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# Regulation and Function of Neuronal Nicotinic Acetylcholine Receptors in Lung Cancer: A Dissertation

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# REGULATION AND FUNCTION OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS IN LUNG CANCER

A Dissertation Presented

By

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**Dedication**

In loving memory of my father, Reynaldo V. Improgo, my grandmother Virginia D. Duenas, and my labmate and friend, Eric M. Hogan – I continue to be inspired by the example of your lives.

**Acknowledgements**

I have come to realize that an endeavor as huge as grad school cannot be undertaken alone. I am humbled by the help and support that I have received. First and foremost, I would like to express my gratitude to my mentor, Paul D. Gardner, for his unwavering support these past years. Thank you, Paul, for being a teacher, a parent, and a friend. I would also like to convey my heartfelt appreciation to my co-mentor, Andrew R. Tapper, whose sharp mind has kept me in my scientific toes. I have such deep respect for your intelligence and your calm and considerate way of dealing with people. I am also sincerely grateful to my thesis committee members - Roger Davis, Brian Lewis, and Alonzo Ross. Thank you for consistently pointing me in the right direction. Your insights have oftentimes been uncanny and have always been valuable. I would also like to thank Jean King and Marissa Ehringer, members of my dissertation examination committee. I really appreciate your going out of your way to help me with the final stages of my dissertation. Huge thanks to David Weaver, chair of the Program in Neuroscience, for always taking the time to listen, to help, and to encourage. Thank you to Andrew Coles for critical reading of my dissertation drafts and for help with my TRAC meeting presentations. I would also like to acknowledge Van Gould of Animal Medicine and the staff of the Worcester State Hospital cafeteria - you guys remind me that sometimes kindness comes from the most unexpected places. Lastly and importantly, thank you to all my family and friends, for providing fun and meaning to this chapter of my life.

**ABSTRACT**

Lung cancer is the leading cause of cancer-related mortality worldwide. The main risk factor associated with lung cancer is cigarette smoking. Research through the years suggests that nicotine in cigarettes promotes lung cancer by activating signaling pathways that lead to cell proliferation, cell survival, angiogenesis, and metastasis. Nicotine's cellular actions are mediated by its cognate receptors, nicotinic acetylcholine receptors (nAChRs). Here, I describe the expression levels of all known human nAChR subunit genes in both normal and lung cancer cells. Of note, the genes encoding the α5, α3, and β4 subunits (CHRNA5/A3/B4) are over-expressed in small cell lung carcinoma (SCLC), the most aggressive form of lung cancer. This over-expression is regulated by ASCL1, a transcription factor important in normal lung development and lung carcinogenesis. The CHRNA5/A3/B4 locus has recently been the focus of a series of genetic studies showing that polymorphisms in this region confer risk for both nicotine dependence and lung cancer. I show that CHRNA5/A3/B4 depletion results in decreased SCLC cell viability. Furthermore, while nicotine promotes SCLC cell viability and tumor growth, blockade of α3β4 nAChRs inhibits SCLC cell viability. These results suggest that increased expression and function of nAChRs, specifically the α3β4α5 subtype, potentiate the effects of nicotine in SCLC. This dual hit from the carcinogens in tobacco and the cancer-promoting effects of nicotine, may provide a possible mechanism for the increased aggressiveness of SCLC. In addition, nAChRs can be activated by the endogenous ligand,

acetylcholine, which acts as an autocrine/paracrine growth factor in SCLC. Increased function of $\alpha3\beta4\alpha5$ nAChRs in SCLC could also potentiate acetylcholine's mitogenic effects. This mechanism, combined with other known autocrine/paracrine growth loops in SCLC, may help explain the ineffectiveness of available therapies against SCLC. In an effort to add to the current arsenal against SCLC, I screened a 1280-compund library using a bioluminescence-based viability assay I developed for high-throughput applications. Primary screening, followed by secondary and tertiary verification, indicate that pharmacologically active compounds targeting neuroendocrine markers inhibit SCLC cell viability.

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List of Abbreviations

| | |
|---|---|
| 5-$HT_3$ | 5-hydroxytryptamine |
| $\alpha$3I5 | $\alpha$3 intron 5 repressor |
| $\alpha$-Ctx | alpha-conotoxin |
| $\alpha$-Btx | alpha-bungarotoxin |
| Acetyl-CoA | acetyl coenzyme A |
| Ach | acetylcholine |
| AChE | acetylcholinesterase |
| ASCL1 | achaete scute complex homolog-1 |
| BAD | Bcl-2-associated death promoter |
| BAX | Bcl-2–associated X protein |
| Bcl-2 | B cell lymphoma 2 |
| BChE | butyrylcholinesterase |
| CDC | cell division cycle |
| Cdk | cyclin dependent kinase |
| ChAT | choline acetyltransferase |
| CHRNA5/A3/B4 | nicotinic acetylcholine receptor $\alpha$5, $\alpha$3 and $\beta$4 genes |
| CNR4 | conserved noncoding region 4 |
| CNS | central nervous system |
| Cyc D/E | cyclin D or E |
| DA | dopamine |
| DMSO | dimethyl sulfoxide |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EMT | epithelial-mesenchymal transition |
| ERK | extracellular signal-regulated kinase |
| ETS | E-twenty six |
| FGF | fibroblast growth factor 2 |
| GABA | $\gamma$-aminobutyric acid |
| GWAS | genome-wide association study |
| HIF-1$\alpha$ | hypoxia-inducible factor-1 alpha |
| HTS | high-throughput screening |
| LOPAC | Library of Pharmacologically Active Compounds |
| MAPK | mitogen activated protein kinase |
| MEK | MAPK/ERK kinase |
| mTOR | mammalian target of rapamycin |
| MTT | 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide |
| nAChR | nicotinic acetylcholine receptor |
| NF-$\kappa$B | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NNK | 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| NNN | N-nitrosonornicotine |
| NSCLC | non-small cell lung carcinoma |

| | |
|---|---|
| PI3K | phosphatidylinositol 3-kinase |
| PKC | protein kinase C |
| PNS | peripheral nervous system |
| PPAR-β/δ | peroxisome proliferator-activated receptor-beta/delta |
| Rb | retinoblastoma |
| SCG | superior cervical ganglion |
| SCLC | small cell lung carcinoma |
| shRNA | short hairpin RNA |
| siRNA | small interfering RNA |
| SNP | single nucleotide polymorphism |
| VEGF | vascular endothelial growth factor |
| VOCC | voltage-operated calcium channels |
| VTA | ventral tegmental area |
| XIAP | X-linked inhibitor of apoptosis protein |
| ZO-1 | zonula occludens 1 |

## Preface

Parts of this dissertation have been published in the following journal articles:

**Improgo MR**, Schlichting NA, Cortes RY, Zhao-Shea R, Tapper AR, Gardner PD. ASCL1 regulates the expression of the CHRNA5/A3/B4 lung cancer susceptibility locus. Mol Cancer Res. 2010 Feb;8(2):194-203. Epub 2010 Feb 2.

**Improgo MR**, Scofield MD, Tapper AR, Gardner PD. The nicotinic acetylcholine receptor CHRNA5/A3/B4 gene cluster: dual role in nicotine addiction and lung cancer. Prog Neurobiol. 2010 Oct;92(2):212-26. Epub 2010 Jun 4.

**Improgo MR**, Scofield MD, Tapper AR, Gardner PD. From smoking to lung cancer: the CHRNA5/A3/B4 connection. Oncogene. 2010 Sep 2;29(35):4874-84. Epub 2010 Jun 28.

**Improgo MR**, Tapper AR, Gardner PD. Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer. Biochem Pharmacol. 2011 May 27. [Epub ahead of print].

**Improgo MR**, Johnson CW, Tapper AR, and Gardner PD. Bioluminescence-Based High-Throughput Screen Identifies Pharmacological Agents that Target Neurotransmitter Signaling in Small Cell Lung Carcinoma. PLoS ONE, *in press*.

## Author Contributions

**Improgo, MR** – designed experiments, performed experiments, performed data analyses, prepared figures, and wrote the manuscript for all published and unpublished material described in this dissertation

**Schlichting, NA and Zhao-Shea, R** – performed some quantitative RT-PCR experiments published in *Mol Cancer Res* article

**Cortes, RY** – performed some siRNA experiments published in *Mol Cancer Res* article

**Scofield, MD** – prepared figures and co-wrote the articles published in *Prog Neurobiol* and *Oncogene*

**Johnson, CW** – performed some viability assays and co-wrote the *PLoS ONE* article

**Tapper, AR and Gardner, PD** – designed experiments and co-wrote all publications

# CHAPTER I.

# INTRODUCTION

*It's easy to quit smoking. I've done it hundreds of times.*
*- Mark Twain*

## I.A.Lung Cancer

More than 1 billion people around the world smoke [1]. Approximately 10 million cigarettes are sold every minute, resulting in more than 5 million deaths per year. This makes tobacco use the leading cause of preventable deaths worldwide. Roughly 600,000 of these deaths are due to second-hand smoke. In the United States, overall tobacco use has been declining, though over 45 million adults still continue to smoke [2].

Based on a recent Surgeon General's report, the list of diseases linked to tobacco use is expanding [3]. Specifically, a causal relationship was described between active smoking and cardiovascular diseases, respiratory diseases, reproductive disorders, and several types of cancer, including cancers of the lung, bladder, cervix, esophagus, kidney, larynx, mouth, pancreas, stomach as well as leukemia. This is not surprising given that cigarette smoke contains 4,000 chemicals, 250 of which are known to be harmful, and at least 50 of which are carcinogens. The most potent of these carcinogens are polycyclic aromatic hydrocarbons and nicotine metabolites such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN). These nitrosamines form DNA adducts that cause mutations leading to cancer [4].

Smoking is estimated to cause more than 80% of lung cancer cases in developed countries [5]. In the United States, 24% of male smokers are expected to develop lung cancer in their lifetime, with a 5- to 10-fold increase in risk compared to non-smokers. The incidence rate for lung cancer in the country is surpassed only by that for prostate cancer in males and breast cancer in females [6].

Lung cancer is one of the most common types of cancer, accounting for approximately 15% of all cancer cases worldwide [5]. It also remains the leading cause of cancer-related mortality around the world, resulting in more than 1 million deaths per year. In the United States, lung cancer poses a substantial economic burden on the healthcare system, averaging $6,250 monthly in total healthcare cost per patient [7]. Annual productivity loss due to the disease is approximately $23 billion for males and $14 billion for females [8]. Overall prognosis for the disease remains dismal, with 5-year survival rates ranging from 6-14% for males and 7-18% for females [9].

Based on histological characteristics, lung cancer is classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC is the most aggressive type of lung cancer and has the poorest survival rate [10]. SCLC arises from pulmonary neuroendocrine cells and can be grouped with other tumors that develop from these precursor cells, namely, typical carcinoid

tumors, atypical carcinoid tumors, and large cell neuroendocrine carcinomas [11]. Pulmonary neuroendocrine cells are either found in isolation throughout the lung and airways or in small groups called neuroepithelial bodies, typically found at bifurcations of small airways. Neuroepithelial bodies synthesize neurotransmitters and neuropeptides as well as growth factors and vasoactive substances [12]. They play a trophic role in lung development and function as oxygen sensors and possibly chemo- and mechanoreceptors [13-15].

NSCLC can be subdivided into adenocarcinomas, squamous cell, and large cell lung carcinomas. Adenocarcinomas can be further classified as acinar, papillary, bronchioalveolar, solid adenocarcinoma with mucin production, and mixed subtypes [9]. Adenocarcinomas develop from small airway epithelial cells and alveolar type II cells while squamous cell carcinomas are derived from large airway epithelial cells. Biomarker-guided therapies against NSCLC target specific cell types and subtypes [9, 16].

**I.B. Nicotinic Acetylcholine Receptors (nAChRs)**

nAChRs are a heterogeneous family of ligand-gated cation channels activated by the endogenous neurotransmitter acetylcholine (ACh) and exogenous chemicals such as nicotine and its metabolites. nAChRs were the first receptors to be characterized at the biochemical, biophysical, molecular, and pharmacological levels and have served as prototypes for all other ligand-gated

ion channels including those activated by 5-hydroxytryptamine (5-$HT_3$), γ-aminobutyric acid ($GABA_A$ and $GABA_C$), and glycine [17, 18]. Binding of a ligand induces a conformational change that causes the channel to open, thereby allowing the flow of $Na^+$, $K^+$, and $Ca^{2+}$ ions down their electrochemical gradients. The propensity of nAChRs to flux intracellular calcium levels is important in the activation of downstream signaling cascades [19].

nAChRs can be classified into two main categories: muscle or neuronal receptors. Muscle nAChRs are expressed primarily in skeletal neuromuscular junctions and are composed of the α1, β1, δ, and ε or γ subunits [20]. In contrast, neuronal nAChRs were originally cloned from neuronal-like cell lines and brain cDNA libraries, hence their name, and are expressed throughout the nervous system where they increase neuronal excitability and facilitate synaptic transmission [20-22].

The structure of nAChRs has been deduced using electron micrographs of nAChRs obtained from the electric organs of *Torpedo* rays, a marine ray that uses electrical discharge for defense and to stun prey [23]. nAChRs from these organs are highly homologous to vertebrate receptors. High-resolution x-ray crystallography structures of the acetylcholine binding protein (AChBP) from mollusks have also been used to study the structure of nAChRs [23-26]. AChBP is a protein that resembles nAChRs but does not contain transmembrane and

cytoplasmic domains. Like nAChRs, AChBP binds acetylcholine and is secreted into cholinergic synapses. More refined details of the nAChR structure have also been provided by crystal structures of ligand-gated ion channels from prokaryotes [27-29].

Neuronal nAChRs form pentameric structures assembled from a family of subunits that include α2-α10 and β2-β4 [24, 30-33]. Each nAChR subunit consists of an extracellular N-terminus, four transmembrane segments (designated M1-M4), a variable intracellular loop (100-200 residues) between M3 and M4, and an extracellular C-terminus (Figure I-1) [34]. The N-terminus contains the ACh-binding domain, with the interface between adjacent subunits forming a hydrophobic pocket that contributes to the binding site [35]. The M2 transmembrane segment of all five subunits forms the conducting pore of the channel, with regions in the M1-M2 intracellular loop contributing to cation permeability and agonist binding affinities [20, 34, 36]. The α subunits contain adjacent cysteines in their large extracellular domain that are important for ligand binding, whereas β subunits lack these residues [21]. Unlike other α subunits, however, α5 does not contribute to ligand binding. Though it contains the viscinal cysteines, it is missing a key tyrosine residue (Tyr198) [37]. However, incorporation of the α5 subunit into a mature receptor alters receptor biophysical properties; it enhances receptor assembly and expression, reduces ligand-

mediated upregulation, facilitates channel closure, and increases calcium conductance of the channel [38-40].

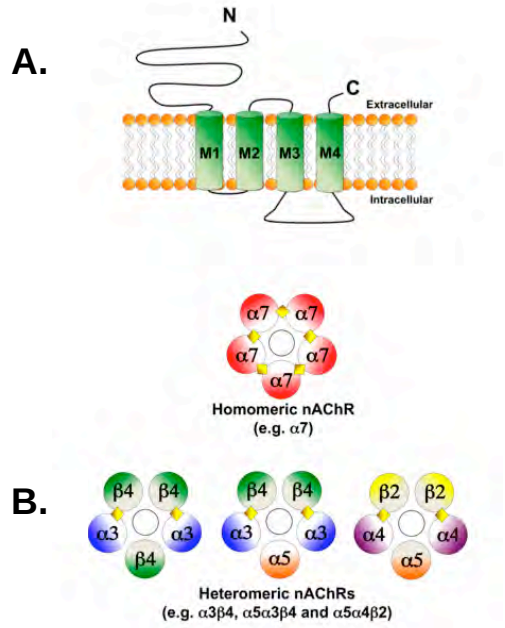


**Figure I-1.** Structure of nAChRs. *A*. Schematic representation of an individual nAChR subunit. Each subunit consists of an extracellular amino (N) and carboxy (C) terminal, four transmembrane domains (M1-M4), and a large intracellular loop between M3 and M4. *B.* Five subunits co-assemble to form homomeric or heteromeric nAChR subtypes. White circles in the center of each pentamer represent the pore region. Diamonds represent ligand binding sites.

Much of what is known about the biophysical and pharmacological properties of nAChRs is based on studies in heterologous expression systems [20]. These systems make use of nAChR mRNA or cRNA injected into *Xenopus* oocytes as well as nAChR cDNA transfected into mammalian cell lines in order to express nAChR subunits singly or in combination. The propensity of nAChR subunits to form either homomeric or heteromeric subtypes was determined by expressing subunits either singly or in combination. When expressed alone, α7,

α8, α9, and α10 are able to form functional receptors that can be blocked by α-bungarotoxin (α-BTx; Figure I-1B) [41-43]. In contrast, other α subunits require the presence of β subunits to form functional receptors [41, 44]. For instance, the α2 - α6 subunits can form heteromeric receptors with the β2 - β4. In addition, α9 can form a heteromeric receptor with α10 [45, 46] and α7 can form a heteromeric receptor with β2 [47]. Each of these receptor subtypes has distinct electrophysiological and pharmacological properties [21, 31, 48, 49]. The functional diversity of the nAChR family offers abundant prospects for the design of novel therapeutics.

**I.C. nAChRs in the Nervous System**

Signaling through neuronal nAChRs underlies several fundamental processes in the nervous system both during development and in the adult [21]. In the central nervous system (CNS), presynaptic nAChRs modulate release of many classical neurotransmitters including acetylcholine (ACh), glutamate, γ-Aminobutyric acid (GABA), and norepinephrine [50]. Postsynaptic nAChRs are intimately involved in fast ACh-mediated synaptic transmission in addition to activity-dependent gene expression, which is critical for synaptic plasticity [21, 22, 51, 52]. Within the peripheral nervous system (PNS), nAChRs mediate fast excitatory transmission in most, if not all, autonomic ganglia and are involved in modulating visceral and somatic sensory transmission [53-58].

The importance of nAChR-mediated signaling is reflected in the many pathologies in which cholinergic signal transduction is compromised. Significant alterations in nAChR expression and function have been documented in several diseases such as Alzheimer's disease, autosomal dominant nocturnal frontal lobe epilepsy, Parkinson's disease, schizophrenia, Tourette's disease, and megacystis-microcolon-intestinal hypoperistalsis syndrome [59-71]. In addition, nAChRs are key players in the development of nicotine addiction [72, 73].

*nAChRs in nicotine addiction*

Nicotine is one of the most widely consumed psychoactive drugs in the world and is the primary reinforcing chemical in tobacco [74]. Nicotine addiction is characterized by heavier smoking, early morning smoking, tolerance and withdrawal. Withdrawal involves both mood-oriented (affective) as well as physical (somatic) symptoms [75]. Withdrawal symptoms account for the high incidence of relapse in people attempting to quit smoking [76].

At the molecular level, nicotine addiction is initiated by the binding of nicotine to nAChRs in the mesolimbic dopaminergic (DAergic) pathway, known as the reward circuitry of the brain [77-79]. Dopaminergic neurons in this circuitry originate in the ventral tegmanetal area (VTA) and project to the nucleus accumbens and the prefrontal cortex. Activation of nAChRs in the VTA causes an increase in the firing of DAergic neurons, resulting in an increase of dopamine

(DA) release in the nucleus accumbens [80-83]. Elevation of dopamine levels in the nucleus accumbens is a phenomenon widely associated with the rewarding properties of drugs of abuse [79, 84]. Increase in dopamine levels is critical for the onset and maintenance of nicotine dependence and inhibition of dopamine elevation via lesions or pharmacological blockade attenuates the rewarding effects of nicotine [85] [86].

Several nAChR subtypes are robustly expressed in mesolimbic dopaminergic neurons, both at the level of the soma and at presynaptic terminals. Expression of α4- and β2-containing receptors in the VTA is necessary and sufficient for nicotine-mediated DA elevation in the nucleus accumbens [87-90]. α4β2* nAChRs are critical for nicotine reward/reinforcement, sensitization, and tolerance [88, 90-92]. α5- and β4-containing nAChRs as well as homomeric α7 nAChRs appear to be involved in the physical symptoms of withdrawal as evidenced by diminished somatic signs in α5, α7, and β4 KO mice [93-95]. Conversely, affective symptoms are readily observable in α5 and α7 KO mice. Affective symptoms, however, are absent in β2 KO mice, suggesting that β2-containing nAChRs may play a role in this component of withdrawal [95, 96]. The various roles that nAChR subtypes play in the different stages of nicotine addiction emphasize the biochemical and functional differences among the nAChR subtypes.

## I.D. nAChRs In Lung Cancer

Though traditionally labeled “neuronal,” it has become evident that neuronal nAChRs are expressed in numerous cell types and tissues including endothelial cells, gastrointestinal tissue, glia, immune cells, keratinocytes, and lung tissue [97-107]. In the lung and airways, nAChR subunit transcripts have been detected using RT-PCR, qRT-PCR, and in situ hybridization and have been shown to be expressed at varying levels in normal and malignant cells [101, 108-111]. Protein expression of a variety of these nAChR subunits has also been investigated using Western blot analysis while assembly of functional nAChRs on the cell surface has been determined using radioligand binding assays and patch clamping [101, 112-114]. The near ubiquitous expression of nAChRs in lung cells underscores the need to elucidate their functional relevance in normal physiology and in disease states.

Through the years, it has become apparent that nAChRs in lung cells act as central mediators in the activation of cancer signaling pathways (Figure I-2) [115]. As early as 1989, Schuller and Hegedus showed that nicotine stimulates proliferation of a neuroendocrine lung cancer cell line, an effect that could be abolished by nAChR antagonists [116, 117]. Shortly thereafter, John Minna’s group showed that lung cancer cells do express nAChRs and that nicotine inhibits apoptosis in these cells [114]. Receptor-mediated effects of nicotine were subsequently recapitulated by other nAChR agonists such as cytisine and

the tobacco nitrosamines 4-(methlynitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN).

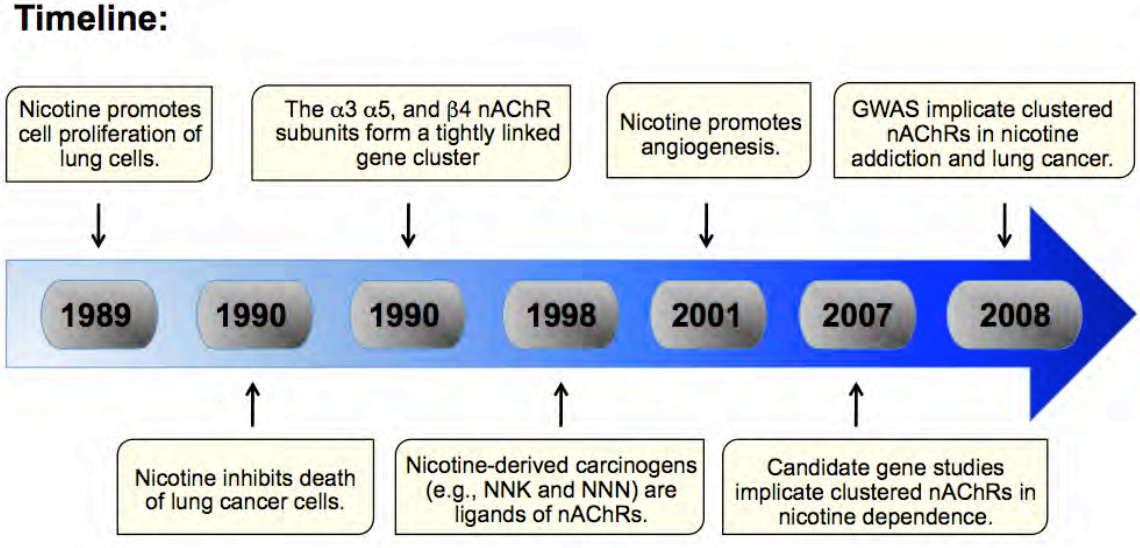


**Figure I-2**. Key research findings implicating nAChRs in lung cancer etiology. Since the first report that nicotine promotes proliferation of lung cells to the recent series of genetic studies implicating nAChRs in lung cancer, mounting evidence suggest that nAChRs play a critical role in the pathogenesis of lung cancer.

nAChR-mediated signaling begins with the binding of agonists to nAChRs, causing a conformational change that leads to the opening of the channel and the influx of cations such as $Na^+$ and $Ca^{2+}$ and the efflux of $K^+$. The resulting membrane depolarization opens the gates of voltage-operated calcium channels (VOCCs), leading to additional flow of $Ca^{2+}$ [115]. Calcium influx triggers the secretion of mitogenic factors and activates signaling cascades involved in cell proliferation, apoptotic inhibition, metastasis, and angiogenesis – processes that are considered hallmarks of cancer (Figure I-3) [115, 118-120].

*nAChRs and cell proliferation*

Nicotine exposure alone does not appear to initiate lung cancer [121]. A more likely scenario is that nicotine promotes cancer after it has been initiated. In NSCLC and their normal cells of origin, nicotine triggers the release of EGF, leading to the binding of EGF to its cognate receptor EGFR, and activation of the Ras-Raf-MAPK cascade, a signal transduction pathway that leads to cell proliferation [122, 123]. Activation of the Ras-Raf-MAPK pathway can be abrogated by the $\alpha$7 nAChR antagonists, $\alpha$-BTx, and $\alpha$-cobratoxin ($\alpha$-Ctx) [124].

Nicotine also induces fibronectin production, which activates the extracellular signal-regulated kinase (ERK), the phosphatidylinositol 3-kinase (PI3-K), and the mammalian target of rapamycin (mTOR), leading to cell growth and survival [125]. In addition, nicotine stimulates the expression of the peroxisome proliferator-activated receptor (PPAR-$\beta/\delta$), an effect that can be blocked by $\alpha$-BTx, $\alpha$7 small interfering RNAs (siRNAs), and PI3-K and mTOR inhibitors [126].

Carcinogenic nitrosamines such as NNK and NNN also promote cell proliferation by activating distinct signaling pathways. NNK has a higher affinity for $\alpha$7 nAChRs while NNN has a higher affinity for heteromeric nAChRs [127]. In SCLC, NNK evokes calcium influx and activates the Raf-MAPK pathway activation, resulting in the phosphorylation of c-myc [128, 129]. Tobacco

nitrosamines can therefore promote lung cancer through direct genotoxic effects or via nAChR-mediated mechanisms [130, 131].

*nAChRs and cell survival*

Nicotine confers resistance to the apoptotic effects of chemotherapeutic drugs, opioids, oxidative stress, and UV radiation [132-134]. The pro-survival effects of nicotine appear to involve the PI3-K-Akt pathway [135]. In non-immortalized human airway epithelial cells, nicotine causes site-specific phosphorylation of Akt [136]. Akt is a known physiological kinase of Bcl-2 family members. Consistent with this, nicotine exposure activates Bcl-2, a key anti-apoptotic molecule while inactivating the pro-apoptotic proteins, Bad and Bax [132, 137, 138]. Similarly, NNK inhibits apoptosis by activating Bcl-2, an effect that can be blocked by inhibitors of PKC and ERK1/2 and by c-myc silencing [139]. Akt activation also leads to the upregulation of the X-linked inhibitor of apoptosis protein (XIAP) and survivin, both inhibitors of apoptosis [140], as well as β-adrenergic receptor and NF-κB activation [141, 142].

*nAChRs and migration/invasion*

Nicotine exposure results in downregulation of the epithelial markers E-cadherin, β-catenin, and the tight-junction protein zonula occludens (ZO-1), with concomitant upregulation of the mesenchymal proteins, fibronectin and vimentin [143, 144]. This epithelial-mesenchymal transition (EMT) is a phenomenon

associated with increased cell mobility and invasion, key events in the process of metastasis. Nicotine treatment, either though intraperitoneal injections or through dermal patches, promotes metastasis in immunocompetent mice [144]. Additionally, NNK exposure leads to increased invasion and migration of lung cancer cells via ERK-dependent phosphorylation of calpains [145]. Pharmacological inhibition of ERK or gene silencing of calpains abolishes this response. The pro-metastatic effects of nicotine and its metabolites may contribute to the aggressiveness of SCLC, a lung cancer type highly associated with cigarette smoking [10].

*nAChRs and angiogenesis*

Angiogenesis, the process of forming new blood vessels, involves 1) activation of endothelial cells by angiogenic stimuli such as hypoxia and cytokine release, 2) degradation of the basement membrane by matrix metalloproteinases, and 3) proliferation and migration of endothelial cells towards the angiogenic stimuli, via a vascular endothelial growth factor (VEGF)-dependent mechanism [146-149]. Vascular endothelial cells express nAChRs as well as other components of the ACh signaling machinery [97, 102]. In these cells, ACh also acts in an autocrine or paracrine fashion and modulates vascularization and remodeling [150, 151]. Nicotine and its metabolite cotinine can induce endothelial cell tube migration by stimulating VEGF expression in lung cancer cells, an effect that can be reduced by $\alpha$-BTx, MLA, and

mecamylamine [151-154]. Inhibition of the MAPK and PI3-K pathways prevents nicotine-induced neovascularization [151]. Nicotine, in combination with estradiol, also enhances growth of lung cancer xenografts via increased VEGF secretion and angiogenesis [155]. Finally, nicotine stimulates accumulation of hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$), a master regulator of angiogenesis [156].

*Autocrine/Paracrine Growth Loops*

Lung cancers secrete ACh and utilize the same ACh signaling components as normal lung cells [157, 158]. In these cells, ACh acts as an autocrine or paracrine growth factor that activates a feedback loop leading to cell proliferation. The non-selective nAChR antagonist mecamylamine inhibits lung cancer growth, suggesting that the ACh machinery can be exploited in the rationale design of therapeutics against lung cancer [159, 160]. Possible pathway points that can be targeted include ACh synthesis, ACh secretion, receptor activation, choline uptake, and downstream pathways [160-164]. Several other neurotransmitters and neuropeptides act as autocrine or paracrine growth factors in lung cancer including bombesin/gastrin-releasing peptide, bradykinin, catecholamines, cholecystokinin, galanin, litorin, neuromedin, neurotensin, ranatensin, serotonin, and vasopressin [165]. Bombesin and serotonin release has been shown to be dependent on protein kinase C (PKC) activation [119]. Interruption of these autocrine or paracrine growth signals may also serve as a viable therapeutic approach against SCLC.

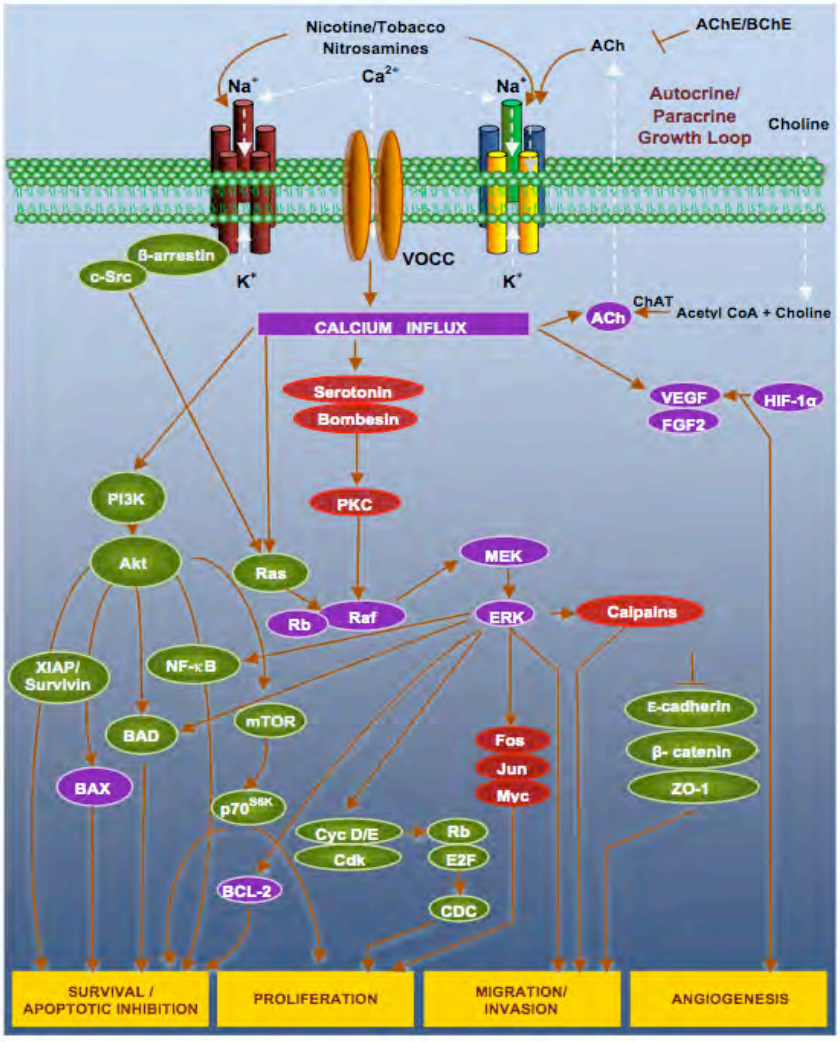


**Figure I-3.** Working model of nAChR-mediated cancer signaling. nAChR agonists such as Ach, nicotine, and tobacco nitrosamines bind to and activate homomeric (red) or heteromeric (multicolored) nAChRs. nAChR activation leads to the opening of the channel and the flow of $Na^+$, $K^+$, and $Ca^{2+}$ ions down their electrochemical gradients. Subsequent membrane depolarization opens voltage-operated calcium channels (VOCCs) and further increases intracellular calcium levels. Calcium influx activates downstream signal transduction pathways leading to lung cancer cell survival, proliferation, migrations/invasion, and angiogenesis. Calcium influx also triggers the release of neurotransmitters and neuropeptides that act as autocrine or paracrine growth factors. Pathway components in red indicate those identified in SCLC and its precursor cells; those in green have been identified in NSCLC and its precursor cells; and those in purple have been identified in both types of lung cancer cells and their corresponding cells of origin.

## I.E. The CHRNA5/A3/B4 Lung Cancer Susceptibility Locus

Recent advances in genetic technology have paved the way for large-scale genome-wide association studies (GWAS), which involve screening hundreds of thousands of single nucleotide polymorphisms (SNPs) across thousands of subjects [166]. Such studies have implicated variants in the chromosome 15q24-25 region in the development of nicotine dependence and lung cancer. This genomic locus, spanning 203 kb, contains the genes encoding the α5, α3, and β4 nAChR subunits (Figure I-4) [167, 168].

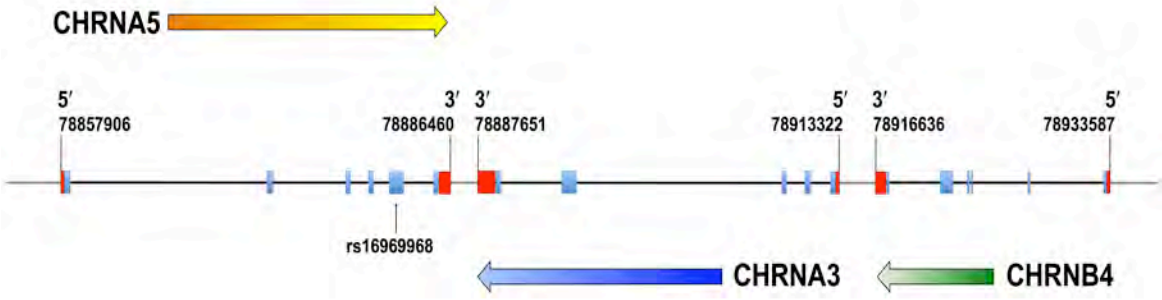


**Figure I-4.** The human CHRNA5/A3/B4 gene cluster**.** The CHRNA5 gene is transcribed in the opposite direction as CHRNA3 and CHRNB4 (colored arrows). Blue boxes represent exons and red boxes represent untranslated regions. Horizontal black lines indicate introns and horizontal gray lines indicate intragenic regions. The boundaries for each gene are labeled with corresponding Genbank annotations. The non-synonymous D398N polymorphism (rs16969968) associated with nicotine dependence and lung cancer is indicated by an arrow under exon 5 of the CHRNA5 gene.

*Expression patterns of the CHRNA5/A3/B4 genes*

Admixtures of the nAChR subunits encoded by the 15q24-25 locus form the predominant nicotinic receptor subtypes expressed in the PNS [169-173] as well as at key sites in the CNS [174, 175]. In the PNS, α3 subunit expression is seen in the dorsal root ganglia [176], facial motoneurons [177], retina [178, 179], adrenal, otic, and spenopalatine ganglia [171], the superior cervical ganglion (SCG), and trigeminal sensory neurons [172, 180]. In the CNS, the α3 subunit is expressed in the brainstem [181], cerebellum [182, 183], cortex [183, 184], hippocampus [99, 183-186], interpeduncular nucleus [174, 185, 187], medial habenula [174, 176], pineal gland [176], spinal cord [188, 189], substantia nigra [190], thalamus [186, 187], and the VTA [187, 191].

Outside of neurons, the α3 subunit is expressed in human oral keratinocytes [192-194] where its mRNA and protein expression levels are increased following exposure to nicotine [193-195]. α3-containing nAChRs are also expressed in bronchial epithelia [97, 101], the gastrointestinal tract [196, 197], lymphocytes [198], oligodendrocyte type-2 astrocyte (O2A) progenitors [199], polymorphonuclear cells [198], and vascular endothelial cells [97, 102].

Similar to the α3 subunit, the α5 subunit is most highly expressed in the PNS, but is also expressed in several key regions of the CNS [191, 200]. In the PNS, α5 is expressed in most autonomic ganglia [180, 196] and the retina [178].

Centrally, α5 is expressed in the cerebellum and thalamus [196] but is also detected in the brainstem, cortex, habenula, hippocampus, interpeduncular nucleus, other midbrain nuclei, and the spinal cord [99, 174, 188-190, 201, 202].

Outside the nervous system, α5 subunit expression has been detected in the gastrointestinal tract, thymus and testis [196, 197]. Furthermore, the α5 subunit is expressed in many of the same cell types as the α3 and β4 subunits including bronchial epithelium [97, 101], O2A progenitors [199], oral epithelium [193, 194], vascular endothelial cells [97, 102], and a variety of immune cells [203].

The β4 subunit gene is widely expressed in the PNS, with relatively high expression in trigeminal sensory neurons [172, 180] as well as sympathetic neurons and the superior cervical, dorsal root, spenopalatine and otic ganglia [171, 176, 204]. The β4 subunit is also expressed in the adrenal medulla [205] with lower expression in the retina [178]. In the CNS, β4 expression is particularly high in the interpeduncular nucleus, medial habenula, olfactory bulb, and pineal gland [174, 185, 206] with lower expression in the cortex, cerebellum, hippocampus, midbrain, spinal cord, and thalamic nuclei, [99, 182, 187-190, 207].

Significant overlap of β4 expression with α3 and α5 expression is also observed outside the nervous system, β4 is expressed in multiple cell types of the bronchial epithelium [97, 101], intestines [196, 197], O2A progenitors, oral keratinocytes [193, 194], polymorphonuclear cells [198], and vascular endothelial cells [102], [199]. Finally, as discussed in detail in Chapter II, β4 is co-expressed with α3 and α5 in the lung and is upregulated in lung cancer [208-210].

*Transcriptional regulation of the CHRNA5/A3/B4 genes*

The co-expression of the CHRNA5/A3/B4 genes coupled with their genomic clustering hinted early on that they may share common regulatory mechanisms in addition to specific regulation of each gene. Further support for this idea comes from several observations. First, nucleotide sequencing of the individual gene promoters revealed that they each lack classical CAAT and TATA boxes [168]. Instead, the promoters are GC-rich and contain several binding sites for the transcription factors, Sp1 and Sp3 (Figure I-5). Both Sp factors positively regulate transcription of each of the clustered subunit genes through multiple binding sites in each individual promoter [211-221]. Chromatin Immunoprecipitation (ChIP) experiments demonstrated Sp1 binding activity in the context of native chromatin for all three promoters [222, 223]. It is likely that Sp1 is involved in tethering the basal transcription machinery to the TATA-less nAChR subunit gene promoters [224]. Second, in addition to the Sp factors, the

CHRNA3/A5/B4 promoter regions can directly interact with and be trans-activated by the more spatially restricted regulatory factors Sox10 and SCIP/Tst-1/Oct-6 [225-227]. Third, the mRNA levels of the CHRNA3/A5/B4 genes are coordinately up-regulated during neural development [228-230] and coordinately down-regulated following denervation [231]. Perhaps the most compelling evidence for a coordinated regulatory scheme comes from the Deneris lab, which showed that two transcriptional regulatory elements, termed β4 3' enhancer and conserved noncoding region 4 (CNR4), play key roles in directing expression of the clustered nAChR genes in a tissue-specific manner. The β4 3' enhancer is critical for expression in the adrenal gland and CNR4 is critical for expression in the pineal gland and SCG [232]. CNR4 is likely to play an important role in directing nAChR gene expression in the brain as well [232]. In addition to these shared regulatory features, the CHRNA3/A5/B4 genes are subject to gene-specific regulation.

*In vitro* experiments have shown that the paired-like homeodomain transcription factor, PHOX2A, regulates transcription at the α3 promoter [223]. PHOX2A does not appear to bind directly to DNA, however, as the DNA-binding domain does not need to be completely intact for PHOX2A to regulate transcription [223]. Co-immunoprecipitation experiments demonstrate a physical interaction between Sp1 and PHOX2A, suggesting that PHOX2A is tethered to

the α3 promoter through its interaction with Sp1, similar to the interactions of Sp1 with homeodomain transcription factors observed in other systems [233].

The POU domain factor SCIP/Tst-1/Oct-6 has been shown to positively regulate transcription from the α3 promoter in a cell-type-specific manner [227]. Similar to PHOX2A, the POU domain factor SCIP/Tst-1/Oct-6 does not require DNA binding for transactivation of the α3 promoter [227]. Deletion analysis of the SCIP/Tst-1/Oct-6 transcription factor demonstrated that only the POU domain is needed for transactivation. This transactivation does not depend on the presence of an Sp1 motif in the promoter region and is likely occurring through protein-protein interactions with other transcriptional machinery [225]. The transcription factor Brn-3a also trans-activates the α3 promoter, while the other members of the Brn-3 family, Brn-3b and 3c, modestly repress α3 promoter activity [234]. The positive regulation by Brn-3a is thought to be a result of protein-protein interaction as the α3 promoter lacks an obvious octamer or octamer-related binding site for Brn-3 factors [234].

The β4 3' enhancer lies upstream of the α3 promoter in a region that overlaps with a 3'-untranslated exon of the β4 gene [235]. This enhancer element consists of two identical 37-base pair repeats separated by a 6-base pair spacer. The β4 3' enhancer acts in a cell-type-specific manner and enhances transcription from the α3 promoter in neuronal cultures [236]. The enhancer

contains several E-twenty six (ETS) factor-binding sites, mutation of which dramatically decreases, but does not completely abolish, α3 promoter activity. The ETS-domain binding factor, Pet-1, has been shown to activate reporter gene transcription in a manner that is both cell type - and β43' enhancer - dependent [237]. Taken together, these studies suggest that Pet-1 interacts directly with the α3 promoter to activate transcription, though it likely requires additional cell-type-specific co-factors.

*In vivo* experiments using transgenic mice show that a larger DNA fragment between the α3 and β4 genes, containing both the β4 3′ enhancer and the α3 promoter, is capable of directing expression of a reporter gene to several areas of endogenous α3 expression in the brain [238, 239]. Surprisingly however, this DNA fragment did not direct reporter gene expression anywhere in the peripheral nervous system, in which the α3 gene is highly expressed, suggesting that elements in this fragment may be acting as repressors or that other sequences are necessary for peripheral expression.

The presence of an intronic repressor element in the fifth intron of α3 has been reported [240]. The sequence of this α3 intron 5 repressor (α3I5) is highly conserved and is capable of bidirectional repressor activity *in vitro.* Notably, cell-type-specific repression of promoter activity was observed to be more potent in non-neuronal cell lines than in neuronal cell lines [240]. These data suggest that

this segment of DNA and the factors with which it interacts function to restrict expression of α3 to neuronal cell types. The protein-DNA interactions that mediate this effect have yet to be elucidated. The mechanisms regulating α3 expression in non-neuronal cells remain largely obscure. In Chapter II, we show that the transcription factor, achaete-scute complex homolog-1 (ASCL1), regulates the expression of α3 and β4 and modestly of α5 in lung cancer cells [208].

The α5 promoter region has been described in several genomic contexts including those in rodents and humans. Transcription of α5 occurs in the opposite direction as α3 and β4, suggesting that in addition to transcription factors that regulate the entire cluster, distinct mechanisms may govern α5 expression. However, apart from the regulatory factors described above, little is known about these mechanisms. SCIP/Tst-1/Oct-6 does not appear to regulate α5 though it regulates α3 and β4. No other transcription factors regulating α5 expression have been reported, underscoring the need for more research efforts in this area.

In addition to Sp1/2, Sox10 and SCIP/Tst-1/Oct-6, the β4 promoter is positively regulated by c-Jun [217]. Trans-activation by all of these factors is abolished when the Sp-binding site on the β4 promoter (referred to as the CA box) is mutated. Conversely, synergistic activation of the β4 promoter is

observed when Sp1 is supplied in concert with Sox10, Sp3 or c-Jun [217, 218]. Co-immunoprecipitation experiments demonstrated that all of these factors physically interact [241] and ChIP experiments confirmed that these interactions occur in the context of native chromatin [222]. These findings suggest the existence of a positively-acting multi-subunit transcriptional regulatory complex that assembles on the β4 promoter. This is consistent with the hypothesis that Sp1 is critical for transcription at the β4 promoter and likely nucleates the regulatory complex that drives expression of the β4 gene.

Two additional transcription factors have been shown to interact with the β4 promoter, Purα and heterogeneous nuclear ribonucleoprotein K (hnRNP K) [242, 243]. These proteins interact with another motif, the CT box, located directly upstream of the CA box. hnRNP K is capable of repressing Sp factor-mediated trans-activation of the β4 promoter [243] and also physically interacts with Sox10 [241]. Similar to hnRNP K, Purα physically interacts with Sox10 [241]. Moreover, Purα and hnRNP K themselves physically interact [241]. These proteins may participate in the multi-subunit complex described above to modulate expression of the β4 gene in the appropriate cellular context. *In vitro* binding experiments demonstrated that each factor binds preferentially to the opposing single strand elements of the CT box, suggesting that some local DNA helix unwinding may occur [244]. Interestingly, Purα and hnRNP K have been shown to function together to negatively impact transcription of genes in other

systems and the same may be occurring at the β4 promoter [245]. *In vivo* experiments have also shown that a 2.3-kb fragment of the β4 promoter, containing the CA and CT boxes, is capable of directing reporter gene expression to brain regions that endogenously express β4, further supporting the importance of these elements in regulating β4 gene expression [246].

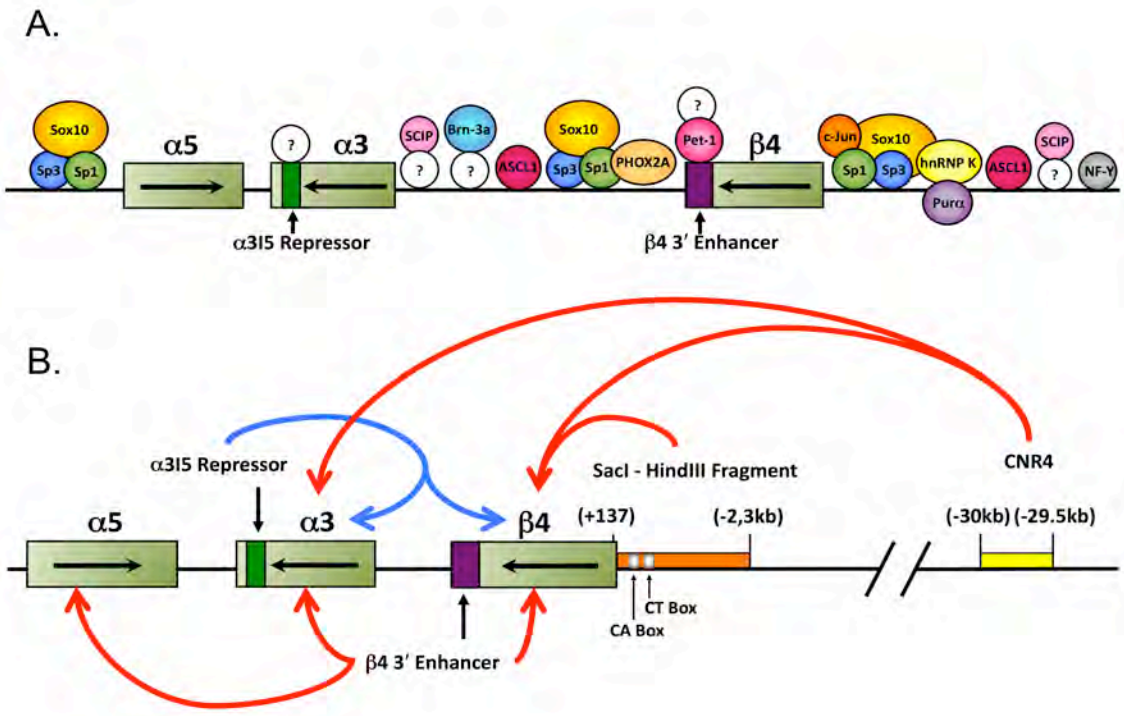


**Figure I-5.** Transcriptional regulation of the CHRNA5/A3/B4 gene cluster. *A.* Protein-DNA interactions at the CHRNA5/A3/B4 locus. Colored circles indicate known transcription factors while circles with a question mark indicate transcription factors that have yet to be identified. Regulation by the ASCL1 transcription factor is described in Chapter II. *B.* Positive (red arrows) and negative (blue arrows) regulation of the CHRNA5/A3/B4 genes. The β4 3' enhancer, the β4 promoter region (SacI – HindIII fragment), and the distal CNR4 regions contain positive regulatory elements. The fifth intron of the CHRNA5 gene (A3I5) contains a repressor element.

*Function of the CHRNA5/A3/B4 genes*

Knockout (KO) mouse models have been generated to determine the function of the clustered nAChR subunits. Mice that do not express the α3 subunit usually die within a week of birth due to multi-organ dysfunction [247]. α3 KO mice develop enlarged bladders causing bladder infection, dribbling urination, and urinary stones – a phenotype resembling that of a rare human condition called megacystis-microcolon-intestinal hypoperistalsis syndrome [247]. Consistently, patients with this disease do not appear to express α3 mRNA [60]. α3 KO mice also display extreme pupil dilation and lack of pupil contraction in response to light. Retinal wave activities have altered spatiotemporal properties delaying the refinement of retinal ganglion cell dendrites [247, 248]. Bladder contraction in response to nicotine is also lost. Lastly, electrophysiological characterization shows that nicotine-induced whole-cell currents are abolished in the SCG of α3 KO mice.

In contrast to the α3 KO mice, α5 and β4 KO mice are both viable and lack any gross abnormalities [247, 249, 250]. α5 KO mice do exhibit abnormal cardiac parasympathetic ganglionic transmission and are less sensitive to acute nicotine treatment. Loss of α5 selectively affects axonal nAChRs in the SCG while leaving somatodendritic receptors unaffected [251]. Similarly, ganglionic transmission is impaired in β4 KO mice, attenuating ileal and bladder contractile responses to nicotinic agonists. Nicotine-induced whole cell currents in the SCG

of β4 KO mice are also reduced but still present, suggesting that compensation from another subunit (i.e., β2) may be occurring [252]. Consistent with this notion, nicotine-induced currents in the SCG are abolished in double β2-β4 KO mice. Moreover, double β2-β4 KO mice exhibit similar bladder and pupil dysfunction as α3 KO mice. Taken together, these studies indicate that the clustered nAChR subunits are essential for normal ganglionic function and that compensation by β2 can occur with the loss of β4.

In addition to their PNS-specific phenotypes, α3, α5 and β4 KO mice also exhibit CNS-centric abnormalities compared to WT mice. For example, α3, α5 and β4 KO animals are resistant to nicotine-induced seizures compared to their respective WT littermates and are not as sensitive to nicotine-induced inhibition of locomotion [253, 254]. β4 KO mice also appear less anxious compared to WT mice in two specific anxiety assays suggesting a role for β4* nAChRs in modulating anxiogenic stimuli [255]. These mice also have a lower core body temperature which is less responsive to modulation by acute nicotine infusion [256].

*Risk alleles in nicotine addiction and lung cancer*

Genetic association between the CHRNA5/A3/B4 genes and nicotine dependence was first reported in an association study that compared nicotine-dependent smokers versus those without symptoms of dependence [257].

Multiple SNPs were correlated with nicotine dependence, including rs16969968, located in the CHRNA5 coding region (Figure I-4). This polymorphism changes an amino acid from aspartic acid to asparagine at position 398 (D398) in the major cytoplasmic loop of the $\alpha$5 subunit. This highly conserved aspartate residue is invariant across species such as frogs, chickens, rodents, cattle, and nonhuman primates [258]. Follow-up studies showed that one copy of the risk allele confers a 1.3-fold increase in risk for developing nicotine dependence, whereas subjects homozygous for the risk allele have almost a 2-fold increase in risk [259]. Since then, many groups have found rs16969968 and several other SNPs to be associated with different smoking behaviors using a variety of approaches and target populations [258-266].

Analogous large-scale genetic studies in lung cancer yielded results converging on the same variants in chromosome 15q24-25 [267-269]. This locus was found to account for 14% of lung cancer cases in a European population [267] and for 18% of cases in an Icelandic population [268]. rs16969968 was again found to be among the SNPs with the strongest disease association [269]. Consistently, a candidate gene study showed that the rs16969968 risk allele is associated with increased risk for lung adenocarcinomas in an Italian population [270]. Another (synonymous) SNP highly associated with lung cancer is rs1051730, found in the coding region of CHRNA3 [267, 268].

Because the chromosome 15 region is associated with both nicotine dependence and lung cancer, it raises the question of whether lung cancer is directly influenced by the genetic variants or merely the consequence of smoking behaviors. Genetic evidence for the two lines of reasoning exist. Increased lung cancer risk in non-smokers supports the view that the polymorphisms have a direct effect on lung cancer [271]. Additionally, the polymorphisms were not associated with other smoking-related cancers such as head and neck cancers including those of the oral cavity, larynx, pharynx, and esophagus [271]. Association with lung cancer also persists even after accounting for cigarette consumption [167, 272, 273]. However, one study argues that cigarette consumption per se might not be a proper measure as individuals who smoke the same number of cigarettes per day exhibit varying levels of carcinogen and toxin exposure [274]. In fact, carriers of some risk variants ingest more tobacco toxins, possibly by inhaling more frequently and more deeply while smoking. Additionally, the CHRNA5/A3/B4 genes have also been linked to other smoking-related disorders such as chronic obstructive pulmonary disease (COPD), peripheral arterial disease, and alcoholism [268, 275, 276].

To distinguish between the two possibilities, direct evaluation of the biological function of specific SNPs needed to be performed. A reasonable candidate for initial studies was the rs16969968 SNP, as it encodes a non-synonymous SNP in CHRNA5. Indeed, heterologous expression of the $\alpha$5 cDNA

containing this SNP, along with α4 and β2, was carried out in HEK293T cells [258]. In this study, agonist-induced changes in intracellular calcium were measured using an aequorin-based luminescence assay. α4β2α5 nAChRs with the asparagine variant exhibited lower maximal response to the nAChR agonist, epibatidine, indicating that the α5 risk allele is associated with reduced function of α4β2α5 nAChRs. This work provides direct evidence that a variant associated with nicotine dependence and lung cancer alters biological function.

Additional mechanistic evidence for the role of nAChR variants in nicotine dependence is provided by work showing that rs3841324, a SNP found in the non-coding region of α5, is associated with altered α5 mRNA levels in the brain [277]. rs3841324 is characterized by an insertion/deletion located upstream of the α5 coding region. In this study, individuals homozygous for the minor allele (deletion) exhibit a 2.9-fold increase in CHRNA5 mRNA levels. rs3841324 is in high linkage disequilibrium with other SNPs in this region. Consistently, these SNPs are also associated with altered CHRNA5 mRNA expression.

Combined analyses of the above biological mechanisms demonstrate that the risk allele of rs16969968 primarily occurs on a low CHRNA5 expression background [277]. Moreover, a combination of low mRNA expression and the presence of the non-risk allele confer protection for both nicotine dependence and lung cancer. In normal lung tissue, rs16969968 also correlated with

CHRNA5 mRNA levels (i.e., an inverse relationship was observed between risk allele dosage and mRNA levels) [270]. The same study showed a 30-fold upregulation of CHRNA5 mRNA levels in lung adenocarcinoma compared to normal lung tissue. In contrast, no differences in expression between cancer and normal samples were observed for the other genes in chromosome 15 outside the CHRNA5/A3/B4 gene cluster. Taken together, the aforementioned studies offer two mechanistic bases for the association of nAChR variants with nicotine dependence and lung cancer: altered receptor function and aberrant gene expression.

In this dissertation, the overarching goal is to elucidate the mechanisms underlying the role that $\alpha5\alpha3\beta4$ nAChRs play in lung cancer, with particular emphasis on SCLC, the most aggressive type of lung cancer. Here we show that the CHRNA5/A3/B4 genes are over-expressed in SCLC and are regulated by ASCL1, a transcription factor of the Notch signaling pathway that directs the neuroendocrine phenotype of SCLC. We then show that genetic depletion or pharmacological blockade of CHRNA5/A3/B4 depletion inhibits SCLC cell viability. Consistently, silencing of CHRNA5 *in vivo* inhibits tumor growth. Finally, we show that the neuroendocrine phenotype of SCLC allows its targeting by pharmacologically active compounds.

# Chapter II:

# EXPRESSION AND REGULATION OF THE CHRNA5/A3/B4 GENE CLUSTER

*The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny..."*
*- Isaac Asimov*

## II.A.Introduction

Lung cancer is the leading cause of cancer-related mortality across the globe [5]. Cigarette smoking and second-hand smoke are the major etiologic factors associated with lung cancer, accounting for nearly 90% of all lung cancer deaths. Given that 25% of adults smoke, a considerable number of people are presently at risk for the disease.

Lung cancer is classified into two main histological types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The latter can be further divided into large cell carcinoma, adenocarcinoma and squamous cell carcinoma. SCLC, a neuroendocrine tumor, is the most aggressive among the various types of lung cancer and has the poorest prognosis, with a 5-year survival rate of 15% [10]. This can reach as low as 2% for patients diagnosed with late-stage disease. Though most patients respond to initial cycles of chemotherapy, they eventually become chemoresistant.

Nearly all SCLC patients (>95%) have a history of cigarette smoking [10]. This strong etiologic link is not surprising given the fact that tobacco contains at least 55 carcinogens, the most potent of which are nicotine-derived nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [131]. Increasing

evidence also suggest that nicotine itself may directly contribute to carcinogenesis by inducing cell proliferation, transformation, apoptotic inhibition, and angiogenesis [115].

Nicotine and NNK are both exogenous ligands of nicotinic acetylcholine receptors (nAChRs) [127]. nAChRs are transmembrane ligand-gated ion channels that have been extensively studied with respect to their role in fundamental physiological processes such as muscle contraction, attention, arousal, anxiety and learning and memory [278]. They are key players in the nicotine reward pathway, making them attractive drug targets for smoking cessation therapies [83, 84, 88, 279].

nAChRs have traditionally been referred to as either “muscle” or “neuronal” based on their expression patterns and subunit composition. Muscle nAChRs are made up of $\alpha$1 subunits combined with $\beta$1, $\gamma$, $\delta$, or $\varepsilon$ subunits. Here we focus on neuronal nAChRs, pentameric proteins made up of homomeric or heteromeric combinations of $\alpha$ and $\beta$ subunits that include $\alpha$2 – $\alpha$10 and $\beta$2 – $\beta$4 [278]. The precise combination of subunits determines the pharmacological and biophysical properties of the receptor [20, 32]. While the complete repertoire of native nAChRs has not been fully elucidated, it is clear that a staggering diversity of receptor subtypes and functions may exist [32].

The neuronal nAChRs have also been found in non-neuronal tissues [105, 107, 203]. In particular, they are expressed in normal as well as lung cancer cells [209, 210]. The two most well-characterized nAChRs in this system are the homomeric $\alpha$7 and the heteromeric $\alpha$4$\beta$2 subtypes [280]. Recently, however, a series of genome-wide association studies pointed to a possible role for the nAChR $\alpha$3$\beta$4$\alpha$5 subytpe in the etiology of lung cancer [267-269, 281]. These studies identified a lung cancer susceptibility locus in the long arm of chromosome 15 (15q24/15q25.1), a genomic region containing the genes encoding the $\alpha$5, $\alpha$3, and $\beta$4 subunits (CHRNA5/A3/B4). Single nucleotide polymorphisms found in the gene cluster were also found in independent studies to be associated with nicotine addiction [282-288]. It is not yet clear how variants in this locus may modulate the function of mature nAChRs but these studies do prompt further investigation on the role of these nAChR subunits in lung cancer.

To address this gap in knowledge, we first examined the expression profile of these genes as well as all other neuronal nAChR genes in lung cancer cell lines and patient samples. Here we describe the over-expression of the clustered nAChR genes in SCLC. Furthermore, we identified a transcription factor, ASCL1, that regulates the CHRNA3/A5/B4 gene cluster in this tumor type. ASCL1 (termed Mash1 in rodents, hASH1 in humans) is a basic helix-loop-helix transcription factor that binds to DNA recognition motifs known as E-boxes [289]. It is over-expressed in SCLC and other neuroendocrine tumors. ASCL1

expression appears to be important for SCLC tumor initiation while its knockdown causes cell cycle arrest and apoptosis [290, 291]. In addition, transgenic mice that constitutively express ASCL1 and the SV40 Large T antigen develop aggressive lung tumors with neuroendocrine features [292]. Over-expression of ASCL1 in SCLC may thus lead to corresponding over-expression of the clustered nAChR genes, providing a mechanism by which nicotine's effects may be potentiated in SCLC, contributing to its increased tumorigenicity.

**II.B.Materials and Methods**

*Cell lines.* Cell lines were obtained from the American Type Culture Collection (ATCC). The SCLC cell lines used were DMS-53, DMS-114, NCI-H69, NCI-H82, NCI-128, NCI-146, NCI-H209 and NCI-446. The NSCLC cell lines used were the large cell lung carcinoma cell lines NCI-H460, NCI-H661, NCI-1581 and NCI-H1915; the lung adenocarcinoma cell lines A549, NCI-H838, NCI-H1395, NCI-H1734 and NCI-H1793; and the squamous cell lung carcinoma cell lines NCI-H520, NCI-H1869, NCI-H2170, SK-MES-1 and SW-900. The normal lung cell lines used were BEAS-2B, HBE4-E6/E7, LL-24 and WI-38. Cell lines were maintained in ATCC-recommended media at 37$^{o}$C and 8% $CO_2$.

*Patient samples.* Tissue samples were obtained from the UMass Cancer Center Tissue Bank and the Cooperative Human Tissue Network. Approval from the University of Massachusetts Medical School Institutional Review Board was

obtained prior to sample collection. To date, a total of 123 cancer and normal lung tissues have been collected consisting of 53 normal, 7 SCLC and 63 NSCLC tissues including 19 adenocarcinomas, 32 squamous cell lung carcinomas and 12 large cell lung carcinomas (Table II-1). Samples were either snap-frozen surgically resected tissues or fresh pleural effusions. Available normal attached tissues or age and sex-matched normal tissues were used as controls.

**Table II-1. Clinical characteristics of lung cancer patient samples**

| Specimen ID | Histology | Sex | Age | Stage | Smoking History |
|---|---|---|---|---|---|
| 143C | SCLC | M | 54 | Extensive | + |
| 985T | SCLC | M | 71 | T1N0M0 | + |
| 1662T | SCLC | M | 59 | Extensive | + |
| 1090251A2 | SCLC | M | 57 | NA* | NA |
| 08-02-A280a | SCLC | M | 61 | T2N2MX | NA |
| 06-11-A306aa | SCLC | F | 72 | NA | NA |
| MAD09-131T | SCLC | M | 53 | T2N0MX | + |
| 350T | Large Cell | M | 51 | T2N0MX | + |
| 808T | Large Cell | F | 45 | T2N1M0 | + |
| 849T | Large Cell | M | 74 | T3N1M0 | + |
| 1722T | Large Cell | F | 74 | T2N0MX | NA |
| MAD02-1005T | Large Cell | M | 76 | T2N0MX | + |
| MAD05-467T | Large Cell | F | 61 | T1N0MX | + |
| MAD07-597T | Large Cell | M | 73 | T1N0MX | + |
| MAD07-661T | Large Cell | M | 71 | T1N2MX | + |
| MAD07-809T | Large Cell | F | 70 | T2N0MX | + |
| MAD08-469T | Large Cell | M | 61 | T2N0MX | + |
| MAD08-638T | Large Cell | M | 44 | T2N1MX | + |
| Z4312A1E | Large Cell | M | NA | T2N2MX | NA |
| 343T | Adenocarcinoma | M | 62 | T1N0MX | + |
| 363T | Adenocarcinoma | F | 56 | T1N2MX | + |
| 423T | Adenocarcinoma | M | 64 | NA | + |
| 457T | Adenocarcinoma | M | 64 | T4N1MX | + |
| 43089A1C | Adenocarcinoma | F | 81 | NA | NA |
| 43464A1C | Adenocarcinoma | F | 59 | NA | + |
| 43471A1C | Adenocarcinoma | F | 26 | T3N2MX | - |
| 44833A1A | Adenocarcinoma | F | 82 | NA | + |
| 45139A1D | Adenocarcinoma | F | 61 | NA | + |

| | | | | | |
|---|---|---|---|---|---|
| 45151A3CA | Adenocarcinoma | NA | NA | NA | NA |
| 45514A1A | Adenocarcinoma | NA | NA | NA | NA |
| 45607A1BA | Adenocarcinoma | M | 78 | NA | + |
| 46127A1BA | Adenocarcinoma | M | 77 | NA | + |
| 46244A1A | Adenocarcinoma | F | 66 | T4N1MX | NA |
| 46598A1A | Adenocarcinoma | M | 67 | NA | + |
| 1081210A1 | Adenocarcinoma | NA | NA | NA | NA |
| 1090694A1 | Adenocarcinoma | F | 66 | NA | NA |
| 08-04-A123A | Adenocarcinoma | M | 67 | NA | NA |
| Z4364A1A | Adenocarcinoma | F | NA | T2N1MX | NA |
| 258T | Squamous | F | 74 | T1N0MX | + |
| 318T | Squamous | M | 81 | T2N1MX | + |
| 43312T | Squamous | F | 76 | NA | NA |
| 43751T | Squamous | NA | NA | T2N1MX | NA |
| 43057A1I | Squamous | M | 68 | NA | + |
| 45843A1F | Squamous | M | 41 | NA | NA |
| 46215A1F | Squamous | F | 75 | NA | NA |
| 46830A1A | Squamous | NA | NA | NA | NA |
| 1082331B2 | Squamous | F | 69 | NA | NA |
| 1090147A2 | Squamous | M | 72 | NA | NA |
| 3081395A3 | Squamous | F | 51 | NA | NA |
| 3081583A5 | Squamous | F | 70 | NA | NA |
| 3090415A2 | Squamous | M | 56 | T2N0MX | NA |
| 08-01-A310A | Squamous | M | 61 | NA | NA |
| 08-02-A290A | Squamous | F | 58 | T2N0MX | NA |
| 08-05-A023B | Squamous | F | 75 | NA | NA |
| 08-07-A078A | Squamous | F | 65 | T2N0MX | NA |
| 08-08-A097B | Squamous | M | 58 | T2N1MX | NA |
| 08-09-A190A | Squamous | M | 58 | NA | NA |
| 08-11-A001B | Squamous | M | 63 | T1N1MX | NA |
| 08-12-A011A | Squamous | M | 74 | T1N0MX | NA |
| 09-03-A012B | Squamous | M | 70 | T1N2MX | NA |
| 09-04-A019A | Squamous | M | 75 | NA | NA |
| MAD06-482T | Squamous | M | 65 | T1NXMX | + |
| MAD06-552T | Squamous | M | 73 | T1NXMX | + |
| MAD06-597T | Squamous | F | 73 | T1N0MX | + |
| MAD06-603T | Squamous | F | 71 | T1N0MX | + |
| MAD06-625T | Squamous | M | 74 | T2N1MX | + |
| Z3770A1A | Squamous | NA | NA | T3N0MX | NA |
| Z4129A1D | Squamous | M | NA | T2N1MX | NA |
| Z4363A1A | Squamous | M | NA | T2N1MX | NA |
| Z4640A1A | Squamous | F | NA | T2N0MX | NA |

* NA – information not available

*Quantitative RT-PCR.* Total RNA was isolated from the cell lines and patient tissues using a RiboPure Kit (Ambion). cDNAs were generated using a RETROscript Kit (Ambion). Quantitative RT-PCR was performed using an Applied Biosystems 7500 Real-Time System and TaqMan assays for nAChR α2-α7, α9-α10 and β2-β4 (Applied Biosystems, see Table II-2). α8 gene expression was not analyzed because its expression has only been observed in avian species. Samples containing no reverse transcriptase were used as negative controls. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene β2-microglobulin was used as the endogenous control.

Table II-2. TaqMan assays used to measure mRNA expression of nAChRs

| Target Gene | TaqMan Assay |
|---|---|
| CHRNA2 | Hs00181237_m1 |
| CHRNA3 | Hs00609519_m1 |
| CHRNA4 | Hs00181247_m1 |
| CHRNA5 | Hs00181248_m1 |
| CHRNA6 | Hs00610231_m1 |
| CHRNA7 | Hs01063373_m1 |
| CHRNA9 | Hs00214034_m1 |
| CHRNA10 | Hs00220710_m1 |
| CHRNB2 | Hs00181267_m1 |
| CHRNB3 | Hs00181269_m1 |
| CHRNB4 | Hs00609523_m1 |
| β2-microglobulin | Hs00187842_m1 |

*ASCL1 knockdown.* Knockdown of ASCL1 expression was performed in a SCLC cell line, DMS-53 and a NSCLC cell line, A549. To control for off-target effects, three different siRNAs against ASCL1 were used namely s1656, s1657, and s1658 (Applied Biosystems). Transient transfections were performed using Lipofectamine™2000 (Invitrogen). Knockdown levels were determined using

quantitative RT-PCR. A negative control siRNA (Applied Biosystems) that does not target any known human, mouse, or rat gene was used to normalize gene expression. Untransfected samples were also analyzed for baseline gene expression. Corresponding changes in nAChR α3, α5, and β4 gene expression was measured using quantitative RT-PCR with β2 microglobulin as endogenous control. To determine specificity, gene expression of β2 was also measured. GAPDH levels were measured as a negative control. Samples were analyzed in triplicate and at least two independent experiments were done for each siRNA.

*Western Blot Analysis.* Western blot analysis was performed using standard procedures to determine ASCL1 knockdown levels. Briefly, 50 µg of DMS-53 lysates were loaded into 10% SDS-PAGE gels then transferred to nitrocellulose membranes. Membranes were incubated with ASCL1 and β2-microglobulin antibodies followed by goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology). Bands were visualized using a SuperSignal West Dura Extended Duration Substrate chemiluminescence kit (Pierce) and a VersaDoc Imaging System (Bio-Rad).

*$^{3}$[H]-Epibatidine Binding*. Radioligand binding assays were performed in collaboration with Dr. Scott Roger's laboratory (University of Utah School of Medicine). SCLC cells that were transiently transfected with ASCL1 siRNA s1656 were harvested. Membrane fractions were collected by centrifugation for

20 minutes at 20,000 x g at 4$^{o}$C. Pellets were resuspended and homogenized in 50 mM Tris on ice. Samples were divided into equal portions; to one set was added 500 µM nicotine (Sigma-Aldrich) to block all nAChRs. Readings from these samples would therefore represent non-specific epibatidine binding and serve as background control. After 30 min, $^{3}$[H]-epibatidine (Perkin-Elmer) was added to both the nicotine-blocked and unblocked samples at a final concentration of 5 nM. Membranes were incubated in a 25$^{o}$C water bath for 4 hours to ensure equilibrium was achieved. Bound ligand was separated from free ligand by vacuum filtration through GF/C filters (Whatman International) Filters were washed with 50 mM Tris (pH 7.4). Filters were dried completely, submerged in scintillation fluid and counted for retained $^{3}$[H] on a Beckman scintillation counter. Specific binding was calculated by averaging the total binding minus the background (nicotine-blocked) binding.

*Statistical analysis.* The mean relative expression values of each gene in the different samples were calculated and subjected to statistical analysis using the GraphPad Prism software. Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test was performed as appropriate.

## III.C.Results

*Overexpression of the CHRNA5/A3/B4 genes in small cell lung carcinoma*

Quantitative RT-PCR was performed to compare mRNA expression of all known human neuronal nAChR genes across normal and lung cancer cell lines. Cell lines derived from each of the major lung cancer types (i.e., SCLC, large cell lung carcinoma, adenocarcinoma and squamous cell carcinoma) were used in this analysis. Differential expression of the nAChR genes was observed across the different cell lines as depicted in the following heat map (Figure II-1).

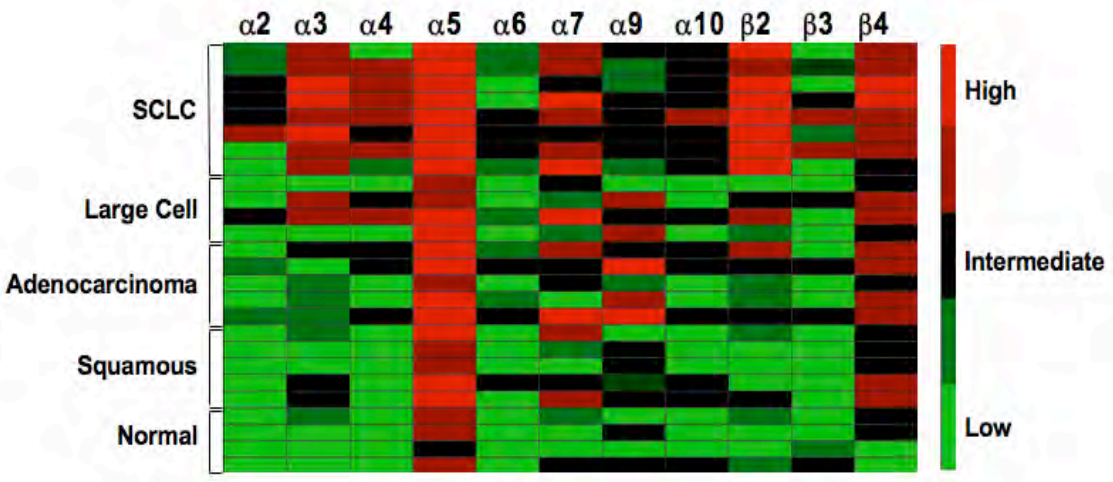


**Figure II-1**. Differential expression of nAChR genes across different lung cancer and normal cell lines. Quantitative RT-PCR was performed to determine mRNA levels of all human nAChR subunits. Red boxes indicate high gene expression while green boxes indicate low to no gene expression. Samples were analyzed in triplicate.

Gene expression in cancer cells relative to normal cells was then calculated using the $2^{-\Delta\Delta Ct}$ method [293]. Two of the clustered nAChR genes, those encoding the α3 and β4 subunits, were significantly over-expressed in

SCLC lines compared to normal lung cell lines (Figure II-2). Interestingly, expression of the α5 subunit gene was high in all the cell lines studied, including normal lung cell lines, with no significant differences in α5 expression observed in any of the cell lines. Conversely, the α3 and β4 subunits had low expression in large cell, adenocarcinoma and squamous cell carcinoma lines, similar to that in normal lung cell lines.

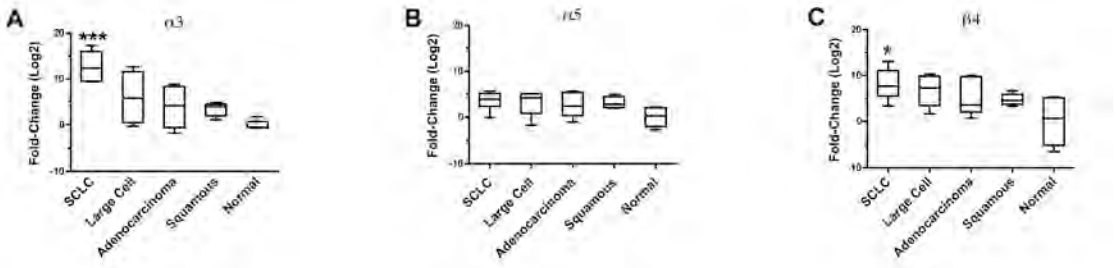


**Figure II-2**. Over-expression of the nAChR α3 and β4 genes across different cell lines. The $2^{-\Delta\Delta Ct}$ method was used to compared expression levels of the CHRNA5/A3/B4 genes in cancer cell lines compared to normal lung cell lines. The line within each box represents the median fold change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentiles whereas the upper and lower bars represent the maximum and minimum values, respectively. * $p < 0.05$, *** $p < 0.001$; n=3; ANOVA-Tukey.

With respect to the non-clustered nAChR subunit genes, the α4, α7, α10, and β2 genes were also significantly over-expressed in SCLC compared to normal lung cell lines (Figure II-3, see following page). In addition, the α7 gene was significantly over-expressed in large cell carcinoma cell lines.

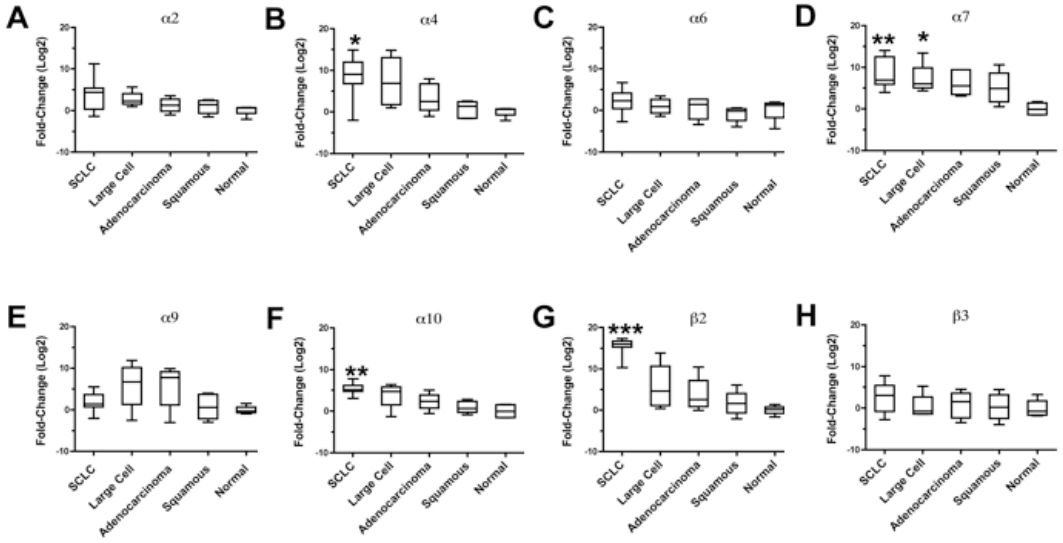


**Figure II-3**. Differential expression of the nAChR α2, α4, α6, α7, α9, α10, β2, and β3 genes across different lung cell lines. The $2^{-\Delta\Delta Ct}$ method was used to compared expression levels of nAChR genes in cancer cell lines compared to normal lung cell lines. The line within each box represents the median fold change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentiles whereas the upper and lower bars represent the maximum and minimum values, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n=3; ANOVA-Tukey.

Using a more physiologically relevant approach, we analyzed mRNA expression of the same set of genes in normal and lung cancer patient samples. The samples were from patients with SCLC, large cell lung carcinoma, adenocarcinoma and squamous cell carcinoma. Expression of all of the nAChR subunit genes was low in normal lung tissue. In comparison, all three of the clustered nAChR genes were significantly over-expressed in SCLC (Figure II-4). The α5 subunit gene was also significantly over-expressed in all NSCLC samples

(Figure II-4B) while the β4 subunit gene was significantly over-expressed in adenocarcinoma and squamous cell carcinomas (Figure II-4C).

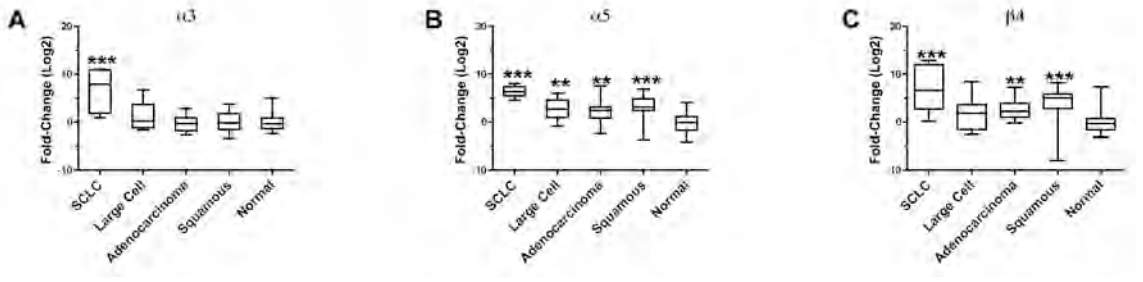


**Figure II-4**. Over-expression of the nAChR α3, α5, and β4 genes in lung cancer patient samples. The $2^{-\Delta\Delta Ct}$ method was used to compared expression levels of nAChR genes in cancer cell lines compared to normal lung cell lines. The line within each box represents the median fold change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentiles whereas the upper and lower bars represent the maximum and minimum values, respectively. ** = $p < 0.01$, *** = $p < 0.001$; n =3; ANOVA-Tukey.

In SCLC samples, the nAChR α9 and β2 subunit genes were significantly over-expressed compared to normal lung tissue (Figure II-5E and G). With respect to non-small cell lung cancer, the β2 subunit gene was significantly over-expressed in adenocarcinoma and squamous cell carcinoma (Figure II-5G). In contrast, nAChR α2 subunit gene expression was significantly lower in all lung cancer tissues compared to normal lung tissue (Figure II-5A).

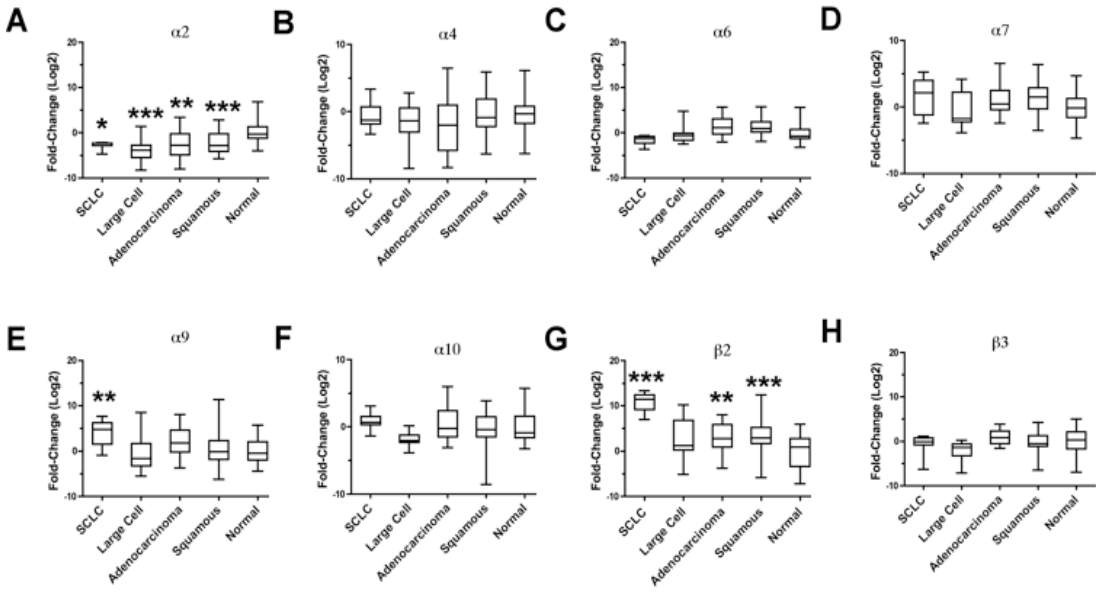


**Figure II-5**. Differential expression of the nAChR α2, α4, α6, α7, α9, α10, β2, and β3 genes across different lung cancer patient samples. . The $2^{-\Delta\Delta Ct}$ method was used to compared expression levels of nAChR genes in cancer cell lines compared to normal lung cell lines. The line within each box represents the median fold change relative to normal. The upper and lower edges of each box represent the 75th and 25th percentiles whereas the upper and lower bars represent the maximum and minimum values, respectively * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; n=3; ANOVA-Tukey.

*E-boxes are present in the promoters of the clustered nicotinic receptor genes*

The high expression of the α3, α5, and β4 genes in SCLC as well as their genomic clustering suggests that they may be coordinately regulated [168]. As an initial approach to identifying regulatory factors of this gene locus, we analyzed the promoter region of each gene for potential transcription factor binding sites. A number of putative binding sites for basic helix-loop-helix transcription factors were identified (Figure II-6).

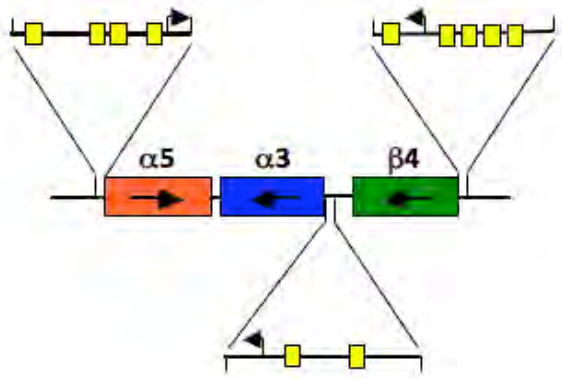


**Figure II-6**. The CHRNA5/A3/B4 gene cluster contains putative ASCL1 binding sites. E-boxes (yellow), containing the consensus sequence CANNTG, are present in the promoter regions of the α5, α3, and β4 genes (orange, blue, and green boxes, respectively). Straight arrows indicate the directions of transcription while bent arrows indicate major transcription initiation sites.

These sites are referred to as E-boxes and have the core sequence 5'-CANNTG-3'. The α3 gene promoter contains two E-boxes with the sequences CAGGTG and CACCTG. The α5 gene promoter contains four E-boxes with the sequences CAAATG, CAGCTG, CACCTG, and CACATG while the β4 gene promoter contains five E-boxes with the sequences CATTTG, CACATG, CAGCTG, and two CAGGTGs. With the exception of one E-box in the β4 promoter, all E-boxes are located upstream of reported major transcription initiation sites [196, 219, 294].

*ASCL1 regulates expression of the clustered nicotinic receptor genes*

Although there is a large family of basic helix-loop-helix transcription factors, we focused on ASCL1 because of its critical role in SCLC, as described above. To determine whether ASCL1 regulates expression of nicotinic receptor genes, knockdown experiments were done in SCLC cell lines using small interfering RNAs (siRNAs) against ASCL1. To control for off-target effects, three distinct siRNAs were used. The most potent siRNA, s1656, reduced ASCL1 mRNA expression by approximately 87% leading to an 89% decrease in $\alpha$3 gene expression, a 45% decrease in $\alpha$5 gene expression and a 78% decrease in $\beta$4 gene expression (Figure II-7A, left). The second siRNA, s1657, reduced ASCL1 mRNA expression by 64% leading to a 77% decrease in $\alpha$3 gene expression, an 18% decrease in $\alpha$5 gene expression and a 66% decrease in $\beta$4 gene expression (Figure II-7A, middle). The third siRNA, s1658, reduced ASCL1 mRNA expression by 65% leading to a 78% decrease in $\alpha$3 gene expression, a 17% decrease in $\alpha$5 gene expression and a 41% decrease in $\beta$4 gene expression (Figure II-7A, right). Decreases in $\alpha$5 expression were not found to be statistically significant. In addition, ASCL1 knockdown did not significantly affect the expression of the genes encoding the $\alpha$7 and $\beta$2 subunit genes, two other nAChR subunits implicated in lung cancer, indicating specificity of $\alpha$3 and $\beta$4 subunit gene regulation by ASCL1. ASCL1 knockdown also did not affect the expression of the housekeeping gene, GAPDH (data not shown). Furthermore, knockdown of ASCL1 in a non-small cell lung carcinoma cell line, A549, did not

reduce expression of the α3, α5, and β4 subunit genes (Figure II-7B). Expression of the β2 subunit gene, however, appears to increase in this cell line upon ASCL1 knockdown.

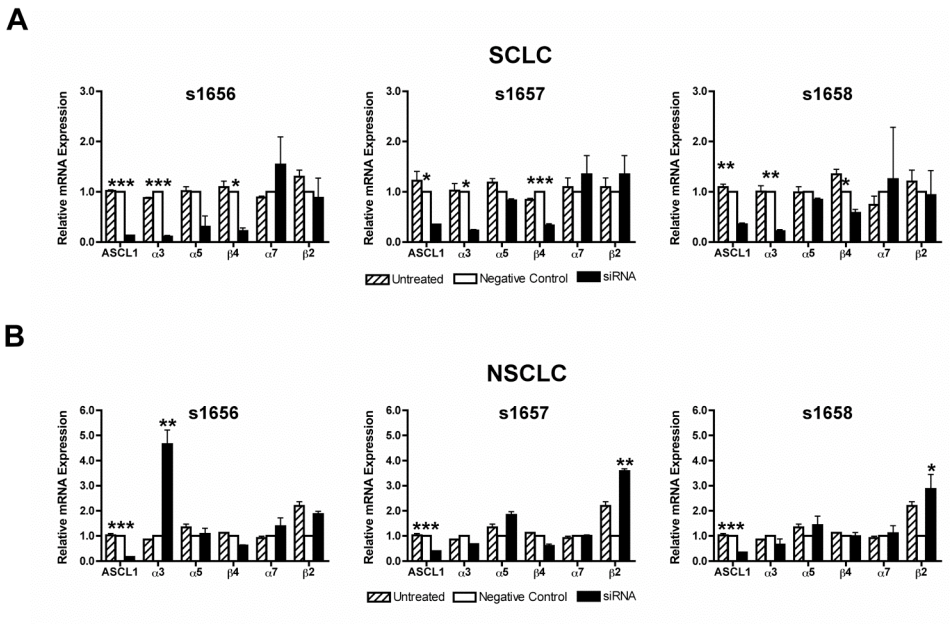


**Figure II-7**. Knockdown of ASCL1 leads to a decrease in CHRNA3/B4 gene expression in SCLC but not NSCLC. ASCL1 knockdown was achieved in SCLC (A) and NSCLC (B) cells using three different siRNAs: s1656, s1657, and s1658. Changes in gene expression were determined using quantitative RT-PCR. Samples treated with a negative control siRNA (white boxes) were used as calibrator (i.e., expression levels for these samples was set at 1). Error bars indicate SEMs. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, compared to negative control; n = 4; ANOVA-Tukey.

Western blot analysis confirmed that ASCL1 knockdown was achieved at the protein level using all three siRNAs (Figure II-8A). Furthermore, $^{3}$[H]-epibatidine-binding assays show that ASCL1 knockdown leads to a decrease in the total amount of nAChR binding sites on the cell membrane (Figure II-8B).

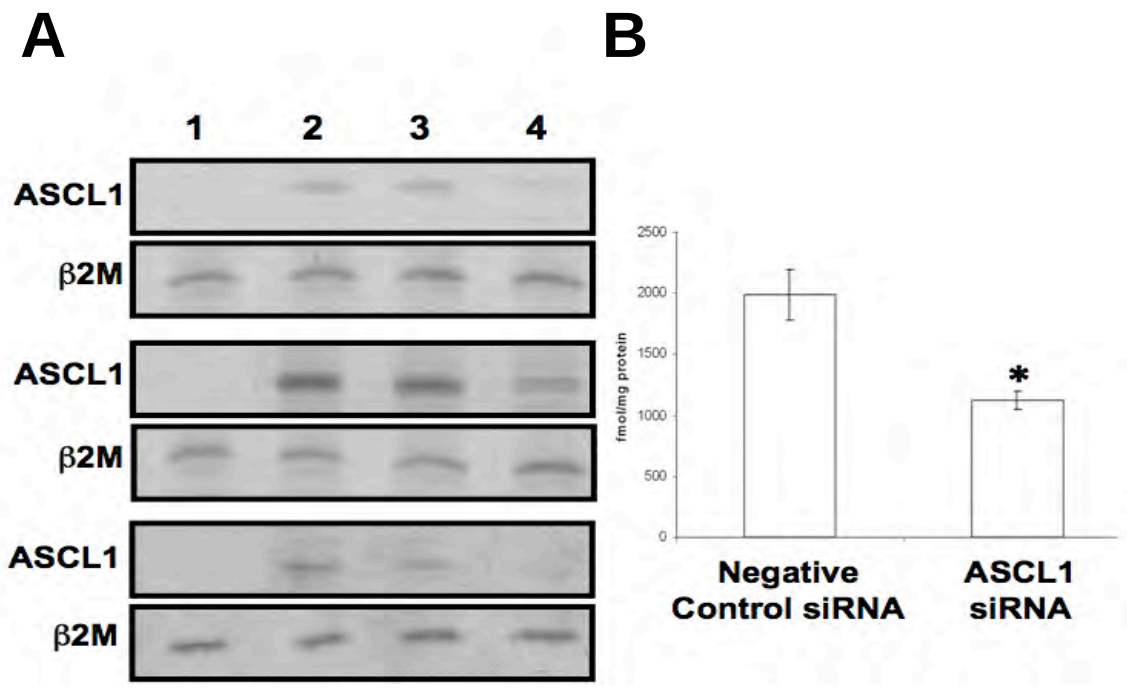


**Figure II-8**. Total nAChR binding sites decrease upon ASCL1 knockdown. *A*. Western blot analysis shows decrease in ASCL1 protein expression upon treatment with ASCL1 s1656, s1657, and s1658 siRNAs (top, middle, and bottom panels, respectively). Lane 1: No ASCL1 primary antibody; Lane 2: Untreated SCLC cells; Lane 3: Negative control siRNA-treated cells; and Lane 4: ASCL1 siRNA-treated cells. β2-microglobulin (β2M) was used as loading control. *B*. A radioligand-binding assay was performed using epibatidine, a nAChR ligand, to determine total nAChR levels at the cell surface upon ASCL1 siRNA treatment. Error bars indicate SEMs. * = $p < 0.05$, n=3, Student's t-test.

### III.C.Discussion

Our observation that the nAChR α3, α5 and β4 subunit genes are over-expressed in SCLC is particularly intriguing in light of the recent genome wide association studies implicating the CHNRA5/A3/B4 gene locus in lung cancer susceptibility [267-269, 281]. Over-expression of the clustered nAChR genes in lung cancer cells supports the notion that these genes play a role independent of

the nicotine addiction pathway. Extrapolating on data gained from work in the nervous system and our own observations, the possible nAChR subtypes that can form in SCLC include $\alpha3\beta2$, $\alpha3\beta4$, $\alpha3\beta4\alpha5$, and $\alpha3\beta2\beta4\alpha5$ [22]. These subtypes are believed to be involved in ganglionic neurotransmission in the peripheral nervous system [295]. A thorough investigation of functional nAChR subtypes in lung cancer has yet to be done but there is evidence that specific subtypes mediate distinct processes. For example, $\alpha3$-containing nAChR subtypes have been implicated in nicotine-mediated activation of the Akt pathway [136] whereas the $\alpha7$ subtype is thought to mediate nicotine-induced angiogenesis and NNK-induced apoptotic inhibition [136, 296]. $\alpha7$ nAChRs also have high calcium permeability and binding of NNK results in calcium influx, which triggers signaling pathways that result in cell proliferation, increased cell migration, apoptotic inhibition, and angiogenesis [280]. These two examples indicate the need to identify all of the precise nAChR subtypes in lung cancer cells as this may be important for design of targeted therapeutics given the unique pharmacological and functional properties of each nAChR subtype.

As nAChRs are the cognate receptors for nicotine and NNK, their activation is likely the first step in signal transduction cascades involving these ligands. Persistent activation of cancer-promoting pathways has been shown to result from nicotine and NNK exposure and may facilitate SCLC development [128, 297]. While these pathways remain to be completely elucidated, they

appear to involve the mitogen activated kinases ERK1 and ERK2, protein kinase C (PKC), the serine/threonine kinase RAF1 and the transcription factors FOS, JUN and MYC [280]. In addition, exposure to nicotine has also been shown to reduce the efficacy of anti-cancer agents by inhibiting apoptosis [114]. Pharmacological approaches suggest that these effects are mediated at least in part by homomeric $\alpha$7 nAChRs [280] but the role of other nAChR subtypes cannot be ruled out due to the lack of specificity of currently available pharmacological agents.

That nAChRs may function in SCLC is not totally unexpected given their important role in the nervous system. SCLC is believed to develop from pulmonary neuroendocrine cells. As the name suggests, these cells share properties with neurons such as the expression of ion channels and neuropeptides and have been referred to as paraneurons [298].

From a regulatory standpoint, the over-expression of the clustered nAChR genes also yields some interesting insights. Several laboratories have previously identified regulatory features shared by these genes [211, 212, 214, 215, 219, 221, 232, 242, 243]. Based on these studies, it is believed that expression of the clustered nAChR genes results from interactions between ubiquitously expressed and cell-type-specific transcription factors with cis-acting regulatory elements located within or near the cluster. To date, only one cell-type-specific factor,

Sox10, has been identified and shown to regulate nAChR gene expression [218, 226]. Sox10 activates the promoters of the clustered genes in neuronal cell lines but not in non-neuronal cells. However, we have observed that Sox10 is not expressed in any of the lung cancer cell lines we used in this study (data not shown). This suggests that other transcription factors must be involved in the expression of nAChR genes in lung cancer. As mentioned above, the transcription factor ASCL1 is an interesting candidate given its role in SCLC [289-292]. ASCL1 is also known to activate neuroendocrine differentiation markers while suppressing putative tumor suppressor genes [299]. In addition, ASCL1 is required for the proper development of peripheral sympathoadrenal tissues, the same tissues where the clustered nAChR genes are abundantly expressed [300].

The knockdown experiments presented here indicate that ASCL1 robustly regulates the expression of the $\alpha$3 and $\beta$4 genes while $\alpha$5 gene expression was, at most, modestly affected. These regulatory differences are likely due to the fact that each gene has its own promoter. Hence, although the three genes share common regulatory elements, each gene may have additional mechanisms that allow fine-tuning of its specific expression. Moreover, the $\alpha$5 gene is transcribed in the opposite direction as the $\alpha$3 and $\beta$4 genes raising the possibility that transcription factors that bind to the $\alpha$3 and $\beta$4 promoters may be differentially utilized by the $\alpha$5 promoter and vice versa. Nevertheless, the effect

of ASCL1 on nAChR subunit gene expression in SCLC appears to be specific for the clustered subunit genes, as expression of the α7 and β2 genes was not affected by ASCL1 knockdown. In contrast, ASCL1 knockdown does not reduce the expression of the clustered subunit genes in NSCLC whereas it increases the expression of the β2 gene, suggesting cell-type specificity of ASCL1 regulation.

Control of nAChR gene expression by ASCL1 may provide a mechanism for the role of nicotine in lung cancer. Nicotine has been shown to induce cellular processes that may lead to the development of cancer including activation of cell proliferation and survival pathways [280]. Acetylcholine, the endogenous ligand for nAChRs, is also thought to act as an autocrine growth factor in lung cancer cells [301]. Over-expression of their cognate receptors via transcriptional control by ASCL1 may thereby potentiate the effects of these ligands, providing a mechanism by which cigarette smoking can promote the growth and aggressiveness of SCLC.

# CHAPTER III:

# α5-CONTAINING NICOTINIC ACETYLCHOLINE RECEPTORS MEDIATE SCLC TUMOR GROWTH

*I have had my results for a long time: but I do not yet know how I am to arrive at them.*
*- Carl Friedrich Gauss*

## III.A.Introduction

Lung cancer is the most frequent cause of cancer deaths, causing over a million deaths annually [5]. Clinically and histopathologically, lung cancer is divided into two groups: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). NSCLC consists of a heterogenous group of tumors that account for 80% of lung cancer cases [302]. SCLC is a highly malignant form of lung cancer, thought to arise from primitive neuroendocrine cells [303]. Though SCLC is more rare, it is characterized by unfavorable prognostic factors such as early and widespread metastases, chemoresistance, and relapses [304].

Tobacco use is the major risk factor associated with lung cancer. Research through the years has forged connections between nicotine in tobacco and various cancer-related events such as cell proliferation, apoptotic inhibition, angiogenesis, and metastasis [115, 305]. Nicotine acts through its cognate receptors, nicotinic acetylcholine receptors (nAChRs), for which acetylcholine is the endogenous ligand. nAChRs are pentameric, ligand-gated ion channels assembled from homozygous or heterozygous combinations of $\alpha$ and $\beta$ subunits [306]. To date, eleven genes have been identified encoding human nAChR subunits ($\alpha$2-$\alpha$10 and $\beta$2-$\beta$4).

Linkage and association studies have implicated the genes encoding the α3, α5, and β4 nAChR subunits in lung cancer etiology [167, 267, 269]. These genes encode nAChR subunits that frequently co-assemble. The three genes lie in a cluster in chromosome 15q25 and harbor single nucleotide polymorphisms (SNPs) that increase risk for developing both nicotine dependence and lung cancer. Of particular interest is a non-synonymous SNP (rs16969968) in the CHRNA5 gene that decreases protein function in heterologous systems [258, 307]. It is unclear if the genetic association with lung cancer is simply a reflection of higher levels of nicotine dependence in carriers of risk alleles and consequently greater exposure to tobacco carcinogens [274]. A case for direct association, however, is supported by studies showing increased risk in never-smokers and in smoking populations after adjustment for smoking behaviors [272, 308]. Furthermore, the polymorphism is not associated with other smoking-related cancers such as head and neck cancers (e.g., cancers of the mouth, larynx, pharynx, and esophagus) [267].

We sought to address this quandary by directly investigating the role of the CHRNA5/A3/B4 genes in lung cancer. We have previously shown that these genes are expressed at aberrantly high levels in SCLC [309]. Here, we demonstrate that CHRNA5/A3/B4 depletion leads to a decrease in SCLC cell viability. In addition, we show that nicotine treatment increases cell viability while treatment with α-conotoxin AuIB, a selective antagonist of α3β4 nAChRs [310],

decreases cell viability. Using a xenograft tumor model, we also show that nicotine promotes tumor growth while CHRNA5 silencing inhibits tumor growth *in vivo.*

### III.B.Materials and Methods

*siRNA knockdown.* Cells were seeded in black, clear bottom 96-well assay plates and allowed to grow overnight. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) with 10 nM of a Silencer Select Negative Control #1 or CHRNA3 (s3043, s3044, s3045), CHRNA5 (s3049, s3050, s3051), and CHRNB4 (s3064, s3065, s3066) siRNAs (Applied Biosystems). After 48 hours, cells were harvested and subjected to a bioluminescence-based cell viability assay (described in detail in Chapter IV.B). To determine knockdown efficiency, cells were seeded in parallel onto 6-well cluster plates and transfected as above. After 48 hours, cells were harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed using RETROscript reagents (Applied Biosystems). Samples without reverse transcriptase were used as negative controls. CHRNA5/A3/B4 transcripts were amplified using Applied Biosystems TaqMan assays (Hs00181248_m1, Hs00609519_m1, and Hs00609523_m1, respectively) and the PRISM 7500 real-time PCR system (Applied Biosystems). Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method [293]. β2-microglobulin

(Hs00187842_m1) was used as the endogenous control to normalize gene expression levels.

*Drug Treatment*. DMS-53 cells were seeded onto black, clear bottom 96-well assay plates and allowed to grow overnight. Cells were then treated daily for one week with the 1 µM nicotine (Sigma-Aldrich) or 2 µM $\alpha$-conotoxin AuIB (a gift from J. Michael McIntosh, University of Utah School of Medicine). Cells were then harvested and subjected to a bioluminescence-based cell viability assay.

*CHRNA5 Gene Sequencing*. DMS-53 cells were grown in a 10-cm plate and harvested. Genomic DNA was isolated from the cells using a Qiagen DNeasy Kit. A region of CHRNA5 containing the rs16969968 SNP was then amplified using the following primers: Forward - 5'-CCATCATCTTCAAAAGTCATACCTC-3' and Reverse - 5'-AGTTCACCCACTGCCCTCAC-3'. PCR was carried out with Phusion High-Fidelity DNA polymerase (Finnzymes/Thermo-Scientific) using the following thermal cycling parameters: 98$^{o}$C for 3 min; 35 cycles of 98$^{o}$C for 10 sec, 67$^{o}$C for 30 sec, 72$^{o}$C for 25 sec; and 72$^{o}$C for 3 min. PCR products were then purified using a Qiaquick PCR purification kit (Quiagen) and sent out for direct sequencing (Genewiz). Sequences were analyzed using Sequence Scanner Software (Applied Biosystems).

*shRNA knockdown.* Two lentiviral pGIPZ CHRNA5 shRNAmir constructs (Open Biosystems; CHRNA5 A Clone ID: V3LHS_367770; CHRNA5 B Clone ID: V3LHS_367772) were used to transfect HEK293T cells for viral packaging using the Trans-Lentiviral Packaging System (Open Biosystems). A non-silencing shRNAmir (Open Biosystems) was used as a negative control (this control is processed by the endogenous RNA interference pathway but the processed siRNA does not target any RNA in the mammalian genome). Viral particles were harvested and used to transduce SCLC cells in the presence of 4 μg/ml polybrene (Sigma). To select for cells stably expressing the shRNAmirs, cells were treated with 6 μg/ml puromycin dihydrochloride for 5 days.

*Xenograft Tumor Model*. All animal experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council [311] as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Assurance Number A-3306-01). DMS-53 cells supplemented with Matrigel (BD Biosciences) were implanted subcutaneously onto the hind flanks of 6-week old athymic nude mice (Charles River Laboratories). After 60 days, tumors were harvested and weighed. Tumor size was determined by measuring the length and width of tumors at the longest axes using a digital caliper. To measure CHRNA5 knockdown levels, total RNA was

isolated from representative samples and quantitative RT-PCR was performed as described above.

*Chronic Nicotine Treatment via Minipumps*. Athymic nude mice carrying tumor xenografts were implanted subcutaneously with osmotic minimpumps (Alzet). Mice were anesthetized using ketamine/xylazine before minipump implantation. Minipumps delivered either saline or 24 mg/kg/day of nicotine (Sigma-Aldrich) at a rate of 0.25 µl per hour and last for about a month. After one month, tumors were harvested, measured for size, and weighed.

**III.C.Results**

To determine the role of CHRNA5/A3/B4 in SCLC, we silenced the expression of these three genes in the SCLC cell line, DMS-53. Three distinct siRNAs against each gene in the cluster were used. Treatment with CHRNA3 a, b, and c resulted in 97%, 97%, and 93% decrease in mRNA levels, respectively; treatment with CHRNA5 a, b, and c resulted in 71%, 93%, and 77% decrease in mRNA levels, respectively; and treatment with CHRNB4 a, b, and c resulted in 96%, 91%, and 95% decrease in mRNA levels, respectively (Figure III-2A). Using a bioluminescence-based viability assay, we found that all siRNAs that yielded >90% knockdown also significantly decreased SCLC cell viability (Figure III-2B). Interestingly, the two siRNAs that yielded the least knockdown levels, CHRNA5 a

and c, did not significantly affect cell viability, suggesting that certain CHRNA5 depletion levels may need to be reached to obtain an observable phenotype.

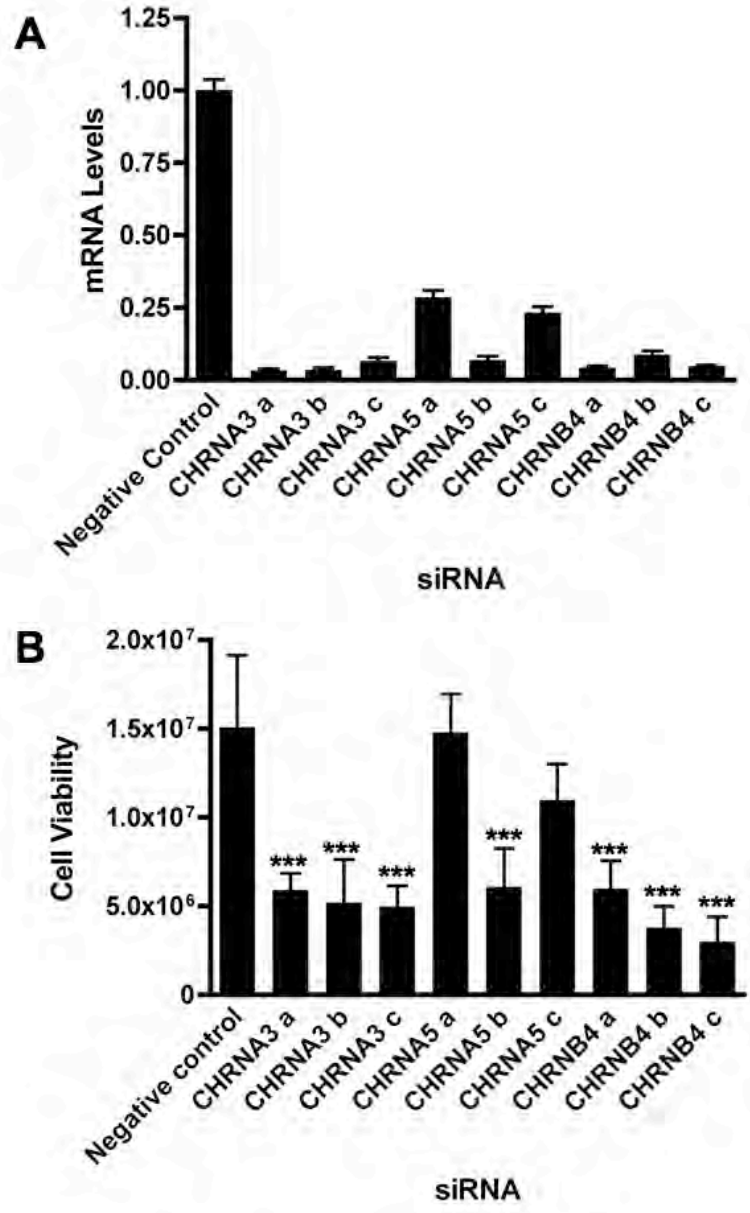


**Figure III-1.** CHRA5/A3/B4 depletion decreases viability of SCLC cells. SCLC cells were treated with negative control or CHRNA5/A3/B4 siRNAs. *A*.

qRT-PCR was performed to determine mRNA levels upon knockdown. *B*. A cell viability assay was performed to determine the effect of siRNA treatment on SCLC cell viability (*** = p value < 0.01; n = 3 for quantitative RT-PCR, n = 5 for viability assay; ANOVA-Tukey.

Using the same bioluminescence-based assay, we then tested the effect of nAChR ligands on SCLC cell viability. Nicotine treatment increased SCLC cell viability (Figure III-2). Notably, treatment with the $\alpha3\beta4$ nAChR-selective antagonist, $\alpha$-conotoxin AuIB, showed the opposite effect (i.e., blocking $\alpha3\beta4$-containing nAChRs decreased SCLC cell viability).

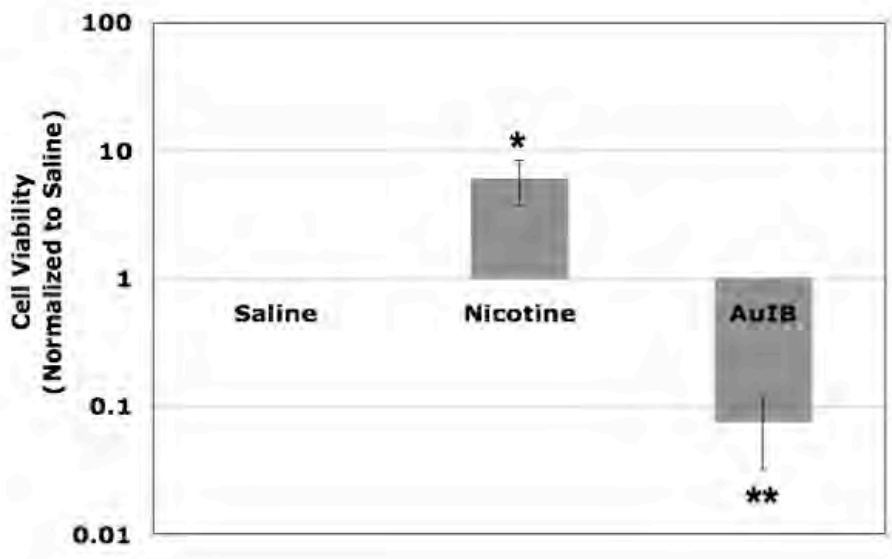


**Figure III-2.** Exposure to nAChR ligands regulates SCLC cell viability. DMS-53 cells were treated daily for 1 week with 1 μM nicotine (nAChR agonist) or 2 μM a-conotoxin AuIB ($\alpha3\beta4$ nAChR antagonist). Cell viability was then measured. Values were normalized to those of saline-treated samples. Asterisks indicate statistical significance compared to saline controls (* = p value < 0.05, ** = p value < 0.01; n = 5; one way ANOVA followed by Tukey's multiple comparison post-test).

For *in vivo* propagation of tumors, we utilized the tumor xenograft model. We focused on CHRNA5 as it harbors the non-synonymous SNP, rs16969968. To determine the rs16969968 allele status of the SCLC cell line, DMS-53, used in the xenograft assays, we directly sequenced the CHRNA5 region containing this SNP. The following chromatograms show that this cell line is heterozygous at this locus.

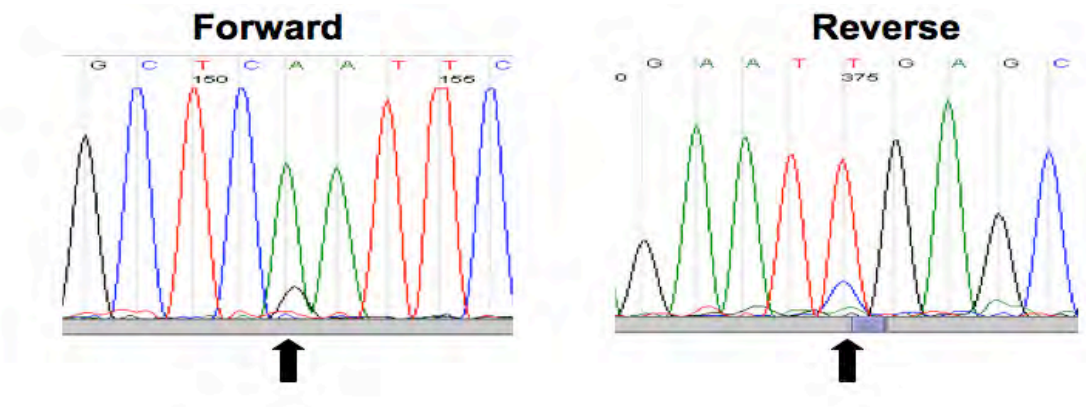


**Figure III-1**. Forward and reverse sequence chromatograms of the rs16060068 locus. Genomic DNA was isolated from the SCLC cell line, DMS-53. The CHRNA5 exon 5 region containing the rs16969968 SNP was PCR amplified and sequenced. Arrows indicate the double A/G (T/C) peaks, indicating heterozygosity at this locus.

This cell line was used to stably express two distinct CHRNA5 shRNAmirs via lentiviral delivery. Cells were then implanted subcutaneously into immunodeficient mice and allowed to grow. The cells were supplemented with

Matrigel to provide the complex extracellular environment found in many tumors. After tumors were harvested, CHRNA5 knockdown levels were determined.

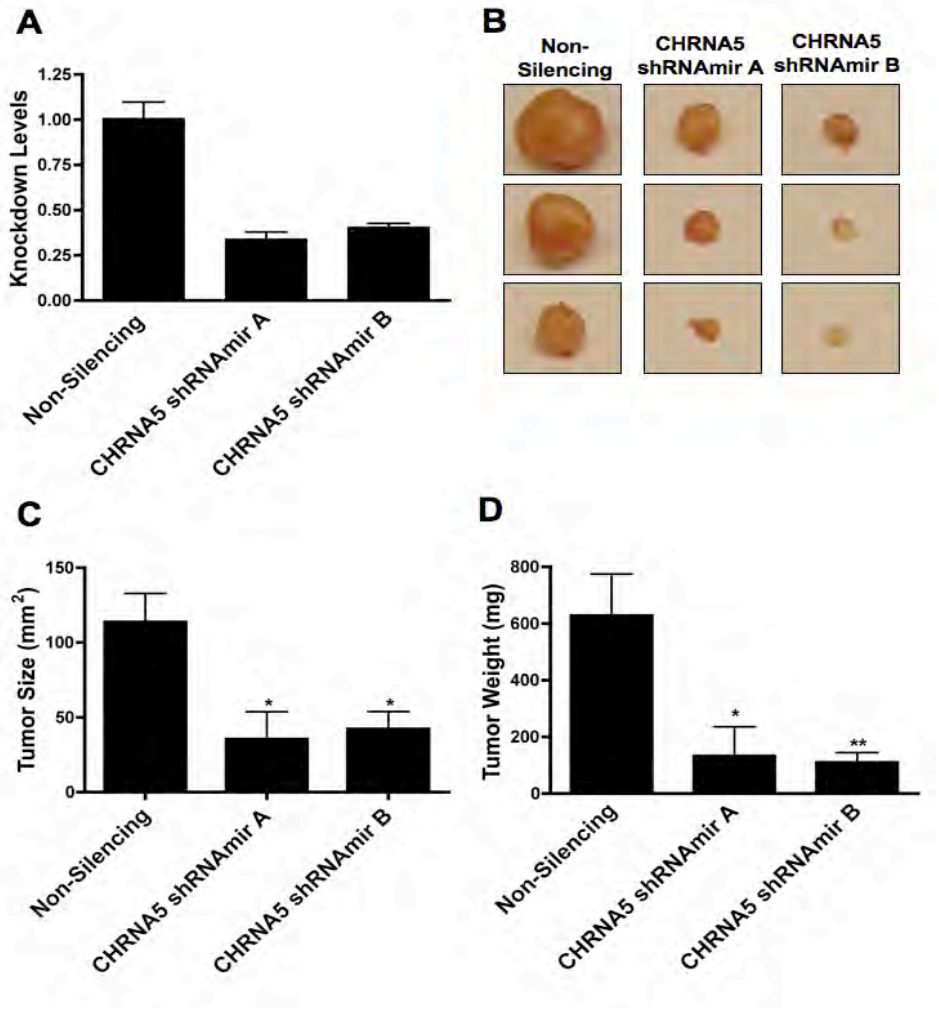


**Figure III-4**. CHRNA5 depletion inhibits growth of tumor xenografts. DMS-53 cells stably expressing CHRNA5 shRNAmirs were subcutaneously injected into the hind flanks of immunocompromised mice *A*. CHRNA5 shRNAmir A and B knockdown levels in harvested tumors. *B*. Representative tumor images. *C-D*. Tumor sizes and weights, respectively. * = $p < 0.05$, ** = $p < 0.01$; n = 9 for non-silencing, n = 5 for CHRNA5 shRNAmir A, n = 9 for CHRNA5 shRNAmir B; ANOVA –Tukey.

Treatment with CHRNA5 shRNAmir A resulted in a 59% decrease in mRNA levels while treatment with CHRNA5 shRNAmir B resulted in a 66% decrease in mRNA levels (Figure III-4A). CHRNA5 knockdown led to a decrease in tumor size and weight (Figure III-4B-D), suggesting that CHRNA5 is important for SCLC tumor growth. In addition, while all cells treated with the non-silencing shRNAmir formed tumors, two out of the nine samples treated with CHRNA5 shRNAmir B did not form tumors, suggesting that CHRNA5 may also impact rates of tumor incidence.

Finally, we tested the effect of chronic nicotine treatment on the growth of SCLC tumors *in vivo*. We used osmotic minipumps to deliver nicotine as these devices allow continuous dosing of drugs, while eliminating repeated injections and minimizing animal handling. This treatment paradigm has not been previously used to test the effect of nicotine on tumor growth. As shown in Figure III-5, chronic nicotine exposure promotes the growth of tumors compared to saline-treated mice.

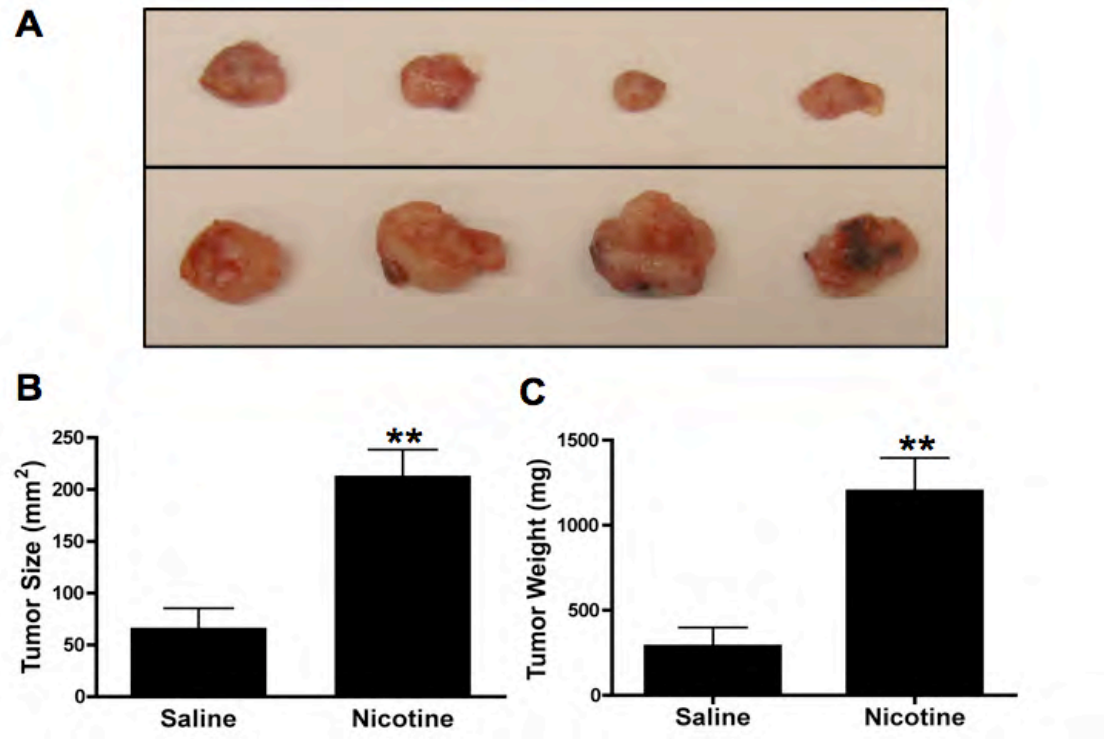


**Figure III-5**. Chronic nicotine exposure increases SCLC tumor growth. *A.* SCLC cells were injected subcutaneously onto the hind flanks of athymic nude mice. Mice were then implanted with osmotic minipumps to deliver either saline (top panel) or 24 mg/kg/day of nicotine (bottom panel). After 4 weeks, tumors were harvested and tumor size (*B*) and weights (*C*) were measured. ** = $p < 0.01$; n = 4; Student's t-test.

## III.D. Discussion

Our findings that CHRNA5/A3/B4 depletion inhibits SCLC cell viability provide mechanistic support to the correlative link between these genes and lung cancer susceptibility. It suggests that the clustered genes perform a vital function in the maintenance of SCLC cell viability. That the CHRNA5/A3/B4 genes play a role in

lung cancer, outside of their role in nicotine addiction, attests to the pleiotropic function of these genes.

Consistent with our genetic approach, we found that pharmacological activation and blockade of α5α3β4 nAChRs modulates SCLC cell viability. Treatment with nicotine promotes SCLC cell viability, in line with previous findings on the proliferative effect of nicotine on SCLC cells [116, 117, 134]. This result suggests the presence of functional nAChRs in DMS-53 cells and confirms its suitability for the experiments described in this study. This result also verifies the reliability of our bioluminescence viability assay as a tool for studying the effects of genetic and pharmacological agents against SCLC.

To perform the converse experiment, we utilized the α3β4-selective ligand, α-conotoxin AuIB. α-conotoxins are derived from the venom of carnivorous cone snails, which have proved to be a valuable source for disulfide-bonded peptides that target nAChRs in a highly subtype-selective manner. α-conotoxin AuIB, in particular, was isolated from the snail-eating cone *Conus aurilicus* and blocks α3β4 nAChRs with > 100-fold higher potency than other nAChR subtypes [310]. Treatment with α-conotoxin AuIB leads to decreased viability of DMS-53 cells, indicating that functional α3β4 nAChRs are present in SCLC cells and are important for maintenance of SCLC cell viability. The unavailability of selective α5α3β4 nAChR ligands limits our ability to determine

the role of this subtype in SCLC cell viability. Nevertheless, the co-expression of CHRNA5/A3/B4 in DMS-53 cells and the proclivity of $\alpha3\beta4$ nAChRs to form mature receptors with $\alpha5$, suggests that the observed reduction in cell viability is due in part to blockade of $\alpha5\alpha3\beta4$ nAChRs.

We next turned to the xenograft tumor model, one of the most commonly used mouse models for studying cancer, for a more physiologically relevant approach. The key advantages of this model include 1) the use of actual human cells, containing the genetic and epigenetic peculiarities of human samples; 2) availability of results within a couple of months; and 3) its relative ease and simplicity [312]. We focused on CHRNA5 given the presence in its coding region of the only non-synonymous SNP associated with both nicotine dependence and lung cancer. CHRNA5 knockdown in this cell line decreased tumor growth and incidence, indicating the importance of CHRNA5 in these processes.

Sequence analysis revealed the heterozygous nature of rs16969968 in DMS-53 cells. Since CHRNA5 shRNAmirs decrease levels of total CHRNA5 mRNA, our results illustrate the effect of decreasing both allelic versions. Hence, we cannot infer from our data whether the reduction in tumor growth is the effect of a reduction in a specific allele or the effect of a global loss of CHRNA5.

It is also worth noting that the knockdown levels achieved using the shRNAmirs were not as robust as those seen using the siRNAs. However, shRNAmirs are stably expressed due to their integration into the genome. This relatively low but stable knockdown appears to be sufficient to yield a measurable phenotype.

In conclusion, our results indicate a role for the CHNRA5/A3/B4 genes in SCLC and supports the hypothesis that allelic variations in these genes are directly associated with lung cancer susceptibility. The added association with nicotine dependence suggests a mechanism for exacerbation of risk (a double whammy effect), possibly providing an explanation for the tight linkage between tobacco use and SCLC [10]. Moreover, association with other diseases such as alcoholism, COPD, and peripheral arterial disease [268, 275, 276] suggests pleiotropy at this locus and warrants further investigation into the role of the CHRNA5/A3/B4 genes in other pathological states. Finally, inhibition of cell viability and tumor growth by genetic and pharmacological disruption of CHNRA5/A3/B4 suggests the utility of these genes as therapeutic targets for SCLC.

# CHAPTER IV:

# HIGH-THROUGHPUT SCREEN IDENTIFIES PHARMACOLOGICAL AGENTS THAT TARGET NEUROTRANSMITTER SIGNALING IN SCLC

*We have two options, medically and emotionally: give up or fight like hell.*
*- Lance Armstrong*

## IV.A.Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, resulting in over 1.3 million deaths per year [5]. In the United States, lung cancer incidence rates are second only to rates for breast cancer in females and prostate cancer in males [6]. Tobacco use is the major risk factor associated with lung cancer. Histopathological classification divides lung cancer into two main types: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC can be further subdivided into adenocarcinoma, squamous cell, and large cell lung carcinoma.

SCLC is the most aggressive type of lung cancer, as demonstrated by its rapid doubling time and early development of widespread metastases [313]. In fact, SCLC is so aggressive that by the time it is diagnosed, metastasis has usually already occurred such that surgical resection of tumors is rarely an option. Hence, chemotherapy and radiation are the treatments of choice for these patients. Most patients exhibit robust initial response to treatment but eventually become chemoresistant [304]. Relapses occur almost without exception and five-year survival rates range from 31% (for patients diagnosed at Stage I) to 2% (for patients diagnosed at Stage IV) [6]. Advances made in the past three decades have resulted in only a slight improvement in treatment outcome for

SCLC [314]. Identification of novel SCLC therapies is therefore of prime importance.

Cell viability assays are indispensable tools in drug discovery efforts. Measurement of cell viability is a simple and rapid approach for determining a cell population's response to endogenous factors such as hormones and growth factors as well as external stimuli such as drugs and environmental stress [315]. A classic approach for measuring cell viability involves the use of vital dyes (e.g., trypan blue) for probing membrane integrity. This method, however, is tedious and prone to experimenter bias [315]. Another traditional method relies on the reduction of tetrazolium salts such as MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), resulting in the formation of colored products that can be quantified via spectrophotometry [316]. However, such assays have limited sensitivity, narrow dynamic ranges, and are subject to variability [315].

Bioluminescence-based assays are a favored approach due to their broad linearity and robustness to library compounds and complex biological samples [317]. These assays exploit the ability of luciferase to catalyze oxidation of a luciferin substrate, a reaction that generates light as a by-product [317]. Light generated by this reaction has the highest quantum efficiency of any known chemiluminescent reaction [318]. Combined with low bioluminescence signals in mammalian cells, this approach allows for highly sensitive assays.

Here, we developed a cell viability assay employing bioluminescence to screen for pharmacological compounds against SCLC. From a library of 1,280 pharmacologically active compounds, we identified several classes of drugs that target classic cancer signaling pathways as well as neuroendocrine markers in SCLC.

**IV.B.Materials and Methods**

*Animals.* All animal experiments were conducted in accordance with the guidelines for care and use of laboratory animals provided by the National Research Council [311] as well as with an approved animal protocol from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School (Assurance Number A-3306-01). Specifically, mice were exposed to 2% isofluorane before being imaged. During imaging, mice lay on a temperature-regulated stage and were continually exposed to isofluorane.

*Cell culture.* DMS-53 and DMS-114 SCLC cell lines were acquired from the American Type Culture Collection (ATCC) and grown in RPMI 1640 containing L-glutamine and 25 mM HEPES (Cellgro), supplemented with 10% fetal bovine serum (PAA). HEK293T cells were acquired from Open Biosystems and grown in Dulbecco's Modified Eagle's Medium containing L-glutamine and 4.5 g/L glucose (Cellgro), supplemented with 10% fetal bovine serum. Cells were

maintained at 37°C and 8% $CO_2$. Cell line authentication is performed by the American Type Culture Collection using cytochrome oxidase subunit I (COI) analysis for interspecies identification and STR analysis (DNA profiling) for intraspecies identification.

*Cloning and viral production.* A luciferase cassette was subcloned from pGL3-Basic (Promega) into the multiple cloning site of the lentiviral expression vector pLEX-MCS (Open Biosystems) using *SpeI* and *MluI* (New England Biolabs) restriction sites. The construct, pLEX-lucSM, was transfected into HEK293T cells for viral packaging using the Trans-Lentiviral Packaging System (Open Biosystems). Viral particles were harvested and used to transduce DMS-53 or DMS-114 cells in the presence of 4 µg/ml polybrene (Sigma). To select for cells stably expressing luciferase (designated DMS-53 luc+ and DMS-114 luc+), cells were treated with 6 µg/ml puromycin dihydrochloride for 5 days.

*Luciferase assays.* Cells were lysed using 50 µl Reporter Lysis Buffer (Promega) and placed on a shaker at room temperature for 5 minutes. To snap-freeze, cells were placed at -80°C for 15 minutes. Cells were then allowed to thaw and equilibrate to room temperature for 15 minutes. Plates were returned to the shaker for another 5 minutes before placing into a luminometer (Bio-Rad Lumimark). The luminometer was set to dispense 50 µl of the luciferase substrate (Promega Luciferase Assay Reagent). Integration time was set for 10

seconds with a 2-second lag time. Non-luciferase expressing cells were used as negative controls, where indicated.

*MTT assay.* Cells were seeded from 0 - $1x10^6$ cells/well in black, clear bottom 96-well assay plates and allowed to grow overnight. Cells were then treated with 10 µl MTT Reagent (ATCC) and incubated for 4 hours. After ensuring that purple precipitates where visible, 100 µl of Detergent Reagent (ATCC) was added. Samples were allowed to incubate at room temperature for another 2 hours. Absorbance readings at 570 nm were taken using a SpectraMax M2 microplate reader (Molecular Devices).

*Bioluminescence imaging.* For imaging of luciferase-expressing cells *in vitro*, cells were seeded onto black, clear bottom 96-well assay plates (Costar). Before imaging, cell culture media were removed. The firefly luciferase substrate D-luciferin (Gold Biotechnology) was added at a final concentration of 150 µg/ml per well. After 15 minutes of incubation, cells were imaged using a Xenogen IVIS 100 imager (Caliper Life Sciences), which makes use of a supercooled charge-coupled device (CCD) camera to detect light-emitting cells. For *in vivo* work, male athymic nude mice were obtained from Charles River Laboratories. For xenograft assays, cells were implanted subcutaneously onto the hind flanks of 6-week old mice. For the lung colonization model, cells were injected into tail veins of 6-week old mice. Mice were injected intraperitoneally with 150 mg/kg D-

luciferin 15 minutes prior to imaging. Quantification was performed using the acquisition and analysis software Living Image (Caliper Life Sciences).

*Pharmacological treatments.* All drugs were purchased from Sigma. For pre-validation of the bioluminescence assay, $1x10^4$ DMS-53 luc+ and DMS-114 luc+ cells were seeded in black, clear bottom 96-well assay plates and allowed to grow overnight. Cells were treated with 0, 2, and 4 µM K252c (staurosporine aglycone) for 0, 12, and 24 hours or 0, 25, and 50 µM cis-diammineplatinum (II) chloride (cisplatin) for 0, 12, and 24 hours. Cells were then harvested and subjected to luciferase assays.

*RNA interference.* Cells were seeded in black, clear bottom 96-well assay plates and allowed to grow overnight. Cells were transfected with 5-10 nM of a Silencer Select Negative Control #1 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA (Applied Biosystems) using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen). Samples treated only with Lipofectamine 2000 were also used as controls. After 48 hours, cells were harvested and subjected to luciferase assays. To determine knockdown efficiency, cells were seeded in parallel onto 6-well cluster plates and transfected as above. After 48 hours, cells were harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed using RETROscript reagents (Applied Biosystems). Samples without reverse transcriptase were used as negative

controls. GAPDH amplicons were generated using GAPDH TaqMan assays (Applied Biosystems) and the PRISM 7500 real-time PCR system (Applied Biosystems). GAPDH levels were quantified using the $2^{-\Delta\Delta Ct}$ method [293]. β2-microglobulin was used as the endogenous control to normalize gene expression levels.

*Large-scale compound screen.* For primary screening, 5 x $10^4$ DMS-53+ cells were seeded in black, clear bottom 96-well assay plates and allowed to grow overnight. The following day, compounds from the Library of Pharmacologically Active Compounds, LOPAC$^{1280}$, (Sigma) were added to each well at a final concentration of 50 μM in 1% dimethyl sulfoxide (DMSO). For each plate, one column of cells (n=8 wells) was treated for 24 hours with equal concentrations of cisplatin as positive control and another column was treated with 1% DMSO as negative control. Tolerance of cells for 1% DMSO was confirmed prior to screening (Appendix I). Media aspiration and addition of compounds, lysis buffer, and luciferase substrate were performed with a Te-Mo (Tecan) automated system at the University of Massachusetts Medical School Small Molecule Screening Facility. Luciferase readouts were taken using a Victor plate reader (Perkin Elmer). For secondary screening, selected hits from the primary screen were retested using DMS-53 luc+ cells and further confirmed using DMS-114 luc+ cells. For tertiary verification, DMS-53 luc+ cells were treated with increasing doses (0, 25, 50, and 100 μM) of the representative drugs cortexolone

maleate/ST-148 (Sigma) and fluoxetine hydrochloride (Sigma) for 24 hours, followed by luciferase assays.

*Analysis.* Assay quality was measured using three statistical parameters [319]. Signal-to-background ratios (S/B) were calculated using the equation: $S/B = \mu_{max} / \mu_{min}$. Signal-to-noise ratios (S/N) were calculated using the equation: $S/N = (\mu_{max} - \mu_{min})/\sigma_{min}$ of treated controls. For S/B and S/N, values > 2 are considered acceptable. Z'-factor values were calculated using the equation Z' factor = $1 - (3\sigma_{max} + 3\sigma_{min})/|\mu_{max} - \mu_{min}|$. For all equations, $\mu$ represents means and $\sigma$ represent standard deviations (SD). For Z'-factor interpretation, we used the scale developed by Zhang and colleagues [320], wherein a score of 1.0 is considered ideal; scores between 0.5 and 1.0 represent excellent assays; scores between 0 and 0.5 represent marginal assays; and scores less than 0 represent assays that are essentially impossible to use for screening purposes.

## IV.C.Results

*Dose-dependent luciferase expression*

A lentiviral delivery approach was used to stably integrate a luciferase gene into the genome of two SCLC cell lines. Serial dilutions of these cells were then prepared to determine assay sensitivity. DMS-53 luc+ cells could be detected above background from as few as 10 cells using luminometry (Figure IV-1A).

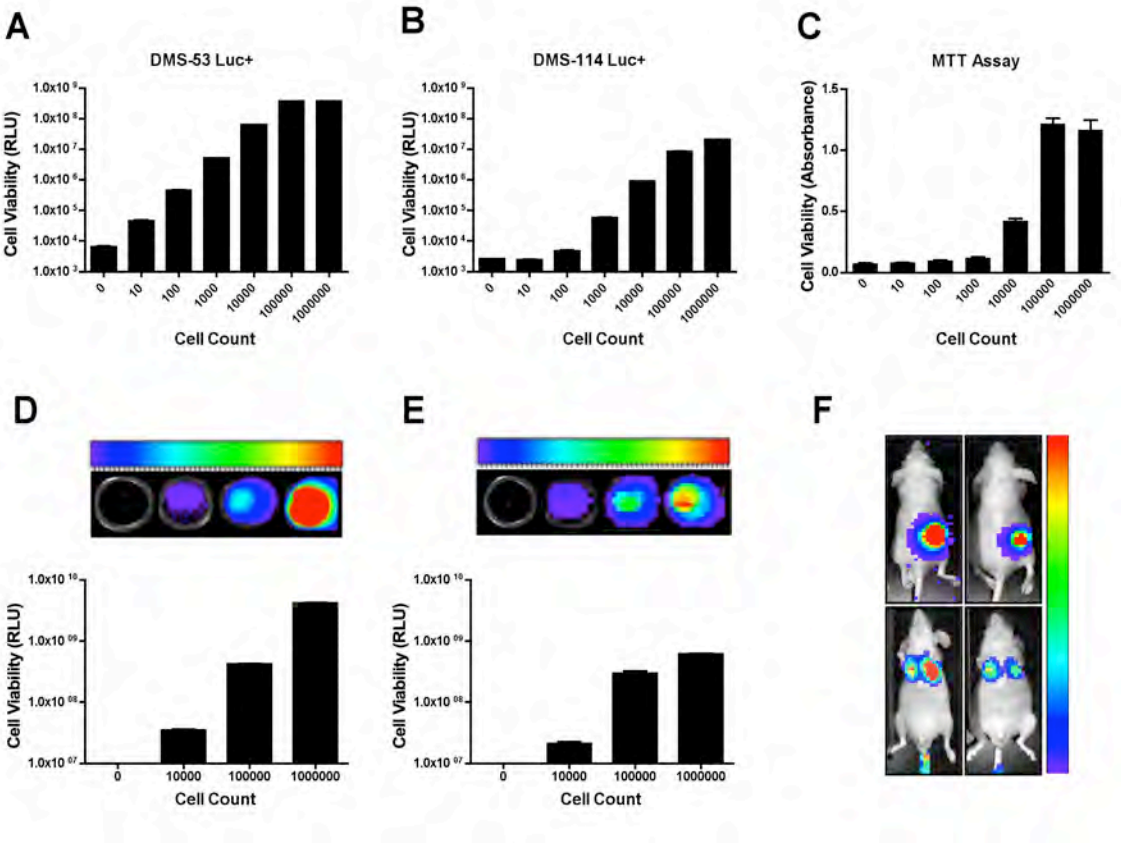


**Figure IV-1.** Establishment of luciferase-expressing SCLC cell lines for *in vitro* and *in vivo* assays. *A-B*. Serial dilutions of DMS-53 luc+ and DMS-114 luc+ cells were prepared. Wells containing medium alone or $1x10^6$ DMS-53 and DMS-114 non-luciferase expressing cells were used as negative controls (0 cells). Cell viability was measured using a luminometer. *C*. A traditional MTT assay was performed for comparison of sensitivity and dynamic range. Cell viability was measured using a spectrophotometer. *D-F*. Cell viability was measured using a Xenogen IVIS 100 imager. Colors represent clusters of CCD pixels while color scale represents luminescence intensity from lowest (violet) to highest (red). Instrument gain was set at min=$5x10^7$ photons/sec to max=$5x10^8$ photons/sec for DMS-53 luc+ *in vitro* (*D*) and at min=$25x10^6$ photons/sec to max=$25x10^7$ photons/sec for DMS-114 luc+ *in vitro* (*E*). For *in vivo* imaging (*F*), mice were injected subcutaneously with DMS-53 luc+ (upper left) or DMS-114 luc+ (upper right) cells. For the lung colonization model, DMS-53 luc+ (lower left) or DMS-114 luc+ (lower right) cells were injected into the tail vein of mice. Instrument gain was set at min=$1x10^5$ photons/sec to max=$1x10^7$ photons/sec for the xenograft model and at min=$1x10^3$ photons/sec to max=$1x10^4$ photons/sec for the lung colonization model. Columns represent mean values and error bars represent standard error of mean; n=5 for luminometry, n=8 for spectrophotometry; n=4 for bioluminescence imaging. RLU - relative light units.

Background readings were taken from wells containing medium alone or wells containing $1x10^6$ DMS-53 cells that do not express luciferase. The linear range of detection for DMS-53 luc + cells was between $1x10^1$ to $1x10^5$ cells. DMS-114 luc+ cells could be detected above background from as few as 100 cells (Figure IV-1B). The linear range of detection for these cells was between $1x10^2$ to $1x10^5$ cells. For comparison, serial dilutions of DMS-53 luc+ cells were subjected to a traditional MTT assay (Figure IV-1C). This approach required as many as $1x10^4$ cells to achieve absorbance values distinguishable from background. In addition, the linear range of detection for the MTT assay was only between 1 $x10^4$ and $1x10^5$ cells/well. Importantly, the MTT assay required at least 6 hours to run versus 45 minutes for the bioluminescence assay.

An additional advantage of using bioluminescent cell lines is their direct applicability to *in vivo* bioluminescence imaging. To confirm the utility of the luciferase-expressing cells for bioluminescence imaging, the Xenogen IVIS 100 imaging system was used, wherein the number of emitted photons is proportional to the number of bioluminescent cells. *In vitro*, the linear range of detection for DMS-53 luc+ was between $1x10^4$ and $1x10^6$ cells, yielding bioluminescence signals between $3x10^7$ to $4x10^9$ photons/sec (Figure IV-1D). In comparison, the linear range of detection for DMS-114 luc+ was between $1x10^4$ and $1x10^5$ cells, yielding bioluminescence signals between $2x10^7$ and $3x10^8$ photons/sec (Figure IV-1E). No luminescence signals could be detected in wells containing $1x10^6$

DMS-53 or DMS-114 cells that did not express luciferase. *In vivo*, DMS-53 luc+ and DMS-114 luc+ cells were used in a xenograft tumor model and a lung colonization model (Figure IV-1F). For the xenograft model, $1x10^6$ DMS-53 luc+ and DMS-114 luc+ cells were detectable 15 minutes after injection of a luciferase substrate (upper left and right panels, respectively). Similarly, in the lung colonization model, $1x10^6$ DMS-53 luc+ and DMS-114 luc+ cells were detectable in the lung area after injection of a luciferase substrate (lower left and right panels respectively). Mice that were implanted with cells that do not express luciferase did not yield luminescence signals (data not shown).

*Response of bioluminescent cells to pharmacological and genetic agents*

To test the hypothesis that luciferase expression reflects cell viability, we measured the responsiveness of the engineered SCLC cell lines to treatment with a known apoptosis-inducing agent, staurosporine. DMS-53 luc+ and DMS-114 luc+ cells were treated with staurosporine at varying doses (0, 2 and 4 μM) and time points (12 and 24 hours). As shown in Figure IV-2A and IV-2B, luciferase activity of DMS-53 luc+ and DMS-114 luc+ cells decreased with increasing staurosporine concentration. Correspondingly, luciferase activity for both cell lines decreased with increased exposure time (Figure IV-2C and IV-2D). A similar strategy was employed to determine whether the engineered cells would also be responsive to a known chemotherapeutic agent, cisplatin. DMS-53 luc+ and DMS-114 luc+ cells were treated with cisplatin at varying doses (0,

25 and 50 μM) and time points (12 and 24 hours). An inverse relationship was observed between luciferase activity and cisplatin concentration (Figure IV-2E and IV-2F). Similarly, luciferase activity decreased for both cell lines with increased exposure time (Figure IV-2G and IV-2H).

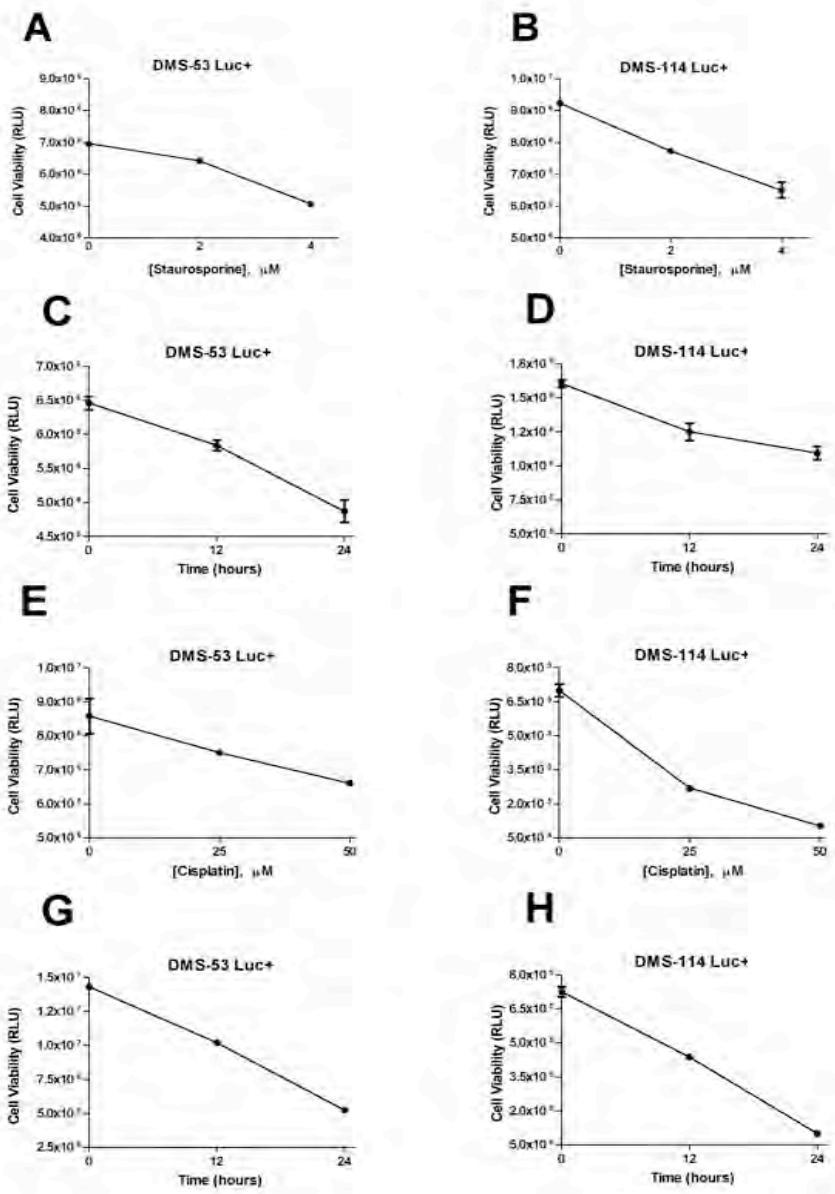


**Figure IV-2.** Bioluminescent SCLC cell lines respond to pharmacological agents in a dose- and time-dependent manner. DMS-53 luc+ and DMS-114 luc+ cells were treated with 0, 2, and 4 µM staurosporine, an apoptotic drug, for 24 hours (**A,B**) or with 4 µM staurosporine for 0, 12, and 24 hours (**C,D**). DMS-53 luc+ and DMS-114 luc+ cells were treated with 0, 25, and 50 µM cisplatin, a chemotherapeutic drug, for 24 hours (**E,F**) and 50 µM cisplatin for 0, 12, and 24 hours (**G,H**). Luciferase assays were then performed to measure cell viability. Data points represent mean values and error bars represent standard error of means (n=5). **RLU** – relative light units.

Finally, to test whether the viability of the engineered SCLC cells can be modulated by genetic manipulation, DMS-53 cells were treated with a siRNA against GAPDH, a known housekeeping gene. siRNA treatment caused a 91% decrease in GAPDH expression (Figure IV-3A). GAPDH knockdown resulted in decreased luciferase activity (Figure IV-3B). Taken together, these results indicate that the bioluminescence viability assay is a feasible assay for screening anti-SCLC therapies.

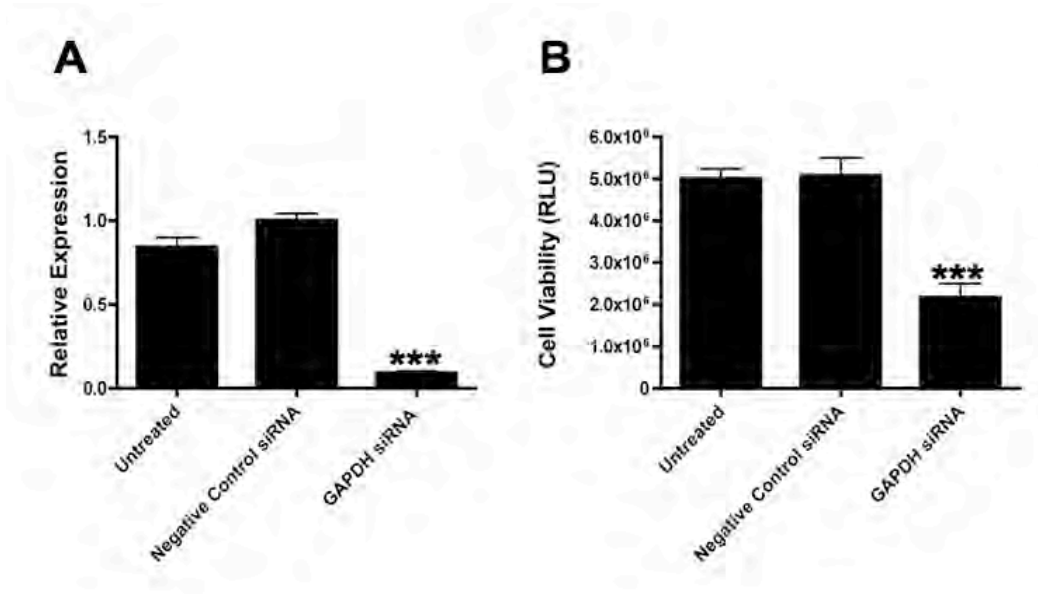


**Figure IV-3**. Bioluminescent cells respond to GAPDH depletion. *A.* DMS-53 luc+ cells were treated with a siRNA against the housekeeping gene, GAPDH. GAPDH levels after knockdown were measured using quantitative RT-PCR. Approximately 91% knockdown was achieved for DMS-53 luc+. Negative controls included untreated cells and cells treated with a negative control siRNA *B*. Cell viability upon GAPDH silencing was measured. *** = $p < 0.001$, n=3 for quantitative RT-PCR, n=4 for viability assay; ANOVA-Tukey.

*High-throughput screening (HTS) of compound library*

The bioluminescence viability assay protocol was modified for implementation in a high-throughput setting using the DMS-53 luc+ cell line. Assay quality was first verified using three different statistical parameters: S/B ratio, S/N ratio, and Z'-factors (see Materials and Methods). An S/B ratio of 3.1 and an S/N ratio of 18.6 were obtained. Both values lie within acceptable range (> 2-fold). A Z'-factor value of 0.7 was also obtained, indicating that the assay was excellent for screening.

The assay was then used to evaluate a library of 1,280 compounds. In the primary screen, numerous compounds reduced cell viability (Figure IV-4). Compounds that reduced cell viability at an efficiency greater than or equal to cisplatin (~77% reduction) were considered positive hits. A total of 237 hits were identified, comprising a diverse class of compounds (Table IV-1). The classes with the most number of hits (≥15) included compounds directed at phosphorylation, dopamine signaling and serotonin signaling.

Because phosphorylation is generally involved in a variety of physiological and pathological processes, we focused secondary screening on hits from the dopamine and serotonin classes of compounds. We retested these compounds first using DMS-53 luc+ cells. Of the 27 dopamine compounds, 24 were

confirmed during secondary screening and of the 15 serotonin compounds, 12 were confirmed.

**Table IV-1. Classes of compounds that inhibit SCLC cell viability**

| Class | Number of Hits |
|---|---|
| Adenosine | 4 |
| Adrenoreceptor | 10 |
| Angiogenesis | 1 |
| Antibiotic | 2 |
| Apoptosis | 6 |
| Benzodiazepine | 1 |
| Biochemistry | 9 |
| $Ca^{2+}$ Channel | 10 |
| Cannabinoid | 2 |
| Cell Cycle | 5 |
| Cell Stress | 2 |
| Cholinergic | 7 |
| Cytokines and Growth Factors | 1 |
| Cytoskeleton | 4 |
| DNA Metabolism | 3 |
| Dopamine | 27 |
| GABA | 5 |
| Gene Regulation | 1 |
| Glutamate | 5 |
| G-protein | 4 |
| Histamine | 6 |
| Hormone | 7 |
| Immune System | 3 |
| Intracellular Calcium | 4 |
| Ion Channels | 2 |
| Ion Pump | 5 |
| $K^+$ Channel | 5 |
| Leukotriene | 5 |
| Lipid | 4 |
| Lipid Signaling | 2 |
| Multi-drug Resistance | 2 |
| Neurodegeneration | 1 |
| Neurotransmission | 7 |
| Nitric Oxide | 4 |
| Opioid | 5 |
| P2 Receptor | 1 |
| Phosphatase | 1 |
| Phosphorylation | 39 |
| Serotonin | 15 |
| Sphingolipid | 1 |
| Tachykinin | 3 |
| Transcription | 3 |
| Vanilloid | 2 |
| | Total = 237 |

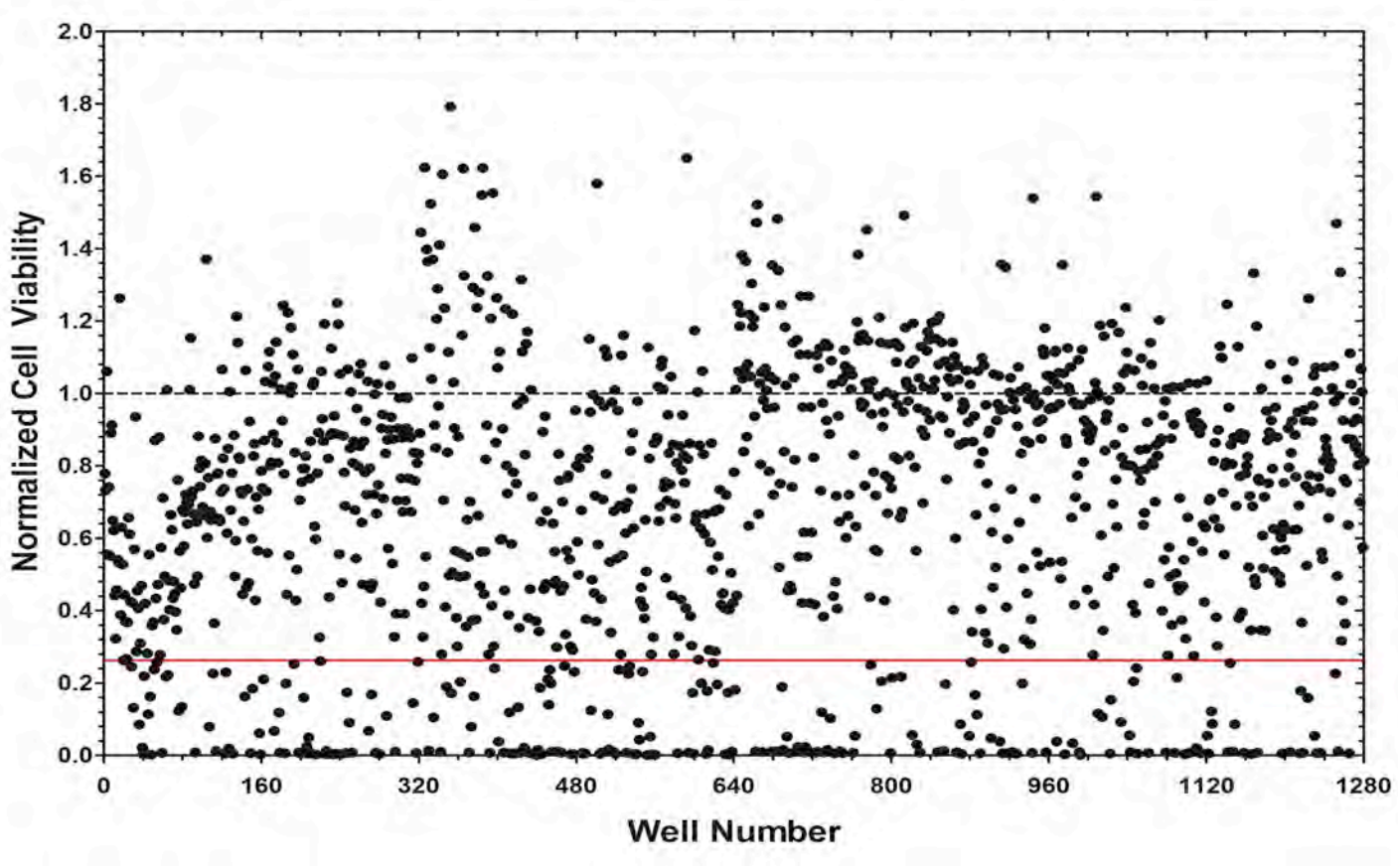


**Figure IV-4.** 237 compounds inhibit SCLC cell viability. DMS-53 luc+ cells were treated with compounds from the LOPAC$^{1280}$ library. Luciferase assays were then performed to measure the effect of the compounds on cell viability. Luciferase values were normalized to the mean luciferase values of the negative control, DMSO (dotted line). The solid red line indicates the mean value for the positive control, cisplatin. Compounds that resulted in inhibition greater than or equal to that of cisplatin were considered hits.

To ensure that reductions in viability caused by the various compounds were not specific for DMS-53 luc+ cells, the confirmed compounds were retested using DMS-114 luc+ cells. Of the 24 confirmed dopamine compounds, 22 caused reduction of viability in both DMS-53 luc+ and DMS-114 luc+ cells. Of the 12 confirmed serotonin compounds, all 12 reduced viability of DMS-53 luc+ and DMS-114 luc+ cells. Table IV-2 lists the compounds that were effective in reducing viability of both cell lines along with their specific pharmacological actions.

Finally, for tertiary verification, we performed dose-response assays of two representative compounds, one from each class. As shown in Figure IV-5A, treatment of DMS-53 luc+ cells with increasing doses of cortexolone maleate, a D2 dopamine receptor antagonist, resulted in corresponding decreases in cell viability. Similarly, increasing concentrations of fluoxetine hydrochloride, a serotonin uptake inhibitor, resulted in corresponding decreases in cell viability (Figure IV-5B).

**Table IV-2. Pharmacological agents that target neurotransmitter signaling in SCLC**

| Class | Name | Action | Selectivity |
|---|---|---|---|
| Dopamine | BP 897 | Agonist | D3 |
| | Chlorprothixene hydrochloride | Antagonist | D2 |
| | Cortexolone maleate* | Antagonist | D2 |
| | (±)-Butaclamol hydrochloride | Antagonist | D2>D1 |
| | R(+)-6-Bromo-APB hydrobromide | Agonist | D1 |
| | BTCP hydrochloride | Blocker | Reuptake |
| | Chlorpromazine hydrochloride | Antagonist | - |
| | R(-)-N-Allylnorapomorphine hydrobromide | Agonist | - |
| | Dihydroergocristine methanesulfonate | Agonist | - |
| | R(-)-Propylnorapomorphine hydrochloride | Agonist | D2 |
| | R(-)-2,10,11-Trihydroxyaporphine hybrobromide | Agonist | D2 |
| | GBR-12909 dihydrochloride | Inhibitor | Reuptake |
| | R(-)-2,10,11-Trihydroxy-N-propylnoraporphine hydrobromide | Agonist | D2 |
| | Fluspirilene | Antagonist | D2/D1 |
| | cis-(Z)-Flupenthixol dihydrochloride | Antagonist | - |
| | Fluphenazine dihydrochloride | Antagonist | D1/D2 |
| | GBR-12935 dihydrochloride | Inhibitor | Reuptake |
| | (±)-Octoclothepin maleate | Antagonist | D2 |
| | Perphenazine | Antagonist | D2 |
| | Pimozide | Antagonist | D2 |
| | Prochlorperazine dimaleate | Antagonist | - |
| | Thiothixene hydrochloride | Antagonist | D1/D2 |
| Serotonin | Amperozide hydrochloride | Ligand | - |
| | Paroxetine hydrochloride hemihydrate | Inhibitor | Reuptake |
| | CGS-12066A maleate | Agonist | 5-HT1B |
| | S-(+)-Fluoxetine hydrochloride | Inhibitor | Reuptake |
| | Fluoxetine hydrochloride* | Inhibitor | Reuptake |
| | SB 228357 | Antagonist | 5-HT2B/2C |
| | Metergoline | Antagonist | 5-HT2/5-HT1D |
| | GR 127935 hydrochloride hydrate | Antagonist | 5-HT1B/1D |
| | Sertraline hydrochloride | Inhibitor | Reuptake |
| | Parthenolide | Inhibitor | - |
| | Ritanserin | Antagonist | 5-HT2/5-HT1C |
| | SB 224289 hydrochloride | Antagonist | 5-HT1B |

* Representative drugs tested for tertiary verification
- Unknown selectivity

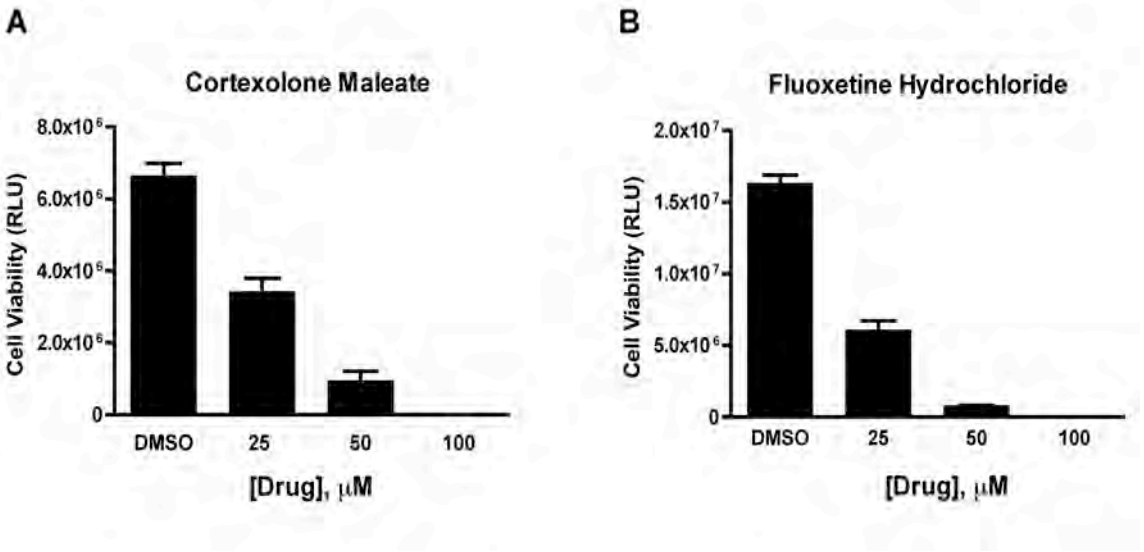


**Figure IV-5.** SCLC cells respond to cortexolone maleate and fluoxetine hydrochloride in a dose-dependent manner. DMS-53 luc+ cells were treated with 0, 25, 50, and 100 μM cortexolone maleate (**A**) or fluoxetine hydrochloride (**C,D**) for 24 hours. Luciferase assays were then performed to measure cell viability. Data points represent mean values and error bars represent standard error of means (n=8). **RLU** – relative light units.

## DISCUSSION

With the aim of uncovering novel therapeutic strategies against SCLC, we developed a bioluminescence-based cell viability assay for high-throughput screening of compound libraries. Phenotypic assays such as the one described here expedite primary screening of large numbers of chemicals, while limiting the use of animals in research. In this study, we used two cell lines, DMS-53 luc+ and DMS-114 luc+, that were originally derived from mediastinal biopsies of

SCLC patients who had not received prior therapy, allowing delineation of specific effects of novel compounds [321].

We demonstrated broad dynamic range of detection for both cell lines. Increased sensitivity of the bioluminescence assay was also observed compared to a traditional MTT-based cell viability assay. Moreover, a direct relationship between luminescence signals and cell number was observed for both cell lines using two approaches, luminometry and bioluminescence imaging. The use of live animal bioluminescence imaging provides a more physiologically relevant context and allows for non-invasive, longitudinal monitoring of animals, again avoiding the use of large numbers of animals for research. These advantages notwithstanding, cell-based assays remain indispensable for large-scale screens.

Prior to performing a large-scale screen, we assessed the responsiveness of the two engineered cell lines to standard pharmacological and genetic agents. Staurosporine, a member of the K252 family of compounds known to inhibit protein kinases [322], was used to show sensitivity of the engineered cells to an apoptosis-inducing drug. Cisplatin, a platinum-containing, broad activity anti-neoplastic and alkylating agent [323], was used to demonstrate the sensitivity of cells to a classic chemotherapeutic agent. Finally, RNA interference using siRNAs against GAPDH, a gene involved in vital metabolic functions [324], illustrated the utility of these cells for studies involving genetic treatments.

The assay was then implemented in a large-scale screen of the LOPAC$^{1280}$ compound library. This library contains 1,280 pharmacologically active compounds. This annotated collection of small molecule modulators and FDA-approved drugs impacts most cellular processes and covers all major drug target classes. The LOPAC screen serves as an excellent starting point for validating high-throughput assays. Moreover, it potentially allows the identification of drugs that have available human dosage and toxicity information as well as the discovery of lead structures for drug development. Our primary screen identified several classes of drugs that reduced SCLC cell viability (Table IV-1). Of these, many have been implicated in fundamental processes associated with the etiology of cancer, such as angiogenesis, calcium signaling, cell cycle progression, and protein phosphorylation [120].

Interestingly, our screen identified several drug classes that impact neuroendocrine pathways known to be involved in SCLC pathogenesis. SCLC cells are characterized by neuroendocrine features such as the expression of ion channels, neuropeptides, and neurotransmitters and, as a consequence, are electrically excitable [325]. Here, we identified drugs that target adrenoreceptors, calcium channels, cholinergic receptors, dopamine signaling, GABA signaling, glutamate signaling, $K^+$ channels, $Na^+$ channels, opioid signaling and serotonin signaling [326].

We focused the follow-up screen on compounds that target dopamine and serotonin signaling as they yielded the highest number of hits. We did not pursue compounds in the protein phosphorylation class given the ubiquitous role protein phosphorylation plays in both normal and disease states. The secondary screening results essentially overlapped with those of the primary screen, indicating the reliability of the assay. Furthermore, the dose-dependent reduction in cell viability induced by the D2 dopamine receptor antagonist, cortexolone maleate, and the serotonin reuptake inhibitor, fluoxetine hydrocholoride, is consistent with the critical role of neurotransmitter signaling in the pathogenesis of SCLC [327].

Dopamine signaling has previously been implicated in SCLC [328]. In particular, the D2 receptor agonist, bromocriptine, has been shown to have an anti-proliferative effect on SCLC cells *in vitro* and inhibits growth of SCLC tumor xenografts [329]. Unexpectedly, we observed that cortexolone maleate, which blocks the D2 dopamine receptor, also has an anti-proliferative effect. In addition, serotonin has been shown to act as a mitogenic signal in SCLC, activating an autocrine growth loop in these cells [330, 331]. However, we found that the serotonin reuptake inhibitor, fluoxetine hydrochloride, known to increase serotonin levels, inhibits SCLC growth. Another serotonin reuptake inhibitor, imipramine, has previously been shown to inhibit the development of SCLC [128]. Taken together, these findings suggest that SCLC growth may rely on the

maintenance of specific levels of neurotransmitters rather than their simple absence or presence or alternatively posit functional selectivity of neurotransmitter receptors in SCLC.

In conclusion, we have described a bioluminescence-based assay for drug discovery in the field of SCLC therapeutics. Such an assay has not been previously applied to SCLC, a disease with very poor prognosis and limited treatment outcomes. The simplicity and speed of the workflow we developed not only allows for routine laboratory use but also lends itself to high-throughput applications and adaptability to automation. We have validated this assay against a library of pharmacologically active compounds. That positive hits included compounds targeting classic cancer signaling pathways suggests internal consistency. Compounds that target neurotransmission also emerged from the screen, reflecting the neuroendocrine nature of SCLC and underscoring the role of neurotransmitter signaling in this disease. In particular, perturbation of dopamine and serotonin signaling inhibits SCLC cell viability, suggesting the utility of these classes of drugs as therapeutic agents against SCLC.

# CHAPTER V:

# DISCUSSION

*That theory is worthless. It isn't even wrong!*
*-Wolfgang Pauli*

The cloning of nAChRs from brain cDNA libraries in the mid-1980s was a watershed event as it opened the window not only to a molecular understanding of cholinergic signaling but also to a structural appreciation of how members of the superfamily of ligand-gated ion channels function. As the cloning frenzy subsided, tremendous effort was put forth to characterize the pharmacological and biophysical properties of nAChRs. As a result, nAChRs are among the most well understood allosteric membrane proteins from a structural and functional point of view. Following the cloning of nAChR subunits, development of novel nAChR ligands – agonists, antagonists, and allosteric modulators - for CNS disorders became a focus for many drug companies. Varenicline (Chantix®), a partial agonist of $\alpha$4-containing nAChRs, is a product of this endeavor and is now a clinically approved drug for smoking cessation [332]. Several other nAChR-targeted drugs for Alzheimer's disease, anxiety, depression, pain, schizophrenia, and ulcerative colitis are now in clinical trials [333].

Despite all this progress, one major challenge that remains in the field is deciphering the native nAChR subtypes responsible for the myriad functions that nAChRs play both in and out of the nervous system. Identification of these subtypes is complicated by the existence of several subunits (i.e., eleven human subunits), the numerous ways these subunits can combine (i.e., various

homomeric and heteromeric receptors), and the different stoichiometries for each subtype (e.g., $(\alpha4)_2(\beta2)_3$ vs. $(\alpha4)_3(\beta2)_2$) [334]. Nevertheless, delineating the role of specific subtypes is critical to the understanding the function of nAChRs in general and is essential in drug discovery efforts for subtype-selective drugs.

For years, the $\alpha4\beta2$ and $\alpha7$ nAChR subtypes have been the focus of most studies in the nAChR field for the reasons that follow. First, the $\alpha4\beta2$ subtype is highly expressed in the brain and binds with the highest affinity to nicotine and other common nAChR agonists [335]. It is also the subunit most strongly upregulated by nicotine exposure [336]. Specifically, chronic nicotine treatment increases the number of $\alpha4\beta2$ nAChRs in rodent brain, consistent with increased $\alpha4\beta2$ expression in post-mortem brains of human smokers. The $\alpha7$ subtype is also highly expressed in the brain, where it undergoes rapid activation and desensitization in response to agonists [175]. In addition, the availability of an irreversible and highly selective ligand for $\alpha7$, $\alpha$Bgtx, has facilitated studies with this receptor.

A major advance towards understanding the role of other nAChR subtypes is the discovery of allelic variations in the genes encoding the $\alpha5$, $\alpha3$, and $\beta4$ nAChR subunits that increase risk for nicotine dependence and lung cancer (see Chapter I). That polymorphisms in these genes influence risk for these and other disorders suggests a pleiotropic role for nAChRs in different cell types [167]. It is

quite compelling that results of both hypothesis-free GWAS and hypothesis-driven candidate-gene studies converged on this gene cluster and that these results have been replicated many times over by independent groups using different tools and different populations.

In Chapter II, we describe the over-expression of CHNRA5/A3/B4 in SCLC. The high expression of nAChR subunit genes in SCLC is consistent with the neuroendocrine phenotype of these cells. Pulmonary neuroendocrine cells fare believed to be the precursor cells of SCLC [11]. They exist either as solitary cells in the tracheobronchial tract or as clusters called neuroepithelial bodies in the intrapulmonary airways. These cells are normally found in the fetal or neonatal lung, where they play a role in lung morphogenesis [337]. They act as chemoreceptors that sense oxygen and carbon dioxide levels and respond by releasing secretory substances. The existence of pulmonary neuroendocrine cells in the adult suggests a recapitulation of embryonic processes, a common theme in carcinogenesis [338].

Intriguingly, the over-expression of the CHRNA5/A3/B4 genes is also consistent with the paraneoplastic syndromes commonly associated with SCLC. These syndromes are usually neuroendocrine or neurological in nature; the former is caused by ectopic production of bioactive substances such as hormones and the latter is due to the production of autoimmune antibodies [339].

Antibodies against nAChRs in the autonomic ganglia are thought to cause autonomic neuropathies that accompany SCLC [340]. Speculatively, the over-expression of $\alpha$5, $\alpha$3, and $\beta$4 nAChRs in SCLC cells triggers the production of these autoimmune antibodies. The consequent immune-mediated tissue destruction is, in fact, associated with favorable prognostic outcomes [339], possibly due to the concurrent clearance of cancer cells expressing the autoantigens.

As also described in Chapter II, the over-expression of the CHRNA5/A3/B4 genes is regulated in part by ASCL1. Our work on CHRNA5/A3/B4 regulation is the first report of a transcription factor regulating expression of nAChRs in cancer cells. It also provides a link between nAChR signaling and the Notch pathway, a signaling pathway involved in normal morphogenesis as well as malignant transformation [341]. Notch receptors are activated by multiple alternative ligands (e.g., Delta, Jagged). Ligand binding leads to the proteolytic cleavage of the intracellular domain of the receptor, which in turn, translocates to the nucleus and forms part of a transcriptional machinery that regulates target genes. One of the best-described Notch target is the transcriptional repressor, Hes (and Hes-related proteins) [341]. Hes represses the expression of ASCL1, such that cells that express Hes do not express ASCL1, and vice-versa. For instance, neuroendocrine cells in the lungs express ASCL1, while non-neuroendocrine cells express Hes-1. Similarly, SCLC cells

express ASCL1 but lack Hes-1 expression, while the opposite is true for NSCLC cells [342].

The use of genetically engineered mouse models has further shed light on the role of ASCL1 in lung development. mASH1 mutant mice lack neuroendocrine cells and die within 12 hours of birth from hypoventilation [343]. In contrast, Hes-1 null mice exhibit precocious neuroendocrine differentiation [344]. Transgenic expression of ASCL1 in non-neuroendocrine cells of the airways, along with SV40 large T antigen, induces lung tumors with a NSCLC-neuroendocrine phenotype [345]. Constitutive expression of ASCL1 alone leads to extensive cell proliferation in the airways without neuroendocrine differentiation, suggesting that ASCL1 expression with concomitant loss of p53 and Rb (due to the T antigen) promotes the development of neuroendocrine lung tumors.

Analysis of ASCL1 transcriptional targets hints at mechanisms by which ASCL1 functions in lung cancer. One study using pancreatic neuroendocrine tumors described components of the Wnt signaling pathway as targets for ASCL1 [346]. Another group using embryonic brain reported ASCL1 regulation of a large number of genes involved in cell cycle regulation [347]. In SCLC cells, the stem cell markers CD133 and aldehyde dehydrogenase 1A1 (ALDH1A1) were identified as ASCL1 targets, advocating the involvement of ASCL1 in tumor

initation [348]. Another group describes ASCL1 repression of E-cadherin, suggesting a role for ASCL1 in epithelial-mesenchymal transition [349]. Our findings add CHRNA5/A3/B4 to the list of ASCL1 responder genes. We propose a mechanism by which upregulation of ASCL1 in SCLC cells causes a corresponding upregulation of $\alpha5\alpha3\beta4$ nAChRs, leading to the potentiation of the tumorigenic effects of nicotine and acetylcholine.

Importantly, the over-expression of CHRNA5/A3/B4 in SCLC supports the hypothesis that these genes play a direct role in lung cancer. As described in Chapter III, their function involves maintenance of SCLC cell viability and modulation of tumor growth and incidence. We envisage a mechanism wherein $\alpha5\alpha3\beta4$ nAChRs act as upstream mediators of nicotine and/or acetylcholine-activated cancer signaling pathways (Figure V-1). A number of such signaling pathways have been described, including the proliferative Ras-Raf-MAPK pathway and the anti-apoptotic PI3K-Akt-Bad pathway. Investigation of the specific pathways mediated by $\alpha5\alpha3\beta4$ nAChRs should expound on the role of this subtype in SCLC.

SCLC remains one of the deadliest types of cancers, for which no major advancements in terms of therapy have been made in the last three decades [314]. In terms of SCLC research, part of the reason may involve the difficulty in obtaining human SCLC tumor samples. Not only is SCLC less common than

other types of lung cancer, SCLC patients also present with metastasis at the diagnosis, precluding surgical resection of tumors. From a biological standpoint, the neuroendocrine phenotype of SCLC may be a huge factor in the aggressiveness and chemoresistant nature of the deisease. The numerous neurotransmitters and neuropeptides produced by SCLC triggers its own proliferative state. Furthermore, the anti-apoptotic pathways evoked by nicotine and/or acetylcholine may interfere with the actions of chemotherapeutic drugs. Results from our high-throughput screen suggest that targeting these neuroendocrine pathways directly may be a viable therapeutic approach for SCLC.

*Future Directions*

One of the major limitations in the nAChR field is the absence of high quality antibodies for use in immunoassays. A recent evaluation of nAChR antibodies showed that several supposedly subunit-specific antibodies against nAChRs immunoreact with both wild-type and subunit knockout mice [350]. This precludes the use of these reagents for immunodetection methods and calls into question previously published work that made use of these antibodies. In terms of our own research, the lack of this valuable tool hindered us from performing Western blot or immunostaining experiments to determine protein expression of nAChR subunits in normal and lung cancer cells. Future development of high quality antibodies against nAChRs would allow immunodetection experiments to

be carried out. Furthermore, these antibodies would be useful in co-immunoprecipitation experiments for identification of nAChR subtypes.

We were able to circumvent this problem by using the epibatidine radioligand assay to determine the effect of ASCL1 knockdown on the total nAChR levels on the cell membrane. Though this results shows that ASCL1 knockdown decreases total nAChR protein levels, it would be interesting to measure the effect of ASCL1 knockdown specifically on $\alpha3\beta4$ nAChR levels. Development of radiolabeled $\alpha$-conotoxin AuIB would facilitate such an experiment. Currently, there are no available $\alpha5\alpha3\beta4$ nAChR-selective ligands, though it is hoped that cone snail venoms or chemical entities being developed by pharmaceutical companies may someday provide this tool.

We have demonstrated that ASCL1 regulates expression of CHRNA5/A3/B4 but we have not shown whether this regulation is via direct binding to the E-boxes found in the CHRNA5/A3/B4 promoter regions. Our preliminary work using chromatin immunoprecipitation (ChIP) assays suggests that ASCL1 directly binds to one of the E-boxes in the CHRNA3 promoter (see Appendix II). This work can be extended to include the other E-boxes in the locus. Additionally, site-directed mutagenesis of the E-boxes should identify functional binding sites for ASCL1. We have also initiated ASCL1 transactivation experiments showing that forced expression of ASCL1 activates the CHRNA3

promoter, while ASCL1 knockdown decreases the activity of the promoter (Appendix III). This work can be extended to include the CHRNA5 and CHRNB4 promoters as well. Co-immunoprecipitation experiments with known transactivators of CHRNA5/A3/B4 would also identify ASCL1 binding partners and elucidate the transcriptional machinery regulating CHRNA5/A3/B4 expression in SCLC.

Though we observed robust regulation of CHRNA3 and CHRNB4 by ASCL1, we only observed a modest regulation of CHRNA5. That CHRNA5 is transcribed in the opposite direction as CHRNA3 and CHRNB4 may offer one possible explanation. A unique set of transcription factors may govern CHRNA5 expression from that direction. The presence of E-boxes in the CHRNA5 promoter suggests other basic helix-loop-helix transcription factors may play a role. One candidate for future studies is N-myc, often amplified in SCLC but not in NSCLC [351].

With regards to our functional work, we have shown that the CHRNA5/A3/B4 genes are necessary for maintenance of SCLC cell viability, an important observation on its own. However, we have not shown whether the decrease in cell viability observed upon CHRNA5/A3/B4 knockdown is due to a decrease in cell proliferation or an increase in cell death. We have begun to set up experiments to address this question. BrdU labeling will be performed to

determine the effect of CHRNA5/A3/B4 knockdown on cell proliferation. Western blot analysis will also be utilized to interrogate the MAPK proliferative pathway. Levels of phosphorylated versions of MAPK pathway components will be determined upon CHRNA5/A3/B4 knockdown. Experiments to determine the effect of CHRNA5/A3/B4 knockdown on cell death would be performed in parallel. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays will be performed to determine effect of CHRNA5/A3/B4 knockdown on apoptosis. The PI3K-Akt apoptotic pathway will also be interrogated using Western blot analysis.

Nicotine and acetylcholine signaling mediate other cancer-related processes and the effect of CHRNA5/A3/B4 knockdown on these processes should also be investigated. For example, soft agar assays can be performed to determine the effect of CHRNA5/A3/B4 knockdown on anchorage-independent growth. Our preliminary work suggests that CHRNA3 knockdown decreases both the number and size of colonies that form in soft agars (Appendix IV). This work can be extended to CHRNA5 and CHRNB4. Other *in vitro* assays include endothelial tube formation assays for angiogenesis or Boyden chamber assays for migration/invasion.

Our *in vivo* work utilizing the xenograft tumor model showed inhibition of tumor growth and incidence with CHRNA5 silencing. To date, we have not

observed significant decreases in tumor growth upon CHRNA3 and CHRNB4 knockdown. The lack of a proper tumor microenvironment may obscure the effects of CHRNA3 and CHRNB4 knockdown. The use of an orthotopic model (i.e., via intrathoracic injections) may be a useful future approach. The lung colonization model may also be used to allow tumor cells to reach the lung via tail vein injections. Though primarily used as a metastasis assay, this model may provide the proper microenvironment for SCLC cells.

The functional assays described here mostly involve loss-of-function experiments. Corresponding gain-of-function experiments should be performed. Similar cell viability and xenograft assays can be performed using transient and stable expression of CHRNA5/A3/B4 expression constructs, respectively, into SCLC cells. One possible problem with over-expression experiments is that CHRNA5/A3/B4 expression may already be at saturation levels, such that further increases in expression may not yield additional measurable phenotypes. Another approach would be to develop knock-in mouse models expressing the rs16969968 SNP, with and without the risk allele. These can then be used to determine susceptibility to lung cancer in the presence and absence of nicotine. Such an experiment would demonstrate whether the risk allele confers lung cancer susceptibility independent of nicotine exposure.

Finally, we have developed and validated a bioluminescence-based viability assay for large-scale screening of compounds against SCLC. However, this project is only at its inception. Further validation of other positive hits in the screen will be performed. Interesting hits will be further investigated using both *in vitro* and *in vivo* assays. Chemical modification of compounds of interest to improve activity and/or other clinically relevant properties may also be worth pursuing. This assay can also be applied to even larger libraries of compounds or for large-scale RNA interference screens. Ultimately, the goal of these experiments is to enhance currently available therapeutics against SCLC.

# APPENDICES

# APPENDIX I

## DMSO Treatment

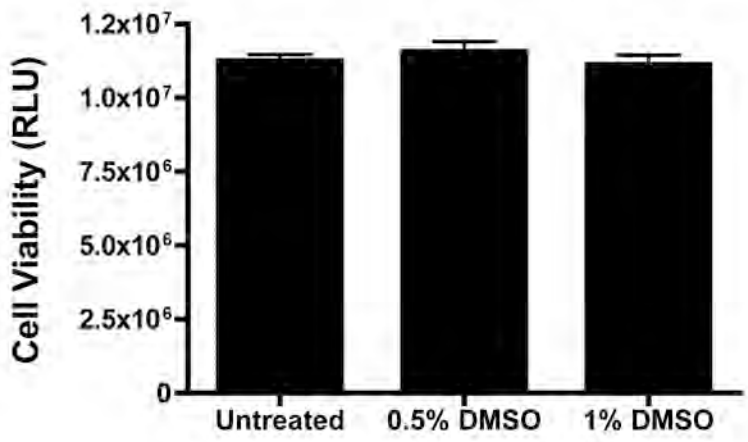


**Figure A-I**. DMSO tolerance of DMS-53 luc+ cells. Cells were treated either with 0.5% or 1% DMSO in complete medium for 24 hours. Cells in medium alone served as untreated controls. No significant difference in cell viability was observed after DMSO treatment.

## APPENDIX II

## Chromatin Immunoprecipitation

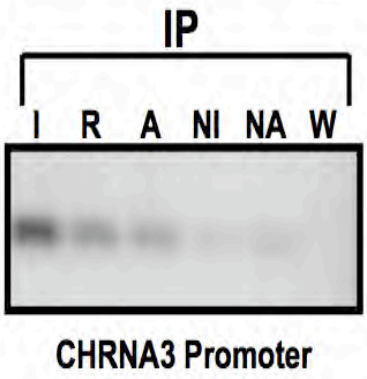


**Figure A-II**. ASCL1 binds to the CHRNA3 promoter. Chromatin immunoprecipitation (ChIP) assay shows enrichment of the CHRNA3 promoter in samples immunoprecipitated with an ASCL1 antibody. ChIP assays were performed using the ChIP-IT Express kit (Activ Motif). Specifically, DMS-53 cells were subjected to formaldehyde-crosslinking followed by sonication to obtain DNA fragments with an average size of 500bp. Chromatin samples were then incubated with an ASCL1 ChIP-grade antibody (Santa Cruz Biotechnology) overnight at 4$^{o}$C. RNA Polymerase II antibodies and normal IgG were used as positive and negative controls for immunoprecipitation. A no-antibody control was also performed. Protein G-coated magnetic beads were co-incubated with the samples for pull-down of chromatin-antibody complexes. Beads were collected using a magnetic bar and washed several times followed by chromatin elution. Reverse-crosslinking and Proteinase K digestion was performed in preparation for PCR. To amplify the promoter region of CHRNA3, the forward primer 5'-GGAAACCTGGGACAGAAACTGA-3' and the reverse primer 5'-GGCGGCGTCTTGACAGAT-3' were used. Input DNA was used as a positive control template while water was used as a no-template control. I - Input; R - RNA polmerase II; A - ASCL1; NI - Normal IgG; NA - No antibody; W - Water.

## APPENDIX III

## Luciferase Reporter Assay

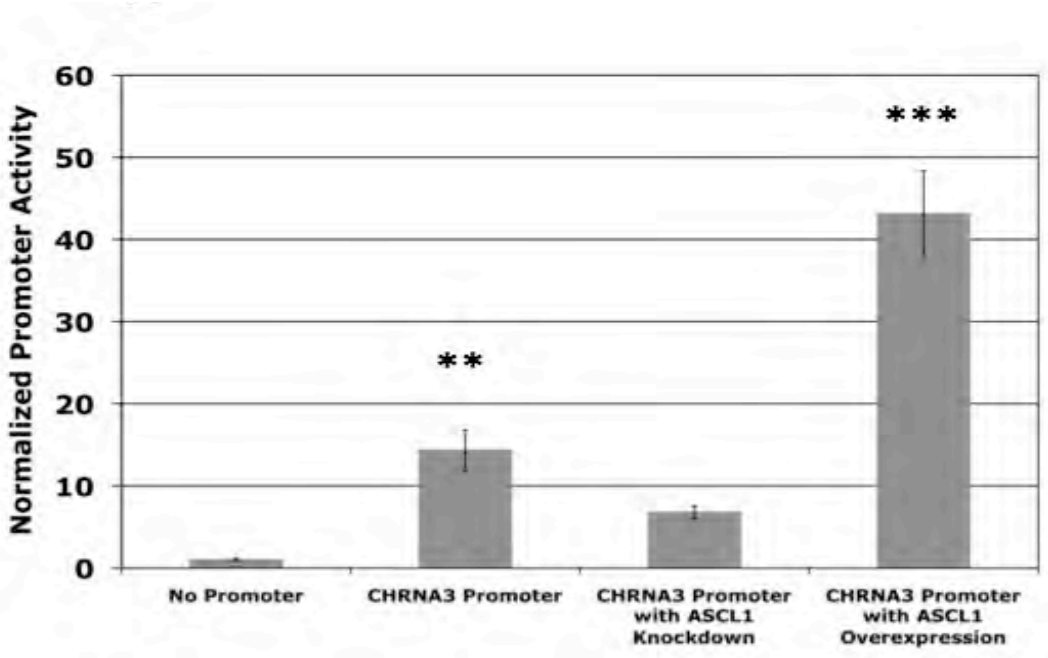


**Figure A-III**. Luciferase reporter assay shows decrease in CHRNA3 promoter activity upon ASCL1 knockdown and increase in CHRNA3 promoter activity upon ASCL1 over-expression. The CHRNA3 promoter region was subcloned upstream of a luciferase reporter gene of the parent vector, pGL3-Basic (Promega). For knockdown experiments, reporter plasmids were transfected into DMS-53 cells along with an ASCL1 siRNA (s1656, Applied Biosystems) using Lipofectamine 2000 (Invitrogen). For transactivation experiments, reporter plasmids were co-transfected with the ASCL1 gene subcloned into the expression vector, pcDNA3.1 (Invitrogen). A β-galactosidase expression vector was co-transfected in each sample for normalization. Cells were harvested 48 hours after transfection and assayed for luciferase (Luciferase Assay System, Promega) and β-galactosidase (Galacto-Star System, Applied Biosystems) activity in a Lumimark microplate luminometer (Bio-Rad). ** = $p < 0.01$, *** = $p < 0.001$ compared to No Promoter sample; n = 4; ANOVA-Tukey.

## APPENDIX IV

## Soft Agar Assay

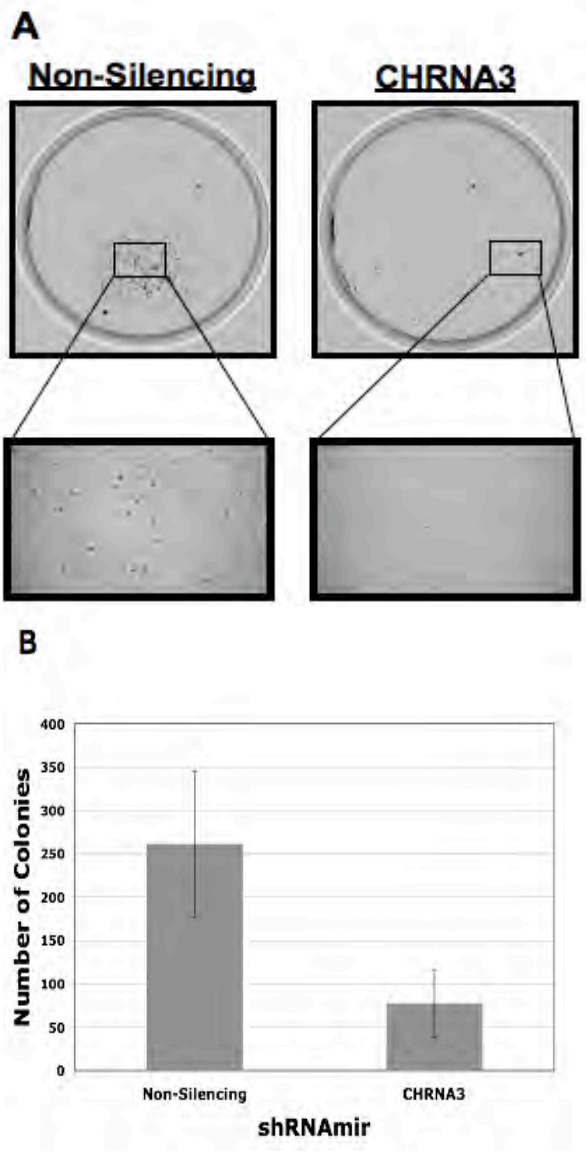


**Figure A-IV**. CHRNA3 knockdown decreases number and size of colonies in soft agar assay. DMS-53 cells treated with a non-silencing or CHRNA3 shRNAmir were grown in soft agar plates at 37$^{o}$C for 21 days. Colonies were visualized by staining with 0.005% crystal violet and counted using the Labworks 4.5 software (UVP Bioimaging Systems).

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