenotype is displayed by a subset of tumor cells based on their ability to efflux Hoechst 33342 dye out of their nuclei; this is due to the expression of the ABCG2 drug transporter on the cell membrane. Stem-like cells from multiple cancers have been found to express elevated levels of ABCG2, allowing the isolation of these cells by flow cytometry (66-68). Nicotine has been found to enhance stem-like properties including self-renewal and expression of stem cell genes in CSCs of multiple cancer types, via mechanisms to be discussed later in the chapter.

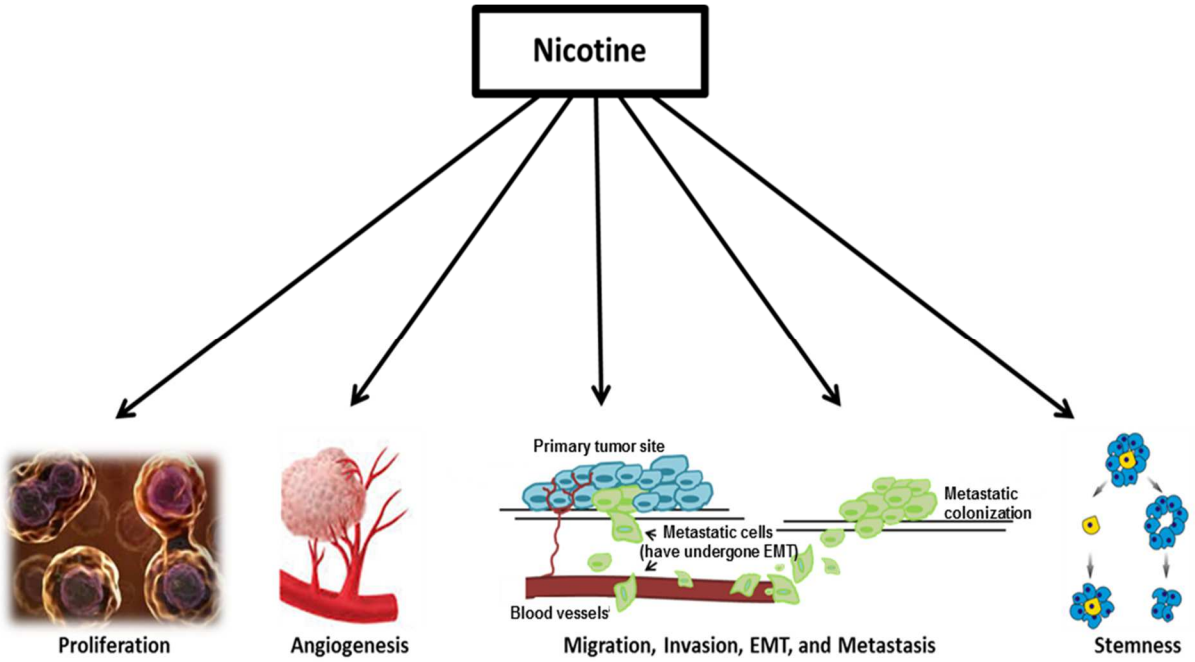


Figure 1.1. Tumor Promoting Effects of Nicotine. Nicotine, while not a carcinogen itself, has been shown to enhance a number of tumor promoting properties including proliferation, angiogenesis, EMT, migration, invasion, and stemness in vitro.

Nicotinic Acetylcholine Receptors

The mechanism by which nicotine exerts its tumor promoting functions is through the binding to and activation of cell surface receptors, specifically nicotinic acetylcholine receptors (nAChRs) and to a certain extent β-adrenergic receptors (β-ARs) (24, 69-71). In addition to nicotine, its oncogenic derivatives NNK and NNN present in tobacco smoke have also been found to bind to and activate nAChRs with high affinity stimulating multiple tumor promoting signaling cascades. Unlike nicotine, its two derivatives can exert carcinogenic effects through the binding of these receptors as well as by inducing DNA damage in a receptor independent fashion (34, 70, 72, 73). Interestingly, while the neurotransmitter acetylcholine (Ach) is the known physiological ligand for nAChRs in the cholinergic systems of the body, nicotine, NNN, and NNK can bind these receptors with greater affinity than Ach and displace Ach, thereby altering the normal functions of the receptors (74).

Classically, nAChRs are neuronal proteins that respond to Ach as well as drugs including nicotine, and are therefore considered cholinergic (75). While Ach binds to both nicotinic and the related muscarinic acetylcholine receptors, nicotine selectively binds to nicotinic acetylcholine receptors alone, giving them their name (75). nAChRs are typically expressed in the central and peripheral nervous systems where they function as ligand gated ion channels to facilitate signal transduction as well as calcium influx, resulting in the release of neutrotransmitters such as γ-aminobutyric acid (GABA), dopamine, serotonin responsible for nicotine addiction, and the catecholamines adrenaline and noradrenaline (76, 77). In the sympathetic and parasympathetic components of the peripheral nervous system, nAChRs relay signals from pre- to post-synaptic cells and act as the receptors facilitating muscle contraction at neuromuscular junctions (77). More recently, nAChRs have been shown to be expressed on non-neuronal cells of epithelial and endothelial origin, including lung cancer cells where they mediate the synthesis and release of growth factors, pro-angiogenic factors, and neurotrophic factors (13, 44, 70, 71, 78).

nAChRs are pentamers, comprised of five identical α subunits (α7, α8, or α9) or a combination of α and β subunits (α2-α6 or α10 subunits, together with β2-β4 subunits (73, 78). Depiction of nAChR organization is detailed in Figure 1.2. To date, nine different types of α subunits (α2-α10) and three types of β subunits (β2-4) have been cloned and characterized (77); they preferentially bind to (-) nicotine over (+) nicotine with approximately 40 fold higher affinity. While multiple nAChR subunits are expressed on non-neuronal cells, the homomeric α7 nAChR has been implicated as the primary receptor facilitating nicotine and NNK mediated cell proliferation (34). Interestingly, the expression of the α7 receptor itself has been shown to increase in response to nicotine stimulation. The proliferative effects of nicotine are reversed by the α7 specific antagonist, α-bungarotoxin (α-BT), or α-cobratoxin (α-CBT), suggesting that the α7 nAChR or its downstream effectors might present a potential target for cancer therapy (22, 44, 70). In contrast to α7 nAChR, the heteromeric α4β2 receptor regulates growth inhibitory responses including the

release of GABA, which typically acts as a tumor suppressor in a number of cancers including lung cancers (73). Both nAChR types are upregulated when exposed to chronic nitrosamine or nicotine stimulation; however, the inhibitory α4β2 nAChRs are desensitized due to constant high affinity stimulation, while the tumor promoting α7 nAChRs are able to remain active to exert their biological function. This is likely due to their lower affinity to these ligands and thus lesser chance of desensitization compared to the α4β2 receptors (24, 79). This may also be one of the reasons that nicotine exerts in cancer promoting effects primarily through the α7 receptors. Studies demonstrating that stimulation with nicotine or tobacco specific nitrosamines could induce cell proliferation through α7 nAChRs utilized concentrations of nicotine ranging from 10nM to 10μM, which is similar to the concentration observed in the bloodstream of smokers; the average steady state level found in the bloodstream of a heavy smoker is typically 1-2μM and can temporarily spike to 10μM or more immediately after smoking (80, 81).

Additional support for the correlation between nAChRs, smoking, and cancer was obtained from genome-wide association studies (GWAS), which identified a lung cancer susceptibility locus on chromosome 15q24-25. Polymorphisms in this region correlated with increased risk for lung cancer development, nicotine dependence, and number of cigarettes smoked per day (82). 15q24-25 contains *CHRNA3*, *CHRNA5*, and *CHRNB4* genes that encode for the α3, α5, and β4 subunits of nAChRs (83, 84). The α5 subunit has been implicated as the primary receptor involved in smoking addiction, and has more recently been associated with smoking behavioral patterns (hung 2008). Further, a non-synonymous variation in *CHRNA5 (D398N)* is strongly associated with increased lung cancer risk (83).

While nAChRs are the primary receptors through which nicotine exerts its effects, β-ARs and EGFRs are also thought to play a role (73, 85, 86). It has been suggested that nAChRs might

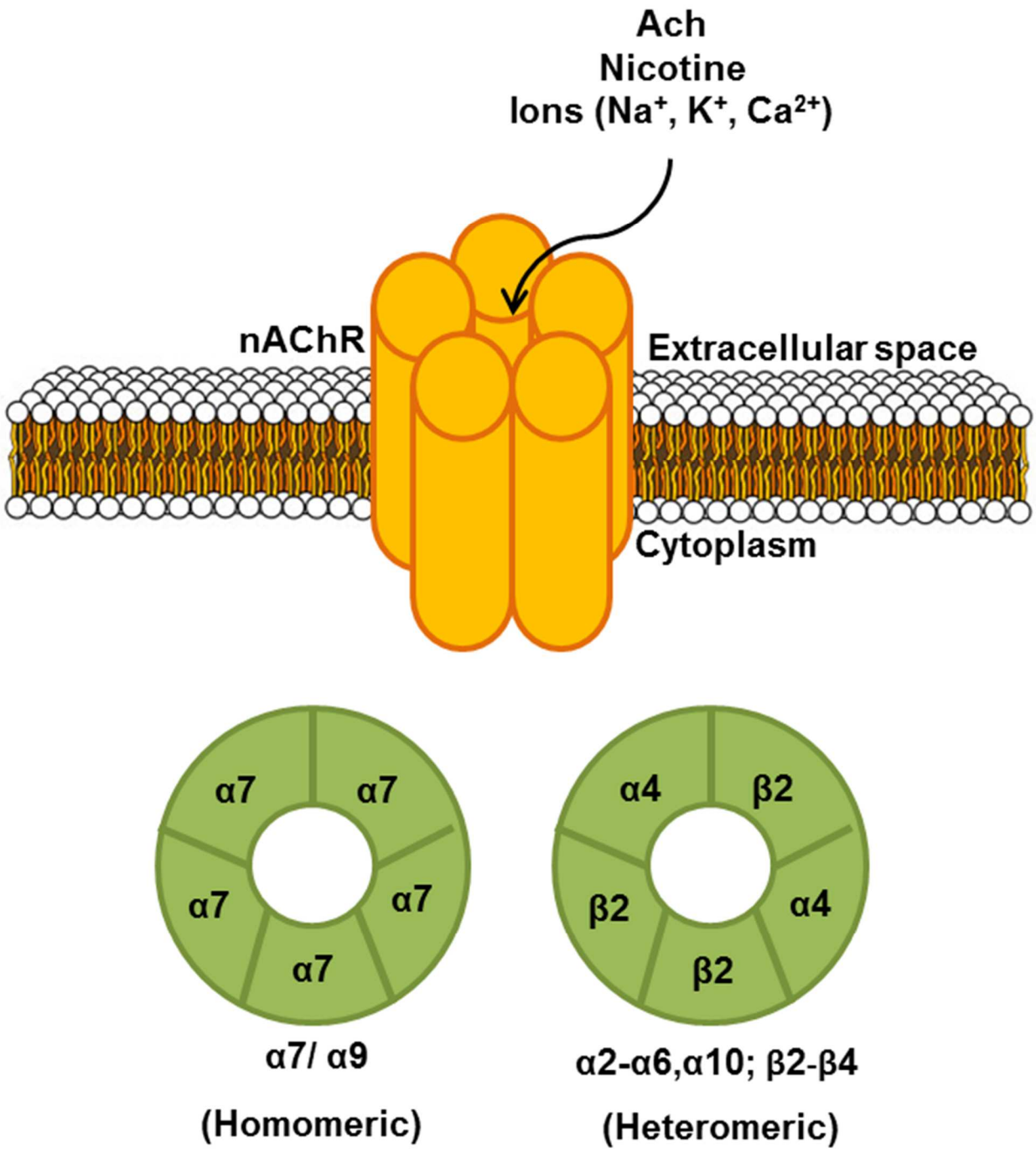


Figure 1.2. Depiction of nAChR Organization. nAChRs are pentameric receptors spanning the plasma membrane, which can be either homomeric or heteromeric in subunit composition. Homomeric receptors have either all α7 or all α9 subunits; while heteromeric receptors can be made up of α2-α6 or α10 in combination with β2-β4. These receptors are classical ligand-gated ion channels, and are activated by the binding of Ach and nicotine.

functionally network with β-AR and EGFR, and all three receptors frequently co-exist on human lung cancer cells, airway endothelial cells, and airway epithelial cells (87) Nicotine binds to α7 nAChRs inducing the secretion of growth factors such as EGF, neurotransmitters such as adrenaline and noradrenaline, and angiogenic factors such as VEGF (86). The nicotine mediated secretion of EGF *via* nAChRs results in the transactivation of EGFR, leading to the activation of mitogenic and antiapoptotic pathways (70, 88). Similarly, the nicotine mediated release of adrenaline and noradrenaline, which are the physiological ligands for β-AR, leads to the binding to and activation of β-AR, which in turn activates proliferative pathways and the release of EGF, VEGF, and arachidonic acid, which contribute to the development and progression of lung cancer (89). As mentioned earlier, nicotine and NNK have been shown to bind to β-ARs as well, stimulating multiple oncogenic and mitogenic signaling cascades (79, 89, 90). This cooperative interplay between these receptor types can be expected to have significant mitogenic effects on cells, contributing to the oncogenic process. The interplay between the different receptors that respond to nicotine and its derivatives is shown in Figure 1.3.

Signaling Cascades Initiated Downstream of nAChRs

nAChR Mediated Induction of ERK/MAPK

A number of studies have been conducted to elucidate the molecular mechanisms involved in nicotine and nitrosamine mediated cell proliferation, and these have revealed the involvement of multiple signaling cascades downstream of the nAChRs; the major pathways are depicted in Figure 1.4.

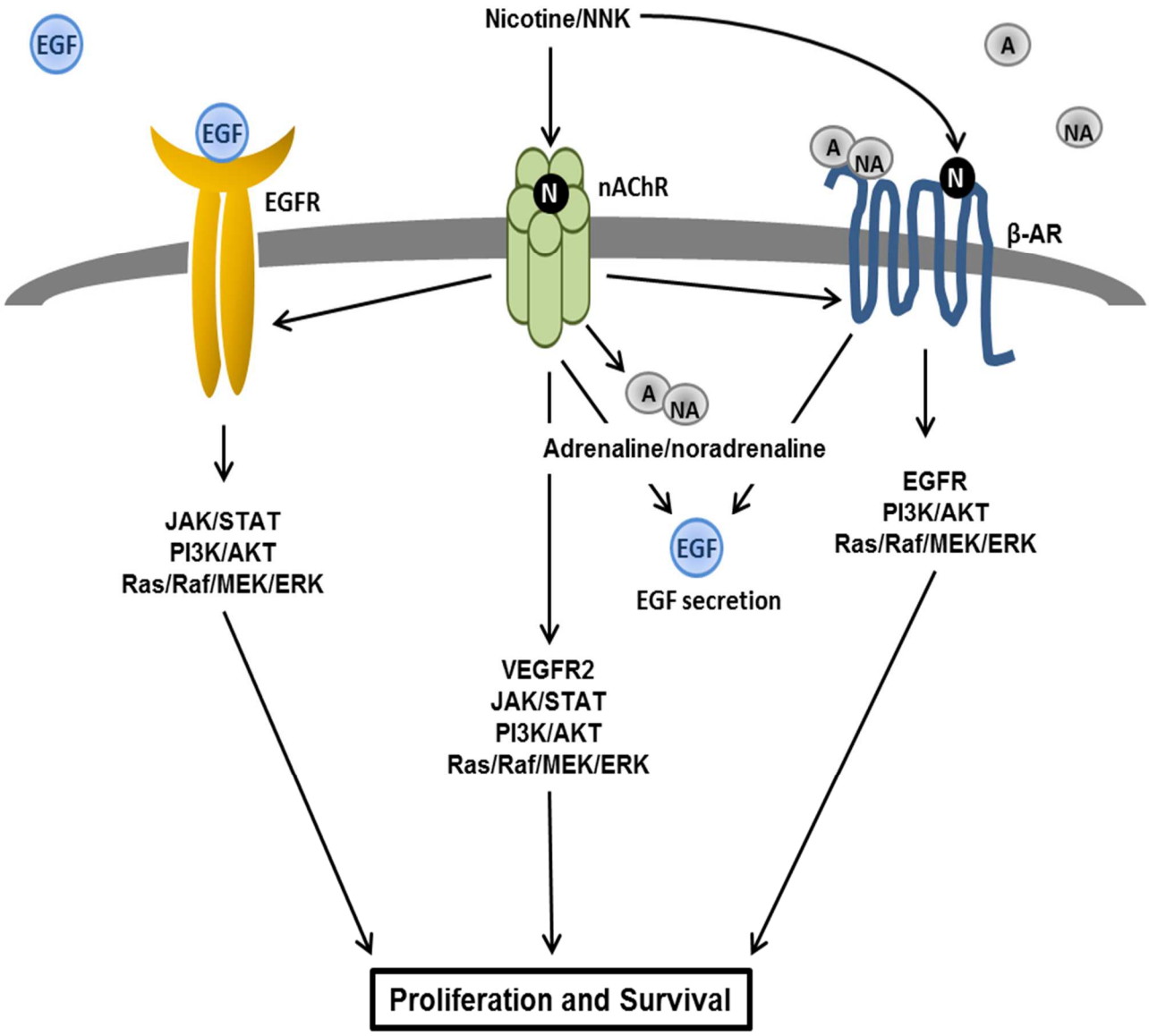


Figure 1.3. Cooperative Receptor Crosstalk Upon Nicotine Stimulation. Components of tobacco smoke induce nAChR signaling, which in turn activates additional cell surface receptors such as β-AR and EGFR, stimulating tumor promoting signaling cascades. In addition, upon NNK or nicotine binding, nAChRs are activated stimulating the secretion of growth factors such as EGF which activate EGFRs, as well as neurotrophic factors such as adrenaline and noradrenaline which bind to and activate β-ARs. Upon activation, β-ARs further stimulate secretion of EGF to further transactivate EGFRs. This receptor crosstalk suggests a cooperative interaction that facilitates tobacco induced cancer progression.(24)

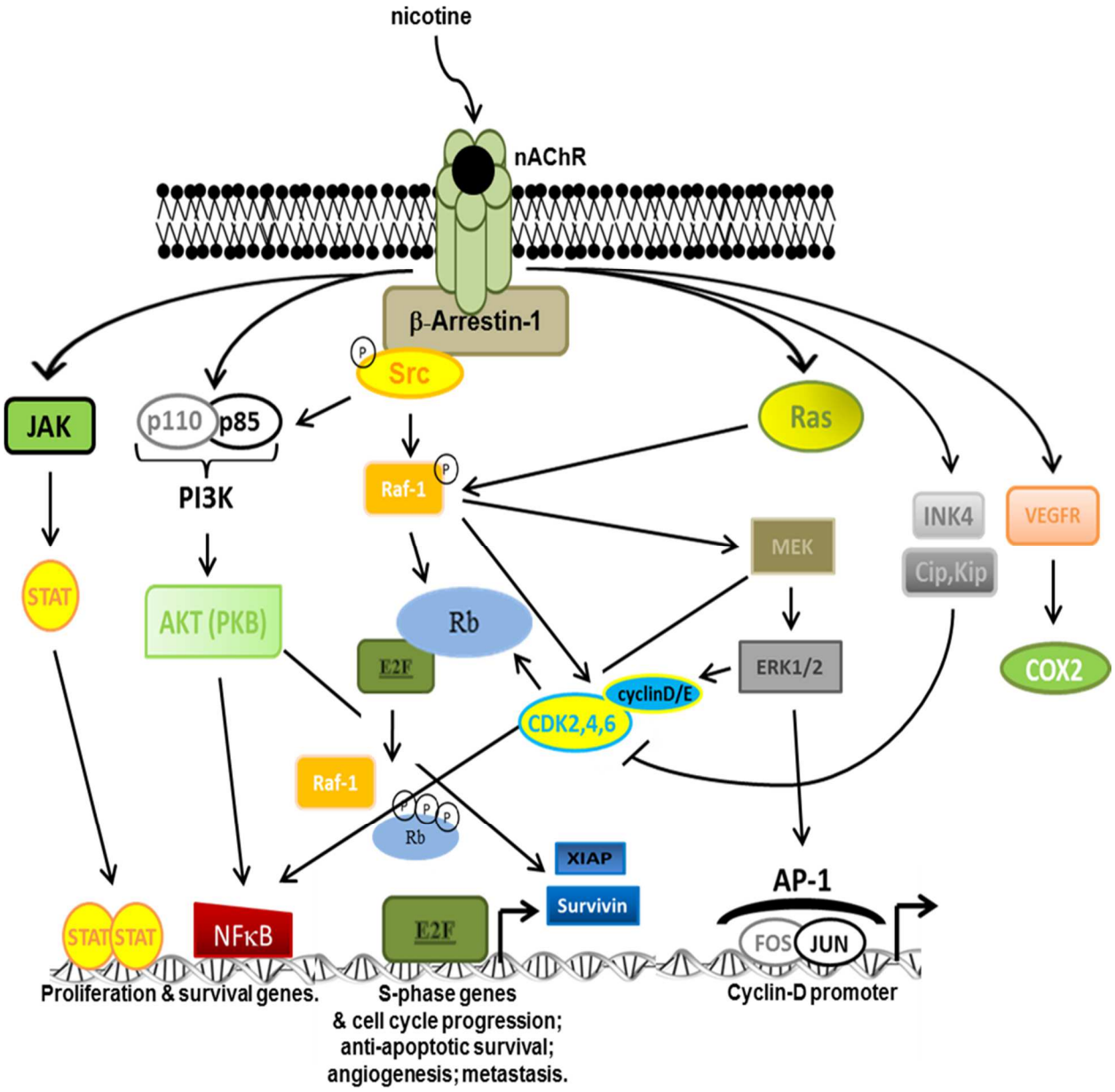


Figure 1.4. Tobacco Smoke Induced nAChR Signaling. A schematic representing nAChR signaling cascades in lung cancer. NNK and nicotine found in tobacco smoke bind to nAChRs with high affinity and induce multiple signaling cascades resulting in cycle progression, proliferation, and survival. Upon activation of nAChRs by ligand binding, b-arrestin-1 is recruited to the receptor and is necessary for further recruitment of Src kinase, which in turn initiates Raf-1 and PI3K/AKT signaling cascades. NNK and nicotine-mediated nAChR activation have also been shown to induce signaling cascades such as JAK/STAT and Ras/Raf/MAPK, and decrease the levels of cell-cycle inhibitors such as p16INK4 and Cip/Kip proteins. (24)

One signaling cascade that is induced by nAChRs across various cell types is the MAPK cascade, which is known to facilitate cell proliferation in a wide variety of tumor types. For example, stimulation of SCLC cells with nicotine was found to induce the MAPK cascade in a dose-

dependent fashion; this was diminished upon treatment with the nAChR inhibitor mecamylamine (91). Similar results were reported in another study on SCLC and NSCLC cells, where proliferation was increased and MAPK signaling was activated in response to nAChR stimulation; interestingly, this study demonstrated that the ERK2 isoform is specifically activated in response to nicotine (92). This activation of ERK2 occurs concomitantly, albeit separately, to the activation of PKC, as seen by a combination of western blotting experiments and MAP kinase assays (92). Multiple studies done on human and murine lung cancer cells have further demonstrated that PKC activity was increased in response to nicotine, and was activated independent of the Ras pathway (23, 93). Additionally, it was demonstrated that nicotine had no apparent effect of the levels of the MAPK family members p38 or JNK, or on the MAPK phosphatases PAC1 and MKP-1, indicating that these had lesser roles in nicotine mediated activation of MAPK signaling (92). Comparable to this finding on nicotine stimulation, it was found that stimulation of SCLC cells or neuroendocrine cells with the nitrosamine NNK resulted in increased DNA synthesis and proliferation, as well as activation of Raf-1 kinase; this activation could be blocked by inhibition of PKC or by the α7 nAChR specific antagonist α-BT (94). These results indicate that the observed effects were mediated through the α7 receptor, and that PKC acts upstream of Raf-1 in this signaling sequence (94). Additionally, it was found that NNK could induce any type of lung tumor including NSCLC through activation of Raf-1 kinase-ERK1/2 cascades in cooperation with PKA-CREB activation downstream of EGFR and β-AR receptors during crosstalk with nAChRs (95). Further, it was demonstrated that NNK stimulation increased the expression of c-Myc protein, suggesting that c-Myc is another target upregulated by NNK, probably through the activation of the MAPK cascade (94). A number of additional studies have validated these results, demonstrating that nicotine and NNK activate the MAPK cascade to induce cellular proliferation, and this can be inhibited by α-BT indicating that this chain of events is mediated specifically by the α7 nAChR. This is true for both cancer cells as well as normal cells (71, 88).

Activation of Src and the Rb-E2F Pathway via nAChRs

The above studies collectively demonstrate the ability of nicotine and NNK to induce the MAPK signaling cascade through the activation of nAChRs; however, unlike growth factor receptors, nAChRs do not possess intrinsic kinase activity (96, 97). A study designed to understand how α7-nAChRs induce MAPK cascade and promote cell proliferation demonstrated that the scaffolding protein β-arrestin-1 is recruited to the receptor upon nicotine stimulation and this was necessary for activation of Src Kinase (98). Src family kinases are known to facilitate signaling from cell surface receptors that lack intrinsic kinase activity and inhibition or depletion of Src could prevent nAChR-mediated downstream signaling as well as cell proliferation (98). This study further demonstrated that the activation of Src upon nAChR stimulation leads to the direct binding of the Raf-1 kinase to the Rb tumor suppressor protein, preceding the cyclin-cdk mediated inactivation of Rb. *In vitro* kinase assays showed an increase in the activity of kinases associated with cyclins D and E when cells were stimulated with 1μM nicotine; further, ChIP assays showed that Rb dissociated from proliferative promoters like Cdc6 and Cdc25A upon nicotine stimulation, leading to their expression (98). The binding of Raf-1 to Rb is thought to facilitate the inactivation of Rb by cyclins and cdks, and Raf-1-Rb interaction was found to be elevated in NSCLC tumor samples compared to adjacent normal tissue, indicating a potential role for this interaction in tumorigenesis (98). Indeed, a disruptor of the Rb-Raf-1 interaction was found to prevent the nicotine-mediated growth and metastasis of lung and pancreatic tumors in mouse xenograft models (99-102).

Interestingly, a subsequent study showed that stable depletion of β-arrestin-1 using shRNA resulted in decreased NSCLC cell proliferation with a corresponding decrease in the levels of phosphorylated Src kinase and ERK1/2 upon nicotine stimulation compared to control cells, indicating a predominant role for β-arrestin-1 in nicotine mediated activation of these signaling events (103). The same study investigated the sequence of signaling events induced by nicotine,

and it was found that phospho-Src levels were increased within 5 minutes of nicotine stimulation, while phospho-AKT1 and phospho-ERK1/2 levels increased after 15 minutes, suggesting that the latter two kinases are activated subsequent to Src activation (103). It was additionally found that a subset of β-arrestin-1 molecules can translocate into the nucleus upon nicotine stimulation where it physically associates with E2F1; β-arrestin-1 recruited the histone acetyl transferase p300 to E2F-regulated proliferative and pro-survival promoters, facilitating histone H3 acetylation and gene expression (103). The potential role for β-arrestin-1 in lung tumorigenesis was further suggested by the detection of elevated levels of β-arrestin-1 on proliferative promoters in human tumor samples compared to adjacent normal tissues. These observations reveal that activation of the Rb-E2F cascade is a major consequence of the nAChR mediated signaling events and that β-arrestin-1 contributes to this process significantly. β-arrestin-1 plays a major role in the de-sensitization of various G-protein coupled receptors; thus, the involvement of β-arrestin-1 also suggests an additional functional link between nAChRs and G-protein coupled receptors like β-ARs and opioid receptors upon exposure to nicotine, which might have a significant impact on the growth of lung cancer in smokers.

Induction of the JAK-STAT Pathway via nAChRs

In addition to activation of the MAPK and Rb-E2F pathways, it has been reported that nicotine stimulation results in the activation of the JAK-STAT signaling pathway and its target genes, which are altered in multiple cancer types (104-107). In a study conducted on oral keratinocytes, stimulation of cells with physiologically relevant concentrations of either nicotine or sidestream cigarette smoke resulted in increased STAT3 mRNA and protein levels; these levels were diminished if the cells were pretreated with the α7 nAChR specific inhibitor α-BT, or if α7 nAChR was depleted using siRNA (108). Further experiments utilizing specific pathway inhibitors revealed that the α7-dependent upregulation of STAT3 occurred through activation of the Ras-Raf-1-MEK-ERK pathway, as well as through the activation of JAK2, indicating the involvement

of two complementary pathways (108). In α7 null mouse cells, nicotine was unable to induce Ras mediated activation of STAT3, further demonstrating that this signaling event is mediated through the α7 nAChR (108). In a separate study done on NSCLC, stimulation with physiologically relevant concentrations of nicotine or NNK resulted in increased phospho-STAT3 levels, leading to an induction of the STAT3 target IKBKE (107). Src kinase is known to activate the JAK-STAT pathway in multiple systems, and nicotine was shown to activate Src kinase which in turn activated the JAK/STAT pathway signaling in cooperation with MEK/ERK1/2 pathway resulting in increased proliferation of pancreatic cancer cells (105). In the same study, nicotine stimulation resulted in increased expression of phospho-JAK2 and enhanced activation of STAT3 through phosphorylation at Y705 in a dose dependent manner, while total STAT3 levels remained unchanged. These effects were abrogated by treatment with the antagonist α-BT, indicating that the signaling was mediated by α7 nAChR (105). Further, nicotine stimulation resulted in an ERK1/2 mediated increase in STAT3 phosphorylation at S727, and this could be reversed by treatment with an ERK inhibitor (105). Immunohistochemical staining of tumors from pancreatic mouse xenograft studies supported these findings, showing increased phosphorylation of STAT3 at Y705 in mice exposed to cigarette smoke (105). Interestingly, similar findings were reported in pancreatic cancer cell lines, where nicotine could induce the mucin MUC4 through activation of the α7 nAChR, in a Tyk2-Src-ERK dependent manner upstream of STAT1 and E2F1 transcription factors (104). This study showed that nicotine-mediated migration and invasion could be abrogated by depletion of MUC4; and that MUC4 gene expression was regulated by STAT1 and E2F1 (104). Upstream of these events, nicotine was reported to increase phosphorylation of Tyk2, in addition to increasing the phosphorylation and dimerization of STAT1, elevating its transcriptional function in the nucleus (104). Nicotine stimulation was shown to activate STAT3 *via* nAChRs as well as β-ARs in bladder cancer cells, leading to overexpression of cyclin D1 driving cell cycle progression, resulting in reduced sensitivity to chemotherapy; sensitivity could

be restored by depletion of STAT3 (109). These studies clearly suggest a role for the JAK-STAT pathway in mediating the proliferative functions of nAChRs.

Upregulation of Cell Cycle Regulatory Molecules

In addition to inducing multiple mitogenic signaling events, nicotine and nitrosamines have been shown to target various components of the cell cycle machinery itself. Numerous studies have shown that the expression of cyclins as well as proteins involved in cell cycle checkpoints are impacted by nicotine and other components of tobacco smoke. In this context, increased expression of cyclin D1 in response to nicotine stimulation has been reported frequently in the literature (47, 110, 111). Initial studies conducted on mouse lung epithelial cells demonstrated the ability of nicotine to induce cyclin D1 promoter as well as protein expression through nAChR-mediated induction of Ras signaling and activation of its downstream effectors Raf/MAPK (112). It was further demonstrated that Ras activation of c-Jun, which is part of the AP-1 transcription factor complex, binds to the cyclin D1 promoter and is necessary for its nicotine mediated induction (112). The upregulation of cyclin D1 in response to nicotine was shown to occur concomitantly with an increase in Rb phosphorylation and E2F transcriptional activity (112), promoting the cell cycle progression of lung epithelial cells. nAChR-mediated proliferation *via* upregulation of cyclin D1 has additionally been reported in mouse pre-osteoblasts (30). In normal human lung epithelial cells, nicotine and NNK have been shown to upregulate cyclin D1 through the ERK1/2 mediated activation of the NFκB, and this signal transduction is facilitated *via* nAChRs (113). Similar results have been reported in human NSCLC cells, where nAChR activation by nicotine stimulation led to an induction of cyclin D-cdk4 and cyclin E-cdk2 kinase activity and Rb phosphorylation, resulting in dissociation of Rb from E2F1 and the induction of E2F1 regulated proliferative genes, including Cdc25A and Cdc6 (47, 111). In addition, nicotine and NNK facilitate cell cycle progression *via* nAChR and β-AR mediated induction of COX2 and prostaglandin E2 (PGE2), with an associated increase in cyclin D1 and cdk4/6 expression and Rb phosphorylation (114). In addition to these cell cycle

regulatory molecules, a study on human head and neck squamous cell carcinomas (HNSCC) showed that nicotine could enhance the expression of FOXM1 transcription factor, which is upregulated in early stages of HNSCC and known to drive cell cycle progression and mitosis; and this was found to happen during malignant transformation (115).

Suppression of Cell Cycle Inhibitors by Nicotine and NNK

It has been established that components of tobacco smoke can also repress negative regulators of cell cycle progression, such as cdk inhibitors (CDKis) (110, 116). CDKis act to regulate cdk activity and are divided into two classes: the INK4 inhibitors including p16, p15, p19, and p18; and the Cip/Kip inhibitors including p21 and p27 (110, 116). Both CDKi types are capable of arresting cells in G1 phase by inhibition of cdks, preventing their phosphorylation of Rb and thus halting cell cycle progression (110, 116). In human gastric cancer cells, p53, p21, and p27 expression levels are significantly reduced in response to nicotine or NNK stimulation (114). In NSCLC cell lines, nicotine has been shown to induce the transcriptional repressor, ID1, in a Src dependent manner through the α7-nAChR (117). ID1 is known to induce proliferation by inhibiting the transcription of CDKis p16, p21, and p27, preventing their normal growth suppressive effect (118). This data implies that tobacco smoke not only activates components driving cell cycle progression, but also inhibits components that arrest cell cycle.

In addition, it has also been shown in murine and human lung epithelial cells that nicotine acts to compromise activation of normal DNA damage checkpoint response at G1/S, but not the G2/M checkpoint (119). DNA damage check point is activated after exposure to γ-radiation or the tobacco carcinogen benzopyrene , which induces DNA double strand breaks; however, when exposed to nicotine, growth restriction was attenuated due to an increase of cyclins D and A, and a decrease in the phosphorylation of the checkpoint kinase 2 (Chk2) (119). Typically ATM and ATR protein kinases are activated in response to double or single stranded DNA breaks respectively, and they stimulate downstream effectors involved in DNA damage checkpoint

response including p53, Chk1, Chk2, and H2AX (116). Activation of these proteins results in cell cycle arrest and DNA repair, or if the damage is beyond repair apoptosis will be induced (116). Once activated, Chk2 phosphorylates Cdc25A phosphatase, preventing it from dephosphorylating and thus activating its target cdk2, resulting in cell cycle arrest (116). The decrease in Chk2 in lung epithelial cells exposed to nicotine suggests that nicotine may be capable of overriding DNA damage checkpoint activation, disrupting genetic surveillance and increasing the risk of oncogenesis (116, 119). These studies show that nicotine as well as tobacco smoke components can modulate multiple components of the cell cycle machinery, facilitating cell cycle progression.

Regulation of Survival Pathways – Anti-Apoptotic Effects

While tobacco smoke carcinogens initiate tumors, the current literature raises the possibility that nicotine additionally confers a survival advantage to already initiated tumors (47, 120-122) by promoting cell cycle progression and by preventing apoptosis (121, 123). This is of particular concern in the context of tobacco smoke components conferring resistance to chemotherapeutic drugs (121, 123) as well as radiation (124). Multiple studies have reported that patients who smoke demonstrate poor response to cancer chemotherapy, and have worse prognosis than their non-smoking counterparts (120, 125, 126). In this context, many attempts have been made to elucidate the pro-survival and anti-apoptotic effects of nAChR signaling.

The PI3K/AKT pathway is a major cancer-associated signaling network that is activated by exposure to nicotine and tobacco carcinogens (127-131). The serine/threonine kinase AKT is a known regulator of key cellular processes such as cell cycle progression, as well as cell survival (127, 131). Both nicotine and NNK were found to induce AKT phosphorylation at S473 and T308 in a time and dose dependent manner in normal human bronchial epithelial cells and small airway epithelial cells (127). This effect was evident as early as 5 minutes post nicotine stimulation and at a range of physiologically relevant concentrations, from 10nM to 10μM; however, response to NNK was not apparent for 15 minutes but was seen at concentrations as low as 1nM (127).

Similarly, downstream substrates of AKT involved in cell cycle progression including GSK-3α, FKHR, and 4EBP-1 were all induced by nicotine, while GSK3-α and 4EBP-1 were induced by NNK (127). Further, this response could be abrogated by the PI3K inhibitor and by the α3/α4 specific nAChR antagonist DHβE, but not by the α7 specific antagonist α-BT, indicating the AKT was being activated through PI3K and this was being facilitated through α3 and α4 containing nAChRs (127). Interestingly, phosphorylated AKT was detected in airway epithelial cells and lung tumors from mice treated with NNK, as well as in human lung cancer samples derived from smokers (127); similar results were observed in a study conducted on NSCLC and SCLC cells (131). In the latter study, stimulation of cells with physiological concentrations of nicotine and NNK induced activation of AKT as well as its downstream substrates GSK-3, FKHR, tuberin, ASK1, 4EBP-1and S6K1; nicotine additionally induced mTOR and MDM2 (131). This study further demonstrated nicotine and NNK could induce proliferation of wild type cells, but not cells transduced with a mutant AKT, indicating that the proliferation seen in response to these tobacco components is AKT dependent (131).

In this context, a direct link between activation of PI3K/AKT pathway by nicotine and induction of chemoresistance has been proposed. Exposure of multiple NSCLC cells to 1μM nicotine conferred resistance to apoptosis induced by cisplatin, gemcitabine and taxol (47). It was found that exposure to nicotine led to an increase in the levels of inhibitor of apoptosis proteins (IAPs) XIAP and survivin in a α3β4 nAChR dependent manner, downstream of AKT signaling (47, 121); interestingly, nicotine did not induce cIAP1 or cIAP2 (47). AKT mediated phosphorylation is known to prevent the ubiquitination and degradation of XIAP (132); reduced ubiquitination and stabilization of XIAP was observed upon nicotine stimulation, correlating with reduced apoptosis (133). Further, induction of survivin by nicotine occurred at the transcriptional level in an E2F1-dependent fashion, suggesting that exposure to nicotine can confer resistance to apoptosis through multiple mechanisms (47). NFκB transcription factor is induced by nicotine and

nitrosamines, promoting cell survival. Nicotine has been shown to induce NFκB *via* the MAPK pathway, promoting anti-apoptotic functions in mesothelioma cells, mesothelial cells, normal bronchial airway cells, and small airway epithelial cells (113, 134). Similarly, nicotine was shown to confer a survival advantage to NSCLC and SCLC cells treated with paclitaxel, and this occurred with a concomitant increase in NFκB levels. This study also showed that the ability of nicotine to promote survival was dependent on NFκB, which was activated downstream of AKT (131). In normal human bronchial epithelial cells, NNK was found to induce nuclear translocation of NFκB and increase its DNA binding activity within 5 to 10 minutes of stimulation, reaching maximal activation within 30 minutes (113). It was further demonstrated that the activation of NFκB occurred through the degradation of its negative regulator IκBα, mediated through ERK1/2 phosphorylation of IκBα (113). In colon cancer cells, NNK has been shown to induce NFκB in association with proliferation, specifically the p65/RelA subunit, with a concomitant decrease in IκB, which is known to have inverse correlation to NFκB levels (135). These results strongly suggest that nAChR stimulation enhances NFκB activity by targeting IκBα for degradation.

Additional survival pathways have also been reported to be induced by nicotine. For example, nicotine was reported to induce autophagy in colon cancer cells through activation of α7 nAChR upstream of PI3K, which resulted in increased levels of Cox2 and PGE2 (136). Conversely, another study found that nicotine could impair autophagy in normal human bronchial epithelial cells (137). The discrepancy between these two papers could be due to differences in cell types studied or due to the concentration of nicotine used, as the study done on bronchial epithelial cells used 5mM of nicotine, which is highly concentrated compared to the study done in colon cancer cells. There is limited literature available on the impact of nicotine on autophagy, and further study is warranted. Additionally, nicotine stimulation could modulate the levels of Bcl-2 family of anti-apoptotic proteins (138). In SCLC cells, nicotine exerts a protective effect against apoptosis induced by cisplatin by inducing the activation of Bcl-2 (121); additionally, nicotine

exposure results in the phosphorylation and inactivation of the proapoptotic proteins Bax and Bad, suppressing cell death (47, 138). This inactivation is mediated by the induction of PKC, PKA, MEK, and P13K signaling cascades (139).

In addition, components of the TGF-β signaling cascade are modulated by nicotine to promote survival. It was found that exposure of NSCLC cells and immortalized bronchial epithelial cells to nicotine decreased the expression of SMAD3, resulting in reduced TGF-β mediated growth inhibition (140). Simultaneously, levels of anti-apoptotic Bcl-2 were increased, resulting in increased cell viability and reduced apoptosis. Interestingly, SMAD3 levels were lower in tumor samples from current smokers compared to never-smokers, and withdrawal of smoking reduced SMAD3 levels (140). Another study conducted on NSCLC cells showed that long-term exposure to nicotine resulted in increased half-maximal inhibitory concentration (IC_{50}) of carboplatin (141). This increase in IC_{50} was associated with elevated Bcl-2 expression and decreased SMAD3 levels (141); the sensitivity to carboplatin was dependent upon this reciprocal relationship (141).

Studies done on pancreatic cancer have also reported the ability of nicotine to confer survival advantage to cells and confer resistance to chemotherapeutic agents. Nicotine was reported to increase proliferation and migration on pancreatic ducal adenocarcinoma cells through the induction of Src kinase and subsequently ID-1 transcription factor, resulting in increased resistance to gemcitabine in vitro (122). Depletion of Src or ID-1 resulted in diminished ability of nicotine to confer resistance to gemcitabine in these cells. It was further demonstrated that nicotine conferred resistance to gemcitabine in mouse pancreatic xenograft models, confirming this contention in vivo (122). These studies clearly show that exposure to nicotine and activation of nAChR signaling not only promotes the proliferative capacity of lung cancer cells, but also renders them resistant to various apoptotic signals and chemotherapeutic agents.

Additional Tumor-Promoting Functions of nAChRs: EMT, Angiogenesis, and Stemness

Nicotine and nAChRs have been found to affect multiple steps in the growth and metastasis of cancers. nAChRs were found to promote the invasion and migration of cells derived from tumors

of the breast, pancreas or the lungs (142). Nicotine has been found to be a strong inducer of angiogenesis and epithelial-mesenchymal transition (EMT), both thought to be precursor steps to cells migrating out of the primary tumor site to metastasize to distal organs. Multiple studies have demonstrated the ability of nicotine to enhance endothelial cell recruitment and new vessel formation, primarily through stimulating increased VEGF and FGF secretion (23, 143, 144). Inhibition of α7 nAChR using a general nAChR antagonist hexamethonium bromide, or the α7 specific antagonist α-BT could reverse this effect, suggesting nicotine induces angiogenesis through these receptors (43, 44, 144). Our lab has shown that depletion of ID1 transcription factor resulted in the inability of human microvascular endothelial cells from the lung to form angiogenic tubules, and this occurred downstream of Src kinase, through α7-nAChRs (46). Interestingly, Src was found to be necessary for nicotine-mediated angiogenic tubule formation by human microvascular endothelial cells of the lung, but not VEGF induced tubule formation (44). Nicotine has additionally been shown to induce Raf-1 kinase-Rb interaction facilitating angiogenic tubule formation in vitro, and that disruption of this interaction could abrogate this effect (44).Nicotine stimulation could further mimic exposure to hypoxia, by increasing accumulation of HIF-1α and VEGF proteins, resulting in increased angiogenesis (145, 146).

In addition to angiogenesis, nicotine has been show to enhance EMT. Our lab has demonstrated that nicotine disrupts tight junctions and alters cell morphology to a more mesenchymal phenotype, facilitating EMT and invasion (21), and this occurred through β-arrestin-1 but not β-arrestin-2 (46). Further, nicotine could suppress expression of epithelial marker E-cadherin, tight junction protein ZO-1, as well as membranous β-catenin, while inducing mesenchymal proteins vimentin and fibronectin in both lung and breast cancer cells lines (21). It was further shown that nicotine-mediated induction of vimentin and fibronectin, and additionally ZEB1 and ZEB2, occurred through β-arrestin-1 mediated regulation of E2F1 transcription factor in the nucleus downstream of Src kinase in NSCLC; and cells lacking β-arrestin-1 had diminished ability to metastasize in a murine orthotopic lung implantation model (46). Further, ID1 transcription factor,

which is induced by nicotine and plays a role in angiogenic tubule formation, could also induce expression of vimentin and fibronectin by downregulating the zinc finger repressor protein ZBP-89 (86).

Another mechanism by which nicotine promotes metastasis is by upregulating the expression of matrix metalloproteinases, including MMP9, MMP14 and MMP15; this occurred in an E2F1 dependent manner. (147). E2F1 was found to bind to and induce expression of MMP9, MMP14, and MMP15 gene promoters which occurred downstream of Raf-1 kinase mediated phosphorylation of Rb, releasing E2F1 from Rb, allowing it to transcriptionally induce these promoters (147). This result could be abrogated by disruption of the Rb-Raf-1 interaction through use of a small molecule inhibitor in vitro, and treatment with this inhibitor also resulted in reduced metastasis of NSCLC tumor cells in vivo (147).

It has also been proposed that nicotine and nAChRs can contribute to stem-like functions of tumor initiating cells, by inducing embryonic stem cell transcription factors including Oct4 and Nanog (56, 57). Nicotine treatment also enhanced the expression of CD44 and BMI-1, and promoted the sphere formation capacity of squamous cell carcinoma cells (56). It has been reported that nicotine enhances ALDH1+ cell populations in oral squamous cell carcinomas as well as normal gingival cells, with a concomitant increase in self-renewal, stemness gene signatures, and EMT markers (57). Similarly, in MCF7 breast cancer cells, nicotine increases CSC populations as indicated by an increase in ALDH1+ cells in a dose dependent manner, through activation of PKC and Notch; an effect which could be abrogated by α-BT indicating this process likely occurs through α7 nAChRs (55). Later studies reported that nicotine enhances breast cancer SP cells as well, through an α9 nAChR-STAT3-galectin-3 mechanism (53).

In pancreatic cancer, nicotine stimulation results in acquisition of gene expression and functional characteristics of CSCs and can induce de-differentiation of acinar cells which frequently give rise to metaplasia (54). Further, nicotine stimulation results in enhanced secretion of the neurotransmitters epinephrine and norepinephrine as well as Sonic Hedgehog (SHH), and

reduced secretion of the inhibitory neurotransmitter GABA with a concomitant increase in self-renewal; these pancreatic CSCs were also reported to express α3, α4, α5, and α7 nAChRs, and their expression increased in response to nicotine (52). Our lab has also demonstrated the ability of nicotine to promote the self-renewal of stem-like SP cells from NSCLC through the induction of stem cell factor (SCF/c-Kit ligand), and the expression of SCF strongly differentiated smokers from non-smokers and correlated with poor prognosis (51). The E2F1 transcription factor could induce expression of SCF at the transcriptional level, and depletion of E2F1 or β-arrestin-1 resulted in diminished ability of nicotine to induce self-renewal in sphere formation assays (51). Further, nicotine could increase secretion of SCF and neutralizing antibodies to SCF also diminished the ability of nicotine to induce self-renewal (51). Overall this data suggests that nicotine induces self-renewal of SP cells through increased expression of SCF in a β-arrestin-1/E2F1 dependent manner.

In addition to nicotine, cigarette smoke condensate has also been found to enhance SP cell populations in both lung and head and neck cancer cells through a phospho-AKT pathway, which was accompanied by an increase in ABCG2 drug transporter (56). Further, we have recently demonstrated that nicotine can enhance NSCLC adenocarcinoma CSC stemness through the embryonic stem cell transcription factor, Sox2. While the above studies indicate additional roles for nicotine in promoting EMT and stemness, further studies in this direction would be needed to obtain a complete picture of these events.

Electronic Cigarettes: A Gap in the Knowledge

While the effects of nicotine and its derivatives in tobacco smoke are well documented, the use of electronic cigarettes, or e-cigarettes, has emerged as a healthier alternative to traditional cigarette smoking; however, due to lack of scientific evidence on short or long term health effects associated with their use, this remains controversial (11, 148). While e-cigarettes do not contain tobacco and the multiple classes of carcinogens found in tobacco, they do still contain nicotine in

addition to other components such as propylene glycol, vegetable glycerol, and flavorings (148, 149). These devices are typically used by pressing a button which activates and internal heating coil which brings the e-liquid containing nicotine to a boil, which is then delivered as a vapor to the user (148). The concentration of nicotine present in e-cigarettes varies by brand and container, but is typically represented as percent nicotine by volume (NBV) on the packaging.

Recent publications have discussed the utility of e-cigarettes for smoking cessation. While e-cigarette use is not encouraged for those who have never smoked, it has been argued that they may be a healthier alternative to traditional cigarette smoking and can be used as a means to attain smoking cessation. It has been known for over a decade that there are considerable benefits to any individual who stops smoking including reduced cancer risk, improved therapeutic efficacy in patients with already established cancers, rapid reversal of adverse cardiovascular effects, and improved health in multiple other areas (150). Nicotine replacement agents to aid in cessation have been shown to double success rates of quitting smoking, and these agents previously included things like transdermal nicotine patches, nicotine gum, and other nicotine replacement therapies (150). While e-cigarettes have been around for roughly ten years, their use has just recently been rapidly on the rise (151) and is just now being regulated by the Food and Drug Administration (FDA). Preliminary studies have shown e-cigarettes to be successful smoking cessation agents in cancer patients. In a recent report discussing the use of e-cigarettes for lung cancer patients, it is stated that the quality of e-cigarettes and e-liquid has been improving and most all constituents are now pharmaceutical grade, most flavorings are food grade, there are reduced impurities, no solid particles or carbon monoxide, and there are no harmful components present in significant levels with the exception of nicotine (149). This report further details that there is 100 fold less nitrosamine content in electronic compared to traditional cigarettes, that e-cigarettes are 20 fold less dangerous (149). It has also been shown that smokers who switch to e-cigarettes show steady improvements in their breathing and respiratory

health as indicated by exhaled breath (FeNo) and exhaled carbon monoxide (eCO) normalization (152).

Studies have begun to look at impact of e-cigarettes on gene expression; in nasal epithelial cells it was reported that biopsies from e-cigarette users showed decreased expression of a number of immune and inflammatory response genes identical to biopsies from traditional cigarette smokers; they also had an additional number of unique genes repressed (153). A recent study has reported that e-cigarette aerosol contains oxidants and copper nanoparticles that impact normal human lung fibroblasts (154). Here they report that e-cigarette aerosols containing these components could increase mitochondrial reactive oxygen species (mtROS) concomitant with reduced OxPhos electron transport chain complex IV subunit, increased nuclear fragmentation, and increased levels of IL-8 and IL-6 (154). This data suggests mitochondrial, genotoxic, and inflammatory stresses occur in response to e-cigarette aerosol, which may lead to deleterious cellular effects (154). While e-cigarettes may be safer than traditional cigarettes in certain aspects, how nicotine present in e-cigarettes impacts the pathophysiology and health of users remains to be elucidated. Further, whether the additional components of the e-liquid might abrogate or amplify the effects of nicotine in the context of cancer has not been determined. In a study done to assess the presence or absence of nicotine exposure after e-cigarette use, it was found that there was no significant difference in urinary cotinine levels (indicative of metabolized nicotine) between e-cigarette users and cigarette smokers, while there was a difference between e-cigarette users and healthy non-smokers, demonstrating that e-cigarette users are exposed to as much nicotine as cigarette smokers (155). Recently in our lab, we have found that three different brands of e-cigarette extracts can enhance a number of tumor promoting properties in a manner similar to nicotine alone (Schaal, C. et al., manuscript under preparation). Each of the three e-cigarettes could enhance the Sox2 embryonic stem cell transcription factor and self-renewal of cancer stem-like cells, and could further enhance mesenchymal markers and migration of NSCLC cells (Schaal, C. et al, manuscript under preparation). Additionally, stimulation with e-

cigarette extracts resulted in increased expression of the α7 nAChR, the receptor primarily responsible for facilitating nicotine-mediated tumor progression. Few studies have been conducted regarding the effects of e-cigarettes at the molecular level specifically in the context of cancer, and this warrants further study which we can expect to be already underway.

Conclusions

While nicotine itself is not a carcinogen, it has been found to promote a wide variety of tumor-promoting properties, almost in a pleiotropic manner, in a number of cancer types. Once tumors are initiated by carcinogens like the nicotine-derivative nitrosamines NNK and NNN, nicotine acts to enhance multiple stages of tumor progression from proliferation, angiogenesis, chemoresistance, and survival of primary tumors, to EMT, migration, and invasion leading to tumor metastasis. More recently, it has been shown that nicotine enhances the self-renewal of cancer stem-like cells, which are implicated in every stage of tumor initiation and progression, and are thought to be resistant to therapies, can remain dormant, and result in recurrence and dissemination. In the context of patients who smoke and whose cancers were initiated as a result of smoking, targeting pathways which nicotine utilizes to exert its tumor-promoting functions seems to hold promise for therapeutic efficacy. Elucidating these pathways and how to target them is therefore, of keen importance. The primary mechanism by which nicotine exerts its effect is through the binding to and activation of α7 nAChRs and the subsequent activation of downstream signaling events. How α7 nAChR is regulated is not well understood. Here we elucidate a mechanism by which nicotine itself induces expression of α7 nAChR, through E2F1 and STAT1 transcription factors in NSCLC. Additionally, little is known regarding the molecular mechanism underlying nicotine-mediated promotion of cancer stem-like cell phenotypes and gene expression. Here we demonstrate that nicotine can upregulate the expression of Sox2 transcription factor which is critical for the maintenance of CSCs in NSCLC, and the mechanism through which this occurs. We believe that these studies fill a gap in our

understanding of nicotine function and how nicotine affects the biology of NSCLC cells exposed to this agent.

CHAPTER TWO:

MATERIALS AND METHODS

Kaplan Meier Survival Plots

The Kaplan Meier survival curves were generated using KM-plotter online analysis tool (http://kmplot.com/analysis), which has previously been described in detail (156-158). KM-plotter is a meta-analysis based method of assessing biomarkers in breast, ovarian, lung, and gastric cancers using data derived from publicly available microarray databases GEO (Gene Expression Omnibus), TCGA (The Cancer Genome Atlas), and EGA (European Genome-phenome Atlas). To date, it is capable of assessing the effect of 54,675 Affymetrix gene-chips on survival outcome using 10,888 patient samples; including 2,437 lung cancer cases. For our analysis, parameters were set using the 2015 version of the analysis tool, univariate cox regression analysis was used, and biased arrays were excluded. Gene symbols *CHRNA7*, *CHRNA3*, and *CHRNA5* were assessed in NSCLC for all histological subtypes, smoking status, and gender differences. Gene expression which correlated with survival with a p value <0.05 was considered statistically significant.

Cell Lines

Human non-small cell lung adenocarcinoma cell lines A549, H460, and H1650 were obtained from the American Type Culture Collection (ATCC). A549 cells were maintained in Ham's F12K medium (Cellgro, Mediatech, Inc.) supplemented with 10% fetal bovine serum (Atlas Biologicals), and H460 as well as H1650 cells were maintained in RPMI 1640 (Gibco, Life Technologies, Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum. Normal human bone marrow derived mesenchymal stem cells (hMSCs) were purchased from Lonza and maintained in their mesenchymal stem cell basal growth medium (MSCGM) designed to

maintain these cells in a proliferative but not differentiated state. A549 and H1650 cell lines have been validated by ATCC and subsequently by Moffitt Core Facilities by STR analysis. Primary human mesenchymal stem cells (hMSCs) were pre-validated by Lonza, and were only used up to passage 10.

Generation of Stable Cell Lines

A549 cell line was used for generating stable overexpression cells. The overexpression of Sox2-core-luc and YAP1 (Addgene #15682) expression plasmids was achieved transfecting these plasmids using FugeneHD reagent transfection (Promega) per manufacturer's protocol, in addition to pCDNA2 vector containing G418 selection marker per manufacturer's instructions. The transfected cells were selected using G418 and maintained in Ham's F12K medium, single colonies were selected and expanded for use in experiments.

Nicotine, E-cigarettes, and Inhibitor Studies

(-)-nicotine (N3876; Sigma-Aldrich) or e-cigarettes (local stores) were used in these studies. A549, H460, or H1650 cells were rendered quiescent by serum starvation in media containing 0.1% fetal bovine serum for 24 hours, following which cells were stimulated with 1.5μM or 2μM nicotine (which are the physiologically relevant concentrations detected in the bloodstream of smokers (hukkanen pharmological reviews 2005) or e-cigarette extracts for the indicated time points. For studies using nicotine or e-cigarette extracts in hMSCs, cells were not serum starved but were stimulated with 2μM nicotine or e-cigarette extracts 24 hours after plating, for indicated time points. For studies using signal transduction inhibitors/anti-cancer drugs, cells were rendered quiescent by serum starvation for 24 hours, were treated with inhibitors for 30 minutes, and then stimulated with 2μM nicotine in the presence or absence of inhibitors. The inhibitors used were AZD0530/Saracatinib (Sellekchem) at 10μM, NVP-BKM120/Buparsilib (Chemietek) at 20μM, GSK1120212/Trametinib (Chemietek) at 10μM, LEE001/Ribociclib (Chemietek) at 20μM, RRD251 at 10μM, α-bungarotoxin (Sigma) at 10μM, or visudyne (Sigma) at 2μM.

Three different brands of e-cigarettes were used to demonstrate the effects observed were not specific to just one type; these included Fin, Njoy, and Mistic (which will be referred to as E-cig 1, E-cig2, or E-cig3, respectively). E-cigarette liquid was obtained through extraction of an internal liquid-soaked sponge within the devices for E-cig 1 and 2, or by syringe extraction for E-cig 3. E-cig 1, 2, and extracts were 1.6% nicotine by volume (NBV) or 16mg/ml, 1.5% NBV or 15mg/ml, and 1.8% NBV or 18mg/ml respectively as indicated on the manufacturer's packaging. Molarity of extracts from each brand was calculated based on the molecular weight of nicotine of 162.23, and the working concentration of 2μM was achieved by serial dilutions of 1:10, 1:9, or 1:11 for E-cig 1, 2, or 3 respectively, to achieve 10mM, then diluted 1:100 to achieve 100μM, and 1:75 for a final concentration of 1.5uM or diluted 1:50 for a final concentration of 2μM.

siRNAs and Antibodies

siRNAs used were purchased from Santa Cruz Biotechnology including Oct3/4 (sc036123), TEF4/Tead2 (sc45232), α7 nAChR (sc42532), E2F1 (sc29297), STAT1 (sc44123), c-Src (sc44250), Sox2 (sc38408), Yap1 (sc38637), c-Yes (sc29860), and β-arr-1 (sc29741).

Antibodies used for western blot against Sox2 (3579s), p-Src (2101s), p-AKT (9018p), pan-AKT (C67E7), Oct4 (2750s), p-ERK1/2 (9101s), and total ERK1/2 (9102s) were purchased from Cell Signaling Technologies; against c-Src (05-184) from EMD Millipore; against E2F1 (sc251) from Santa Cruz Biotechnology; against Yap1 (53-161) from Abnova, α7 nAChR (ab23832 and ab10096) from Abcam; and Actin from Sigma Aldrich (A1978).

Antibodies used for chromatin immunoprecipitation (ChIP) assays included E2F1 (sc193), E2F2 (sc633), E2F3 (sc879), E2F4 (sc1082), E2F5 (sc999), Rb (sc50), STAT1 p84/p91 (sc346), and STAT3 (sc8109) from Santa Cruz Biotechnology; Yap1 (ab56701), H327Kme3 (ab9045), and H327Kme1 (ab6002) from Abcam; a rabbit anti-mouse secondary antibody from Pierce was used as a negative control IgG.

Antibodies used for immunofluorescence included Sox2 (3579s) from Cell Signaling Technologies; ZO-1 (339100) from Invitrogen; E2F1 (sc193), Crm1 (sc5595), and e-cadherin (sc8426) from Santa Cruz Biotechnology; α7 nAChR (ab10096) Yap1 (ab56701) from Abcam.

ChIP-PCR Experiments

Chromatin immunoprecipitation assays were conducted using previously described protocols (103, 159). Cells were treated according to experimental parameters; at the time of harvest, cells were treated with 37% formaldehyde for 20 minutes at room temperature for cross-linking the DNA to the proteins of interest. The cells were scraped, washed in ice-cold PBS, and centrifuged at 4,000rpm at 4°C for 5 minutes. Subsequently, the pellet was resuspended in cell lysis buffer (44 mM Tris–HCl [pH 8.1], 1% SDS, 1 mM EDTA [pH 8.0]). The cells were sonicated thrice for 15 seconds each, with 30 seconds of rest in between. Subsequently, the cell lysates were centrifuged at 12,000rpm at 4°C for 15 minutes. The precleared chromatin was diluted with ChIP dilution buffer (16 mM Tris–HCl [pH 8.1], 250 mM NaCl, 0.1% SDS, 1% Triton-X-100, 1.2 mM EDTA) and rotated overnight with primary antibody at a concentration of 25ug/ml. The next day, 60 µL of 1:1 protein G-Sepharose was added to the immune complexes, and the mixture was rotated at 4°C for three hours. The beads were washed three times with low salt ChIP dilution buffer followed by two times with high salt ChIP dilution buffer. The cross-links were reversed by incubation at 65°C overnight. DNA was isolated by ethanol precipitation. The associated proteins with the DNA were digested with 50 µg Proteinase K at 37°C for 30 minutes. DNA was purified by phenol chloroform extraction followed by ethanol precipitation. Purified DNA was resuspended in 30 µL MilliQ water. Protein binding to promoter DNA was assessed using polymerase chain reaction (PCR). Interactions of the proteins with specific regions of the α7 promoter were detected by PCR amplification using the following primer sequences: α7-F1- 5'-TCGGGTCTGTTTTGTCTGGTT-3'; α7-R1-5'CAGAAGCTGCGCTGGGCACTC-3'; α7-F2-5'-GTACCCAGCGCCGGGAGTAC-3'; α7-R2-5'-GCTCGCGCGCCTTTAAGGAG-3'. PCR results were quantitated using ImageJ software and

are represented as percent of input control as graphical data. Association on the Sox2 promoter was assessed using PCR, and the ChIP primer sequences are as follows: F1-5'-GAAAAGGCGTGTGGTGTGAC3-3'; R1-5'- CGCTGATTGGTCGCTAGAAAC -3'; F2-5'-GGGAGTGCTGTGGATGAGC-3'; R2-5'-GTGGGTAAACAGCACTAAGACTACGTG-3'; F3-5'-TGTGCGCTGCCTGCACCTGTG-3'; R3-5'-ACTCCAGCAGAACCAGCCCTG-3'; F4-5'-ACGTGCTGCCATTGCCCTC-3'; R4-5'-CGGGTTAGAGGAGGATGAGA-3'.

Transient Transfections and Luciferase Assays

A549, H460, or H1650 cells were cultured to 70% confluency and transfected in Opti-MEM medium (Gibco, Life Technologies) using Fugene HD (Promega) transfection reagent following the manufacturer's protocol. The 235bp and 1115bp α7-luciferase gene promoter constructs were kindly provided by Dr. Sherry Leonard (University of Colorado, Denver, CO). Sox2-core-luciferase was kindly provided by Dr. Angel G. Martin (InBiomed, Spain). The mutSox2-core-luc construct containing a mutated Oct4 binding site was previously generated in our lab using Quikchange Lightening multi-site-directed mutagenesis kit (Agilent Technologies). The predicted binding site of Oct4 in the Sox2-core-luciferase was ATGCAAAA at position -95 to -102. The sequence ATGC from position -99 to -102 was deleted to abrogate the binding of Oct4 to Sox2-core-luciferase (Borasinghal). The expression vectors used were pcDNA3-HA-E2F1, pcDNA3-E2F2, pcDNA3-E2F3, pcDNA3-E2F4, pcDNA3-E2F5, pcDNA3-STAT1, pcDNA3-STAT3, Yap1 (Addgene #18978). Empty vector pcDNA3 was used as a control. Luciferase assays were conducted 24 to 48 hours after transfection per manufacturer's protocol using the Dual Luciferase Assay system (Promega). Results are reported as relative luciferase activity (RLA) based on the ratio of RLUs1 firefly luciferase to RLUs2 Renilla luciferase (normalization control) values as measured on a Turner Biosystems luminometer.

siRNA Transfections and Quantitative Real-Time PCR

A549 or H460 cells were grown to 70% confluency and transfected in Opti-MEM (Gibco Life Technologies) with 100pmol of siRNAs using Oligofectamine reagent (Invitrogen) per

manufacturer's protocol. 4-6 hours after transfection, media was replaced by complete medium containing 10% FBS. RNA was isolated using Qiagen RNEasy miniprep kit (Hilden, Germany) according to manufacturer's protocol. First strand cDNA was synthesized using Bio-Rad iScript cDNA synthesis kit (Hercules, CA). mRNA expression was assessed using quantitative real-time PCR (qRT-PCR)(Bio-Rad CFX96 Real Time System) and data were analyzed using the CFX96 software. RT-primers used were as follows:

α7(F)5'-TCCTGCACGTGTCCCT-3';

α7(R)5'-CTTGGTTCTTCTCATCCACG-3';

GAPDH(F)5'-GGTGGTCTCCTCTGACTTCAACA-3';

GAPDH(R)5'-GTTGCTGTAGCCAAATTCGTTGT-3'.

Vimentin(F)5'-GGACCAGCTAACCAACGACA-3';

Vimentin(R)5'-AAGGTCAAGACGTGCCAGAG-3';

Fibronectin(F)5'-TAGATGTACAGGCTGACAGA-3';

Fibronection(R)5'-TCTTTCTTAAGCCCTTTGCT-3';

Yap1(F)5'- CCCAAGACGGCCAACGTGCC-3';

Yap1(R) 5'- ACTGGCCTGTCGGGAGTGGG-3';

Sox2(F)5' – GGGAAATGGGAGGGGTGCAAAAGA-3';

Sox2(R)5'- TTGCGTGAGTGTGGATGGGATTGG-3';

ZEB1(F)5'-AGCAGTGAAAGAGAAGGGAATGC-3';

ZEB1(R)5'-GGTCCTCTTCAGGTGCCTCAG-3';

ZEB2(F)5'-ATCTGCTCAGAGTCCAATGCAGCAC-3';

ZEB2(R)5'-AACAGTATTGTCCACAATCTGTAG-3'.

Data was normalized using GAPDH as an internal control, and fold change was determined using the $2^{-\Delta\Delta CT}$ method.

Lysate Preparation and IP/Western Blotting

Cells were washed twice with cold 1xPBS, scraped off the plates, collected by centrifugation for 5 minutes at 6,000rpm, and lysed in M2 lysis buffer (20mM Tris-HCl pH6.0, 0.5% NP-40, 250mM NaCl, 3mM EGTA, and 3mM EDTA) containing protease inhibitors as described in our previous work (103). After lysis, protein concentration was measured using Bradford assay (BioRad) and equal amounts of proteins were resolved on 8% SDS-page polyacrylamide gels, and transferred onto nitrocellulose membranes using BioRad semi-dry transfer unit. Membranes were blocked using 5% nonfat dry milk in 1xPBS containing 0.1-0.5% Tween20. After rinsing with 1xPBST, membranes were incubated overnight at 4 degrees Celsius with primary antibodies, washed again in 1xPBST, incubated for one hour at room temperature in HRP-conjugated secondary antibodies at 1:3000 dilutions, and protein was detected using ECL reagent from GE Healthcare or Pierce Biotechnology according to standard protocols. Results were quantitated using ImageJ software where protein expression was normalized to the corresponding Actin control, and represented as fold change relative to the control as graphical data.

For co-immunoprecipitation assays, 200 µg of total protein lysate from A549 and H1650 cells were incubated with 4 µg of indicated antibodies. An equal amount of non-specific IgG from rabbit or mouse serum (Sigma-Aldrich) was used as a negative control. The interacting proteins were detected by western blotting.

In-cell Western Blots

A549 cells were washed three times with PBS, fixed for 20 minutes using 10% buffered formalin, permeabilized using 0.2% Triton-x, blocked for 1 hour at room temperature, and incubated in primary antibody overnight. Following primary antibody incubation, cells were incubated in rabbit 700cw IRDye (Li-Cor) and mouse 800cw (Li-Cor) secondary antibodies for 1 hour at room temperature, washed with 1xPBS, and imaged using Li-Cor Odyssey 9120

Infrared Imaging System. Quantitative analysis was conducted using the Odyssey analysis software.

Immunofluorescent Analysis and Confocal Microscopy

A549 cells were plated onto poly-D-lysine coated 8-well glass chamber slides (LabTek), washed three times with PBS, fixed for 20 minutes at room temperature in 10% buffered formalin, permeabilized using 0.2% Triton-X 100, blocked for one hour in 5% goat serum, and incubated overnight in primary antibody. Subsequently, cells were incubated in anti-rabbit Alexa Fluor-488 or anti-mouse Alexa Fluor-594 secondary antibody (Life Technologies) for 1 hour at room temperature, and mounted using DAPI Vectashield (Vector Labratories). Cells were visualized with a DM16000 inverted Leica TCS SP5 tandem scanning confocal microscope at 630x or 1890x magnification

Proximity Ligation Assays

PLA studies were conducted as previously described (160, 161). Cells were fixed using 10% buffered formalin and permeabilized with 0.5% Triton-X-100 in PBS for 10 min. The cells were blocked with 5% normal goat serum. The diluted primary antibodies were added to the cells and incubated at 4^{o}Cs overnight. For the remaining steps the Duolink assay system was used (Sigma-Aldrich) following the manufacturers' protocol (161). Briefly, the slides were washed twice with buffer A (0.15 M NaCl, 0.02 M Tris, 0.05% Tween 20, pH 7.4) for 5 min. Plus and minus PLA probes (secondary antibodies conjugated to oligonucleotides) were added to the cells and incubated for 1 h. The cells were washed again in wash buffer A with gentle agitation. Amplification was carried out by incubating in the amplification solution with nucleotides and fluorescently labeled oligonucleotide detection probes along with polymerase at 37^{o}C for 120 min. The cells were washed twice in wash buffer B (0.1 M NaCl in 0.2 M Tris, pH7.5) for 10 min. The slides were washed with 0.01% wash buffer B in water, dried and mounted in mounting medium with DAPI (Vectashield). The images were taken in Leica TCS SP5 confocal microscope (Leica Microsystems) at x630 and x1890 magnification.

Isolation of SP Cells and Self-Renewal

SP cells were sorted from heterogenous cell populations using flow cytometry based on Hoechst 33342 dye efflux, and were then plated for self-renewal assays using protocols described in detail earlier (59, 160). For experiments involving nicotine or e-cigarette extracts, these were added directly into stem-cell media at the time of plating post-sort. For depletion experiments, cells were transfected using siRNA and cells were sorted 24 hours later. Briefly, asynchronously growing cells were harvested using Accutase reagent (Sigma Aldrich), washed once with PBS and re-suspended in DMEM:F12K medium (Gibco, Life Technologies) with 2% FBS at 1 x 10^6 cells/ml. Cells were incubated with 4 µg/ml of Hoechst 33342 dye (Life Technologies) for 90 min at 37^{o}C in the presence or absence of 1 µM Fumitremorgin C (Sigma Aldrich) which is used as a gating control. Cells were analyzed using LSRII (BD Biosciences) and sorted using FACS Vantage (BD FACSDiVa) or Aria (BD FACSAria) cell sorter. Data analysis was conducted by Moffitt Cancer Center's Flow Cytometry Core Staff using the FlowJo software (Tree Star).

Self-renewal was assessed by sphere formation assay by plating 1,000 sorted SP or MP cells per well in stem cell selective media on ultralow attachment 96 well plates (Corning Inc.). Stem cell selective media was composed of DMEM/F12K supplemented with 1x N2 supplement (Invitrogen), 10ng/ml EGF, and 10ng/ml bFGF (Sigma Aldrich). Spheres were allowed to grow for 10 days; in the experiments utilizing nicotine, fresh nicotine was added after 5 days. Spheres were imaged using Evos FL microscope system (Life Technologies).

Wound Healing Assays

Wound healing or scratch assays were conducted by plating A549 cells in 6-well plates (BD Biosciences) at confluency, and allowed to attach overnight. Cells were serum starved for 24 hours prior to making the scratch wounds; the scratch was made using a sterile pipette tip in

each well, each well was washed 1x with PBS, and nicotine and e-cigarette extracts were added. Images were taken every 24 hours for 48 hours, using EVOS FL microscope system (Life Technologies) at 10x magnification.

CHAPTER THREE:

NICOTINE-MEDIATED REGULATION OF NACHRS IN NSCLC BY E2F1 AND STAT1 TRANSCRIPTION FACTORS

Note to Reader

Portions of this chapter have been previously published in *PLoS One*; 11(5); e056451 and have been reproduced with permission from *PLoS One* which is an open access journal, and is distributed under the Creative Commons Attribution License. Author contributions: Schaal, C. (writing, design, figures, experiments), Chellappan, S. (writing, concept, and design).

Abstract

Cigarette smoking is the major risk factor for non-small cell lung cancer (NSCLC), which accounts for 80% of all lung cancers. Nicotine, the addictive component of tobacco smoke, can induce proliferation, migration, invasion, epithelial-mesenchymal transition (EMT), angiogenesis, and survival in NSCLC cell lines, as well as growth and metastasis of NSCLC in mice. This nicotine-mediated tumor progression is facilitated through activation of nicotinic acetylcholine receptors (nAChRs), specifically the α7 subunit; however, how the α7 nAChR gene is regulated in lung adenocarcinoma is not fully clear. Here we demonstrate that the α7 nAChR gene promoter is differentially regulated by E2F and STAT transcription factors through a competitive interplay; E2F1 induces the promoter, while STAT transcription factors repress it by binding to an overlapping site at a region -294 through -463bp upstream of the transcription start site. Treatment of cells with nicotine induced the mRNA and protein levels of α7 nAChR; this could be abrogated by treatment with inhibitors targeting Src, PI3K, MEK, α7 nAChR, CDK4/6 or a disruptor of the Rb-Raf-1 interaction. Further, nicotine –mediated induction of α7 nAChR was

reduced when E2F1 was depleted and in contrast elevated when STAT1 was depleted by siRNAs. Interestingly, extracts from e-cigarettes, which have recently emerged as healthier alternatives to traditional cigarette smoking, can also induce α7 nAChR expression in a manner similar to nicotine. These results suggest an autoregulatory feed-forward loop that induces the levels of α7 nAChR upon exposure to nicotine, which enhances the strength of the signal. It can be imagined that such an induction of α7 nAChR contributes to the tumor-promoting functions of nicotine.

Introduction

Genome-wide association studies (GWAS) have identified a susceptibility locus for human lung cancer at chromosome 15q24-25, which contains *CHRNA3*, *CHRNA5*, and *CHRNB4* genes encoding the α3, α5, and β4 subunits of nAChRs (82-84). Polymorphisms in this region were found to correlate with nicotine dependence, number of cigarettes smoked per day, and increased risk for lung cancer development (82). The α5 subunit has been implicated in smoking-related lung cancer, implicated as the primary central nervous system receptor involved in smoking addiction and behavioral patterns, and additionally has been strongly associated with increased lung cancer risk via a nonsynonymous variation in *CHRNA5 D398N* (83, 162, 163). Methylation status of *CHRNB4* has prognostic value for NSCLC, as demethylation correlates with tumor progression and poor survival in patients with this disease (164). While multiple nAChRs have been found to be expressed on non-neuronal and NSCLC cells, nicotine-mediated tumor progression is facilitated predominantly through the α7 subunit (22, 24, 46, 70, 98, 165). Consistent with this, α7 levels are found to be elevated in mice that were administered nicotine, and nicotine-mediated effects on cell proliferation, invasion, migration and angiogenic tubule formation are abrogated in the presence of the α7-specific inhibitors (22, 122, 166). Given this information, study of nAChRs and their regulation in tumor progression is warranted. While α7 nAChR regulation has been reported to be mediated

through Sp1/GATA pathway in squamous cell carcinomas of the lung (33), not much information is available on its regulation by other transcription factors or in lung adenocarcinoma. Since nicotine is known to activate E2F transcriptional activity, we sought to elucidate whether E2F as well as other transcription factors play a role in the regulation of α7 in NSCLC cells, in response to exposure to nicotine.

Mitogenic signaling cascades are aberrantly altered in tumors due to mutations or over activation of upstream receptors (167). Our lab had previously shown that in NSCLC, stimulation of α7 nAChR with nicotine results in the activation Src kinase through the recruitment of the β-arrestin-1 scaffolding protein, followed by the subsequent activation of Raf-1 kinase which phosphorylates Rb tumor suppressor protein (46, 98, 103). Upon phosphorylation by Raf-1 and cyclins/CDKs, Rb becomes hyperphosphorylated and dissociates from E2F transcription factors allowing them to activate expression of E2F target genes, including those involved in cell cycle and tumor progression (24, 98). Upon nicotine stimulation the β-arrestin-1 scaffolding protein itself can translocate to the nucleus where it binds to E2F transcription factors and increases expression of target genes (46, 103). The studies presented here show how nicotine upregulates the levels of the α7 nAChR subunit through the involvement of E2F1 transcription factor, which suggests the existence of a feed-forward mechanism by which the downstream signals mediated by nicotine might be amplified.

Results

Levels of α3, α5, and α7 nAChRs Correlate with Lung Cancer Patient Survival

The Kaplan-Meier plotter web-based tool available on KMplot.com was used to analyze a database of 2,437 NSCLC patient cases (156) to assess whether patient survival outcome correlated with expression of the α7 nAChR subunit which is functionally implicated in lung cancer progression, or α3 and α5 nAChR subunits which were identified to correlate with lung cancer incidence in GWAS studies (82-84). Expression threshold was defined by KMplot.com using various quantile expression of proposed biomarkers. High expression of α7 correlated

with increased survival across all histological subtypes and variants of NSCLC cases, with a *p* value of 0.00043. High expression of α3 significantly correlated with decreased survival probability in all histological subtypes and variants of NSCLC, with a *p* value of 0.0015. Similarly, high levels of α5 correlated with decreased survival probability across all histological subtypes and variants of NSCLC with a *p* value of 2.6e-09. This data is shown in Figure 3.1

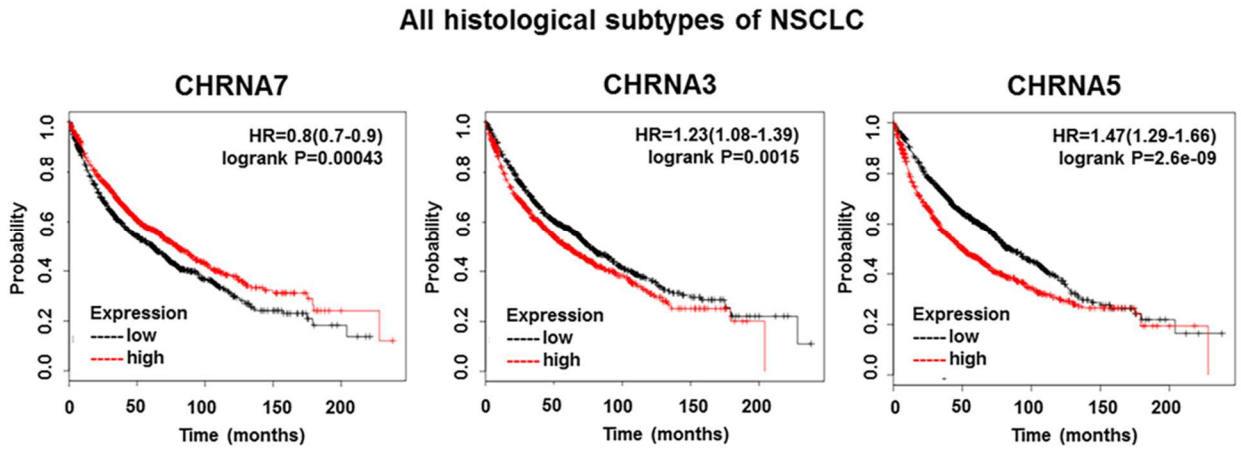


Figure 3.1. Association of *CHRNA*7, *CHRNA*3, and *CHRNA*5 Expression with Survival Outcome Across All Histological Subtypes of NSCLC.

When survival outcome and expression of α7, α3, and α5 were analyzed for gender criteria, we found that high levels of α7 expression correlated with increased survival while high levels of α3 and α5 expression correlated with decreased survival in men (*p*-values=0.079, 0.012, and 6.1e-05 respectively); surprisingly, no correlation was found in women. The basis for this difference is not clear at this time, but will be discussed later. This data is shown in Figure 3.2.

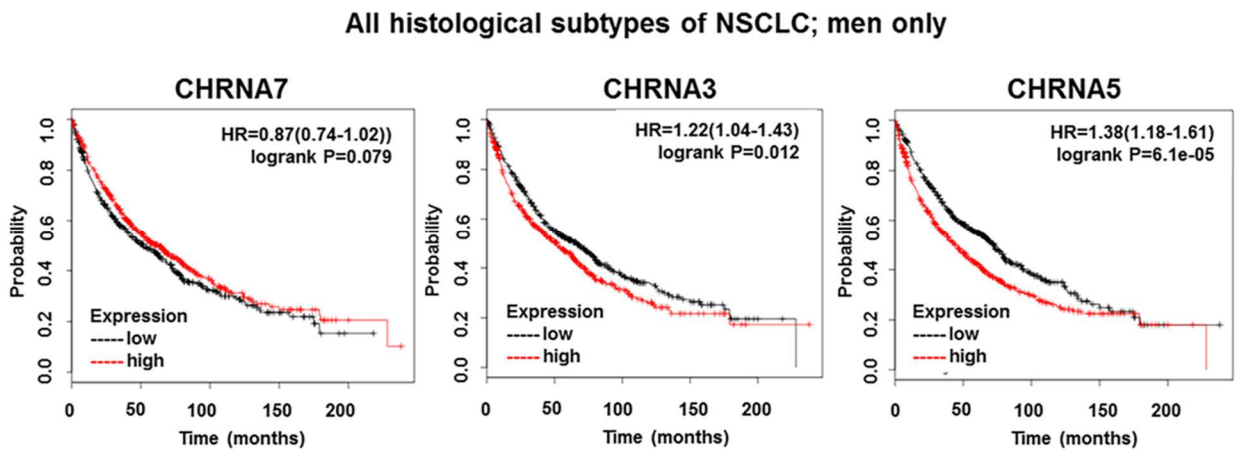


Figure 3.2. Association of *CHRNA7*, *CHRNA3*, and *CHRNA5* Expression with Survival Outcome Across All Histological Subtypes of NSCLC, in Men.

NSCLC frequently occurs in patients who are current or former smokers, and nAChRs are implicated in smoking-related NSCLC progression (168), so we next analyzed correlation of survival outcome and α7, α3, and α5 expression in male patients who were smokers. High α7 levels correlated with increased survival in male smokers, while high levels of α3 and α5 correlated with decreased survival outcome in male patients who smoked (*p*-value=0.00043, 0.024, and 0.00069 respectively). This data is shown in Figure 3.3. No differences were seen when histological subtype or stage were analyzed.

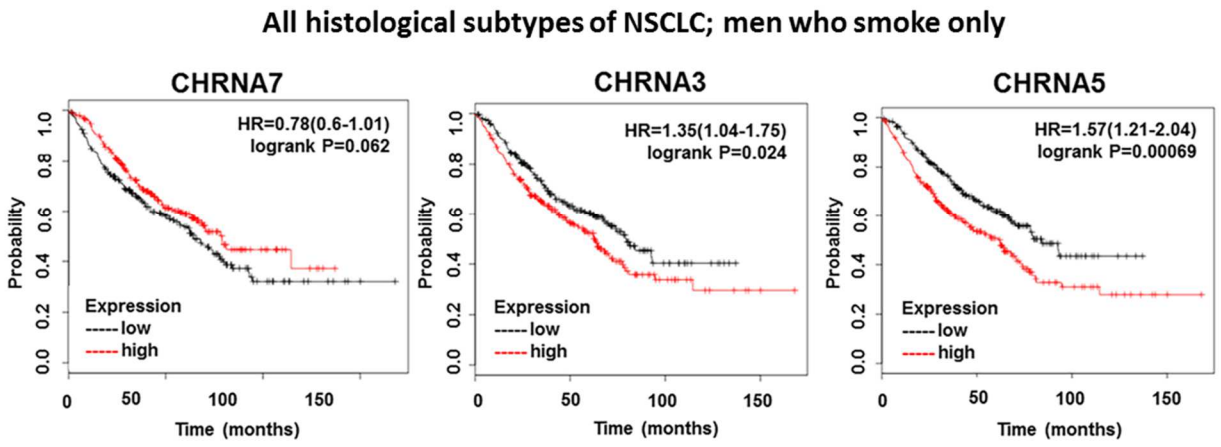


Figure 3.3. Association of *CHRNA7*, *CHRNA3*, and *CHRNA5* Expression with Survival Outcome Across All Histological Subtypes of NSCLC, in Men Who Smoke.

Collectively, these data show that high levels of α7 expression correlates with increased survival probability, which was unexpected, while α3 and α5 expression correlate with decreased survival probability across all histological subtypes of lung cancer, in men, and in men excluding never smokers. Here we report that E2F and STAT transcription factors regulate α7 nAChR gene expression.

E2F and STAT Transcription Factors Regulate α7 nAChR Gene Expression

We first sought to determine whether E2F transcription factors were involved in the activation of the promoters of the α3, α5, and α7 genes, since these subunits have been implicated in smoking related lung cancer and E2Fs are activated downstream of nAChRs in response to nicotine stimulation (82-84). Genomatix MatInspector analysis of 1000 base pair (bp) regions upstream of the transcription start sites (TSS) of each promoter revealed multiple putative E2F binding sites including four sites on the α3 promoter, ten sites on the α5 promoter, and nineteen sites on the α7 promoter (Figure 3.4). E2F transcription factors are known to preferentially bind the consensus sequence TTTSSCGC (where S is either a C or a G) (169).

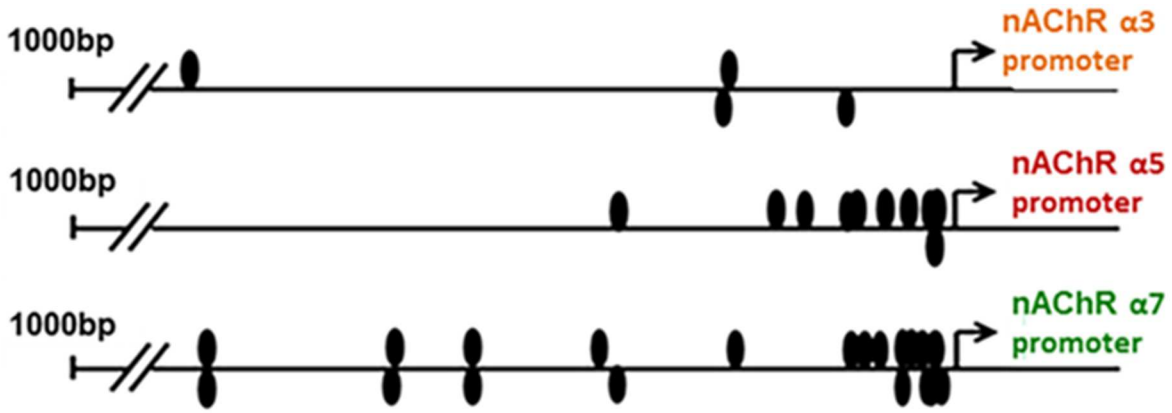
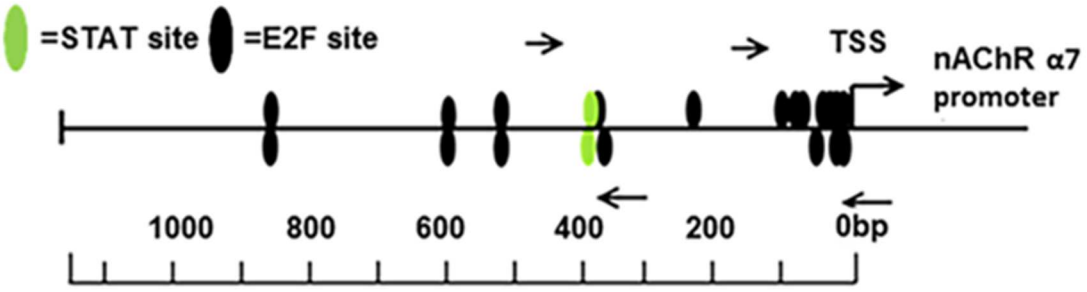


Figure 3.4. Analysis of nAChR Gene Promoters Implicated in Lung Cancer. Schematic representation of 1000bp promter regions of the α3 nAChR, α5 nAChR, and α7nAChR gene showing potential E2F binding sites as black ovals.

During analysis of the α7 promoter, it was noted that there were also two predicted STAT transcription factor binding sites which overlapped with two of the predicted E2F sites at regions

-337 through -380bp upstream of the TSS (Fig 3.5). STAT1 transcription factors preferentially bind the consensus sequence TTCnnnGAA (170). This was of interest as we have previously found that nicotine stimulation could induce STAT activation thereby activating a number of STAT target genes involved in tumor progression. Interestingly, when we analyzed the α3 and α5 nAChR promoter regions 1000bp upstream of TSS, there were no predicted STAT binding sites. Since α7 is the primary subunit facilitating nicotine-mediated tumor progression and was found to contain the greatest number of predicted E2F binding sites, we focused our studies primarily on this subunit.



	TF	Binding Site	Strand	Matrix Sim.	Sequence
12	E2F1	-353 thru -337	-	0.79	gaaaGCCGggacaattc
1	STAT1	-378 thru -360	+	0.856	gggagtccgGGAAgactgg
13	E2F1	-377 thru -361	+	0.76	ggagtccggGAAGactg
2	STAT3	-380 thru -362	-	0.955	agtcTTCCcggactccctc

Figure 3.5. Predicted E2F and STAT Binding Sites on the α7 nAChR Promoter. Schematic representation of 1100bp region of nAChR α7 gene promoter showing potential E2F binding sites as black ovals and potential STAT binding sites as green ovals. Arrows represent the position of primers spanning E2F and STAT binding sites used for ChIP assays. Sequence data of overlapping E2F and STAT binding sites is detailed.

To validate whether E2F and STAT transcription factors could bind to the α7 promoter, chromatin immunoprecipitation (ChIP) assays were conducted on A549 and H460 cells, both of which harbor K-Ras mutations, which frequently occur in lung cancer patients who smoke. It was found that E2F1, E2F2, E2F3, E2F4, and E2F5 could bind at region -177 through +13bp upstream of TSS, where 10 predicted E2F sites were clustered on the promoter, in both A549

and H460 cell lines (Figure 3.6A); quantification of the bands are shown in the bottom panel. E2F1 and E2F4 additionally were shown to bind at region -294 through -463bp upstream of the TSS in A549 where the two predicted E2F sites overlap with the two predicted STAT sites; and E2F1, E2F2, E2F3, E2F4, and E2F5 were shown to bind this site in H460 (Fig 3.6B); the bands are quantified in the lower panel. STAT1 and STAT3 also bound the promoter at region -294 through -463bp upstream of the TSS where the two predicted STAT sites overlap with two E2F sites in A549; STAT1 but not STAT3 bound to this region in H460 cells (Fig 3.6C); the results are quantified in the lower panel.

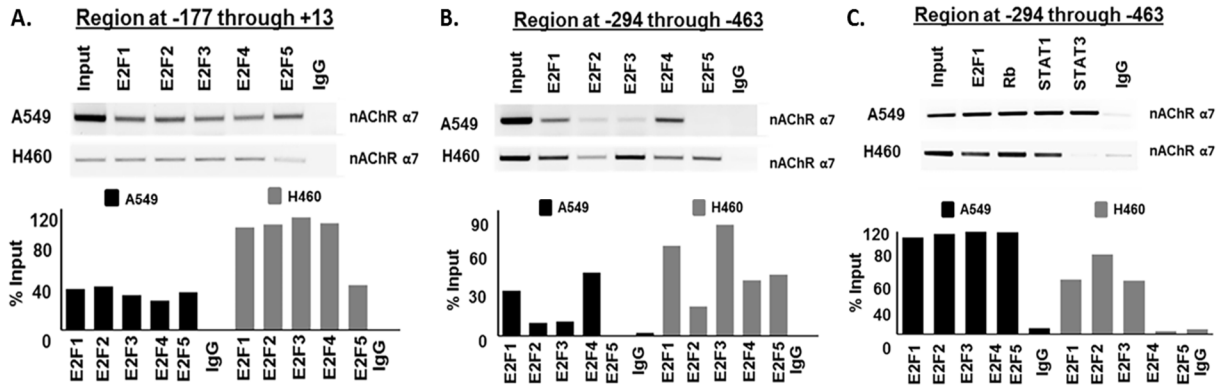


Figure 3.6. ChIP Analysis of α7 nAChR Promoter. (A) Assays showed binding of E2F1-5 to the α7 nAChR promoter region -177 through +13 in A549 cells and binding of E2F1-4 in H460 cells. (B) E2F1 and E2F4, and to some extent E2F2 and E2F3 could also bind region -294 through -463 in A549 cells while E2F1-5 could bind this region in H460cells . (C) STAT1 and STAT3 additionally could bind region -294 through -463 in A549 cells, while STAT1 alone could bind in H460 cells. Sonicated DNA was used as input control, and there was no detectable amplification from irrelevant IgG, used as negative control. Quantification of the data is depicted as percent of input, in the corresponding graphs.

Experiments were conducted to determine whether E2F and STAT transcription factors which bound the α7 promoter could regulate its expression. Transient transfection experiments were carried out using an 1115bp α7-luciferase promoter construct which we received from the lab of Sherry Leonard at the University of Colorado, Denver (171). Co-transfection using 0.5μg of the reporter construct with 1μg of E2F1, E2F2, E2F3, or E2F4 expression vectors resulted in 5.0, 5.4, 3.9, or 1.2 fold induction of α7-luciferase expression respectively; conversely co-

transfection with 1μg of E2F5, STAT1, and STAT3 expression vectors resulted in 70%, 50%, and 40% reduction in expression of α7-luciferase reporter in A549 respectively, demonstrating the ability of these proteins to differentially regulate the α7-luciferase promoter (Figure 3.7A and B). Similar results were observed in H460 cells where co-transfection of α7-luciferase promoter with E2F1, E2F2, or E2F3 resulted in 2.6, 1.7, or 1.4 fold induction respectively; E2F4 had little effect on expression at 1.1 fold induction, E2F5, STAT1, and STAT3 resulted in 10%, 60%, and 40% reduction of expression respectively. To further assess the role of E2F1 and STAT1 in the regulation of α7 promoter, E2F1 or STAT1 were depleted in A549 or H460 cells by transient transfection using 100pmol of small interfering RNAs (siRNA) and expression of α7 mRNA was assessed using qRT-PCR. α7 mRNA was reduced by 20% in A549 or 30% in H460 upon depletion of E2F1, while α7 mRNA levels were increased by 1.5 fold in A549 or 1.2 fold in H460 upon depletion of STAT1, further suggesting that E2F1 induces α7 expression while STAT1 represses it (Figure 3.7C and D).

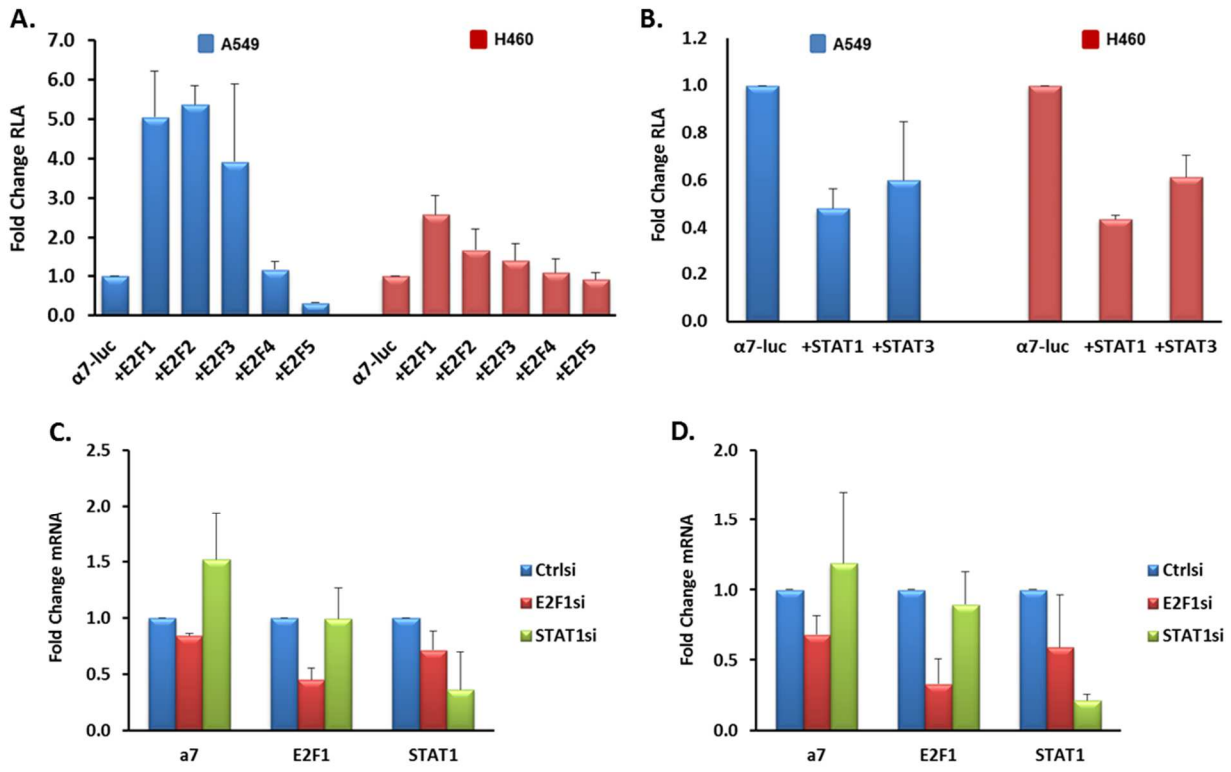


Figure 3.7. E2F and STAT Transcription Factors Regulate α7 nAChR Expression. (A and B) Transient transfection experiments showing that E2F1-3 can induce the α7 promoter, E2F4 has no effect, and E2F5, STAT1, and STAT3 act repress it in A549 and H460 cells. (C and D) Depletion of E2F1 by siRNA results in reduced expression of α7 mRNA, while depletion of STAT1 results in increased expression in A549 and H460 cells.

E2F1 and STAT1 Differentially Regulate α7 nAChR

Since E2F1 and STAT1 were found to have the greatest effect on α7 expression, we focused on these two factors for further studies. The two predicted STAT binding sites overlapped with two putative E2F sites in the region -294 through -463bp upstream of the TSS on the α7 promoter; so we next sought to determine whether the differential regulation of α7 expression observed was occurring *via* competitive interplay between E2F1 and STAT1 transcription factors binding to this region (Figure 3.8A). Transient transfection experiments were conducted using α7-luciferase co-transfected with 1μg of E2F1 and increasing concentrations (0.5μg, 1.0μg, and 2.0μg of STAT1); STAT1 could repress E2F1 mediated induction of α7-luciferase in a dose dependent manner (Fig 3.8B). Reciprocally, 0.5μg, 1.0μg, and 2.0μg of E2F1 could alleviate repression mediated by 1μg of STAT1 on α7-luciferase expression in a concentration

dependent manner (Fig 3.8C). Thus each transcription factor could abrogate the effect of the other, suggesting a competitive interplay occurring at this region of the α7 promoter.

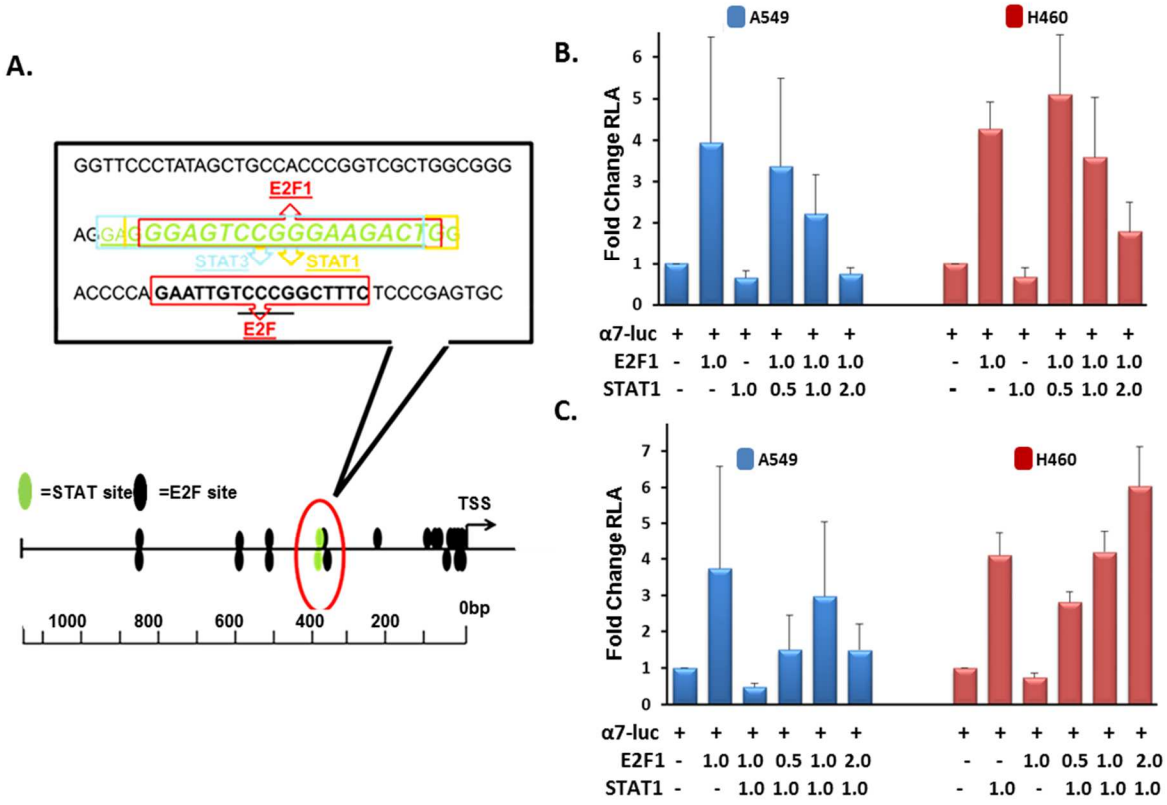


Figure 3.8. E2F and STAT Have Overlapping Binding Sites on the α7 Promoter, and Differentially Regulate its Expression. (A) Schematic representation of overlapping E2F1 and STAT1/3 binding sites on α7 promoter region -294 through -463. (B and C) Transient transfection show increasing concentrations of STAT1 repress E2F1-mediated induction of α7 promoter; reciprocally, increasing concentrations of E2F1 can alleviate STAT1-mediated repression of α7 promoter in A549 cells.

Additional experiments were conducted to validate these findings. Towards this purpose, site directed mutagenesis was conducted. Nucleotides critical for STAT1 binding on the α7-luciferase promoter at position -368, -373, -374, -375, -376, -381, and -382bp upstream of TSS were mutated to disrupt STAT1 binding; these sites coincided with the overlapping E2F binding site (Figure 3.9A). Transient transfections were conducted using 0.5μg of α7-luciferase or α7-luciferase-STAT-mutant promoter constructs with 1μg of E2F1 and STAT1; STAT1 had diminished ability to repress α7 when the binding site was mutated (Figure 3.9B). STAT1 reduced α7-luciferase promoter activity by 86% and 83% in A549 and H460 cells respectively,

while STAT1 reduced α7-luciferase-STAT-mutant promoter activity by 0.02% and 39% in A549 and H460 cells, respectively. E2F1 could induce α7-luciferase promoter activity 3.1 and 3.7 fold in A549 and H460 cells, and could induce the α7-luciferase-STAT-mutant promoter activity 2.3 and 3.0 fold in A549 and H460 cells, respectively. Further, transient transfections were conducted using 0.5μg of a truncated 235bp α7-promoter construct lacking the two STAT binding sites (but retaining ten E2F binding sites), or the full length 1115bp α7-promoter construct 1μg of STAT1 could repress the 1115bp α7-promoter construct by 70%, while STAT1 resulted in a 10% repression of the 235bp α7-promoter construct. E2F-mediated induction of both the 1115bp and 235bp α7-luciferase promoters remained similar at 2.56 and 2.69 fold induction, respectively (Figure 3.9C). This data suggest that region -294 through -463bp upstream of the TSS on the α7 promoter is critical for STAT-mediated repression; E2F1 could still induce the promoter, through additional sites close to the TSS.

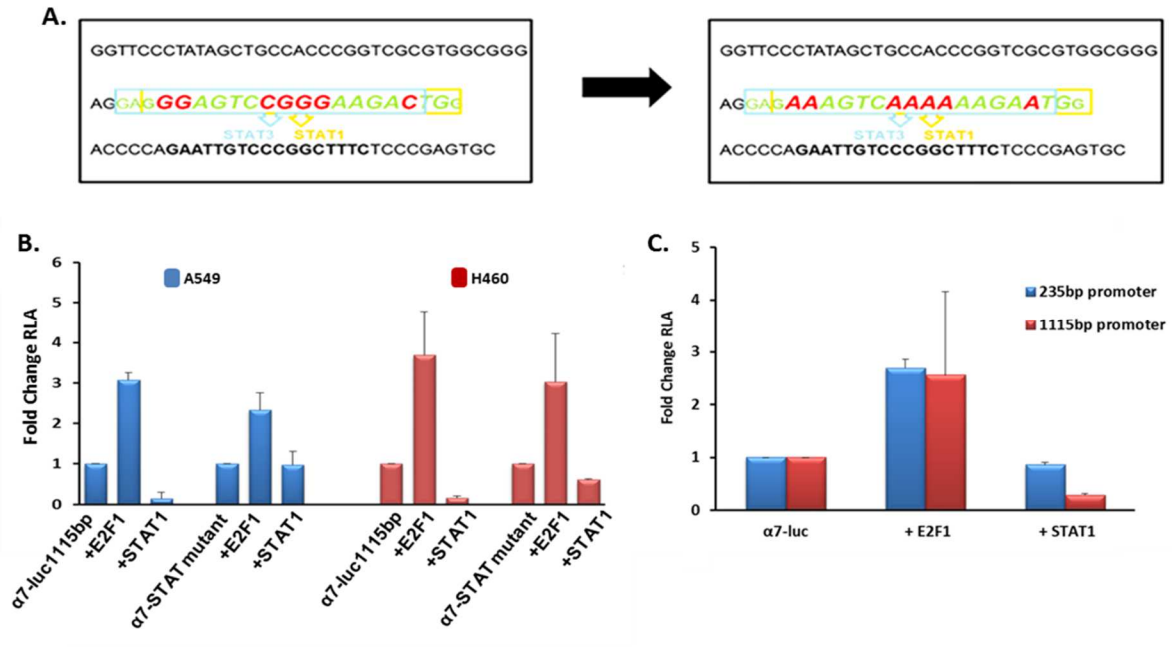


Figure 3.9. Region -294 Through -463bp Upstream of TSS is Critical for STAT-Mediated Repression of α7-luc. (A) Schematic representation of DNA sequence of overlapping E2F and STAT binding sites. Nucleotides which were mutated to disrupt STAT binding on α7 promoter are depicted in red on the left and the resulting mutated sequence is depicted in red on the right. (B) Transient transfections showed that STAT1 could repress the α7 promoter but not the α7-STAT-site-mutant promoter in A549 and H460 cells. (C) Transient transfection showed that STAT1 could repress the 1115bp α7 promoter, but not 235bp α7 promoter lacking predicted STAT binding sites.

Nicotine and E-cigarette Extracts Enhance α7 nAChR Expression

Nicotine has been shown to enhance α7 in adenocarcinoma and squamous cell carcinoma of the lung in vitro as well as in mouse lung adenocarcinoma models (22, 98, 104, 165). Transient transfection experiments were conducted to assess whether nicotine induces the α7 promoter. Towards this purpose, A549 and H460 cells were transiently transfected with 1μg of 1115bp α7-luciferase promoter, serum starved for 24 hours, and stimulated with 2μM nicotine for 24 hours. Upon nicotine stimulation, α7-luciferase activity increased by 1.9 and 2.6 fold in A549 and H460 cell lines respectively, compared to unstimulated cells (Figure 2.10A). Time course experiments showed that in A549, mRNA levels of α7 decreased by 50% at 18 hours and 90% at 24 hours, but increased at 48, 72, 96 and 120 hours reaching a 5.1 fold increase by 120 hours (Figure 2.10B, top panel). To determine whether the other nAChRs implicated in lung cancer are also impacted by nicotine, α3 and α5 mRNA levels were additionally assessed by qRT-PCR after 18, 24, 48, 72, or 96 hours of nicotine treatment. α3 was reduced by 92% and 76% at 18 and 24 hours, but induced by 2.25 and 6.1 fold by 72 and 96 hours post stimulation (Figure 3.10B, middle panel). α5 levels were shown to increase across all time points of nicotine stimulation, ranging from 1.5 to 2 fold (Figure 3.10B, bottom panel). Similar results were observed when cells were examined by immunofluorescence followed by confocal microscopy; α7 was increased upon nicotine stimulation for 48 hours (Figure 3.10C).

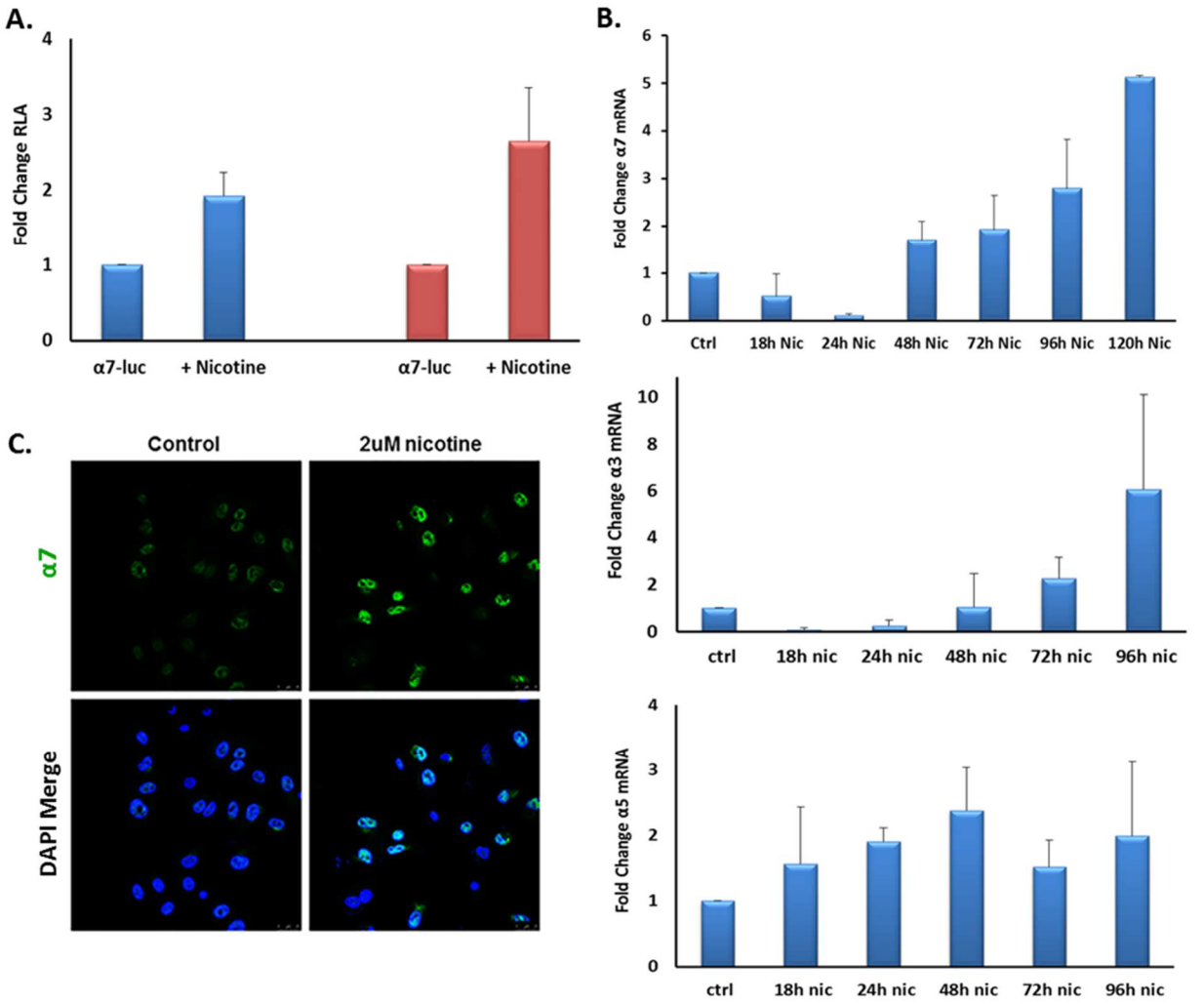


Figure 3.10. Nicotine Can Induce α7 Expression by 48 Hours. (A) Transient transfection showed that 2μM nicotine could induce the α7 promoter after 48 hours of stimulation in A549 cells. (B) qRT-PCR analysis showed that 2μM nicotine induced α7 mRNA levels at 48, 72, 96, and 120 hours, but not at 18 or 24 hours. 2μM nicotine reduced α3 mRNA expression at 18 and 24 hours, little effect was seen at 48hrs, and expression was induced by 72 and 96 hours. 2μM nicotine induced α5 mRNA levels at 18, 24, 48, and 96 hour time points. (C) Immunofluorescent staining showed that 2μM nicotine could induce α7 at the protein level after 48 hours in A549 cells.

To conduct time course experiments on protein levels, cells were rendered quiescent by serum starvation for 24 hours, and stimulated with 2μM nicotine for 18, 24, 48, 72, 96, or 120 hours. α7 protein levels were assessed using SDS-page western blot analysis or α7 mRNA expression was assessed by qRT-PCR. In A549 cells, α7 protein level increased beginning at 48 hours through 120 hours (Figure 3.11A); quantification of the blot is shown in the bottom panel. In

H460 cells, α7 protein level increased beginning at 18 hours through 120 hours (Figure 3.11B), and the blot is quantified in the bottom panel.

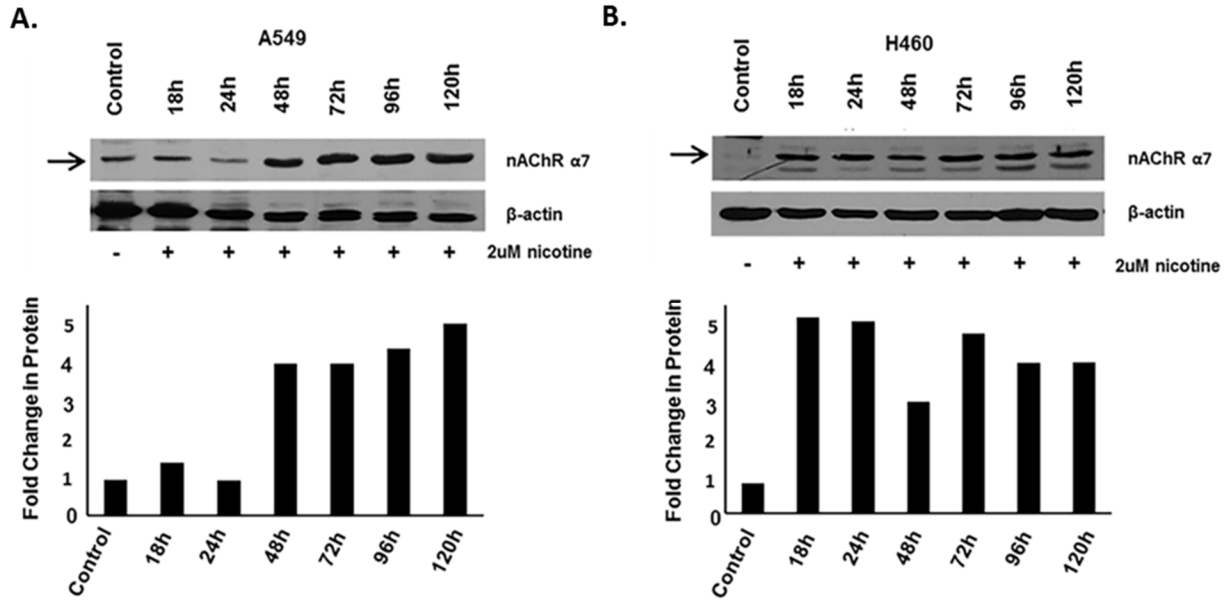


Figure 3.11. Nicotine Induces α7 nAChR in NSCLC. (A) Western blot analysis showed 2μM nicotine induced α7 protein levels at 48, 72, 96, and 120 hours; an effect not seen at 18 or 24 hour time points in A549 cells. (B) In H460 cells, 2μM nicotine induced α7 protein levels at 18, 24, 48, 72, 96, and 120 hour time points. Arrows indicate the α7 protein band of interest, at approximately 50 kD. Quantification of the western blot data is depicted in the corresponding graphs.

The health impact and implications of tobacco smoke and nicotine have been well documented; however, more recently electronic-cigarettes (e-cigarettes) have emerged as an alternative to cigarette smoking (172). While e-cigarettes are advertised as less harmful tobacco-free alternatives that can also be used for smoking cessation, these products still contain nicotine in addition to other chemical compounds and their use is currently unregulated (173, 174). Here we examined whether liquid extracts (containing nicotine) from three common brands of e-cigarettes Fin, Njoy and Mistic, had similar effects as nicotine on α7 nAChR expression. After 48 hours of stimulation with e-cigarette liquid, levels of α7 were assessed and were shown to increase at 1.5μM concentration. Western blot analysis and immunofluorescent staining demonstrated that 1.5μM of each of e-cigarette extract could enhance α7 at the protein level (Figure 3.12A and B). Western blot results were quantitated and are depicted in Figure 3.12C.

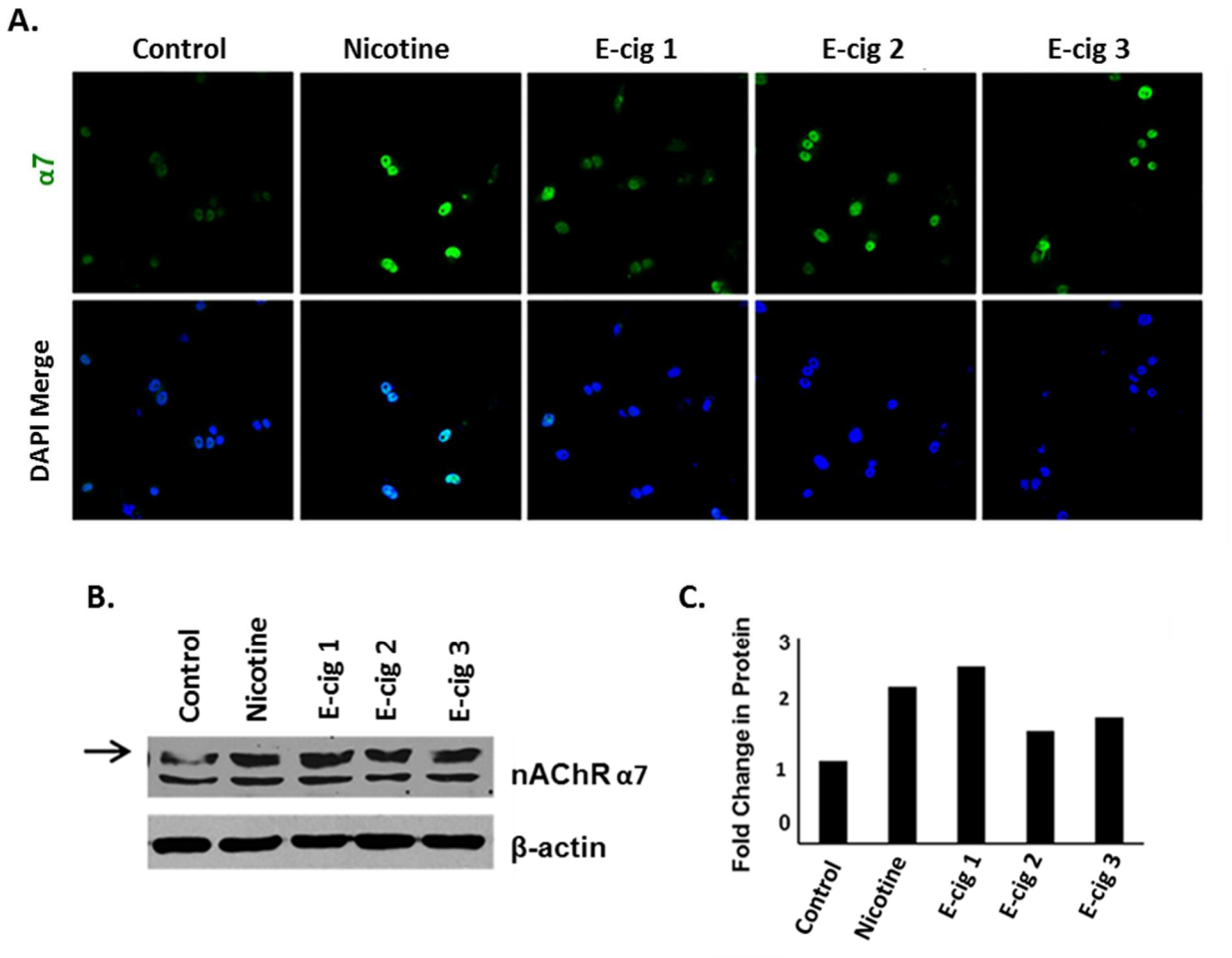


Figure 3.12. E-cigarette Extracts can Induce α7 Protein Expression. (A and B) Immunofluorescent staining and western blot analysis showed that 1.5μM of e-cigarettes could induce α7 at the protein level after 48 hours in A549 cells, to a similar extent as nicotine. (C) For western blot, arrow indicates the α7 protein band and quantification of the data is depicted in the corresponding graphs.

Transient transfection assays demonstrated 1.5μM of each e-cigarette extract to induce α7-luciferase promoter activity, as well (Figure 3.13).

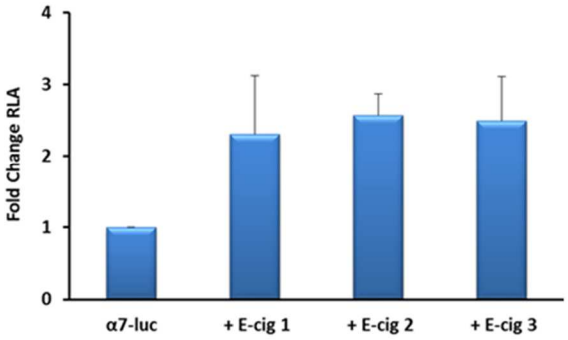


Figure 3.13. E-cigarette Extracts Can Induce α7-luciferase Promoter Activity. 1.5μM concentrations of extracts from each of the three brands of e-cigarettes could induce α7-luciferase.

At the mRNA level, Fin induced α7 by 2.3 fold, Njoy induced α7 by 3.2 fold, and Mistic induced α7 by 2.7 fold (Figure 3.14). To assess the impact of e-cigarettes on the other nAChRs implicated in lung cancer, qRT-PCR analysis was conducted. α3 mRNA levels were decreased after 48 hours of e-cigarette stimulation and α5 mRNA levels were increased after 48 hours of e-cigarette stimulation, both effects similar to that seen after 48 hours of nicotine stimulation (Figure 3.14).

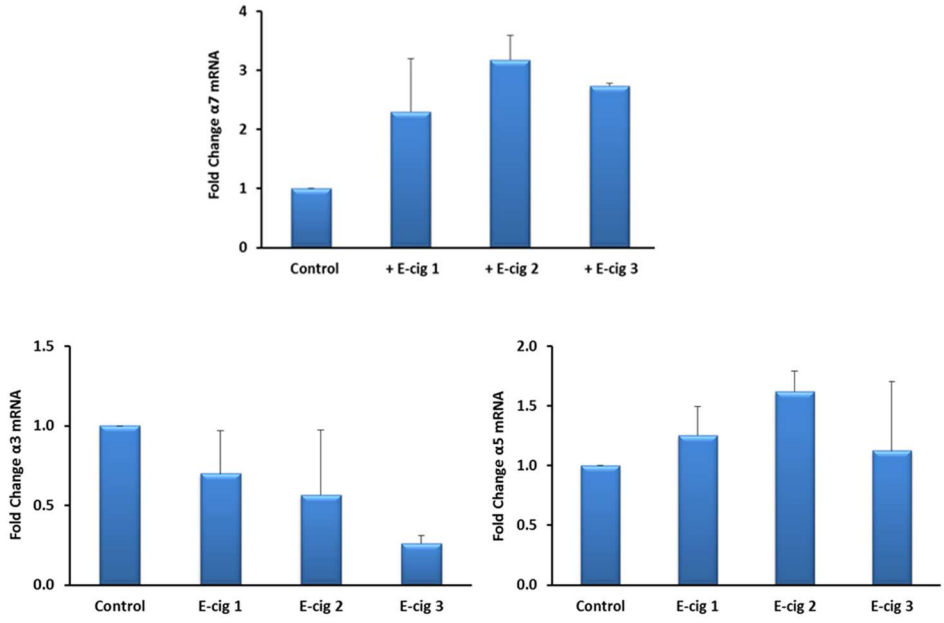


Figure 3.14. E-cigarette Extracts Impact Expression of α7, α3, and α5 nAChRs at the Transcriptional Level. qRT-PCR analysis showed that 1.5μM of extracts from each of the three brands of e-cigarettes resulted in increased α7 mRNA levels after 48 hours, decreased α3 mRNA levels after 24 hours, and increased α5 mRNA levels after 48 hours.

Nicotine-Mediated Induction of α7 is Abrogated by Inhibition of Src, PI3K, MEK1/2, CDK4/6, Rb-Raf Interaction, and α7 nAChR

To elucidate the mechanism by which nicotine induces α7 expression, experiments were conducted using inhibitors to signaling pathways that are activated in response to nicotine stimulation. Previous studies have identified that Src kinase is activated upon nicotine stimulation and subsequently activates downstream kinases including PI3K and Raf-1 (24, 175). PI3K is classically known to activate AKT/PKB, followed by activation of transcription factors such as NFκB, which initiates transcription of a number of tumor promoting genes. Similarly upon nAChR activation, Raf-1 and cyclin/CDKs phosphorylate Rb tumor suppressor protein, resulting in the induction of E2F-mediated transcription of proliferative genes. Studies have also shown that nicotine stimulation activates the MAPK cascade. To elucidate whether these pathways are involved in nicotine-mediated induction of α7 expression, cells were stimulated with nicotine in the presence or absence of the following inhibitors: AZD0530/Saracatinib targeting Src, BKM120/Buparlisib targeting α, β, δ, and γ isoforms of PI3K, GSK1120212/Trametinib targeting MEK1/2, LEE001/Ribociclib targeting CDK4/6, RRD251 targeting Rb-Raf-1 interaction, and α-bungarotoxin/α-BT, which is an α7 nAChR antagonist. Western blot analysis demonstrated all six inhibitors abrogated nicotine-mediate induction of α7 protein levels, although the Src inhibitor AZD had a modest effect (Figure 3.15A). These results were confirmed at the mRNA level by qRT-PCR, which showed nicotine to induce α7 mRNA levels by 1.6 fold, while AZD, BKM, GSK, LEE, RRD, or α-BT reduced α7 levels by 61%, 58%, 18%, 14%, 15%, and 39%, respectively (Figure 3.15B).

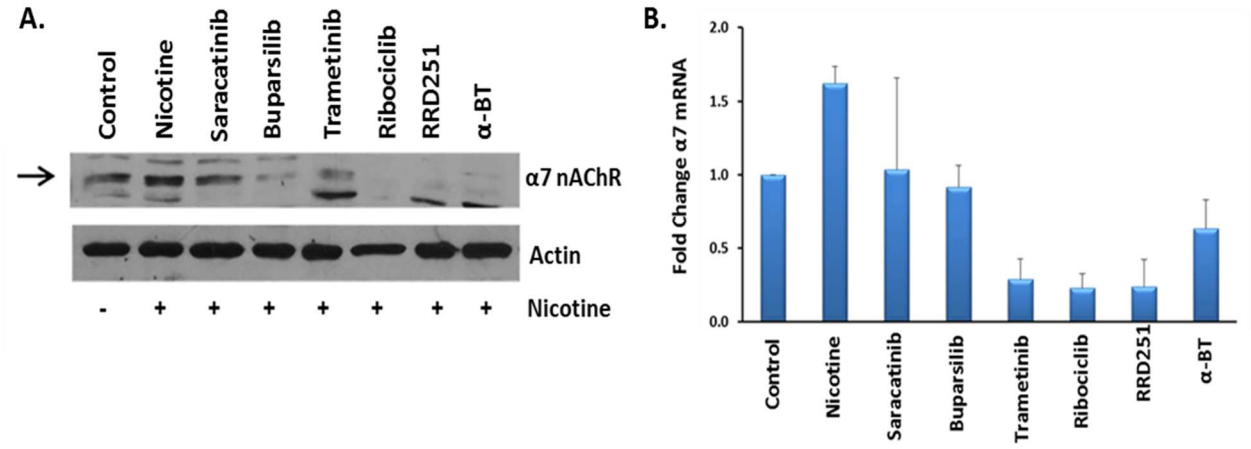


Figure 3.15. Nicotine-Mediated Induction of α7 could be Abrogated by Inhibitors of Src, PI3K, MEK, CDK4/6, Rb/Raf, or α-BT. (A and B) Western blot and qRT-PCR analysis showed that treatment with indicated inhibitors for 30 minutes prior to nicotine stimulation could abrogate the nicotine-mediated induction of α7 levels in A549 cells. For western blots, arrows indicate the α7 protein band.

These results were additionally confirmed by in-cell western blotting which quantitates protein levels. 48 hours of nicotine stimulation induced α7 expression 2 fold, while treatment with Saracatinib, Buparsilib, GSK, Riboiclib, RRD251, or α-BT 30 minutes prior to nicotine stimulation reduced α7 levels by 67%, 32%, 45%, 34%, 34%, and 40%, respectively; quantification of signal intensity is included in the right panel (Figure 3.16).

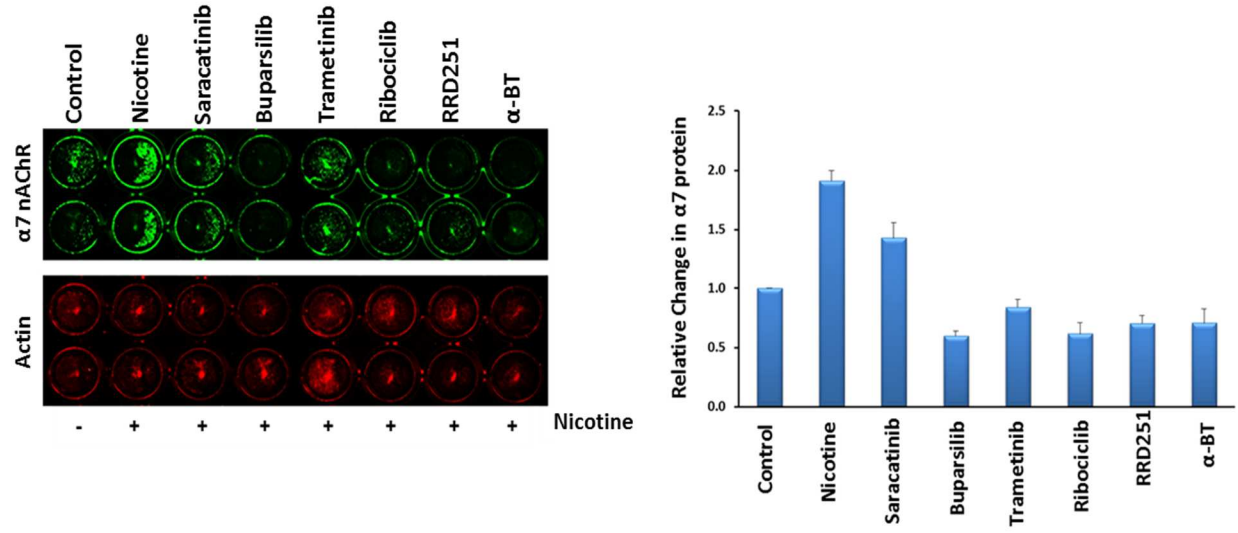


Figure 3.16. Licor In-Cell Western Blot Confirmation of Inhibition. In-cell western analysis further showed that treatment with indicated inhibitors for 30 minutes prior to nicotine stimulation could abrogate the nicotine-mediated induction of α7 levels in A549 cells.

E2F1 is Necessary for Nicotine-Mediated Induction of α7

Since we find that E2F1 and STAT1 regulate α7 nAChR expression, we sought to determine whether the effect of nicotine on α7 occurs through E2F1 and STAT1. To test this, cells were transfected with 1μg of α7-luciferase which increased 2.1 fold upon 48 hours of nicotine stimulation, an effect which was reduced by 33% upon E2F1 depletion and increased by 33% upon STAT1 depletion in A549; similarly, in H460 cells nicotine increased α7-luciferase 1.7 fold, which was reduced by 41% upon E2F1 depletion, and increased by 29% upon STAT1 depletion (Figure 3.17A). This was further confirmed when cells were transfected with small interfering RNA (siRNA) to deplete E2F1 or STAT1, and subsequently stimulated for with 2μM of nicotine for 48 hours, and α7 levels were assessed. We found that when E2F1 was depleted there was diminished ability of nicotine to induce α7, and when STAT1 was depleted nicotine further induced α7-luciferase or α7 at the protein or mRNA level in A549 cells. At the RNA level, nicotine induced α7 by 2.3 fold, which was reduced to by 61% upon E2F1 depletion, and increased by 13% upon STAT1 depletion (Figure 3.17B).

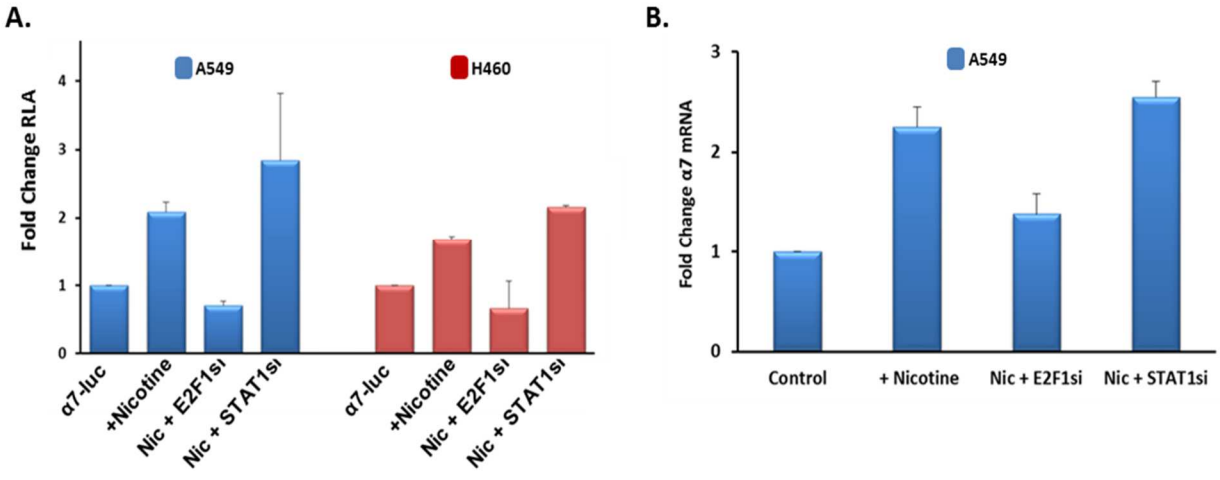


Figure 3.17. Nicotine-Mediated Induction of α7 is abrogated by siRNA Depletion of E2F1. (A) Transient transfection showed that nicotine-mediated induction of the α7 promoter was decreased when E2F1 was depleted using siRNA, but was increased when STAT1 was depleted using siRNA in A549 and H460 cells. (B) Similar results were seen by qRT-PCR analysis which showed that nicotine-mediated induction of α7 mRNA was abrogated when E2F1 was depleted using siRNA, but enhanced when STAT1 was depleted using siRNA.

Collectively, This data suggests that E2F1 as well as the pathways targeted by inhibitors to Src, PI3K, MEK1/2, CDK4/6, RRD251, or α-BT play a role in nicotine-mediated induction of α7 nAChR, as detailed in the schematic presented in Figure 3.18, and if impeded by inhibition (or depletion in the case of E2F1) result in abrogation of nicotine-mediated induction of α7.

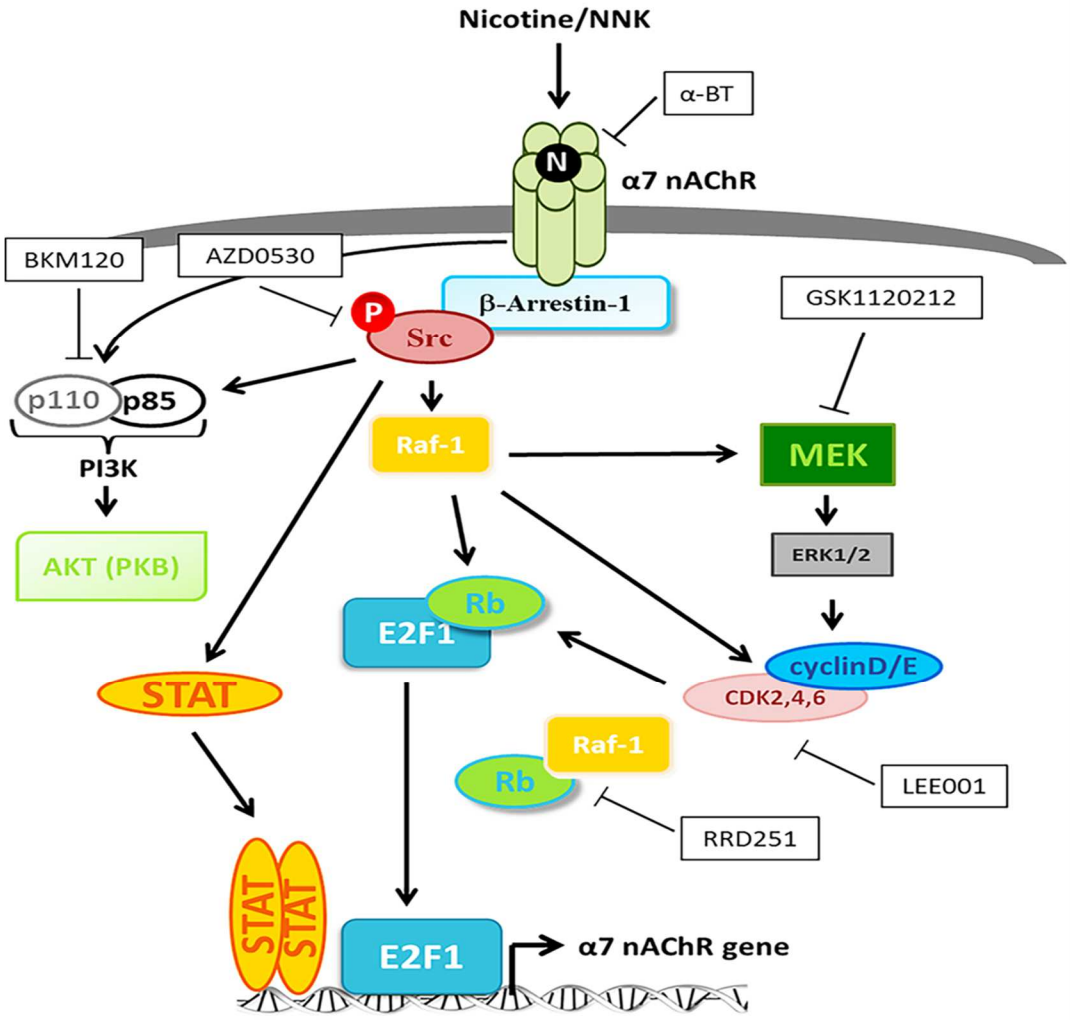


Figure 3.18. Schematic Depicting Signaling Cascades Initiated by Nicotine-Mediated Activation of the α7 nAChR and Inhibitors used to Target these Pathways. Upon nicotine binding to α7 nAChR, oligomeric complexes form including the receptor, β-arrestin-1 scaffolding protein, and Src kinase. This activates Src, resulting in activation of Raf-1 kinase which acts along with activated CDK/cyclins to hyperphosphorylate the Rb tumor suppressor, resulting in its dissociation from E2F transcription factors, allowing them to activate their target genes including a number of genes involved in multiple aspects of tumor progression. The PI3K-AKT and MAPK signaling pathways are also known to be activated by nicotine-mediated α7 nAChR activation subsequent to Src phosphorylation; and activation of MEK-ERK is known to result in activation of CDK/cyclin complexes. AKT1 activation downstream of PI3KC in response to nicotine has additionally been shown to result in activation of NFκB transcription factors. Further, Src activates STAT proteins up nicotine-mediated activation of α7 nAChR. Inhibitors used in this study are depicted in black and white boxes, and their targets are indicated accordingly.

Discussion

Cigarette smoking is highly correlated with NSCLC and accounts for 80-90% of all lung cancer deaths (10). Our lab and others have previously reported that nicotine, while not able to initiate tumors itself, can enhance a number of tumor promoting properties (52, 53, 176, 177). These tumor promoting effects have been shown to occur primarily through the binding to and activation of nAChRs; and proliferation, migration, invasion, and EMT have been shown to occur through the α7 nAChR subunit in specific, implicating it in tumor progression. The α7 nAChR subunit is overexpressed on human NSCLC tumors compared to normal adjacent, and nicotine can enhance expression of the α7 receptor itself, further demonstrating the importance of α7 in nicotine-mediated pathophysiological effects; however, how this occurs and how the α7 nAChR gene is regulated is less understood.

In squamous cell carcinoma of the lung (a subset of NSCLC), it has been previously reported that nicotine increases α7 nAChR expression which accelerates tumor growth and results in worse clinical outcomes (165). In this context, nicotine increased α7 expression through transcriptional mechanisms involving Sp1 and GATA4/6 proteins. Nicotine enhances association of these proteins to the promoter, and they are required for its nicotine-mediated induction. The transcriptional regulation of the α7 nAChR promoter in the context of adenocarcinoma of the lung, as well as the mechanisms by which this occurs, still remains elusive. In this study we demonstrate that E2F1 and STAT1 transcription factors act to differentially regulate the α7 gene promoter and disruption of STAT binding sites can enhance α7 expression. Further, we find that nicotine-mediated induction of α7 can be abrogated by inhibitors to Src, PI3K, MEK1/2, CDK4/6, Rb-Raf-1 interaction, or α7 nAChR suggesting the pathways targeted by these molecules play a role in nicotine-mediated induction of α7 and their disruption inhibits this. As depicted in Figure 3.18, stimulation of cells with nicotine activates Src via β-arrestin-1, facilitating the downstream signaling events. These signaling events lead to the

enhanced expression of α7 nAChR, which in turn amplifies the same signaling cascades. Our earlier studies had shown that signaling via α7 nAChR leads to the expression of genes involved in cell proliferation, like Cdc25, thymidylate synthase and Cdc6 (98); those involved in epithelial-mesenchymal transition like ZEB-1 and ZEB2 as well as fibronectin and vimentin (21, 46, 86) and genes involved in invasion and metastasis, including multiple matrix metalloproteinases (24, 147). Overall, it is highly likely that cells exposed to nicotine for extended periods of time will overexpress α7 nAChR, which we had observed in a mouse model (22); this will lead to the induction of genes involved in tumor progression and metastasis, thus facilitating the tumor promoting functions of nicotine.

Interestingly, we also demonstrate that increasing amount of one transcription factor could abrogate the effect of the other on α7 promoter expression, suggesting a competitive interplay between E2F1 and STAT1. A number of previous studies have reported similar findings of competition between transcription factors to differentially regulate gene expression, across various contexts. For example, in macrophages it has been shown that AP-1, STAT1, and STAT3 compete with NFκB to bind a gene encoding aralkylamine N-acetyltransferase and differentially regulate its expression to control the synthesis of melatonin and inflammatory response (178). In colorectal cancers it has been shown that β-catenin/TCF4 complexes compete with TCF3 to bind to the MYC promoter, and β-catenin/TCF4 induces its expression while TCF3 represses it (179). HNF4α has also been found to compete with TCF4 and AP-1 at overlapping motifs to regulate distinct genes involved in proliferation and inflammation in colorectal cancer (180). Notably, a number of reports of competitive transcription factor interplay involve TCF, AP, SP-1, and STAT transcription factors (178-182). While this suggests that such a dynamic to differentially regulate gene expression may be a common mechanism occurring in response to environmental stimuli, this is the first demonstration of such a mechanism involved in the regulation of nAChR expression. What determines how E2F1 and

STAT1 transcription factors compete to differentially regulate α7 nAChR is unclear at this time. It could be time dependent, where STATs repress expression at early time points while E2Fs activate expression at later time points. It could also be context dependent, and environmental stimuli may cue which transcription factor mediates expression. Further studies in this direction are warranted.

Recently, the use of e-cigarettes has emerged as an alternative to traditional cigarette smoking and as a smoking cessation agent. These nicotine-delivery devices represent a public health concern, and mounting evidence suggests that the liquid containing nicotine found within the cartridge of e-cigarettes, which is delivered as a vapor/aerosol to the user, elicits detrimental health effects similar to those of nicotine from traditional cigarettes (148). There has been an increase in their use among both youths and adults (183). Typically, e-cigarettes consist of a battery, a heating element, an air flow sensor, and a cartridge containing liquid which includes nicotine in addition to other components such as propylene glycol, glycerol, and other additives. When activated the liquid is heated and is delivered as an aerosol in a vapor form to the user (148). Each brand varies in concentration of nicotine present in the liquid, but it is typically indicated as percent nicotine by volume (NBV) on the packaging of the item. While e-cigarettes are thought to be better than traditional cigarettes since they lack tobacco which is known to contain multiple classes of carcinogens, the presence of nicotine may still largely impact pathophysiology and health in users. In this study, we report that e-cigarette extracts could induce α7 nAChR levels in a manner similar to nicotine. Further research is underway to elucidate whether these products can also enhance other tumor-promoting properties, and whether this occurs through the same mechanisms as nicotine-mediated tumor progression.

Of interest, our Kaplan Meier analysis revealed that high levels of the *CHRNA7* gene encoding α7 nAChR correlated with increased patient survival in NSCLC, despite the known role of α7 to facilitate tumor progression. It is unclear why this is; however, we can speculate that there may be a number of factors at play. First, it is possible that tumors may have higher levels of α7 in

early stages of tumorigenesis (while the tumor has better prognosis and therefore correlates with increased survival) which would enhance the ability of the tumor to progress, but as the tumor advances to late stages α7 levels decrease and other factors are driving tumor progression. Another possibility is that patients with higher levels of α7 may respond better to treatment modalities for undefined reasons, resulting in increased patient survival despite high levels of this receptor; while α3/β2 receptor subunits were involved in conferring resistance to chemotherapy drugs, α7 did not appear to have a role (184). It could also be that when there are higher levels of α7 present in a tumor, the receptors become desensitized to stimulation and are no longer able to be activated to exert their biological function; or alternatively they could become hyperactivated resulting in desensitization in a manner similar to the tumor inhibitory nAChR α4β2 which becomes desensitized in response to constant high affinity stimulation (79). Interestingly, we also found that high levels of α7 correlated with increased survival in men, but no difference was seen in women. Why this is remains unclear at this time, but could be due to differences in the expression levels of the receptor in each gender or due to differences in other factors that might affect receptor function, such as nicotine uptake. Studies have shown that men and women metabolize nicotine at different rates, and women have greater susceptibility to carcinogens than men (185). Factors such as these may be involved in the differences observed. The A549 and H460 cell lines used in the present study were both derived from male patients. Further studies are warranted to answer these questions.

The elucidation of regulation of nAChRs involved in nicotine-mediated cancer progression and the signaling mechanisms involved presents new opportunities to develop agents targeting these pathways, which could potentially improve therapeutic outcome for patients with smoking-related cancers. This study helps us to better understand the pathophysiology of such disease, specifically in the context of nicotine-mediated promotion of NSCLC.

CHAPTER FOUR:

NICOTINE AND E-CIGARETTES REGULATED SOX2 AND STEMNESS IN NSCLC

Note to Reader

Portions of this chapter are included in a manuscript in preparation to be submitted to Molecular Cancer Research in August 2016. Author contributions: Schaal, C. (writing, concept, design, figures, experiments), Namrata Bora-Singhal (concept, experiments), Durairaj Mohan Kumar (experiments), Chellappan, S. (writing, concept, and design).

Abstract

Lung cancer is the leading cause of cancer death and is highly correlated with cigarette smoking. Nicotine, the addictive component of tobacco smoke, cannot initiate tumors, but can promote proliferation, migration, and invasion of cells in vitro and promote growth and metastasis of tumors in vivo. This nicotine-mediated tumor progression is facilitated through activation of nicotinic acetylcholine receptors (nAChRs), specifically the α7 subunit. More recently, nicotine has been implicated in promoting self-renewal of stem-like side-population cells from lung cancers. This subpopulation of cancer stem-like cells has been implicated in tumor initiation, generation of entire heterogeneous tumor population, metastasis, dormancy, and drug resistance. Here we demonstrate that nicotine can induce the expression of embryonic stem cell factor Sox2, which is indispensable for self-renewal and maintenance of stem cell properties in non-small cell lung adenocarcinoma (NSCLC) cells. We further demonstrate that this occurs through a nAChR-Yap1-E2F1 signaling axis downstream of Src and Yes kinases. Data suggests Oct4 may also play a role in this process. Over the past few years, electronic cigarettes (e-cigarettes) have emerged as healthy alternatives to traditional

cigarette smoking as they do not contain tobacco; however, they do still contain nicotine. To this extent we have investigated whether e-cigarettes can enhance tumor promoting properties similar to what we have reported with nicotine alone, and find that they can induce expression of Sox2 and mesenchymal markers as well as enhance migration and stemness of NSCLC cells.

Introduction

While our lab and others have demonstrated the ability of nicotine to enhance processes involved in the growth and metastasis of NSCLC, we have shown more recently that nicotine can enhance stemness in a subset of lung adenocarcinoma cells enriched in stem-like cell populations through the induction of c-Kit ligand/Stem Cell Factor (SCF). SCF is known to promote self-renewal and differentiation of multiple stem cell types through the binding of its receptor, c-Kit (51, 186-188), and this finding reveals a novel mechanism by which nicotine can potentially regulate tumor progression.

Tumors were traditionally thought to be a disease of clonal origin where a single transformed cell has the ability to give rise to heterogeneous tumor cell populations, with each daughter cell having the same capacity to give rise to more tumor cells. More recently, growing evidence supports the cancer stem cell model, which proposes that cancer stem-like cells (CSCs) arise through a reprogramming mechanism and these cells are responsible for tumor initiation, progression and maintenance (58-60). The model proposes that only CSCs are able to initiate tumors; these stem-like cells resemble traditional stem cells in that they are able to self-renew, divide asymmetrically, and are slow cycling (60), and have been implicated not only in tumor initiation, progression, and maintenance; but also in therapeutic resistance, dormancy, recurrence, and metastasis (60, 61). Given these properties, understanding and targeting CSCs has become an important area of cancer research.

Over the past decade or better, CSCs have been characterized and isolated using cell surface markers, which are differentially expressed in CSCs compared to non-stem-like, differentiated cancer cells. Various markers such as aldehyde dehydrogenase 1 (ALDH1) and CD133

positivity are effectively used for CSCs from cancers such as breast, colon, brain, pancreas, head and neck (62-65); however, there is no single marker ubiquitously expressed and used to identify lung cancer CSCs. A subset of tumor cells enriched in CSCs can be isolated based on their ability to efflux Hoechst 33342 dye out of their nuclei through the ABCG2 drug transporter expressed on the cell membrane of the stem-like cells, and have been termed as side-population cells, based on their distribution in flow cytometric sorting (59, 67, 68). Our lab and others have shown that non-small cell lung cancer CSCs can be isolated using SP phenotype from cell lines as well as human tumor xenografts. Such cells were highly tumorigenic and produced highly invasive tumors in mice compared to the non-SP cells, and displayed stem-like properties such as the ability to self-renew, expressed epithelial-to-mesenchymal transition (EMT) markers as well as the classic embryonic stem cell (ESC) transcription factors Sox2, Oct4, and Nanog (59, 160). We further demonstrated that EGFR-Src-AKT signaling axis acts upstream of Sox2 and inhibition of this pathway downregulates Sox2 and self-renewal ability of SP cells. Sox2 itself was critical to maintain self-renewal of SP cells from NSCLC cell lines, compared to Oct4 and Nanog (59). Since we find that Sox2 ESC transcription factor is required to maintain NSCLC CSC stemness and that nicotine acts to enhance stemness, we sought to determine whether this nicotine-mediated induction of stemness acts through induction of Sox2. Here we report that nicotine can induce Sox2 through a Yap1/E2F1/Oct4 signaling axis. We further report that e-cigarette extracts containing nicotine can induce self-renewal in a manner similar to nicotine, and e-cigarette extracts could induce Sox2 expression suggesting that exposure to nicotine, either through tobacco smoke or through the use of e-cigarettes, might have deleterious effects.

Results

Nicotine and E-cigarettes Enhance Sox2 Expression and Self-Renewal of SP Cells

Our lab had reported that the embryonic transcription factor Sox2 is indispensable for the maintenance of SP cell self-renewal and stemness (59), while Oct4 and Sox2 had a relatively lesser role. Further, stimulation with nicotine could enhance the self-renewal of SP cells in a receptor dependent manner (51). We first sought to elucidate whether nicotine may enhance self-renewal through Sox2 using A549 and H1650 lung adenocarcinoma cell lines. Cells were serum starved for 24 hrs and stimulated with 2μM nicotine; induction of Sox2 was examined by immunofluorescence microscopy. Nicotine could induce Sox2 after 21 hours of stimulation in A549 cells (Figure 4.1A; similar induction was observed in human mesenchymal stem cells (Figure 4.1B), suggesting that the induction of Sox2 by nicotine is not restricted to cancer cells.

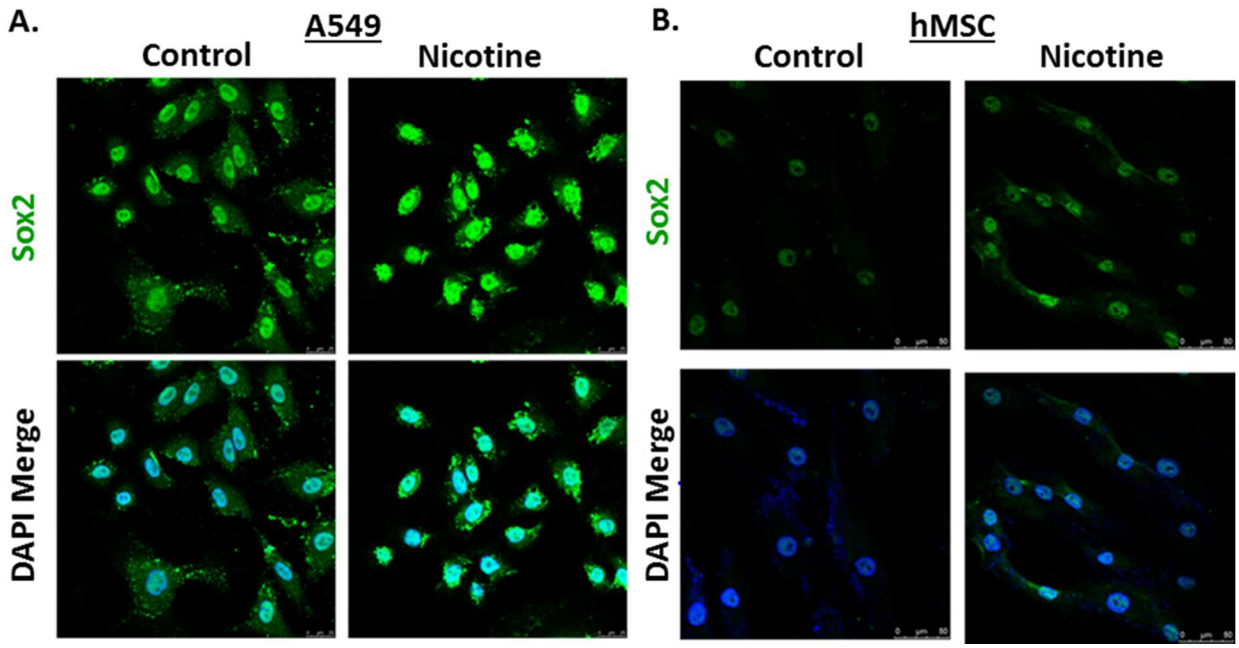


Figure 4.1. Nicotine Enhances Sox2 Expression in NSCLC Cells and hMSCs. (A and B) Immunofluorescent staining of A549 NSCLC cells and hMSCs shows increased levels of Sox2 after 21 hours of stimulation with 2μM nicotine compared to the control untreated cells.

To determine the time course of the nicotine-mediation induction of Sox2, A549 cells were stimulated with nicotine for 18, 24, 48, 72, 96, and 120 hours and Sox2 expression was assessed by qRT-PCR and by western blotting. 2μM nicotine could induce Sox2 mRNA as well as protein

at 18 and 24 hours, an effect which was diminished by 48 hours and completely abolished by 72 hours (Figure 4.2A and B). To further confirm this induction, a Sox2 core promoter luciferase construct (Sox2-core-Luc) was tested in transient transfection experiments. This construct contains the region -530bp upstream through +238 of the transcription start site (TSS)(where TSS=0) on the Sox2 gene promoter driving the luciferase reporter. Nicotine induced Sox2-core-luc activity by 2.7 and 2.2 fold after 21 hours in A549 and H1650 cells (Figure 4.2C). Since we saw the peak induction of Sox2 occurring after 18-24 hours of nicotine stimulation, we used 21 hours as the time point for the majority of experiments to follow.

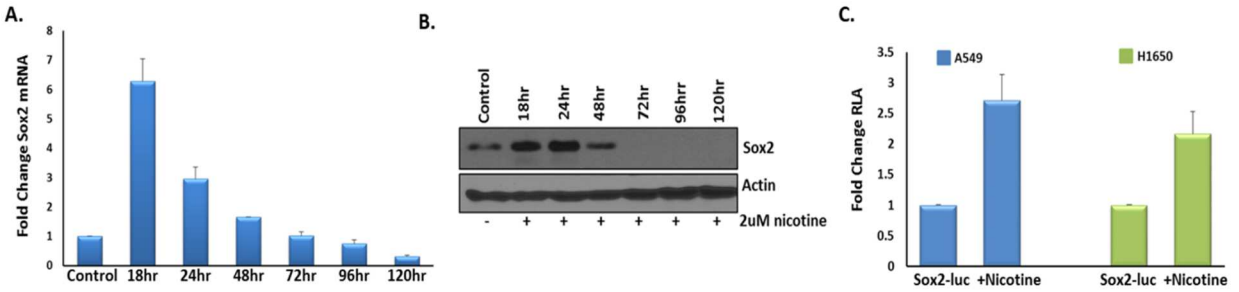


Figure 4.2. Nicotine Enhances Sox2 Expression at the Promoter, Transcript, and Protein Levels. (A) qRT-PCR of A549 cells treated with 2μM nicotine for 18, 24, 48, 72, 96, or 120 hours show increased Sox2 mRNA levels at 18 and 24 hours compared to untreated control cells; an effect diminished by 48 hours. (B) Western blot analysis showing similar results in A549 cells at the protein level. (C) Transient transfection of Sox2-core-luciferase promoter showing enhanced luciferase activity in response to 24 hours of 2μM nicotine stimulation in A549 and H460 cells.

Similar experiments were conducted to assess whether e-cigarette extracts had similar ability to induce Sox2 levels comparable to nicotine. Immunofluorescence microscopy assays in A549 cells showed that extracts from all three brands of e-cigarettes, E-cig 1, E-cig2, and E-cig 3 could induce Sox2 after 21 hours of nicotine stimulation; similar induction was also observed in hMSCs, demonstrating that the induction is not specific to CSCs (Figure 4.3A and B).

qRT-PCR and western blot experiments confirmed that E-cig1, E-cig2, and E-cig3 could induce Sox2 mRNA and protein expression in A549 cells, after 21 hours of stimulation at levels comparable to nicotine (Figure 4.4A and B). Transient transfection assays further demonstrated that extracts from each of the three brands of e-cigarettes could also induce Sox2-core-luc activity

after 21 hours of stimulation by 2.3, 2.2, and 2.3 folds in A549 cells, and by 2.2, 2.1, and 2.5 fold in H1650 cells (Figure 4.4C).

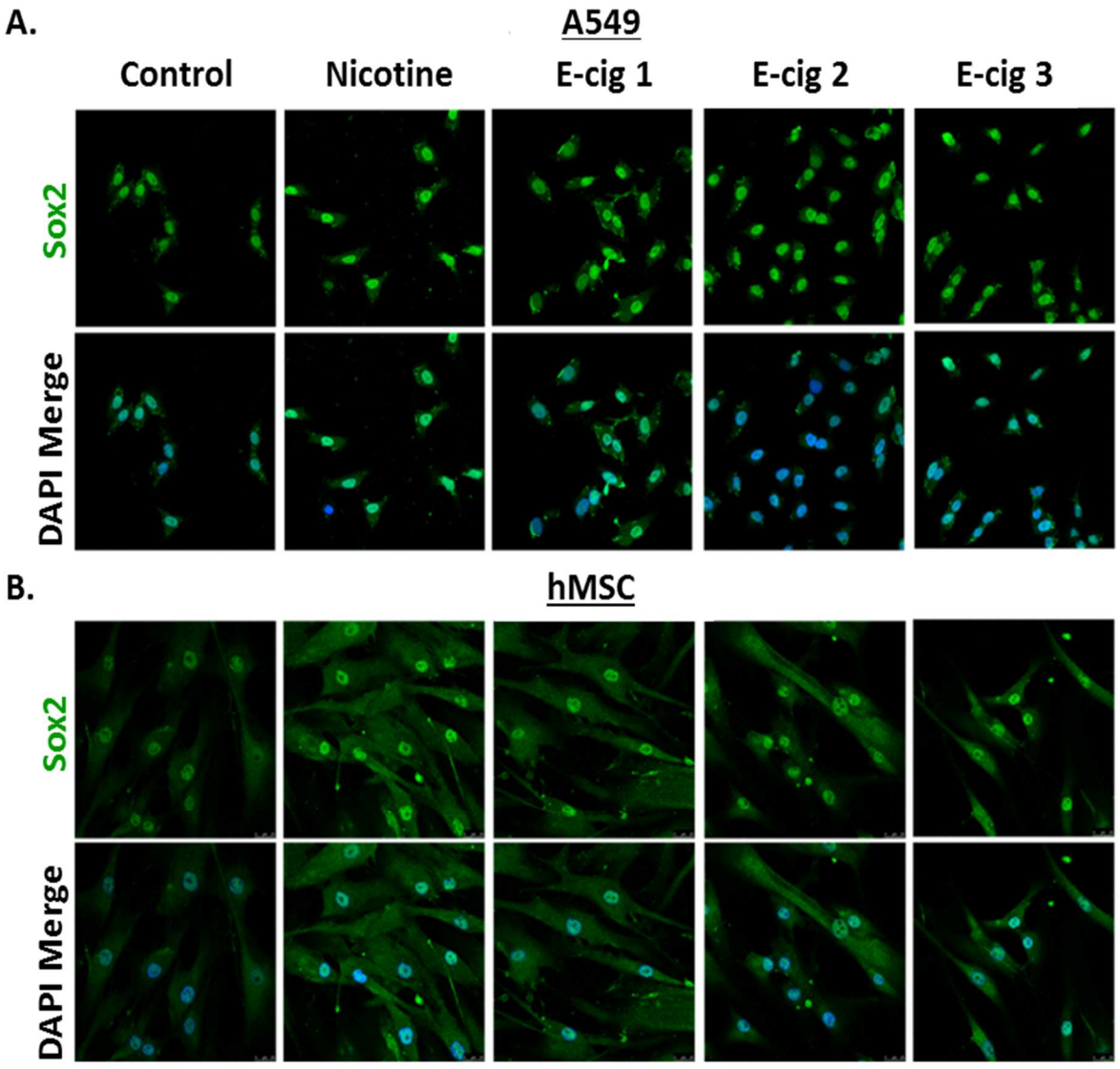


Figure 4.3. E-cigarette Extracts Enhance Sox2 Expression in A549 Cells and hMSCs. (A and B) Immunofluorescent staining showing that 2μM nicotine or 2μM of extracts from each of three brands of e-cigarettes can enhance Sox2 expression levels after 24 hours, as compared to untreated control cells.

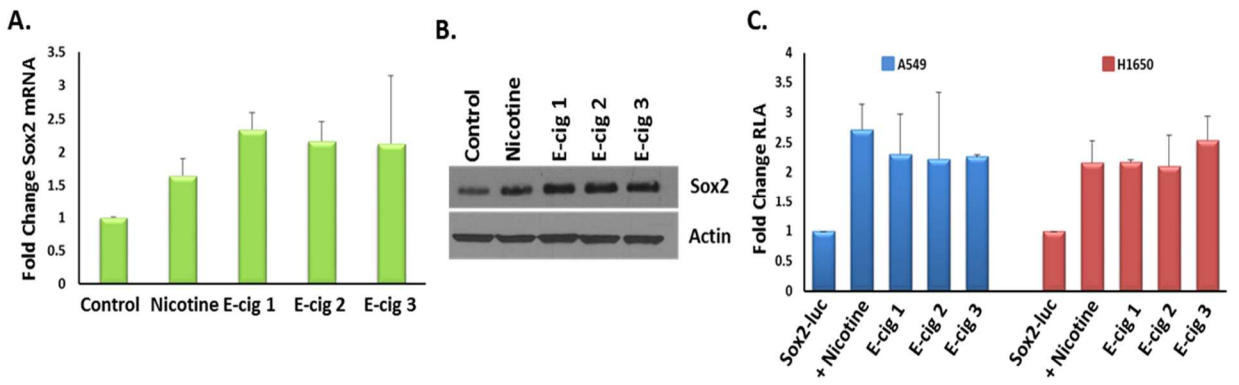


Figure 4.4. E-cigarette Extracts Enhance Sox2 Expression at the Promoter, Transcript, and Protein Levels. (A) qRT-PCR of A549 cells treated with 2μM nicotine or 2μM of each brand of e-cigarette for 24 hours shows increased Sox2 mRNA compared to untreated control cells. (B) Western blot analysis showing similar results in A549 cells at the protein level. (C) Transient transfection of Sox2-core-luciferase promoter showing enhanced luciferase activity in response to 24 hours of 2μM nicotine or 2μM e-cigarette extract stimulation in A549 and H460 cells.

Since we had shown that nicotine could enhance the stemness and self-renewal ability of SP cells, we next determined whether this was observed with e-cigarette extracts. A549 and H1650 cells were sorted as described in materials and methods, and SP and MP cells were plated separately. Cell sorting and self-renewal is depicted in Figure 4.5.

At the time of plating, nicotine or extracts from each of the three brands of e-cigarettes was added to the cell media in the corresponding wells. After 10 days cells were imaged to asses sphere formation as an indication of self-renewal using microscopy. These experiments showed that e-cigarette extracts could enhance sphere formation and self-renewal in a manner similar to nicotine in A549 and H1650 cells (Figure 4.6A and B). We further demonstrated that if similar experiments were conducted after Sox2 was depleted using siRNA, the ability of nicotine or e-cigarette extracts to enhance self-renewal was diminished in A549 or H1650 cells (Figure 4.7A and B).

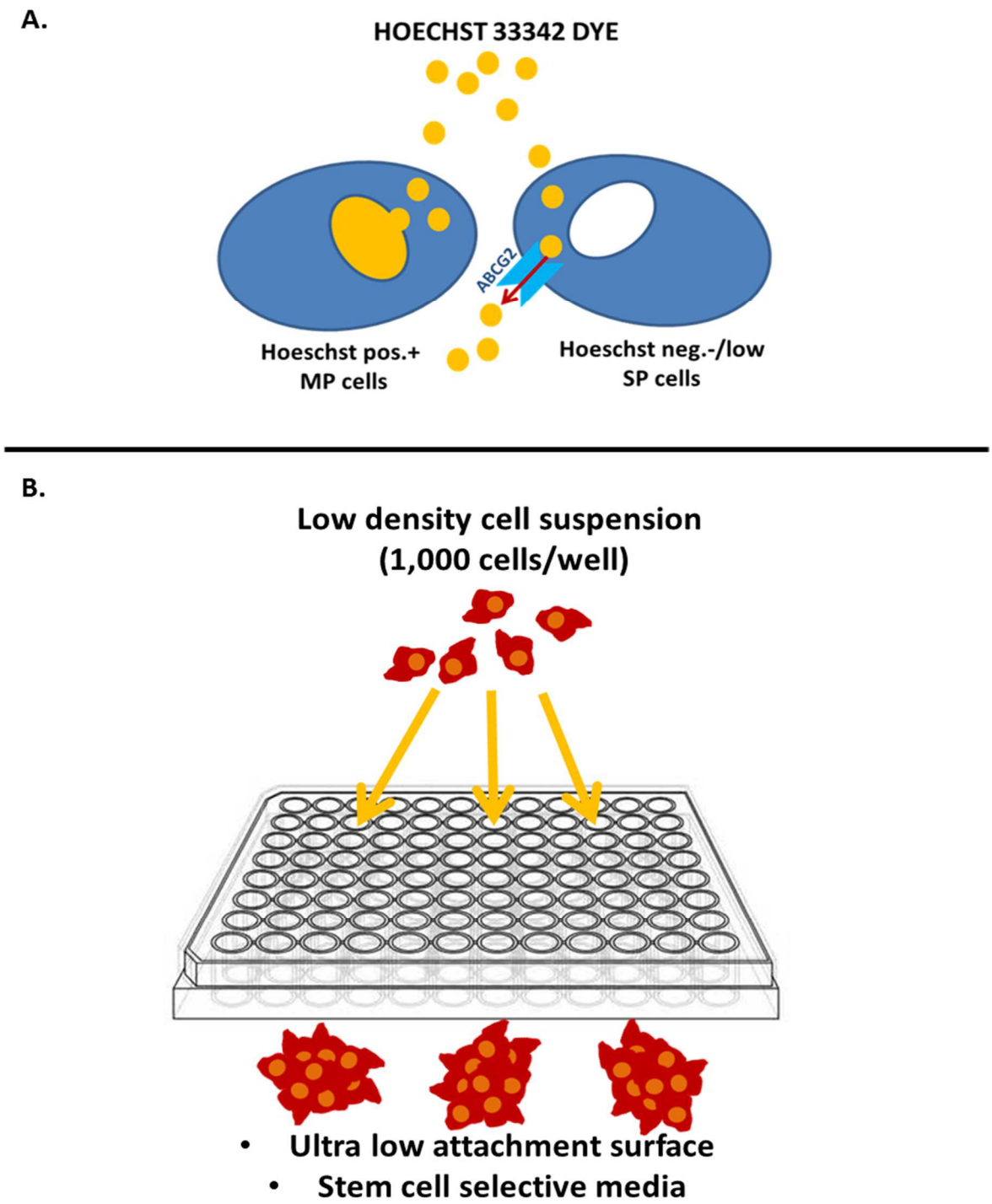


Figure 4.5. Depiction of SP Cell Sort and Self-Renewal Assay Parameters. (A) Heterogenous cell populations are stained with Hoechst 33342 nuclear dye. SP cells, enriched in cancer stem cell populations, have increased expression of the ABCG2 drug transporter, and are able to efflux the dye so the nucleus is not stained while the MP cells retain the dye. Cells are then sorted by flow cytometry based on nuclear staining. (B) To assess self-renewal capability, sphere formation assay are conducted by plating sorted cells at low density, onto ultra-low attachment surfaces, in stem cell selective media, and allowed to form spheres for 10 days.

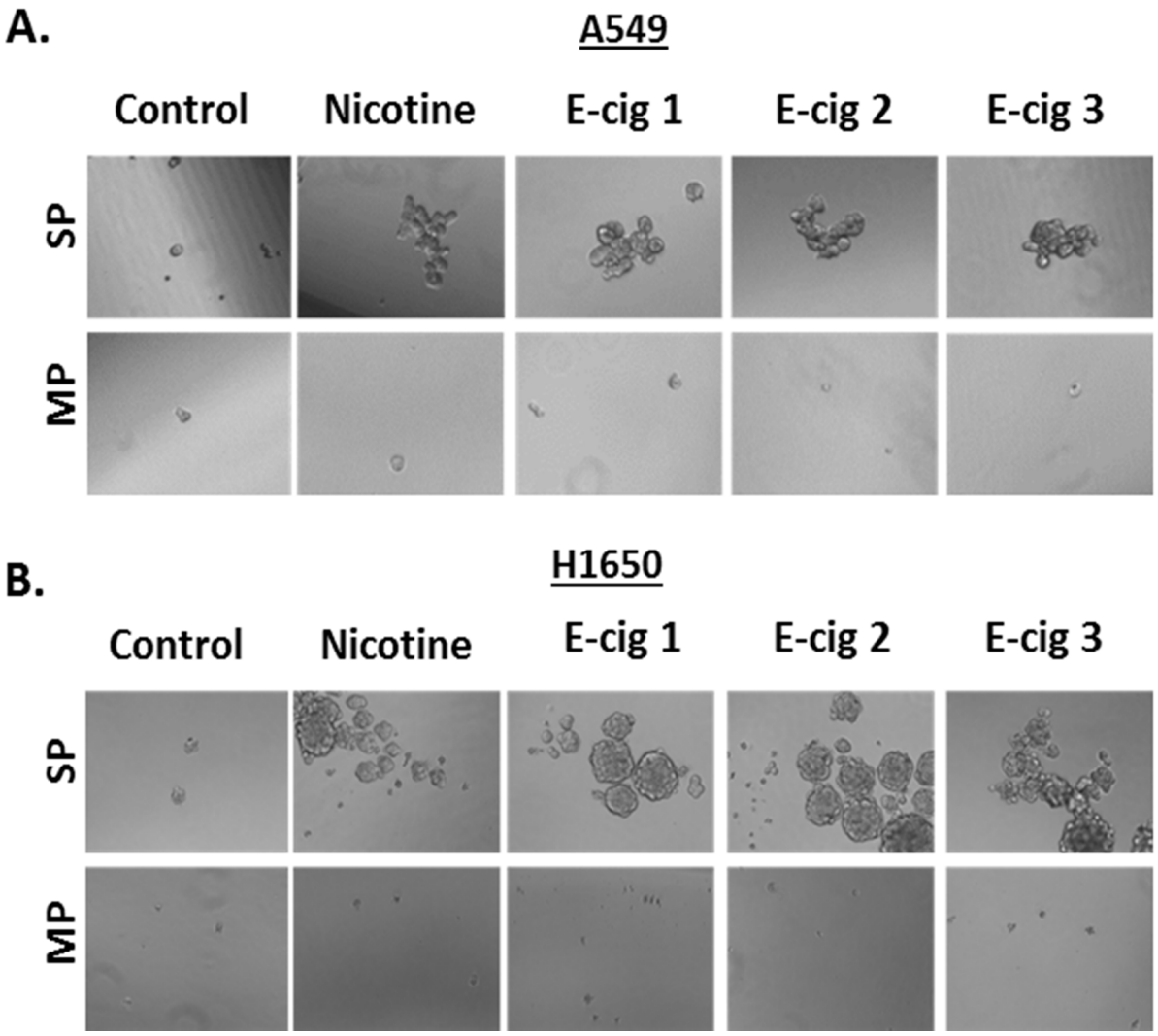


Figure 4.6. E-cigarette Extracts Enhance Self-Renewal Ability of SP Cells. (A and B) Self-renewal assays using A549 and H1650 NSCLC cells demonstrated that 2μM of nicotine or each of the three e-cigarette extracts could enhance sphere formation, indicative of self-renewal, compared to untreated controls.

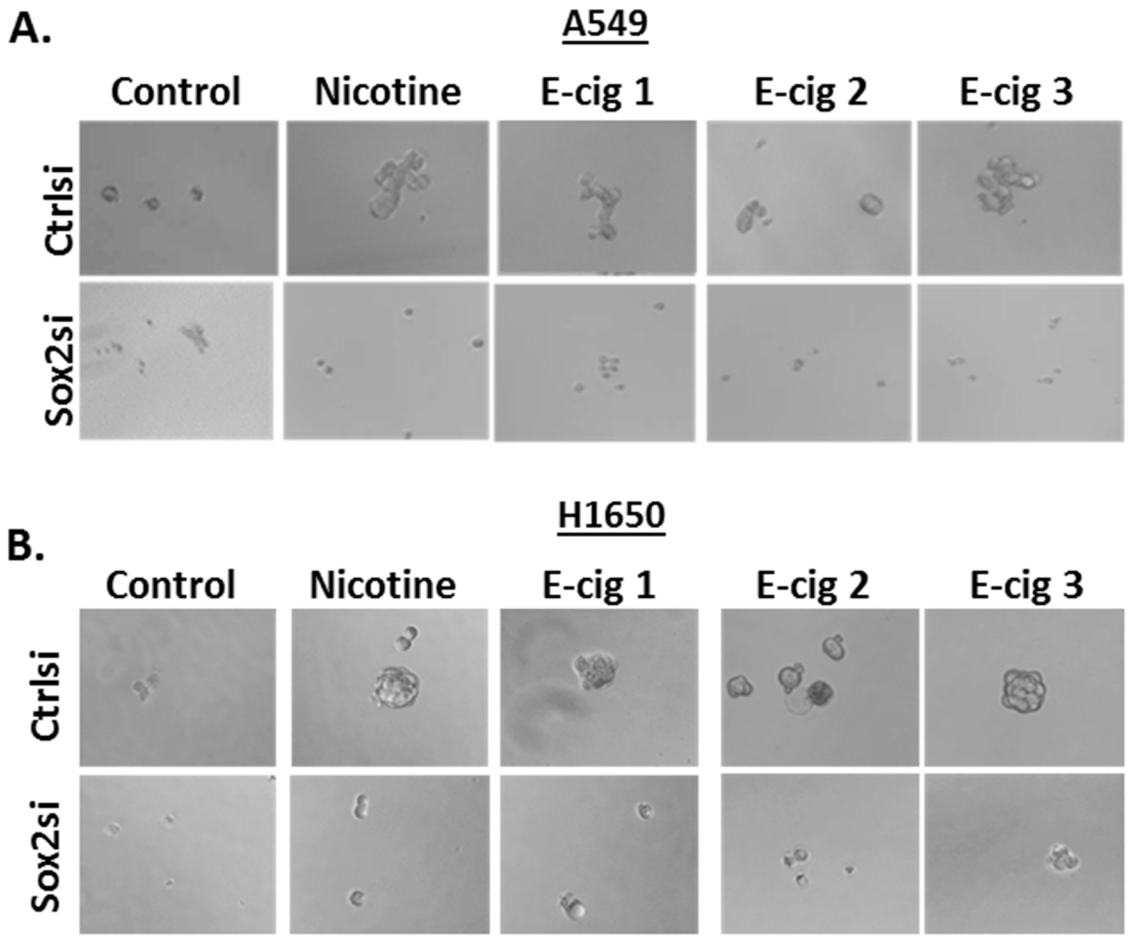


Figure 4.7. Depletion of Sox2 Abrogates the Nicotine or E-cigarette Mediated Induction of Self-Renewal of SP cells. (A and B) SP cells from A549 or H1650 cells treated with Sox2 siRNA show diminished ability to form spheres in the presence of 2μM nicotine or e-cigarette extracts in self-renewal assays.

Nicotine-Mediated Induction of Sox2 Occurs Through a nAChR-Yap1-E2F1 Axis

We next sought to elucidate the mechanism by which nicotine induces expression of Sox2. Previous studies have shown the nicotine exerts a number of tumor promoting properties such as proliferation, migration, and invasion through the binding to and activation of α7 nAChR, and that the nicotine-mediated activation of these receptors results in the transcriptional activity of E2F1 transcription factor (21, 22, 24, 159). We have also previously reported that Yes Associated Protein 1 (Yap1), which is a transcriptional co-activator and the major effector of the Hippo signaling pathway, binds to Oct4 embryonic stem cell transcription factor on the Sox2 promoter to regulate both Sox2 expression as well as the stem-like functions of cancer stem-like

cells. Induction of Sox2 by Yap1 occurs independent of TEAD2 transcription factor, which is a well documented binding partner and mediator of Yap1 functions (160). In this context, we depleted α7 nAChR, E2F1, Yap1, Oct4, or Tead2 using siRNA in A549 cells, stimulated with nicotine, and conducted western blot experiments; it was found that depletion of α7 nAChR, E2F1, or Yap1 could abrogate nicotine-mediated induction of Sox2 at the protein level (Figure 4.8A). To verify that the siRNAs used could reduce the expression of the indicated factors, transient transfection experiments were conducted in untreated A549s cells demonstrating that siRNAs targeting α7 nAChR, E2F1, Yap1, Oct4, Sox2, or Tead2 effectively reduced the corresponding protein level of each, as shown by western blot (Figure 4.8B-E). This led us to hypothesize that perhaps downstream of nicotine binding to the α7 nAChR, Yap1 is being activated to bind E2F1 on the Sox2 promoter to induce its expression.

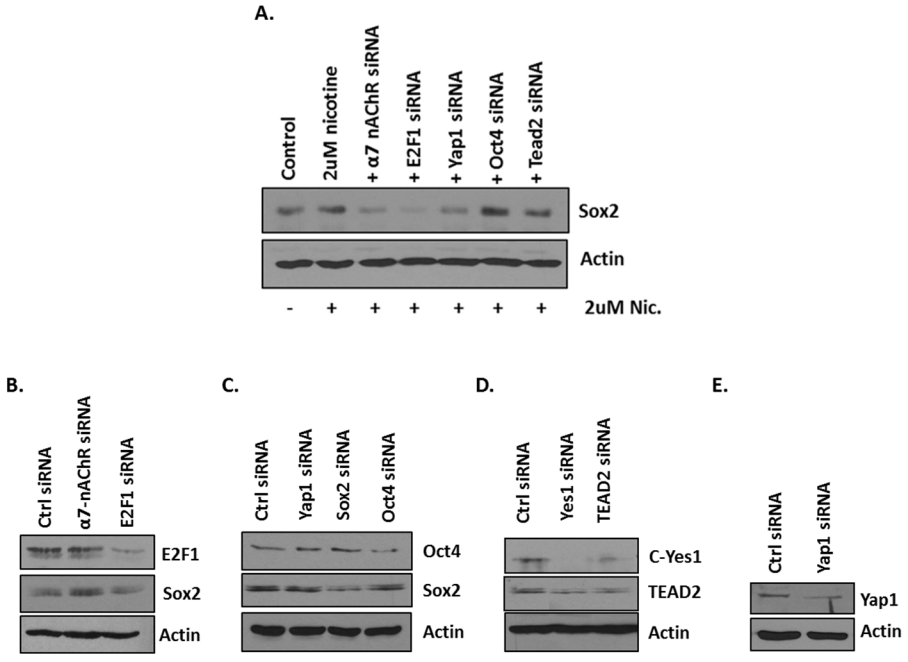


Figure 4.8. Depletion of α7 nAChR, E2F1, or Yap1 Could Abrogate Nicotine-Mediated Induction of Sox2. (A) Western blot analysis showing the protein levels of Sox2 to be reduced after transfection using siRNA targeting α7 nAChR, E2F1, or Yap1 followed by nicotine stimulation, compared to control scrambled siRNA transfected nicotine stimulated A549 cells. (B-E) Western blot analysis verifying the siRNA mediated depletion of E2F1, Sox2, Oct4, Yes1, TEAD2, and Yap1 in A549 cells. Upon depletion of each factor, corresponding protein levels are seen to be reduced as compared to scrambled siRNA transfected control cells.

Sox2 Gene Expression is Regulated by E2F1, and Nicotine Enhances E2F1 Binding to Sox2

To determine whether E2F transcription factors bind to the Sox2 gene promoter, Genomatix MatInspector Analysis Software was used to analyze a 1500bp region upstream of the Sox2 TSS for potential E2F binding sites; 19 predicted E2F binding sites were detected on this promoter region (Figure 4.9A). Primers were designed spanning four different regions of the Sox2 promoter and chromatin immunoprecipitation assays were carried out to validate whether E2Fs bound to the predicted regions. E2F1 and to a lesser extent E2F2 and E2F3 could bind to a region –104 through –259bp upstream of TSS (denoted as F1R1) in A549 cells; E2F1, E2F2, and E2F3 could also bind to a region could bind to a region –266 through –497bp upstream of TSS denoted as F2R2; E2F1 and to a lesser extent E2F2 and E2F3 could bind could to a region –1078 through –1233bp upstream of TSS denoted as F3R3; and E2F1 and to some extent E2F2 could bind to a region –1414 through –1433bp upstream of TSS denoted as F4R4. An irrelevant IgG antibody was used as a negative control for these experiments, and showed no amplification for any region (Figure 4.9B).

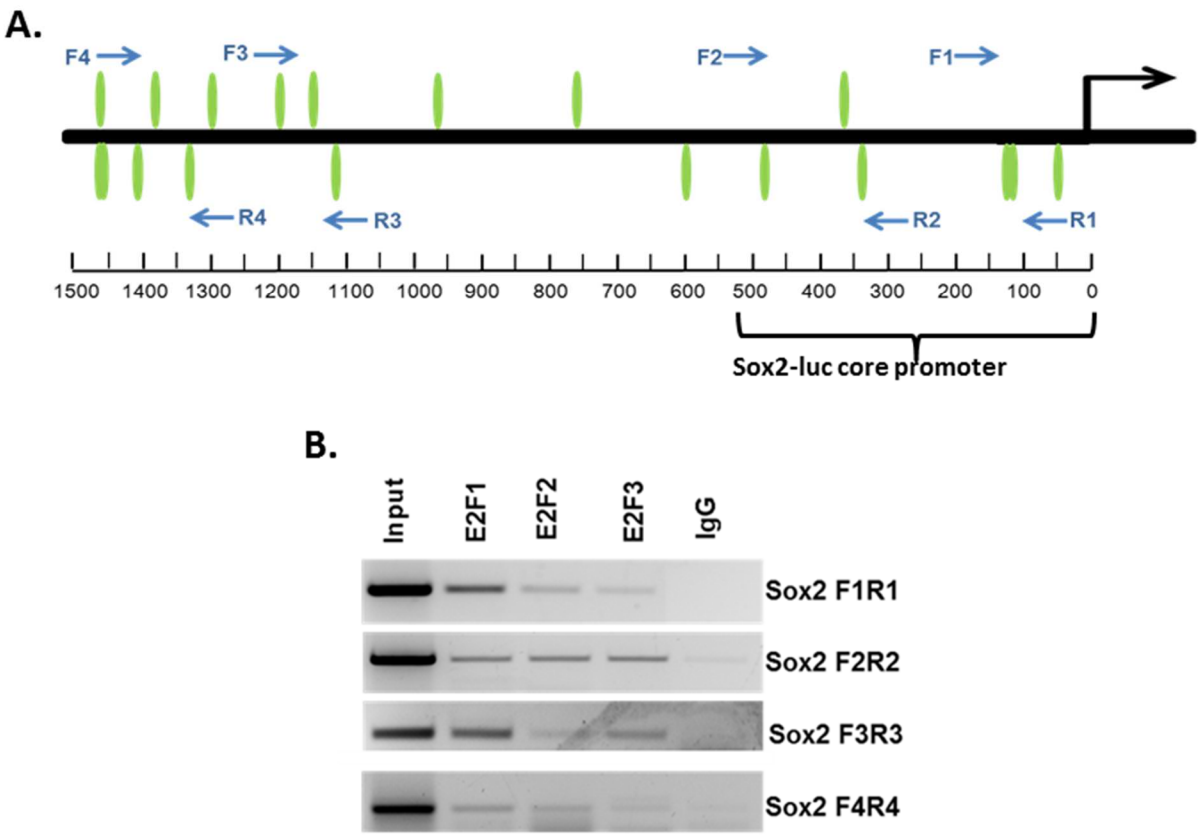


Figure 4.9. E2Fs have Multiple Predicted Binding Sites and can Associate with the Sox2 Promoter. (A) Schematic representation of a 1500bp region of the Sox2 gene promoter showing potential E2F binding sites as green ovals. Arrows represent the position of primers spanning E2F and STAT bindig sites used for ChIP assays. (B) ChIP-PCR assays in A549 cells showing binding of E2F1 and to a lesser extent E2F2 and E2F3 could bind to a region –104 through –259bp upstream of TSS (F1R1); E2F1, E2F2, and E2F3 could bind to a region–266 through –497bp upstream of TSS (F2R2); E2F1 and to a lesser extent E2F2 and E2F3 could bind could to a region –1078 through –1233bp upstream of TSS (F3R3); and E2F1 and to some extent E2F2 could bind to a region –1414 through –1433bp upstream of TSS (F4R4).Sonicated DNA was used as input control, and there was no detectable amplification from irrelevant IgG, used as negative control.

Given that E2Fs could bind to the Sox2 promoter, transient transfection experiments were conducted to determine whether E2Fs could regulate Sox2 expression. It was found that the transcriptionally active E2F family members (E2F1-3) could induce Sox2-core-luc activity by 2.3, 1.8, and 1.8 fold respectively, while little or no effect was seen with the inactive E2F4 and E2F5 (0.7 and 1.0 fold respectively) in A549 cells (Figure 4.10).

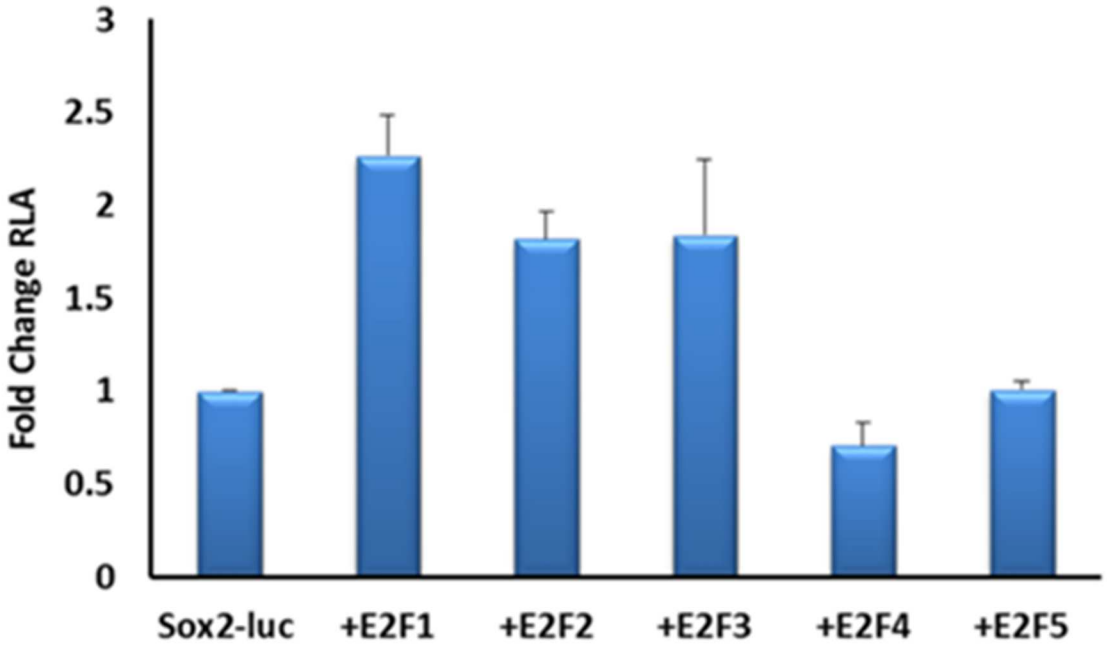


Figure 4.10. E2F1-3 can Induce Sox2-luciferase Activity. Transient transfection of A549 cells using 0.5ug of Sox-core-luciferase showed that co-transfection using 1.0ug of E2F1, E2F2, or E2F3 could induce promoter activity, while little to no difference was seen with E2F4 or E2F5.

To further assess whether the association of E2F1 with the Sox2 promoter was responsive to nicotine stimulation, A549 cells were serum starved and treated with nicotine, and ChIP assays were conducted. It was found which that nicotine could enhance recruitment of E2F1 to the Sox2 promoter after 18 hours of stimulation, and both the F1R1 region -104 through -259bp and the F2R2 region -266 through -497bp upstream of TSS (Figure 4.11A and B).

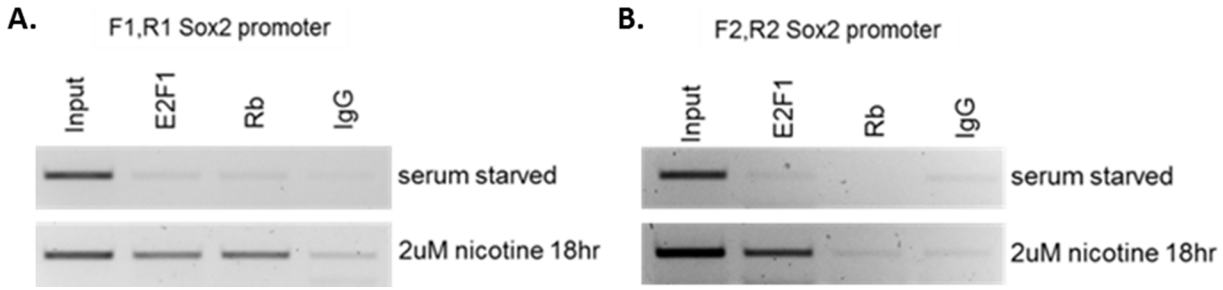


Figure 4.11. Nicotine Enhances the Recruitment of E2F1 Transcription Factor to the Sox2 Promoter. (A and B) ChIP-PCR assays demonstrated that after 18 hours of stimulation with 2μM nicotine, there was increased association of E2F1 to the F1R1 and F2R2 regions on the Sox2 promoter in A549 cells compared to control serum starved cells.

Binding of Yap1 to E2F1 is Enhanced with Nicotine

It has been suggested that Yap1 could regulate E2F1-mediated transcription. Given that Yap1 can regulate Sox2 expression, and since E2F1 was found to associate with the Sox2 promoter, we next examined whether Yap1, could co-localize or interact with E2F1 and if this was sensitive to nicotine or e-cigarette extract stimulation. Double immunofluorescence experiments showed that Yap1 and E2F1 co-localized in A549 cells: treatment with nicotine or extracts from each of the three e-cigarette brands enhanced this interaction (Figure 4.12).

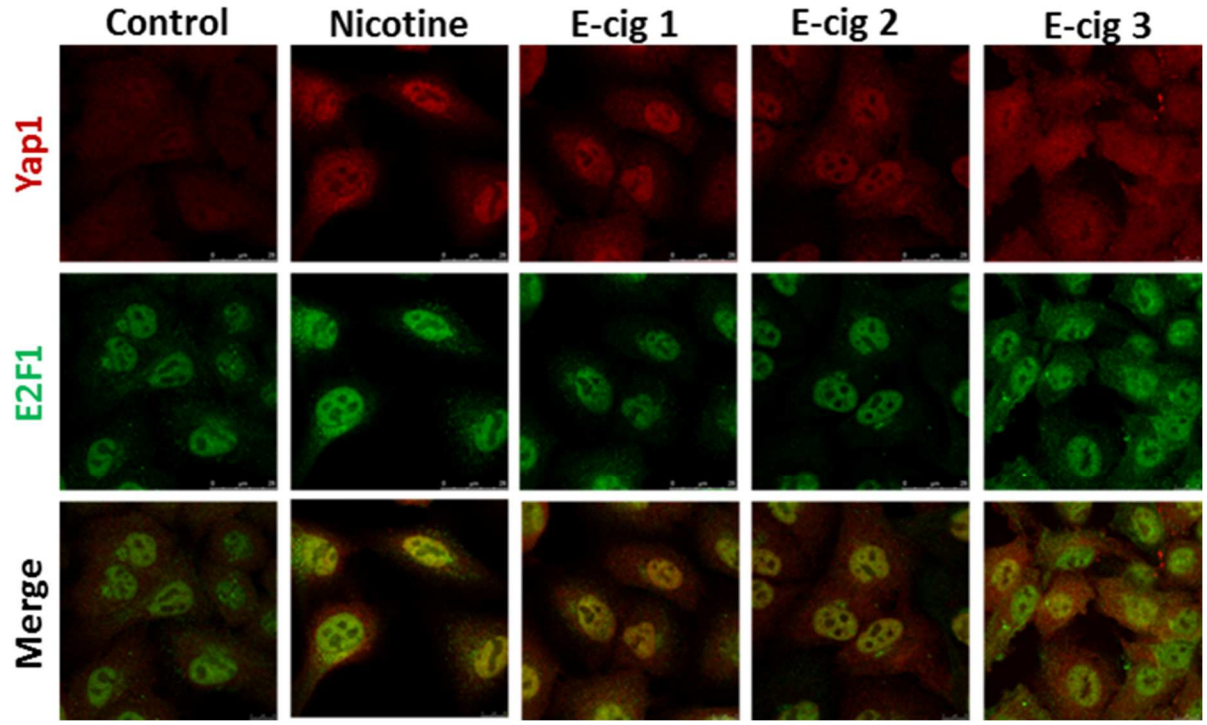


Figure 4.12. Yap1 and E2F1 Colocalize in Cells, and this Interaction is Increased in the Presence of Nicotine or E-cigarette Extracts. Double immunofluorescent staining of E2F1 (green) and Yap1 (red) showing colocalization of the two proteins in A549 cells. Stimulation with 2μM nicotine or e-cigarette extracts enhanced colocalization after 24 hours.

Similarly, proximity ligation assays (PLA) (161) were conducted to determine whether Yap1 and E2F1 proteins exist in close proximity within the cell, and at what point in time this occurs. PLA is able to detect proteins within a 40nm range of one another, which generally indicates a direct physical interaction, and this can be visualized as individual foci by confocal microscopy. These assays showed that Yap1 and E2F1 existed in close proximity of one another, and the

interaction was enhanced by nicotine as well as extracts from each of the three brands of e-cigarettes. We further find that this effect increased from 6 to 12 to 18 to 24 hours of nicotine or e-cigarette extract stimulation, coinciding with the time points at which Sox2 is induced (Figure 4.13).

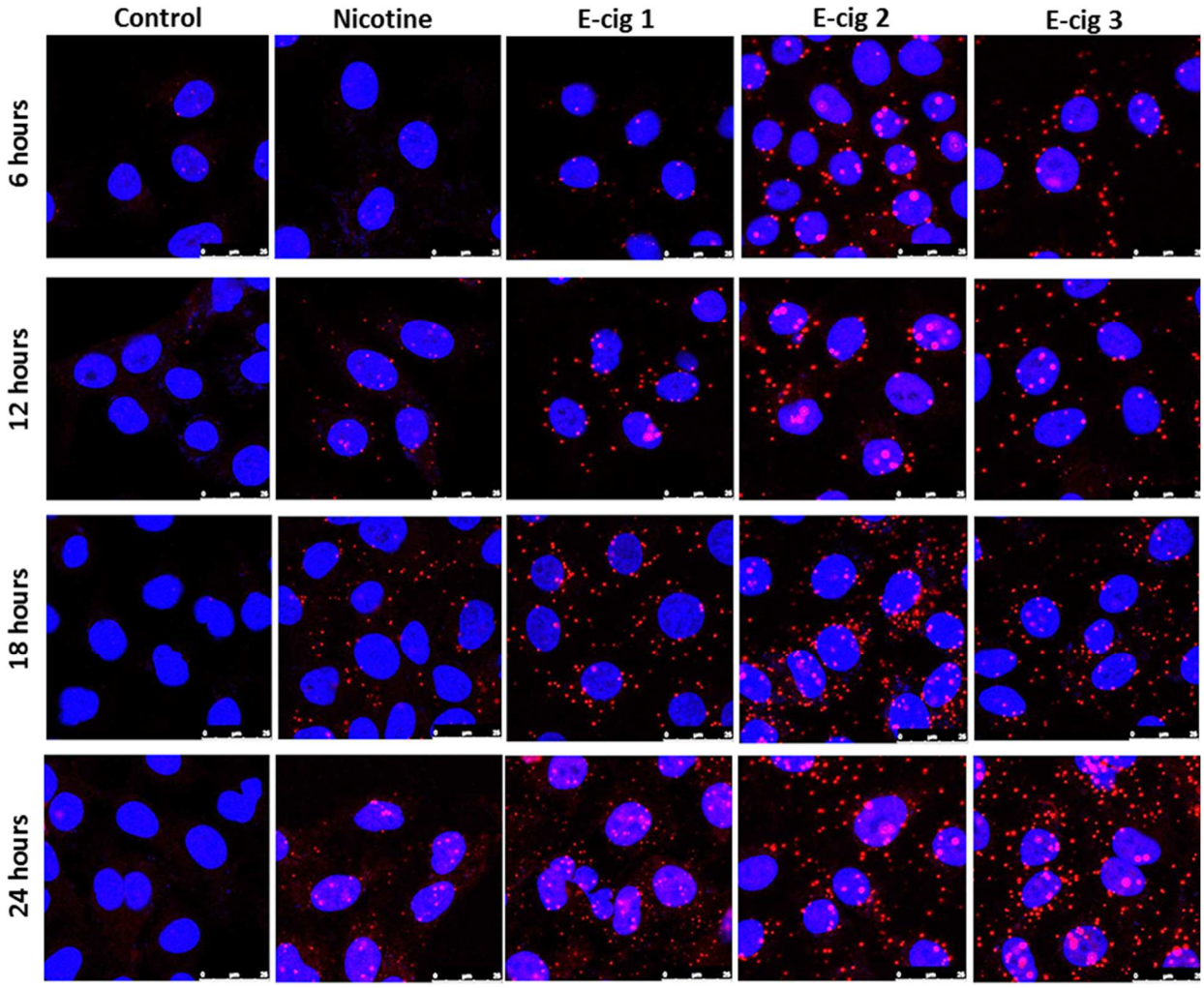


Figure 4.13. Yap1 Associates with E2F1 Transcription Factor within Close Proximity and this is Enhanced by Nicotine or E-cigarette Extracts. Proximity ligation assay (PLA) conducted on A549 cells demonstrated association of Yap1 and E2F1 proteins indicated as red foci; this association was enhanced in response to 2μM nicotine or e-cigarette stimulation and increased over time beginning at 6 hours through 18 and 24 hours (the time points at which we find Sox2 to be induced by nicotine and e-cigarette extracts).

To further confirm the physical interaction of Yap1 and E2F1 proteins, immunoprecipitation-western blot experiments were conducted; these showed that Yap1 could physically interact

with E2F1 in untreated A549 and H1650 cell lines. Yap1 could be detected in E2F1 immunoprecipitates, by western blotting, and vice versa. An irrelevant IgG was used as a negative control in both cases to establish the specificity of the assay (Figure 4.14A and B).

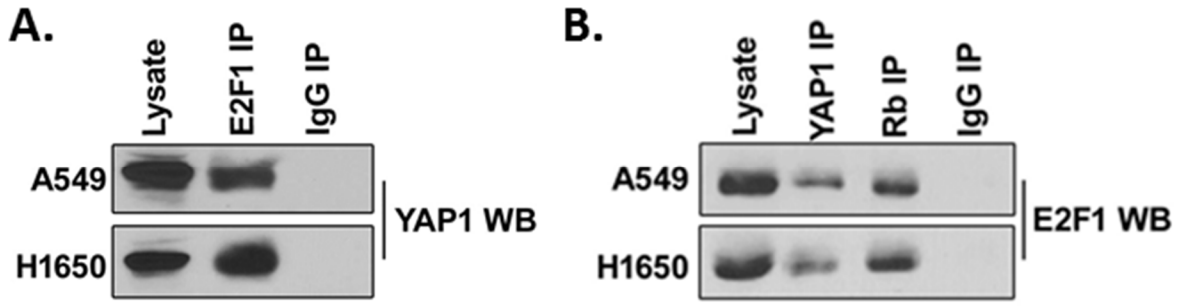


Figure 4.14. Yap1 and E2F1 Proteins Physically Interact. (A) Yap1-E2F1 binding is detected by immunoprecipitation with E2F1 antibody followed by western blot with Yap1 antibody in A549 and H1650 cells. (B) Reciprocally, this is shown by immunoprecipitation with Yap1 antibody followed by western blot with E2F1 antibody. Normal IgG was used as a negative control.

Since we had previously reported that Yap1 could induce Sox2 expression and here we find that E2F1 not only induces Sox2 expression but can also interact with Yap1, we next sought to determine whether Yap1 and E2F1 cooperatively induce the Sox2 promoter. Transient transfection experiments showed that Yap1 or E2F1 alone could induce Sox2-core-luc activity by 1.8 and 2.7 fold respectively; when both were transfected together, they showed an 6.2 fold induction of Sox2-core-luc activity, indicating a co-operative effect (Figure 4.15).

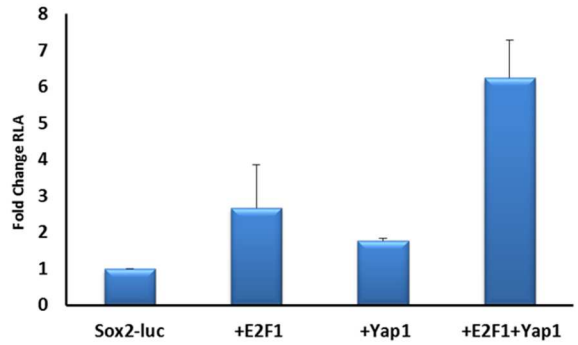


Figure 4.15. Yap1 and E2F1 have an Additive Effect on Induction of Sox2-luciferase Activity. A549 cells transfected with 0.5ug Sox2-core-luciferase showed increased activity when co-transfected with 1.0ug E2F1 or Yap1 separately, and this effect was further enhanced when E2F1 and Yap1 were co-transfected together.

Yap1 and E2F1 Association with the Sox2 Promoter is Enhanced with Nicotine

To further interrogate whether Yap1 and E2F1 mediate Sox2 expression in response to nicotine stimulation, we conducted ChIP experiments on A549 cells stimulated with nicotine for 21 hours. It was found that association of both E2F1 and Yap1 proteins to the Sox2 promoter was enhanced in response to nicotine, compared to unstimulated, serum-starved cells. Interestingly, we also found that the presence of known chromatin modifiers on the promoter was altered with nicotine. Histone 3 tri-methylation of lysine 27 (H3K27me3) is known to contribute to gene repression and is present on silent chromatin, while histone 3 mono-methylation of lysine 27 (H3K27me1) is conversely known to be associated with gene activation (189, 190). Here we report that after 21 hours of nicotine stimulation in A549 cells the repressive marker H3K27me3 has diminished association with Sox2 promoter while the activation marker H3K27me1 has enhanced association indicating that this promoter becomes activated in response to nicotine (Figure 4.16). Further, previous literature has postulated that H3K27me3 repression is associated with maintenance of ESC pluripotency. Studies mapping polycomb repressive complex 2 (PRC2) (which catalyzes H3K27me3 modifications) across the entire human ESC genome found that PRC2 is distributed across large portions of genes occupied by Sox2, Oct4, and Nanog transcription factors and these gene are heavily occupied by trimethylated H3K27 nucleosomes acting to repress their expression until activated for differentiation (189, 191, 192). The key finding here is that the presence of K3K27me3 is not indicative of maintaining pluripotency through the Sox2 gene itself, but rather by co-occupying genes which Sox2 transcription factor (as well as Oct4 and Nanog) turns on during differentiation; thus, the reduced association of H3K27me3 seen in response to nicotine is likely involved in the nicotine-mediated activation of Sox2 expression, and Sox2 expression is known to be critical for maintaining stem-cell phenotypes in both NSCLC and ESC cells (59, 193).

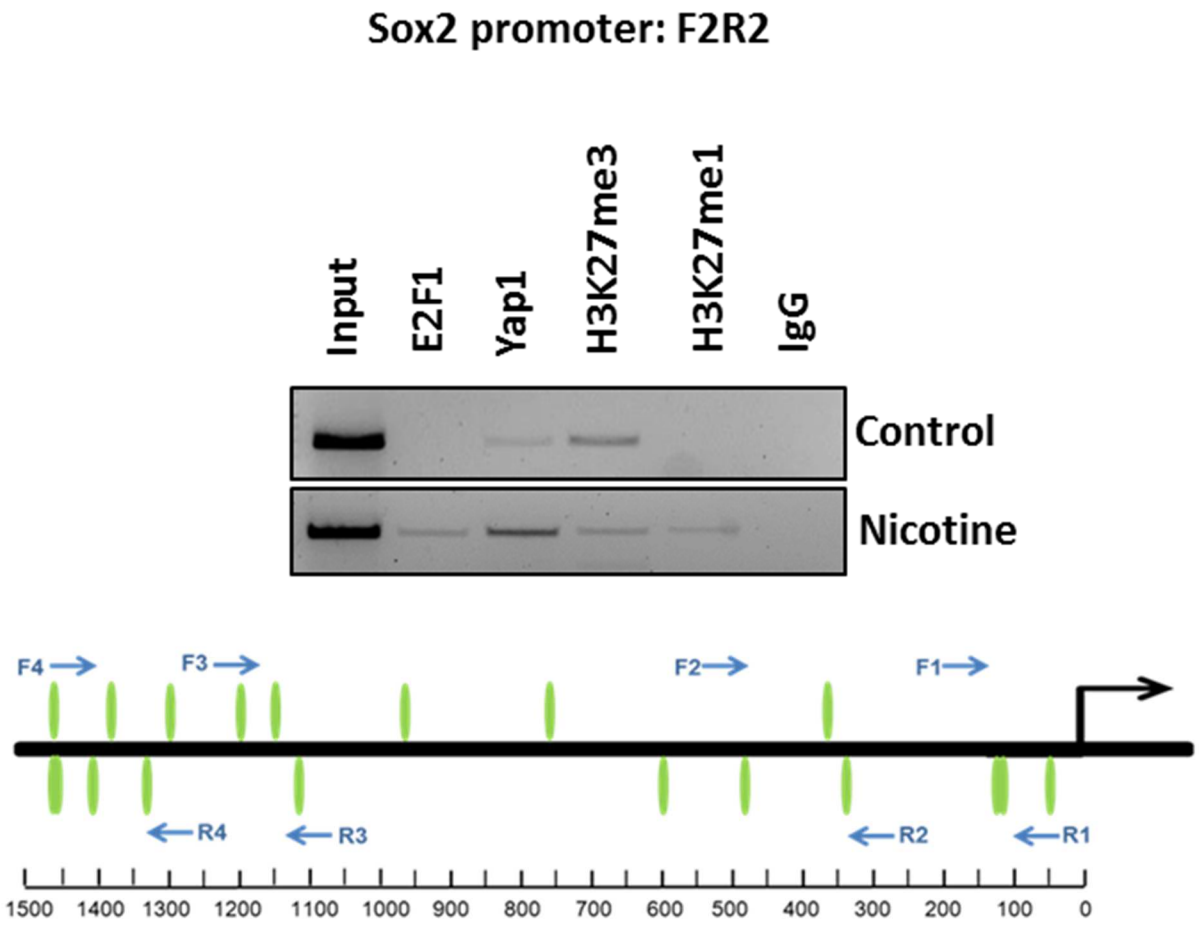


Figure 4.16. Nicotine Enhances Recruitment of E2F1, Yap1, and H3K27me1 while Reducing H3K27me3 Recruitment to the Sox2 Promoter at Region -266 Through -497bp Upstream TSS. ChIP-PCR assays showed that 21 hours of stimulation with 2μM nicotine could enhance association of E2F1, Yap1, and H3K27me1 (chromatin activation marker) to the Sox2 promoter, with a concomitant reduction in association of H3K27me3 (chromatin repressive marker) in A549 cells.

Nicotine and E-cigarette Extracts Enhance Yap1 Expression

During our experiments to determine the role of Yap1 and E2F1 in regulating Sox2 expression in the context of nicotine, we observed that the expression of Yap1 itself was enhanced upon nicotine stimulation. Indeed, it has been reported that nicotine could induce Yap1 in certain cell types (194). Additional immunofluorescence microcopy experiments in A549 cells showed that nicotine and all three brands of e-cigarette extracts induced Yap1 levels after 21 hours. Nicotine could induce Yap1 expression in hMSCs as well, demonstrating that the induction is not specific to NSCLC cells (Figure 4.17A and B).

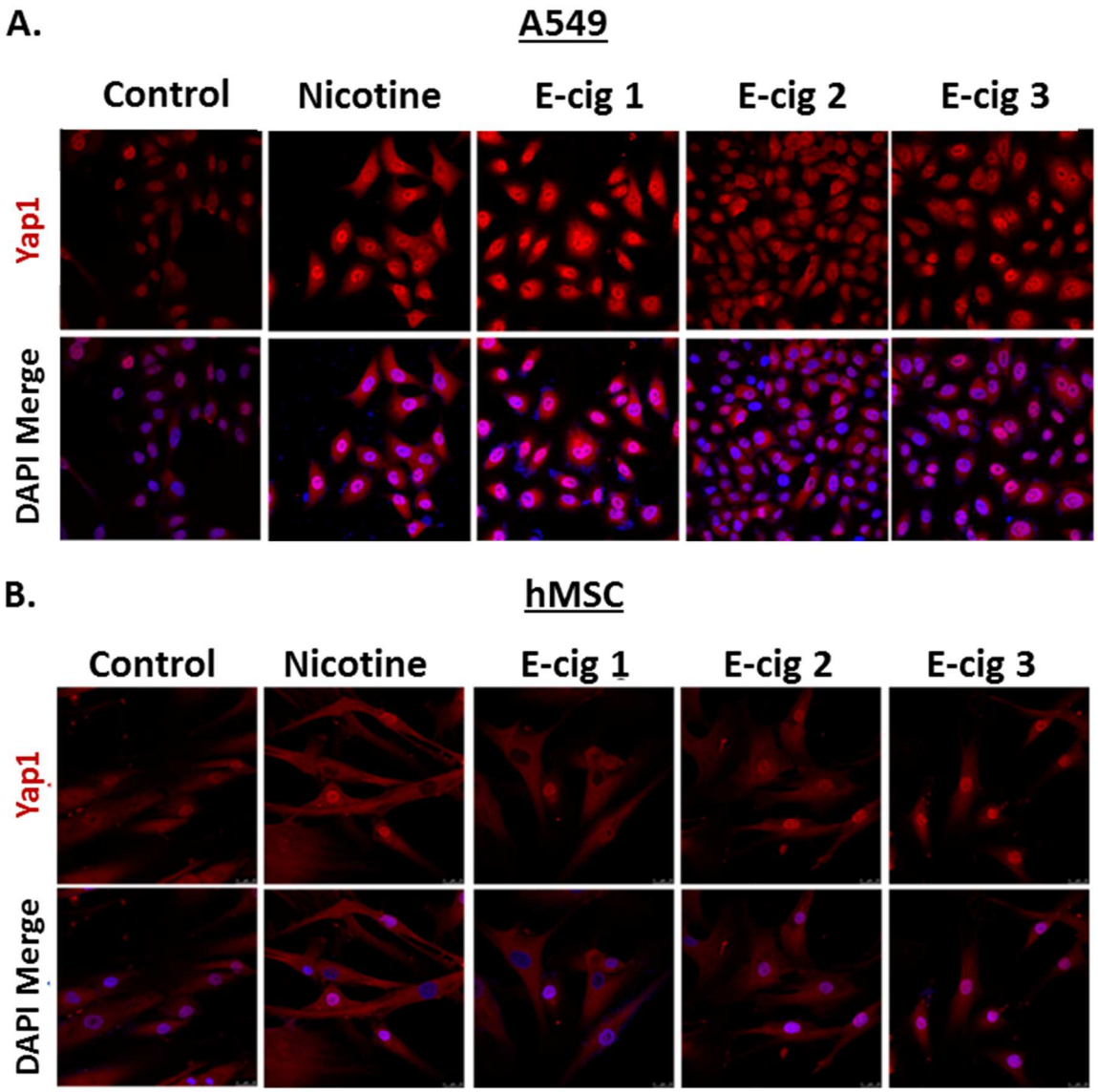


Figure 4.17. Nicotine and E-cigarette Extracts Enhance Yap1 Expression in A549 and hMSCs. (A and B) Immunofluorescent staining showing that 2μM nicotine or 2μM of each of three brands of e-cigarette extracts can enhance Sox2 expression levels after 24 hours, as compared to untreated control cells.

These results were confirmed at the mRNA level by qRT-PCR, which showed that nicotine and each brand of e-cigarette extracts induced Yap1 transcripts compared to serum starved control cells (Figure 4.18).

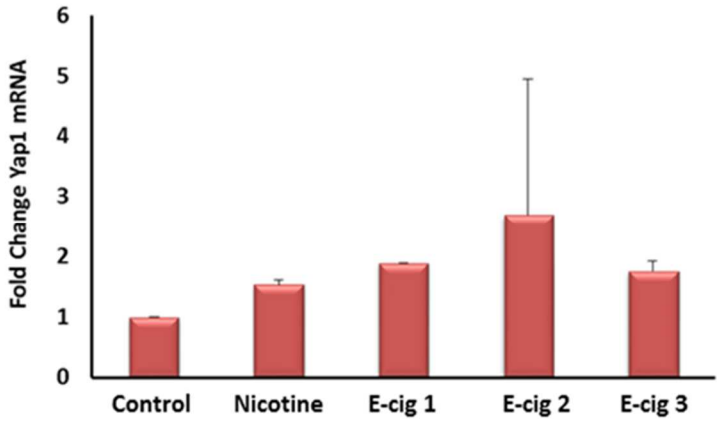


Figure 4.18. Nicotine and E-cigarette Extract Enhance Yap1 mRNA Levels. A549 cells treated with 2μM nicotine or e-cigarette extracts for 21 hours showed increased Yap1 mRNA expression compared to the untreated control cells as seen by qRT-PCR.

Nicotine-Mediated Induction of Sox2 Occurs Through Src Kinases

The Yap1 protein is known to be oncogenic as it promotes growth while inhibiting apoptosis, and is amplified or overexpressed in a number of cancer types (195-197). It is named Yes-associated-Protein 1 due to its ability to associate with SH3 domains of Src family tyrosine kinases which include Src, Yes, and Fyn (195). Typically in quiescent cells, Yap1 is phosphorylated downstream of the tumor suppressive Hippo pathway, and when phosphorylated it is sequestered in the cytoplasm by 14-3-3 proteins resulting in degradation, thereby preventing its nuclear import (198). Conversely, Yap1 is phosphorylated by Yes1 in embryonic stem cells, leading to its activation (2011). Multiple studies have now reported that Yap1 has functions independent of the Hippo pathway, and it has further been shown that Yap1 is a direct phosphorylation target of Src in a number of cancer cell lines, independent of the canonical Hippo pathway (199-201). Our lab has previously reported that when nicotine binds to α7 nAChR, the β-arrestin-1 (β-arr-1) scaffolding protein is recruited and required to then activate phospho-Src kinase (p-Src), which mediates a number of downstream pathway effectors, including E2F1 transcriptional activity (46, 103). We have additionally reported that

inhibition of the EGFR pathway including Src and PI3K could strongly inhibit Sox2 expression, thereby suppressing the self-renewal of SP cells enriched in NSCLC stem-like cells; and this occurred in a β-arrestin-1 (but not β-arrestin-2) dependent manner (59, 202). In this context we next sought to determine whether nicotine-mediated regulation of Sox2 by Yap and E2F1 was a result of the upstream activation of Src or Yes kinases. Initial studies in A549 cells using inhibitors to Src/Yes/Fyn (Saracatinib), PI3K (Buparsilib), MEK1/2 (Trametinib), CDK4/6 (Ribociclib), Rb-Raf interaction (RRD251), α7 nAChR (α-bungarotoxin), and Yap1 (visudyne) were carried out to determine which molecules known to be activated downstream of nicotine of might be involved. Cells were treated with inhibitors as described in materials and methods, stimulated with nicotine for 21 hours, and Sox2 protein expression was assessed by western blotting. It was found that treatment with inhibitors to Src/Yes/Fin, PI3K, CDK4/6, or Yap1 could significantly abrogate nicotine-mediated induction of Sox2 (Figure 4.19).

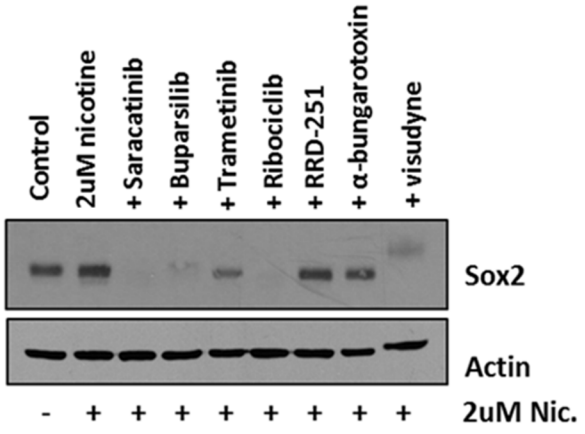


Figure 4.19. Inhibition of Src Family Kinases, PI3K, MEK1/2, CDK4/6, α7 nACHR, or Yap1 Could Abrogate Nicotine-Mediated Induction of Sox2. A549 cells were serum starved, pre-treated with indicated inhibitors (Saracatinib, Buparsilib, Trametinib, Ribociclib, RRD-251, α-bungarotoxin, or visudyne), followed by stimulation with 2μM nicotine. Western blot analysis showed that inhibitors targeting Src, PI3K, MEK1/2, CDK4/6, α7 nACHR, or Yap1 resulted in reduced protein levels of Sox2 compared to untreated, nicotine stimulated control cells.

To further confirm whether Src or Yes played a role, we conducted depletion experiments in A549 cells using siRNA targeting β-arr-1, Src, or Yes, following which cells were stimulated with

nicotine for 21 hours and protein levels of Sox2 were assessed by western blotting. Depletion of β-arr-1, Src, or Yes could reduce the induction of Sox2 following nicotine treatment (Figure 14.20A). To verify siRNA targeting β-arrestin-1, c-Src, or c-Yes1 could deplete the corresponding proteins, transient transfections of each factor in untreated A549 cells followed by western blot analysis was carried out demonstrating that the siRNA transfections resulted in reduced levels of protein expression (Figure 4.20 B-C).

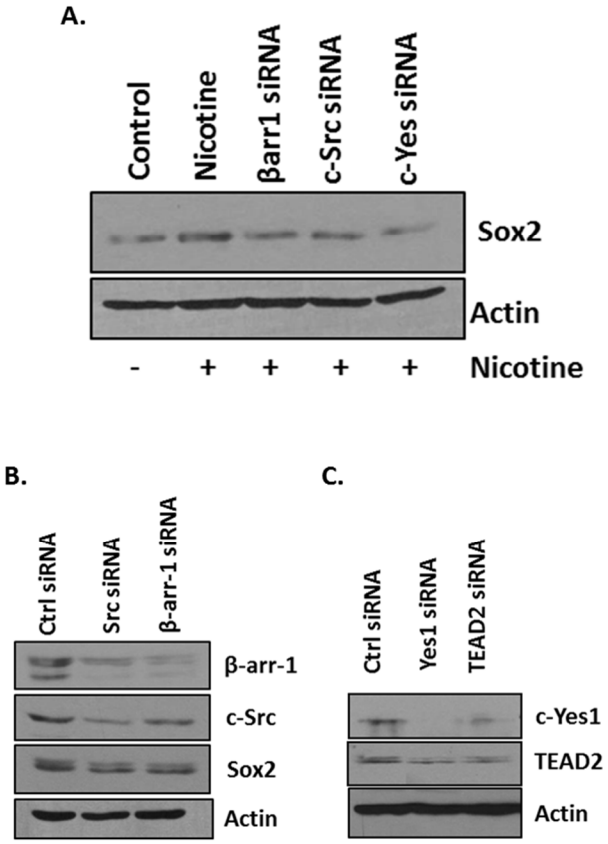


Figure 4.20. Depletion of β-arrestin-1, c-Src, or c-Yes Abrogated Nicotine-Mediated Induction of Sox2. (A) A549 cells transfected using siRNA, serum starved, and treated with 2μM nicotine for 21 hours. Western blot anlysis showed depletion of -arrestin-1, c-Src, or c-Yes abrogated nicotine-mediated induction of Sox2. (B andC) siRNA transfection followed by western blotting showing that depletion of Src, Yes1, or TEAD2 using siRNA effectively resulted in reduced protein levels of each of these factors.

Additionally, double immunofluorescence experiments showed that depletion of Src using siRNA or inhibition using Saracatinib could abrogate the co-localization of Yap1 and E2F1 in response to nicotine stimulation after 21 hours (Figure 4.21).

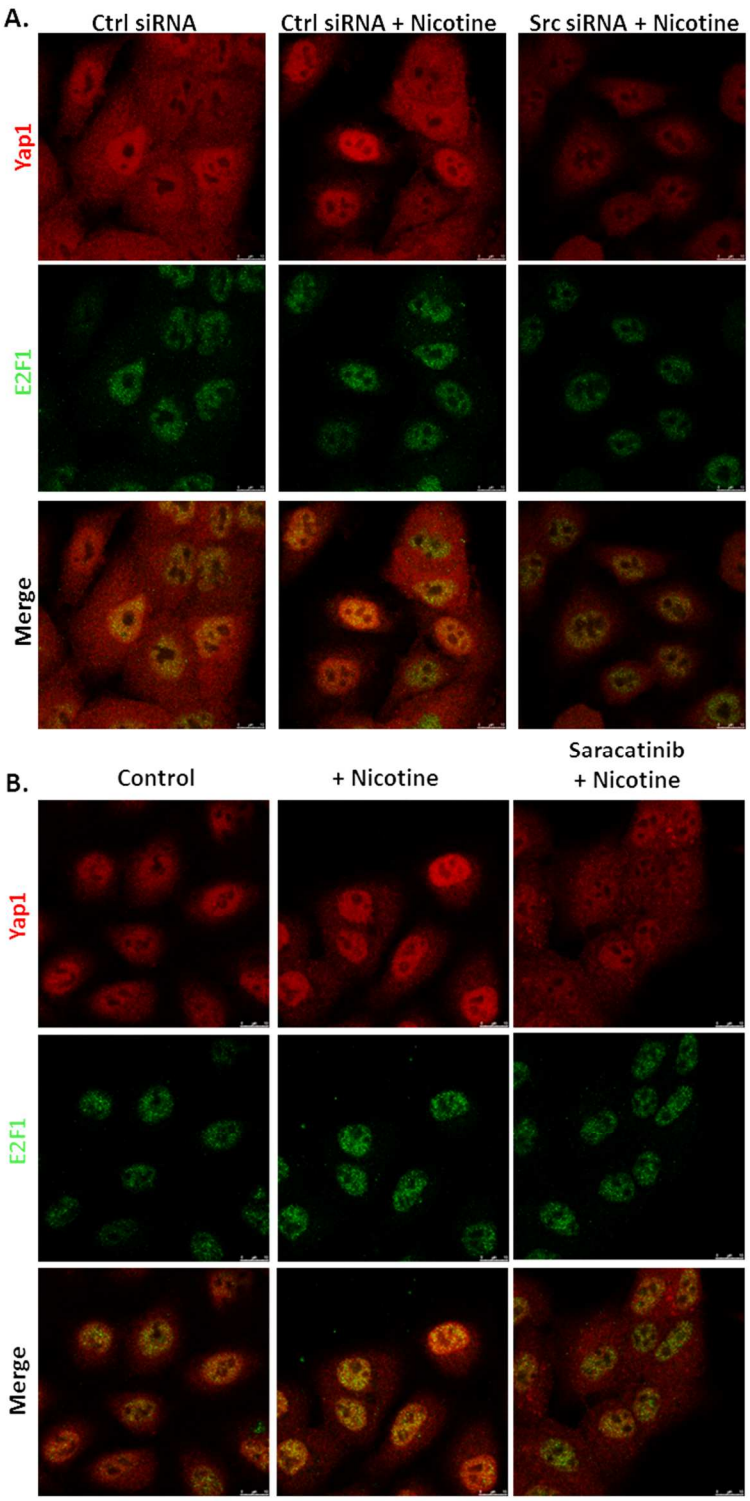


Figure 4.21. Depletion or Inhibition of Src Kinase Abrogates Nicotine-Mediated Yap1/E2F1 Colocalization. (A) Double immunofluorescent experiments showed that depletion of Src using siRNA could abrogate the enhanced Yap1/E2F1 colocalization seen in response to 2μM nicotine stimulation for 21 hours. (B) Similarly, if Src family kinases were inhibited using Saracatinib (targeting Src, Yes, and Fyn), the nicotine-mediated induction of Yap1/E2F1 colocalization was diminished.

These results were recapitulated in PLA experiments conducted in the same manner (Figure 4.22). Together, this data suggests that Src family kinases act upstream of Yap1 and E2F1 to regulate induction of Sox2 expression in response to nicotine.

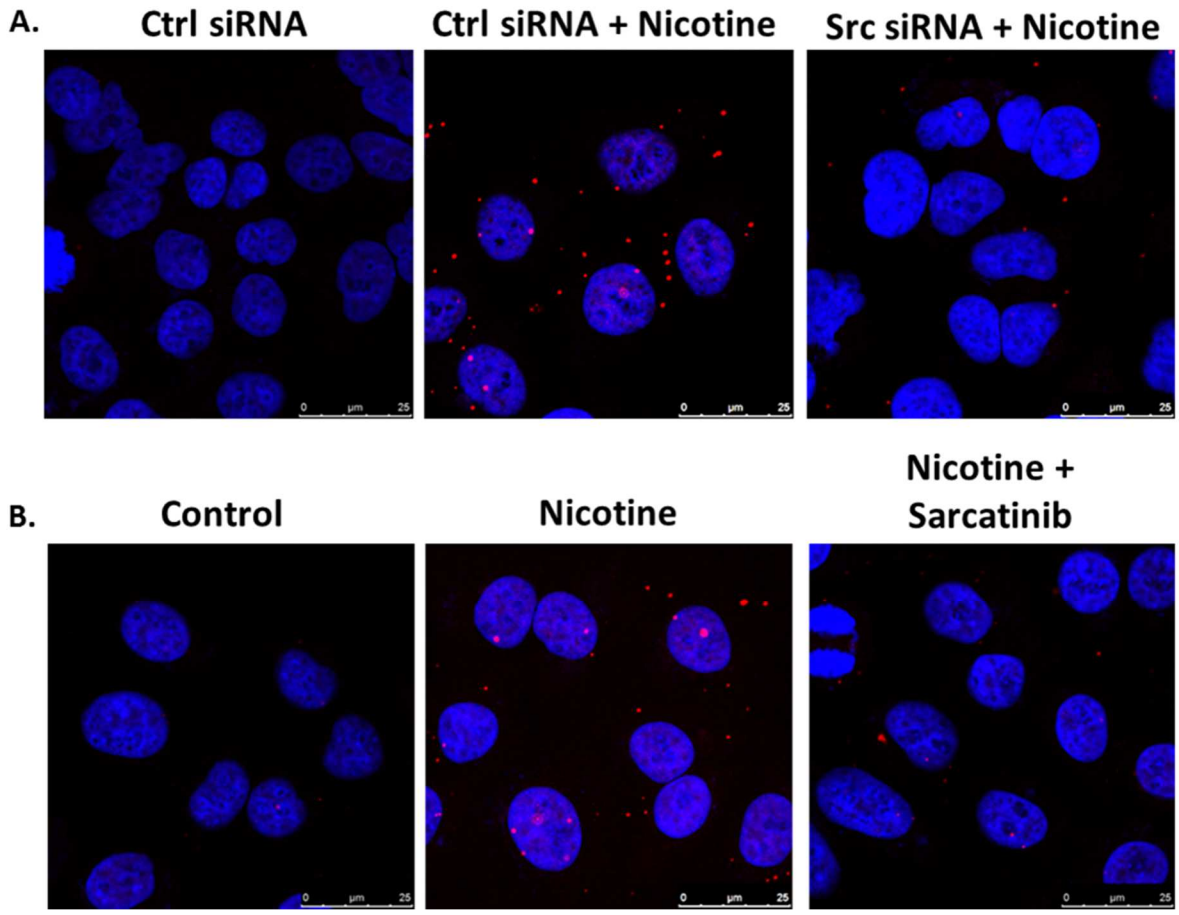


Figure 4.22. Depletion or Inhibition of Src Kinase Disrupts Nicotine-Mediated Yap1/E2F1 Interaction by PLA. (A) PLA experiments showed that siRNA targeting Src could diminish the interaction of Yap1 and E2F1 proteins which was enhanced by 2μM nicotine after 21 hours, as indicated by red foci. (B) Similarly, if Src family kinases were inhibited using Saracatinib (targeting Src, Yes, and Fyn), the nicotine-mediated induction of Yap1-E2F1 protein-protein interaction was diminished.

Oct4 may also Contribute to Nicotine-Mediated Induction of Sox2

We had previously shown that Yap1 was elevated in cancer stem-like cells from NSCLC and was necessary for their self-renewal and ability to form angiogenic tubules; and these effects of Yap1 were mediated through the induction of Sox2 (160). This was shown to occur through the

physical interaction of Yap1 with the Oct4 transcription factor on the Sox2 promoter independent of TEAD2 transcription factor (typically the primary mediator of Yap1 transcriptional effects); and Yap1-Oct4 interactions were found to be elevated in patient tumor samples compared to normal (160). Further, depletion of Yap1 resulted in the inability of NSCLC cell lines to form tumors and metastasize in murine orthotopic lung implantation models, and the overexpression of Sox2 could rescue this effect (160). While this supports the important role Yap1 plays in modulation of stem-like functions through the regulation of Sox2, it lead us to question whether Yap1 interaction with Oct4 might also play a role in nicotine-mediated induction of Sox2, in addition to Yap1-E2F1. PLA experiments were conducted to determine whether nicotine or e-cigarette extracts had an effect on the interaction of Yap1 with Oct4 in A549 cells (Figure 4.23). These assays showed that Yap1 and Oct4 existed in close proximity of one another, and the interaction was enhanced by nicotine as well as extracts from each of the three brands of e-cigarettes similar to what we found for Yap1-E2F1 interaction.

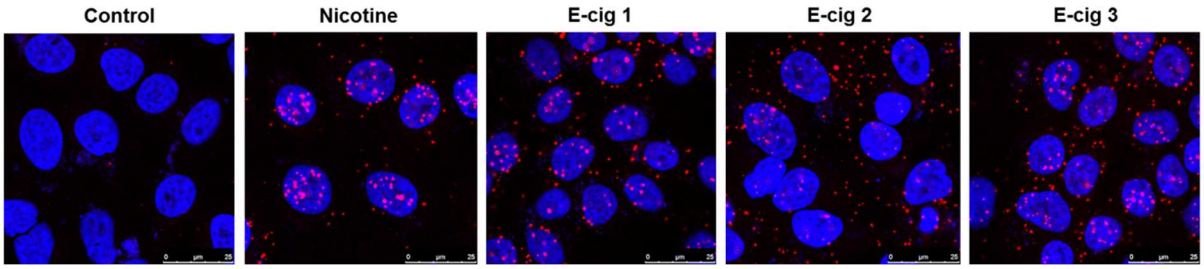


Figure 4.23. Yap1-Oct4 Association is Enhanced by Nicotine or E-cigarette Extracts. Proximity ligation assay conducted on A549 cells demonstrated association of Yap1 and Oct4 proteins indicated as red foci; this association was enhanced in response to 2μM nicotine or e-cigarette extract stimulation for 21 hours.

We next sought to determine whether Oct4 had a role in nicotine induction of Sox2 itself. Transient transfection experiments were conducted using A549 cells and either the Sox2-luc reporter or a Sox2-luc reporter with the Oct4 site deleted to prevent binding (mutSox2-luc), followed by 24 hours of serum starvation and subsequently stimulation with 2μM nicotine or E-cig 1 for 21 hours. The Oct4 binding site is located in a region -266 through -497bp upstream of

the TSS on the Sox2 promoter, a region which additionally contains two predicted E2F binding sites. These assays showed that while nicotine or E-cig 1 could enhance Sox2-luc expression, neither could enhance expression of the mutSox2-luc suggesting that the Oct4 binding site is important for nicotine-mediated induction of Sox2-luc activity (Figure 4.24A). Similar experiments were carried out using A549 cell lines stably expressing either Sox2-luc, mutSox2-luc, or mutSox2-luc plus Yap1 overexpression. These results additionally showed that in cells lacking an Oct4 binding site, nicotine or E-cig 1 could not induce Sox2-luc expression (Figure 4.24B).

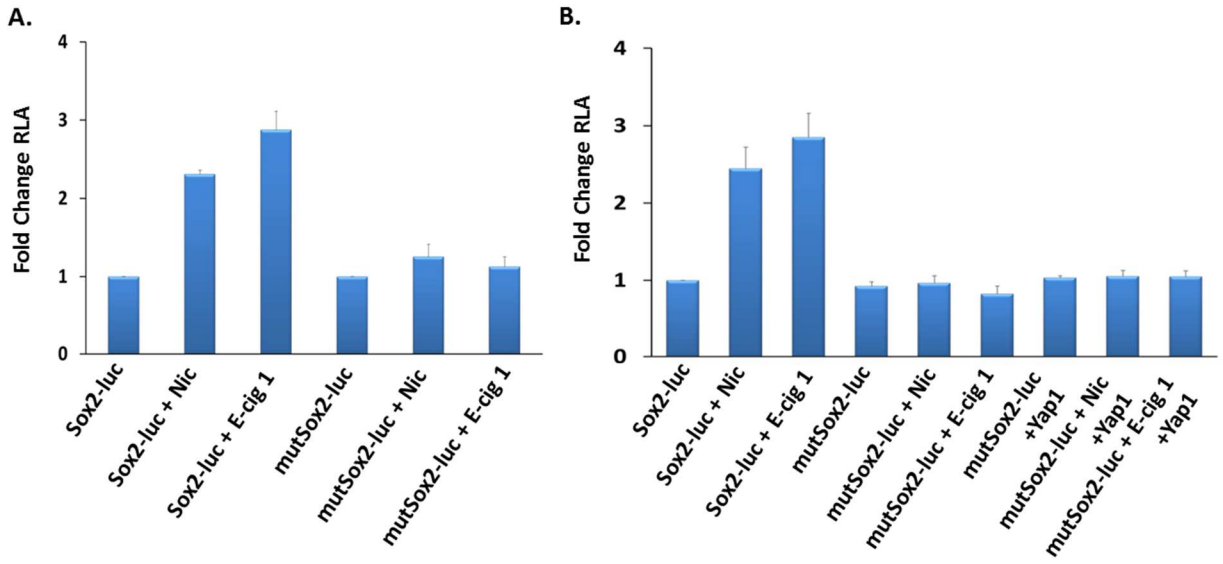


Figure 4.24. Deletion of an Oct4 Binding Site on the Sox2 Promoter Abolishes the Ability of Nicotine or E-cig 1 to Induce Sox2-luc Expression. (A) Transient transfections of A549 cells showing that 2μM nicotine or E-cig 1 can induce Sox2-luc activity after 21 hours, an effect diminished using the mutSox2-luc reporter which has the Oct4 site deleted. (B) 2μM nicotine or E-cig 1 can induce Sox2-luc activity in cells stably expressing the construct after 21 hours; an effect which no longer occurs in cells stably expression mutSox2-luc or mutSox2-luc plus Yap1 overexpression.

Since these results demonstrated the inability of nicotine to induce Sox2 expression when the Oct4 binding site was absent on the promoter, we next sought to determine whether depletion of Oct4 protein in addition to depletion of E2F1 protein could abrogate nicotine-mediated induction of Sox2, further implicating their roles in this process downstream of Yap1. Transient transfections were conducted in A549 cells where Yap1, E2F1, or Oct4 were each depleted

using siRNA, were then transiently transfected using the Sox2-luc reporter, serum starved for 24 hours, and stimulated with 2μM nicotine for 21 hours. These experiments demonstrated that depletion of each of the three proteins could abrogate nicotine-mediated induction of Sox2, further supporting their roles this signaling cascade (Figure 4.25). Overall, Yap1 seems to have an integral role in the regulation of Sox2 and its induction by nicotine, an effect which is moderated through is transcriptional collaboration with E2F1 and likely Oct4 as binding partners.

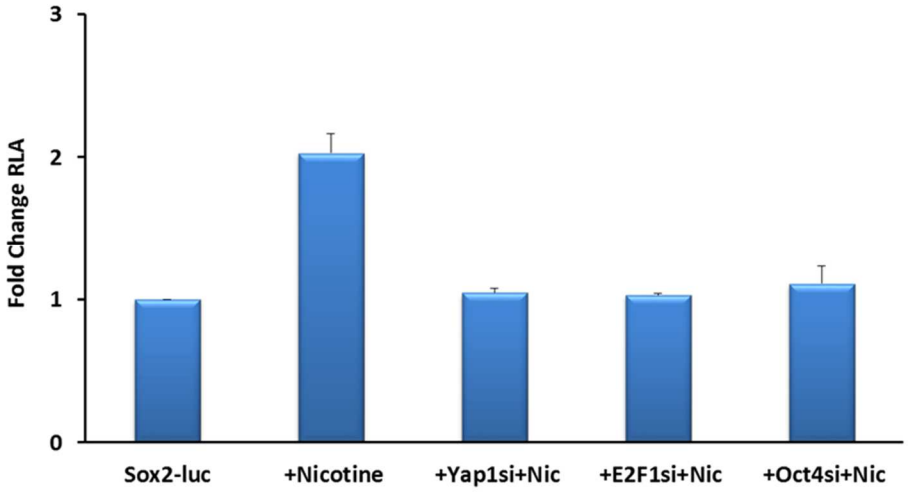


Figure 4.25. Depletion of Yap1, E2F1, or Oct4 Each could Abrogate Nicotine-Mediated Induction of Sox2-luc. A549 cells were transiently transfected using siRNA to deplete Yap1, E2F1, or Oct4 followed by subsequent transfection with Sox2-luc reporter. After 24 hours of serum starvation followed by 21 hours of 2μM nicotine stimulation, results showed that depletion of each of the three proteins resulted in the inability of nicotine to induce Sox2-luc activity.

E-cigarette Extracts Enhance an Epithelial-Mesenchymal-Transition (EMT) Phenotype

Epithelial-to-mesenchymal transition (EMT) is a normal process during development that is frequently activated during tumor progression allowing for a more migratory and invasive phenotype (203, 204). Growing evidence suggests that activation of EMT induces the acquisition of stem cell properties in epithelial cells and that the induction of EMT and emergence of CSCs is strongly linked (203, 204). We have previously reported that, in addition to nicotine enhancing CSC properties, it can induce a number of factors involved in EMT including the activation of mesenchymal markers such as vimentin, fibronectin, Zeb1, and Zeb2; suppression of epithelial markers such as e-cadherin; disruption of the tight junction protein ZO-

1 thereby allowing for cell motility; and increased cell migration and invasion (21, 46). Since we also find e-cigarette extracts are capable of enhancing CSC properties, we next interrogated whether e-cigarette extracts could further enhance EMT phenotypes, perhaps as a precursor to the acquisition of CSC phenotype. At the mRNA level, we found that each of the three e-cigarette brands could induce the mesenchymal markers vimentin, fibronectin, Zeb1, and Zeb2 (Figure 4.26). Immunofluorescent assays further demonstrated that e-cigarette extracts could disrupt ZO-1 tight junction protein with a concordant decrease in ZO-1 staining overall, and could additionally reduce levels of e-cadherin in A549 cells (Figure 4.27A and B).

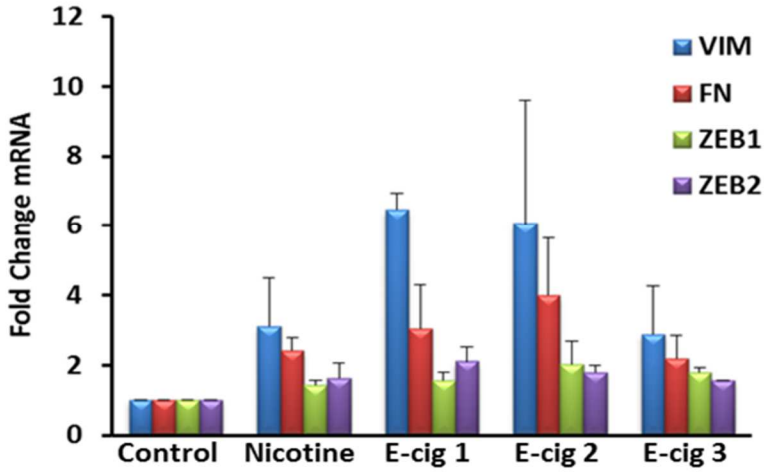


Figure 4.26. E-cigarette Extracts Induce mRNA Expression of Mesenchymal Markers Vimentin (VIM), Fibronectin (FN), ZEB1, and ZEB2. qRT-PCR analysis of A549 cells serum starved followed by stimulation with 2μM nicotine show increased expression of VIM, FN, ZEB1, and ZEB2 mesenchymal markers which are upregulated during epithelial-to-mesenchymal transition.

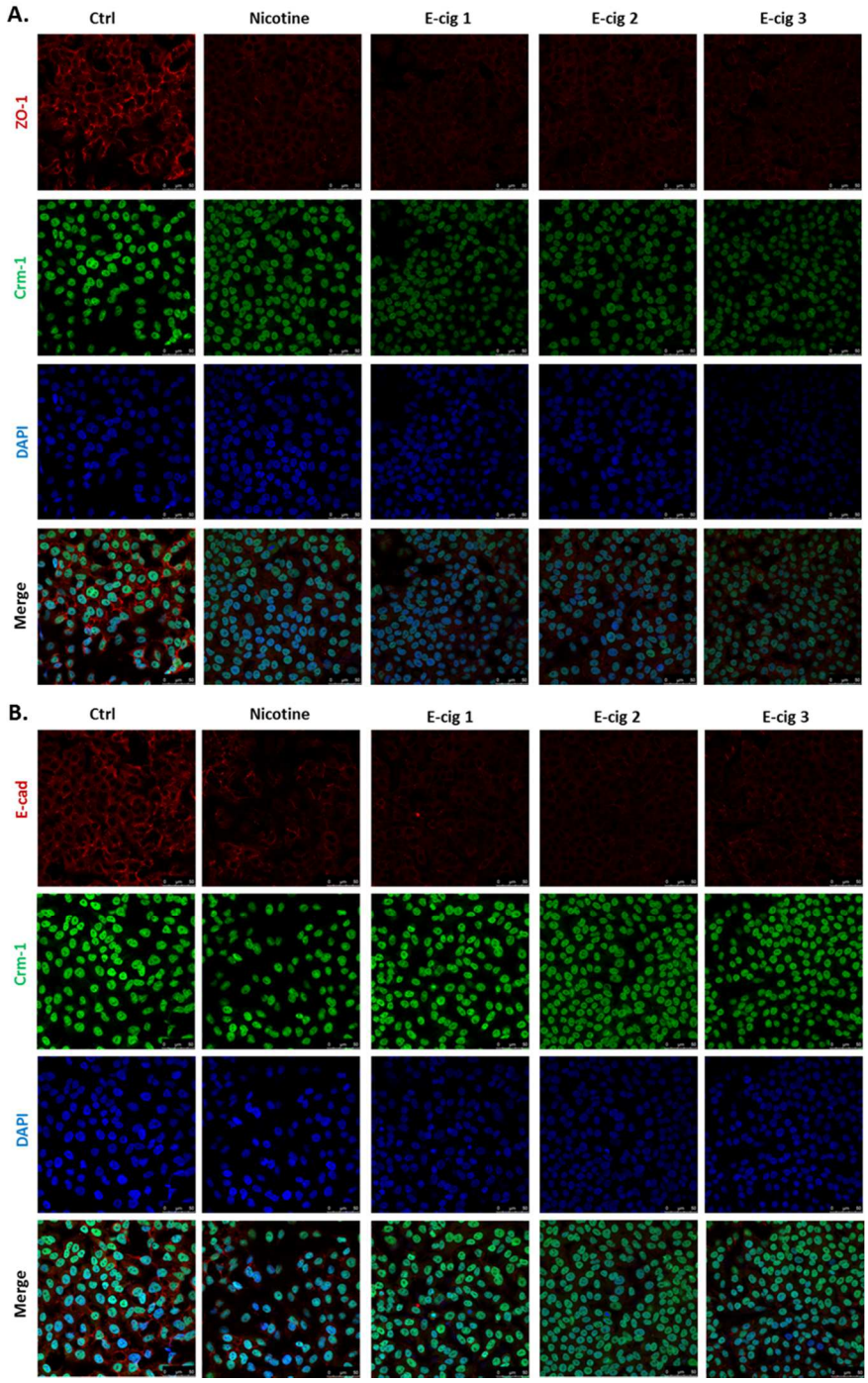


Figure 4.27. E-cigarette Extracts Disrupt Tight Junction Protein ZO-1 and Epithelial E-cadherin (E-cad), with a concomitant decrease in expression of these two proteins. (A) Treatment with 2μM of nicotine or each of the three e-cigarette extracts resulted in reduced expression of and translocation of ZO-1 from the cell membrane in A549 cells. (B) Treatment with 2μM of nicotine or each of the three e-cigarette extracts resulted in reduced expression of and translocation of E-cadherin from the cell membrane in A549 cells.

Wound healing assays additionally demonstrated that e-cigarette extracts could enhance cell migration to a modest extent (Figure 4.28).

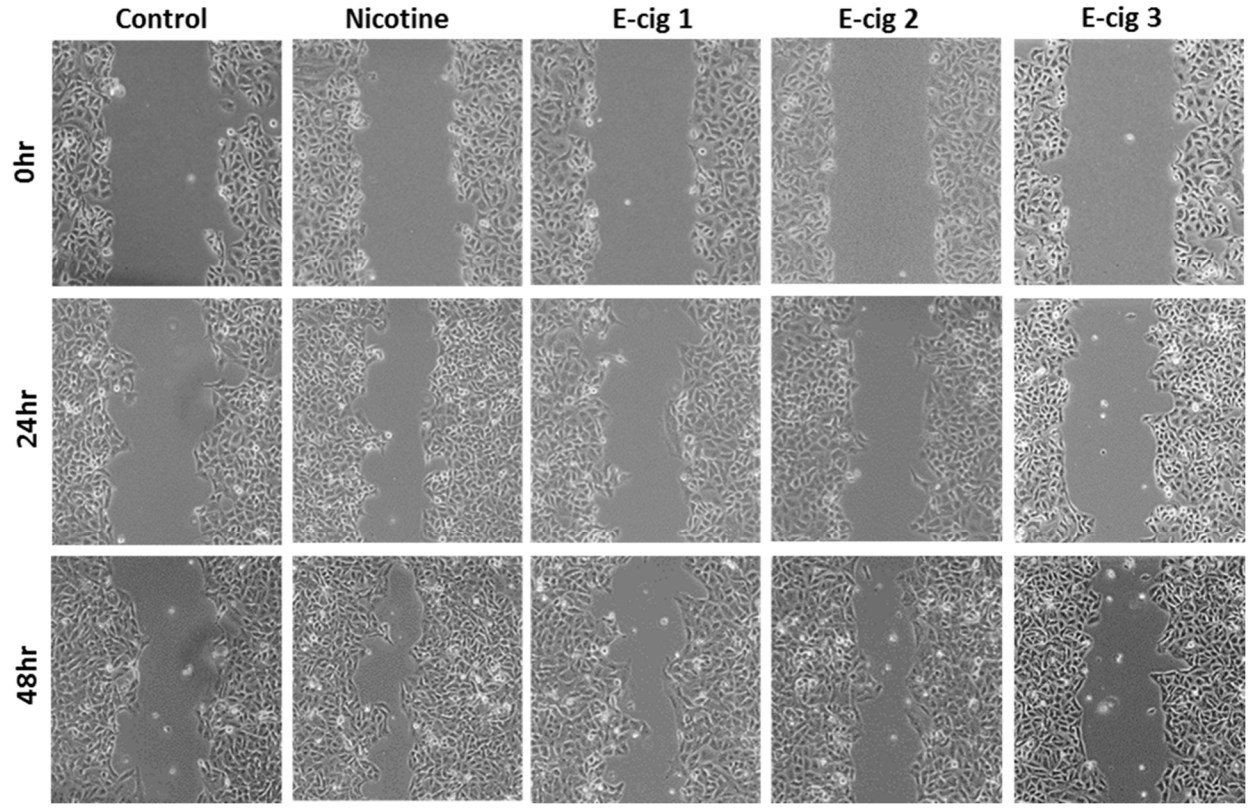


Figure 4.28. E-cigarette Extracts Induce A549 Cell Migration in a Manner Similar to Nicotine. Cells were plated at confluency, serum starved, and stimulated with 2μM nicotine or each of the three e-cigarette extracts. Results demonstrate that e-cigarette extracts enhance migration to a modest extent.

Overall, this data lays the ground work to suggest that e-cigarette extracts induce EMT phenotypes in a manner similar to what has previously been observed with nicotine.

Discussion

CSCs represent a subpopulation of tumor cells with increased tumor-initiating capability. They can divide asymmetrically to replenish the heterogenous tumor bulk, and are highly efficient in initiating tumors upon implantation in animal models (61). CSCs are resistant to various treatment modalities in part due to their enhanced ability to efflux drugs; additional reasons include the fact that they are slower cycling, and they express higher levels of anti-apoptotic proteins. At the same time, complete mechanisms underlying the drug resistance of these cells

are not fully understood (61). Additionally, these cells are thought to remain dormant and facilitate tumor recurrence and metastasis (61, 64). Not surprisingly, based on these properties of CSCs, efforts are being made to elucidate mechanisms underlying the biology of CSCs in order to target this subpopulation. CSCs have different gene regulatory programs, including epigenetic changes, than the bulk tumor cells; understanding what these differences are, how the programs are regulated, will open up new opportunities for therapeutic targeting.

We have previously reported that nicotine could enhance self-renewal of NSCLC SP cells. Here we find a new mechanism through which this occurs, where nicotine induces Sox2 expression at 18 and 24 hour time points in NSCLC cells, through the Hippo pathway effector, Yap1. We also find that nicotine induces expression of Yap1 itself, and that the nicotine-mediated induction of Sox2 and Yap1 is not just specific to lung cancer cells but is also observed in human mesenchymal stem cells. One previous report has demonstrated the ability of nicotine to induce Yap1 in esophageal squamous cell carcinoma (ESCC), and this occurred through nAChRs (194). Interestingly, they find that Yap1 physically interacts with nAChRs and stimulation with nicotine could induce nuclear translocation and activation of Yap1 by disrupting its association with a negative regulatory complex in the cytoplasm composed of α-catenin, β-catenin, and 14-3-3 proteins (194). The molecular mechanisms regulating this process are not completely understood.

Our prior studies have shown that Yap1 regulates Sox2 through the binding to Oct4 transcription factor, facilitating self-renewal and vascular mimicry (160). Here we report that E2F1 transcription factor can regulate the Sox2 promoter, and that Yap1 binds to E2F1 likely modulating this effect. Further, we also find that nicotine or e-cigarette extracts can increase the binding of Yap1 to both E2F1 and Oct4. Nicotine has been shown to induce E2F1 transcriptional activity through a sequence of signaling events mediated downstream of nAChRs (103). Upon nicotine binding, β-arrestin-1 scaffolding protein is recruited to the receptor and activates Src kinase which subsequently activates Raf-1. Raf-1 then acts to phosphorylate the

Rb tumor suppressor protein which is typically bound to E2F1 during cellular quiescence, but once hypophosphorylated Rb dissociates from E2F1 allowing it to turn on a number of promoters involved in proliferation and survival (98). We now find that this pathway might contribute to the induction of stemness, by facilitating the expression of Sox2.

Our studies also suggest that Yap1 is induced by a non-canonical signaling mechanism in response to nicotine. The Hippo signaling pathway has been demonstrated to have tumor suppressive roles, but is aberrantly altered in multiple cancers including those of the lung (205). Typically the activation of this pathway by upstream mediators Mst1/2 and Lats1/2 results in the inactivation of Yap1 through its phosphorylation, leading to cytoplasmic sequestration and degradation by 14-3-3 protein (205). Our results in NSCLC cells suggest that Yap1 is activated through Src and Yes kinases in response to nicotine; the role of the canonical Hippo signaling pathway in the induction remains unclear.

Overall these studies suggest that upon nicotine binding to α7 nAChR, Src is activated and subsequently leads to Yap1 binding to E2F1 and/or Oct4, upregulating Sox2 expression, thereby enhancing self-renewal of CSCs. However, the role of Oct4 in this process is not fully clear. When endogenous expression of Oct4 is knocked down, nicotine could still induce Sox2; in contrast, in transient transfection experiments using Sox-luciferase reporters showed that Oct4 binding sites on the Sox2 promoter were critical for nicotine to induce its expression. This was also found in cell lines stably transfected with a Sox2-Luciferase promoter, where mutation of the Oct4 site prevented nicotine-mediated induction of Sox2-luciferase. The molecular basis for the difference in the induction of endogenous Sox2 versus induction of a transfected Sox2-luciferase reporter remains elusive at this time. It could be that other proteins are forming complexes with Oct4 or E2F1 to regulate Sox2, and these is somehow disrupted by mutation of the Oct4 binding site. It is also possible that post-translational modifications of the proteins involved or histone modifications on Sox2 promoter around the Oct4 binding site play a role.

Our lab had shown that nicotine induces the translocation of β-arrestin-1 scaffolding protein to the nucleus where it binds to E2F1 transcription factors to enhance transcription of E2F target genes (103). This was found to occur through the formation of an oligomeric complex consisting of β-arrestin-1, E2F1, and p300 histone acetyltransferase proteins which facilitated the acetylation of histones and E2F1, acting to induce transcription of genes involved in proliferation and survival (103). Our initial experiments show that depletion of β-arrestin-1 reduced endogenous levels of Sox2; this raises the possibility that Yap1 is recruited to a complex with β-arrestin-1 and E2F1 on the Sox2 promoter. Alternately, β-arrestin-1 might be recruiting p300 to E2F1 independent of Yap1. It is additionally worth noting that other E2F family transcription factors may be involved, and their role is worth investigating. These are novel findings that might have a significant impact on our understanding of how nicotine promotes self-renewal of stem-like cells from non-small cell lung cancer. Full elucidation of these mechanisms will shed light on the pathophysiology of smoking-related cancers, and reveal new pathways involved in promotion of CSC populations that can potentially be therapeutically exploited.

CHAPTER FIVE:

SUMMARY AND CONCLUSIONS

Summary of Novel Findings

- Nicotine induces α7 nAChR promoter as well as mRNA and protein expression.
- E2F1 and STAT transcription factors, which are activated downstream of nicotine, act to differentially regulate α7 nAChR through an overlapping binding site on the promoter. Nicotine induces Sox2 expression in NSCLC and hMSCs, through a Src-Yap1-E2F1 axis; β-arr-1 and Oct4 may also be involved.
- E2Fs bind to and induce Sox2 promoter, and nicotine enhances recruitment of E2F1 and Yap1 to the Sox2 promoter.
- Depletion of Sox2 results in decreased self-renewal of SP cells.
- E-cigarette extracts can enhance expression of α7 nAChR, Sox2, Yap1, and mesenchymal markers FN, VIM, ZEB, and ZEB2; while disrupting ZO-1 and E-cadherin.
- E-cigarette extracts also enhance self-renewal ability of SP cells.

Future Directions

- Determine whether the differential regulation of α7 nAChR by E2F1 and STAT1 is time dependent and signal dependent. Do agents that induce STAT1 suppress α7 nAChR expression?
- Analyze patient samples for E2F1, STAT1, and α7 nAChR interactions and levels in smokers compared to non-smokers.

- Conduct orthotopic mouse experiments using wild type cells compared to α7 nAChR null cells with two treatment groups: nicotine vs. control; to demonstrate whether α7 nAChR is required for nicotine-mediated induction of tumor growth and metastasis.
- Elucidate the involvement of other E2F family members in nicotine-mediated induction of Sox2 as well as α7 nAChR.
- Determine whether other proteins may interact with Yap1, E2F1, and Oct4 to regulate Sox2 expression in response to nicotine (ie: p300, β-arr-1).
- Determine whether histone modifications or post-translation modifications play a role in nicotine-mediated induction of Sox2.
- Analyze patient samples for E2F1, Yap1, and Sox2 interactions and levels in smokers compared to non-smokers.

Conclusions

Tobacco smoke is the single greatest preventable risk factor for cancer (206), yet is still responsible for an estimated 160,848 cancer related deaths each year in the United States, let alone globally. The current literature has shed light upon the multiple molecular mechanisms by which components of tobacco smoke can initiate tumor formation, impact cell cycle progression and proliferation, and promote tumor progression in multiple cancer types. In a review of 10 studies, it was found that people who continue to smoke after diagnosis of early stage lung cancer nearly double their risk of dying (207), and cessation of smoking after diagnosis improves multiple aspects of lung cancer including decreased risk of second tumors, increased overall well-being, improved immune response, and increased response to chemotherapeutic agents (125). There are a number of smoking cessation aids available, including varenicline, bupropion as well as various nicotine replacement therapy (NRT) agents such as transdermal patches, chewing gums, and now e-cigarettes.

Given that nicotine has tumor promoting properties, concerns have been raised regarding the deleterious effects of NRT on cancer therapy and survival. There have been limited efforts made to examine how exposure to nicotine through NRT products affects tumor growth or normal physiology including immune function in human subjects. The advantage of NRT is that while tobacco smoke contains thousands of carcinogens, these are absent in NRT products, and hence NRT products are unable to initiate tumors. Supporting this contention, a model was developed to estimate mortality patterns associated with NRT use and success in smoking cessation; this compared the risks and benefits of NRT population-wide taking into account cancer-related safety of these products (208). This study found that the benefits from successful cessation through the use of NRT outweighed the risks in terms of mortality and survival (208). Further, although a significant amount of additional studies need to be conducted to assess whether NRT affects the response to cancer therapy in general, it is reasonable to conclude that nicotine replacement therapy is significantly less harmful than smoking; nevertheless, it might be prudent to reduce the exposure to nicotine in any form for prolonged periods of time.

While smoking cessation is the most effective method to reduce the risk of lung cancer, the elucidation of the signaling mechanisms as well as genetic alterations that facilitate smoking-induced lung cancers has opened up new opportunities to develop novel therapeutic agents that target these pathways. While this process could be increasingly complex and painstakingly long, such efforts could hold promise for the treatment of tobacco-related cancers in the future.

Given the multifaceted roles of nAChRs in initiation of tumors, promotion of cell proliferation, tumor growth and metastasis, and the additional association of the gene cluster encoding for the α3, α5, and β4 subunits of nAChRs with lung cancer and nicotine addiction, attempts have been made to target nAChRs to combat smoking induced lung cancers (157, 209). Small molecule inhibitors and other agents have been under interrogation for use in the context of nAChRs in smoking related cancers, and the number of patented compounds selectively targeting nAChR subtypes are becoming increasing available; however, complete functional studies of these agents are still

limited; whether they work as agonists, antagonists, or partial agonists still needs to be elucidated; and which receptor subunits they target still needs to be determined (210). Antagonists to the receptors facilitating oncogenic drivers as well as agonists to these receptors could both be viable therapeutic modalities, as antagonists block the receptor mediating pro-tumorigenic signaling events while agonists can lead to the release of factors such as GABA which can inhibit nicotine-mediated tumor promotion (85). As can be imagined, one major concern regarding the use of nAChR antagonists is the potential deleterious effect on the brain, central nervous system, neuromuscular system, and other vital cell and organ functions where these receptors are highly expressed and act to initiate signaling events (211). Further, many nAChR antagonists are known toxins such α-cobratoxin and α-bungarotoxin present in snake venom; although these have been found to abrogate nicotine-mediated tumor promotion in multiple contexts. Since the main physiological roles of nAChRs are in the brain, long term treatment targeting these receptors may lead to things such as cognitive impairment, alterations in memory, alterations in sleep, and alterations in personality (210). Improved specificity of nAChR targeting agents in both the context of organ site as well as receptor subunit type could prove valuable for intervention of smoking-related cancers.

These results raise the intriguing possibility that targeting nAChRs, specifically α7 nAChR, might be a viable strategy to combat NSCLC, but a significant amount of additional studies would be needed to pursue this further. Given the potential toxicity of α7 antagonists and given the neuronal function of these receptors, targeting the downstream effectors of nAChRs might be an alternate approach that is worth considering; and therefore understanding detailed molecular pathways involved is of importance. There is substantial literature detailing key oncogenic pathways such as the MAPK, JAK/STAT, PI3K, and other pathways which are activated downstream of the receptor; however, how expression of the receptor itself is regulated is not well understood. Here we sought to elucidate the mechanism by which α7 nAChR is regulated. We report that E2F1

and STAT1 transcription factors act to differentially regulate α7 nAChR expression through an overlapping binding site, perhaps by competitive interplay. Nicotine itself has been shown to enhance expression of α7 nAChR, and we found that this additionally occurs through E2F1 and depletion of this transcription factor could abrogate nicotine-mediated induction of the receptor.

We also discuss a subset of cells, cancer stem-like cells, which are a rare subpopulation within a tumor capable of giving rise to the entire heterogenous tumor. These cells have gained attention as they are thought to be the cells which persist after therapeutic treatment, remain dormant, are responsible for recurrence, and metastasize to distal organs leading to mortality. New therapeutic strategies are being developed to target this subset of cells in addition to the tumor bulk, with the intention of completely eradicating the tumor. Since CSCs exhibit distinct molecular profiles, elucidating these profiles and the molecular underpinnings of the function of these cells is critical. It has been shown that nicotine acts to enhance gene expression and phenotype of CSCs, which raises the possibility that targeting the mechanisms by which nicotine mediates these events in smoking-related lung cancer may be beneficial. Here we find that in NSCLC cells, nicotine induces the expression of Sox2 embryonic transcription factor, and this occurs through a Src family kinase-Yap1-E2F1/Oct4 signaling axis. Further studies are warranted to elucidate whether targeting this pathway might effectively disrupt cancer stem cells specifically in the context of smoking related cancers, and potentially hold clinical utility.

Interestingly, while the use of e-cigarettes has been gaining popularity and continues to be evaluated as an efficacious smoking cessation agent, we find that the nicotine present within the e-cigarette extracts acts to enhance a number of tumor promoting functions. We find that extracts from three different brands of e-cigarettes can induce expression of α7 nAChR, Sox2, and Yap1; and also can enhance mesenchymal markers while repressing tight junction protein ZO-1 and epithelial E-cadherin as well as leading to their translocation from the cell membrane; features associated with EMT and cancers acquiring more aggressive phenotypes. We

additionally show that e-cigarette extracts can enhance self-renewal of CSCs as well as migration of NSCLC cells in vitro. Thus, while e-cigarettes may prove viable cessation agents, the use of these devices may act to promote progression of disease in patients with pre-existing tumors. While a vast amount of information is known and continues to be generated regarding the role of nicotine in cancer progression, we have not found methods to effectively disrupt its ability to exert these effects so far; such an advance would have great utility for cancer patients who have history of smoking or who continue to smoke. For the agents that have already been developed, further studies are needed to evaluate their efficacy, specificity, and toxicities in pre-clinical models to progress them more rapidly towards use in the clinic, and ultimately benefit to patients with smoking-related cancer.

In summary, nicotine promotes a wide variety of tumor-promoting properties, almost in a pleiotropic manner, in a number of cancer types. It acts to enhance multiple stages of tumor progression from proliferation, angiogenesis, chemoresistance, and survival of primary tumors, to EMT, migration, and invasion leading to tumor metastasis. More recently, it has been shown that nicotine enhances the self-renewal of cancer stem-like cells, which are implicated in every stage of tumor initiation and progression, and are thought to be resistant to therapies, can remain dormant, and result in recurrence and dissemination. In the context of patients who smoke and whose cancers were initiated as a result of smoking, targeting pathways which nicotine utilizes to exert its tumor-promoting functions seems to hold promise for therapeutic efficacy. These pathways are depicted in Figure 5.1.

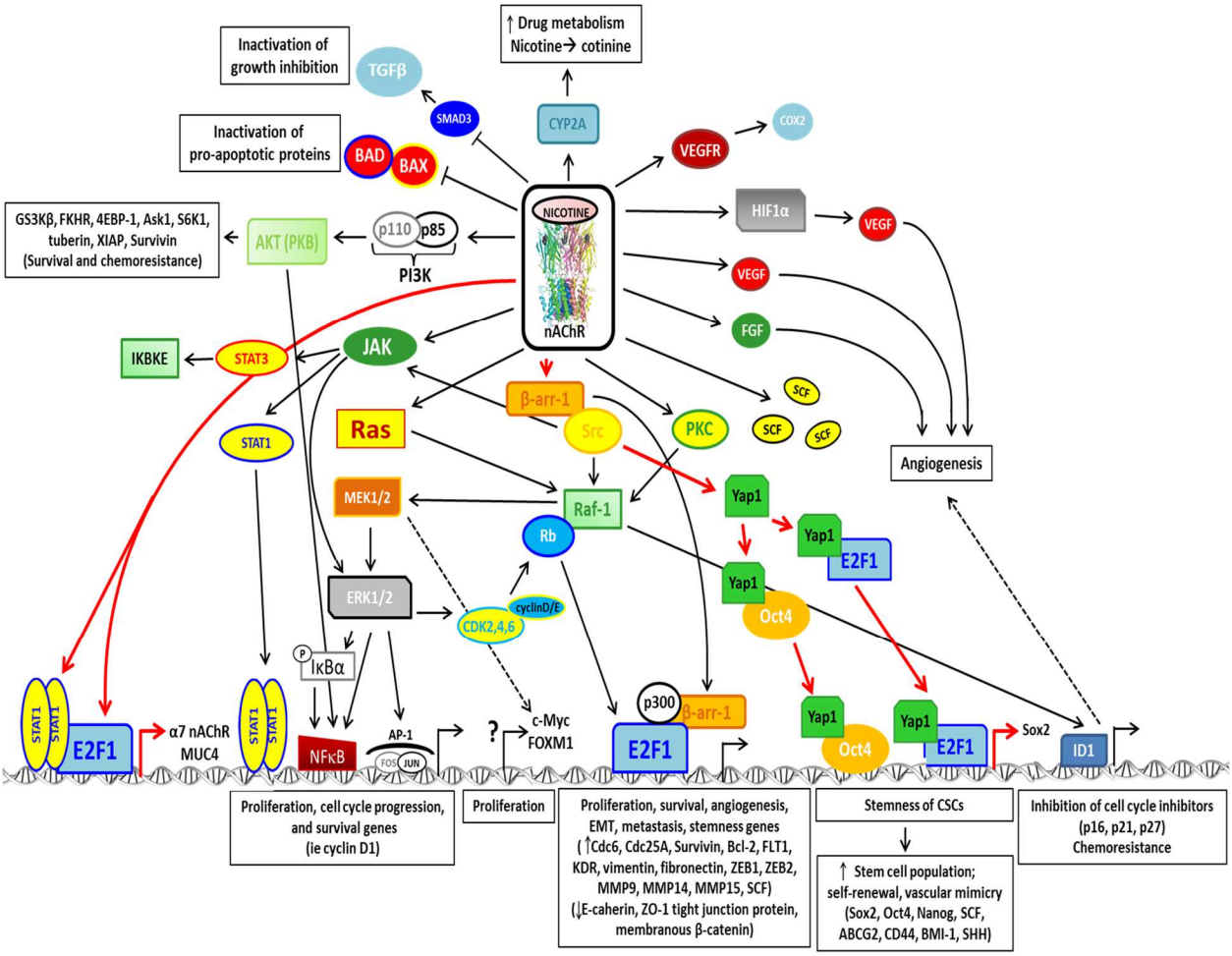


Figure 5.1. Nicotine-Mediated Signaling Pathways Involved in Multiple Aspects of Tumor Initiation, Maintenance, and Progression. Nicotine has been found to promote a variety of tumor promoting properties in multiple cancer types. This occurs through the activation of molecular signaling cascades known to promote key processes in cancer progression including cell cycle progression, proliferation, survival, hypoxia, angiogenesis, EMT, cancer stem cell populations, drug resistance, and cancer stem cell populations. Nicotine-mediated activation of these signaling cascades results in altered gene expression and altered secretion of factors such as VEGF, FGF, and SCF which in turn enhance these tumor promoting properties.

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